STUDIES IN DIABETES:
METABOLISM OF MUSCLE AND AORTA

by

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A THESIS

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INTRODUCTION

"The theory that there is an ultimate truth, although very generally held by mankind, does not seem useful to science except in the sense of a horizon toward which we may proceed, rather than a point which may be reached."

Gilbert Lewis

The Anatomy of Science, 1926. (46)

This thesis is concerned both with certain basic problems of lipid metabolism and also with the application of basic knowledge to the better understanding of two diseases, diabetes mellitus and athero-sclerosis. The field of lipid metabolism is in a phase of development in which new information is being supplied very rapidly so that comprehension and application of this field alone is a formidable undertaking. An extension of the basic findings on lipid metabolism to two poorly understood clinical conditions is done with the realization that only a small beginning can be made on two of the great problems that face medical science.

It is understandable that the amount of work done in studying the clinical aspect of a disease has been proportional to the importance of the disease to mankind. The condition of diabetes mellitus has been known for several centuries. Until 1788 when Cowley published a paper in the London Medical Journal relating the condition to the pancreas, the disease had only been described (12) with no attempt

at explaining its cause. However, at that time Cowley did not offer any experimental proof for his finding.

While work progressed on the relationship of the disease to specific tissues, some of the concomitant metabolic alterations of diabetes were also extensively reported on. Towards the middle of the 19th century the ketone bodies, acetone, acetoacetic acid and B-hydroxy butyric acid were found in the urine of diabetics and considered with great interest. The work of Magnus Levy and others as early as 1901 pointed to the fatty acids as the main precursors of the ketone bodies. Later it was shown that certain amino acids could also be converted to ketone bodies (11). In normal animals the ketone bodies were found to accumulate to only a slight extent, i.e. the normal human being has only small amounts in the circulation. Until the relatively recent experiments of Banting and Best, that permitted the isolation of insulin from the islets of Langerhans of the pancreas, the disease could only very inadequately be treated by special diets.

As with the early history of diabetes, there is evidence of atherosclerotic lesions in the arteries of Egyptian mummies, and lesions of this kind were discussed in early medical writings. It is obviously impractical and beyond the scope of this thesis to describe in any detail all the ramifications of the two diseases or to present an evaluation of the economic importance of them, however interesting these may be.

In the voluminous literature on factors which predispose to atherosclerosis we find a common, although as yet obscure, link between both atherosclerosis and diabetes. If only one looks deeply into the problem of diabetes and attempts to localize the disease at

the molecular level, or to "sites" in intermediary metabolism, it will be seen that of the many steps that show alterations, several occur in the field of lipid metabolism. If the same detailed search is carried out in the case of atherosclerosis, some of the alterations found are also seen to be related to problems of lipid metabolism. Yet another common link appears fairly certain: among the many factors conditioning for the development of atherosclerosis (high blood pressure, diet, points of arterial branching, etc) diabetes also appears as an important predisposing state.

The approach to the study of these diseases, through the use of experimental animals has been made difficult because of certain problems inherent in the use of animals. Species of animals vary in response from one to the other, and within one species also, tissues may differ from one another in composition and in metabolic activity. The liver is considered to be the primary organ for synthesis of structural and metabolic products and also for the catabolism of many substances. Muscles are considered to be concerned mainly with energy production. The circulation of blood, so brilliantly described by Andrea Cesalpino and later by Harvey, at the time when so few aids for the study of physiology were yet known, until recently was considered only as an elaborate and amazing distributing system. The role played by the walls of the arteries as metabolizing systems, themselves able to synthesize compounds and, inversely, to catabolize others, has been minimized until current years. There is literature from only one decade ago (33) that cites the inability of the vessel wall to synthesize cholesterol. Since then, this statement has been disproved by various workers. The experimental work to follow will

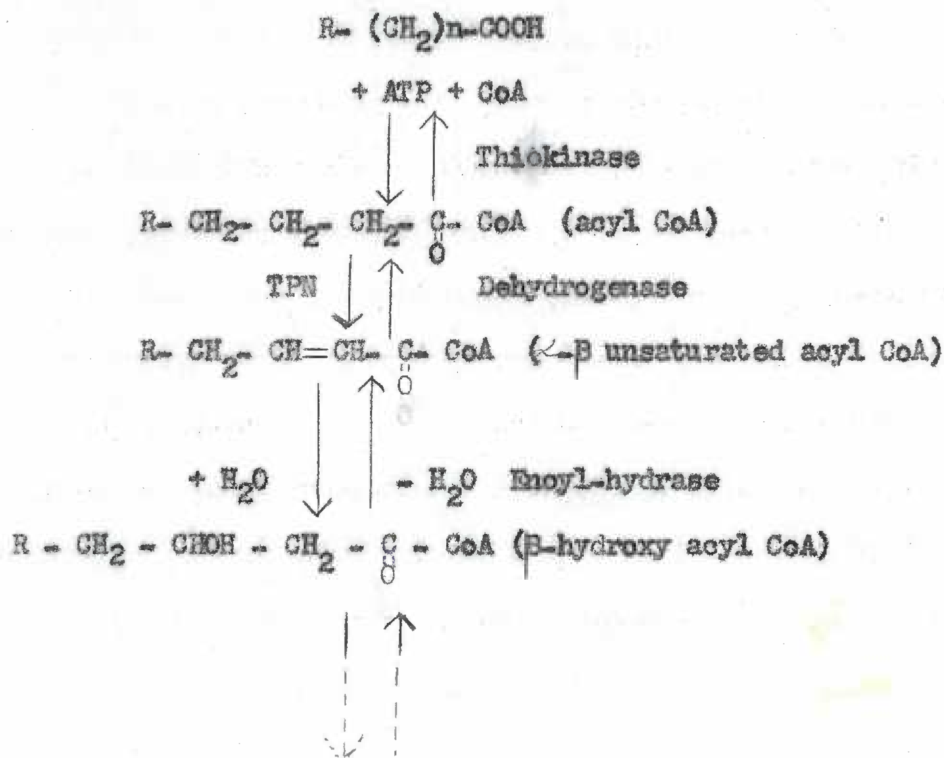
illustrate the metabolic role of the aorta.

As was said before, the clinical aspects of disease have long been studied, but the metabolic errors present have been neglected until these last years. In recent years, due largely to the use of radioactive labeled compounds and advanced instrumentation, more attention has been directed to this field. In general then, this metabolic area will be the one to be considered in the experiments described in this thesis.

The synthesis and oxidation of cholesterol and fatty acids have been well covered in review articles and in books by leading investigators in the field. (10,68) We, therefore, present here only an outline sequence of the reactions that are believed to occur. This is done to enable the reader to localize within an overwhelmingly large and complex scheme, the particular sequence under discussion.

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

<u>Fatty acid breakdown and enzymes involved:</u>



DPN β-hydroxy acyl-dehydrogenase

$R - CH_2 - \underset{\|O}{C} - CH_2 - \underset{\|O}{C} - CoA$ (β-keto acyl CoA)

CoA β-keto acyl thiolase

$R - (CH_2)n-2 - CO-CoA + CH_3 - \underset{\|O}{C} - CoA$

$CH_3 - CH_2 - CH_2 - \underset{\|O}{C} - CoA$ (butyryl CoA)

TPN Butyryl dehydrogenase

$CH_3 - CH = CH - \underset{\|O}{C} - CoA$ (crotonyl CoA)

$+ H_2O$ $- H_2O$ Crotonase

$CH_3 - CHOH - CH_2 - \underset{\|O}{C} - CoA$ (β hydroxy butyryl CoA)

OPN β-hydroxy butyryl dehydrogenase

$CH_3 - CO - CH_2 - \underset{\|O}{C} - CoA$ (acetoacetyl CoA)

The acetyl CoA formed may undergo the following reactions.

$$2\ CH_3-\overset{O}{C}-CoA \longrightarrow \text{acetoacetyl CoA} + CoASH$$

and

$$\text{acetyl CoA} + \text{Acetoacetyl CoA} \longrightarrow \text{sterol}$$

and

$$\text{acetyl CoA} + \text{oxalacetate} \xrightarrow[\text{cycle}]{\text{Krebs}} CO_2 + H_2O$$

<u>Fatty acid Biosynthesis</u>: 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:

$$CH_3 - \overset{O}{\overset{\|}{C}} - S\text{-}CoA + HCO_3^- \longrightarrow HOOC - CH_2 - \overset{O}{\overset{\|}{C}} - S\text{-}CoA \quad \text{Malonyl CoA}$$

$$CoA\text{-}S\text{-}\overset{O}{\overset{\|}{C}} - \overset{COOH}{CH_2} + O = \underset{CH_3}{C} - S\text{-}CoA \longrightarrow H_3C - \underset{H}{\overset{OH}{C}} - \underset{H}{\overset{COOH}{C}} - \overset{O}{\overset{\|}{C}} - CoA \quad \alpha\text{ Carboxy } \beta \text{ Hydroxy Butyryl CoA}$$

$$H_3C - \underset{H}{C} = \overset{COOH}{C} - \overset{O}{\overset{\|}{C}} - CoA \quad \text{Unsaturated } \alpha \text{ Carboxy Compound}$$

$$H_3C - \underset{H}{\overset{H}{C}} - \underset{H}{\overset{COOH}{C}} - \overset{O}{\overset{\|}{C}} - CoA \quad \alpha\text{ Carboxy Butyryl CoA}$$

$$H_3C - CH_2 - CH_2 - \overset{O}{\overset{\|}{C}} - Co - A + CO_2$$

Butyryl CoA

It may well be the initial product of condensation of malonate and acetate is not the α carboxy compound shown but is in reality the decarboxylated form.

<u>Cholesterol biosynthesis</u>: 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:

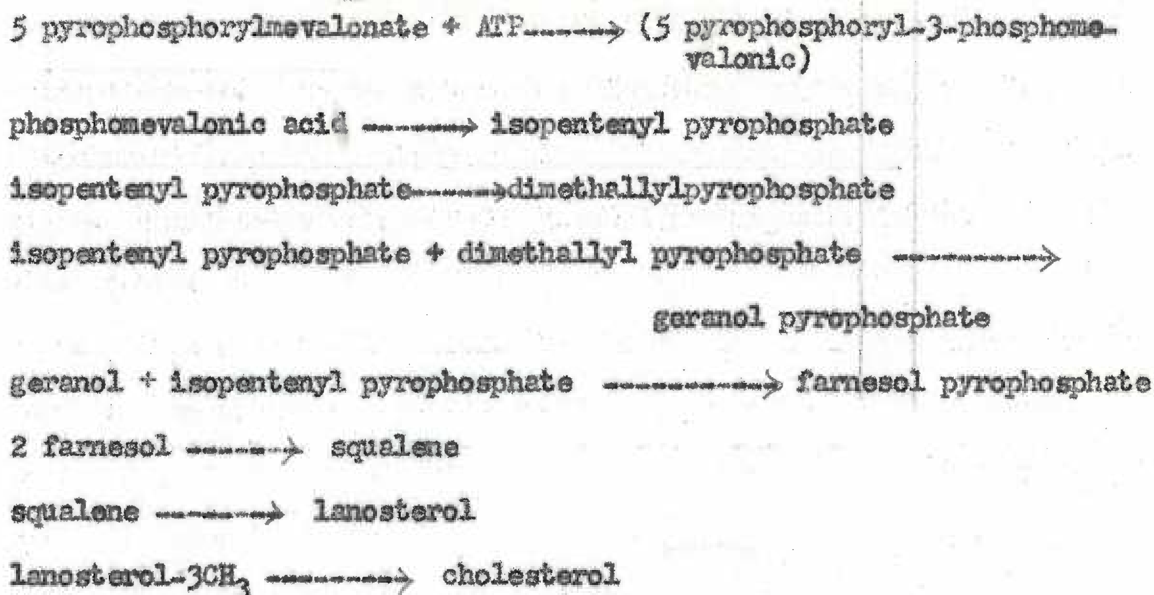
2 acetyl CoA $\rightleftharpoons$ acetoacetyl CoA + CoA

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

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

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

5 phosphomevalonic acid + ATP $\rightleftharpoons$ 5 pyrophosphorylmevalonic-acid + ADP



5 pyrophosphorylmevalonate + ATP ——→ (5 pyrophosphoryl-3-phosphomevalonic)

phosphomevalonic acid ——→ isopentenyl pyrophosphate

isopentenyl pyrophosphate ——→ dimethallylpyrophosphate

isopentenyl pyrophosphate + dimethallyl pyrophosphate ——→ geranol pyrophosphate

geranol + isopentenyl pyrophosphate ——→ farnesol pyrophosphate

2 farnesol ——→ squalene

squalene ——→ lanosterol

lanosterol-$3CH_3$ ——→ cholesterol

Tissues Used

In the previous studies done by this writer (52), liver slices, adductor muscle strips, and diaphragm muscle were used. These studies then compared two kinds of muscle; a complex, continually active structure, the diaphragm; and a more homogenous preparation, muscle strips from the adductor muscle, which are functionally relatively inactive in confined laboratory animals. It was considered of interest to include heart muscle slices with the tissues being studied because of several unique properties of heart muscle. Cardiac muscle is an involuntary muscle although it is also striated, but, unlike skeletal muscle, it has central nuclei and the appearance of a syncytium. Heart muscle shows structures known as intercalated disks (Eberth lines) which develop late in fetal life and then increase in number, their function not being well understood; they may well be a separation of segments to act as cellular units, since they stain like membranes and are attached to the sarcolemma (53,1,69). Unlike voluntary muscle, cardiac muscle has abundant lymphatic vessels in its interfiber

connective tissue. It is the most active of the three kinds of muscle studied and it is unique in never ceasing its rhythmic contractions from early embryonic life until death. The heart, considered as specialized blood vessel, has the usual 3 layers of the vessel: a) its internal coat or endocardium, b) the middle coat or myocardium, c) the external membrane or epicardium. The morphological characteristics of these coats point to the myocardium as responsible for most, if not all, the metabolic reactions to be discussed. Of the three muscles studied it is the one with the highest mitochondrial density (37) (56). As compared to other muscle tissue it is incapable of performing work under an oxygen debt. To survive and perform work it can utilize carbohydrates and non-carbohydrates from the arterial circulation. (8) After a meal rich in carbohydrates these substances become the main source of energy for the myocardium while in the fasting state, or after a fatty meal, it draws principally on the circulating fatty acids (as free fatty acids) with a greater utilization of the saturated rather than the unsaturated acids. (3)

The study of arteries as organs having active metabolic processes was delayed partially due to a lack of reliable methods. However, experiments initiating such studies were begun in 1910 (73) with the quantitative determinations of some components. Since then, interest has increased and methodology has progressed rapidly so that more dynamic studies have now been carried out, and good reviews on the subject are becoming available (45). Probably one stimulating factor in research on the aorta is the realization of the high place of atherosclerosis in the list of fatal diseases of man.

In describing the morphology of the arteries we distinguish

three concentric coats:

1) The tunica intima (innermost, i.e. in contact with, the lumen of the vessel).

2) The tunica media (in the middle).

3) The tunica adventitia (outer layer or farthest from lumen).

The vessel relies on oxygen and other metabolites, obtained by diffusion from the blood it is transporting, for its own metabolic needs. In those vessels of more than 1 mm in diameter, thickness of the wall would be a limiting factor for access of these compounds to the outer portions of the vessel wall. Nature has provided these larger vessels with their own circulatory system, these "vessels of vessels" known as "vasa vasorum" are present in the adventitia and in approximately the outer half of the media.

Of the aortic tunica intima we shall mention its endothelial polygonal cells lining the lumen, providing it with a smooth surface adequate for helping to maintain the fluidity of the circulating blood, while, at the same time this cell layer offers but small resistance for diffusion of oxygen and nutrients from the blood to the metabolic sites in the arterial wall. If the intima suffers a damaging reaction and a lesion develops, as in atherosclerosis, a circulatory network is formed in the lesion as it increases in size.

As was true in the case of heart muscle, it is probably the media tissue that is mostly responsible for the metabolic activity that will be reported for aorta preparations. In the aorta the media is the thickest of the three tunics and has a large amount of elastic and collagenous tissue with a few muscle cells. Generally, as arteries progress further away from the heart the proportion of muscle cells

increases as compared to the content of elastic fibers. This change in cellular composition is not concerned in the experiments of this thesis since the same segment of artery was removed from all animals. The description is for the purpose of pointing out the very acellular structure of the aortic wall: a relatively few fibroblasts, mast cells and muscle cells then, are responsible for its metabolic reactions.

Several authors have shown the presence of an active oxidative cycle (16,21) for the aorta, also, the ability to utilize intermediates of the tricarboxylic acid cycle and carbohydrates has been reported (42,54). We have found studies on the metabolism of fatty acids and cholesterol by the aortic wall to range from statements on the inability of the wall to synthesize these compounds (33), to more recent studies proving that these reactions do take place in situ (19).

As is also the case for other tissues, the study of the metabolism of the aorta is complicated by possible deposition on the artery wall of substances synthesized by some other organ and transported by the general circulation; in the case of the artery this possibility might appear greater since transport is done through the arterial tree itself and the lipid composition of the arterial wall is very similar to that of plasma (40,65).

In addition to considering the differences between tissues it is necessary to look within the tissues for possible deficiencies. When substrates cross physiological barriers and arrive at the level of the interior of the cell, the picture of the fate of the substrate is not as simple as we might be led to believe. Lately more and more importance is being given to the different sites in the cell, where metabolic processes occur. Even if the cell, because of its reduced

size, tends to give us the impression of a common "container" for many different compounds, it has become more evident, as better methods of study have been made available, that compounds do not mix homogenously inside the cell. Molecules of the same nature in some cases appear to keep within certain definite cellular boundaries so that the overall concept of one "pool" for each intermediate would, in many instances be erroneous, according to Green et. al, (35). It has even become possible to separate experimentally the coupling of oxidative phosphorylation from the electron transport system and cellular sites have been established using the morphology developed with the use of the electron microscope; oxidative phosphorylation resides on a double membrane structure in the mitochondria while the electron transport system is related to the number of cristae per unit area.

Ketosis and Diabetes

Diabetes has been mentioned as a predisposing factor for the development of atherosclerosis, the reason for this being as yet unknown. Composition of the atheromatous plaque (largely cholesterol) points to abnormal lipid metabolism among other possible aberrations of intermediary metabolism. A condition of irregular carbohydrate and lipid metabolism also is present in the diabetic condition. The faulty metabolism of fatty acids and cholesterol, as shown for other tissues (26,74), is also true for the arterial wall in vitro as will be shown in a following section.

By considering the sequence of reactions that take place in the breakdown of fatty acids one can imagine that in the diabetic state, where an impaired metabolism of carbohydrates requires a greater breakdown of fatty acids as a compensatory source of energy,

a condition of ketosis might be favored. It has been shown (14,34) that the liver is the chief organ in the production of ketone bodies, and that these compounds are carried by the circulation and then utilized mainly by the extrahepatic tissues as a source of energy. When the rate of removal from the blood cannot cope with an overproduction of ketones or when there is underutilization (even with a normal production) the condition of ketosis may be produced. There are other organs capable of the formation of ketone bodies; such is true of the kidneys, but there is a difference in their overall contribution to ketonemia as compared to the liver. Kidney, for example, also has a considerable power to utilize acetoacetate so that the net amounts it produces do not grossly contribute to that already existing in the circulation.

Let us consider briefly some aspects of the chemistry of the ketone bodies (acetoacetic acid, acetone, and β-hydroxybutyric acid):

Acetone can be formed by the decarboxylation of acetoacetate, this reaction taking place non-enzymatically in the body and in the urine. Acetoacetate can also be converted to β-hydroxybutyrate by a reversible enzymatic reaction linked to the DPN system. The enzyme L-β-hydroxybutyric dehydrogenase, necessary for the reaction, is present in many tissues but is found in greater quantities in muscle and kidney, and to a lesser extent in liver. As a condition of ketosis develops the rise in total ketone bodies usually is reflected in a rise of the β-hydroxybutyrate fraction. Some investigators have considered β-hydroxybutyrate the storage form of ketone bodies and acetoacetic acid that form which is ready to be utilized in metabolic reactions. Some of the in vitro experiments presented here are an

attempt to explain certain aspects of the derangement in ketone body metabolism during the condition of alloxan diabetes in rats.

Activation of metabolites

It must be kept in mind that molecules must acquire a certain energy level before they can go on to a new arrangement of their atoms. The product of that necessary energy level is what is commonly referred to as the "active form" of the substrate. It is well to review, because of its importance in this field, the activation mechanism involving coenzyme A. These were studied by Krebs, Lynen, Berg, and many others (7,44,49), after the isolation of the coenzyme by Lipman and Snell (47,61).

In the transformation of fatty acids to their thiol esters the thiokinase enzyme necessary for the reaction can be of three different kinds: a) acetic thiokinase, b) C_4 to C_{12} thiokinase (including branching hydroxy acids and unsaturated acids), c) C_8 to C_{18} thiokinase. Berg (6) (after synthesizing acetyl AMP) showed that acetic thiokinase catalyzes the following reactions:

The second reaction has a requirement for Mg. Therefore Berg believes the enzyme catalyzes a two step reaction:

The process of activation becomes very important for experiments like those described in this thesis since substrate was used in the free form, i.e. non-active form. The lack of utilization of a radioactive tracer for labeling a product could well be due to the lack of the

proper activating system in that particular tissue. The case of aceto-acetate is a good example if we consider that the liver is known to be a good source of ketone bodies because it favors the reaction:

This reaction was believed to be a simple deacylation mediated by a deacylase enzyme, but more recent studies (50) show it to consist of a condensation of acetoacetyl-CoA with acetyl CoA to form β-hydroxy-β-methylglutaryl CoA (HMG CoA) which in turn is broken down by an HMG-cleavage enzyme to acetoacetate plus acetyl CoA and free coenzyme A. It appears that with the proper enzymes present, both of the above mechanisms can take place. The reversible reaction, that is, activation of the acetoacetate molecule is important for tissues like muscle where such activation can occur by a thiophorase or by a thiokinase reaction. In heart muscle another activation becomes of importance and that is the net transfer of the coenzyme molecule from succinyl-CoA to aceto-acetate. Activation of acetoacetate (36) continues to be reported as being absent in liver but that it does occur, although to a much lesser degree, will be shown by some of the following results.

The use of hydroxylamine as a method for determining levels of these reactive acyls, is very valuable, since it is really these active forms of the compounds being studied that would show the true dynamic status for that particular tissue.

II EXPERIMENTAL

Animals used.

Normal rats. As used in previous work in this laboratory, "normal" animals were male rats, of the Sprague Dawley strain weighing approximately 200 g. The rats were trained to feeding (17) and then were fasted for three hours before use.

Diabetic rats. Animals were made diabetic by a single intramuscular injection of alloxan given after a 48 hour fasting period. They weighed approximately 200 g and the 10% alloxan solution (in 0.9% saline) was freshly prepared each time. The amount injected was calculated on the basis of 0.06 ml. (6 mg) per 100 g weight. At the same time 5 ml. of 0.9% saline was injected intraperitoneally. The rats were not fed again until approximately 4 hours after the injection and were given saline solution in place of drinking water for 3 or 4 days after being injected. As the criteria for diabetes a minimum fasting blood sugar level of 200 mg. per cent present three weeks after the injection of alloxan.

By this method, usually 2 rats from a group of 12 or 14 animals, died one or two days after the injection of alloxan. Of the surviving group approximately 75% became diabetic animals.

These animals, because of their increased water consumption and low resistance to stress were not trained to feeding but were fasted approximately 8 hours and then

were fed for one hour. By this method the animals always ate well when food was offered. After feeding, the animals were fasted for three hours prior to sacrifice, these conditions being the same as for the normal animals.

Normal goats. Tissue from these animals were made available through the kindness and the cooperation of the Surgery Department of the University of Oregon Medical School. Sections of aorta were removed during studies on surgical prosthesis. The aortas were placed in special containers and immediately taken to the Department of Biochemistry. The goats were adult animals of mixed sex and were generally 1 to 4 years of age. The animals were maintained on a regular grass diet.

Tissues

Liver slices, adductor leg muscle fibers and diaphragm. The use of the Stadie Riggs tissue slicer for liver tissue and the method of obtaining the two muscle preparations has already been previously described. (5)

Heart slices. The heart was obtained after removal of the diaphragm and the major vessels were cut as close as possible to the organ. The heart was left for a few minutes in a beaker with ice-cold Krebs-Ringer-phosphate solution so that by its own pumping action it would empty itself of blood. The organ was then cut transversely through its central axis and from this area further slicing was done using the Stadie-Riggs slicer.

Aortas. In the following experiments two kinds of vessel

wall preparations have been used.

Rat aortas. In these cases aortas have been dissected from normal and diabetic rats; prior removal of the kidney and liver facilitated the procedure considerably. The artery was clamped off as near as possible to the heart and was removed rapidly, together with a large amount of surrounding connective and fatty tissue, and put in a beaker with ice cold buffer. The aorta was freed of all extraneous material and the preparation checked under the dissecting microscope. In removing the adjacent connective tissue the adventitia layer was also discarded. The clamped segment was discarded because it differed from the rest of the vessel due to prolongations of heart muscle fibers into the initial portions of the aorta (64). The artery was cut longitudinally to check the lumen for remaining blood clots. It was assumed that for the rat, the thickness of the two remaining tissue layers (intima and media) was not a barrier to the exchange of metabolic intermediates and products. The tissue was blotted on filter paper and weighed. The amount obtained from an animal varied between 25 and 60 mg. Incubation of these samples at 37°C was started before 30 minutes had elapsed from the time the animal was sacrificed.

Goat aortas. In these experiments, since the tissue was obtained after a considerably more difficult surgical procedure, the time elapsed from the moment the

aortic segment was removed from the animal until incubation was begun, was somewhat longer than it was for rat aortas. Approximately 1 gram of normal aorta tissue was made available by the Department of Surgery. As soon as the segment of the artery was removed from the living animal under Sodium Barbital anesthesia, it was put in a beaker with Krebs-Ringer-phosphate buffer solution at room temperature and taken to the laboratory.

The goat aortas had a thick, fairly rigid wall with a distinctly visible vasa vasorum and a diameter of approximately 8 mm. Theouter layer was stripped off and sections where the vasa vasorum were visible were discarded. Rings approximately 1 mm. thick were cut from the tissue and incubated at 37°C. Incubation time was extended to two hours for these preparations for, as will be observed, metabolic activity was much lower than that seen for the rat aorta. Prior to extending the incubation time it was necessary to determine whether the metabolic activity of the tissue continued at the same rate during the second hour of incubation as compared to the first hour. This was found to be so, therefore the longer period was preferred in order to have a greater use of tracer and a larger activity for the radioassay of isolated products. Aortas were used in the present work less than one hour after the removal from the experimental animal.

<u>In vitro studies, incubation technique and C^{14} labels.</u> In vitro experiments were all done using a conventional Warburg incubation procedure at 37°C.(66) Krebs-Ringer-phosphate buffer solution was used as the suspending media at a total volume of 3 ml of fluid in the 17 ml Warburg flasks. Incubation time was either one or two hours. The acetoacetate 3-C^{14} available commercially had a specific activity of 0.92 mc per millimole, a concentration of label considerably lower than that of the acetate generally used in the laboratories. Although it is generally desirable to use such small amounts of labelled compounds that the tracer will not exert a mass effect, it was necessary to use the acetoacetate at a level of 1.5 mg %. This level is close to the concentration of acetoacetate found in normal plasma. The acetate-1-C^{14} used was added to tissues at a level of about 1 μc per 100 mg. tissue, the specific activity of this acetate was 0.21 mc per mg.

The DL mevalonate-2-C^{14} used in a limited number of studies had a specific activity of 2.26 mc per millimole and the material was added at a level of 0.042 μc per 100 mg tissue.

<u>In vivo time course experiments.</u> The time course experiments were done on a group of normal rats which was injected with acetate-1-C^{14} and then sacrificed at varying time intervals in order to follow the labeling of the fractions: fatty acid, cholesterol and hydroxamic acids formed from reactive acyl derivatives present in the tissue. In these studies there were four animals at each time period; two of these animals were used in determining the fatty acid and the cholesterol fractions and

the other two were used for the determination of the acyl derivatives. These determinations were carried out both in liver and in aortic tissue.

In vivo - in vitro studies on the influence of diet. The animals used in this group of experiments were those kept on different fat diets in this laboratory for a study of another aspect of fat metabolism. The small amount of tissue represented by the aorta in comparison to total body weight made it possible to obtain the vessel without interfering with the other work.

The animals were male rats receiving different fatty diets at three levels of fat (5% - 10% - 30%) and with two kinds of fat, saturated and unsaturated. The animals were part of another experiment in which it was necessary to label their body cholesterol with as much activity as possible. Because of its specific and direct position in the pattern of cholesterol synthesis, mevalonate-2-C^{14} was used for injection. The time of injection prior to sacrifice was either 2 hours or 4 days.

It was chosen to use aortas to study the in vitro incorporation of activity from acetate-1-C^{14} into the carbon dioxide, fatty acid and nonsaponifiable fractions in order to determine if these reactions had been influenced by the diet to which the animal had been subjected to during the 4 weeks prior to the experiment. In addition to the tracer study, the non-saponifiable fraction was measured quantitatively. Since the animals had previously been injected with labeled mevalonic acid, one aorta from each group was incubated without labeled acetate in order to see if activity from the mevalonic acid was present in

the tissue. If this turned out to be true this value would have to be subtracted from the activity found after the use of acetate-1-C^{14}.

Measurements reported.

Oxygen consumption: Oxygen utilization was calculated by the usual Warburg method using the flasks' constants and the serial readings of the manometers during incubation.

Carbon dioxide activity. Carbonate, formed by the reaction of tissue carbon dioxide with sodium hydroxide solution in the center well of the Warburg flask was plated (41) as an infinitely thick sample of barium carbonate after the addition of carrier carbonate.

Amounts of carbon dioxide. Due to the small amounts of tissue used in these experiments it was not possible to determine the quantity of carbon dioxide put out by the tissue. A manometric technique was then tried but with unsatisfactory results. It is considered of general interest, however, to report these negative results together with the possible reasons for their failure.

A procedure which is adequate for working with bacterial suspensions consists of working with duplicate flasks for each experiment, one of which has the usual filter paper with sodium hydroxide in its center well while the other does not. In the first flask the manometer readings will indicate oxygen consumption while in the second flask the reading will reflect the net pressure resulting from the consumption of oxygen and the evolution of carbon dioxide.

By difference (using oxygen consumption from the 1st manometer) theoretically one should be able, using the proper flask constants for carbon dioxide, to calculate the amount of carbon dioxide produced. As can be seen from Table I, the ranges of values obtained by this method are wide and are unsatisfactory. It is possible to suggest reasons to explain these results.

TABLE I

Micromoles CO_2/HR/Gram[(1)] of Tissue

Normals

	LIVER	LEG MUSCLE	DIAPHRAGM	HEART
Mean	-------	37.9	63.6	66.9
Range	-------	(28.1-47.6)	(61.5-67.3)	(26.2-89.7)
No. of Samples	-------	2	3	3

DIABETICS

	LIVER	LEG MUSCLE	DIAPHRAGM	HEART
Mean	86.6	43	64.2	84
Range	(77.4-92.2)	(29.8-51.7)	(47.5-79.5)	(36.6-110.3)
No. of Samples	4	6	6	6

(1) Wet weights.

The method presumes that the tissue is respiring under the same conditions in both flasks, otherwise any change in the respiratory rate inside one flask will be reflected as an error in the amount of carbon dioxide as determined by difference. This assumption may not be valid here for the tissue

preparation is, in one case, respiring in the presence of the carbon dioxide produced while the other sample is respiring in an atmosphere free of carbon dioxide, since the CO_2 was removed by absorption in the center well.

It is also assumed that the tissue samples are exactly the same in the two flasks. With a complex biological structure, this may not be the case for, even if the same worker prepares the samples for each experiment, there will always be a variation both in the technique of the experimenter and in the trauma suffered by the tissue. Besides this variation it is necessary to introduce a correction for the difference in tissue weight, since for these preparations, it is seldom that identical tissue weights will be obtained for different flasks.

A further limitation of this method is the need for a double sample of tissue which in itself defeats part of the purpose involved in using small amounts of the tissue in the small Warburg flask. There is, furthermore, a loss of carbon dioxide from the flask without the center well NaOH, so that in the case of a required radioassay of the carbon dioxide fraction, one cannot even use the extra flask as a duplicate control.

It was mentioned this double flask approach is a fairly common technique in work related to bacterial physiology. Because of the nature of the preparations used in those instances it is easy to see how a homogenous bacterial suspension measured in equal amounts into 2 flasks can be assumed to contain practically the same number of viable cells in each one of the aliquots. It is probable that the lack of identical tissue

preparations is the main source of error rather than the presence or absence of carbon dioxide from the gaseous phase of the flask.

Fatty acid determination. The fatty acid fraction was separated by extraction with petroleum ether following a technique previously described (28). By this method the fatty acids are obtained in an alcohol solution. The evaporation of an aliquot of this solution in a tared vial allowing the determination of the weight of fatty acid. The procedure had originally been used for extracts of one gram portions of liver but it was found that with 100 mg or less of tissue, the method gave inconsistent and falsely high results. It was realized that with these small amounts of tissue, even if the total volume of the alcohol extract were to be evaporated, (which was seldom the case since a certain volume was needed for the determination of radioactivity) still the net weight of the fraction would be on the order of 1 mg. It became apparent then that, whereas in the case of the larger liver sample, a small contamination with extraneous material could still yield acceptable results, for the small tissue samples the error was unacceptably large. With the "constantly loaded" balance used, and with the elimination of contaminants, there should be no reason for the failure of the technique. Two possibilities for reducing the weight of the extraneous material have been considered: 1) It was thought possible that the alcohol used in the tissue extract might have a non-volatile residue. 2) in the extraction procedure, after acidification of the saponified

fatty acids with HCl, the salt KCl is present in the water phase. If not enough time is allowed for separation of both phases any small droplets of water in the petroleum ether layer would be carrying some KCl which, upon drying, would add false weight to the fatty acid fractions.

Working with blank extractions and with a standard solution of palmitic acid, recovery experiments were done as shown in Table II.

TABLE II

Procedure	Residues
1) 10 ml. of alcohol measured into tared vial and evaporated under nitrogen	0.0005 grams
2) 10 ml. of petroleum ether measured into vial and evaporated under nitrogen	0.0000 grams
3) 1 boiling stone and 15 ml. 20% KCl sol.+ 1 ml. alcohol	0.0023 grams
4) 1 boiling stone + 15 ml. 20% KCl sol. + 10 ml. alcohol.	0.0011 grams

The last two tubes were made acid to Congo Red paper with 0.5 N HCl and extracted with two 10 ml portions of petroleum ether. This solvent was evaporated almost to dryness, some alcohol added and evaporation continued to remove the last traces of petroleum ether. The volume was made up to 10 ml. with alcohol and 5 ml of this alcohol extract evaporated in a tared vial under N_2 and weighed.

With the use of labeled and unlabeled palmitic acid we tested for the recovery of fatty acid when different kinds of alcohol were used, and also after allowing 30 minutes for the separation of the water-petroleum ether phases after each period in the shaker.

A group of 11 samples was set up:

1) 5 ml. alcohol (undistilled) + 20 ml. H_2O

2) 10 mg unlabeled palmitic acid in 5 ml alcohol + 20 ml H_2O + KOH + HCL until acid reaction.

3) 10 mg unlabeled palmitic acid in 5 ml alcohol + 20 ml H_2O + KOH + HCl + 0.25 μc palmitic acid.

4) 2 mg unlabeled palmitic acid in 1 ml alcohol + 4 ml alcohol + 20 ml. H_2O + KOH + HCl + 0.25 μc palmitic acid.

5) 2 mg unlabeled palmitic acid in 1 ml alcohol + 4 ml alcohol + 20 ml H_2O + KOH + HCl + 0.25 μc palmitic acid.

These 5 samples were extracted 3 timeswith 10 to 12 ml. portions of petroleum ether and made up to a volume of 10 ml. with alcohol; 5 ml of the extracts were then evaporated in tared vials. In this way the actual tissue procedure was followed. With the two concentrations of the fatty acid used, one of these was in the range of the small samples of tissue, while the other resembled the case where more tissue was available.

The next 6 samples were made up to test the influence of distillation of the alcohol and also of its storage in plastic or glass containers. Some of the standard fatty acid solution was measured directly into the vials in order to determine that its concentration was as calculated:

6) 5 ml. dist. alcohol not extracted but measured directly into vials.

7) 10 mg unlabeled palmitic acid in 5 ml alcohol not extracted but measured directly into vials.

8) 2 mg unlabeled palmitic acid in 1 ml alcohol not extracted but measured directly into vials.

9) 5 ml undistilled alcohol (glass bottle) not extracted but measured directly into vials.

10) 5 ml undistilled alcohol (stored in polyethylene bottle) not extracted, but measured directly into vials.

11) 5 ml undistilled alcohol (stored in polyethylene bottle) extracted as were the first 5 tubes.

Table III records the recoveries from these trials.

TABLE III

	Weights (mg)			Counts		
Samples	Added	Found	%Recovery	Added	Found	%Recovery
1	0.0	0.1	---	---	---	---
2(lost)	---	---	---	---	---	---
3	10.0	9.8	98	105,842	105,160	99.36
4	2.0	2.0	100	105,842	100,804	95.24
5	2.0	2.0	100	105,842	97,416	92.04
6	0.0	0.0	---	---	---	---
7	20.0	19.9	99.5	---	---	---
8	4.0	4.0	100.0	---	---	---
9	0.0	-0.2	---	---	---	---
10	0.0	-0.1	---	---	---	---
11	0.0	-0.1	---	---	---	---

From these results it can be concluded that the procedure was an efficient one for the recovery of fatty acids and that the use of stock alcohol without special distillation was justified. It became clear that it is much more likely that the main cause of the previously obtained high values was electrolyte contamination due to the insufficient time allowed for the separation of the two solvent phases.

Radioassay of fatty acids: The alcohol extract of the fatty acid fraction which was obtained as described was also used for its

radioassay. 0.5 ml portions were measured into 1"1/4 copper shallow planchets and allowed to dry under an infra red lamp. By this method, counting was done at infinite thinness using as criteria for the upper margin of the infinitely thin zone 0.3 mg lipid per plate.

Amounts of non-saponifiable fraction and/or cholesterol: In following the extraction technique for obtaining the fatty acids as has been described, the first product of the extraction with petroleum ether after saponification is a non-saponifiable fraction which, if determined quantitatively by colorimetric procedures (77)(59), will consist of cholesterol and other sterols.

For most purposes it is adequate to consider the non-saponifiable fraction to be the equivalent of "cholesterol", but it is well to keep in mind that this fraction may contain material other than cholesterol.

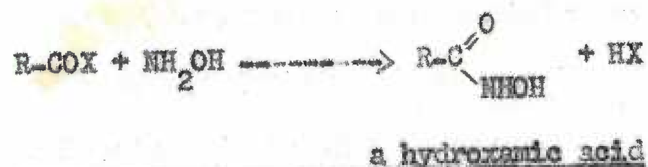
Precipitating the 3β sterols of the non-saponifiable fraction with digitonin (62) makes it possible to determine the color reaction on a more specific fraction and which is more justifiably regarded as cholesterol. The results presented in this thesis in some cases refer to the less specific non-saponifiable fraction while others fefer to the digitonide but each will be specified in the appropriate places.

Radioassay of Cholesterol. Determination of the radioactivity was carried out at infinite thinness either directly on the alcohol extract of the non-saponifiable fraction or on the solution obtained by dissolving the digitonide.

Acyl derivatives. In order to determine the presence of some reactive intermediates of lipid metabolism the derivatives formed with hydroxylamine were studied.(31).

By varying the conditions of the reaction, hydroxylamine can

be acylated by almost any substance of the general structure R-COX



$$R\text{-}COX + NH_2OH \longrightarrow R\text{-}C\begin{matrix}{}^{\nearrow O}\\ {}_{\searrow NHOH}\end{matrix} + HX$$

a hydroxamic acid

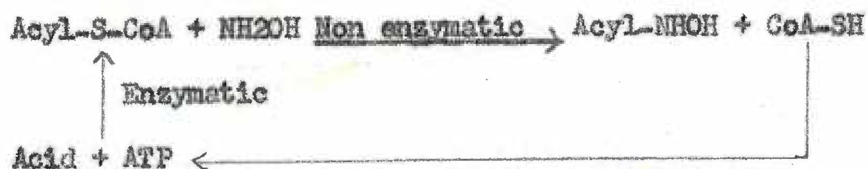
The rates for the formation of different hydroxamic acids vary according to the acyl compound in the reaction and according to the kind of grouping attached to the acyl.

The formation of hydroxamic acids by a non-enzymatic reaction of hydroxylamine with the tissue extract containing the expected acyl intermediates, is of great interest because it offers a good method for detection and separation of the different acyls. One can assume that the biological system being studied will contain, at any one time, many acyl compounds so it is valuable to be able to also separate the various hydroxamic acids. This separation can be accomplished by chromatographic techniques.

The experimental work in this thesis followed the procedure that had been previously established for the optimum concentration of hydroxylamine, the time of reaction, the temperature of inactivation of enzymes, etc. (27,67) It is considered useful to detail one of the present biological experiments since, due to the small quantities of tissue used, the details of the original technique had to be readjusted. The following method was developed to use optimum conditions for the selective reaction of hydroxylamine with thiol acyls.

The procedure consisted generally of three parts: (1) formation of hydroxamic acids. Hydroxylamine can be used as a "trapping" agent for acyl derivatives being formed enzymatically by the tissue during a certain time period; the general equation for such a reaction

is:



By addition of NH2OH at the beginning of a tissue incubation one can obtain certain information as to the total hydroxamic acids formed during the incubation period. One cannot assume that this represents a "normal" figure since the tissue has probably altered its metabolism in the presence of the reagent. It is also quite certain that the rate for this general reaction is not the same as that of the untreated sample since one product is being "trapped" while another is regenerated and can again take part in the reaction, nevertheless, if one keeps in mind these limitations, hydroxylamine can be used in this way to yield useful information. In the determinations of hydroxamic derivatives described below a different use has been made of this reaction. The problem here was mainly concerned with the quantitative and qualitative evaluation of those intermediates of the type acyl-S-CoA, present in a tissue preparation at any one moment. This required the breaking of the reaction sequences just discussed, so that there would be no feed-back of the liberated CoA-SH. Since the recycling of the coenzyme A molecule, by which it can convert more acid to the active acyl form, is an enzymatic reaction, the present technique uses the property of heat inactivation of enzymes to stop this part of the reaction. The tissue samples used were those from a one hour Warburg incubation, without the addition of H_2SO_4 to displace dissolved CO_2. The same procedure was carried out as for those _in vitro_ experiments in which labeling of carbon dioxide,

cholesterol and fatty acid was determined. In some of the experiments tracer amounts of acetate 1-C^{14} were added and labeled hydroxamic acids were separated by chromatography. Our purpose was to obtain a picture of the pool size for these intermediates as well as to gain an idea of rates for the different reactions. In other cases, incubation was carried out with no added radioactive material and the tissues were used only for the estimation of amounts of hydroxamic acids. For these latter cases the incubation period might have been unnecessary yet it was used so as to duplicate the other _in vitro_ experiments as closely as possible. In this way, any change in the metabolic activity of the sample, within that one hour period, would also be reflected in the quantitative determination of hydroxamates. Experiments such as these were run with liver and with aortas of normal and diabetic male rats. This technique was also used in the incubation of goat aortas, and in running a time course study on liver samples and aortas of a group of 20 normal rats.

The conditions for formation of the hydroxamic derivatives were as follows: when the incubation period at 37°C (with or without addition of acetate 1-C^{14}) was completed, the tissue was rinsed free of the suspension media by washing it with Krebs-phosphate buffer delivered from a polyethylene wash bottle. The tissue was then rapidly homogenized with a teflon pestle homogenizer for the liver and a ground glass tissue grinder for the aorta. At this point the tube of the homogenizer had in it the tissue and only 2 or 3 drops of buffer. The tube was kept in a beaker with cold water, and the grinding was carried out as fast as possible. It was thus necessary to start the incubation with appropriate time intervals between flasks.

Enzyme inactivation was then carried out by placing the homogenizer tube in a water bath for 10 minutes at 70°C. The next step was the addition of an equal volume of 0.6 M (pH 7) hydroxylamine solution. The mixture was allowed to react for 30 minutes at room temperature. Because of the small samples it became necessary to sacrifice the convenience of separating the derivatives into two groups as described in the original procedure in favor of a method involving less transfers, and also in order to keep the volume of the extract as small as possible.

The treated homogenates were now transferred to 10 ml screw cap tubes using 2-3 ml ethanol in the process (proteins precipitate at this point). These tubes were placed in the shaker for 15 minutes, then centrifuged for 10 minutes at 1200 rpm. The alcoholic solutions were then transferred to conical tip graduated centrifuge tubes. Two more extractions with alcohol were carried out in the same way on the protein precipitate and each time the alcoholic supernatant decanted into the same graduated tube. The volume of this alcohol extract was then reduced to 1 or 2 ml in a water bath at 60°C under a stream of nitrogen. As will be described, the colorimetric determination of the hydroxamate required a larger volume, while the solution for paper chromatography was preferred more concentrated to facilitate the application of material to the paper.

Quantitative determinations of hydroxamic acids. The assay for hydroxylamine derivatives was based on the original method of Lipmann and Tuttle (48), which uses the color reaction studied by Feigl (29), which consists in measuring, by optical density readings, the amount of colored (brownish pink) complex these compounds form

with trivalent iron. Again in this case there was need to reduce the volumes of the reagents in order to get a maximum concentration of the complex in the minimum volume necessary for reading in a Bausch and Lomb Spectronic 20 colorimeter.

The hydroxamic acids used in preparing standards were synthesized by Dr. J. C. Elwood. Acetohydroxamate was prepared from acetyl glutathione by reaction with hydroxylamine for 30 minutes.

Some results will be presented for what may be a derivative of malonic acid, therefore the preparation of the standard material used in this case will be described (39). Preparation of the monoethyl ester of malonic acid was done by dissolving 8.7 g (0.31 equivalents) of KOH in 100 ml of absolute alcohol and gradually adding this solution to a well stirred solution of 25 g (0.31 equivalents) of diethylmalonate in 100 ml of absolute.alcohol. The mixture was kept at room temperature for about one hour, or until its alkaline reaction disappeared. It was then heated to boiling and filtered while hot. Neutral potassium malonate remained on the filter and the filtrate contained the potassium salt of the monoethyl ester of malonic acid, which crystallized out in large flat needles as the filtrate cooled. The yield was approximately 61%. The derivative with hydroxylamine was made by dissolving some of the ester in water, in the appropriate concentration, and leaving an aliquot to react with NH2OH at an alkaline pH for one hour. Further reference will be made as to characterization of the Rf value of this compound in the discussion of our results.

<u>The colorimetric reaction.</u> This is a simple reaction, that according to the original procedure consisted in adding 5 ml of a 1%

alcoholic solution of $FeCl_3$ (0.1 N in HCl) to 2 ml of an alcoholic solution containing the derivative. The color produced by the reaction is stable and is read at a wavelength of 520 Mu.

Since it was expected that very small quantities of hydroxamates would be found in the biological reactions, a standard curve was prepared, using the same ratio of reagents for the reaction, but with a total volume of only 5 ml. It was not possible to use smaller volumes since, upon eluting a spot, the filter paper absorbs a considerable quantity of the solution. Another variation introduced in the colorimetric assay and for which a calibration curve was made, was the use of a 2% solution of $FeCl_3$ in only a 2.5 ml volume. This permitted the completion of the assays of tissue extracts in a shorter time since there was less need to reduce the volume of the extract. By this modification, 2.5 ml of 2% $FeCl_3$ solution were added to 4.5 ml of extract. A new calibration curve coincided exactly with that determined using the original amounts and concentrations. There was also no difference between a calibration curve determined using a standard solution of palmitohydroxamate and that determined with acetohydroxamate.

With the colorimetric method it was possible to measure as little as 0.1 μm of hydroxamate per colorimeter tube.

Recoveries from tissues. To test for the quantitative extraction of hydroxamates from a biological system, sets of reactions with added amounts of hydroxamic acids were measured for recoveries. An example for this kind of assay is described below:

1) 1 g liver 70°C (10 minutes) + 1.2 μm acetylglutathione + NH_2OH (30 min.)

2) " " 1.0 " palmitohydroxamate

3) " " 1.2 " acetohydroxamate

4) 1 g liver 70°C (10 minutes) + 1.2 μm acetylglutathione + NH_2OH + 1 μm palmitohydroxamate

5)** " nothing added

6)** " NH_2OH (30 minutes)

**these tubes were considered "blanks".

For these levels of hydroxamic acids (1-2.2 μmoles) recoveries from tissues were 95% or higher. A similar series of recoveries were run with smaller amounts i.e. amount compatable with tissue levels.

1) 100 mg liver + 1 μmole palmitohydroxamate

2) " " 00.6 " acetylglutathione + NH_2OH

3) " " 0.6 " palmitohydroxamate

4) " " 0.4 " " "

5) " " 0.2 " " + 0.2 μmole acetohydroxamate

6) " " 0.1 " "

7) " " + NH_2OH -------

8) " " =-----------

Recoveries at these concentrations of hydroxamic acids were above 80% except for the 0.1 μmole level. Recovery of 0.1 μmole was tested repeatedly and was never found to be above 60%-80%.

<u>Paper Chromatographic Separation of Hydroxamic Acids</u>. The hydroxamate derivatives obtained by allowing hydroxylamine to react with tissue area mixture. Therefore, if the tissue extract is analyzed directly colorimetrically the result will represent the total amount of a complex mixture of the derivatives.

Ascending paper partition chromatography was used in an attempt to separate different fractions from these tissue extracts. In partition chromatograph, the solute is fractionated according to its

partition coefficient for a non-miscible two solvent system, in which the more polar phase is held "stationary" by a supporting structure. The mobile phase is the other solvent, and the solute partitions itself between the two as if a considerable number of plates were present as by Craig's counter-current method. The two kinds of supporting media used were paper and silica gel; in both instances water was the stationary phase. The mobile phase for the ascending paper chromatography was butanol. The solvent system consisted of butanol, acetic acid and water in the ratio of 4:1:5, and the paper used was Whatman #3 filter paper.

An aliquot of the tissue extract was chromatographed on paper after concentration to a small volume. A stream of warm air was used to speed up the drying of the spot at the origin. The paper strip was left hanging in the butanol:acetic acid:water solvent system for 12 to 15 hours, and was then left to dry after marking of the distance covered by the solvent front. Once dry the chromatogram was radioassayed or sprayed. Radioassay was done in a Nuclear Actigraph Assembly with a D47 detector. Spraying with 1% $FeCl_3$ made visible spots of the hydroxamates if 0.3 μmoles or more was present.

Elution of a spot from the paper consisted in cutting out that portion of the strip and placing it in a screw cap tube with 4.5 ml ethanol and then shaking the tube for a few minutes. 2.5 ml of 2% $FeCl_3$ reagent were added to the tube, and the contents mixed and decanted into a colorimeter tube. In cases where some turbidity developed, the colorimeter tubes were centrifuged before reading the optical density. Recoveries after elution of known amounts of palmito- and acetohydroxamates, were around 95% when the amount chromatographed

was at the 1 µmole level, and from 80 to 90% if amount was approximately 0.1 µmole.

The following list in Table IV of R_f values was obtained with the indicated standard substances, those marked * are values reported by other authors (67).

TABLE IV

Substance	R_f
Ethylcarbanohydroxamate*	0.76
Carbanohydroxamate*	0.30
Acetohydroxamate	0.52-0.58
Free acetoacetic acid	0.73-0.75
"Malonylhydroxamate"	0.21-0.23
Myristohydroxamate	0.95
Laurohydroxamate	0.95
Palmitohydroxamate	0.91
Stearohydroxamate	0.94
Fumarohydroxamate*	0.31-0.50
Succinohydroxamate*	0.24-0.53

RESULTS AND DISCUSSION

Although all the experimental work of this thesis is related to the same general problem, the studies can be divided in two main groups: 1) those related to ketosis using acetoacetate-3-C^{14} as tracer, and 2) those pertaining to the metabolism of the aorta.

Experiments with acetoacetate

Of the three ketone bodies, acetoacetic acid is the form which is generally stated to be directly utilized in metabolic reactions. This is not altogether true, for by reviewing the scheme for fatty acid metabolism, one can observe that the acetoacetate takes part in these reactions only in its active form, the thio-acyl derivative. The breakdown of fatty acids produces the acetoacetyl CoA molecule and the reaction responsible for hydrolyzing the acetoacetic acid from its active form takes place mainly in the liver. It is also generally stated that the opposite reaction, i.e. activation, does not take place in hepatic tissue. From the data presented here it is clear that activation of acetoacetate does take place in the liver.

Utilization of acetoacetyl CoA takes place principally in extra hepatic tissue which has been represented in these experiments by several kinds of muscle. As with liver, after the addition of the labeled material to the muscle tissue, activation must take place if acetoacetate is to be used in a metabolic pathway. In this case activation is favored and mediated through the thiokinase and thio-

phorase enzymes of muscle tissue.

Considerable controversy in the field of metabolism and diabetes has revolved around the problem of the production and utilization of ketone bodies. In the absence of normal carbohydrate metabolism, there is an increase of fatty acids and a potentially large increase in the formation of ketone bodies. An increase in ketogenesis would not necessarily result in the condition of ketosis developing if there was present an increased rate of utilization of the ketones formed. Both phases of the problem of ketosis, i.e. production and utilization, have been extensively studied, and the various theories on overproduction or under utilization have ardent proponents. The problem of ketone body utilization has been the chief aspect studied in the present experiments.

Studies by others with intact animals (63) measured blood levels of the ketones before and after circulation through the liver or muscle, but the _in vitro_ preparation has hardly been used. In the intact animal, it has also been reported that exercise will increase utilization of ketone bodies as measured by their decreased concentration in the blood of rats made ketotic by fasting. Exercise will, however, also increase their production as is observed by a later rise in the blood level (22) (4). Among other conditions affecting the utilization of acetoacetic acid which have been considered are age (72) and certain compounds with chemical groups believed to yield better oxidants for acetoacetate and thus act as antiketogenic substances (18). As far back as 1928, Chaikoff (13), by measuring the fall of ketone body levels in the circulation, saw that this occurred at a lower rate in the diabetic animals (dogs) as compared to the

normal. If however, the liver was eliminated from the circulation, the diabetic animals showed the same rate of removal of ketones as the control group. This observation was taken as substantiating the theory that utilization by the extra-hepatic tissue was the same for the diabetic preparation, and that ketosis developed only because of the overproduction by the liver of the diabetics.

In the following group of experiments the results are expressed as "per cent incorporation of the labeled material into the fraction studied, per hour incubation and per gram of tissue." In order to compare the percent incorporation figures the fatty acids and non-saponifiable fraction of the tissues were also quantitatively evaluated. Amounts of the two lipid fractions, per unit weight of tissue, for the normal and diabetic group of animals were found not to be significantly different statistically for any one tissue. The activity of the carbon dioxide fraction is measured as evidence of an active tricarboxylic acid cycle, but, as was explained in the experimental part, the quantity of carbon dioxide has not been measured.

By looking at the comparison of the percent incorporation into carbon dioxide from acetate into the fractions studied, we can observe that the normal muscle preparations follow the general concept that the more active the oxidative cycle, the higher the activity of the muscle (which has also been related to a higher mitochondrial density). In otherwords the heart slices show the greatest activity in the CO_2 fraction; leg muscle and diaphragm are lower, but in turn much higher than liver, which is considered an organ of synthesis.

TABLE V

Rat Tissues in vitro. Comparison of Normals to Diabetics.

% Incorp.* of Acetate-1-C^{14} into CO_2.

NORMALS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	11.9	24.38	26.36	34.04
± St. Dev.	3.44	3.7	5.62	9.4
Range	(8.5-17.39)	(19.32-29.76)	(18.9-34.7)	(21.1-41.5)
No. of Animals	8	8	8	4

DIABETICS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	13.22	31.49	26.08	39.35
± St. Dev.	3.1	9.06	5.8	5.9
Range	(9.0-17.8)	(22.66-47.5)	(11.7-32.4)	(32.8-50.9)
No. of Animals	10	10	11	6
P	>0.1	>0.1	>0.1	>0.1

* per hour per gram wet weight

TABLE VI

Rat Tissues in vitro. Comparison of Normals to Diabetics.

% Incorp.[1] of Acetate-1-C^{14} into Cholesterol[2]

NORMALS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	1.220	0.026	0.057	0.005
± St. Dev.	0.41	0.006	0.043	0.003
Range	(0.51-1.72)	(0.018-0.04)	(0.02-0.14)	(0.002-0.009)
No. of Animals	8	8	8	6

DIABETICS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	0.041	0.006	0.012	0.005
± St. Dev.	0.019	0.005	0.002	0.003
Range	(0.007-0.114)	(0.002-0.016)	(0.006-0.016)	(0.003-0.009)
No. of Animals	10	9	9	6
P	< 0.01	< 0.01	< 0.01	> 0.1

(1) per hour per gram wet weight.
(2) non-saponifiable fraction.

TABLE VII

Rat Tissues: in vitro. Comparison of Normals to Diabetics.

Per cent incorporation* of acetate-1-C^{14} into fatty acids

NORMALS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	0.23	0.08	0.67	0.026
± St. Dev.	0.14	0.044	0.43	0.0115
Range	(0.10-0.52)	(0.043-0.14)	(0.28-1.42)	(0.012-0.042)
No. of Animals	8	8	8	6

DIABETICS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	0.011	0.010	0.062	0.012
± St. Dev.	0.003	0.006	0.049	0.007
Range	(0.006-0.015)	(0.001-0.021)	(0.004-0.127)	(0.006-.022)
No. of Animals	9	9	10	6
p	< 0.01	< 0.01	< 0.01	< 0.05

* per hour per gram wet weight

In tables VI and VII the normal preparations with acetate-1-C^{14} show that muscle does carry on an active lipogenesis as evidenced by the activity determined in the two lipid fractions of all muscle preparations. Although these figures are much smaller than those for liver slices, if one considers the importance of muscle tissue in terms of percentage body weight, its contribution to lipid metabolism is not negligible.

If results for the diabetic tissues are compared to the normal preparations, data of percent incorporation into CO_2 from acetate-1-C^{14}. (Table V, page 41) shows no statistically significant difference for any of the 4 tissues. In the case of the liver this result would still support the theory of a decreased acetate pool formulated through previous work in this laboratory (25). In that set of experiments, amounts of carbon dioxide for liver tissue were determined gravimetrically and found to be reduced as compared to the normal animals. If we assume the same tendency for the amounts of carbon dioxide of the present two groups of animals, the same percent incorporation figure with the $C^{14}O_2$ contained in a smaller amount of $C^{12}O_2$ would indicate a more concentrated or smaller pool of acetyl-CoA. Labelling of the 2 lipid fractions from acetate 1-C^{14} was found greatly reduced for the liver as was shown previously in this laboratory (25). The same significant decrease in percent incorporation figures was found for the muscle tissues, although the greatest decrease was for the liver slices indicating that this tissue, specially in the synthesis of cholesterol, is much more affected by the diabetic condition as compared to similar reactions in muscle. The only tissue showing no significant change in percent incorporation for these fractions during

diabetes was the heart preparation regarding its non-saponifiable fraction however it should be noted that cholesterologenesis of heart muscle is insignificant. It also is the tissue with the least decrease in labeling of the fatty acid fraction since its approximately two fold decrease is only significant at a 5% probability level while the other preparations show decreases ranging from 8 to 20 fold. It could very well be that the heart is least affected since it is an organ that must draw directly on metabolites in the circulating blood for its own energy requirements (3). In spite of being essentially muscle tissue the heart cannot withstand anoxic conditions and function under an oxygen debt, like other muscle tissue. One important class of substrates it extracts from blood are the free and esterified fatty acids. It could be possible in this case that the one hour incubation period was not long enough to exhaust the supplies of this material that remained within the tissue.*

Acetoacetate-3-C^{14} has been added to essentially the same kind of tissue preparations. Work done by other workers in the field (5) indicates that there is no dilution of the label, as a result of acetoacetate being added to the media by the muscle tissue, either in the normal or the diabetic preparations. These same authors have determined uptake of the labeled material by the normal and the diabetic (depancreatized) muscle tissue. Uptake by the diabetic was found to be about one half of the normal. This is an important figure to bear in mind when interpreting results as the percent incorporations since in this case incorporation figures should have been calculated by dividing

*In general it is observed that incorporation of radioactivity from acetate-1-C^{14} into fatty acids is much more affected by the diabetic state than is the activity in the cholesterol fraction.

by one half of the added dose. Therefore, the percent incorporation figures as presented here might actually be approximately twice as large for the diabetic group.

When acetoacetate was added, the results in $C^{14}O_2$ that were obtained were in the same range as those reported by Krebs and Eggleston (43). These authors, after comparing sheep heart minces to the other tissues, found that the former have the highest rate of acetoacetate removal. Table VIII lists activity from acetoacetate-3-C^{14} in the carbon dioxide fractions. It is shown that the liver CO_2 fraction has activity, so there must be a way for liver to activate the acetoacetate molecule. The possibility that the compound has been broken down non-enzymatically to acetate molecules and used as the source of activity in the liver CO_2 fraction is ruled out because we would then expect the same degree of change for the lipid fractions (Tables IX and X) as compared to the results for acetate in Tables VI & VII. We see this is not the case because the percent incorporation figure for acetoacetate into CO_2 is approximately 1/7 that of acetate, while the unsaponifiable fraction is approximately 1/2.5 and for fatty acids approximately 1/2. In comparing the figures for the CO_2 incorporation of the diabetic and of the control group, they appear significantly decreased in the case of the diabetic condition. Nevertheless if one remembers the fact that uptake of the labeled substrate has been reduced to approximately 50%, then these figures could be corrected and they then appear to have no difference from those of the normal group. Labeling of the lipid fractions also appears reduced but these figures would also approach those for the normal preparation if they are approximately doubled to account for the decrease in uptake. Also in

TABLE VIII

Rat Tissues in vitro. Comparison of Normals to Diabetics.

% incorp.* from Acetoacetate-3-C^{14} into CO_2.

NORMALS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	1.62	14.57	18.57	37.95
± St. Dev.	0.56	3.55	3.19	4.47
Range	(1.06-2.85)	(9.35-19.67)	(14.73-26.45)	(32.6-43.3)
No. of Animals	8	8	8	4

DIABETICS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	1.78	8.92	13.63	25.26
± St. Dev.	0.3	2.66	4.57	5.15
Range	(1.3-2.16)	(4.8-14.04)	(7.3-19.9)	(21.0-34.9)
No. of Animals	10	9	8	5
P	>0.1	<0.01	<0.05	<0.01

*per hour and per gram of wet weight.

TABLE IX

Rat Tissues in vitro. Comparison of Normals to Diabetics

% incorp.[1] from Acetoacetate-3-C^{14} into Cholesterol[2]

NORMALS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	0.49	0.033	0.056	0.003
± St. Dev.	0.27	0.014	0.051	0.001
Range	(0.18-1.01)	(0.01-0.05)	(0.02-0.17)	(0.001-0.004)
No. of Animals	8	8	8	4

DIABETICS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	0.018	0.008	0.016	0.006
± St. Dev.	0.011	0.006	0.015	0.001
Range	(0.005-0.045)	(0.002-0.018)	(0.005-0.049)	(0.004-0.007)
No. of Animals	10	9	10	6
P	< 0.01	< 0.01	< 0.05	< 0.01

(1) per hour and per gram of wet weight.
(2) non-saponifiable fraction.

TABLE X

Rat Tissues in vitro. Comparison of Normals to Diabetics

% incorp.* from Acetoacetate-3-C^{14} into Fatty Acids

NORMALS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	0.11	0.08	0.35	0.020
± St. Dev.	0.07	0.017	0.129	0.005
Range	(0.05-0.21)	(0.05-0.10)	(0.14-0.50)	(0.014-0.026)
No. of Animals	8	8	8	4

DIABETICS

	Liver	MUSCLE		
		Leg Adductor	Diaphragm	Heart
Mean	0.020	0.025	0.051	0.020
± St. Dev.	0.008	0.009	0.024	0.004
Range	(0.007-0.030)	(0.008-0.044)	(0.032-0.088)	(0.013-0.028)
No. of Animals	10	10	10	6
P	< 0.01	< 0.01	< 0.01	> 0.1

* per hour and per gram of wet weight.

this case the heart is in discrepancy by having an increased activity of its unsaponifiable fraction for the diabetic and showing no difference for the case of the fatty acids.

In conclusion: The results for this group of experiments can be listed as: (1). Amounts of the two lipid fractions and µm of oxygen consumed are the same for the normal and the diabetic preparations. (2) Muscle tissue does show evidence of lipogenesis from acetate, and if considered in relation to percent of the body weight constituted by muscle, this property of the tissue becomes fairly important. (3) A comparison of the utilization of acetate by the diabetic and the normal groups supports the theory of a decreased acetate pool in the liver of the diabetic and also shows decreased labelling of both lipid fractions by liver and muscle tissue. Of the different kinds of muscle, heart muscle seems to be least affected by the diabetic condition. (4) The diabetic muscle preparation shows a decreased (50%) uptake for depancreatized rats. If this same phenomenon occurs for alloxan diabetes, then the per cent incorporation figures for the diabetic animals should be approximately doubled before comparison to determine ability of extrahepatic tissue for utilization of ketone bodies. (5) These results confirm the work of other authors on the fact that heart slices utilize acetoacetate at the highest rate. (6) A mechanism by which acetoacetate is activated to a small extent by liver slices is shown to occur and it has been pointed out that the data suggests this mechanism cannot originate from a non-enzymatic breakdown of acetoacetate giving rise to labeled acetate which could then be used by the liver slices. (7) The per-cent incorporation data from acetoacetate into CO_2 for different kinds

of muscle shows a significant decrease of approximately two fold in the labeling for the diabetic. If one again applies a theoretical correction for the decreased uptake of the diabetic, utilization in vitro of this ketone body by the normal and the diabetic muscle preparation would be the same. If the term utilization refers to the overall processes of uptake and use, the diabetic preparation reported here shows a decreased utilization of acetoacetate.

Experiments with arterial tissue

In vitro studies with normal and diabetic rat aortas, and normal goat aortas.

Viable preparations of rat aortas were incubated for one hour with acetate-1-C^{14} and the incorporation of radioactivity into CO_2, fatty acids and cholesterol was measured. Measurements of oxygen consumption, as shown in Table XI, are slightly higher than figures reported by Briggs et al (9) who showed that arterial tissue of the rat respires at a rate about one tenth that of rat liver slices. When the oxygen consumption of the normal group is compared to the diabetic there is no significant difference, although a significant decrease for the diabetic group has been reported (70). It has been found that in atherosclerosis, changes in blood proteins and lipoproteins take place (20,32); the most common changes are a decrease in albumin, an increase in α_2-, β- and γ globulins, serum cholesterol, total lipids and β lipoproteins and some of these changes, especially the last two, are present in diabetes. Since the lipid composition of the arterial wall is similar to that of blood plasma (40) it has been assumed that the fatty deposits in atherosclerosis are produced by deposition from the blood. The plaques formed during the atherosclerotic process have also been analyzed (65) and found to resemble the composition of blood lipids so closely that this finding is offered as further proof for the theory of lipid deposition from the circulating blood. As mentioned, above, it has been found that in diabetes certain lipid fractions of blood plasma are increased but whether this increase is thought to cause a greater deposition of lipids in the artery wall, is not mentioned in the articles. Table XI shows a significant increase in the

TABLE XI

Rat aortas. Oxygen consumption and amounts of lipids.

Comparison normals to diabetics.

NORMALS

	Oxygen consumption Um/hr/g wet weight	Fatty acids mg/g wet weight	Cholesterol* mg/g wet weight
Mean	12	13.3	0.98
±St. Dev.	4.82	1.88	0.3
Range	(6.02-21.3)	(10.12-17.87)	(0.59-1.4)
No. of animals	6	6	6

DIABETICS

	Oxygen consumption Um/hr/g wet weight	Fatty acids mg/g wet weight	Cholesterol* mg/g wet weight
Mean	6.17	23.62	1.42
±St. Dev.	3.36	8.88	0.35
Range	(2.23-11.57)	(14.54-35.74)	(1.06-1.96)
No. of animals	8	5	8
P	> 0.05	< 0.05	< 0.05

* cholesterol digitonides.

aorta lipid fractions of the diabetic over the normal. Even if this significant increase in lipids for the diabetic would seem to agree with this hypothesis of precipitation of lipids from blood serum on the artery wall, one hesitates to ignore the part that might be played by endogenous synthesis. The use of P^{32} has permitted research on the endogenous synthesis of phospholipids (15). Experimental atheromas produced in rabbits by a cholesterol rich diet have been shown to have the major portion of their phosphatides synthesized _in situ_ by the aorta (60). An endogenous synthesis by the aorta of the rat, and other species, also occurs for fatty acids and cholesterol as has been determined by a number of investigators (24,71,23,55). The difference in species for reactions of this type is pointed out repeatedly by these authors. The author of this thesis believes that this difference might well be due to problems in technique such as lack of a proper counting device for the low activity of the lipid compound, the incubation of the tissue after a considerable lapse of time, etc. This time factor is specially true for the work done with human autopsy samples in which in some cases reported (2) as unable to convert acetate-C^{14} into cholesterol _in vitro_, eight hours had elapsed since the removal of the tissue.

Table XII shows a comparison of radioactivity incorporated from acetate-1-C^{14} into fatty acid and cholesterol by rat aortas from normal and diabetic groups. One can observe a decreased incorporation into the two lipid fractions by the diabetic similar to what has been shown for other tissues. Both groups of animals convert appreciable amounts of acetate to CO_2 which suggests the presence of an active tricarboxylic acid cycle, which should in turn provide energy for the synthesis of fatty acids and cholesterol. Considering the large amount of relatively

TABLE XII

Rat Aortas in vitro. Comparison Normals to Diabetics.

% Incorp. From Acetate-1-C^{14}/Hour/G.(1) of Tissue

NORMALS

	CO_2	Fatty Acids	Cholesterol (2)
Mean	10.65	0.22	0.036
± St. Dev.	2.75	0.11	0.023
Range	(8.42-15.4)	(0.11-0.41)	(0.02-0.08)
No. of Animals	6	6	6

DIABETICS

	CO_2	Fatty Acids	Cholesterol (2)
Mean	17.95	0.029	0.012
± St. Dev.	6.27	0.025	0.007
Range	(11.85-28.35)	(0.007-0.07)	(0.004-0.02)
No. of Animals	5	5	5
P	< 0.05	< 0.01	< 0.05

(1) Wet Weight.

(2) Unsaponifiable Fraction.

inert extracellular, structural material, this represents an active metabolic role for the small number of cells involved. Results for these experiments are reported as for one gram of wet weight. Samples were pooled and then dried to determine their water content. No difference was found for the 2 groups of animals (28% dry weight for the normals and 30.3% for the diabetics as calculated from pooled tissue from 3 rats each. This small difference in composition would not significantly change values reported as per wet or dry weights.

Arterial lipid synthesis by aortas from diabetic animals has not been extensively studied. A paper on the subject has rather controversial results (30) for it reported that increased cholesterogenesis "occurred in this preparation as it did in livers from diabetic rats". In this laboratory it has never been shown that the diabetic condition causes increased lipid labeling by the liver. Table XII shows that the three % incorporation values reported, differ significantly if the corresponding normal and diabetic fraction are compared. The degree of labeling exhibited by the fatty acids of the normal group is pointed out as an unexpectedly high figure since it lies in the range of values found for liver slices (Table VII). This fatty acid value also is the one that appears most influenced by the diabetic state and the same tendency was previously mentioned for muscle tissue.

The increased activity in the carbon dioxide fraction of the diabetic prompted the investigation of the amounts of intermediates for reactions as studied by the use of hydroxylamine. Activity was too low to be measured after paper chromatography of the extract of hydroxamic acids from duplicate flasks. By the colorimetric assay of

the extract at a specific R_f for acetohydroxamate from several paper strips the following figures were obtained: for the diabetic--0.20 μmoles and for the normals 0.30 μmoles for 1 gram of tissue.

Although these figures were obtained only after pooling a considerable number of samples they represent only one determination for normal tissue and one for diabetic tissue. Therefore from this experiment one can only conclude that the difference between the 2 figures is in the right direction to postulate a decrease in acetyl-CoA concentration for this tissue.

TABLE XIII

Normal Rat Aortas in vitro.

% Incorp. From Mevalonate-2-C^{14}/hr/g Tissue (1) and Oxygen Consumption.

	CO_2	Fatty Acids	Cholesterol (2)	μm O_2/hr/g (1)
Mean	0.24	0.096	0.07	15
± St. Dev.	0.25	0.039	0.04	4.63
Range	(0.05-0.68)	(0.05-0.14)	(0.06-0.09)	(10.97-22.7)
No. Animals	5	5	5	5

(1) Wet weight.

(2) Unsaponifiable fraction.

Table XIII presents data from normal rat aortas incubated with mevalonate. Mevalonic acid requires phosphorylation prior to becoming an active metabolite in the pathway to cholesterol synthesis (57). These results indicate that rat aortic tissue does have the capacity to incorporate the mevalonate molecule into cholesterol.

Table XIV presents results for incubation of goat aortas with acetate-1-C^{14}. Although the lipid components are not grossly different in amounts, to those of the rat, the oxygen consumption has decreased

to approximately 1/4 that determined for the rat.

TABLE XIV

Normal Goat Aortas.

Oxygen Consumption, Amounts Lipids

	Oxygen Consumption (1)	Fatty Acid (1)	Cholesterol (2)
	Umoles/Hour/G. Tissue	MG/Gram Tissue	
Mean	2.77	13.83	1.24
± St. Dev.	1.64	5.44	0.48
± St. Error	0.47	2.05	0.18
Range	(1.02-7.23)	(6.34-21.90)	(0.61-2.16)
No. of Animals	12	7	7

(1) Wet Weight.

(2) Digitonide.

TABLE XV

Normal Goat Aortas in vitro

	% Incorp. From Acetate-1-C^{14}/Hour/G of Tissue (1)		
	CO_2	Fatty Acids	Cholesterol (2)
Mean	2.54	0.0032	0.0031
± St. Dev.	0.64	0.0018	0.0016
± St. Error	0.17	0.0005	0.0004
Range	(1.24-3.62)	(0.001-0.007)	(0.0007-0.006)
No. of Samples	14	13	13

(1) Wet Weights

(2) Digitonide

Table XV shows % incorporation figures for goat aortas and these are also decreased to about one fourth the values reported for the rat. This could well be a species difference as has been cited from the literature or be due solely to the difference of the preparation of the tissue preparations (see Experimental part). Although for these experi-

ments with goats, more tissue was available than in the case of the rat, the low metabolic activity did not permit radioassay of hydroxamates and the colorimetric readings were in a range of amounts of hydroxamates at which recoveries were considered unreliable.

Due to the inability to evaluate the hydroxamic acids from the in vitro preparations a group of rats was studied in vivo.

In conclusion it can be said for this group of experiments that arterial tissue from rats and goats has an active oxidative cycle, and can synthesize in situ fatty acids and cholesterol from acetate, although to a much lesser extent by the goat aorta than by that of the rat. This difference is thought to be due to structural differences in the artery wall of the two species. Considering the small number of cells that are present per unit wet weight of arterial tissue, this metabolic activity for the rat is high.

The normal rat aorta is capable of phosphorylating mevalonate in vitro and of utilizing it in the synthesis of cholesterol.

In comparison with the normal tissue it was found that the aorta from the diabetic rat had a higher content of both fatty acids and cholesterol. Labeling of these two fractions from acetate-1-C^{14}, as has been found for other tissues, was greatly reduced for the diabetic group which can indicate a decreased turnover rate of the end product or dilution of the label at some of the intermediate steps in the synthesis of these components. Parallel to these findings there was an increased labeling of the carbon dioxide of the diabetic group; one determination of acetohydroxamate separated by paper chromatography of many pooled samples gave a lower value for the diabetic lending support to the theory of a smaller pool for this compound.

Time course study of hydroxamic acids from rat livers and aortas

Description of method. In the previous *in vitro* work with hydroxylamine, the approximate level of hydroxamic acids determined represented the amounts of reactive acyls present at a particular moment in the intermediate metabolism of that tissue.

The present studies on liver and aorta tissue hydroxamates have as their goal the eventual description of metabolic processes in terms of the size and turnover of reactive components of these processes. It is our belief that measurements reported here represent the first such studies to be reported. It is acknowledged that the present data are limited, and that interpretation must also be limited. The material is presented then as an example of a new experimental approach rather than as a completed study.

A group of 20 normal, male rats was divided into 5 sub-groups of four animals each. The smaller groups represented 5 different time intervals measured as the time elapsed from the moment the animals were injected intraperitoneally with acetate-1-C^{14} until they were sacrificed. These five time periods were: 15 minutes, 30 minutes, 1 hour, 2 hours, 6 hours. Because each animal was injected with approximately 100 μc of acetate it was necessary to place the animal in a metabolism chamber to collect respiratory carbon dioxide in a solution of sodium hydroxide.

Within one group of four animals, two were used to determine the amounts and the radioactivity of fatty acids and cholesterol digitonide from liver and arterial tissue. The other two of the same group were used in determining the content and radioactivity of the hydroxamic

acids of the aorta and liver after a partial separation by paper chromatography. The data that can be obtained from such an experiment can be extensive and complex, so that it is necessary to decide on specific steps to be followed in deriving the information which had the greatest significance. An outline is presented so that the reader can follow the sequence of operations.

<u>Outline</u>. (1) separation, radioassay, and determinations of amounts of hydroxamate derivatives. The procedure for formation of hydroxamic acids in a biological system and their separation into more specific groups of compounds by paper chromatography was followed as has already been described. All paper strips were radioassayed in a Nuclear Actigraph with a D47 Detector so that this procedure resulted, for this experiment, in 10 graphical representations of the distribution of radioactivity on the paper chromatography strip which permitted their evaluation as follows:

(a) Each radiochromatogram was divided into equal "zones" corresponding to 0.05 Rf units in width.

(b) The highest point of the radioactivity curve for each "zone" was measured in milimeters and normalized to the same set of counting characteristics for the machine (slit width, scanning speed, etc.).

(c) The former results were converted, by using a predetermined factor, to activity counted as $BaCO_3$ at infinite thickness at 4.63% efficiency. This operation facilitated calculations of % incorporation figures because the injected dose had been counted at the same efficiency.

(d) All data from (c) were standardized to the same unit of

tissue weight (1 gram wet weight).

(e) Results from the previous step were multiplied by the proper dilution factor considering the size of the aliquot used for chromatography.

(f) Per cent incorporation of the injected acetate into each one of the 20 segments in one strip, was now calculated.

Results and Discussion:

After following the steps for the analysis of the data, figure 1 was obtained for the liver by plotting the percent incorporation figures against time, permitting a comparison of this measurement, at a specific Rf value, made at the 5 different time intervals. Figure 2 is a representation of the decay in radioactivity with respect to time for several of the hydroxamates found at specific Rf values; the diagram being presented to show the direction and magnitude of the changes for some of the intermediates.

The Rf values selected for these calculations are:

(1) 0.55, because it is assumed that one of the major components in this part of the paper is acetohydroxamate which represents the activated form of the labeled material injected.

(2) 0.75 for acetoacetate. The product of acetoacetyl-CoA and hydroxylamine reaction has an Rf value very close to that of the free acid, therefore, differentiation of the two forms is not possible with this solvent system.

(3) 0.35, around this area was recorded one of the largest concentrations of radioactivity occuring in the liver samples from the 15 minute animals. In this area would be the largest part of a carbanohydroxamate derivative.

Figure 1

Time course of hydroxamate labeling of liver tissue

The percent incorporation of acetate-1-C^{14} is presented for 20 Rf zones at five time periods. The hydroxamic acids chromatographed were obtained from rat liver tissue. As described in the text, the figure records, in some cases, the build up of radioactivity, and in some others, the loss or decay of radioactivity during the 15 minute to 6 hour period of study.

Figure 1.

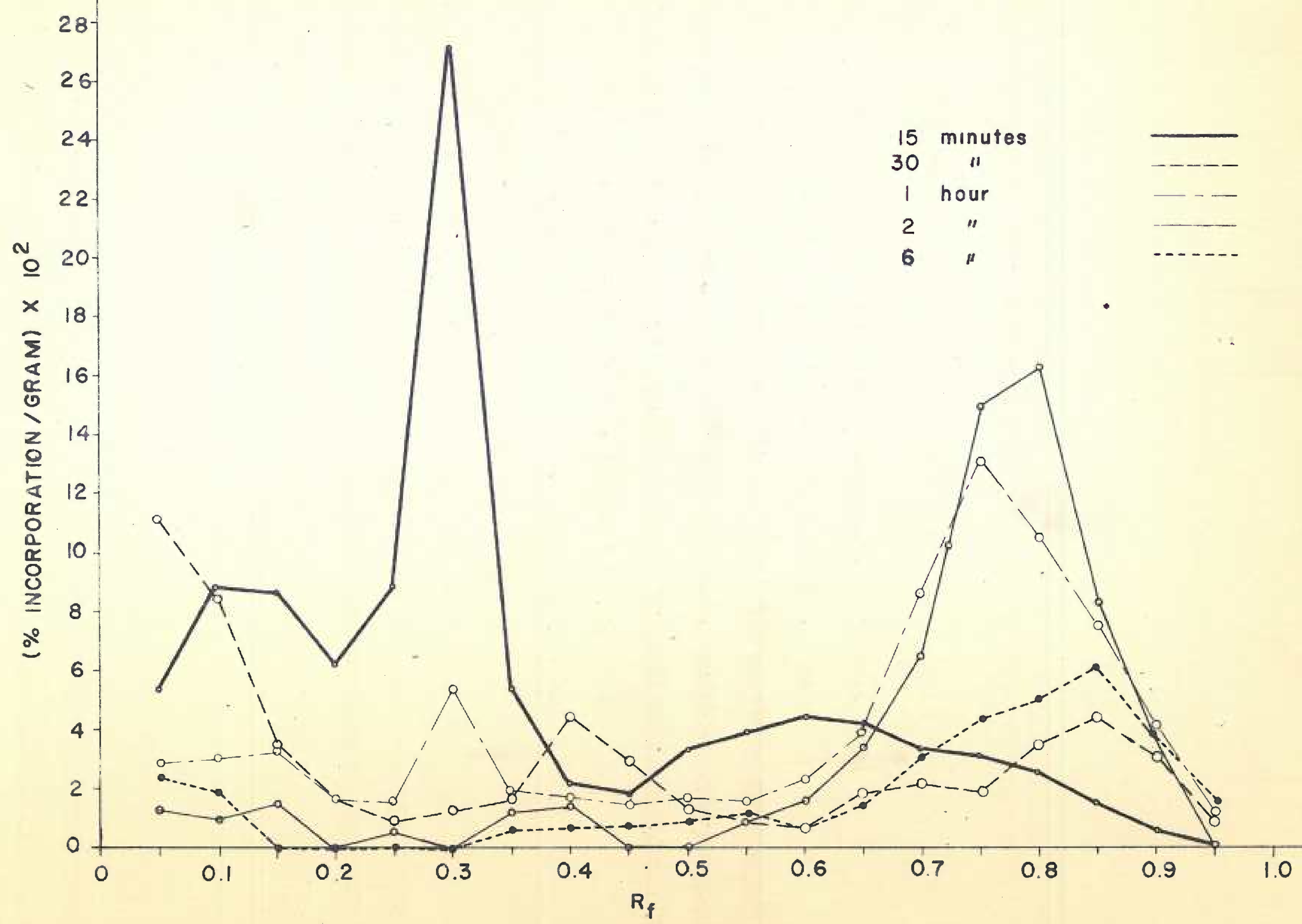
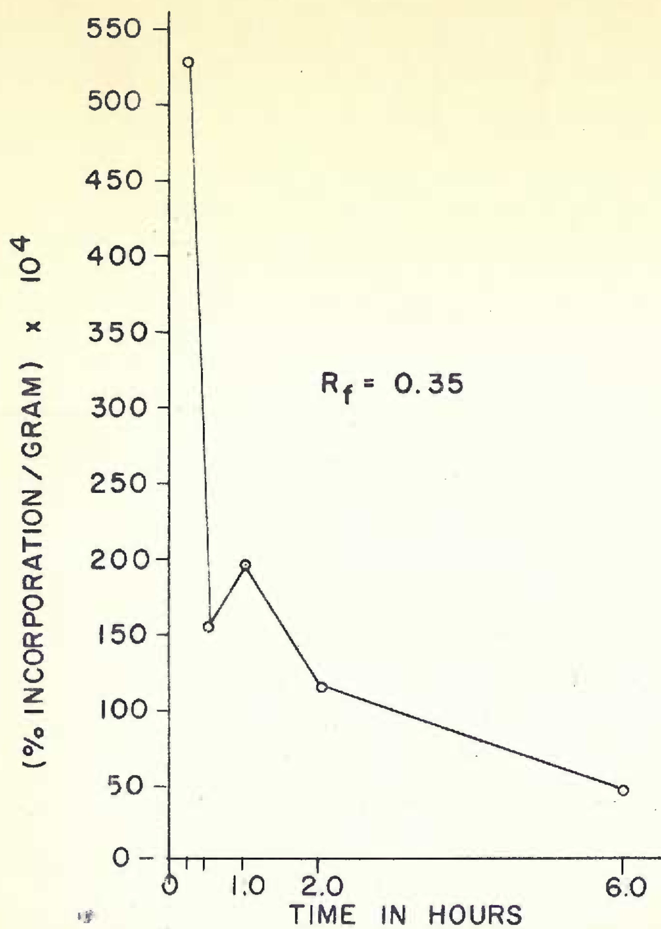


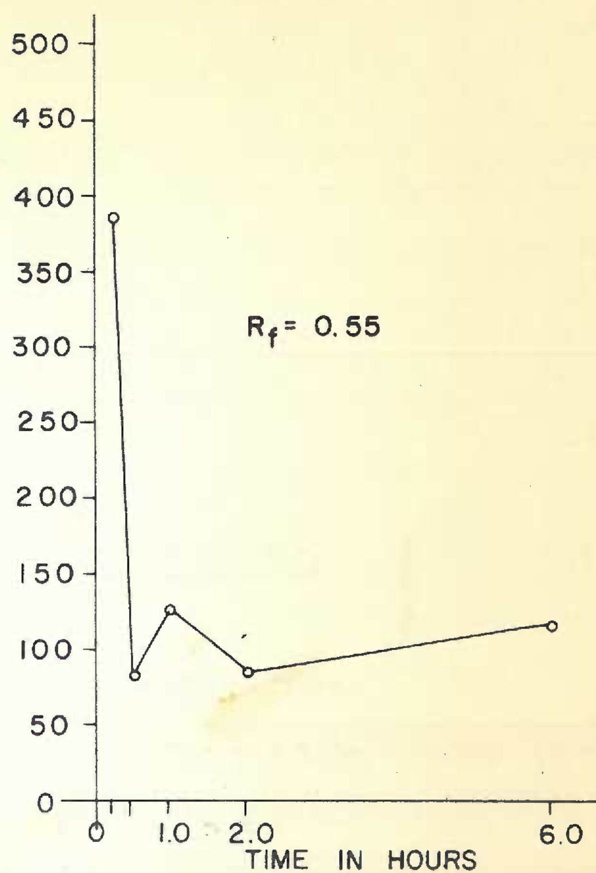
Figure 2

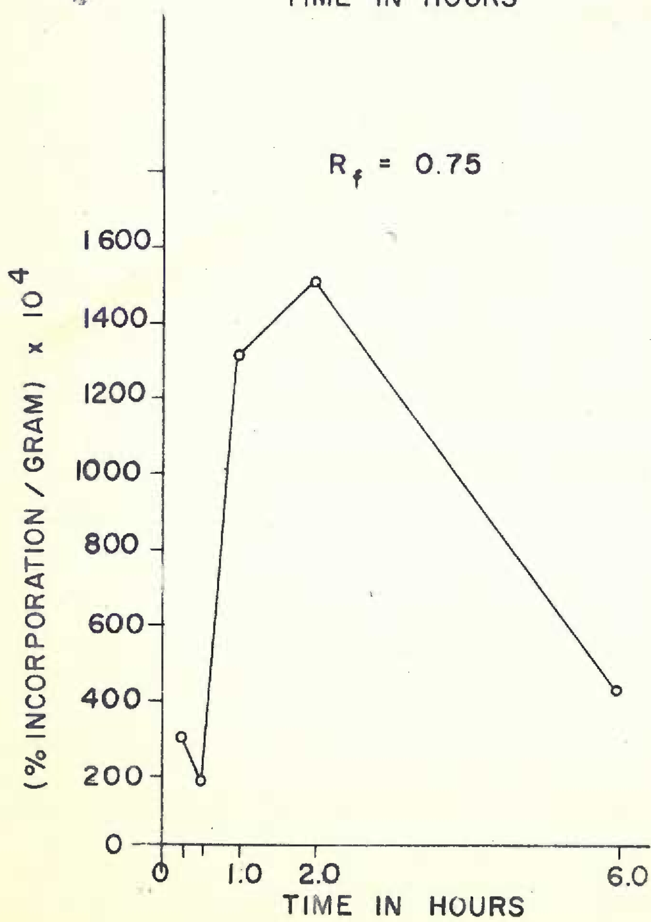
Increase or decrease of radioactivity at specific Rf values.

For any one Rf value, percent incorporation of acetate-1-C^{14} is plotted with respect to time and this represents the change in radioactivity (i.e. turnover) of the specific fraction.

Figure 2.







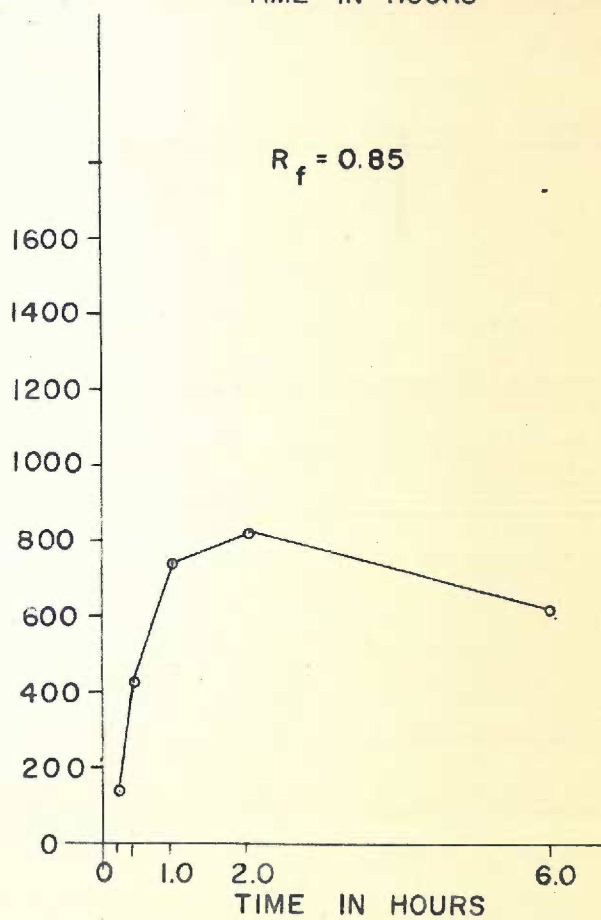


Figure 3

Time course of hydroxamate labeling of arterial tissue

The percent incorporation of acetate-1-C^{14} is presented at various Rf values at four time periods. As described in the text, the plotted data indicates the comparative "turnover" of radioactivity in arterial tissue hydroxamate fractions.

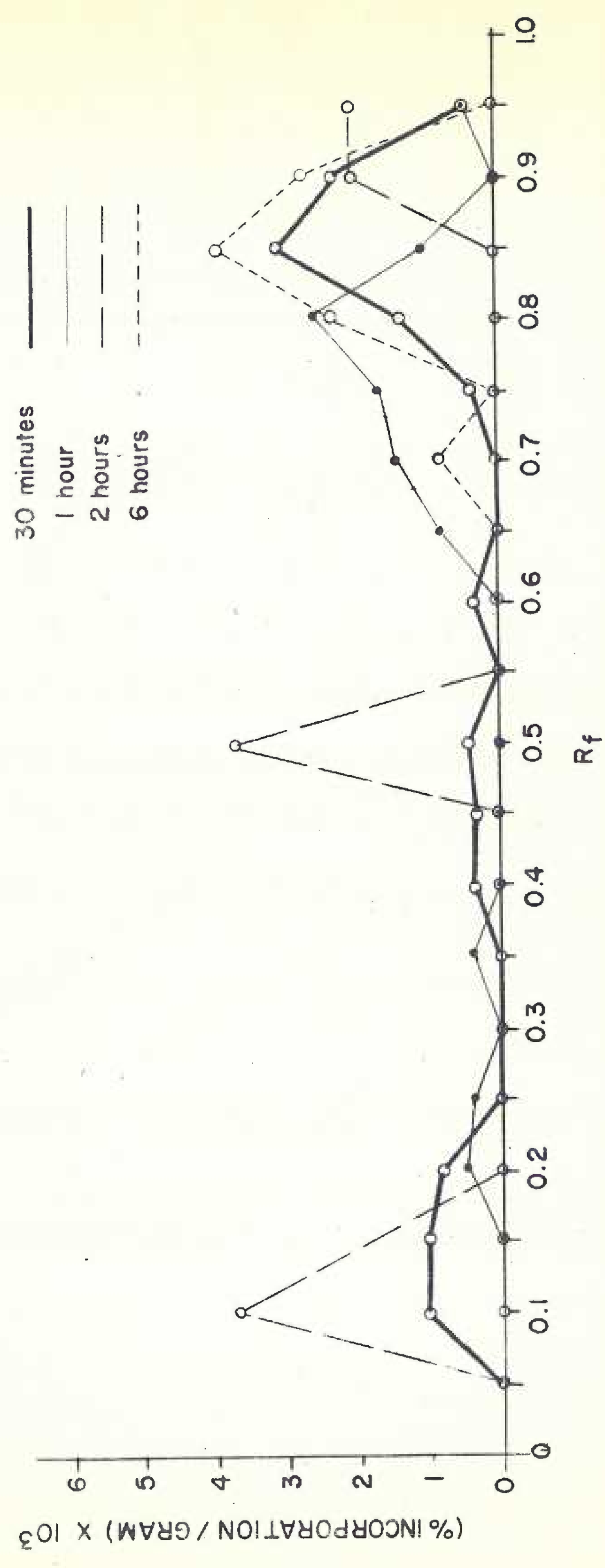


FIG. - NO. 3

(4) 0.85, as an example of an area with acyls of long chain fatty acids.

(5) Calculations were also done for values at R_f 0.22, but, due to its low activity, the graphical representation has been omitted. At this R_f value one would expect to find the malonyl or the succinyl derivatives, but Elwood and Van Bruggen have found (27) that by the heat treatment method of extraction of hydroxamic acids from tissues that succinyl CoA is completely hydrolyzed.

Figure 3 represents the same kind of information for the aorta. (The 15 minute samples for arterial tissue were lost).

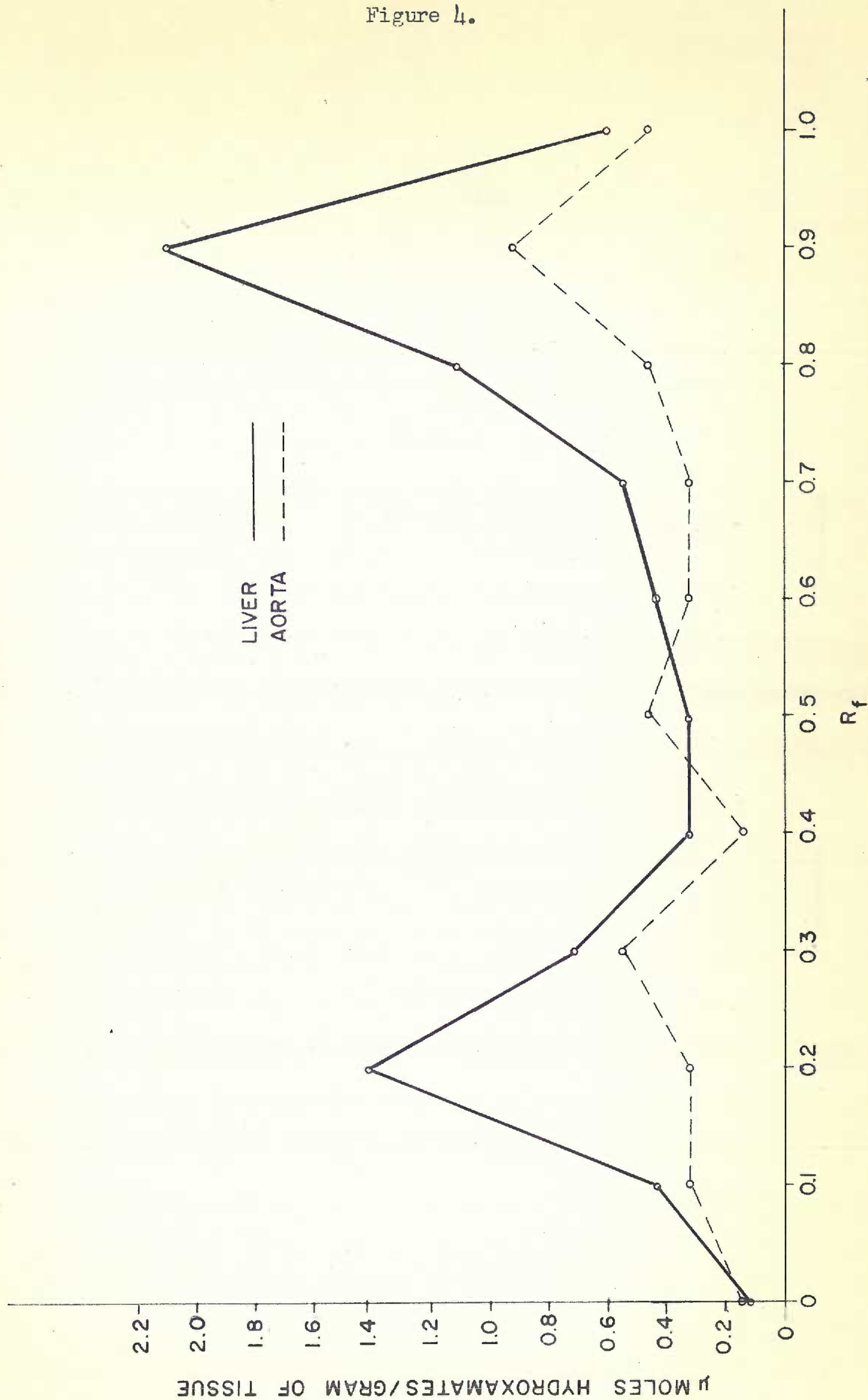
All calculations of turnover times of compounds using radioactive precursors require knowledge of the specific activity of the compounds analyzed; that is, amounts of these compounds must be measured. For this group of animals, and keeping in mind the unsatisfactory results obtainedafter eluting portions of the paper strip from the _in vitro_ studies, the assumption was made that all animals had, at the time of sacrifice, a comparable level of acyl derivatives of similar composition. All 10 strips (5 for each tissue) were cut into fragments representing 0.1 R_f units, and all strips from one tissue and for a same 0.1 R_f zone were eluted in the same tube regardless of the different time intervals. Elution was done as usual and the colorimeter assay determined on the extracts. Special care was needed when correcting values for a certain unit of tissue weight, for in this case, the hydroxamate derivatives in each tube represented the amounts for that R_f contained in all of the tissue samples. Figure 4 presents a quantitative evaluation of hydroxamate derivatives of different R_f values for liver and aorta expressed as μmoles per 1 gram wet weight of tissue.

Figure 4

Amounts of hydroxamic acids present in tissues.

The two lines represent the μmoles of hydroxamic acids present in one gram of liver or aorta tissue plotted against the position of the derivatives on the paper strip.

Figure 4.



The results obtained from the colorimetric analysis have been plotted (figure 4) as the ordinate at the R_f value of the particular range. The concentration of acetohydroxamate (R_f 0.55) is found to be 0.38 μmoles per gram of tissue both for liver and the aorta. As can be observed for the liver, the aceto-derivative is one of the hydroxamic acids found in the lowest concentrations, a finding which would be in accord both with the function of the organ studied, that is, the great variety of synthetic processes which occur in hepatic tissue, and with the key position of acetyl-CoA in intermediate metabolism. Acetyl CoA is a common intermediate for important sequences i.e. the tricarboxylic acid cycle, fatty acid synthesis, cholesterol synthesis, acetylating reactions, etc. That part of figure 2 corresponding to the 0.55 R_f zone shows a rapid decrease in activity already taking place 15 minutes after injection of acetate-1-C^{14}.

Intraperitoneally injected acetate is absorbed into the general circulation and from it is rapidly cleared in order to be metabolized at many different sites. If at such sites, it goes through the tricarboxylic acid cycle the carbon dioxide produced must be transported, via the blood, to the lungs. The whole process, as evidenced by radioactivity in the respiratory carbon dioxide, is very rapid and $C^{14}O_2$ is detectable less than 2 minutes after the injection of acetate.(75)

It has been observed, for the rat, that approximately 90% of the activity from injected acetate-1-C^{14} is found in the expired carbon dioxide within 1 hour after injection (75), therefore, the rapid decrease of activity of acetyl CoA observed in Figure 2 is probably an indication of its incorporation into the tricarboxylic acid cycle. If the chromatographic fraction of R_f 0.35 is considered to be made of

the fumaric acid derivative, one would expect the same kind of rapid decrease as is actually observed here. It must be pointed out however, that the formation of a fumarohydroxamate implies the existence of an active form of fumaric acid. Such an active form is not now recognized. The large amount of radioactivity in this fraction and the rapid turnover of it possibly suggests the presence of a compound related to active carbon dioxide, the derivative being carbanohydroxamate.

Although it is acknowledged that the number of observations presented do not justify a rigorous calculation of turnover rates, of any of the intermediary "pools", it does seem profitable to use the data in preliminary calculations. In order to be able to compare the rates of the different reactions, these were treated as first order reactions, and the rate of change per hour calculated from the tangent to the lines obtained by plotting the logarithm of percent incorporation against time. That rate obtained for the rapid decrease in activity of the acetohydroxamate fraction has been taken as the unit figure for all the other values. By this treatment the values have been unified and serve as comparative values rather than absolute ones. R_1 refers to the change taking place between 15 and 30 minutes after injection of the label, except for the case of acetoacetate where it has been calculated from the thirty to one hour period. R_2 represents the same kind of evaluation for the two to six hour period. Plotting results that decrease with time yields a negative number, while the opposite is true for an increase. The following figures in Table XVI were determined

TABLE XVI

	R_1	R_2
R_f of 0.55	-1	no slope
0.22	-1.29	-0.07
0.35	-0.81	-0.03
0.75	+0.68	-0.06
0.85	+0.40	-0.01

Considering now the amounts of hydroxamates formed and the comparative rates of turnover it is seen that for the liver, the amount of hydroxamates found at the R_f 0.10-0.20 region, 1.4 μmoles/g, is high when compared to the quantities of other derivatives. At this R_f one finds the malonyl derivative but with the area covered i.e. R_f 0.1-0.2, it cannot be stated that the material found here is only the malonyl derivative.

At the R_f zone 0.70-0.75 one would expect to find the acetoacetyl derivative or the free acid. It is probable that in the case of the liver the free acid is contributing some of the observed radioactivity for in this tissue the rate of the thiolase reaction is displaced towards the formation of the free acid from its active form. The amount of derivatives present at this zone is 0.8 μmoles per gram of hepatic tissue, while for the aorta it is half this amount. Activity in this fraction for the liver increases rapidly in the 30 minutes to one hour period, while from the two hour to six hour period there is a great loss in activity but at a slower rate, indicating that if this were acetoacetyl CoA it has, within a four hour period, starting approximately two hours after injection time, gone through almost one complete turnover of its metabolic pool, either by being transported to other organs or by going on to other synthetic processes, such as fatty acid synthesis.

The curve in figure 2 for the R_f of 0.85 shows an increase in activity at a rapid rate and a slow decay in activity from the two hour to the six hour period. Around this area and at somewhat higher R_f values one finds acyl-derivatives of long-chain fatty acids. The amounts of these components are in the level of 2 μmoles/gram for liver, and 0.90 μmoles/gram for the aorta. The radioactive acyls at this R_f zone are more probably those from the synthetic sequence for fatty acids, ready to be cleaved into the free acid and a coenzyme A molecule, rather than those originating from the first reaction of oxidation of fatty acids, that is, activation of the acid to its active acyl form. It has been shown (68) that once the oxidation of the long chain fatty acid begins, it is usually carried out until complete breakdown to two-carbon units so that intermediates probably do not mix and equilibrate in a common pool with those of synthesis of fatty acids.

For the aorta, only at the R_f zones from 0.6 to 0.95 does one find considerable radioactivity for all the time periods studied. However, it cannot be certain if this is due to endogenous synthesis or transport to the artery of these labeled intermediates.

Figures 5 and 6 show results for those animals used for isolating fatty acids and cholesterol from both tissues. In figure 5, the highest level of activity for the fatty acids of liver tissue, is reached within 30 to 60 minutes after the injection of labeled acetate. To obtain an approximate turnover time for one mg of fatty acid for this organ, the slope of the line between the one hour and the six hour period was used, and the turnover time was found to be 0.26 hours/mg of fatty acid. In the case of the aorta, a small level of activity was found to be maintained constant throughout the six hour interval, indicating a slow

Figure 5

Increase or decrease of radioactivity in fatty acids with time.

The lines for rat liver and aorta represent the percent incorporations of acetate-1-C^{14} into fatty acids of one gram of tissue at different time intervals after the injection of acetate-1-C^{14}.

Figure 5.

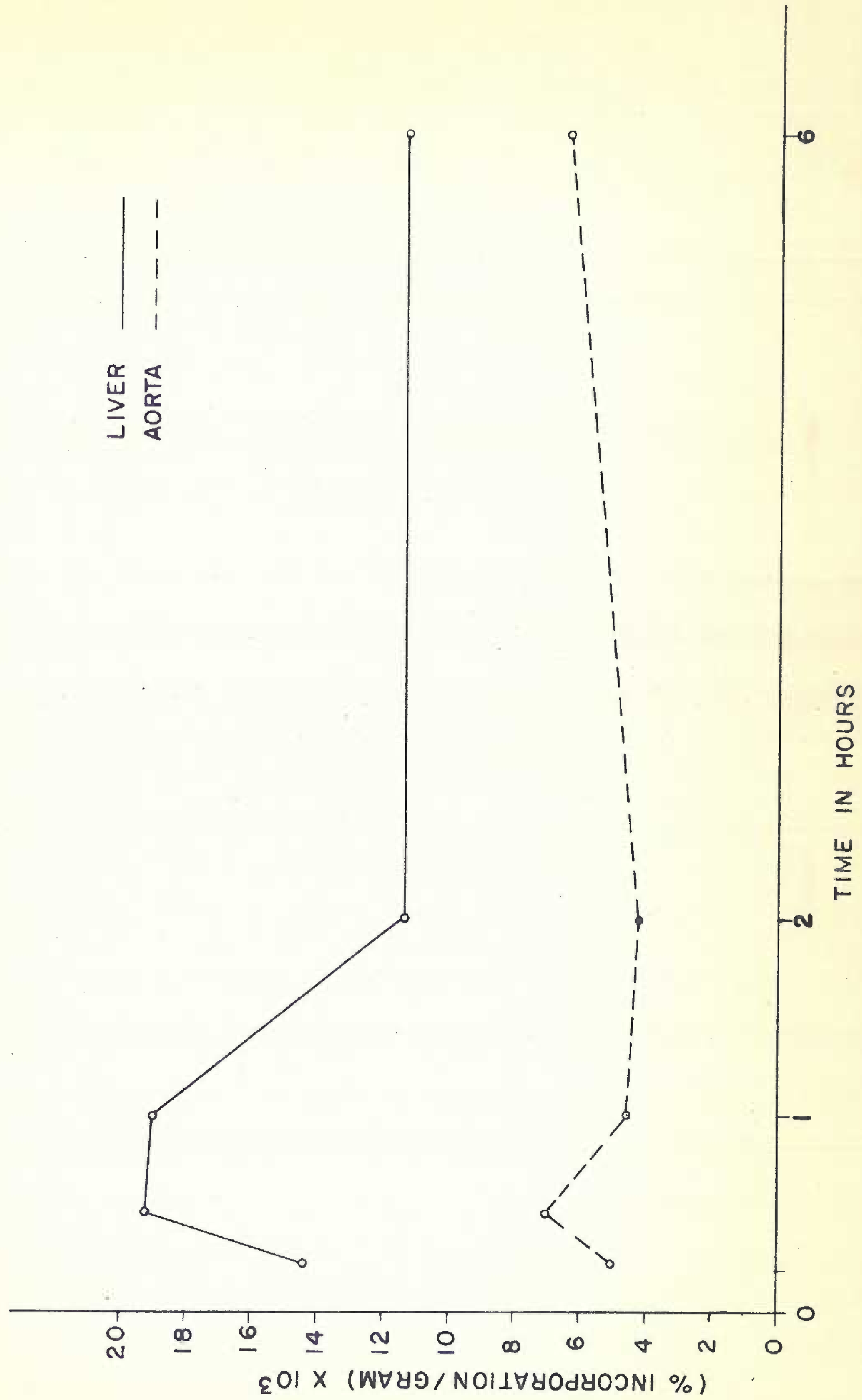
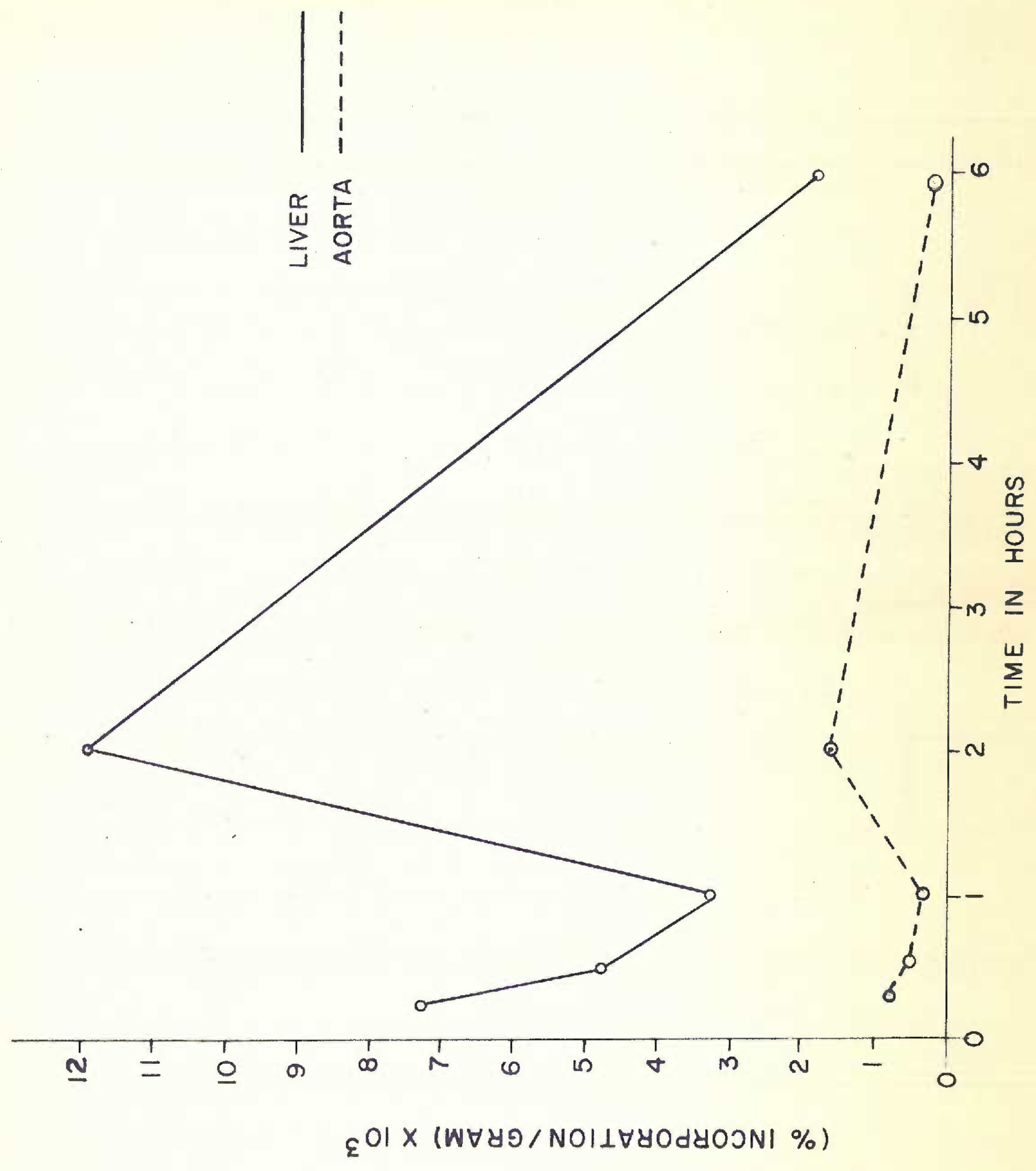


Figure 6

Increase or decrease of radioactivity in the cholesterol fraction with respect to time.

The line for rat liver represents the percent incorporation of acetate-1-C^{14} into cholesterol plotted against time elapsed after injection of the label. Radioactivity determined in the cholesterol digitonide or in the nonsaponifiable extract produced similar curves for liver. The line for the rat aorta represents the same change with time, as described for liver, but refers only to the nonsaponifiable fraction.

Figure 6.



metabolic process which again cannot be distinguished from deposition from the circulation.

Figure 6 represents similar studies for the digitonide fraction of liver. The nonsaponifiable fraction in this case gave a similar curve. The curve for the aorta represents only the activity from the nonsaponifiable fraction since activity was too low to obtain similar assays for the digitonide. For both tissues, activity was highest at the two hour point. Calculations of turnover rates resulted in a figure of 1.67 hours per mg of cholesterol for liver and 1.28 hours/mg for the artery. The figure for liver agrees with one found before (25) as a rapid component in a biphasic decay curve. The rate found for the artery seems high as compared to liver.

Conclusions:

The values found for the amounts of aorta hydroxamates and the radioactivity associated with them turned out to be so low that it was not possible to make reliable calculations of turnover rates. It is clear, however that the aorta does have active metabolic systems capable of activating metabolites. The net contribution of these systems to the variations in tissue lipids seen in disease processes is unknown.

Analytical information obtained for liver tissue was more amenable to evaluation and several approximations were made of turnover rates. In general, the turnover of the various compounds studied, agrees well with existing concepts of metabolic pathways. To account for the rapid appearance of $C^{14}O_2$ in the respiratory air after a ministration of acetate, a rapid flux of C_{14} through the metabolic pools is required. Turnover rates were also calculated for fatty acids and cholesterol extracted from both tissues. One cannot say for the artery that this value refers only to endogenous synthesis; Dayton (19) has

calculated that approximately one half of the artery wall cholesterol is due to transport from other sites. Nevertheless the approximate values obtained are important because this active moblization plus endogenous synthesis could be one of the factors that are deranged in those conditions that predispose to the development of lesions. The turnover rates found for the liver tissue agree fairly close to the rapid-component rates previously determined in this laboratory.

The influence of different diets on the in vitro capacity of the rat aorta to incorporate acetate 1-C^{14} into its cholesterol, fatty acid, and carbon dioxide fractions. The experimental conditions for these groups of animals have been described in the Experimental section and the results of these studies are presented in Table XVII. Although it is realized that the number of samples in several of the following groups is small, the information of the table is presented for two chief reasons.

The preparation of a group of animals on different diets for 4 weeks prior to sacrifice is cumbersome and time consuming. These animals were being utilized in another metabolic study (58), the results of which would not be hampered by removal of the aorta. It was thought of interest to use these samples to study the influence of diet on the in vitro metabolism of acetate by the arterial wall. If the results turned out to be essentially the same for the four diets, or if they showed a difference, the results could be used to determine if continuation of these studies would be of value and of interest.

As was also mentioned in the experimental description for this set of rats, these animals had been previously injected with mevalonate -2-C^{14} so that it was necessary to incubate one aorta from each group without added acetate 1-C^{14} in order to determine the presence of any activity from the mevalonate. If activity were to be found, besides being subtracted as a "blank", it would be of interest in showing in vivo labeling of that fraction from the mevalonate activity. The activity of the fraction would not necessarily however mean endogenous

TABLE XVII

COMPARISON OF AORTAS FROM RATS SUBJECTED TO

LOW FAT AND TO HIGH FAT DIET

DIETS		% Incorporation from Acetate-1-C^{14}/Hour/G of Tissue, in vitro							MG Cholesterol/G of Tissue	
		CO_2		Fatty Acids		Cholesterol (Non-Sap. Fraction)				
Kind of Fat	Amount of Fat	Individual Values	Means	Individual Values	Means	Individual Values	Means	Specific Activity CPM/MG Chol.	Individual Values	Means
Sat.	5-10%	15.10 20.42 18.74 18.30	18.14	0.09 0.16 0.32	0.19	0.013 0.037 0.070	0.04	1560	1.31 1.77 3.65 2.59 3.25 2.83	2.57
Sat.	30%	13.16 18.91 19.25	17.1	0.03 0.08 0.035	0.05	0.015 0.019 0.016	0.017	790	2.05 2.16 2.44 1.94	2.15
Un-Sat.	5-10%	15.40 10.40 8.42 10.00 9.03 13.26	11.09	0.41 0.16 0.23 0.20 0.11 0.22 0.07	0.2	0.028 0.031 0.029 0.030 0.020 0.080 0.014	0.033	2100	1.11 (6 pooled) 1.88 1.81	1.6
Un-Sat.	30%	13.50 17.41 17.88	16.26	0.11 0.12 0.33	0.19	0.084 0.033 0.080	0.067	2430	2.21 4.10 2.31 2.36	2.75
F Values			2.40		1.79		3.00			3.10

TABLE XVIII

In vivo incorp. from mevalonate-2-C^{14}.

Aortas incubated with no addition of acetate 1-C^{14}

rat #	Diet: Amount	Diet: Kind of Fat	Mevalonic Ac. Inj.: 2 Hrs.	Mevalonic Ac. Inj.: 4 days	Fractions: CO_2	Fractions: F.A.	Fractions: Chol.
	5%	Un-sat.	x		-----	-----	-----
	5%	"		x	-----	-----	-----
(12)	10%	"	x		None	None	None
	10%	"		x	-----	-----	-----
(16)	30%	"	x		None	0.006	0.015
(20)	30%	"		x	-----	-----	-----
(1)	5%	Sat.	x		0.08	None	None
(5)	5%	Sat.		x	0.025	None	None
(3)	10%	"	x		0.02	None	None
(7)	10%	"		x	0.34	None	None
(9)	30%	"	x		None	None	None
(14)	30%	"		x	None	None	0.002

synthesis by the arterial wall, but could represent transport and deposition at that site. Table XVIII presents these blank values for each group and shows activity to be present in only two cholesterol samples: that from the animal of the 30% unsaturated fat diet (2 hour mevalonic acid injection) and that for the 30% saturated fat diet (4 days mevalonic acid injection). CO_2 samples from most animals of the saturated fat diet showed a small amount of activity which was disregarded because even the largest value represented only 2% of the value usually found for this kind of preparation. Fatty acid labeling was found only in one sample belonging to the 30% unsaturated fat group (2 hours mevalonate injection).

Additional information to be kept in mind in the evaluation of the results in Table XVII is the fact that the original experiment consisted of 6 groups of rats each receiving diets of saturated fat and unsaturated fat in three different concentrations for each class (5% - 10% - 30%). In tabulating and analyzing the data we have regrouped samples as to amounts of fat in the diet so that by pooling the first 2 original groups (5-10%) we obtain a "low fat diet" group which is compared to a "high fat diet" group represented by the 30% levels.

It should be kept in mind that if additional numbers of animals were to be available for each of the groups, differences between even the 5 and 10 percent levels of feeding might become apparent.

Statistical analysis of data from group of diet study animals.

The F values given in Table XVII were calculated by an analysis of variance for the means of the different groups. These F values are smaller than those required for the 0.05 confidence level therefore

no differences are shown for the means within each group ("groups" in this case are: (1) CO_2 - % inc., (2) F.A.-% inc., (3) CHOL -% inc., (4) CHOL - mg/gram). Since both the F test and t test for comparison of means assume equal variances we decided to apply some t determinations to this data because the t test is not too susceptible to unequal variances while the F test is. We elected to apply the t test only to those means that appeared to be grossly different:

(1) <u>% incorporation into CO_2</u>

(a) Unsaturated 5-10% vs the mean of all other samples pooled. Significantly different at 1% level.

(b) Unsaturated 5-10% vs. unsaturated 30%: Significantly different at 5% level.

(2) <u>% incorporation into F.A.</u>

(a) Saturated 30% vs the mean of all other samples pooled. Significantly different at 5% level.

(3) <u>% incorporation into Cholesterol.</u>

(a) Saturated 30% vs unsaturated 5-10%: Not significant.

(b) Saturated 30% vs saturated 5-10%: Not significant.

(c) Saturated 30% vs unsaturated 30%: Significantly different.

(d) Saturated 5-10% vs unsaturated 30%: Not significant.

(e) Unsaturated 5-10% vs unsaturated 30%: Significant at 1% level.

(4) <u>Amounts Cholesterol.</u>

(a) Saturated 30% vs unsaturated 5-10%:

Not significant.

(b) Unsaturated 5-10% vs mean of all other samples:

Not significant (almost significant at 5% level)

(c) Unsaturated 5-10% vs saturated 5-10%:

Not significant.

(d) Unsaturated 5-10% vs unsaturated 30%:

Not significant at 5% level (Significantly different at 10% level)

From this statistical evaluation of the data, results considered to be the more significant will be briefly discussed:

1) The group on a 30% saturated fat diet showed a significantly smaller incorporation figure for fatty acids as compared to the mean of all the other samples pooled.

2) The 30% saturated fat group also shows a significantly smaller incorporation into cholesterol as compared to the 30% unsaturated group.

3) The 30% unsaturated group has a significantly larger incorporation into cholesterol than does the 5 to 10% unsaturated group. When amounts of cholesterol are also considered, the specific activities of the cholesterol fraction turn out to be the same.

Wood and Migicovsky (76) have compared effects of 20% oils in the diet of rats with others containing 9% fatty acids present in these oils; the workers have concluded that it is essentially the fatty acids of the diet that determine its action. They have also found that unsaturated oils increased total cholesterol in the liver and stimulated incorporation of acetate-1-C^{14} into cholesterol both in vivo and in

vitro by liver homogenates. Saturated oils, on the other hand, decreased liver cholesterol and in vivo incorporation of acetate-1-C^{14} into that same fraction in vivo (this effect was not shown in vitro). It is interesting to observe that the two 30% fat groups above show similar results with aorta tissue to those of Wood et al. The specific activity figure for the 30% saturated diet is lower than that of the 30% unsaturated. This could indicate a decreased lipogenesis for the aortas of the 30% saturated fat diet group. Such results would agree with the finding of Wood and Migicousky as to incorporation by liver in vitro but do not agree in that the amounts of cholesterol are the same. At a 90% confidence level, the low fat diet animals have less aortic cholesterol than those of the 30% diets, and this finding appears to be more related to amount of fat than to the class of fat in the diet.

SUMMARY

Because this thesis presents several different approaches to the study of the metabolism of diabetic tissue, the results of the various approaches have been summarized in the respective sections.

For the study on utilization of ketone bodies, the biological preparations that have been used are three kinds of muscle tissue, leg adductor, diaphragm and heart muscle. These tissue preparations represented the extrahepatic tissue for the study of ketone body utilization. Liver slices from the same animals were also incubated with the labeled compounds. It was found that of the three muscle preparations, the heart muscle slice is the most active in the oxidation of acetate. The literature suggests that a high degree of activity in a muscle indicates a great mitochondrial density and a more active tricarboxylic acid cycle. These suggestions lend support to the present results. Amounts of lipid in muscle were found to be the same for the normal and diabetic preparation. Lipid labeling from acetate was greatly reduced in the diabetic as has been previously shown for hepatic tissue. With acetoacetate-3-C^{14} in the incubating media, radioactivity of the CO_2 fraction showed a significant decrease in the diabetic preparation as compared to the normal. Before interpreting these results to mean a decrease in utilization of ketone bodies by muscle tissue it is important to define terms. It has been shown previously that the uptake of, or that amount of the labeled material that is actually made available to the inside of the cell, is less in the diabetic condition. Approximately half of the acetoacetate is available as a metabolite to the diabetic muscle fibers when compared to normal fibers. If incorporation figures,

which are reduced to one half of the value of the normals, are compared on the basis of what is in contact with the interior of the cell, utilization of this ketone in vitro by rat tissue would be the same for normal and diabetic animals. If, however, the concept of utilization involves the step of crossing from an extracellular to an intracellular environment, then utilization is decreased in the diabetic animals.

For the study of metabolism by the aorta, arterial wall tissue has been used from rats and from goats. In the case of the rat aorta the vessel was stripped of the adventitia, sliced open longitudinally and incubated, making the assumption that diffusion was not a limiting step for this kind of preparation. For the goat aorta, which is a much larger vessel than that of the rat, it was necessary to cut rings of approximately 1 mm thickness or less. The aorta, considered as an organ in these metabolic experiments, is quite unique in having much connective tissue and very few cellular elements. These cells consist of some fibroblasts, some mast cells and a small number of smooth muscle fibers. The relative number of these elements varies according to the proximity of the segment to the heart. In all cases, the same segment of aorta was used and rats were all of one age group and, within one experimental diet group. Experiments on arterial tissue were divided into three groups and the results will be summarized following this classification -- In vitro experiments: In this case it was observed that aortic tissue of the rat respired quite actively for such an acellular structure, putting considerable activity from acetate-1-C^{14} into the carbon dioxide fraction. In analyzing for amounts of lipids

it was found that the diabetic tissue had increased amounts of lipids as compared to the normal which might be a factor favoring the consequent development of a lesion in the diabetic. This finding brought out the importance of the possibility of studying (a) transport of the lipid material to the vessel wall since the present experiments do not permit conclusions on in situ synthesis independent of that amount which is transported from other sites; (b) some measurement of the degree of turnover for intermediates and end products of the normal animal so that in later studies these values would be used as a parameter for the diabetic.

Work with the rat artery is difficult because of the small amount of tissue that is obtained from each animal. The possibility of obtaining a larger sample of aorta from the goat through the kindness of the Surgery Department of the University of Oregon Medical School at first appeared as a solution to the problem. Unfortunately, the artery of the goat being a much larger vessel needs more structural material to maintain its lumen so that the ratio of cells to connective tissue is, in this case, even smaller. Incubation of this preparation with acetate-1-C^{14} gave very low figures for radioactivity in the end products. The presented results therefore, only indicate a viable tissue which, either because of the histological differences just mentioned, or because of the use of rings instead of the total longitudinally sliced organ as for the rat aorta, has a much smaller metabolic activity than that of the rat.

In our experiments a method was developed to isolate hydroxamic acids from tissue which would represent those activated acyl derivatives present at a finite moment in the tissue preparation. The purpose here

was to attempt to establish the approximate level of such compounds, or groups of compounds, and to, if possible, calculate turnover rates of these fractions after injecting acetate-1-C^{14} and allowing several time intervals for its metabolism. Determinations were done with livers and aortas of normal rats at 5 different time intervals. The amounts of 10 fractions, spread out in paper strip 0.1 R_f units apart, were determined colorimetrically. From these values the lowest amounts for liver were obtained in the zone where the acetohydroxamate would be found. The largest amounts found for liver were in the zone of the long chain fatty acids and also in the lower R_f values where malonyl derivatives might be present. In following the time course of activity for liver, the greatest concentration of activity for all samples and zones was at the 15 minute period in the area where the carbano-derivative might be found. A number of compounds were analyzed as to their rate of decay. These were pooled samples from only a few animals and the data cannot be taken as being complete. Amounts of hydroxamates, for the aorta, were within the range of good recoveries by the colorimetric procedure, but the radioactivity found in these fractions was very small. The greatest build-up of activity for the aorta was secured at the zone of the long chain fatty acids and acetoacetate.

From the amounts and the activity in the end products, turnover rates were calculated. Those for liver coincide with the findings of a rapid component that has previously been described in this laboratory. The curve for the liver non-saponifiable fraction and for the cholesterol digitonides was essentially the same. For the aorta, only the non-saponifiable extract could be analyzed. Rates of turnover were calculated

for this tissue also and from the high values obtained, it is clear that the transport of lipids from other sites could well account for one half or more of the lipid found.

Influence of diet on arterial metabolism of acetate: The differences in the diets of this group of animals were both in the amount of fat and in the degree of saturation or unsaturation. Arterial tissue from the animals was studied for its capacity to incorporate acetate-1-C^{14} *in vitro* into CO_2, fatty acids and cholesterol. The number of animals does not permit applying high statistical significance to the results, but it can be commented that the higher amount of fat in the diet seems to parallel an increase in cholesterol in tissue. A decreased labeling of fatty acids and cholesterol seems to accompany the saturated fat diet indicating the possibility of a decreased rate of synthesis for these compounds.

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