THIOL ACYLS AND

ACETATE METABOLISM

by

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A THESIS

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ABSTRACT

The formation of hydroxamic acids from biological material may proceed by either enzymatic or non-enzymatic reactions. Conditions are presented that allow the use of hydroxylamine in biological systems to measure non-enzymatically the amount of reactive anhydride and thiol acyls present, with subsequent separation of these intermediates. Reaction of hydroxylamine with the high energy thiol acyls is shown to be rapid and quantitative. Evidence is presented showing the optimum concentration of hydroxylamine in determining anhydrides and thiol acyls while eliminating carboxylic esters from reaction. A method is described for specifically inhibiting the enzymatic reactions, and also a complete procedure for the quantitative recovery of hydroxamates formed is given. The procedure consists of heat inactivation of the enzymes with subsequent quantitative fractionation of intermediates. Alcoholic filtrates of reaction mixtures allow the preparation of concentrates suitable for chromatographic analysis and radioassay. Procedures are presented describing two different methods of assay for radioactivity on paper chromatograms. Experiments are described which show the amount of thiol acyls present in liver tissue of fasting rats and the degree of incorporation of radioactive acetate-1-C^{14} into various fractions of rat liver homogenate. Conditions are presented that reflect the need for more precise definitions in relation to our concepts of the size

of a tracer dose of radioactive material. Attempts are made to define a tracer dose and data are presented to verify the statements made. Part of this study is a continuation of work presented by R. J. Emerson in a thesis for the Master of Science Degree (1957) in which he conducted an acetate-1-C^{14} concentration study and followed the incorporation of this isotope into CO_2, cholesterol, and fatty acid fractions of rat liver slices.

CHAPTER I

INTRODUCTION

Preliminary Remarks

The history of science will long record the date 24 February, 1896. It was on this day that the Frenchman, Henri Becquerel, reported his first evidence of the phenomenon that was later to be termed radioactivity. The problem of determining the nature of the emitted radiations and the subsequent development of our present concepts is a familiar story to most. That scientific horizons have been greatly extended by these developments in radioactivity is questioned by none. The present thesis is a result of the application of radioisotopes to metabolic pathways.

Within a relatively short period three main avenues have been opened for the application of radioisotopes to biology and its related sciences. The first of these avenues has been the medical use of radioisotopes in the diagnosis of disease. The diagnosis of thyroid dysfunction from data obtained by the "uptake" of I^{131} by the thyroid gland may be cited as an example. The second avenue of application in medicine has been in the actual treatment of disease, such as the bombardment of cancerous tissue by some radioactive source. The third avenue, and the one from which the first two had their beginnings, is the use of radioisotopes in basic biological research.

The mechanisms of intra-cellular synthesis and reactions of complex molecules in metabolic pathways are almost universally being studied in some aspect by the use of stable or radioactive isotopes. The ability to label organic and inorganic compounds with isotopes has made possible the elucidation of problems which could not have been attacked prior to this new era.

The use of isotopes has been in the past and promises to be in the future a most powerful tool in the hands of the competent scientist. It must be remembered, however, that this new method of study does not make the problems studied any less complex, on the contrary, it adds complexity in new analytical techniques to already complex situations. From this complexity are beginning to come well defined areas of research and well defined methods or techniques of approaching biological problems. This thesis is concerned with one of these areas; lipogenesis, and the defining of tracer conditions or "tracer methodology".

Review of Lipogenesis

The productive research of lipid metabolism started in 1904 with Knoop's hypothesis of the beta-oxidation of fatty acids, but it was a full fifty years before his hypothesis was born out by studies of individual enzymes. Green[1] has divided this fifty years into three periods:

1- 1904-1939, when fatty acid oxidation could be studied only at the level of organized or intact systems such as the whole animal, the perfused organ, or the tissue slice.

2- 1939-1954, when fatty acid oxidation could be studied at the mitochondrial level.

3- 1952 onwards when fatty acid oxidation could be reconstructed in non-mitochondrial and soluble enzyme systems.

Knoop's experiments are well known, but being classics, are worthy of repetition. Knoop fed omega-substituted phenyl fatty acids to animals and then isolated various metabolic end products from the urine. Depending on the chain length of the fatty acids fed, odd or even, he could isolate hippuric or phenylaceturic acid in the urine of the experimental animals. These products being benzoic or phenyl-acetic acid conjugated with glycine. Knoop explained the appearance of these end products as the result of degradation of the alkyl side chain by two carbon cleavage as follows:

$$Phenyl-CH_2CH_2CH_2CH_2COOH \longrightarrow Phenyl-CH_2CH_2COOH \longrightarrow Phenyl-COOH$$

Knoop then postulated the following sequence (arrived at independently by Dakin[1] in 1912):

$$R-CH_2CH_2COOH \longrightarrow R-CH(OH)CH_2COOH \longrightarrow R-CH=CHCH_2COOH$$

$$R-CH=CHCH_2COOH \longrightarrow R-\overset{O}{\overset{\|}{C}}CH_2COOH \longrightarrow R-COOH + CH_3COOH$$
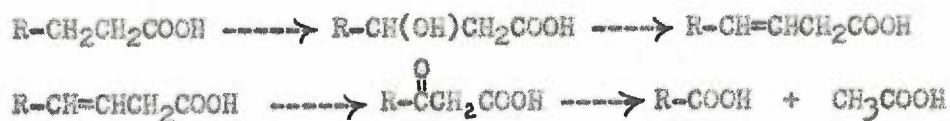
During the interim of Knoop's hypothesis of beta-oxidation and the reconstruction of soluble enzyme systems by numerous workers[1,2,3,4] a great deal of research has been recorded. Nowhere in this recorded work is there unequivical proof that fatty acid synthesis is the reverse of fatty acid oxidation. Green has shown that the reactions in his reconstructed enzyme systems are reversible, thus providing a means of building long chain fatty acids from C_2 units. Most workers consider, as a working hypothesis, that synthesis is the reversal of oxidation, and until someone shows the contrary it will

undoubtably remain a common hypothesis. There have been attempts to show that oxidation and synthesis take place at different geographical locations within the cell[5], but this experimental work is open to some question.

Acetate Activation

The oxidation of fatty acids according to current concepts involves the formation of an "active acetate" which can be defined as a C_2 unit which is capable, as one of its functions, of condensation with oxalacetate to form citrate and so enter the tricarboxylic acid cycle[6].

Lipmann[7,8] and his colleagues announced the participation in acetylation reactions of an entity which they termed Coenzyme-A (1948-49), and later Lynen, Reichert, and Rueff[9] identified acetyl-S-CoA as the "active" form of acetate (1951). These discoveries and the subsequent extensive studies of beta-oxidation of fatty acids led to the realization that fatty acids are metabolized as Coenzyme-A derivatives[10,11]. The finding of Lynen et al coupled with those of Lipmann and Ochoa and others, that established acetyl-S-CoA as the active form of acetate, have also shown it to be an ester in which the acetyl group is bound in a thiol ester linkage to the thiol-ethanolamide of pantothenic acid. The accepted formula for Coenzyme-A is as follows[12].
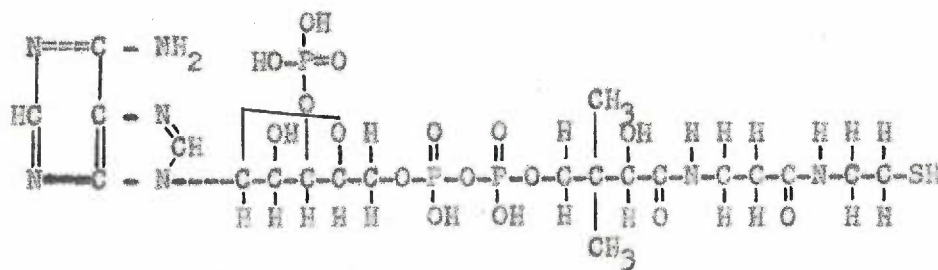
Once a fatty acid is converted to its acyl derivative and is acted upon by a mitochondrial system, the next recognizable products are citrate or acetoacetic acid. It is presumed by most that the acyl-S-CoA intermediates are enzyme bound. The dissociation of the fatty acyl-S-CoA's from the surface of the enzyme is so slight that it does not allow their equilibration with their free fatty acids and consequently no intermediary acyl-S-CoA derivatives accumulate in significant amounts[13]. In a soluble enzyme system it is possible to isolate most any desired acyl-S-CoA derivative. The predominance of C_{16} and C_{18} fatty acids in mammalian lipides may be due to the fact that the acyl-S-CoA of only these particular acids dissociate to a sufficient degree from combination with the enzymes of the fatty acid oxidizing system so as to become available for carboxylic ester synthesis[14].

The current concepts of fatty acid synthesis involves the activation of acetic acid to "active acetate" or acetyl-S-CoA and condensation of the latter to form fatty acids and cholesterol. Common to several of these pathways are activated Coenzyme-A intermediates of various acids similar to acetyl-S-CoA.

A number of different mechanisms are known for the activation of acetate. One pathway found in microorganisms only is initiated by the phosphorylation of acetate with adenosine triphosphate (ATP). This reaction is catalyzed by the enzyme acetokinase[15,16]. Another enzyme, phosphotransacetylase, is responsible for the transfer of the acetyl group to Coenzyme-A[17,18]. This reaction is as follows:

ATP + Acetate $\longleftrightarrow$ Acetyl-PO_4 + ADP

Acetyl-PO_4 + CoASH $\longleftrightarrow$ Acetyl-S-CoA + PO_4

Activation in mammalian tissue and yeast have been shown to occur by
way of a different reaction. This involves the reaction of adenosine
triphosphate, acetate, and Coenzyme-A with the formation of acetyl-
S-CoA, adenosine diphosphate and pyrophosphate and has been termed
the aceto-CoA-kinase reaction

$$ATP + Acetate + Coenzyme\text{-}A \longleftrightarrow Acetyl\text{-}S\text{-}CoA + A5P + PP$$

From a recent investigation by Berg[19] into the mechanism of this
reaction has come the new acyl-adenylate method of acetate activa-
tion. Berg proposed a primary reaction of adenosine triphosphate
and acetate to form a compound "adenyl-acetate" and its subsequent
reaction with Coenzyme-A to form acetyl-S-CoA as:

$$ATP + Acetate \longleftrightarrow Adenyl\text{-}acetate + PP$$
$$Adenyl\text{-}Acetate + Coenzyme\text{-}A \longleftrightarrow Acetyl\text{-}S\text{-}CoA + A5P$$

Jenks[20] has reported the formation of adenyl-octanoate from pig
liver enzymes. It seems possible that acyl-adenylate formation may
represent a general mechanism for the activation of fatty acids.

Enzymes of Lipid Synthesis

Three enzymes in animal tissue have been demonstrated that
catalyze the following reaction:

$$Fatty\ acid + ATP + Coenzyme\text{-}A \longleftrightarrow Fatty\text{-}acyl\text{-}S\text{-}CoA + AMP + PP$$

These three enzymes are classified by Green[1] as:

1- Acetate activating enzyme (specific).

2- Short chain activating enzyme ($C_4 - C_{12}$).

3- Long chain activating enzyme (greater than C_{12}).

There is one other activating enzyme and it is specific for aceto-acetic acid and other keto acids. The short chain activating enzyme works on the alpha-beta unsaturated acids and to some extent on beta-hydroxy acids, but beta-keto acids are unaffected.

With the foregoing information a schematic diagram of fatty acid synthesis in mammalian tissue can be devised which is acceptable to most workers in the field. As stated earlier, this scheme is nothing more than the reverse of fatty acid oxidation and will serve as a working hypothesis.

The next step after activation of acetate is its condensation to form acetoacetyl-S-CoA which is mediated through the acetoacetyl thiolase enzyme or more commonly called the condensing enzyme[21,22].

$$2 \; CH_3CO\text{-}S\text{-}CoA \;\longleftrightarrow\; CH_3\overset{O}{\overset{\|}{C}}CH_2CO\text{-}S\text{-}CoA \;+\; CoASH$$

Acetoacetyl-S-CoA is then converted to the beta-hydroxy acid by the addition of a hydrogen and the enzyme involved in this sequence is beta-hydroxybutyryl dehydrogenase[23].

$$CH_3\overset{O}{\overset{\|}{C}}CH_2CO\text{-}S\text{-}CoA \;\underset{DPNH}{\overset{DPN}{\longrightarrow}}\; CH_3CH(OH)CH_2CO\text{-}S\text{-}CoA$$
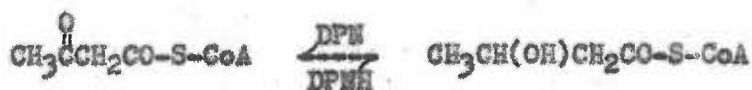
The next step in fatty acid synthesis is the formation of crotonyl-S-CoA which is the alpha-beta unsaturated form of butyryl-S-CoA and is promoted by the enzyme crotonase[24].

$$CH_3CH(OH)CH_2CO\text{-}S\text{-}CoA \;\underset{-\;H_2O}{\overset{+\;H_2O}{\longrightarrow}}\; CH_3CH{=}CHCO\text{-}S\text{-}CoA$$

Crotonyl-S-CoA is next converted to butyryl-S-CoA via the enzyme system butyryl dehydrogenase[25].

$$CH_3CH=CHCO-S-CoA \xleftarrow[\text{TPNH}]{\text{TPN}} CH_3CH_2CH_2CO-S-CoA$$

This sequence of events is then repeated adding acetyl-S-CoA units to the preexisting acyl-S-CoA moities and proceeding on to long chain fatty acids.

Because of the central position of acetate in intermediary metabolism almost any metabolite may be considered as a potential source of carbon for fatty acid formation.

Scheme of Fatty Acid Synthesis from Acetate

CH_3COO^-
Acetate

$CH_3CO-S-CoA$
Acetyl-S-CoA

$CH_3COCH_2CO-S-CoA$
Acetoacetyl-S-CoA

Tricarboxylic $CH_3CH(OH)CH_2CO-S-CoA$ Cholesterol
Acid   beta-OH-butyrl-S-CoA
Cycle

$CH_3CH=CHCO-S-CoA$
Crotonyl-S-CoA

$CH_3CH_2CH_2CO-S-CoA$
Butyrl-S-CoA

$CH_3(CH_2)_nCOCH_2CO-S-CoA$
Beta-keto-acyl-S-CoA

$CH_3(CH_2)_nCHOHCH_2CO-S-CoA$
L-beta-OH-acyl-S-CoA

$CH_3(CH_2)_nCH=CHCH_2CO-S-CoA$
Trans-a,b-dehydro-acyl-S-CoA

$CH_3(CH_2)_nCO-S-CoA$
Acyl-S-CoA

Acyl-adenylate

Fatty Acid

Biological Thiol Acyls

As can be seen from the above scheme, there are a variety of thiol acyl intermediates in the synthesis and oxidation of fatty acids according to the accepted pathway. In our study of intermediates in lipogenesis, it was desired to obtain means of quantitatively determining some of these thiol acyls common to this pathway and isolating them if possible. It was also desired to measure the specific activity of some of the thiol acyl precursors of fatty acids using radioactive acetate-1-C^{14}.

Hydroxylamine and the Formation of Hydroxamic Acids

Hydroxylamine has been used by many investigators as a trapping agent in enzymatic reactions[27,28,29,30]. The literature also contains considerable information on the relative reactivity of carboxylic derivatives in organic solvent systems. An investigation was conducted in this laboratory to ascertain if this reagent could be used to convert, non-enzymatically, some of the thiol acyl derivatives in an aqueous medium to their respective hydroxamic acids.

Hydroxamic acids were first described by Lossen in 1869 when he observed that they could rearrange to form iso-cyanates (Lossen rearrangement). The analytical possibilities of hydroxamic acids came to the fore in 1935 when Feigl and coworkers reported on a spot test for esters and anhydrides. His test was based on the color reaction of hydroxamic acids with ferric chloride solutions to form purple colored complexes. Since that time the reaction with hydroxylamine to form hydroxamic acids has been used to detect and determine many types of esters, amides, anhydrides, nitriles, sulfonic acids, aldehydes, etc.[31,32,33,34,35].

Evidence has been presented[36] that hydroxylamine under specified conditions will selectively form hydroxamates of water-dispersed thiol esters while the conditions will exclude from reaction, carboxylic esters. It was felt that this particular reaction, coupled with the formation of a colored complex with iron, and the separation of hydroxamic acyds by paper chromatography would give us a sensitive tool for studying the acyl-S-CoA intermediates formed during the intracellular synthesis of lipides.

Acetate-1-C^{14} and "Tracer Conditions"

Work in this laboratory has shown that the amount of acetate-1-C^{14} incorporated into the lipides of rats varies with the period of fasting prior to the administration of acetate. This is true in in vivo experiments[37] as well as in vitro[38]. This bears out the fact that fasting, as a tool in the study of fat metabolism, is a variable to be accounted for in any study of lipogenesis. A portion of the experimental data of this thesis shows the effect of fasting upon the concentration of acyl-S-CoA intermediates in the liver slice of rats.

Further work in this laboratory[38] has shown that a more critical evaluation of "tracer" conditions is needed if the total flux of acetate carbon into various lipid fractions is going to be measured and compared with other work. It has become apparent that much lower concentrations of acetate are needed than have been in common use in most laboratories if the systems being studied are not to be influenced by the tracer being employed. Part of this work is to elucidate the meaning of "tracer conditions" and another part is an attempt to show where the pathways in lipogenesis are affected when

tracer conditions are violated by the increased concentration of acetate. It was thought that if radioactive acetate-1-C^{14} could be presented to liver slices of animals which had been trained to feeding, that the radioactive intermediates trapped through the use of hydroxylamine would give insight into the mechanism of intra-cellular synthesis. At the same time, quantitization of one or a few of these intermediates might allow some treatment of the rela-tive rates of synthesis and the variables which affected these rates.

Summary of Objectives Concerning this Work

The first problem considered was the quantitization of thiol acyls by the formation of hydroxamic acid derivatives using hydroxyl-amine as reagent. This reaction is shown to be rapid and quantita-tive. Conditions will be presented that will allow enzymatic or non-enzymatic formation of hydroxamic acids depending on the purpose of the study; the trapping of intermediates or the determination of a-mount present at a given time. The problems of isolation and separa-tion of these derivatives from biological tissue were undertaken with good results. Solvent extraction was found to be satisfactory for isolation and paper chromatography gives a good method for separa-tion of the hydroxamic acids formed. Methods for determining the amount of radioactivity associated with various derivatives on the paper chromatograms is presented and discussed.

The next large area of work involves the following of tagged molecules, acetate-1-C^{14}, into the various intermediates of fatty acid and cholesterol synthesis. The purpose of this study was an attempt to better understand lipid synthesis, but also to demon-strate the intermediates which are most susceptible to increasing

concentration of acetate. It was felt that if we could "push" our system to its capacity, as regards its utilization of acetate, then we could isolate intermediates which were increasing in total amount of C^{14} incorporated from the administered acetate-1-C^{14}. Studies were also made that give verification to the fact that a compartment size can actually be increased by allowing this particular system access to "larger" quantities of acetate. These "larger" quantities are shown to be much less than many people would consider to be sufficient to affect the system. With this information it is felt that more definitive statements can be made as regards tracer conditions and perhaps a better understanding of tracer methodology will ensue.

CHAPTER II

EXPERIMENTAL

Methods and Materials

Preparation of Derivatives

To initiate a study of biological intermediates by the formation
of some derivative it is desirable to have at hand samples of the der-
ivatives which are expected to be encountered in the biological system.
That carboxylic esters can be converted to hydroxamic acids has been
shown by many authors.[39,40,41,42]

$$R\text{-CO-OCH}_2\text{CH}_3 \quad + \quad NH_2OH \longrightarrow \quad R\text{-CO-NHOH} \quad + \quad CH_3CH_2OH$$

From Feigl[43] came the information that the product of this
reaction gave a characteristic red-violet color when complexed with
iron. Thus, there is available a method of preparing the hydroxamic
acids and also a method for detecting and determining the amount
formed. The actual preparation of a variety of hydroxamic acids was
carried out using the methods of Y. Inoue and H. Yukawa[44]. Since
we modified the original procedures and since the original papers
are in Japanese*, an outline of a procedure for synthesizing these
compounds will be presented.

The ratio of molar quantities used in these preparations were
as follows: ester:NH_2OH:KOH, 1:1:2. The volumes of solvent ethanol
and methanol was varied, but the ratio of the methanol to ethanol
was maintained at approximately 1:2.

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* We are grateful to Dr. Takahashi for his kind assistance in
translation.

1- Stearohydroxamic Acid, Molecular Wt. = 299, (C_{18}).

Hydroxylamine hydrochloride, 0.64 grams, is dissolved in 13
ml. of absolute methanol and 30 ml. of absolute ethanol. Methyl
stearate, 2.6 grams, is added to the solution and then 1.0 grams
of potassium hydroxide is dissolved in a minimum of absolute
alcohol and added slowly with shaking. A white precipitate (KCl)
occurs instantly. The flask is sealed with a rubber cork covered
with para-film and left at room temperature. A more copious
white precipitate, the potassium salt of the hydroxamate, forms
on standing. After a sufficient length of time, 36 hours, this
solution and precipitate are cooled in ice and filtered while
cold. The precipitate is washed with water and dried. The dried
precipitate is dissolved in 10 ml. of ethanol and neutralized,
first with alcoholic HCl 2 N and then alcoholic acetic acid mix-
ture until the solution becomes slightly acid to congo red.
Petroleum ether, 15 ml., is added to this solution and the mix-
ture cooled in ice resulting in crystallization. After recrys-
tallization two more times as described above, stearohydroxamic
acid was obtained with a melting point of 106 degrees. (Theo-107).
Stearohydroxamic acid is easily soluble in alcohol and acetone
when warmed, but insoluble in petroleum ether and water.

2- Palmitohydroxamic Acid, Molecular Wt. = 271, (C_{16}).

Palmitohydroxamic acid was prepared by the same procedure as
presented for stearohydroxamic acid. After recrystallizing
twice with alcohol and petroleum ether, palmitohydroxamic acid
was obtained having a melting point of 103 degrees. (Theo-102).

Solubility of this compound is about the same as stearohydroxamic acid.

3- Myristohydroxamic Acid, Molecular Wt. = 243, (C_{14}).

This acid was prepared as the previous ones except water was used as the precipitating agent rather than petroleum ether. After recrystallizing twice with water, myristohydroxamic acid was obtained having a melting point of 100 degrees. (Theo- 98). Myristohydroxamic acid is soluble in alcohol, methanol and acetone. It is insoluble in petroleum ether and water.

4- Laurohydroxamic Acid, Molecular Wt. = 215, (C_{12}).

The same procedure was followed as for myristic with the exception that the first reaction mixture was neutralized with cold alcoholic HCl and the KCl was filtered off. The filtrate was condensed at a low temperature and pressure and acidified to congo red by the addition of alcoholic acetic acid. The filtrate was then cooled down rapidly and cold water added. The precipitate formed was filtered and washed with water and dried. After recrystallizing twice with alcohol and water, laurohydroxamic acid was obtained with a melting point of 95 degrees. (Theo- 94). Laurohydroxamic acid is easily soluble in alcohol, methanol and acetone; soluble in ether; insoluble in petroleum ether and water.

5- Caprichydroxamic Acid, Molecular Wt. = 187, (C_{10}).

The same procedure was followed as for laurohydroxamic acid. The acid was precipitated twice with water and washed with petroleum ether. Caprichydroxamic acid was obtained with a melting point of 89 degrees. (Theo- 88). Caprichydroxamic acid is easily

soluble in alcohol and acetone and highly soluble in ether and insoluble in petroleum ether and water.

6- Caprylohydroxamic Acid, Molecular Wt. = 159, (C_8).

The same procedure was followed as for the previous two acids. Caprylohydroxamic acid was precipitated with petroleum ether and water. The acid was recrystallized twice and a compound obtained with a melting point of 81 degrees. (Theo-80). The derivative was soluble in alcohol and acetone, slightly soluble in water and insoluble in petroleum ether.

7- Caprohydroxamic Acid, Molecular Wt. = 131, (C_6).

The same procedure was followed as for the previous two acids. However, no solid caprohydroxamic acid was obtained. After carrying out the entire procedure and filtering off the KCl, a solution of caprohydroxamic acid was obtained. Later analysis showed this solution to contain 8.2 uMols of hydroxamic acid per ml. of stock solution.

8- Acetohydroxamic Acid, Molecular Wt. = 75, (C_2).

The preparation of acetohydroxamic acid was the most difficult so the procedure will be given in detail. Hydroxylamine hydrochloride, 1.2 grams, was dissolved in 32 ml. of absolute methanol and 40 ml. of absolute ethanol. To 28 ml. of ethanol was added 2.5 grams of potassium hydroxide. Two grams of ethyl acetate was added slowly and with shaking. A white precipitate formed immediately. The precipitate was filtered off and gave no hydroxamate test with ferric chloride color reagent. The filtrate gave a strong test.

The filtrate was made slightly acid to congo red and a precipitate formed immediately which was cooled and filtered. The filtrate gave a strong test with ferric chloride test reagent, but the precipitate gave none. The filtrate was placed in the refrigerator and cooled after the addition of a few ml. of petroleum ether. No precipitate was evidenced after 30 minutes of freezing temperature. Acetone was added to the solution, and a milky precipitate formed immediately. The solution was again placed in the cold. After standing two hours the precipitate was filtered and there was no reaction of the precipitate with the color reagent. The filtrate gave a strong positive test for hydroxamate. The filtrate was again placed in the cold and allowed to stand over night. No further precipitate was obtained after 12 hours in the cold. The petroleum ether was blown off and the alcohol volume reduced under pressure. Acetone was added to this solution and the solution was then made acid to congo red. After a considerable quantity of acetone had been added a curdy, milky precipitate formed and the flask was immediately placed in the cold. After two hours the precipitate was filtered. The precipitate gave no test with ferric chloride, but again the filtrate gave a strong positive reaction. The volume of solution was again reduced under pressure to about six ml. A small amount of petroleum ether was added to this solution and placed in the refrigerator. After three weeks in the refrigerator a small amount of precipitate was noted in the flask which now contained only a small volume of a viscous brown solution. The precipitate was filtered and gave a strong test with ferric chloride color

reagent. The precipitate was washed four times with petroleum ether and the brownish color diminished. The precipitate was washed four times with petroleum ether and the crystals appeared clear. The precipitate was dried and gave a melting point of 88 degrees. (Theo- 88). Acetohydroxamic acid is soluble in alcohol, acetone, ether, and water. It is slightly soluble in petroleum ether.

The hydroxamic acids described above show sensitive purplish-red color complexes with ferric chloride in alcohol solution. By the addition of a neutral alcoholic solution of copper acetate, a voluminous green amorphous copper salt can be precipitated. This precipitate then goes back to the original hydroxamic acid by the addition of H_2S. Strong acids will hydrolyze the hydroxamic acids back to their original fatty acids.

Determination of Rf Values

After the preparation of the purified hydroxamic acids the Rf values for many of them were determined using the chromatographic procedure of Wainfan and Van Bruggen[36,45]. This procedure uses butanol, acetic acid and water as the solvents in the ratio of 4:1:5. Whatman #3 filter paper was used and was pretreated by washing with glacial acetic acid prior to use.

According to a number of investigators[46,47], and also from our own observations, acetoacetic acid or the thiol acyl of acetoacetic acid does not form a hydroxamate as do the other members of the series. There also appears to be a shift to the enol form on addition of ferric chloride, but there is not a definite purple color formed. On paper chromatograms, 24 hours are necessary for

the development of a colored spot at Rf-.78,.84 corresponding to
acetoacetic acid or the derivative thereof. Free acetoacetic acid
has an Rf-.70 in a butanol:acetic acid: water system. Table I gives
some Rf values of the acids tested.

Table I

Rf Values of Some Important Biological Hydroxamates	
Hydroxamic Acid	Rf
Carbanohydroxamate	.30
Formohydroxamate	.43
Acetohydroxamate	.54
Acetoacetic derivative	.77
Laurohydroxamate	.94
Myristohydroxamate	.96
Palmitohydroxamate	.94
Stearohydroxamate	.94
Fumarohydroxamate	.31, .50
Succinohydroxamate	.24, .53
Ethylcarbanohydroxamate	.76

When diethyl carbonate is reacted with hydroxylamine and chroma-
tographed in butanol, two spots can be identified. The first spot,
Rf-.31, shows a transient pink color with iron reagents low in
hydrogen ion concentration. This pink, on standing, or in the
presence of high hydrogen ion concentration turns to a yellow color.
The hydroxamate of carbonic acid appears to be unstable, particular-
ly in acid solution. The second spot, Rf-.76 is of normal purple
color and is stable, likely being ethyl carbanohydroxamate.[49]

Colorimetric Determination of Hydroxamates

Use of Standard Solutions

The next requirement was one of obtaining a standard curve for quantitatively determining the amount of hydroxamate present in a sample. The following procedure is a modification of Lipmann and Tuttle[34]. Known amounts of the purified acids were weighed out and standard solutions were prepared. Known quantities of palmito and acetohydroxamate were placed in colorimeter tubes and brought to 2 ml. volume with alcohol. Five ml. of 1% alcoholic ferric chloride (0.1 N HCl) was added and 5 minutes allowed for color development. The samples were then read in the Bausch and Lomb Spectronic 20, modified as by Creamer[48], and the optical density recorded. Table II shows the summary of this information.

Table II

Standard Curve for Hydroxamic Acids		
uMols Hydroxamate Added	Optical Density*	
	Palmito	Aceto
0.2	.07	.07
0.4	.14	.16
0.6	.22	.24
0.8	.27	.30
1.0	.35	.38
1.5	.52	.60
2.0	.67	.74
3.0	1.02	----

* All readings made at 520 mu.

Elution of Derivative from Paper

It was desired to ascertain if the hydroxamic acids could be quantitatively eluted from chromatographic strips. Known amounts of laurohydroxamic acid were spotted on Whatman #3 filter paper. Single spots of aceto and myristohydroxamate were also made. These spots were then cut from the paper and added directly to a colorimeter tube containing 5 ml. of ferric chloride color reagent and 2 ml. of alcohol. The tube was stoppered, shaken for three minutes and centrifuged. The color density was read as before in the Basuch and Lomb Spectronic 20 at a wavelength setting of 520 mu. The amount of color produced was at each level consistent with the amount expected on the basis of the previously prepared standard curve. It is concluded that the elution of these spots from chromatographic strips is a quantitative procedure as outlined above. Table III is the results of this experimental procedure.

Table III

Elution of Laurohydroxamate from Paper Strips	
uMols Hydroxamate Added	Optical Density
0.2	.08
0.4	.14
0.6	.22
0.8	.29
1.0	.36
1.0 (Acetohydroxamate)	.37
1.0 (Myristohydroxamate)	.35

Stability of Laurohydroxamate Ferric Complex

It now became necessary to know if time would alter the color developed upon addition of the iron color reagent. Two ml. of a standard solution of laurohydroxamate containing 1 uMol of the acid was placed in a colorimeter tube and 5 ml. of the color reagent was added. Optical density readings were taken at various time intervals and the results as shown in Table IV were obtained. From these results it is obvious that five minutes is sufficient for full color development and that the color is stable for at least two hours under these conditions.

Table IV

Stability of Laurohydroxamate Ferric Chloride Complex

| uMols Hydroxamate Added	Time (minutes)	Optical Density
1	1	.355
1	2	.356
1	4	.358
1	10	.360
1	15	.360
1	30	.360
1	60	.360
1	120	.360

Enzymatically Formed Hydroxamic Acids

After establishing a method for quantitatively determining thiol acyls, a second problem immediately presented itself. Hydroxylamine added to a biological system may form hydroxamic acyds by enzymatic exchange and addition as well as by a recycling mechanism. To

illustrate: radioactive acetate-1-C^{14} is used as a tracer in our study of lipogenesis. As acetate is made available to the system the following reaction may take place:

$$ATP + Acetate \longleftrightarrow Adenyl\text{-}acetate + PP$$
$$Adenyl\text{-}acetate + CoASH \longleftrightarrow Acetyl\text{-}S\text{-}CoA + A5P$$

If, to this system we add hydroxylamine the following reaction or recycling can occur:

$$Acetyl\text{-}S\text{-}CoA + NH_2OH \longrightarrow Acetyl\text{-}NHOH + CoASH$$
$$Acetate + ATP \longleftarrow$$
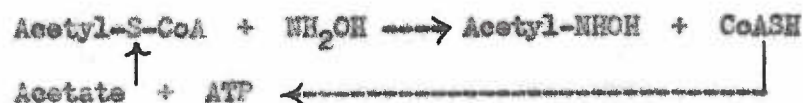
With each turn of this cycle we generate another molecule of aceto-hydroxamic acid. If higher thiol acyls are present, they too may be formed in a similar manner.

Quantitative Conversion of Thiol Acyls

To illustrate an increase of enzymatically formed hydroxamic acids, let us first look at the data obtained when hydroxylamine was reacted with known amounts of acetyl-glutathione for varying periods of time in the absence of tissue enzymes. In Figure I there is plotted the uMols of acetohydroxamate formed as the per cent of the anticipated conversion versus time of reaction with hydroxylamine. From this figure it is evident that a 30 minute reaction time is sufficient for the complete reaction of acetyl-glutathione to form theoretical amounts of acetohydroxamic acid.

The experimental data obtained when hydroxylamine is similarly added to rat liver homogenate is shown in Figure 2. On this graph is shown the uMols of hydroxamic acids formed per gram of rat liver

Figure 1

Quantitative Conversion of Thiol Acyls-Hydroxylamine added to aqueous solutions of acetyl-glutathione forms theoretical amounts of acetohydroxamate. This conversion is plotted in figure 1 as the % converted versus time of reaction with hydroxylamine.

Figure 2

Rat Liver Homogenate Thiol Acyls-Hydroxylamine added to rat liver homogenates forms increasing amounts of thiol acyls as the reaction time is extended. This increase is indicative of enzymatic formation of hydroxamates.
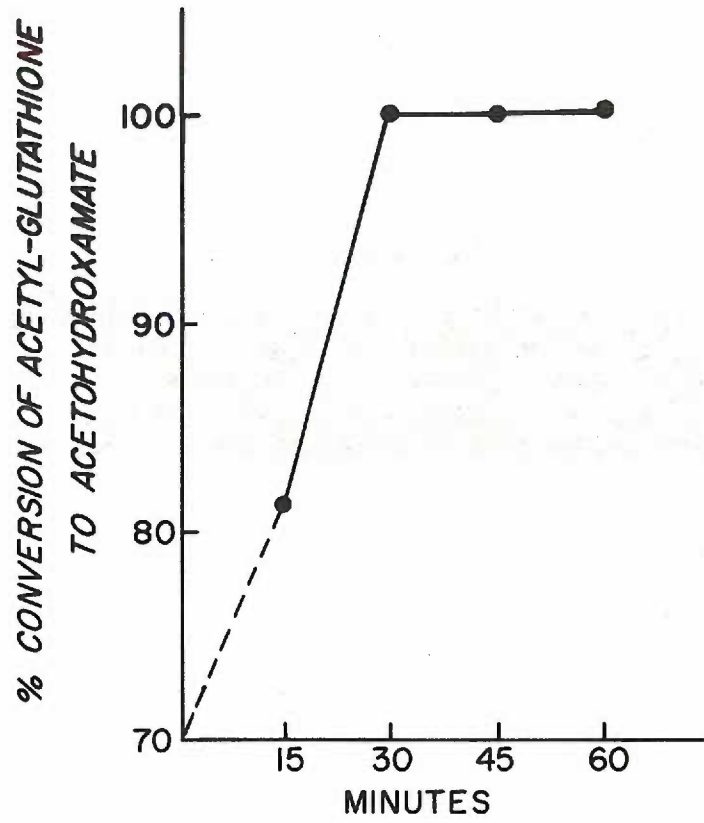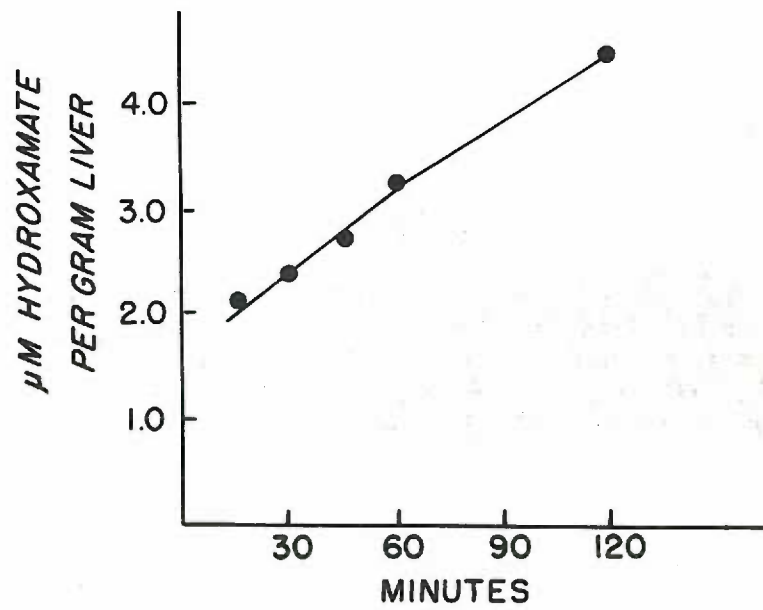
FIG. 1

FIG. 2

plotted against reaction time with hydroxylamine. From this figure
it is apparent that in an enzymatically functioning tissue there is
an increasing amount of derivative being formed beyond the 30 minute
time, for in the control experiment, 30 minutes was sufficient reac-
tion time for the thiol acyl to react. Since the purpose of this
study was not to continuously trap intermediates, but was to look at
the relative amounts of intermediates at a given time, it became im-
perative to inhibit this enzymatic activity. The inhibition of en-
zymes is not a difficult task and there are a number of methods avail-
able. One of the most common methods, that of heavy metal enzyme
poisons, was not felt desirable in this system since hydroxamic acids
have a tendency to form metal complexes with heave metals[50]. It
was decided that heat inactivation would be the simplest means at
our disposal if in the process the acyl-S-CoA intermediates were not
altered. A number of authors have reported on the stability of a
few acyl-S-CoA intermediates[51,52] and those tested were stable at
100 degrees for 15 minutes at neutral pH. Also, acetoacetic thiol
esters are known to be stable if not kept at alkaline pH[46].

Temperature Stability of Thiol Acyls

Acetyl-glutathione was again chosen as a representative thiol
acyl and the following experiment was conducted. Known amounts of
acetyl-glutathione in aqueous solution were subjected to 70 degree
heat treatment in a water bath for 10 minutes and other aliquots
were maintained at 26 degrees for the same period of time. The
values obtained represent a 96 - 100 per cent recovery of the hy-
droxamic acid derivative showing that the 70 degree temperature for

the period of 10 minutes did not hydrolyze or interfere with the formation of hydroxamic acids.

Inhibition of Tissue Enzymes

Figure 3 shows the quantities of hydroxamates formed from liver tissue homogenates when these homogenates were maintained at one of the indicated temperatures for 10 minutes prior to reaction with hydroxylamine. Curve D shows the linear increase of derivative with time when the homogenate was kept at 26 degrees and then reacted with hydroxylamine. Curve C indicates a decrease in slope and in total amount at 60 minutes after being incubated for 10 minutes at 60 degrees. When the incubation was at 70 degrees for 10 minutes we find that there was no increase of derivative with increasing reaction time. That is to say, the same amount of hydroxamate was formed at 30 minutes as after 60 minutes indicating that being determined were reactive acyls present after incubation and not reactive acyls being formed during the reaction period.

The one determination recorded after incubating liver homogenate at 80 degrees for 10 minutes showed a slight loss in recovered derivative indicating likely hydrolysis of the thiol acyls or at least partial destruction of a portion of these intermediates.

Figure 4 shows data obtained when acetyl-glutathione was reacted with hydroxylamine in aqueous solution and in rat liver homogenate after being subjected to heat treatment (70 degrees) compared to the amount formed at 26 degrees. Curve A represents the data obtained where known amounts of acetyl-glutathione were reacted in aqueous solution after 26 and 70 degree heat treatment for 10 minutes. Curves B and C represent the data obtained from rat liver homogenate

Figure 3

<u>Temperature Effect on Enzymatic Formation of
Hydroxamates</u>-Temperature incubation at the
temperatures indicated on the graph show a
marked change in the rate and total amount
of derivative being formed after increasing
reaction time. It is shown with this data that
70 degree incubation is sufficient, if maintained
for 10 minutes, for complete enzymatic inhibition.

Figure 4

<u>Temperature Effect on Acetyl-Glutathione, In
Aqueous Solution and Rat Liver Homogenate</u>-
The reaction of acetyl-glutathione with Hydrox-
ylamine at 70 degree temperature incubation in
aqueous solution and in rat liver homogenate is
presented. It is apparent that there was no
loss in reactive acyls at this temperature and
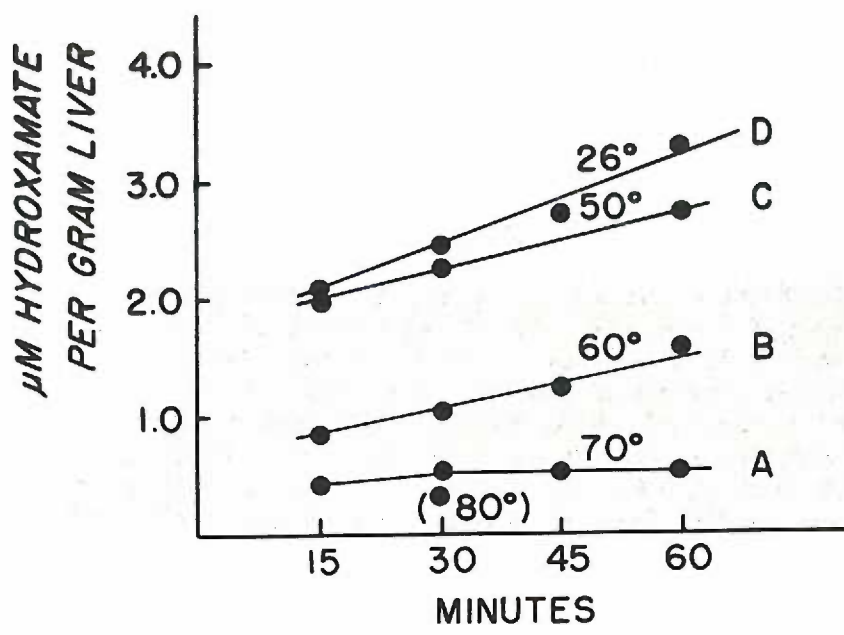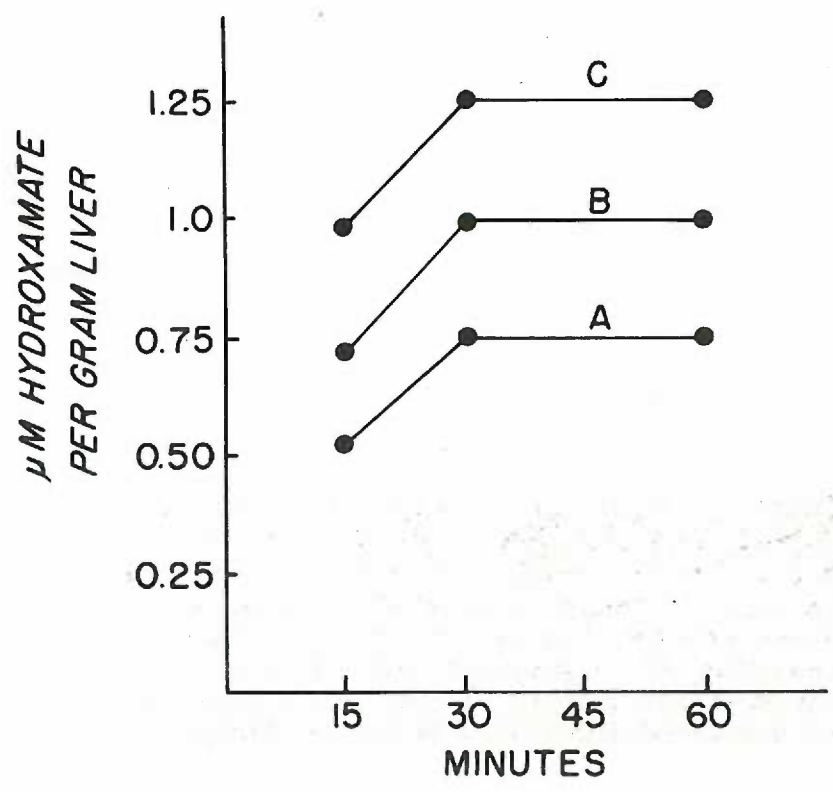that enzymatic formation has been eliminated.

FIG. 3

FIG. 4

to which had been added 0.50 and 0.75 uMols of acetyl-glutathione respectively. The vertical distance between curve B and C represents the difference in amount of acetyl-glutathione added, i.e. 0.25 uMols. The error of this difference is 4 per cent. Thus, 70 degree treatment eliminated the unwanted enzymatic formation of hydroxamic acids without concurrent loss of reactive acyls.

Hydroxylamine Concentration Study

The reaction of hydroxylamine to form hydroxamic acids has been used by many investigators to detect many types of compounds. The reactivity of these compounds is dependent on NH_2OH concentration and upon the pH of the reacting medium. After establishing the desired conditions for non-enzymatically measuring the amount of reactive thiol ester, it was felt necessary to do a concentration study with NH_2OH. It had already been determined in this laboratory that a pH of 7.4 and a concentration of 0.25 M NH_2OH[36] was required for complete reactivity of anhydrides and thiol acyls. With this information the following experiment was performed. Rat liver tissue was sliced and placed in homogenizing tubes. The moist tissue was then heat treated at 70 degrees for 10 minutes. Table V is the data collected after 30 minutes reaction time with NH_2OH at different concentrations. As can readily be seen, 0.30 M NH_2OH is just maximal for the optimum reactivity and a plateau is maintained until a concentration of 1 Molar is obtained. The increased amount of derivative formed at the concentration of 1 Molar is likely due to the reactivity of carboxylic esters. Since it was desired to keep the ionic strength at a minimum the 0.3 Molar NH_2OH solution was chosen as the concentration to be used in subsequent experiments with

biological systems. The carboxylic esters, tripalmitin and tri-laurin were reacted with 0.3 M NH_2OH at room temperature for 30 minutes and the amount of hydroxamic acid formed determined. Under these conditions there was no hydroxamate formed from the triglycerides confirming Wainfan and Van Bruggen[36].

Table V

Hydroxylamine Concentration Study	
Hydroxylamine Conc.	uMols Hydroxamate formed per gram of Tissue
0.1 Molar	1.75
0.3 "	2.70
0.5 "	2.70
0.8 "	2.70
1.0 "	4.00

Recovery of Hydroxamates from Tissue

Now that a technique for quantitatively converting thiol inter-mediates into hydroxamic derivatives was available, it became necessary to develop a method for quantitatively recovering formed deriva-tives from tissue. Fresh rat liver was obtained, sliced in the usual manner and homogenized with known amounts of preformed hydroxamic acids. The homogenate was then transferred to a screw cap culture tube with a total volume of 10 ml. of ethanol. The ethanol precipitated the protein and this solution was centrifuged. The supernatent was de-canted and the tissue residue was extracted three more times with 5 ml. volumes of ethanol with 15 minutes shaking between centrifugations. The amount of hydroxamate in the alcohol filtrate was determined.

Table VI is a summary of that data.

Table VI

Recovery of Hydroxamic Acids from Rat Tissue

Hydroxamic Acid Added	Amount Added	Amount Recovered	Per Cent Recovered
Palmitohydroxamate	20 uM	19.30 uM	96
Palmitohydroxamate	10 "	9.50 "	95
Laurohydroxamate	10 "	10.00 "	100
Laurohydroxamate	10 "	9.30 "	93

Current concepts of fatty acid metabolism involve the activation of various acids to thiol acyls varying in chain length from C_2 to C_{18}. We felt it would be desirable to separate, if possible, these intermediates into at least two fractions prior to attempting isolation or purification. Various solvents and pairs of solvents were tried, the following qualitative experiment giving an indication that a separation was feasible. A two phase butanol and water system was prepared in three different test tubes. Tube #1 contained acetohydroxamate; tube #2 contained aceto and palmitohydroxamate, and tube #3 contained palmitohydroxamate only. After shaking each tube for three minutes, 1 per cent ferric chloride solution in 0.1 N HCl, alcoholic, was added. The hydroxamic acid formed its usual red-purple colored complex with the iron. In tube #1 all of the color was in the aqueous layer; in tube #2 the color was distributed equally between both layers; and in tube #3 all of the color was present in the butanol layer. It appeared that at least qualitatively, a separation of acetohydroxamic acid from palmitohydroxamic acid had been made.

With this information in mind the following experiment was conducted. Known amounts of hydroxamic acids were added to rat liver slices, the tissue was homogenized, and the protein was precipitated and extracted with butanol. The butanol fraction was then extracted with water in the manner described in the section, Liver Slice Technique. A total of 5 water extractions were made and the data obtained listed in Table VII.

Table VII

Recovery of Hydroxamate from Butanol-Water Extractions

Hydroxamate Added		Amount Added		% Recovery of Added Compound	
				Butanol	Water*
Acetohydroxamate		5-10 uM		99-101	97-102
Capro-	"	"	"	99-100	93- 97
Caprylo-	"	"	"	98- 99	6- 13
Capri-	"	"	"	97-100	0
Lauro-	"	"	"	98- 99	0
Myristo-	"	"	"	98- 99	0
Palmito-	"	"	"	98-100	0
Aceto, Lauro-		5 uM each		98- 99**	98**
Myristo, Palmito-		5 uM each		98-100	0

* The butanol extract of the tissues was analyzed and then an aliquot extracted with water as above.

**In this experiment the butanol was not analyzed until after the water extract was made. The 98-99% in the butanol is that amount of the 10 uMols expected and the 98% in the water is that amount of the 5 uMols expected.

It is quite evident from this data that a good separation of $(C_2 - C_6)$ from $(C_8 - C_{18})$ is possible with this technique.

The protein residue from this experiment was further extracted
with 25 ml. of butanol. This solution was warmed to 60 degrees for
10 minutes and then shaken on the shaking machine for 30 minutes.
It was then centrifuged for 15 minutes and the supernatent transferred
to 50 ml. flasks and blown down to approximately 2 ml. volume with
nitrogen in a 60 degree water bath. Two ml. of this solution was
quantitatively determined for hydroxamates as described previously.
The results obtain are shown in Table VIII.

Table VIII

Recovery of Hydroxamates from Protein Residue

Sample No.	Optical Density	uMols Hydroxamate
1	.035	0.0
2	.030	0.0
3	.035	0.0

These results indicate that all of the hydroxamate present had been
extracted from the tissue with the methods described.

The Liver Slice Technique

The validity of the utilization of the tissue slice technique
has been argued pro and con by the best of investigators for many
years so no attempt will be made to condemn or uphold our choice of
this technique. The tissue slice was developed to a great extent
by Warburg[53] and modified by a great many other workers. Emerson[38]
has given quite a complete account of the development of this tech-
nique and since his modified procedure was used in these studies,
all of the arguments presented in his work holds also for the present
investigation.

Kreb-Ringer phosphate buffer[54] is used as the incubating
medium in all of the experiments reported in this thesis. The follow-
ing table shows the concentration of reagent used in its preparation.
Stock solutions of 5 times the desired concentration are made up and
kept frozen until used. The diluted solutions are prepared and used
within a week. This solutions are kept chilled in the refrigerator
prior to use. The buffer solution is prepared on the day of the ex-
periment from the diluted solutions. The pH is adjusted to 7.4 with
0.1 N HCl or 0.1 N NaOH.

Table IX

Stock Solutions for Preparing Krebs-Ringer Phosphate Buffer

Reagent	% Needed	5 X % Needed	5 X %* Amount	Quant. to Dilute	Parts per 200
NaCl	.90	4.50	100 ml.	5/25 ml	200
KCl	1.15	5.75	1.4375	"	8
CaCl$_2$	1.22	6.10	1.5250	"	6
KH$_2$PO$_4$	2.11	10.35	2.6375	"	2
MgSO$_4$	3.82	19.10	4.7750	"	2
NaH$_2$PO$_4$**	--	--	--	--	24

* Amount in grams to be diluted in 25 ml. distilled water.
** Made NaH$_2$PO$_4$ at time of preparing buffer. Weigh out 0.71 grams
and dilute to 50 ml. Use 24 ml. of this freshly prepared solution.
This gives a total volume of buffer of 242 ml.

The following procedure has been adopted for the metabolic
studies and for the quantitative determination of water soluble
thiol acyls in liver tissue. The animals are maintained on some
nutritional regime for a defined period of time. The animal is
killed by decapitation and its liver dissected as quickly as possible

and placed in chilled buffer. The liver is sliced using the Stadie-Riggs slicer (microtome) and approximately one gram samples are randomly placed in previously weighed plastic vials which contain 5 ml. of chilled buffer solution. The plastic vials are reweighed and the exact amount of tissue present is determined.

The slices are then transferred to 125 ml. Warburg vessels with the aid of a funnel and the funnel is then rinsed with 20 ml. of buffer. This gives a total volume of 26 ml., 25 ml. of buffer and 1 gram of tissue. The flasks are flushed with 100 % oxygen for one minute and the CO_2 center well traps are placed in the flasks and the flask is then mounted in the Warburg bath.

The slices are allowed to equilibrate for 30 minutes and then the acetate is tipped in from the sidearm and the sidearms are rinsed three times with the buffer in the flask. The total time from decapitation to placing of flasks in the Warburg is 28 to 30 minutes. Two minutes after addition of acetate the manometer is adjusted to 150 mm and the stopcock closed. Pressure readings are obtained every 15 minutes for a total period of 60 minutes. This allows one hour for the utilization of the added acetate-1-C^{14} by the liver slices. In an effort to provide equal incubation periods for all flasks it is necessary to tip in the acetate at 2 to 3 minute intervals thus allowing time for the reading of the manometers and for the stopping of the reaction at the end of the incubation period.

After 60 minutes of incubation the flasks are removed from the bath and the slices are removed and washed with normal saline solution. The substrate is collected from the flasks and stored for future analysis.

The moist, washed slices are placed in homogenizing tubes and are then heat inactivated at 70 degrees in a hot water bath for a period of 10 minutes. At the end of this period, 2 ml. of 0.3 M NH_2OH is added to the tube and the contents are homogenized with a Teflon homogenizer.

The contents of the tube are quantitatively transferred with 15 ml. of butanol to a screw cap tube (Teflon lined cap) and placed on a shaking machine for thirty minutes. The tube is centrifuged for 10 minutes at 1200 rpm and the supernatent decanted into a 50 ml. graduated centrifuge tube. The liver tissue is extracted twice more with 10 ml. portions of butanol with shaking and centrifuging each time. The supernatent is blown down to approximately 10 ml. in volume on a 60 degree water bath with a stream of nitrogen. The butanol is next made alkaline to phenopthalein with 0.1 N NaOH. Five ml. of water is added to the tube and the contents shaken. Time is allowed for the emulsion to break, this being aided by the addition of a drop or two of ethanol. The aqueous layer is drawn off with a long needle and syringe and transferred to a 10 ml. volumetric flask. The butanol is extracted with water until the pink color has been extracted; this takes about five, 1 ml. extractions. The aqueous extract is made to a total volume of 10 ml. Two ml. of this solution is used for the colorimetric determination of hydroxamates with ferric chloride.

The following has been adopted for the procedure of colorimetrically determining the amount of hydroxamate present in the sample. Two ml. of the sample to be determined are placed in a colorimeter tube. Five ml. of a 1% alcoholic ferric chloride

solution that is 0.1 N in HCl is added to give a total volume of 7 ml. Five minutes is allowed for reaction and the optical density is then determined on the Basuch and Lomb spectronic 20. The concentration of the sample is obtained from the standard curve described previously. Occasionally a precipitate will form on the addition of the ferric chloride solution. This can be centrifuged down without apparent loss of color. The precipitate has the appearance of a salt and is probably precipitated because of its low solubility in the alcoholic solution.

One half ml. of the aqueous extract is used for radioassay and the rest of the extract is blown down to one ml. in a 60 degree bath and with a nitrogen stream. Two tenths to 0.3 of this remaining extract is spotted on Whatman #3 filter paper and dried by a stream of warm air. The strips are chromatographed, dried, and radioassayed.

Radioassay of Chromatograms

After the aqueous extraction of the butanol fraction is complete or after protein precipitation of the substrate from the Warburg flasks is obtained, solutions are spotted on Whatman #3 filter paper and chromatographed. After chromatography the strips are allowed to dry and then they are assayed for radioactivity. Two methods have been devised for this analysis and will be described.

The moving strip assay consists of passing the chromatogram under a Geiger-Mueller tube at a constant rate and recording the activity. The scaler is a Berkely decimal scaler and the counter is an unsheilded thin end-window Tracer Lab-TGC-1 G-M tube with a background count of 39 counts per minute. The recording apparatus

consists of a rate-meter and an Esterline-Angus Recorder. The chromatogram is connected to the Esterline-Angus recorder paper by the use of scotch tape and is pulled into the roll of recorder paper at the same rate as the recorder is moving. Under the G-M tube is a polyethylene shield with a previously selected specified slit in place. This slit width can be varied from 1 mm to 10 mm. Most of our work was done with the 5 mm slit width. Figure 5 is a typical scan of a chromatographic strip.

The second method of assay for a chromatogram is to cut the strip into 1 cm widths and paste these strips on aluminum planchets that are cut to fit in the D-47 Nuclear counter. These strips can be counted for any specified time and/or for some specified total number of counts, and the statistical accuracy desired can be obtained. Figure 9 is a graph of the data obtained from this type of assay.

Column Chromatography

It was felt that a chromatographic column would be advantageous in that larger quantities of derivative could be handled. An attempt was made to separate hydroxamic acids using column chromatography. A silicic acid column was prepared in the following manner: to 20 grams of silicic acid there was added 12 ml of water. The water was thoroughly mixed with the silicic acid using a mortar and pestle. This material was then slurried with 80 ml. of chloroform. The slurry was added in 10 ml. portions into a previous prepared glass column that had a sand base upon which rested a piece of filter paper. The slurry was aided in settling by putting pressure, 2 pounds per square inch, upon the column. A vibrator tool was used with a cork attached to the vibrating end to dislodge air bubbles. The columns packed

Figure 5

Moving Strip Assay-A typical radioactivity
scan of a chromatographic strip obtained by
feeding the strip into an Esterline-Angus
Recorder after passing under a thin end-window
Tracer Lab-TGC-1 G-M Tube. The scaler is a
Berkely decimal scaler which has attached to
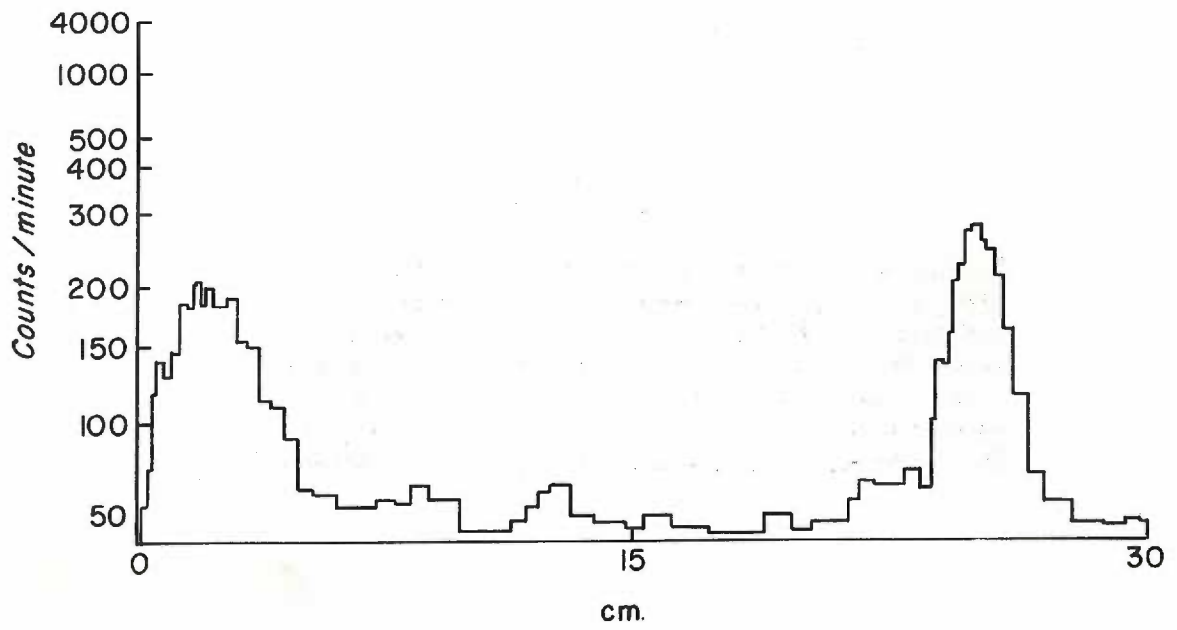it a Berkely rate-meter feeding to the recorder.

Figure 5

tightly and quite uniformly. To this column was added 10 uMols of palmitohydroxamic acid, 5 uMols of acetohydroxamic acid and 10 uMols of formohydroxamate in 2 ml. of solution. This was washed in with a chloroform solution saturated with water and the developing solvent was then added. The developing solvent in this instance being 40 % chloroform and 60 % butanol saturated with water. Figure 6 shows the results of this separation. It is apparent that long chain hydroxamic acids can be satisfactorily separated from short chain or water soluble hydroxamic acids, however, it is also apparent that there was no separation of the C_2 from the C_1 acid. Many attempts were made utilizing column chromatography to separate the homologous series of hydroxamic acids, C_1 - C_{16}, but the separation presented in Figure 9 was not improved upon. The solvent ratio of chloroform and butanol, and benzene and butanol were varied all the way from 0:1 to 1:0, but these two systems do not appear to be satisfactory in the separation of hydroxamates that differ by only a C_2 unit.

Determination of Ketone Bodies

The following is the method employed for determining ketone bodies in the tissue and substrate of the metabolic experiments to follow. This procedure is from Bessman and Anderson (personal communication) and since it was not published in detail[55] it will be included here as received by them. (Courtesy of Dr. Clarissa Beatty).

Reagents

1. Saturated barium hydroxide.
2. 5 % zinc sulfate hydrate ($ZnSO_4 \cdot 2 H_2O$).
3. 13.6 N sulfuric acid.
4. 0.4 % sodium dichromate hydrate ($Na_2Cr_2O_7 : 2 H_2O$)

Figure 6

Column Chromatography Separation of Hydroxamates-
Solvent separation employed was a 40 % chloroform
and 60 % butanol system saturated with water on a
silicic acid column. Two ml. fractions were coll-
ected using a Gibson Medical Electronics fraction
collector.
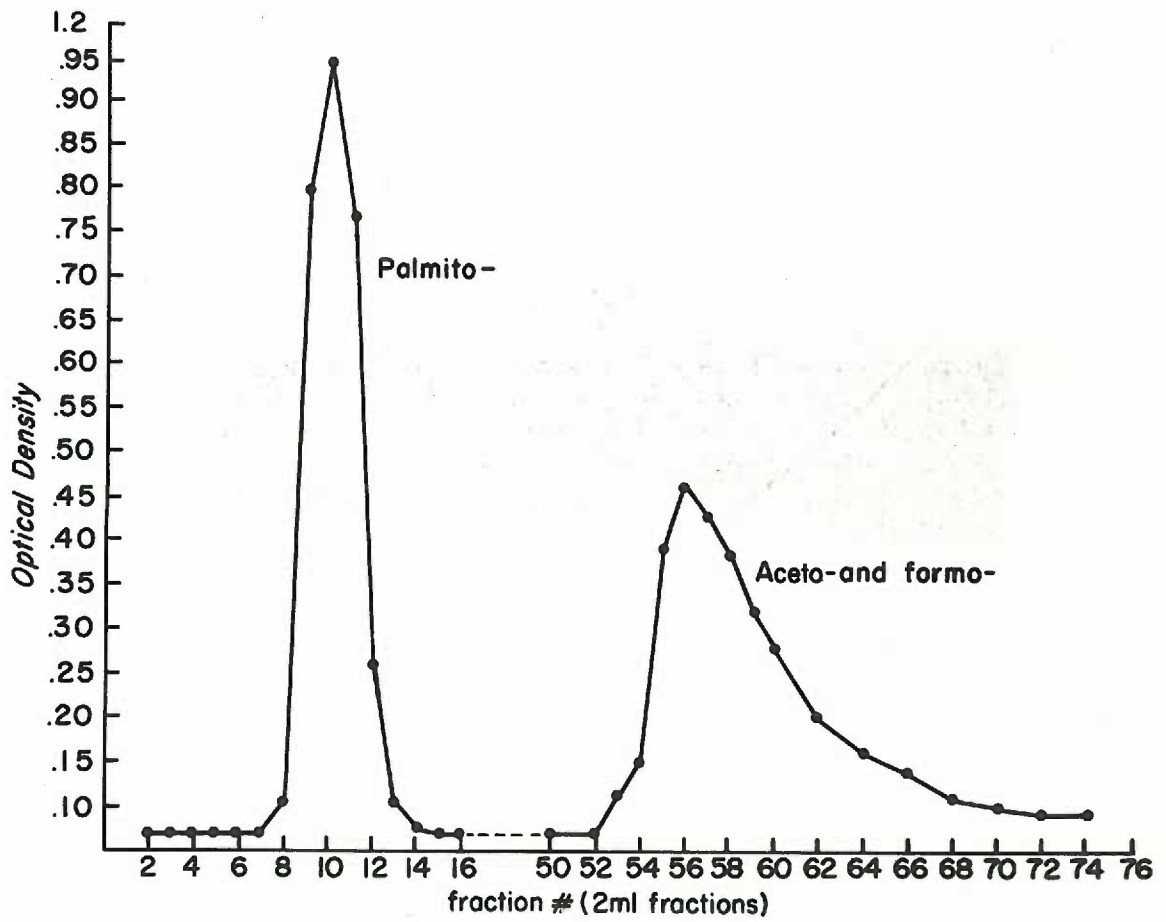
Figure 6

5. Salicylaldehyde, 10% (volume/volume) in acetone free methanol.

6. Saturated potassium hydroxide.

7. 50% (volume/volume) ethanol.

Procedure

1. Prepare a Somogyi filtrate of sample material by adding 0.5 ml. each of the barium hydroxide and zinc sulfate to 4 ml. of tissue extract. The precipitation is aided by shaking for two minutes and then centrifuging.

2. Add 1 ml. of filtrate to a screw-top culture tube and then 0.25 ml. of the 13.6 N H_2SO_4.

3. Seal the tube with a plastic cap containing a Teflon liner. Hand tightening is sufficient, being careful to protect the hand in case the tube snaps.

4. Heat in an autoclave or boiling water bath for ten minutes.

5. Cool and add 0.25 ml. of the dichromate solution. Reseal.

6. Heat an additional thirty minutes.

7. Cool and add 0.05 ml. of methanolic salicylaldehyde and then, by blowing in, add 2 ml. of saturated potassium hydroxide.

8. Allow thirty minutes for color development keeping the tubes cooled to 16 degrees during this period of time. Add 2 ml. of the 50% ethanol after thirty minutes. More of this solution may be added to allow sufficient volume in the cuvette, however, the volume suggested is practically a minimum for detecting normal blood levels of the ketones.

9. Mix well and centrifuge this solution until clear. Read at 500 millimicrons.

Notes:

1. Recovery of acetone is not quantitative, ranging from 50%
   to 80%. The loss occurs in the preparation of the filtrate.

2. Standards may be prepared from acetone or (fresh) solutions
   of sodium acetone bisulfite. A 0.1 uMol standard is usually
   carried through the determination. This gives a reading on
   the Bausch and Lomb Spectronic 20 of .240 optical density.
   Standards are not carried through the filtration step. The
   standard curve deviates slightly from linearity. For ex-
   treme accuracy, a standard curve should be run, especially
   if very low or high values are expected. Recovery of aceto-
   acetic and beta-hydroxybutyric acids is quantitative.

3. No interfering substances have been observed other than
   glucose. The interference is at the oxidation step, and
   in the presence of 200-500 mgm per cent of glucose, the re-
   covery of beta-hydroxybutyric acid is reduced. The amount
   of interference varies with the sample. This may be diluted
   out in diabetic blood since the ketones are in such high
   concentration as to make dilution necessary in the first
   place. If the dichromate is visably reduced,(a blue solu-
   tion), the run should be repeated using lesser amounts of
   filtrate. For a rough estimation this error is not too
   great. About 85 % recovery is obtained in the presence of
   glucose equivalent to 1,000 mgm per cent, though the di-
   chromate was completely reduced.

4. Combined acetoacetic acid and acetone may be separately de-
   termined by omitting the oxidation procedure (steps 5 and 6).
   Separate standards must be run omitting these steps also.

We have found in our lab that alcohol is an interfering substance in the beta-hydroxybutyric acid determination because of the conversion of the alcohol to aldehyde on heating with potassium dichromate[56].

$$CH_3CH_2OH \quad + \quad (O) \quad \xrightarrow[H_2SO_4]{K_2Cr_2O_7} \quad CH_3CHO \quad + \quad H_2O$$

The conditions of this reaction are those which prevail in the tubes we have prepared for determining ketone bodies. The aldehyde is capable of condensing in the same manner as acetone with the color developing reagent. The following reaction is that postulated for the development of the yellow color which is indicative of ketones and aldehydes.

Acetoacetic Acid + Sulfuric Acid ⟶ Acetone + Carbon Dioxide

Acetone + Salicylaldehyde + Potassium Hydroxide ⟶ Color

Pure acetoacetic acid was prepared according to Krebs and Eggleston[57]. Pure acetoacetic acid was used as a standard rather than acetone. The procedure listed gives a 1 Molar solution which is stored at this concentration. Dilutions are made at the time of use.

It was later found that the preparation of pure acetoacetic acid was not necessary and that purified ethyl acetoacetate would work quite satisfactorily and this was used as standard in subsequent experiments. A stock solution of about 0.3 mg per ml. was kept frozen and dilutions of this stock solution were made at the time needed.

Studies with Metabolizing Systems

Acetate Concentration Study

The procedure to be described was used in an acetate concentration study which was carried out to uncover the block in the metabolic cycle which precludes the utilization of acetate-1-C^{14} as tracer when the concentration is increased beyond certain limits[38]. This study was constructed so there would be a 1,000 fold concentration increase of acetate presented to the liver slice per experiment. The acetate alloted for this experiment was #109 and was prepared in this laboratory from barium carbonate[58]. The acetate was assayed for radioactivity on a D-47 Nuclear counter and the stock solution was shown to contain 2×10^6 counts per minute per ml. as infinitely thick barium carbonate. The D-47 counter is fitted with a Micro-Mil window. The concentration of this stock solution was 6.3 uMols of acetate per ml., as determined by titration of a distillation and the per cent yield from barium carbonate. Ten fold dilutions were made of the stock solution giving the following activities: 2×10^6, 2×10^5, 2×10^4, and 2×10^3 counts per minute per ml. This represents a 1,000 fold concentration range from 6.3 to 0.0063 uMols of acetate per ml.

The following protocol is an outline of the procedures previously described in detail. Each experiment included two rats and there was a total of eight experiments. Therefore, 16 rats were used in these experiments and duplicate samples were ran for each concentration studied.

Summary of Procedure

1. Trained feeding of animals.

2. Preparation of buffer and equipment.

3. Sacrificing of animal and dissecting liver.

4. Slicing of liver and preparing Warburg flasks for equilibration.

5. Manometric determinations and incubating of tissue for one hour in the presence of acetate-1-C^{14}.

6. Obtaining tissue and substrate from incubating flask.

7. Inactivating enzymes and formation of hydroxamic acid derivatives.

8. Fractionation of tissue with butanol and subsequent isolation of the water soluble derivatives from the butanol.

9. Colorimetric determination of amount of hydroxamate formed.

10. Chromatographic determination of type of derivative formed.

11. Radioassay of the aqueous extract, both chromatographically and by direct plate assay.

12. Interpretation of data.

The following data sheet is taken from experiment #C:8 and is presented in detail. Table 10 is a summary of all the data collected from the individual experiments. The purpose of presenting a data sheet in total is to show the manner in which the data was collected at the time of experimentation. All eight experiments were conducted in a similar manner with all variables as closely controled as possible.

Experiment #C:8   Four Hour Fast   9-3-57

Acetate-1-C^{14} Concentration Study

Wt. of Rat = 233 gm	Sacrificed-1000	Acetate In-1055	Reaction
Wt. of Rat = 234 "	In Warburg-1025	Slices Out-1155	30 Mins.

Wt. of Slices:

13.4590	13.4409	13.4420	13.4600	13.4100	13.3951	13.4345	13.4041
12.4308	12.4300	12.4300	12.4303	12.4302	12.4300	12.4301	12.4300
1.0282	1.0109	1.0120	1.0297	lost	.9651	1.0044	.9714

Wt. of Pooled Samples:

1 & 2	3 & 4	5 & 6	7 & 8
2.0391	2.0417	.9651	1.9758

Sample No.	Acetate Conc.	Vol.	cpm/ ml.	O.D.	uM HA	Total uM HA	Total uMols HA/gm Tissue
1	6.300 uM	1 ml.	2 X 10^6	.220	.51	2.55	1.24
2	.630 "	"	2 X 10^5	.220	.51	2.55	1.25
3	.063 "	"	2 X 10^4	.135	.20	1.00	1.04
4	.006 "	"	2 X 10^3	.190	.40	2.00	1.01

Count Data Sample No.	Pre-Count	time	cpm	cpm-Bkg	Total cpm	cpm/ gm	cpm/ uMol HA
1	6400	0.73	3699	3684	184,200	90,334	72,267
2	6400	6.30	1023	1008	20,160	9,874	7,899
3	1600	22.92	70	54	1,086	1,086	1,082
4	1600	66.34	28	13	257	257	129

Oxygen Utilization Data From Warburg Incubation

Flask No.	0 Min	15 Min	30 Min	45 Min	60 Min	Total mm	mm/gm
1	150	130	104	77	47	103	102
2	150	129	102	73	44	106	103
3	150	130	102	73	44	106	102
4	150	125	94	67	41	109	106
5	150	128	104	79	54	96	98
6	150	125	97	68	40	110	114
7	150	129	105	78	51	99	99
8	150	130	110	86	61	89	92

Table X

Summary of Acetate-1-C^{14} Concentration Studies

Experiment No.	Sample No.	uMols HA /gm.	% Incorporated /gm.	counts per minute /gm.
C-2	1	2.10	4.41	42,068
"	2	1.57	3.16	4,020
"	3	1.70	5.00	593
"	4	1.36	5.83	86
C-3	1	1.00	5.24	104,820
"	2	1.34	5.66	8,450
"	3	—	—	—
"	4	1.24	5.28	166
C-4	1	.88	3.81	86,691
"	2	1.05	4.35	8,287
"	3	.98	—	627
"	4	1.01	6.55	130
C-5	1	1.80	6.70	74,539
"	2	1.51	5.90	7,869
"	3	1.63	5.30	653
"	4	1.55	7.20	93
C-6	1	1.12	7.00	124,200
"	2	.97	6.40	13,089
"	3	1.08	6.50	1,291
"	4	1.10	9.50	172
C-7	1	1.31	9.50	144,347
"	2	1.38	9.00	13,030
"	3	1.29	9.70	1,497
"	4	1.31	10.50	161
C-8	1	1.25	4.50	72,267
"	2	1.25	4.90	7,899
"	3	1.04	5.60	1,082
"	4	1.10	6.50	129
C-9	1	1.57	7.26	145,325
"	2	1.45	7.21	14,429
"	3	1.34	9.15	1,831
"	4	1.52	10.41	208

Summary of Data

It is apparent from Table X that the uMols of thiol acyl remains relatively constant throughout the concentration range of acetate

employed. That is to say, 0.0063 uMols of acetate to 6.3 uMols per flask does not appear to alter the amount of thiol acyl present in liver tissue.

In Figure 7 is plotted the theoretical amount of radioactivity anticipated versus acetate concentration. This function was obtained by assuming that 0.0063 uMols of acetate was a true tracer dose range and that the subsequent 10 fold increases in dosage would give 10 fold increases in the amount of radioactivity isolated in the aqueous fraction. This gives a straight line function in the tracer range. Plotted with this information is the data actually found by experimentation and it is at once obvious that within the experimental error of this procedure, the anticipated response is being achieved at all levels of acetate studied.

Since there was no deviation of the experimental from the anticipated at any dosage level administered, 0.0063 - 6.30 uMols, tracer conditions are not exceeded in this system.

There appears to some discrepancy in these findings when it is remembered that somewhere in the metabolic scheme of acetate to fatty acids or cholesterol there is a non linear response as measured by the radioactivity arriving in these two fractions. If the amount of acetate is being activated at higher concentrations, as has been concluded from $C^{14}O_2$ measurements, but the amount arriving at the product is not linearly increasing, it would be expected that radioactivity would be found increasing in some metabolite along this scheme of synthesis. We have just concluded, however, that there is a linear increase of activity in the tissue extract but not an accumulation of activity as one might predict. The answer to this problem soon

Figure 7

Per-Cent Anticipated Response- The two
curves illustrated represent the theo-
rectical and the observed response of
incorporation of acetate into the aqueous
extract of rat liver homogenate as the
concentration of acetate is varied from
0.0063 to 6.30 umols. There is no statis-
tical difference between the theorectical
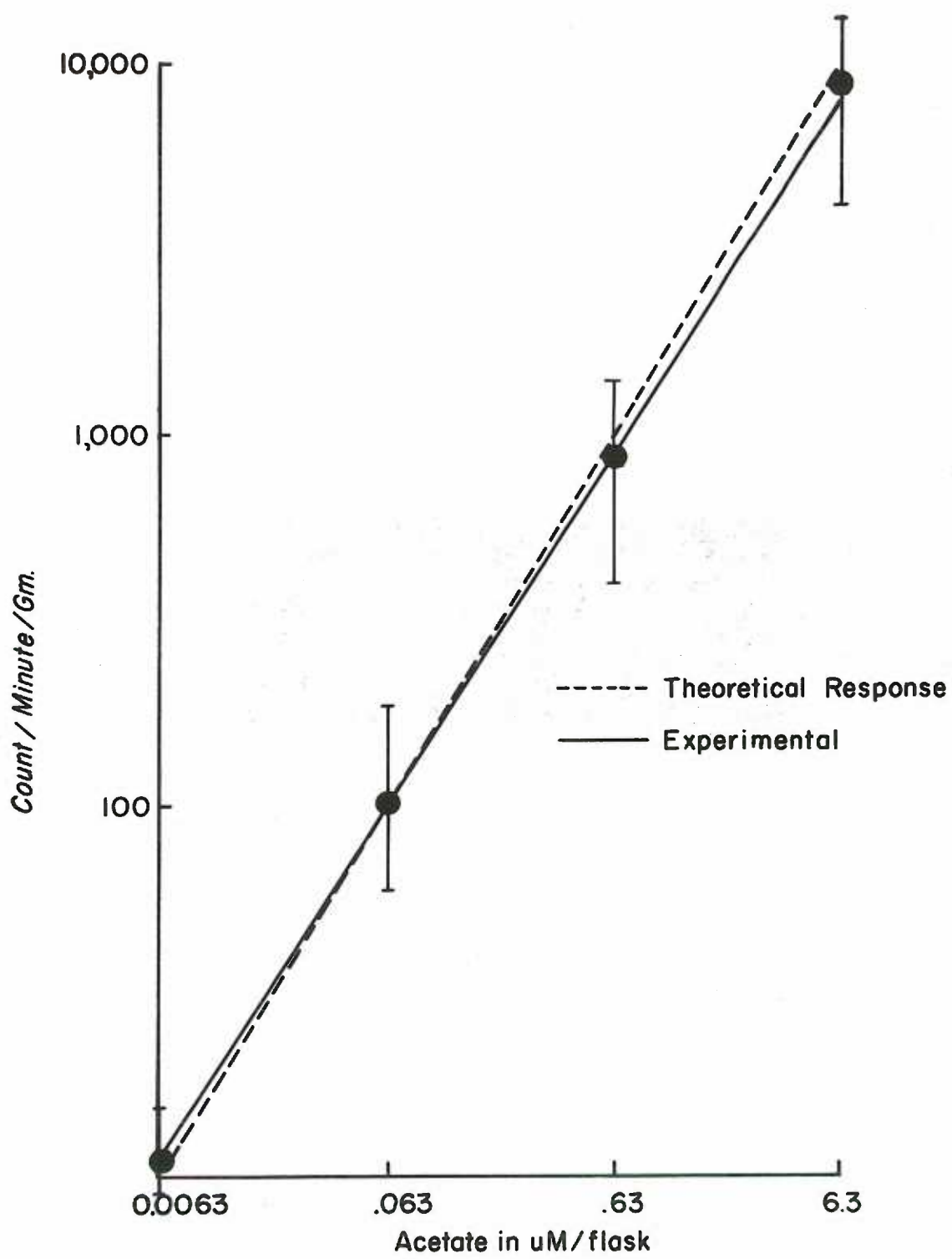and the observed response.

Figure 7

became apparent when the suspending medium of the system was analyzed for activity. When it was ascertained that there was considerable activity in the suspending medium which was associated with ketone bodies it became necessary to carry out a series of investigations related to these particular metabolites.

Ketone Body Analysis, Concentration and Radioactivity

The liver has been implicated for many years as an important site of metabolism of the lipids. The clearing of fat from the blood by the liver was accepted as fact long before isotopic methods proved this to be true. Ketone bodies are found in traces in blood and urine of normal individuals, but during diabetes or starvation there is a pronounced accumulation of these products of metabolism. Ever since the realization that the ketone bodies are products of fatty acid oxidation the liver has been depicted as the villain in the production of ketosis. The problem of the control and significance of ketone bodies in the blood and urine of humans and experimental animals has been one of the larger fields of investigations and has included among its ranks of workers, physicians, physiologists, and biochemists.

When considering the origin of acetoacetic and beta-hydroxybutyric acid there were three alternatives as to the formation mechanism.

1- Condensation of two C_2 (acetyl-S-CoA) units to form acetoacetic acid.

2- Under certain conditions the breakdown of the fatty acid chain can proceed beyond the C_6 to the C_4 stage, but that acetoacetic represents the "unoxidizable stump" of the fatty acid molecule.

3- By primary breakdown of the fatty acid chain into C_4 units rather than C_2 units.

The utilization of isotopes brought about the resolution of these alternatives. Octanoic acid labeled with C^{14} in the carboxyl group is oxidized by cell free preparations. If acetoacetic acid is formed by condensation of two C_2 untis then there should be label equally distributed in the carboxyl and carbonyl carbons. If the acetoacetic acid is only the unoxidizable stump then there should be no label in the acetoacetic, and finally if the acid should be oxidized as C_4 units then all of the carboxyl label of the octanoic acid would remain as carboxyl carbon in the acetoacetic acid. Several workers have carried out experiments such as this and all find label in the carbonyl and carboxyl positions of acetoacetic acid, however, not in the same proportion. This led to much controversy until Lynen[14] suggested that the carbonyl/carboxyl ratio was being affected by the time of incubation as well as by the equilibrium conditions with the enzyme, substrate, and enzyme substrate complex. So we arrived at the conclusion that the C_4 acetoacetic acid is being formed by the condensation of two C_2 units (acetyl-S-CoA), mediated by the acetoacetyl-thiolase enzyme available in liver.

Procedure for Ketone Body and Acetoacetic Acid Determination

The method of determining ketone bodies has already been presented. The following is the experimental procedure adopted in isolating the C_4 units both from tissue and from the suspending medium. All conditions of trained feeding, fasting, sacrificing and incubation of tissue are as outlined previously. The modification of the procedure presented begins with the removal of the tissue from the Warburg flask after incubation with acetate-1-C^{14} for sixty minutes. The tissue is washed with water and immediately placed in a homogenizing tube that has been cooled in ice water. The tissue is then

homogenized in two ml. of water while being kept cold by immersion in ice water. The purpose of the ice water is to minimize loss of volital compounds during homogenization and protein precipitation. The protein of the homogenate is now precipitated using 0.5 ml. of zinc sulfate and 0.5 ml. of barium hydroxide which have been adjusted, equivalent per equivalent, using phenolpthalein as indicator. The mixture is transferred to screw cap culture tubes and shaken for 10 minutes on an Eberback shaking machine and one half speed. The solution is centrifuged and the supernatent collected. The supernatent is then used for the determination of total ketone bodies and for the determination of acetone and acetoacetic acid. The difference between the total ketone bodies and the acetoacetic analysis gives the concentration of beta-hydroxybutyric acid. The incubating medium is treated in the same manner, i.e. protein precipitated, shaken, centrifuged and ketone bodies determined. The supernatent from tissue and suspending medium extracts is spotted on Whatman #3 as described previously and chromatographed in the usual manner. The radioactivity of the C_4 units is then determined by the radioassay of appropriate portions of the chromatographs and total per cent incorporation is calculated.

In this study of substrate components, the dosage range of the acetate study was increased. Control values were obtained by incubating tissue in buffer to which had been added no acetate. The acetate concentration range ran from the highest dose, 63.0 uMols, through a series of ten fold dilutions, to the lowest dose given, 0.063 uMols of acetate. Each experimental run consisted of a

concentration study of at least four different dosages plus a
control. There were a total of six such experimental runs useing
ten animals in all. Each point in Figures 10 and 11 represents
the data from at least four animals and sometimes six.

A Representative Experiment

The total amount of C_4 units within the tissue is small in re-
lation to the amount put out into the medium by the tissue. A typi-
cal experiments will be presented that involves the analysis of the
suspending medium and not that of the tissue. Since the suspending
medium was a reflection of the tissue activity as regards ketone
body production, this will be the only information reported here
for "tissue activity".

Table XI is a summary of an actual experiment. Table XII, which
follows, is the data obtained from the radioanalysis of the chromato-
graphic strips of three different acetate concentrations, 0.63, 6.3,
and 12.6 uMols of acetate per flask. Figure 8 is the graph of the
information obtained when the chromatogram was cut into 1 cm. strips
and mounted on aluminum discs and asseyed on a mechanical D-47 Nuclear
Micro-Mil window counter. Each point on the graph represents a
total of 1600 counts which gives a statistical significance of .95
confidence at a level of $\pm$ 5 per cent accuracy.

Table XI

Acetoacetic Acid Study				1-8-58

Four Hour Fast

Wt. of Rat = 204 gm

Wt. of Samples:	1	2	3	4	5
	13.7088	13.6855	13.7260	13.7026	13.6800
	12.6850	12.6855	12.6850	12.6856	12.6856
	1.0238	1.0000	1.0410	1.0170	.9944

Sample No.	Acetate-1-C^{14} Concen.		cpm/ ml
1	0.0	uM	0
2	0.063	"	2×10^4
3	0.630	"	2×10^5
4	6.300	"	2×10^6
5	12.600	"	4×10^6

Suspending Medium Ketone Body Determination

Acetoacetic Acid:

Sample No.	Optical Density	Totals uMols	uMols/ gm.
1	.228	4.65	4.57
2	.232	4.71	4.71
3	.235	4.63	4.63
4	.240	4.89	4.89
5	.276	5.64	5.64
Std.	.380	--	--
Blk.	.050	--	--

Total Ketone Bodies:

Sample No.	Optical Density	Totals uMols	uMols/ gm.
1	.145	5.54	5.41
2	.180	6.52	6.52
3	.160	5.96	5.73
4	.135	5.28	5.18
5	.185	6.76	6.70
Std.	.230	--	--
Blk.	.040	--	--

Table XII

Chromatographic Radioassay Data

12.6 uMols Acetate-1-C^{14}

Distance Moved	Rf Value	cpm/ Strip
17 cm	.54	36.7
18 "	.58	52.1
19 "	.61	83.4
20 "	.64	192.2
21 "	.67	135.0
22 "	.71	291.0
23 "	.74	925.0
24 "	.77	602.0
25 "	.80	85.6
26 "	.83	53.4
27 "	.87	41.4

Total counts per minute per gm of tissue = 180,671

6.3 uMols Acetate-1-C^{14}

17 "	.57	27.8
18 "	.60	32.8
19 "	.63	56.2
20 "	.66	102.6
21 "	.70	92.6
22 "	.73	170.0
23 "	.76	526.0
24 "	.79	270.0
25 "	.83	40.0

Total counts per minute per gm of tissue = 92,307

0.63 uMols Acetate-1-C^{14}

18 "	.59	0.0
19 "	.62	0.0
20 "	.65	7.6
21 "	.68	9.1
22 "	.72	13.5
23 "	.75	41.4
24 "	.78	42.3
25 "	.81	6.0

Total counts per minute per gm of tissue = 8,000

Figure 8

Radioactivity Assay of Chromatograms from
Varying Acetate Concentrations-The three
graphs represent ten fold increases in
acetate concentration as listed in the
figure. The chromatograms were cut into
1 cm. strips and radioassayed on a D-47
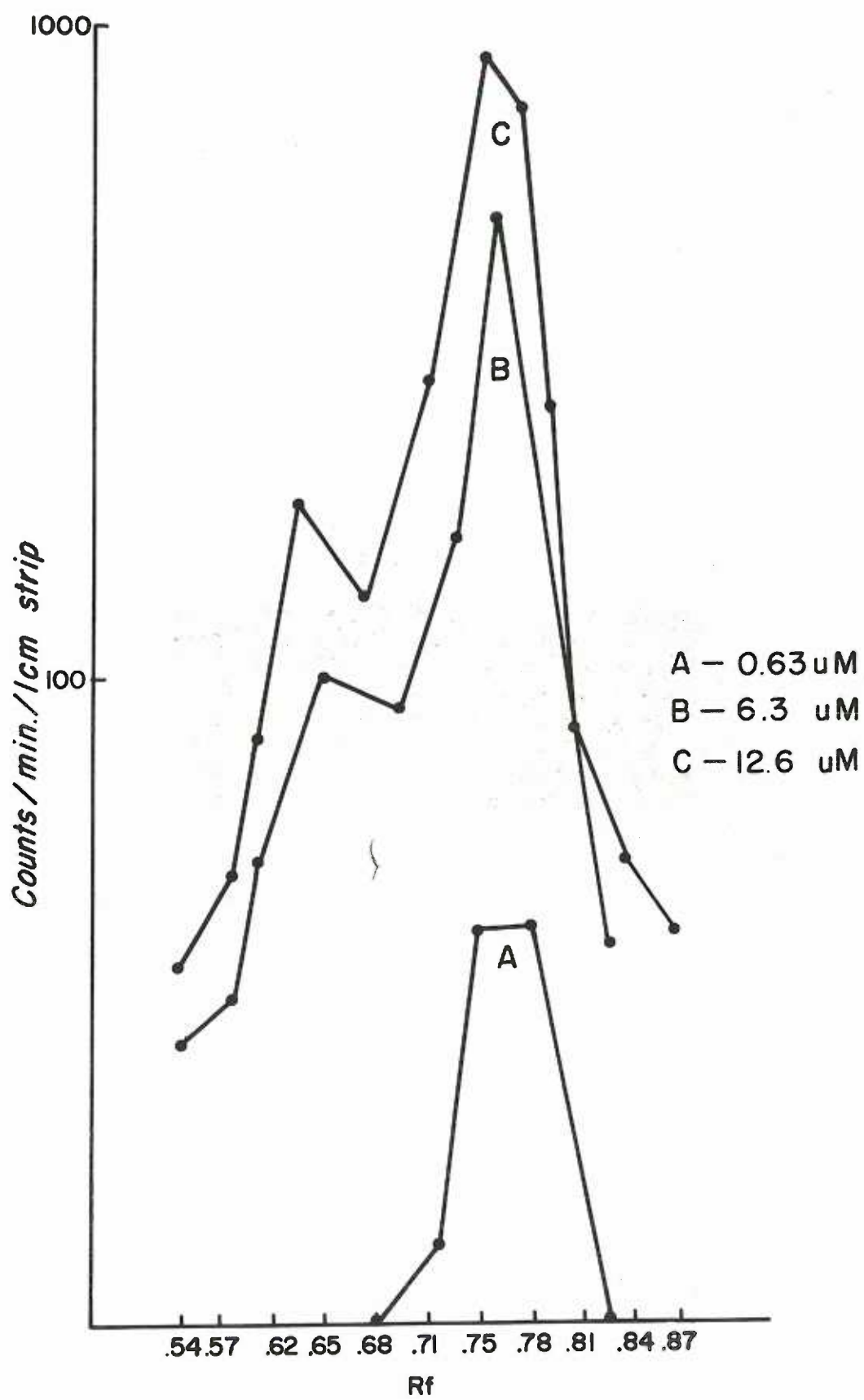Nuclear counter for a total of 1600 counts
per 1 cm. strip.

Figure 8

A typical scan of an entire chromatogram is shown in Figure 9. In this figure is plotted counts per minute per 1 cm. strip of chromatogram versus Rf value. The activity in the upper Rf region is due to acetoacetic acid, but at this time it has not been determined what is responsible for the high accumulation of activity at the lower Rf. The total activity recorded between Rf-.57 and Rf-.82 is considered to be due to beta-hydroxybutyric and acetoacetic acid and the per cent incorporation of labeled acetate is calculated on this basis.

$$\% \text{ Incorporation} = \frac{\text{Total Radioactivity of Specified Rf Range}}{\text{Total Radioactivity of Dose}} \times 100$$

Summary of the Data

Table XIII is a compilation of data obtained from the experiments described above in which the accumulation of ketone bodies in the medium was followed and the radioactivity determined. Recorded in the table are the amounts of total ketone body and acetoacetic acid determined after a specified amount of acetate had been presented to the tissue. Also recorded in this table are the standard deviations and the standard error of the results. The final column in the table is the per cent of the dose of acetate actually incorporated into C_4 units as calculated from the above equation.

When these figures are plotted against the concentration of acetate as in Figure 10, we find that up to concentrations of 6.3 uMols of acetate per gram of tissue per flask there is a linear incorporation of label into product. That is, as the amount of label is increased 10 fold there is found a 10 fold increase in activity in the product, acetoacetic acid. It can be said that this system

Figure 9

Radioactivity Assay of a Complete Chromatogram-A chromatographic strip which was utilized in a metabolic experiment explained in the text, was cut into 1 cm. strips. Each strip was counted for a total of 1600 counts on a D-47 Nuclear counter. The counts per minute obtained were graphed in respect to the Rf value on the chromatogram.
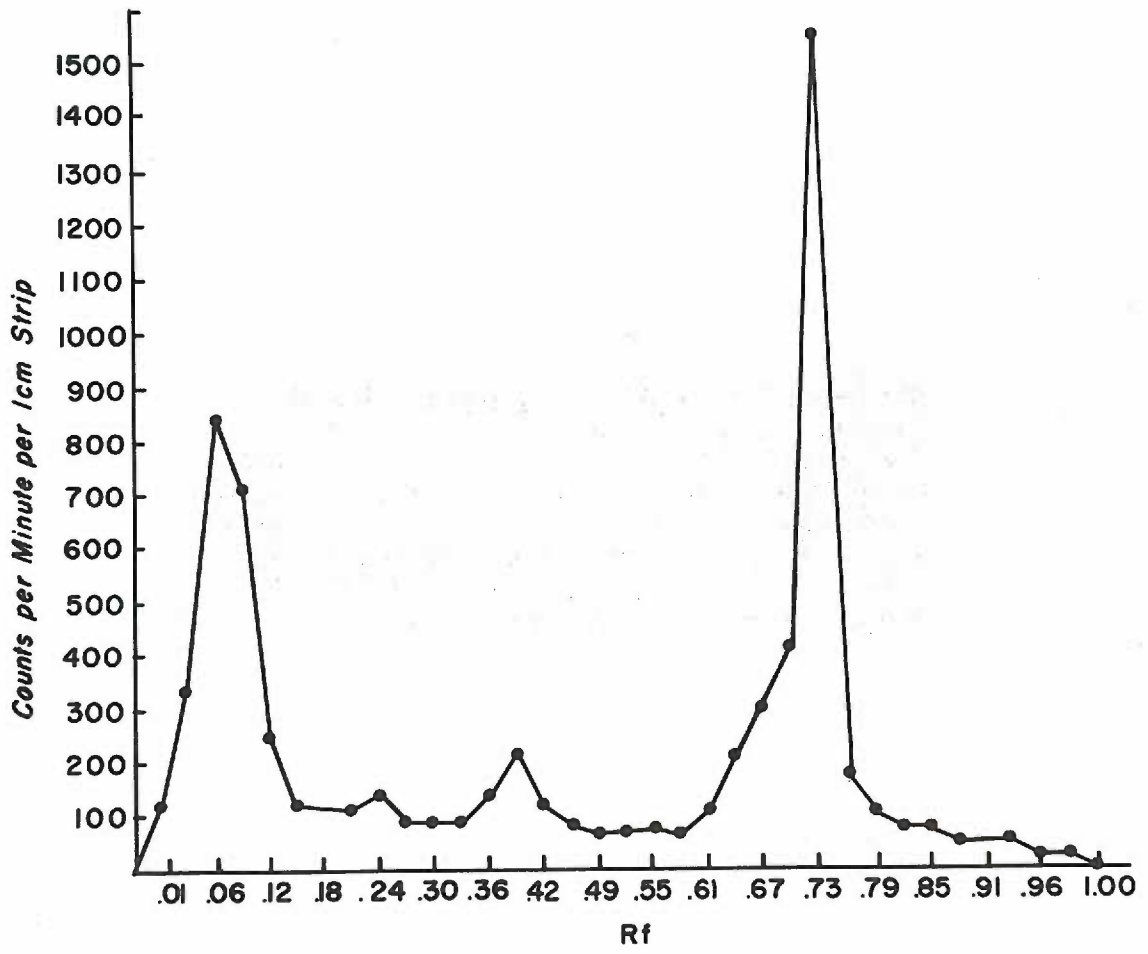
Figure 9

is incorporating acetate into a product without being influenced by
the amount of acetate available, or, that we are working at tracer
levels in the system which includes only the activation of acetate
by the cell and the condensation of two acetyl-S-CoA molecules to
form acetoacetyl-S-CoA. The Coenzyme-A is then cleaved from the C_4
unit by the liver acetoacetyl-S-CoA deacylase enzyme.

Table XIII

Total Ketone Bodies and Acetoacetic Acid Determinations

No. of Animals	Acetate Conc.	Aceto-Ac. Conc.	S.D*	S.E**	Total K.D.	S.D.	S.E.	% In-Corp.‡
6	0.0000	4.2 uM	1.92	.91	5.73 uM	1.50	.75	0.0
5	0.0630	4.1 "	1.70	.78	5.21 "	1.00	.47	5.6
5	0.6300	4.1 "	1.30	.61	5.77 "	.70	.31	5.5
5	6.3000	4.5 "	1.00	.47	5.87 "	.50	.20	5.6
4	12.6000	5.2 "	.68	.34	5.90 "	.80	.39	5.1
4	63.0000	7.5 "	1.36	.78	8.50 "	1.10	.63	3.6

* S.D.= Standard Deviation
** S.E.= Standard Error
‡ Per cent Incorporation into Total Keton Bodies.

Liver isn't the only tissue which contains the acetoacetyl-S-CoA
deacylase, but it appears to be the only tissue which lacks almost
completely the acetoacetate activating enzyme. In other tissues,
deacylation is matched by reactivation and the net result may be
an insignificant accumulation of acetoacetic acid. In the liver
slice preparation, the conditions do not allow the C_4 units pro-
duced to be transported to other tissue and they thus diffuse into
the substrate.

Figure 10

Per-Cent Incorporation-

$$\% \text{ Incorp.} = \frac{\text{Total Radioactivity of Specified Rf Range}}{\text{Total Radioactivity of Dose}} \times 100$$
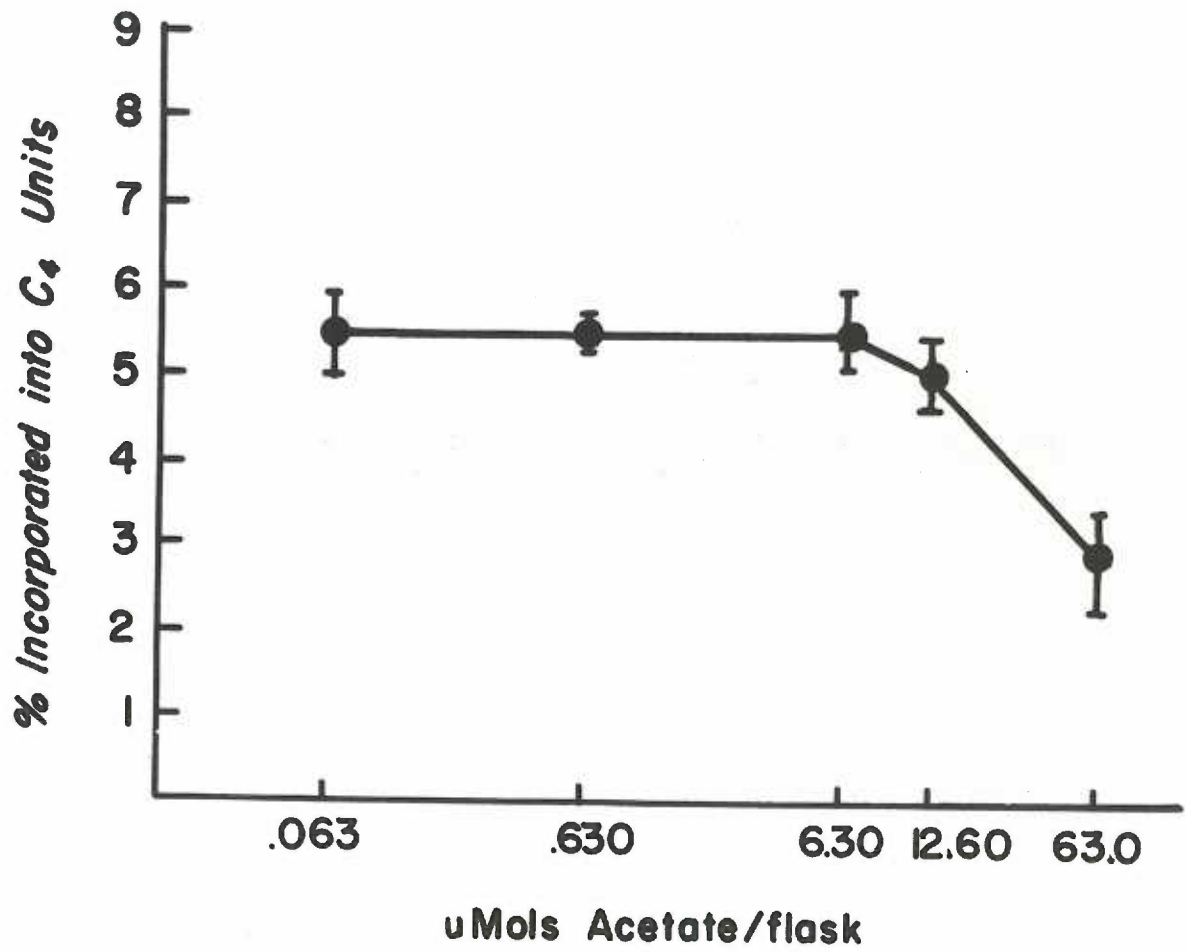
Figure 10

As the amount of acetate-1-C^{14} is increased over 6.3 uMols per flask the per cent incorporation into C_4 units decreases. At 63.00 uMols of acetate the incorporation has decreased to less than 3.6 per cent. The per cent incorporation figure is a composite of two measurements, specific activity and compartment size. As is detailed below, an increase in this last parameter may be a key factor for the present interpretation.

Figure 11 is a graph of the total amount of acetoacetic acid and total ketone bodies present in the substrate as the acetate dosage is increased. The actual increases in total ketone bodies and acetoacetic acid are accountable for by the amount of acetate that was incorporated into this fraction. For instance, the increase in total amount of ketone bodies from the 63 uMol dose of acetate over the control dose is on the order of 2.5 uMols of C_4 units. At this level, 3.6 per cent of the dose was incorporated, which approximates 2.6 uMols of acetate. This, of course, would produce 1.3 uMols of acetoacetic acid, but this is in fair agreement when compared within the limits of standard error.

Here then may be one of the "bottle necks" in the linear incorporation of acetate into fatty acid and cholesterol with increasing concentration of acetate. This is not an unreasonable finding if consideration is given to the previous remarks. Once acetoacetic acid is deactivated it is no longer available to the liver slice for metabolic purposes because there is no activating enzyme in liver capable of forming acetoacetyl-S-CoA from the free acid. The same can be said of beta-hydroxybutyric acid and consequently they both

Figure 11

Total Production of Ketone Bodies at Varying Acetate Concentrations-The upper graph represents total ketone bodies formed per gm. of liver tissue as the concentration of acetate was increased from 0 - 63 uMols per flask. The lower graph represents the amount of acetoacetic acid formed per gm of liver tissue over the same concentration range.
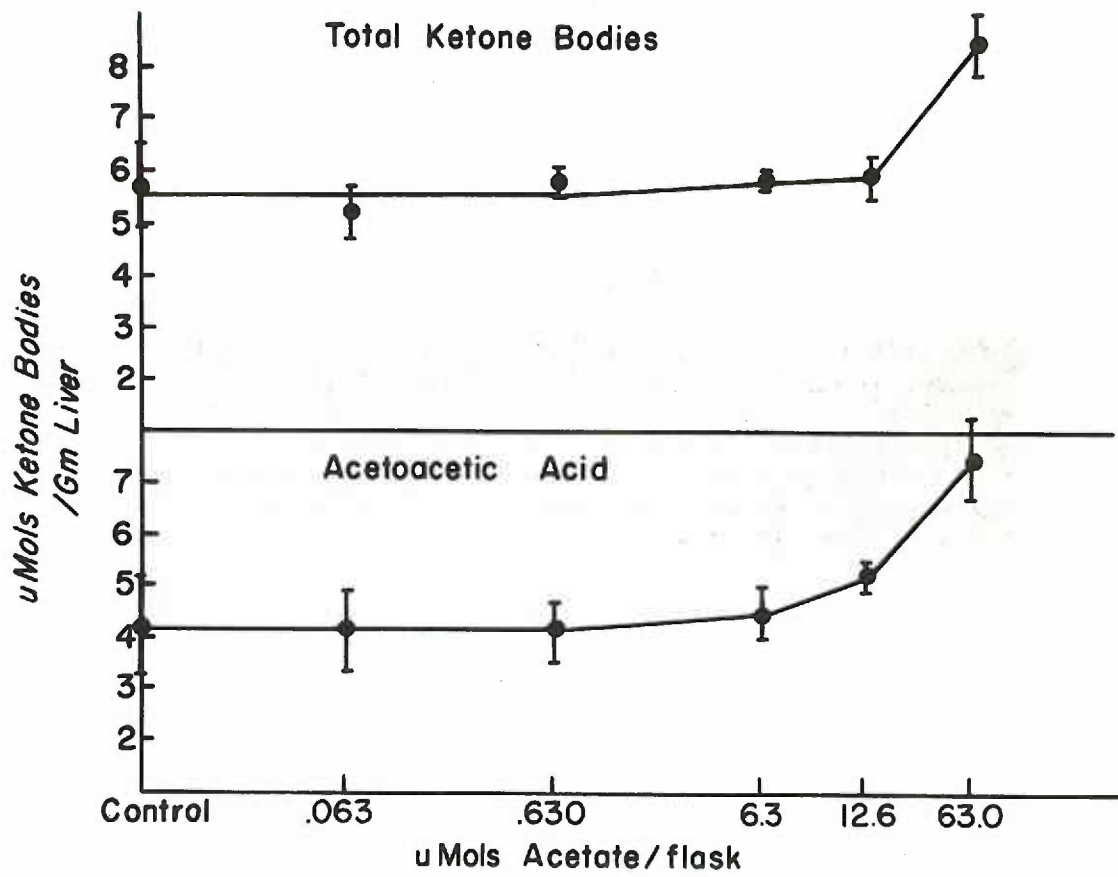
Figure II

accumulate in the suspending medium by diffusion from the cell.
Another point which is evident from these experiments is the fact
that the increase in total ketone bodies is due entirely to aceto-
acetic acid. This, of course, means that the acetoacetic/beta-
hydroxybutyric acid ratio is changing. This again is not too sur-
prising when it is remembered that the conversion of acetoacetic
acid to beta-hydroxybutyric acid is DPNH dependent and that without
an exogenous supply of glucose our system could very well have a low
DPNH/DPN ratio.

CHAPTER III

DISCUSSION

Principles of Tracer Methodology

The use of isotopes as tracers has become a common procedure in recent years and with justification. Many investigators, if asked, define "tracer" as a molecule common to the sequence being studied and non-distinguishable from the common metabolite by the system employing it. A few workers might further qualify their definition to include the statement that the amount of tracer presented to a reaction should not be in amounts sufficient to alter the rate or equilibrium conditions. It is this matter of the amount of added tracer that has been shown by our laboratory[59] to be more critical than heretofore considered.

The assumption must be made in defining a tracer dose that within the limits of tracer conditions, the amount of label appearing in a certain fraction will reflect the amount of label made available to that system. However, when tracer conditions are violated, the concentration of the label per se will affect the rate of the reaction and perhaps the equilibrium position of the reaction or reaction sequenced.

In considering a general approach to the definition of tracer conditions, the following schematic illustrations may be useful. This diagram represents a metabolic pool of A from which there is formed the products, A*B, A*C, and A*D.
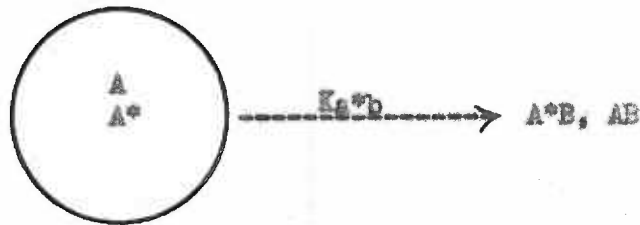
A = Precursor
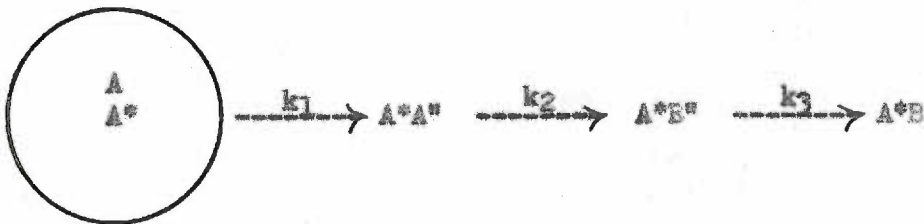
A* = Labeled precursor

K = A rate constant

A*B, A*C, A*D = Products

For general purposes, let us consider only one of these pathways.

In a normal metabolizing system this reaction sequence may be the summation of a number of reactions.

where $Ka*b = \sum_{i=1}^{K} k_i$ and A*A" and A*B" are intermediates in a metabolic stream.

To relate such a scheme to our present discussion it is necessary to add an additional pathway, divergent at one of the intermediate steps.
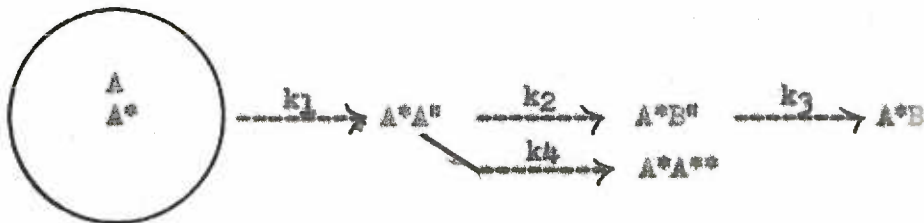
If the amount of A* added to a system is small relative to A, we will find a certain amount of A* in all of the intermediates listed as well as in the product AB. If we increase A*, assuming again that this increase is small compared to total amount of A, a proportionate rise in the amount of A* in each and all of the intermediates and products would be anticipated. These conditions can be described as fulfilling tracer conditions, that is, the system is in no way altered by the presence of A*.

It is apparent that if A* is increased further in amount, there is some concentration which will effect the system. What kind of an influence can be expected and where will the influence first be apparent? These are logical questions and an attempt will be made to answer them. The first influence one might consider is in the rate or rates of the reactions forming a product. One of the earliest changes to be noticed then, might be in the product. Whether the amount of influence would be to increase A*B or to decrease A*B can not be predicted. All that can be said is, if A* is increased to an amount large enough to cause alterations in our system, these alterations should be reflected in the amount of A* appearing in A*B.

Let us suppose that A*B has been measured under "tracer" conditions and then under non-tracer conditions. It is found that there is a decrease in the amount of A* arriving in the product, A*B, as A* was increased beyond tracer limits. This implies that A* is being slowed down or stopped in its movement toward the product. If all of the intermediates, A*B", A*A", and A*A** can be measured, it might be expected that one of these intermediates would have accumulated a greater portion of the label A*. Or to put it another way,

there should be an accumulation of A* in some fraction of our scheme if indeed A* entered the metabolic sequence.

If an accumulation of label in some intermediate could be shown and followed over a concentration range of A*, then limits could be defined for this system. Working within these limits would be working at tracer levels of A*, and going beyond these limits would violate tracer conditions and an indication of the capacity of this system would be ascertained.

A practical obstacle in the above presentation is that none of the intermediates or products from a metabolic system are determined in terms of A^*A'', A^*B'', or A^*B alone, but are evaluated in terms of AB present so that comparisons between systems can be made. The measurement of A^*B as related to AB is a common and accepted practice and a valid one for the term specific activity is widely known and utilized. In this hypothetical system, specific activity is defined as:

$$\text{Specific Activity} = \frac{\text{Activity in terms of } A^*B}{\text{Amount of AB} + \text{Amount of } A^*B}.$$

It is readily apparent from this description that comparisons of specific activities implies a constant amount of product and a constant precursor compartment size. If either of these parameters change in value, the term specific activity loses its comparitive value and needs to be interpreted in terms of the changes just considered.

Before pursuing this line of reasoning further, let us consider a system about which we can be more specific.

When acetate-1-C^{14} is added to rat liver slices incubating in Krebs phosphate buffer there are a number of pathways available for its utilization. Common to all of these pathways is the step of acetate activation to acetyl-S-CoA. The question has been raised that perhaps acetate per se is not a normal metabolite in this tissue, only the activated form. However, since there is a "specific enzyme" present in rat liver for acetate activation, teleological reasoning would suggest that acetate must be a common metabolite within this system, otherwise there would be no reason for the existence of the enzyme. It is also true that acetate constitutes a part of the diet of humans while in ruminants, it may constitute a major source of digested and absorbed carbon. After the activation of acetate to acetyl-S-CoA, it can be diverted in a number of directions. It may condense with oxalacetate and become part of the tricarboxylic acid cycle. It may also condense with another molecule of its own kind to become acetoacetyl-S-CoA. If acetoacetyl-S-CoA is formed, then the system may diverge further for acetoacetyl-S-CoA can be reduced to beta-hydroxybutyric acid in the presence of adequate DPNH and be converted to a fatty acid molecule, or it can condense again with acetyl-S-CoA to form a six carbon moiety common to cholesterol metabolism.

Emerson and Van Bruggen[59] have shown that as the concentration of acetate is raised from 9×10^{-8} to 9×10^{-2} molar, the amount of label incorporated increases with increasing dosage of acetate in all fractions studied; CO_2, fatty acids, and cholesterol, but not to the same extent and not linearly in the case of fatty acids and cholesterol. For the fatty acid forming system of this tissue,

there were no conditions studied where there was a theoretical linear increase of activity into the fatty acids formed on increasing the dosage of acetate. This same situation was found true for the cholesterol fraction and deviation is even more pronounced.

Since the activation of acetate is common to all three major pathways, CO_2, fatty acid, and cholesterol, this was of course the first consideration. However, if in the dosage study, the activation of acetate was the rate limiting step, this would be reflected in the CO_2 producing pathway as well as in cholesterol and fatty acid synthesis. That label can be incorporated into CO_2 in a linear fashion over the same concentration range that appears to limit the amount of label into the other fractions would indicate that the activation of acetate is not the primary rate limiting step. If acetate is being activated, then there should be an accumulation of radioactive metabolite somewhere along the pathway, if indeed there is a block to the flow of acetate into fatty acids and cholesterol.

The sequence of acetate utilization next leads us to the condensation of two acetyl-S-CoA molecules to form acetoacetyl-S-CoA with the liberation of free Coenzyme-A. If this were the rate limiting step, one would expect to find an accumulation of acetyl-S-CoA. That this does not happen has been shown in this laboratory with the use of hydroxylamine to form acetohydroxamic acid from liver tissue with subsequent chromatography. There is no accumulation of color on spraying the chromatogram with ferric chloride color reagent nor any accumulation of radioactivity at the Rf value corresponding to acetohydroxamic acid.

In radioassay tracings of chromatograms prepared from liver tissue incubated with acetate, it was found that there is a consistent accumulation of radioactivity within a certain area. This activity has been shown to be due to acetoacetic acid.

After determining the amount of radioactivity in the C_4 units of tissue extracts, it was still apparent that there was label not being accounted for which under theoretical considerations should have arrived in the product. This was resolved when the suspending medium of our system was analyzed for C_4 units. As presented earlier, most of the activity found in the suspending medium was associated with C_4 units (ketone bodies) which had been formed as acetoacetyl-S-CoA, reduced to the free acid and was no longer available for metabolic purposes because of a lack of activating enzyme in this system.

With the preceding information, the following more specific scheme of metabolism, as compared to the general scheme suggested earlier, is proposed.
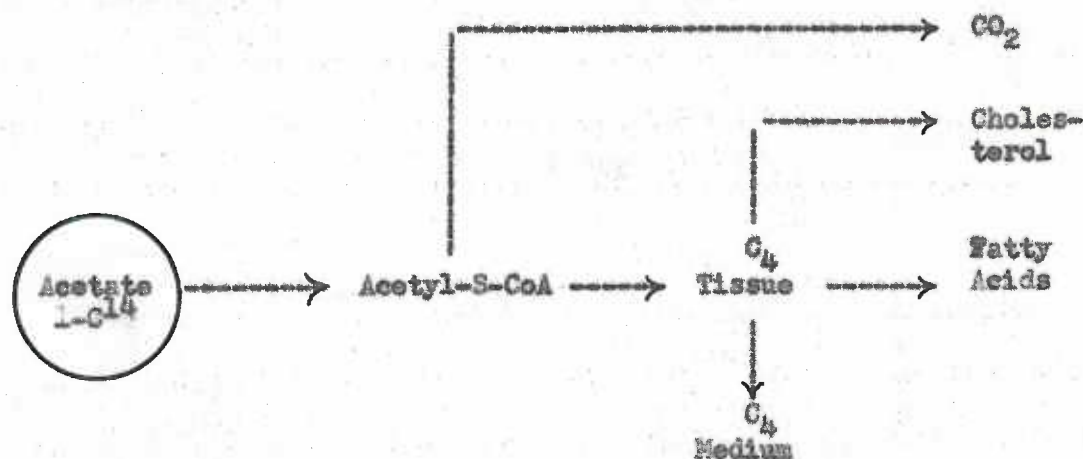
The metabolic pool which was described earlier as A* is now defined as the dose of acetate made available to the liver tissue slice.

From this pool is arising CO_2, cholesterol and fatty acids through
the metabolic pathways described in the scheme of fatty acid synthesis
from acetate described earlier. As has been shown by Emerson and
Van Bruggen[59], and data presented in this thesis, a certain dose of
acetate gives a different response depending on which of the three
large fractions of the above scheme is studied.

That the activation of acetate is not limiting is shown by the
fact that CO_2 responds in a linear fashion as regards incorporation
of acetate up to doses as high as 10^{-4} molar, whereas the incorpora-
tion of acetate into fatty acids and cholesterol is not linear at
the lowest doses studied, 10^{-9} molar. From this information it must
be assumed that the activation of acetate is not a limiting step.

From the scheme above it is evident that the formation of aceto-
acetyl-S-CoA by the condensation of two active acetates is the next
common intermediate in cholesterol and fatty acid synthesis. When
the activity of the C_4 intermediate is analyzed it becomes immedi-
ately apparent that here is at least one of the major pathways for
diverting acetate from its flow into fatty acid and cholesterol syn-
thesis. When the C_4 units of the suspending medium are determined
as to amount and incorporation of label, it is shown that up to doses
as hight as 12.6 uMols of acetate-1-C^{14} per gram of tissue per 27 ml.
of substrate can be incorporated into this system in a linear fashion.
All doses of acetate up to 12.6 uMols caused 5.5 % of that dose to be
incorporated into the C_4 units of the suspending medium. When 12.6
uMols are used there appears to be a decrease in % incorporation and
when the dose is extended to 63 uMols the % incorporation has dropped

to 3.6 %. It is this level of acetate that causes a deviation in the CO_2 response curve and the possibility presents itself that at this level of acetate concentration the system is reaching its limits of acetate activation.

The statement has made earlier that if an accumulation of label in some intermediate could be shown and followed over a concentration range, then limits could be defined for this particular system. We feel that this has been accomplished for the system outlined and that any attempt to determine rates of reaction or flux through a particular compartment are doomed to misinterpretation if the label being used is not functioning under tracer conditions. If rates of reactions are being influenced and/or if compartment sizes are being changed because of the affect of the tracer, then nothing more is being measured than the capacity of the system.

That non-tracer conditions can exist has been shown in the work of this thesis and a system has been described and experimentally developed to point out where in the flow of metabolites the system has been affected under "tracer and "non-tracer" conditions.

In previous remarks the premise was put forth that if a label was presented to a tissue in concentrations not exceeding tracer amounts, this label would be incorporated into a product without in any way influencing the rate or amount of synthesis of this product. If at any time the amount of label is presented in such quantity as to influence this rate or amount of synthesis, then tracer conditions have been violated. As has been shown from the previous data, this amount of label is considerably less, in the case of acetate-1-C^{14}, than has been appreciated by a great many workers.

CHAPTER IV

SUMMARY STATEMENTS

1. The preparation of a variety of hydroxamic acids was carried out using the method of Y. Inoue and H. Yukawa with modifications as listed in the text.

2. The Rf values of a number of important biological hydroxamic acid derivatives were determined.

3. A quantitative determination of hydroxamates was developed using a modification of the Lipmann and Tuttle technique and using standard solutions as prepared from 1 above.

4. Known amounts of laurohydroxamate were eluted from chromatographic paper and determined with the quntitative technique developed above and this elution technique was also shown to be quantitative.

5. The color of laurohydroxamate ferric complex was determined to be stable up to at least two hours under the conditions employed.

6. The enzymatic formation of hydroxamates was demonstrated and compared to the amount formed in an aqueous cell free system.

7. Conversion of thiol acyls in biological systems was shown to be rapid and quantitative as was the recovery of these derivatives formed by the tissue.

8. 0.3 molar hydroxylamine was shown to be optimal for the complete reactivity of thiol acyls while eliminating carboxylic esters from reaction.

9. The inhibition of tissue enzymes concerned with enzymatic formation of hydroxamates was shown to be complete with 70 degree temperature incubation for 10 minutes prior to reacting with hydroxylamine.

10. The liver slice technique was described.

11. Two methods for assaying radioactivity of chromatograms were described as was also the equipment necessary for this assay.

12. The results of an attempt to separate hydroxamates using column chromatography was presented and the conclusion drawn that aceto, C_2, could be separated from palmitohydroxamate, C_{16}.

13. The method of Bessman and Anderson for the determination of ketone bodies was outlined. Interfering substances were listed and the limitations of the method discussed.

14. Many of the above techniques were employed in studying metabolizing systems in relation to their response to increasing dosage of acetate-1-C^{14}. The analysis of ketone bodies and the radioactivity associated with these metabolites was shown. It was concluded that the liver slice technique is much too sensitive to the concentration of acetate for any analysis of rates of reaction to be considered.

15. A theory of tracer methodology is presented based on the experimental data of this thesis. The main conclusions to be drawn from this data is, if at any time the amount of label presented to the tissue is in such quantity as to influence the rate of reaction or the compartment size of a precursor, this changes the interpretation which can be placed on specific activity data. It also implies that tracer conditions have been violated and that the label is no longer being handled as a tracer molecule. It is shown that this a-mount of label is considerably less in the case of acetate-1-C^{14} than has been appreciated by the majority of workers in the field.

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