STUDIES ON THE METABOLISM OF ACETATE AND ACETOACETATE

by

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I. PREFACE

It has become evident in scientific fields that a simple observation can subsequently yield a complex field of investigation. Different branches of one and of many sciences can intermingle so that boundaries are no longer well defined. In undertaking a problem in the laboratory, one of the difficulties that arises is to confine the problem within the boundaries of the parent disicipline.

It would appear that the major metabolic pathways of the mammalian organism have now been well defined, however, the factors influencing the dynamics and interplay of these metabolic pathways have not been equally well defined. We now realize that an alteration in any one of supposedly different areas of investigation (i.e. lipid or carbohydrate metabolism) affects not only one system but many others as well.

In this study, we have worked with one of the "points of intersection" of metabolic pathways. Acetate, in the form of acetyl CoA, is both an end product of, and a building block for, both lipid and carbohydrate chemistry. Acetoacetic acid, on the other hand, is a key intermediate in the lipid field and is not common to the carbohydrate field. However, this C_4 compound, acetoacetic acid, through acetyl CoA as an intermediate, can merge with the flow of C_2 units of carbohydrate metabolism. In these studies, the metabolism of acetate and acetoacetate has been followed in a number of tissues and in both control and in diabetic preparations. In vitro systems were chosen for these studies, for although such systems cannot

be considered to be completely physiological, it is possible to better control a number of the factors known to influence tracer studies.

II. INTRODUCTION

A. Ketosis. The condition of ketosis was first recognized in the XIX century, and its relation to diabetes mellitus was noticed at that time.

We consider the ketone bodies to be acetoacetic acid, β-hydroxybutyric acid and acetone. Normally, there are only small amounts of these compounds in the circulation (1), and where there are excessive amounts, the condition of ketosis is said to be present. Experiments using the perfusion technique prove the liver to be the chief organ concerned with the production of ketone bodies which are secreted into the blood (2). Very small amounts of ketone bodies are utilized by the liver as acetoacetate per se (3). Circulating acetoacetic acid can either be utilized by the extrahepatic tissues or converted to β-hydroxybutyric acid by DPNH in the presence of a β-keto reductase enzyme found in the liver. The formation of β-hydroxybutyric acid may be a mechanism of transport for acetoacetic acid in a less toxic form.

Mechanisms for acetoacetate synthesis and utilization are described in a subsequent section.

In fasting, in diabetes mellitus or in other conditions in which there is an impaired utilization of carbohydrate, there is an insignificant conversion of carbohydrate to fat by the liver. In many of these cases, an excessive breakdown of fats leads to an overproduction of acetoacetic acid, which the extrahepatic tissues are not able to completely utilize.

B. General remarks on the experimental approach. The original idea in this work was to study, within the problem of ketosis, the handling, by muscle and liver, of one of the ketone bodies, acetoacetic acid. Because of the importance of acetate as the C_2 "building block" in so many biochemical reactions, we considered this an interesting opportunity to compare its metabolism to that of acetoacetic acid by these same tissues.

Considerable work has been done previously in this laboratory with liver slices and with tracer acetate in a macro system. Since it was planned to use a considerably smaller system as far as volumes of media and amounts of tissue were concerned, it was considered an unnecessary risk to use previously determined values for comparison with the new values. Investigation of the metabolism of acetate by hepatic tissues, in our smaller system, turned out to be a good confirmation of the adequacy of control of our laboratory techniques since the present work yielded results equivalent to the results obtained on the macro system.

In the selection of muscle tissue for study, it was necessary to choose the muscle that would provide strips easily with small damage to the tissue. A modification of Shorr's technique (4) for obtaining muscle strips from leg adductor muscle was chosen. In addition, diaphragm muscle tissue was used and compared to the leg muscle preparation.

As mentioned before, due to the interrelationships existing among the different metabolic pathways, it was considered that it would be intriguing and exciting to be able to follow labeled substrates into all of these pathways. For obvious practical reasons, it was necessary to confine the work to only those reactions nearest to a particular interest.

For this reason this thesis is concerned with the presentation of information on the incorporation of labeled substrates into the fatty acid, cholesterol and CO_2 fractions of the tissues.

C. Choice of tissues. The theory of the liver slice preparation has been amply discussed by Dr. Robert Emerson in another thesis (5) from this laboratory. Frequent reference was made to this work during the course of these experiments.

Muscle preparation: The mammalian muscular system, formed by its specialized cells called muscle fibers, has contractibility as its primary function, the myofibrils being the contractile elements. With few exceptions, all three classes of muscle fibers: smooth, cardiac and skeletal, arise from myoblasts. The myofibrils appear at an early stage in the cytoplasm (sarcoplasm). Under polarized light, the myofibrils of smooth muscle appear to remain homogenous, while in cardiac and skeletal muscles regions appear giving the characteristic dark (A) and light (I) bands. Krause's membranes (Z lines) continue through the I-bands of the fibrils, across the sarcoplasm, attaching on to the cell membrane or sarcolemma. Later, fibrils become aligned so that all dark bands are next to each other giving the fiber a striped appearance (6). Alongside the myofibrils lie the nuclei and mitochondria.

Human muscle fibers have diameters of 10 to 100 μ, about 20% of their weight is protein, while most of the rest is water. The myofibril is approximately 1 μ in diameter and is formed by still smaller filaments of approximately 50 to 100 $\mathring{A}$ in diameter, which in turn can be of two kinds, one twice as thick as the other and of different lengths. The two kinds of filaments are linked by a system of crossbridges of constant

occurrence. This microstructure of the myofibrils seems to be of great importance in the contraction mechanism.

Close to 90% of the protein of muscle is formed by myosin, actin and tropomyosin; myosin constitutes about half of the contractile protein. Myosin catalyzes the liberation of energy from ATP by removal of a phosphate group (7). Myosin (which composes the dark or A bands) and actin (which makes up the I or light bands) combine, in solution, to form actomyosin. Szent-Gyorgyi completed the picture of the essentials for the contractile system by preparing "fibers" from precipitated actomyosin, and by making them contract in a solution of ATP (8).

"Bridges" between the filaments that form the myofibrils seem to be related to the myosin molecule and permit its combination with actin for the contraction process. For this reaction the "bridges" would oscillate using the energy provided by splitting of phosphate groups from ATP (9,10,11).

This brief description of the units forming the structure of muscle tissue is presented with the purpose of giving an idea of the kind of preparation dealt with in the following experiments.

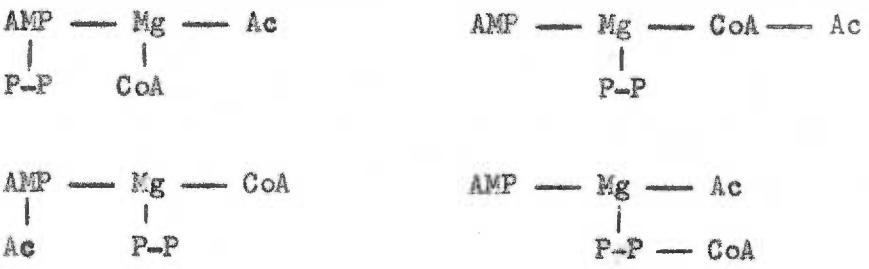
Diaphragm: The diaphragm muscle fibers are radial, running from the wall to a central tendon (phrenic center). This central tendon has been eliminated from our preparation. This also eliminated lymph containing vessels of this portion of the diaphragm. The edge of the diaphragm was also eliminated because of the tendonous insertions present in the area near the thoraxic wall (12, 13).

The average dry weights for fibers are reported as 16.2 $\pm$ 0.3% of the wet weight for control rats and 16.5 $\pm$ 1% for alloxan diabetics. In these same groups, the nitrogen content is given as 2.19 $\pm$ 0.09% for controls and 2.27 $\pm$ 0.13 for diabetics (14).

D. Acetate and acetoacetate as related to lipid metabolism. The importance of acetate as an intersection of many metabolic pathways has already been mentioned. Acetate constitutes the two carbon unit which needs to be "activated" by coenzyme A before it can function in fatty acid and cholesterol synthesis and in CO_2 formation. Acetate is activated by union with coenzyme A as: (15)

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

This reaction has been studied in detail by Lipmann, Ochoa, Lynen, Popjak and Green. The activation is likely the sum of two reactions, but the structure of the intermediates is not known, although it has been proposed that the reaction procedes through acetyl enzyme and adenosine-monophosphoryl enzyme as intermediates (16, 17). Berg (18) has suggested that the active intermediate is acetyl AMP, but participation of this intermediate has been subjected to criticism by David Green (19). Green has suggested that the components of the reaction may exist complexed to Mg^{++} in the various tautomeric forms shown below.



From such a mixture it would be possible to isolate various "intermediates," depending upon the isolation techniques used.

Acetyl-CoA can enter many reactions; it can go to cholesterol synthesis, acetylation reactions, fatty acids synthesis, or enter the tricarboxylic acid cycle and be metabolized to CO_2 and H_2O.

To enter into the synthesis of fatty acids, two molecules of acetyl-CoA condense to form acetoacetyl-CoA. Both coenzyme A and the group of enzymes linked to fatty acid synthesis are found in muscle, but since muscle represents the principal fuel utilizing system in mammalian organisms (20), synthesis of fatty acids and cholesterol is not one of its important functions.

If instead of adding acetate to tissue, acetoacetate was added, it also would need to be activated to acetoacetyl-CoA before it could enter the metabolic sequence. Henry Mahler (21) related and classified the reactions for activation and inactivation of acetoacetate by liver as:

Metabolism of acetoacetic acid by liver

1) Acetoacetyl-CoA is formed by:

Butyryl-CoA ⟶ Acetoacetyl-CoA

β-keto-acyl-CoA ⟶ Acetyl-CoA ↑

also formed by a very weak reaction in liver (present in kidney):

$$\text{acetoacetate} \xrightarrow[(\beta\text{-keto acid + activating E})]{\text{CoA + ATP}} \text{acetoacetyl-CoA}$$

2) Acetoacetyl-CoA is used up by:

a. Reversible reaction

acetyl-CoA ⟷ acetoacetyl-CoA + CoA

(condensing E. of Ochoa) ↓

oxalacetate ⟷ Citrate

b. Irreversible reaction

$$\text{acetoacetyl-CoA} \xrightarrow[\text{(deacylase + } H_2O)]{(22)} \text{acetoacetate}$$

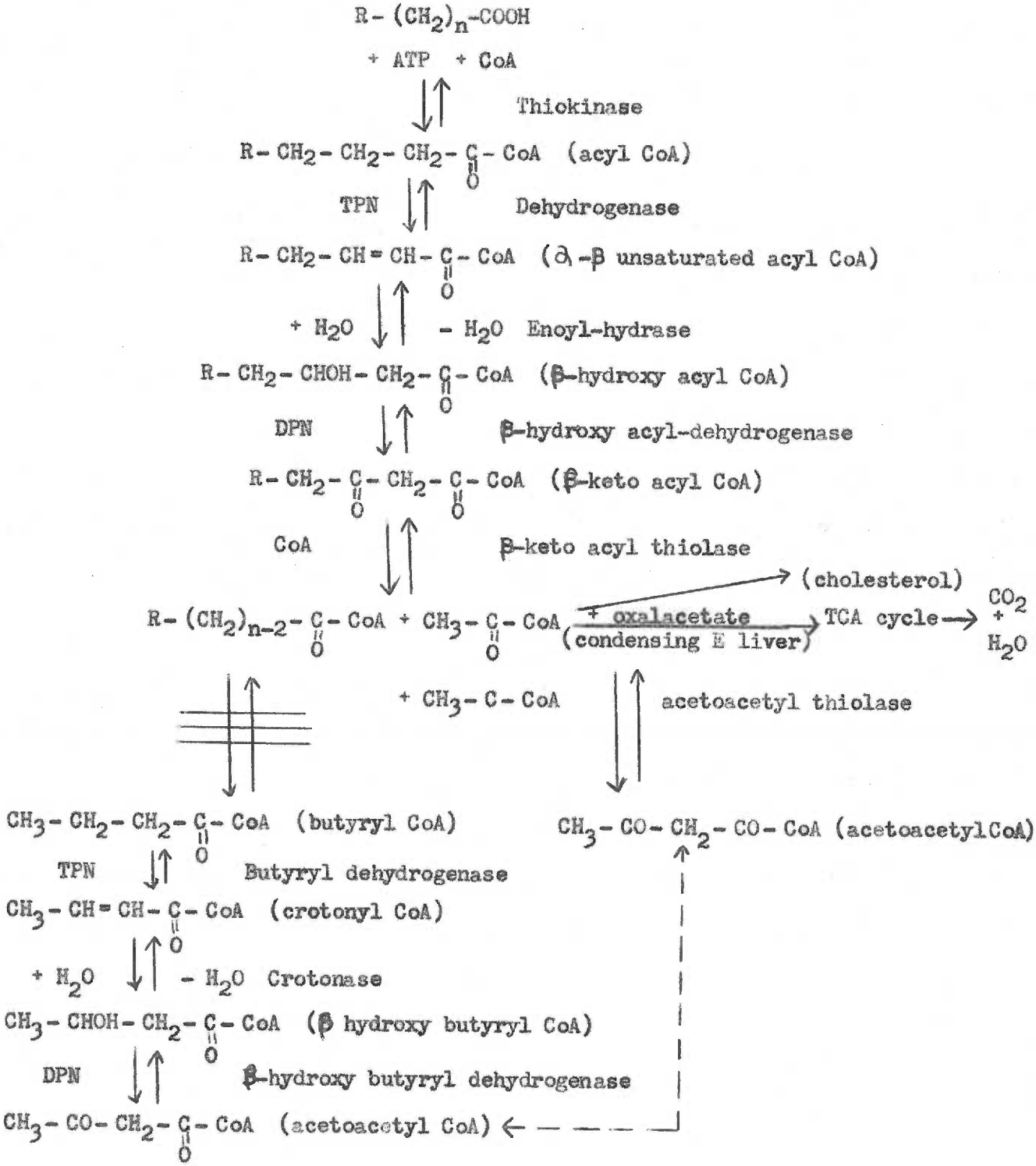
3) Completely absent in liver: transferase reaction (present in heart and kidney):

$$\text{acetoacetate + succinyl-CoA} \xrightarrow[\text{(transferase)}]{(22, 23)} \text{acetoacetyl-CoA + succinate}$$

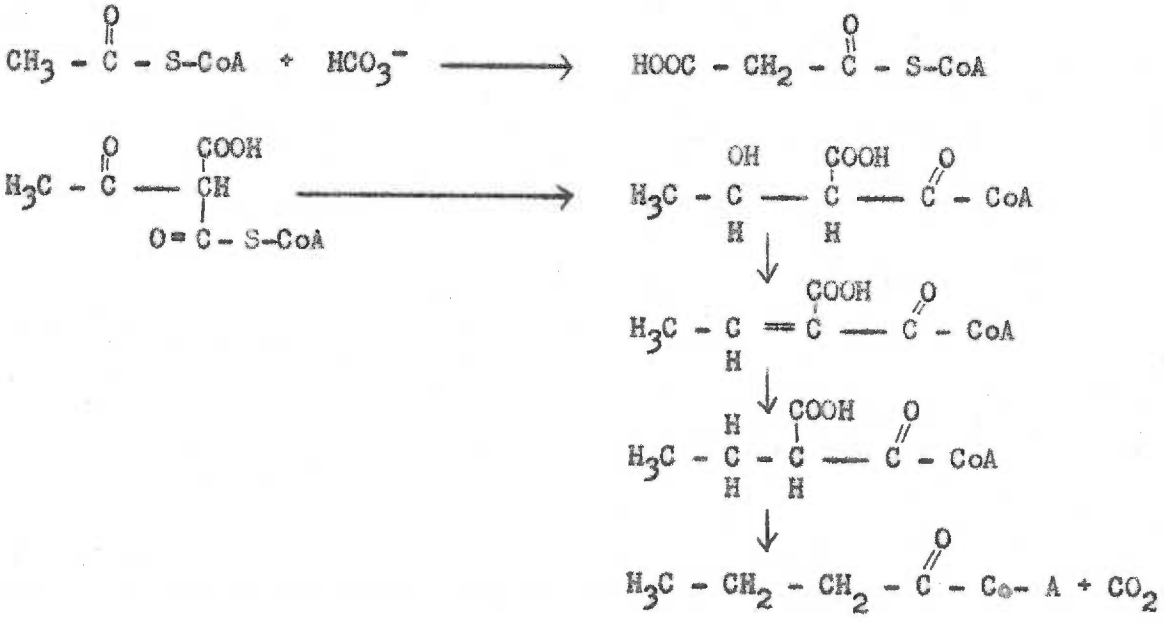
Mahler points out that reactions 2 (a and b) are generally the ones that account for the equilibrium between formation and breakdown of this ketone body. As one would consider the sequence of events in the formation of fatty acid from acetate, the Claissen-type condensation of two acetate molecules, mentioned before as a source of acetoacetyl-CoA, is mediated through a "condensing" enzyme studied by Lipmann. Consideration of this mechanism brought forth the question of the need for the activation of both of the acetate molecules involved. From calculations of free energy of hydrolysis of acetoacetate (16,000 calories) (24), Lipmann believed only one activation to be involved. Later work by Lynen, Ochoa and Green (21,25) showed that both acetyl groups are activated, although the energy of one (the terminal carboxyl-CoA link) is not used in the condensation proper. The importance of this work is in showing that the energy of the terminal-S-CoA link is retained in the acetoacetate molecule formed, thus providing an "activated" state. As pointed out by Lipmann (26) the activated acetoacetate can be hydrolyzed by liver deacylase.

The relationship of acetoacetate metabolism to fatty acid metabolism is presented in the following schemes:

1) Fatty acid breakdown and enzymes involved (27):



2) Fatty acid synthesis: Fatty acids are synthesized by reactions that are not the exact reverse of those of fatty acid breakdown. The outline for fatty acid synthesis shown in a preceding page has recently been modified to a newly suggested sequence presented below. In these recently reported observations, malonyl CoA plays an important semi-catalytic role. The new scheme was discovered by the observation that mitochondrial enzyme systems required bicarbonate for fatty acid formation. The final version of the malonyl scheme below is not completely established:



3) Cholesterol biosynthesis: Early in the use of isotopes as tracers, it was learned that small molecules such as acetate and ethanol could be incorporated into the cholesterol molecule by animal tissue. Although each of the several steps below involved a major piece of work for clarification, the reactions can be summarized as follows:

2 acetyl CoA $\rightleftharpoons$ acetoacetyl CoA + CoA

acetyl CoA + acetoacetyl CoA $\rightleftharpoons$ β-Me, β-OH, glutaryl CoA (HMG CoA)

HMG - CoA + 2 TPNH + $2H^+$ $\longrightarrow$ mevalonic acid + 2 TPN + CoA

mevalonic acid + ATP $\longrightarrow$ 5 phosphomevalonic acid + ADP

5 phosphomevalonic acid + ATP $\longrightarrow$ 5 pyrophosphorylmevalonic acid + ADP

5 pyrophosphorylmevalonate + ATP $\longrightarrow$ [5 pyrophosphoryl-3-phosphomevalonic]

phosphomevalonic acid $\longrightarrow$ isopentenyl pyrophosphate

isopentenyl pyrophosphate $\longrightarrow$ dimethallylpyrophosphate

isopentenyl pyrophosphate + dimethallyl pyrophosphate $\longrightarrow$ geranol pyrophosphate

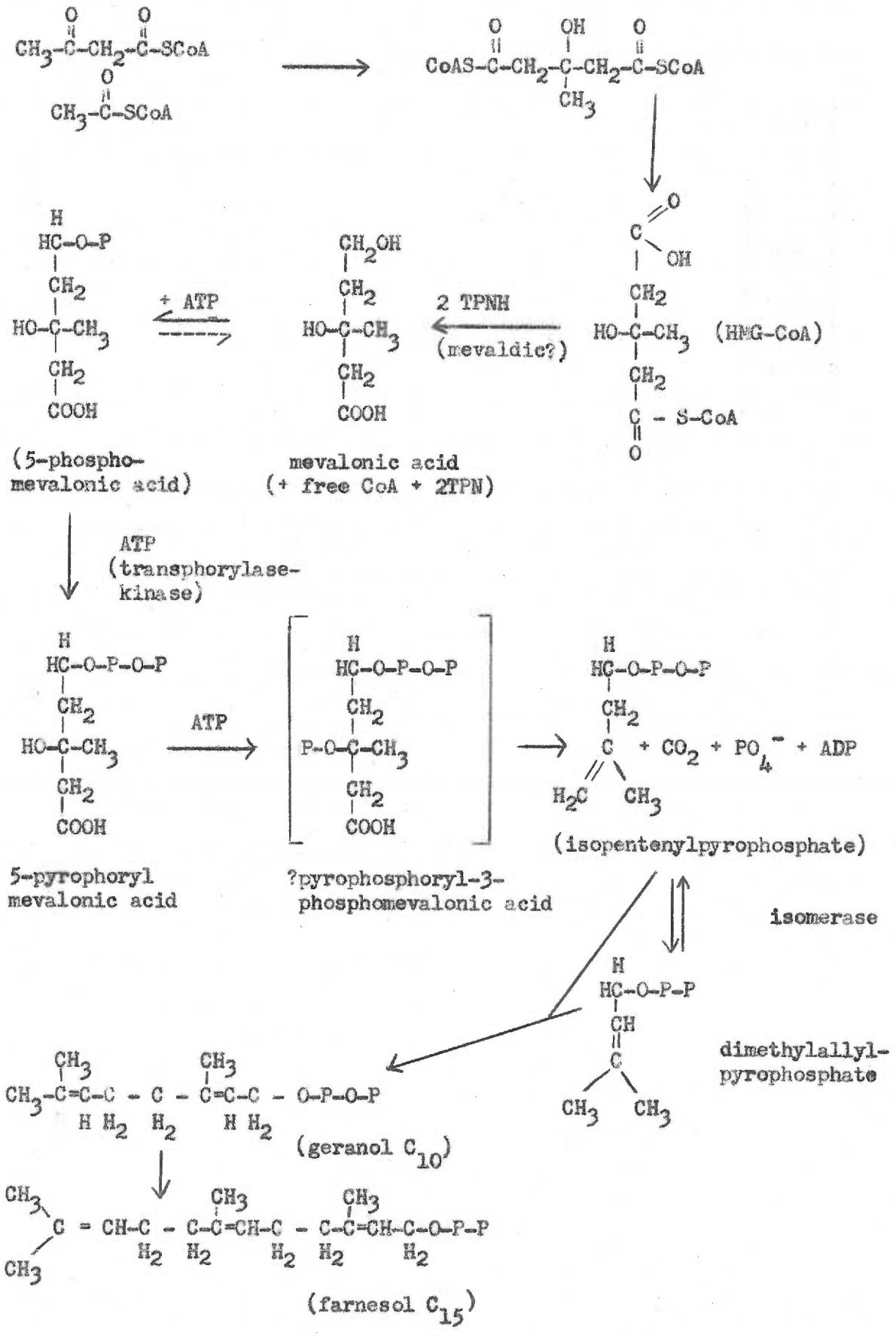
geranol + isopentenyl pyrophosphate $\longrightarrow$ farnesol pyrophosphate

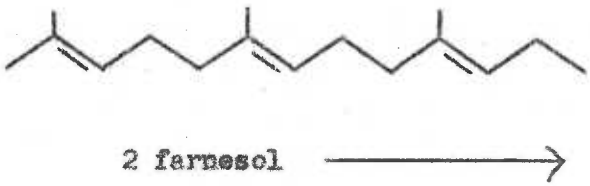
2 farnesol $\longrightarrow$ squalene

squalene $\longrightarrow$ lanosterol

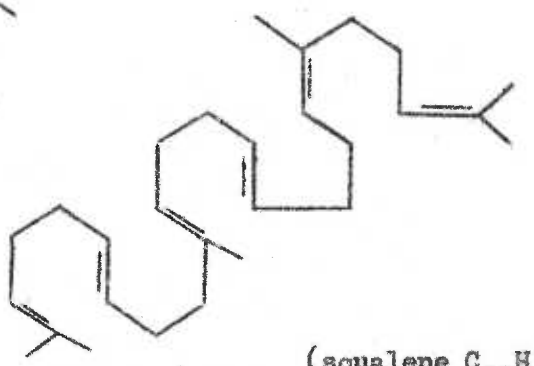
lanosterol-$3CH_3$ $\longrightarrow$ cholesterol

These reactions are shown in structural formulas below:

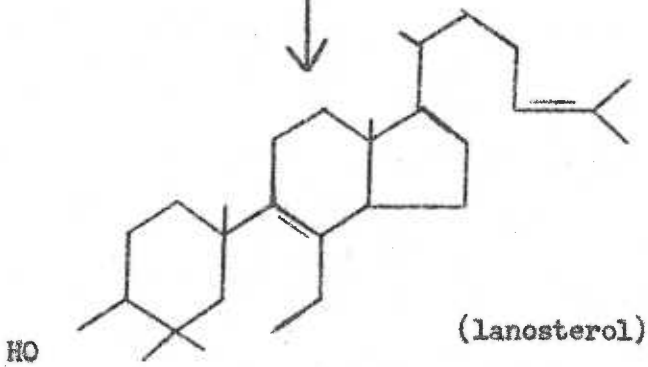




2 farnesol

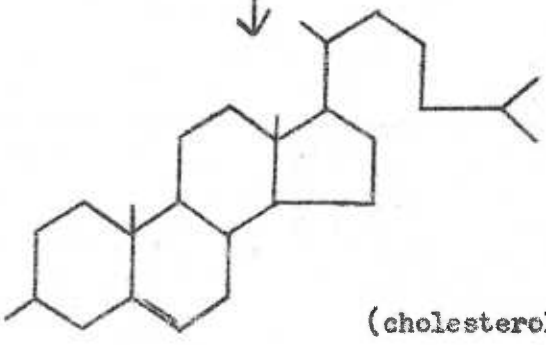


(squalene $C_{30}H_{50}$)



HO

(lanosterol)



HO

(cholesterol)

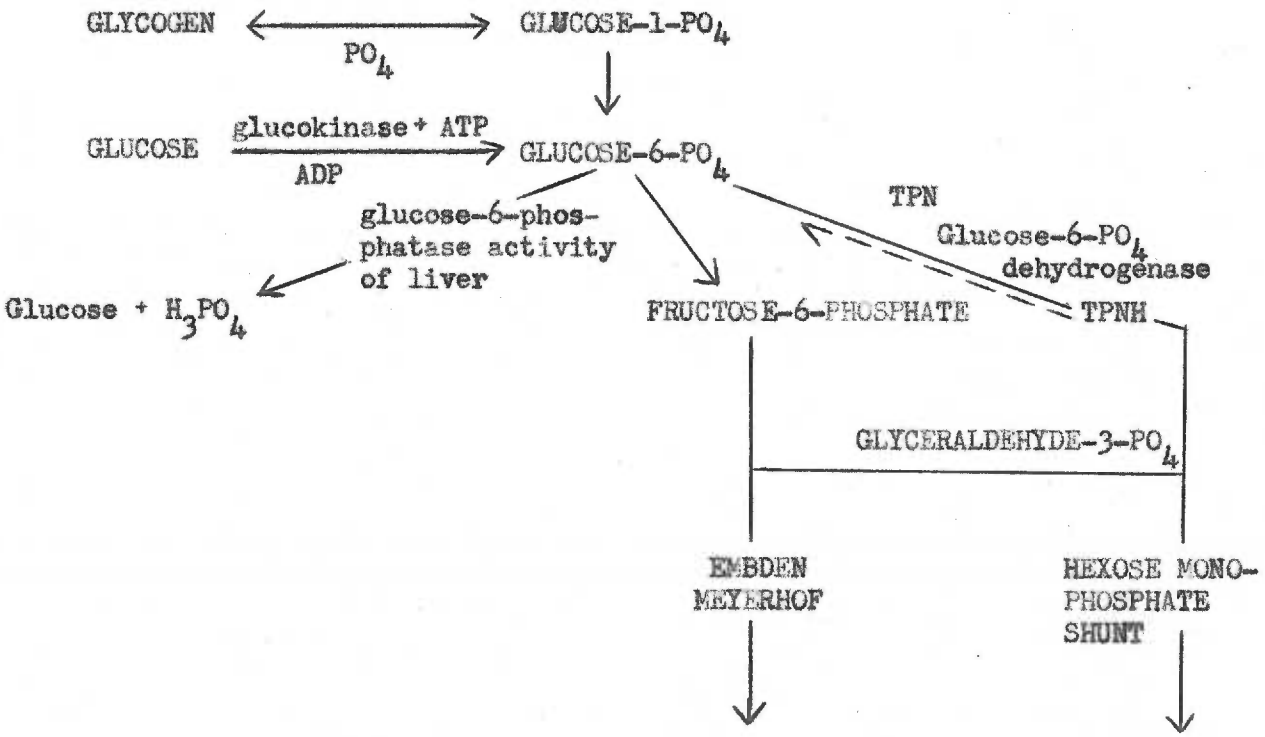
4) Alterations in lipid metabolism in diabetes mellitus: Below, there is presented a summary of some of the alterations involved in carbohydrate metabolism during diabetes. These are presented because they are, indirectly, the cause for the variations in lipid metabolism.

Besides the theory of the existence of decreased permeability to insulin by peripheral tissues in the diabetic state (28, 29, 30), metabolic blocks are also present. These are: 1) Decreased transphosphorylase reaction (glucokinase) for glucose in the liver; formation of glucose-6-PO_4 is thus blocked. The liver fructokinase reaction, from fructose to fructose-1-PO_4, however, can continue unimpaired. 2) Increased glucose production by gluconeogenesis and increased glycogenolysis because of increased glucose-6-phosphatase activity of the liver. These reactions emphasize the importance of glucose-6-phosphate, as it participates in the glycolytic pathway of carbohydrate utilization, as well as in the "hexose monophosphate shunt." Glucose-6-phosphate is an intermediate common to both pathways. Since it is decreased because of increased glucose-6-phosphatase activity, and since glucose-6-phosphate-dehydrogenase catalyzes the reduction of TPN^+, the equilibrium of TPN^+ to TPNH is disturbed and the concentration of the oxidized form of the nucleotide increases (31).

As has been shown in the section on fatty acid synthesis, the step of reducing the acetoacetate molecule to form its B-hydroxybutyryl-derivative is dependent upon reduced diphosphopyridine nucleotide. In later steps, i.e. in going from the crotonyl derivative to butyryl-CoA, there is also a specific requirement for TPNH (32, 33).

In the diabetic preparation there is decreased labeling of both fatty acids and cholesterol when C-14 acetate is added to the system, this may be accounted for by blocks at specific sites due to the decreased availability of the reduced nucleotides coming from the altered carbohydrate pathways.

Scheme of glucose metabolism for sites of alteration during diabetes mellitus



III. EXPERIMENTAL

The results to be presented in the following sections were obtained from in vitro experiments using acetate-1-C^{14} and acetoacetic acid-3-C^{14} as tracers and as substrates for liver, diaphragm and leg adductor muscle strips from control and alloxan diabetic rats. After incubation for a predetermined length of time, the reactions were stopped and the tissues analyzed for the quantity and the radioactivity of the cholesterol and fatty acid fractions. Oxygen consumption was determined during the incubation and CO_2 produced was analyzed for total radioactivity after the incubation. The main steps involved in a typical experiment are detailed in this chapter.

Animals: 200 gram, male Sprague Dawley strain rats obtained from the Northwest Rodent Co., Pullman, Washington, were used in all of the studies.

A. Normal animals. These rats were fed ad libitum (Purina chow) during the 3 or 4 days following arrival at the laboratory after which they were trained to feeding with 10 gm. of food twice a day (34). A rat was judged ready for use after at least 5 days of trained feeding and if it had attained a weight of 200 gm.

B. Alloxan diabetic animals. Rats weighing approximately 200 gm. were fasted 48 hours and then given an intramuscular injection of freshly prepared 10% alloxan solution (in 0.9% saline) in the amount of 0.06 ml. per 100 gm. of rat. At the same time 5 ml. of 0.9% saline was injected intraperitoneally. For the next 3 or 4 days the rats were given 0.9% saline solution in place of drinking water to aid in maintaining their electrolyte balance. After three weeks the animals were fasted 24 hours and a blood sugar determination was made according to the microtechnique

of Somogyi (35). Rats with blood sugar values over 180 mg.% were considered diabetic. Of a group of 13 rats, two died two days after the injection of alloxan and all the remaining animals had fasting blood sugar levels of over 180 mg. %.

C. Fasting period. All the results to be presented are of experiments using 3 hour fasted animals. A "3-hour fast" is defined for control and diabetic animals as follows:

1. For the normal trained fed animal: 10 gm. of chow were given to each animal 4 hours prior to sacrifice and the food was allowed to remain in the cage for 1 hour, during which time a well trained animal ate all or nearly all the food. At the end of this feeding hour, any remaining food was removed. If most of the food had not been eaten the animal was not used. The rat to be used was then "fasted" for 3 hours and killed by decapitation without anesthesia.

2. Three hour fasted diabetic rats: As has been mentioned before, these rats were not trained fed in order to eliminate the addition of the stress of hunger to their pathological condition. The animals were fed ad libitum throughout the days of their preparation until approximately 12 hours prior to the experiment, at which time all food was then removed from their cages. Four hours prior to the time of sacrifice they were allowed access to food for one hour. The subsequent fasting 3-hour period and sacrifice of the animal then proceeded as for the normal rats. Diabetic animals were not used unless they ate the larger part of the food during the one hour eating period.

Preparatory steps for the Warburg incubation technique (36).

Tissue incubation: Since it was thought of interest to compare some of the present studies on acetate metabolism by liver with previous work done in this laboratory, the time elapsed from sacrifice of the animal until beginning of incubation was considered important. This had been held to within 30 minutes in previous work; therefore, it was decided to keep the present experiments within this same limit. Because of the three tissues involved in these studies, an assistant was necessary to make it possible to prepare all tissues within the stated period.

A. Incubating media. Krebs-Ringer phosphate buffer pH 7.4, prepared according to the method of Umbreit, Burris, and Stauffer, was used (36). The solution was chilled in an ice bath and equilibrated for 10 minutes with 100% oxygen; 100 ml. of media per animal was generally prepared each time. If two or three animals were to be run in one day, an adequate additional amount of media for all experiments was prepared and stored in the refrigerator until needed.

B. Flasks. Six 15 ml. Warburg flasks were used per animal with tissue and substrate as follows:

Flask No. 1: liver plus acetate
" " 2: liver plus acetoacetate
" " 3: leg muscle strips plus acetate
" " 4: leg muscle strips plus acetoacetate
" " 5: diaphragm plus acetate
" " 6: diaphragm plus acetoacetate

C. Liquid volume in the flasks. Two ml. of Krebs-Ringer phosphate buffer solution were measured into the flask, and 1 ml. of the solution of tracer compound to be studied was measured into the side arm approximately thirty minutes before sacrifice of the rat, making a total liquid volume of 3 ml.

D. Labeled solutions.

1. Sodium acetate-1-C^{14}: In order to maintain the same ratio of tissue, volume of media, and labeled substrate as the amounts used previously in this laboratory (1 gm. of liver, 27 ml. total fluid volume, 160 ml. Warburg flask) (5, 37), the acetate solution was made up to contain approximately 1.26 µc/ml (0.072 µM/ml). When this amount of labeled acetate was diluted to the 3 ml. total volume in the flask, a concentration of 0.024 µM/ml (or 0.42 µc/ml) was obtained which is the same as in the larger system mentioned.

Stock solution of sodium acetate: Tube No. 301 of sodium acetate prepared in this laboratory (S.A. = 0.2087 millicuries per mg.) was made into a stock solution by the addition of 25 ml. water. This stock solution contained 22 µc/ml. or 1.287 µM/ml. For a single animal experiment, 0.3 ml. of this solution was diluted to 5 ml. with the oxygenated Krebs-Ringer buffer on the same day it was to be used. Every new dilution of tracer was assayed by a liquid counting method.

2. Acetoacetate solution: Ethyl acetoacetate-3-C^{14}, from New England Corp. of S. A. = 0.92 millicuries/millimole, was hydrolyzed with sodium hydroxide and neutralized with hydrochloric acid according to directions of Krebs and Eggleston (38): 0.01 ml. 0.5N NaOH was added to 0.05 ml. of the tracer. The reaction was allowed to stand at 40°C

for 1 hour and the mixture was then neutralized with 0.5N HCl to pH 7, using Nitrazine paper as the indicator.

Hydrolysis of the ester was always carried out on the same day of the experiment, and the desired dilution was made up with Krebs-Ringer phosphate buffer. 0.05 ml. of the stock solution of ethyl-acetoacetate (40.8 µc. or 44.3 µmoles per ml.) diluted to 5 ml. with Krebs-Ringer buffer was adequate for one animal experiment. Each new dilution was counted by the liquid counting method.

One ml. aliquots of the acetoacetate solution, containing approximately 0.41 µc or 0.44 µmoles, was measured into the side arms of the Warburg flasks. When diluted to the 3 ml. total fluid volume in the flask a concentration of 0.14 µc or 0.15 µmoles per ml. was attained. This concentration of acetoacetate gives the tissue a substrate concentration equivalent to approximately 1.5 mg. % acetoacetic acid.

mol.wt. acetoacetic acid =102 g

0.44 µm acetoacetic acid = 0.045 mg.

fluid volume in flask = 3 ml.

0.045 mg/3ml. = 1.5 mg/100 = 1.5 mg. %

E. Center well tubes. 22 x 5 mm tubes for the center well, previously prepared for each experiment, were stored CO_2 free, in a covered container. Pieces of filter paper approximately 2 x 1 cm., folded in small pleats, were placed in each tube and moistened with 0.2 ml. of approximately 9.5 N sodium hydroxide.

Experimental procedure.

The liver was excised immediately after sacrifice of the animal and was placed in a beaker containing about 25 ml. chilled (4° C) Krebs-Ringer

phosphate solution. The diaphragm was then removed by cutting around its edges as an assistant held the thorax in a distended position. The diaphragm was placed on a filter paper moistened with Krebs-Ringer solution, and its central part, free of fatty deposits and veins, was removed. The diaphragm was then placed in a beaker containing chilled Krebs-Ringer solution. One worker started slicing the liver with the Stadie-Riggs slicer while the other dissected out the adductor muscle strips. One leg was skinned by superficially cutting the skin of the distal end of the leg above the ankle with a small pair of scissors and then pulling the skin off the leg. An incision was made with a scalpel over the adductor muscle which was then dissected free of the muscles around it. Part of the adductor muscle was then held near the leg knee joint with fine forceps and strips separated from the intact muscle with the back part of the scalpel blade avoiding excessive damage to the tissue. The muscle strips obtained were approximately 15 mm. in length and less than 1 mm. in thickness which size is within the limits given by Field's data for optimum metabolic conditions (39). After cutting off and discarding the small piece of tissue crushed by the forceps, the strips were placed in another beaker containing 25 ml. Krebs-Ringer phosphate solution. The three beakers of tissues were kept chilled by surrounding them with crushed ice.

During the muscle preparations, the liver had been sliced and weighed on a torsion balance and an amount as close as possible to 111 mg. put into each of two Warburg flasks. The decision to use 111 mg. tissue was adopted in order to maintain the same ratio of reagents to tissue as the system previously used in this laboratory, the present amounts being one-ninth of the former amounts.

The diaphragm and leg muscle preparations, partially dried by blotting on a moist filter paper, were then weighed (approximately 111 mg) and transfered to flasks. All flasks were flushed with oxygen for one minute, and small tubes for the center well (CO_2 trap) were put in place.

In order to check against the possibility of contaminating the filter paper with acetate from the side arm by the stream of oxygen directed into the flask, duplicate flasks were run during an experiment with all reagents mentioned but with no tissue present. Some flasks were first flushed with oxygen and then the tube was placed in the center well while other were flushed with the tube already in place. In no case were any "counts" recovered from the filter paper in the center well, therefore, it was decided that it is not critical if the small tube is put in before or after flushing with oxygen. All the flasks were then placed in the bath at 37° C at one minute intervals. The shaking rate was at 140 strokes per minute and the flasks were allowed to equilibrate for one-half hour with the manometer stopcocks open.

After equilibration, the flasks were removed from the water bath, the contents of the side arm were tipped into the flasks and the flasks were replaced in the bath. After two minutes, manometers were set at 150 (zero reading) and the stopcocks closed. Incubation was continued for one hour and readings of the manometer were made at 30 and at 60 minutes. Immediately after the 60 minute reading, flasks were removed from the bath, the stopcock opened, the flask detached from the manometer, and 3 drops of approximately 12 N H_2SO_4 added to the substrate to stop the reaction and liberate the dissolved CO_2 from the liquid media. The flasks were replaced in the bath and shaken for 30 minutes. A blank

experiment was carried out to determine the optimum time for CO_2 absorption. Recoveries of $C^{14}O_2$ after 5 or 15 minutes of shaking following the addition of H_2SO_4 were poor as compared to the 97% recoveries when a 30 minute period was allowed. A comparison of these recoveries is made in Table I.

Table I

Recoveries of labeled CO_2 from the filter paper in the center well of 15 ml. Warburg flasks.

Time after addition of H_2SO_4	Counts added*	Counts recovered*	% Recovered
5 minutes	1852	917	49.5
5 "	3704	773	21
15 "	1852	1297	70
15 "	3704	2924	79
30 "	102	100	97.8
30 "	184	181	98.4
30 "	205	200	97.7
30 "	205	209	102.1

*These "counts" are expressed as: counts/minute minus background for an infinitely thick $BaCO_3$ plate counted under a GM end window tube at 1.94% efficiency.

A. Contents of the flask after incubation. After the 30 minute CO_2 absorption period, the manometers and flasks were removed from the

bath and the flasks detached. The center well tubes for CO_2 were lifted out with a pair of forceps and put into 40 ml. centrifuge tubes which were then closed with rubber caps. The grease on the joint of the flask was removed with Celluwipes and the contents of the flasks poured through a funnel fitted with a piece of very fine stainless steel mesh. The tissue held on the mesh was washed with distilled water and then dropped into a 150 x 18 mm. screw capped culture tube containing 5 ml. 11% alcoholic KOH and a boiling stone. The media from the flasks was generally discarded.

B. End products studied:

1. Determination of radioactivity of CO_2 collected in the center well.

a. The filter paper strip used to absorb the CO_2 was washed with water (5) and to the Na_2CO_3 now in solution, enough carrier Na_2CO_3 was added to permit plating as infinitely thick $BaCO_3$ samples (40). To check recoveries of CO_2 under the exact conditions of the present Warburg incubation technique, a known amount of radioactive Na_2CO_3* was used in the flask instead of tissue. Na_2CO_3 solution of known activity was measured into the flask, enough water to make 2.5 ml. was added, and 0.5 ml. 13.6N H_2SO_4 measured in the side arm. Flasks were allowed to equilibrate, the contents of the side arm were tipped in, and incubation carried out as described above. The results for this experiment are given in Table I. It is clear that a 30 minute CO_2 absorption period is adequate for the collection of the CO_2 in the center well. The experiments also demonstrate that the total, over-all recovery of CO_2 (and $C^{14}O_2$) from the substrate of a metabolic experiment is a practical and reliable procedure.

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*To make up this solution, $BaCO_3$ from Oak Ridge National Laboratories of isotopic ratio of 3.84% and S.A. = 0.0136 uc/mg was used. A measured amount of $BaCO_3$ was acidified in a closed system and the CO_2 liberated was trapped in a standard NaOH solution.

b. In the final experiments reported in this thesis, the filter paper with NaOH in the center well was omitted and 0.1 ml.of 9.5 N NaOH solution was measured directly into the small tube with a syringe and needle. The alkali was spread around the inside surface of the tube with the needle so as to increase the area for absorption. After the experiment was completed, the small tubes were taken out of the flasks and placed in an upright position in a beaker. As soon as possible (after the tissues were placed in alcoholic KOH) the contents of the small tubes were washed out with a tuberculin syringe and needle as follows: 0.20 ml. of water was added, the contents of the tube stirred with the needle and the solution drawn out completely and placed in a stainless steel cup together with a drop of aerosol solution. Another 0.20 ml. aliquot of water was measured into the tube, stirred, drawn out and added to the contents of the stainless steel cup. This sample was then counted as a liquid sample. Recoveries of CO_2 by this method gave the results shown in Table II.

Table II

% Recoveries of labeled CO_2 collected in NaOH solution in the center well of Warburg flasks.

Flask	Counts added at 4.63% efficiency as $BaCO_3$	Counts recovered: as liquid*	as $BaCO_3$*	% Recovery
2	1852	39.8	1870	101.0
3	1852	36.5	1715	93.0
4	3704	72.6	3410	92.1
5	3704	77.2	3627	98.0
6	3704	75.2	3534	95.4
7	3704	76.2	3580	96.7
8	1852	39.9	1875	101.2

* Counts recovered expressed as liquid samples have been converted to their equivalent infinitely thick $BaCO_3$ plates by the appropriate factors determined in this laboratory.

2. Quantitative determination of CO_2: Amounts of CO_2 were not determined in all of the experiments to be reported since the amount of CO_2 given off by approximately 100 mg. tissue are so small that it is not practical to carry out gravimetric determinations. To determine the amounts of CO_2 produced by the three tissues studied in both normal and diabetic animals, a modified procedure was adopted: three normal and three diabetic animals were used in an experiment similar to those described but using the 160 ml. Warburg flasks and approximately 1 gm. of pooled tissue. To obtain this amount of tissue, muscle strips from both legs of each animal were used, and the three diaphragms were incubated in the same flask. With these larger amounts of tissue, the CO_2 collected in the center well was enough so that it could be determined gravimetrically using carrier Na_2CO_3.

The efficiency of the $BaCO_3$ precipitating procedure, in the writers hands, was checked and the results are shown in Table III.

Table III

Efficiency of the $BaCO_3$ precipitating procedure as shown by the gravimetric determination of different amounts of a carrier solution of unlabeled Na_2CO_3

Flask	ml.carrier	mg $BaCO_3$ for 100% recovery	Actual % recovery
1	0.95	97	90
2	0.95	97	100
3	0.80	81.7	100.5
4	0.80	81.7	100.7
5	0.85	86.8	101.5
6	0.85	86.8	100.2

3. Lipid fraction: After incubation, the tissues from the Warburg flasks were put into screw capped culture tubes (150 x 18 mm) with 5 ml. 11% KOH and a boiling stone. The tissues were digested for 30 minutes in a boiling water bath with a glass marble covering the tubes; the glass marbles were removed for the last 5 or 10 minutes to permit reduction of the volume in the tube to approximately 1 ml. After cooling, 15 ml. of distilled water and 5 ml. of 95% ethanol were added to each tube.

a. Extraction of the non-saponifiable fraction: Approximately 14 ml. of distilled petroleum ether (B.P. 30- 60° C) were measured into each tube and the tubes placed in the Eberbach and Son, Co. shaker at a rate of 280 strokes per minute for 3 minutes. After shaking, the tubes were placed in an upright position for a few minutes to allow complete separation of the two solvent phases. Approximately half of the petroleum ether layer was then drawn out with a 10 ml. Multifit Becton Dickinson syringe fitted with a 19 gauge, 3 inch long blunt ended spinal puncture needle. This portion of the petroleum ether layer was placed in a 10 ml. volumetric flask along with a boiling stone and evaporated on a steam bath without allowing it to go to dryness. The remainder of the petroleum ether layer was drawn out, added to the same volumetric flask and evaporated. Two additional portions of petroleum ether of 7 ml. each were added to the tubes containing tissue digest, and the extraction and evaporations carried out in like manner. The last addition of petroleum ether was evaporated down to about 1 ml. and 3 to 4 ml. alcohol then added to the 10 ml. volumetric flask. Heating over the steam bath was continued until no petroleum ether could be detected by its characteristic form of boiling and by the odor of the vapor phase. After cooling, the

flasks were made up to volume with alcohol and after mixing, the contents were transferred to small dark bottles and stored until analyzed.

b. Extraction of saponifiable fraction: After removal of the non-saponifiable fraction, the tubes were placed in a beaker with ice and made acid to congo-red paper with concentrated HCl. An extraction of this fraction with 3 portions of petroleum ether was then carried out in the same way as for the non-saponifiable fraction.

c. Colorimetric determination of the non-saponifiable fraction: The non-saponifiable fraction will be referred to as cholesterol since it is the principal component. The colorimetric determination of cholesterol was carried out according to the method of Zlatkis and Zak (41). Aliquots of the tissue alcoholic extract (non-saponifiable fraction) containing approximately 0.03 mg. cholesterol were measured into colorimetric tubes. At the same time, tubes containing 0.05 and 0.1 mg. of cholesterol were prepared from alcohol solutions of standard.

The tubes were dried in a 70° C water bath with a stream of nitrogen. After cooling, 0.1 ml. distilled water were added to each and mixed thoroughly, followed by 3 ml. glacial acetic acid. At this time, a blank tube was set up. Two ml. of the color reagent were then carefully added to all tubes. The color reagent consisted of a dilution of 2 ml. of a 10% (in glacial acetic) ferric chloride solution, to 200 ml. with CP concentrated H_2SO_4. The contents of the tubes were well mixed and allowed to cool. A brown color first appears which then changes to purple, remaining stable for a long time. Absorbancy is measured at 560 mu against the reagent blank.

d. Radioactivity of the non-saponifiable fraction: The alcohol solutions of the various fractions were assayed as infinitely thin

samples under a D47 Micromil end window gas flow counter at 29.63% efficiency. 0.3 mg. of material was used as criteria for the upper limit of an infinitely thin sample (5).

e. Gravimetric determination of the saponifiable fraction: The saponifiable fraction will be referred to as fatty acids. Five ml. aliquots of the alcohol solution were evaporated in tared glass vials in a 70° C water bath under a stream of nitrogen. When completely dry, the samples were cooled, left overnight in a dessicator and weighed. Although only some 2 mg. of material were usually weighed, and the glass vials weighed as much as 7 gm, the use of the gramatic balance method of substitutionary weighing allowed good accuracy of measurement.

f. Radioactivity of the saponifiable fraction: These determinations were carried out the same as for the non-saponifiable fraction, and samples counted with the same equipment as infinitely thin samples, but at 27.78% efficiency.

g. Recoveries by this procedure:

(1) Blank extractions without tissue but with an equivalent amount of acetate to that used in the experiments showed no extraction of acetate by this method.

(2) Digestions and extraction were also carried out adding a known amount of both labeled cholesterol and palmitic acid to some fresh tissue in a tube, following the procedure described before, with the recoveries shown in Table IV.

Table IV

% Recoveries of radioactivity in saponifiable and non-saponifiable fractions.

	% recovery of fatty acid	% recovery of cholesterol
1. Cholesterol and palmitic acid added together	99	103.4
2. " " " " " "	94	101.6
3. " " " " " "	88	99.8
4. " " " " " "	93	101.1
5. Only palmitic acid added	90	--
6. Cholesterol and palmitic added to KOH sol. with no tissue present	82	100

4. Liquid counting technique: Stainless steel cups from the Nuclear-Chicago Co., one inch in diameter and 5/16 of an inch high were used. A drop of 1% aerosol solution was placed in the cup to aid in wetting the surface,and 0.5 ml. of the solution to be assayed measured into the cup. To prevent contamination of the G-M tube,all plates were covered with 33 mm. diameter disks of Mylar film (Dupont 0.25 mil Mylar) fastened to the plate with a No.8 rubber band. Placing the rubber band around the plate was facilitated by wrapping it twice around a rubber stopper of a slightly larger diameter at its tapered end to that of the plate. The disk of Mylar film was placed on the stopper by pressing the stopper down firmly on the plate and at the same time rolling the rubber band down, off the stopper, and around the plate. The Mylar film could thus be easily extended over the plate, without creases, being firmly held by

the rubber band. The cups were counted in a holder, always within 30 minutes of setting up the sample in order to prevent interference from the condensation of small drops on the underside of the Mylar film. For this counting, a Tracerlab TGC-2, 1.8 mg/cm^2, GM end window tube was used at 0.1% efficiency. This method offered a rapid and accurate way of assaying liquid material even if efficiency was reduced 22 times as compared to counting infinitely thick $BaCO_3$ samples with the same equipment.

5. Determination of counting efficiency for infinitely thick $BaCO_3$ plates: A standard solution of Na_2CO_3 of known activity (Tracerlab, Boston, Massachusetts) was used. This standard consisted of a 0.047 molar solution of labeled Na_2CO_3; its activity given as calibrated against the National Bureau of Standards C^{14} standard, was 1067 $\pm$ 22 disintegrations/second/ml. Since this 0.047 molar solution of Na_2CO_3 only had 4.98 mg. of Na_2CO_3 per ml. which would precipitate only as 9.28 mg. of $BaCO_3$, a carrier solution of Na_2CO_3 was added in order to obtain infinitely thick $BaCO_3$ plates. A set of eight tubes with different amounts of the standard solution were plated as indicated (40). Results are shown in Tables V and VI.

Table V

Gravimetric recovery of standard solution of labeled Na_2CO_3 plus carrier Na_2CO_3 solution.

Tube	mg carrier	Standard Na_2CO_3 ml.	mg.	Net $BaCO_3$ recovered	Theoretical recovery	Found % recovery
1	98.98	0.25	2.319	103	101.3	101.7
2	"	0.25	2.319	103	101.3	101.7
3	"	0.5	4.638	105.8	103.62	102.1
4	"	0.5	4.638	105.9	103.62	102.2
5	"	0.75	6.957	108.2	105.94	102.1
6	"	0.75	6.957	106.8	105.94	100.8
7	"	1.0	9.276	110.8	108.26	102.4
8	"	1.0	9.276	111.3	108.26	102.8

Table VI

% Efficiency as calculated from counting standard infinitely thick $BaCO_3$ plates on a D47 Micromil end window gas flow counter.

Sample	% Efficiency*
1	4.82
2	5.26
3	4.29
4	4.65
5	4.52
6	4.50
7	4.55
8	4.39

*% Efficiency was calculated as the ratio between counts recorded by this system and actual disintegrations in the sample, multiplied by 100. An average of 4.63 $\pm$ 0.3 was obtained.

6. Determination of infinite thickness corrections for $BaCO_3$ plates: As has been previously mentioned, all CO_2 samples in these studies were plated as infinitely thick $BaCO_3$ plates and counted under an end window GM tube.

The plating area for the disks used measured 5 cm^2, and an infinitely thick plate of $BaCO_3$ weighed 100 mg, i.e. 20 mg. per square cm. This figure that determines the lower limit for infinite thickness can be determined as follows: It is possible to plate and count a very thin coat of $BaCO_3$ and then to increase the number of layers of material with the same $BaCO_3$ (same specific activity). If we then plot the results it will be seen that the counts increased but not in a linear manner. The increase tends to fall off and form a curved line. This phenomenon is due to self-absorption in which beta radiations are being lost by not being able to get through the upper $BaCO_3$ layers. Eventually a point is reached above which no increase of counts would occur as the weight of $BaCO_3$ is increased. From this point on, we would obtain a line essentially parallel to the horizontal axis.

Since in many instances our carrier Na_2CO_3 gave us plates under 100 mg. net weight of $BaCO_3$, we determined the factors that correct the plate's activity to what it would count if the plate were infinitely thick. To determine counting corrections, we plated increasing amounts of a Na_2CO_3 solution of known activity. The carrier and labeled Na_2CO_3 were calculated so that the specific activity was the same on all plates. To obtain the curve, we plotted counts against mg. per cm^2 but were not able to go below 2.5 mg/cm^2 in plate preparation. With the graph so made, the factors for each different weight can be determined, assuming the point of infinite thickness to be unity.

Theoretically if we were able to adequately plate much smaller amounts of $BaCO_3$, we would reach the inferior end of our curve which would be linear; this zone is the region of infinitely thin samples, and it is the place where no self-absorption occurs. In this case, we would obtain a linear response while the increases in weight were within the limits of infinitely thin samples. This is the case with our samples of cholesterol and fatty acids as has been mentioned previously.

IV. RESULTS AND DISCUSSION

A definition of unit used to evaluate tracer results is necessary. When labeled molecules are used as tracers, it is expected that these molecules will mix homogeneously with naturally occurring molecules of the same kind in the biological system and will not influence the rate of or the nature of the reaction.

To determine the amount of acetate-1-C^{14} that would be a tracer level, we have used information obtained from previous determinations in this laboratory.

In the case of acetoacetic acid an experiment on a concentration study was designed to determine the tracer levels.

Results are reported as per cent incorporation. Per cent incorporation was calculated by dividing the total counts* found in CO_2, fatty acid or cholesterol fractions of 111 mg. tissue, by the number of counts added,

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*All radioassays were converted to the equivalent activity of infinitely thick $BaCO_3$ plates counted at 4.63% efficiency.

and multiplying by 100. Expressing results as per cent incorporation has two main advantages. It enables the comparison of different substrates having different specific activities, and it permits comparison with in vitro determinations done previously in this laboratory, i.e. those experiments with 1 gm. of liver tissue. In the following presentation, the significance of apparent differences between groups has been evaluated statistically using the "t" test at a 0.95 confidence level. In general, no significance is attached to differences unless the probability value (p) is less than 0.05.

Oxygen consumptions are given as umoles of oxygen used in 1 hour by 1 gm. of tissue (wet weight) and amounts of fatty acid and cholesterol are reported as mg. per gram of tissue (wet weight).

Oxygen consumption: The oxygen consumption of the tissue was determined to obtain an idea of the viability of the tissue as the experiment proceeded. It also provided a way of determining that the concentration of acetoacetic acid being used did not alter the respiration rate of the tissue. The data for the oxygen consumption of normal animals is presented in Table VII. The mean for liver, 58.3 μM/hr./gm. of tissue agrees with the value of 57 μM given by Robert Emerson (5) for slices from two hour fasted rats.

Table VII

Oxygen consumption of normal animals

μM/hour/gram of tissue

	Liver	Leg Muscle	Diaphragm
J27	59.3	21.15	30.4
JJ28	61.1	29.9	46.1
JJ29J	59.6	26.4	37.36
JJ30J	56.1	26.24	48.35
JJ31J	50.6	30.3	47.81
JJ32J	63.5	24.2	---
Mean	58.3	26.4	42.0
S.D.	± 5.45	± 3.13	± 7.88
S.E.	± 2.23	± 1.28	± 3.53
Range	50.6-63.5	21.2-30.3	30.4-48.35

For diaphragm the mean value of 42 μM/hr./gm. (or 0.9408 μl./hr./mg.) can be compared to a mean of 1.05 μl/hr./mg. wet weight reported by Beatty, Peterson, Bocek and West (14). The leg muscle preparation shows a lower oxygen consumption as is to be expected from the lower activity of this muscle as compared to the constantly active diaphragm. The mean of 26.4 μM/hr./gm. (0.5914 μl./hr./mg.) is close to the value of 0.72 μl/hr./mg. that has been reported (14).

The results on oxygen consumption for the diabetic animals are given in Table VIII. These values are not statistically different from those of the normal preparations as is made clear by the data of Table IX.

Table VIII

Oxygen consumption of diabetic animals

μM/hour/gram of tissue

	Liver	Leg Muscle	Diaphragm
DJ36JJ	60.40	23.70	46.71
DJ37JJ	64.63	18.92	51.0
DJ38JJ	62.04	22.70	---
DJ39JJ	66.17	29.12	41.82
DJ40JJ	72.71	25.14	58.24
DJ45	40.40	24.08	49.17
Mean	61.1	24	49.4
S.D.	± 10.7	± 2.78	± 5.91
S.E.	± 4.37	± 1.14	± 2.64
Range	40.4 - 72.7	18.9 - 29.1	41.8 - 58.24

Table IX

Oxygen consumption of normal animals compared to diabetics

μM/hour/gram of tissue

	Liver	Leg Muscle	Diaphragm
	Normal		
Mean	58.3	26.4	42.0
S.D.	± 5.45	±3.13	± 7.88
S.E.	± 2.23	± 1.28	± 3.53
Range	50.6 - 63.5	21.2 - 30.3	30.4 - 48.35
	Diabetic		
Mean	61.1	24	49.4
S.D.	±10.7	± 2.78	± 5.91
S.E.	± 4.37	± 1.14	± 2.64
Range	40.4 - 72.7	18.9 - 29.1	41.8 - 58.24
p	> 0.1	> 0.1	> 0.1

Data on CO_2 production as obtained in the "pooling experiment" previously described is presented in Table X, but it is not possible to derive useful conclusions from this limited amount of data.

Table X

μM CO_2/hour/gram tissue

	Liver	Leg Muscle	Diaphragm
Normal (J46)	85	35.5	75.4
Diabetic (DJ45)	62.6	38.8	49.2

Amounts of lipid. The results for normal animals are presented in Table XI. Values for liver tissue agree with those previously reported in this laboratory. Values for leg muscle and diaphragm indicate that the lipid concentration of these tissues is lower than that of the liver. If, however, we consider that the muscle mass in a rat represents about 40 % of the body weight and the liver only 4 % of the body weight, muscle tissue appears to have an important fraction of the total body cholesterol and fatty acids. The higher content of cholesterol in the diaphragm as compared to leg muscle per unit weight bears out Bloor's theory (42) that a more active muscle has a higher cholesterol content.

Table XI

Lipid content of normal tissues

mg./gram wet weight of tissue

	Liver		Leg muscle		Diaphragm	
	Fatty acid	Chol.	Fatty acid	Chol.	Fatty acid	Chol.
J27	34.4	1.54	11.5	0.57	27.8	0.61
JJ28	--	2.02	--	0.53	20.2	0.78
JJ29J	--	1.85	24.2	0.43	24.8	1.11
JJ30J	37.8	2.16	16.3	0.48	29.3	0.73
JJ31J	31.4	1.31	18.6	0.42	17.0	0.47
JJ32J	29.9	2.29	24.9	0.49	32.7	0.72
JJ41	--	--	11.2	0.42	39.6	0.97
JJ43J	20	2.14	20.7	0.71	23.1	1.05
JJ44J	10.8	1.76	7.2	0.38	24.5	0.89
Mean	27.4	1.88	16.83	0.49	26.6	0.81
S.D.	±10	±0.36	± 6.43	±0.26	± 6.58	±0.23
S.E.	± 4.08	±0.127	± 2.27	±0.09	± 2.19	±0.076
Range	10.8-37.8	1.31-2.29	7.2-24.9	0.38-0.71	17-39.6	0.47-1.11

The results for the lipid analysis of diabetic animals are presented in Table XII. There is no statistical difference between the diabetic and the normal values (Table XIII) which agrees with previous findings reported from this laboratory (43). Values for cholesterol in leg muscle and in diaphragm agree well with the data of Bloor who found 0.64 and 0.91 mg. cholesterol per gram of tissue of leg and diaphragm muscle respectively.

Table XII

Lipid content of diabetic tissues

mg./gram wet weight of tissue

	Liver		Leg muscle		Diaphragm	
	Fatty acid	Chol.	Fatty acid	Chol.	Fatty acid	Chol.
DJ36JJ	38	2.26	—	0.43	—	0.604
DJ37JJ	—	1.90	20.1	0.59	53.2	0.94
DJ38JJ	35.9	1.98	34.7	0.43	—	1.34
DJ40JJ	27.3	1.82	18.1	0.39	15.4	0.74
DJ42	—	—	—	0.40	34.1	0.89
Mean	33.7	1.99	24.3	0.45	34	0.90
S.D.	± 5.96	±0.19	± 9.1	±0.08	± 19.5	± 0.254
S.E.	± 3.45	±0.096	± 5.24	±0.034	± 11.27	± 0.113
Range	27.3-38	1.82-2.26	18.1-34.7	0.39-0.59	15.4-34.1	0.74-1.34

Table XIII

Lipid content (mg./gm.) of normal animals as compared to diabetics

	Liver		Leg muscle		Diaphragm	
	Fatty acid	Chol.	Fatty acid	Chol.	Fatty acid	Chol.
Normal						
MEAN	27.4	1.88	16.83	0.49	26.6	0.81
S.D.	± 10	± 0.36	± 6.43	±0.26	± 6.58	± 0.23
S.E.	± 4.08	± 0.127	± 2.27	±0.09	± 2.19	± 0.76
Range	10.8-37.8	1.31-2.29	7.2-24.9	0.38-0.71	17-39.6	0.47-1.11
Diabetic						
MEAN	33.7	1.99	24.3	0.45	34	0.90
S.D.	± 5.96	± 0.19	± 9.1	±0.08	± 19.5	± 0.254
S.E.	± 3.45	± 0.096	± 5.24	±0.034	± 11.27	± 0.113
Range	27.3-38	1.82-2.26	18.1-34.7	0.39-0.59	15.4-34.1	0.74-1.34
p	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1

Acetate-1-C^{14} incorporation into liver fractions. The results for normal animals, Table XIV, show the greater part of the label to be found in the CO_2 fraction. This indicates that the acetate molecule is being activated by the proper enzymes in the tissue and is thus available to different metabolic sequences. The synthetic activity of the liver is shown by the incorporation of label into fatty acid and cholesterol.

Table XIV

Liver - Normal

Per cent incorporation of acetate

	CO_2	Fatty acid	Cholesterol
J27	14.46	0.34	1.67
J27	11.3	0.17	1.12
JJ-J29	8.24	0.52	1.72
JJ-J30	10.00	0.14	0.51
JJ-J43	17.39	0.12	1.37
JJ-J44	15.12	0.10	1.14
JJ31J	10.37	0.21	1.49
JJ32J	8.5	0.23	0.72
Mean	11.9	0.23	1.22
S.D.	± 3.44	± 0.14	± 0.41
S.E.	± 1.22	± 0.05	± 0.15
Range	8.5-17.39	0.10-0.52	0.51-1.72

As compared to the results for the diabetic animals (Table XV and XVI), we see a decreased labeling of fatty acid and cholesterol. It is

Table XV

Liver - Diabetic
Per cent incorporation of acetate

	CO_2	Fatty acid	Cholesterol
DJ36JJ	11.33	0.008	0.016
DJ37JJ	16.90	0.01	0.022
DJ38JJ	--	0.008	0.014
DJ40JJ	17.80	0.015	0.016
DJ45	16.0	--	--
DJ45	16.0	--	--
Mean	15.6	0.01	0.017
S.D.	± 2.55	±0.0043	±0.0035
S.E.	± 1.14	±0.0017	±0.0018
Range	11.33-17.8	0.008-0.015	0.014-0.022

Table XVI

Liver - Per cent incorporation of acetate
Normal animals as compared to diabetics

	CO_2	Fatty acid	Cholesterol
		Normal	
Mean	11.9	0.23	1.22
S.D.	± 3.44	± 0.14	± 0.41
S.E.	± 1.22	± 0.05	± 0.15
Range	8.5-17.39	0.10-0.52	0.51-1.72
		Diabetic	
Mean	15.6	0.01	0.017
S.D.	± 2.55	±0.0043	± 0.0035
S.E.	± 1.14	±0.0017	± 0.0018
Range	11.33-17.8	0.008-0.015	0.014-0.022
p	> 0.1	< 0.05	< 0.005

important to remember that it has been shown (Table XIII) that the quantities of cholesterol and fatty acid per unit weight of tissue are the same for the normal and the diabetic group. Impaired carbohydrate metabolism as in diabetes or in fasting decreases the supply of DPNH and TPNH necessary for the synthesis of fatty acid. This may account for at least a part of the defective lipid synthesis shown here.

Incorporation of acetate into muscle fractions. Table XVII contains the results of acetate incorporation for leg muscle and diaphragm from normal rats. The data points out the ability of muscle tissue to activate acetate and oxidize the greater portion of it through the TCA cycle. Lipogenesis is reduced in muscle as compared to liver tissue.

The muscle preparation shows an incorporation of acetate into CO_2 of approximately twice that of the liver slice. Although this is not a large difference when expressed as per unit of tissue weight, if one now considers the percentage of muscle in the total body weight of the rat as compared to the liver, the ability of muscle tissue to oxidize acetate becomes much more evident.

Comparison of the incorporation of acetate by the two muscle preparations. Even if, basically, both the adductor muscle strips and diaphragm are skeletal muscle, they have different degrees of activity and it is possible that they differ metabolically. In a muscle like diaphragm that is constantly active, Paul and Sperling have shown (44) that there exists a greater cyclophorase activity* with a parallel increase of mitochondrial density.

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*Cyclophorase activity refers to the functional aspect of mitochondria in containing the enzymes necessary for the reactions of the TCA cycle.

In comparing the results from the two muscle preparations, we find a statistically significant greater incorporation of acetate into the fatty acid fraction of diaphragm as compared to leg muscle. No significant difference in the per cent incorporation of acetate into the CO_2 and cholesterol fraction was demonstrated. Since both these fractions are larger in the diaphragm, this would tend to agree with the theory of a more active tricarboxylic acid cycle in the diaphragm.

A comparison of acetate incorporation into the muscle fractions of diabetics as compared to normals is presented in Tables XVII and XVIII. It is seen that the incorporation of acetate into CO_2, fatty acid and cholesterol is in each case similar for normal and diabetics, suggesting that lipid synthesis in muscle was not altered by the disease.

Studies with acetoacetic acid. To carry out experiments using labeled acetoacetic acid it was necessary to determine the tracer level for this substrate.

The possibility of dilution of added label by endogenous acetoacetic acid was also considered. Other studies in this laboratory (37) indicate that 1 gm. of liver puts out approximately 5 μmoles of acetoacetic acid in 1 hour, and approximately 6 μmoles of total ketone bodies and that the total amount of C_4 units retained within this tissue is small in relation to amounts put into the media. In later work, there was found a slightly decreased output of ketone bodies for diabetic animals. In the present studies, 0.44 μmoles acetoacetic acid have been added to approximately 100 mg. of tissue. The media surrounding the tissue would then have: 0.44 μmoles added to flask + 0.5 μmoles put out by 100 mg. tissue = 0.94 μmoles in 3 ml. liquid volume or 30 μmoles per cent (3 mg. per cent). In the case of the muscle

Table XVII

Normal - Per cent incorporation of acetate

	Leg Muscle			Diaphragm		
	CO_2	Fatty acid	Chol.	CO_2	Fatty acid	Chol.
J27	19.32	0.043	0.018	18.9	0.28	0.026
J27	22.58	0.14	0.026	20.6	0.42	0.101
JJ-J29	20.59	0.07	0.03	21.32	0.57	0.14
JJ-J30	25.86	0.13	0.02	27.05	1.42	0.03
JJ-J43	22.29	0.05	0.02	28.65	0.38	0.06
JJ-J44	27.14	0.05	0.02	32.00	0.69	0.04
JJ31J	29.76	0.09	0.03	34.7	1.24	0.04
JJ32J	27.5	0.09	0.04	27.62	0.35	0.02
Mean	24.38	0.08	0.026	26.36	0.67	0.057
S.D.	± 3.7	±0.044	±0.006	± 5.62	±0.43	±0.043
S.E.	± 1.31	±0.016	±0.0021	± 1.99	±0.15	±0.015
Range	19.32-29.76	0.043-0.14	0.018-0.04	18.9-34.7	0.28-1.42	0.02-0.14

Table XVIII

Diabetics - Per Cent incorporation of acetate and comparison to normals

	Leg Muscle			Diaphragm		
	CO_2	Fatty acid	Chol.	CO_2	Fatty acid	Chol.
DJ36JJ	28.47	0.021	0.008	28.93	0.09	0.016
DJ37JJ	24.48	0.013	0.065	24.65	0.117	0.013
DJ38JJ	28.13	0.018	0.004	25.3	0.127	0.015
DJ40JJ	36.00	0.04	0.007	28.87	0.120	0.013
DJ45	22.66	—	—	22.42	—	—
DJ45	22.66	—	—	22.42	—	—
Mean	27.07	0.023	0.021	25.43	0.1135	0.0143
S.D.	± 5.07	±0.012	±0.029	± 2.56	±0.016	±0.00164
S.E.	± 2.07	±0.0059	±0.0147	± 1.05	±0.008	±0.0008
Range	22.66-36.00	0.013-0.04	0.004-0.065	22.42-28.93	0.09-0.127	0.013-0.016
p	> 0.1	>0.1	> 0.1	> 0.1	< 0.05	> 0.1

preparations, Beatty et al. (14) reported that only negligible amounts were formed by these tissues from both normal and diabetic animals. Thus there is probably little error caused by dilution of the labeled substrate.

Concentration study. Leg muscle strips were incubated for 1 hour with different levels of acetoacetic acid. In this case, the micromoles of acetoacetic acid used in the production of CO_2 and in the synthesis of fatty acids and cholesterol were also calculated. Appropriate amounts of a non-labeled acetoacetic acid solution were added to attain the desired substrate levels in the flask. The results are presented in Table XIX.

Table XIX

Metabolism of acetoacetic acid by leg muscle

mg.% aceto-acetic	% incorporation			Amount of acetoacetate converted to product*					
				CO_2		Fatty acid		Cholesterol	
	CO_2	F.A.	Chol.	µM	mg.	µM	mg.	µM	mg.
2	22.16	0.14	0.024	13.14	1.34	0.083	0.0085	0.014	0.0015
2	19.6	0.11	0.034	11.62	1.19	0.063	0.0067	0.020	0.0021
4	13.73	0.14	0.037	16.28	1.66	0.166	0.0169	0.044	0.0045
4	17.9	0.03	0.011	21.23	2.17	0.036	0.0036	0.013	0.0013
24	4.58	0.06	0.018	32.59	3.33	0.427	0.0436	0.128	0.0131
44	4.6	0.22	0.014	60.01	5.06	2.87	0.242	0.183	0.0154

* x 10^{-2}

This experiment showed a mass effect in the response to the 24 and 44 mg. % doses in that a larger number of µmoles of substrate were converted to the products measured. The level of approximately 1.5 mg.% used in the subsequent experimental work plus the equivalent of 1.5 mg.% produced by the

liver gives a total of 3 mg. %, which is within limits of the true tracer level, amounts produced by muscle being considerably less than 1.5 mg.%(14).

An additional experiment gave values that agreed with the results of the 24 mg. % level. In this experiment, diaphragm and leg muscle strips from a normal rat and from a diabetic rat were incubated for two hours in a system with 22 mg. % of acetoacetic acid. The specific activity was unitized to the level of that used in the previous concentration study at the 24 mg.% level. Results are given as per cent incorporation per hour.

	Leg Muscle			Diaphragm		
	CO_2	Fatty acid	Cholesterol	CO_2	Fatty acid	Cholesterol
Normal	5.4	0.025	0.006	11.08	0.054	0.012
Diabetic	1.67	0.01	0.004	6.05	0.015	0.005

Utilization of acetoacetate by liver, leg adductor muscle strips and diaphragm. In the early literature concerned with the metabolism of acetoacetic acid, the liver was described as being responsible for production but not for the utilization of this material. After acetoacetate is formed in the liver it can then be added to the circulation. Weinhouse (45) suggested there might be a slight activation mechanism for acetoacetic acid in the liver. Tables XX and XXI present results for the per cent incorporation of acetoacetic acid into CO_2, fatty acid and cholesterol of liver slices from normal and diabetic rats. From these results, we observe some incorporation into all three fraction of both preparations. Activation of acetoacetate in liver by the succinyl CoA transferase mechanism seems unlikely since this enzyme is not considered to be present in liver. The mechanism for activation would seem to be through a specific β-keto acid activating enzyme present in small amounts in the liver.

Table XX

Liver - Normal-Per cent incorporation of acetoacetate

	CO_2	Fatty acid	Cholesterol
JJ28	1.06	0.17	0.34
JJ28	1.22	0.16	0.42
JJ-J29	1.7	0.12	0.73
JJ-J30	1.32	0.06	0.18
JJ-J43	1.88	0.05	0.37
JJ-J44	2.85	0.08	0.49
JJ31J	1.43	0.21	1.01
JJ32J	1.5	0.05	0.41
Mean	1.62	0.11	0.49
S.D.	± 0.56	± 0.07	±0.27
S.E.	± 0.2	± 0.024	± 0.095
Range	1.06-2.85	0.05-0.21	0.18-1.01

Table XXI

Liver - Diabetic- Per cent incorporation of acetoacetate

	CO_2	Fatty acid	Cholesterol
DJ36JJ	2.01	0.03	0.012
DJ37JJ	2.09	0.019	0.013
DJ38JJ	1.91	0.02	0.011
DJ40JJ	2.16	0.007	0.013
Mean	2.043	0.019	0.0123
S.D.	± 0.108	± 0.009	± 0.001
S.E.	± 0.054	± 0.0045	± 0.0005
Range	1.91-2.16	0.007-0.03	0.011-0.013

Incorporation of label into the fatty acids and cholesterol fractions is significantly decreased in the diabetic animals. Table XXII shows the comparison of the normal and diabetic groups.

Table XXII

Liver - Per cent incorporation of acetoacetate
Normals as compared to diabetics

	CO_2	Fatty acid	Cholesterol
	Normal		
Mean	1.62	0.11	0.49
S.D.	± 0.56	± 0.07	± 0.27
S.E.	± 0.2	± 0.024	± 0.095
Range	1.06-2.85	0.05-0.21	0.18-1.01
	Diabetic		
Mean	2.043	0.019	0.0123
S.D.	± 0.108	± 0.009	± 0.001
S.E.	± 0.054	± 0.0045	± 0.0005
Range	1.91-2.16	0.007-0.03	0.011-0.013
p	>0.1	<0.05	<0.0125

Comparing these results to those obtained on the incorporation of acetate into liver fractions (Table XIV) it is seen that less acetoacetate is utilized than acetate. A lower activation of acetoacetate by the liver cells may well be the cause of this decrease. The possibility of some of the acetoacetate being broken down to acetate and then being incorporated as such has been considered. It would seem that if this were the case,

there should be a proportional incorporation from acetoacetate into all three fractions of the diabetic group. Actually, there was a greater decrease in the conversion of labeled acetoacetate to $C^{14}O_2$ than there was in its conversion to lipid.

Utilization of ketone bodies by muscle tissue. Muscle tissue is known to be able to metabolize ketone bodies, obtaining energy by this mechanism. It was previously believed that this primary energy providing function of muscle, oxidation, excluded the possibility for synthesis of lipids by this tissue. It has now been shown that muscle can incorporate a labeled precursor into fatty acid and cholesterol although to a lesser degree than the incorporation into CO_2. Weinhouse (46) has also shown that there exists some degradation of fatty acids in muscle although this is also a "weak" reaction. Table XXIII presents the results for ketone body utilization by the leg muscle and diaphragm preparation from normal rats. Table XXIV shows the utilization of acetoacetic acid by the diabetic muscle preparations. The diabetic muscle preparations appear to metabolize acetoacetic acid to a lesser extent than the normal preparations as evidenced by the p values on Table XXIV.

Comparison of both normal muscle preparations shows a significantly greater incorporation from acetoacetate into the fatty acid fraction of diaphragm as compared to leg muscle. The CO_2 and cholesterol fractions show no significant differences. This same effect was apparent with acetate as substrate.

Comparison of Tables XVII on acetate to XXIII on acetoacetate show, as is to be expected, that the greater part of the label is to be found in the CO_2 fraction. There is also a significant greater incorporation of

Table XXIII

Normals - Per cent incorporation of acetoacetate

	Leg Muscle			Diaphragm		
	CO_2	Fatty acid	Chol.	CO_2	Fatty acid	Chol.
JJ28	14.48	0.1	0.03	16.9	0.27	0.04
JJ28	14.37	0.09	0.02	17.79	0.33	0.17
JJ-J29	14.5	0.05	0.05	15.2	0.47	0.03
JJ-J30	9.87	0.08	0.01	19.1	0.50	0.04
JJ-J43	9.35	0.06	0.03	16.27	0.14	0.03
JJ-J44	17.29	0.05	0.05	22.15	0.29	0.09
JJ31J	19.67	0.10	0.03	26.45	0.50	0.03
JJ32J	17.01	0.10	0.04	14.73	0.30	0.02
Mean	14.57	0.08	0.033	18.57	0.35	0.056
S.D.	± 3.55	±0.017	±0.0141	± 3.19	±0.129	±0.051
S.E.	± 1.25	±0.006	±0.005	± 1.13	±0.046	±0.018
Range	9.35-19.67	0.05-0.10	0.01-0.05	14.73-26.45	0.14-0.50	0.02-0.17

Table XXIV

Diabetics - Per cent incorporation of acetoacetate and comparison to normals

	Leg Muscle			Diaphragm		
	CO_2	Fatty acid	Chol.	CO_2	Fatty acid	Chol.
DJ36JJ	14.04	0.044	0.008	18.21	0.086	0.035
DJ37JJ	10.56	0.034	0.013	9.6	0.083	0.009
DJ38JJ	8.82	0.025	0.008	17.34	0.086	0.01
DJ40JJ	4.8	0.008	0.002	7.3	0.032	0.005
Mean	9.56	0.028	0.008	13.11	0.073	0.015
S.D.	± 3.84	±0.0153	±0.002	± 5.48	±0.0274	±0.0137
S.E.	± 1.92	±0.0077	±0.001	± 2.74	±0.0137	±0.0069
Range	4.8-14.04	0.008-0.044	0.002-0.013	7.3-18.21	0.032-0.086	0.005-0.035
p	≤ 0.05	> 0.05	$< .05$	> 0.05	$< .025$	> 0.1

acetate into CO_2 as compared to acetoacetate. Both substrates have to be activated to their respective CoA derivatives before they can enter any metabolic pathway. In the case of acetoacetate this is done by the succinyl-CoA transferase reaction yielding acetoacetyl CoA which may then break down to two acetyl CoA molecules. These steps before the formation of the acetyl group might account for the decrease in incorporation from acetoacetate.

V. CONCLUSIONS

Using an in vitro technique, liver tissue has been compared with two skeletal muscle preparations, strips from an adductor leg muscle and diaphragm, in respect to the utilization of acetate and acetoacetate.

Liver slices appear to metabolize acetate more easily than they do acetoacetate. Although a definite reason for this difference cannot be given, it may well be that the activation of acetoacetate is the rate limiting step. It is of interest that the difference in degree of metabolism was greater for the formation of CO_2 than it was for the synthesis of lipid.

Acetoacetic acid would seem to be activated to acetoacetyl-CoA by liver tissue, but this reaction is not thought to be quantitatively significant. As far as is known, such activation through CoA is required for any subsequent reaction. After activation the acetoacetyl-CoA can be thiolytically cleaved to two molecules of acetyl-CoA. It is only in this later form that acetoacetate carbon can enter the CO_2 forming reactions of the Krebs cycle. Both fatty acids and cholesterol can, however, be formed from the acetyl-CoA as well as from the acetoacetyl-CoA.

It is possibly because of this fact that there is found proportionally more C^{14} activity in the lipids than in the CO_2 as one compares the metabolism of the two compounds.

A comparison of liver and muscle tissue, with either acetate or acetoacetate as substrates, reveals that muscle has a greater oxidative capacity than does liver, but liver has the greater lipid synthesis capacity. This comparison is true on a unit tissue weight basis but is not true when calculated for the total organ weight. Certainly the liver tissue can produce lipid of a higher specific activity when presented with a labeled substrate, but the muscle tissue can be responsible for a considerable part of the total lipid formed in the body.

These present studies agree with previous studies from this laboratory in that a defect in hepatic lipid synthesis is shown to be present in the diabetic preparation. Use of acetoacetate as subetrate extends the previous studies and indicates that the diabetic liver slice preparation also has a defect in acetoacetate utilization. This finding is in agreement with the current concept regarding the importance of DPNH and TPNH as electron or hydrogen donors in lipid synthesis.

The muscle preparations from diabetic animals show essentially similar metabolism of acetate as compared to the control group. With acetoacetate there is a decreased response of the diabetic as compared to the normal animals. Diaphragm also shows a significant decrease in incorporation of labeled carbon from acetoacetate into its fatty acid fraction. It appears that diaphragm is more active in fatty acid synthesis than is leg muscle, thus supporting the theory that a more functionally active muscle is proportionally more metabolically active.

These results correspond with uptake studies for similar preparations previously reported by Beatty et al. (14).

Since no glucose was used in the incubating media, it can be assumed that the normal preparations are already at a low level of synthetic activity, and for this reason there might appear to be no apparent further reduction of activity in the diabetic tissues. This situation perhaps is nearer to true biological conditions than it appears to be since it has been postulated by Kipnis and Cori (47, 48) that "under steady state conditions (without specific treatment) neither the fed, the fasted, nor the diabetic animals contain free glucose inside the muscle cell."

VI. SUMMARY

1) An in vitro technique has been described for liver and skeletal muscle incubation. Tissues from control and diabetic rats have been used to compare the metabolism of labeled acetate and acetoacetate at tracer levels.

2) The liver appears to metabolize acetate more readily than it does acetoacetate. This might be due to a decreased activation of acetoacetate by liver cells as compared to their activation of acetate.

3) The liver shows considerable activity for lipid synthesis from acetate.

4) The muscle preparations show some degree of lipogenesis taking place, but also a very high oxidative metabolism. This response agrees with the anatomical and physiological function of muscle and its need for energy production.

5) In the diabetic state the liver shows decreased lipogenesis from both acetate and acetoacetate although this decrease is not proportional in the two substrates.

6) Under the experimental procedure followed in this work, the diabetic muscle preparations react with no significant difference as compared to the control group, when acetate is the substrate. Acetoacetate utilization appeats to be decreased in the diabetic state.

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