LIPID TECHNIQUES

AND

ACETATE DOSAGE STUDIES

by

Leonard N. Johnson, B.S.

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CHAPTER I

INTRODUCTION

General Description of the Investigations

A portion of the work embodied in this thesis originated as the result of the need for improved radioassay and lipid separation techniques. An additional portion of the work which is presented is related to earlier studies on tracer methodology.

In radioassay procedures the Geiger-Muller counter is sensitive to any source of ionizing radiation. Any cross contamination of C^{14} labeled lipid fractions will thus lead to erroneous results. A procedure was needed for fatty acid purification that could result in the separation of fatty acids of high radio-chemical purity. Such a separation was doubly important for it was also desired to isolate individual fatty acids from the complex mixtures which occur biologically.

Another need was for an adequate semi-micro lipid extraction procedure. Previous lipid separation procedures required the extraction of the four tissues, liver, gut, carcass and skin of a 200 gm. rat after each tissue fraction was first digested in alcoholic KOH. The extraction of the digested tissue involved the use of some 12 separatory funnels some of which were of the two liter size. These large separatory funnels proved hard to clean and above all, hard to decontaminate. Because of the bulki-

ness of this macro procedure and because of separatory funnel stop cocks that had to be lubricated, loses and/or grease contamination, errors became a problem. This thesis describes a new semi-micro, quantitative method of lipid separation.

With the advent of more sensitive radio assay equipment, a re-evaluation of lipid radioassay techniques was necessitated. The fatty acid plating method previously employed in this laboratory, required about 2 mg. of fatty acid per $cm.^2$ or approximately 10 mg. per plate. Because of the large amounts of fatty acid involved and because of the technique of preparation, each plate had a different geometry (or surface) creating an unwanted variable. Described are new methods of radioassay that eliminate much of the uncertainty described above.

The final study presented attempts to answer the question; what constitutes the size of a tracer dose of labeled metabolite? Few investigators have considered this problem and fewer have studied it, yet it is a very important one to consider when interpreting data on intermediary metabolism. The above question must be asked regardless of the kind of tracer or labeled compound used, for the size of the tracer dose may be different for various labeled compounds and for various routes of administration. The tracer used in this study was sodium acetate labeled in the carboxyl carbon with C^{14}. The purpose of this study was to determine if varying concentrations of sodium acetate had

any effect upon fatty acid synthesis, cholesterol synthesis and/or on carbon dioxide production. Within the dosage range studied the labeled acetate appeared to be tracer in amount.

CHAPTER II

Review of
Acetate Activation and Metabolism

The following is a review of the history of acetate activation and metabolism. This section was assembled and written to help the reader gain a greater appreciation of the role that acetate plays in intermediary metabolism and to aid the writer in his comprehension of the general problem.

Summaries of much of the following material were found in the review entitled "The Metabolism of Acetic Acid in Animal Tissues" written by Bloch[1] and in the paper based on Lipmann's Nobel Prize address[2] entitled "Development of the Acetylation Problem, a Personal Account". For a more comprehensive and detailed review of this subject these two papers may be consulted.

Early Historical Information

The biosynthesis of long-chain fatty acids from small molecules, notably from two-carbon conpounds, is one of the oldest concepts of biochemistry, the idea dating back almost 100 years. Raper[3] wrote in 1907; "The formation of fatty acids in animals from carbohydrates, and the occurrence in natural fats, such as butter, of all the fatty acids containing an even number of carbon atoms, from two to twenty, suggest that these fatty acids are produced by the condensation of some highly reactive substance containing two carbon atoms

and formed in the decomposition of sugar".

This view was formulated after Lawes & Gilbert[4] in 1866 had demonstrated by balance-sheet experiments the conversion of carbohydrate to fat and after Nencki[5] had found in 1878 that two molecules of acetaldehyde, a product of fermentation of sugar by yeast, condensed in an alkaline medium to aldol. Nencki[5] suggested that aldol might be rearranged to butyric acid. Magnus-Levy[6] in 1902 and Raper[3] in 1907 developed this idea further and it was considered that the long-chain fatty acids might be formed by multiple condensations of acetaldehyde. Although it is now known from experiments with isotopic tracers that acetaldehyde as such is not used for fatty acid synthesis, nevertheless, presumable after its conversion to "acetate", acetaldehyde is an effective source of carbon for fatty acid synthesis.

Another important observation was made by Embden and Oppenheimer[7] in 1912. They showed that, when the surviving liver of dogs was perfused with blood to which pyruvate was added, acetoacetic acid appeared in the blood. Also Loeb[8] in 1912 and Friedmann[9] in 1913 demonstrated the formation of acetoacetic acid from acetate.

The first experimental demonstration that two-carbon compounds were indeed used in the synthesis of fatty acids and sterols was made by Smedley, MacLean and Hoffert[10] in 1926, who found that these lipids accumulated in yeast cells grown in a medium in which either ethanol or sodium acetate was the sole source of carbon. Nevertheless, the

real importance of two-carbon compounds in the biosynthesis of fat in animals was not fully appreciated until after 1935, when Schoenheimer and Rittenberg[11] introduced the technique of isotopic labelling for the study of this problem.

Bloch and Rittenberg[12, 13, 14] reported that the small molecules from which cholesterol and fatty acids are synthesized in animals are acetate units, or a more reactive derivative of acetate. Since then the central role of acetate in the metabolism of fat has been well established, almost entirely on the basis of work carried out with the aid of isotopic tracers.

Although acetic acid had long been suspected to be an intermediate in animal metabolism, the significance of its role in biochemical processes has only been recognized for about the past 15 years. The lack of information on acetic acid metabolism during the past is in part attributable to the inadequacy of methods for its identification and for its quantitative determination and partly due to the fact that in some isolated tissues acetic acid appears to possess only limited reactivity. Up until the past 10 or 15 years the principal approach to problems of intermediary metabolism had been the balance experiment, i.e., the measurement of the changes in concentration of the reaction product induced by varying concentrations of suspected precursor. The only reactions of acetic acid in animal tissues which could be reasonably well established by balance experiments were the acetylation of foreign amines[15, 16] and the formation of

acetoacetic acid[8, 17, 18]. A much greater variety of reactions involving acetic acid has since been revealed with the aid of isotopically labeled substances. The utility of the tracer technique for the study of intermediary metabolism derived from the fact that with its aid, biochemical conversions become demonstrable irrespective of whether or not the total quantity of the reaction product undergoes a change. Thus the use of labeled acetic acid has been of particular service in detecting acetate formation and in demonstrating its participation in processes which proceed independently of exogenous acetate supply, viz., the biological synthesis of steroids, fatty acids, porphyrin, glucose and uric acid.

Studies on Acetylation

Acetylation is one of the processes occurring in the metabolism of foreign amines prior to their elimination from the animal body. To what extent acetylation takes place in a given case will depend on the chemical nature and the dosage[19] of the foreign substance and on the species of the test animal. In rats, sulfanilamide is 1.) excreted unchanged, 2.) converted to N-acetyl sulfanilamide and 3.) oxidized to a hydroxyl compound which can subsequently be conjugated with glucuronic acid.

The view that detoxification reactions occur with foreign substances in animal tissues mirror normal events, formed the basis for the classical investigations of Neubauer, Knoop and Dakin, who employed phenyl-substituted

compounds as models for the study of the metabolism of the fatty acids. It attests to the validity of the original concepts that the conclusions arrived at by these investigators some fifty years ago have not required fundamental revision.

Many attempts were made to identify the nature of the acetyl precursor by balance experiments with intact animals but no clear picture was formed. For instance, there were reports both that the dietary addition of acetate depress (20, 21) and increase(22) acetylation of sulfanilamide. The probable acetyl precursors such as acetate, pyruvate, or acetoacetate must be available from metabolic reactions in much greater quantities than are required for the acetylation of the relatively small amounts of foreign amine which can be given to animals so that the dietary addition of precursors is unlikely to influence the yield of acetylated product. Direct evidence concerning the sources of acetyl groups for foreign amines has been obtained from feeding experiments with isotopically labeled test substances. Bernhard has administered to humans and rabbits deuterio acetic acid(23) and deuterio ethanol(24), as sources of acetyl for either sulfanilamide, p-amino benzoic acid or cyclohexyl-alanine(25). In all cases high concentrations of deuterium were found in the excreted acetyl derivatives, demonstrating the conversion of both acetate and ethanol to acetyl groups for aromatic amines and foreign amino acids.

Acetylation of sulfanilamide has been investigated in

slices of guinea-pig liver by Klein and Harris[15] and in homogenate and extracts of pigeon liver by Lipmann[16]. In these investigations the aromatic amines, sulfanilamide and p-amino benzoic acid, were used as acetyl acceptors. In these systems acetate was the most potent acetyl donor, while acetoacetate and pyruvate showed a lesser effect. The results suggest that the immediate reactant is either acetic acid itself or a C_2 compound which under the conditions arises more readily from acetate than from pyruvate or acetoacetate. Five times as much acetylsulfanilamide was formed under aerobic than under anaerobic conditions[16]. Although oxygen does not enter into the formal equation of the acetylation by acetate, aerobiosis is required to supply the energy for the condensation. Lipmann demonstrated that acetylation of sulfanilamide takes place also anaerobically if adenosine triphosphate is added to the reaction mixture [16].

It was evident that whatever the nature of the C_2 compound which is the immediate acetyl precursor, it can readily be formed from acetic acid itself. The effect of adenosine triphosphate on the acetylation reaction observed by Lipmann[16] suggests that the condensation between the carboxyl group of acetic acid and the amine involves the elimination of phosphoric acid rather than of water. Since the effect of adenosine triphosphate cannot be attributed to the intermediary formation of acetylphosphate, the possibility remained to be explored that phosphorylated sulfanil-

amide of an acetyl derivative of adenosine triphosphate may be formed as intermediates.

Formation of Acetic Acid

The first suggestions of the formation of acetic acid itself in quantity in animal metabolism were contained in experiments of Knoop(26) and Dakin(27) on the biological degradation of phenyl-substituted fatty acids. The excretion of either phenylacetic acid or benzoic acid, depending on the number of carbon atoms in the aliphatic chain of the test substance, was most reasonably explained as resulting from the successive removals of two carbon fragments by hydrolysis of an intermediate beta-keto acid. Acetic acid had been proposed repeatedly as an intermediate in the main path of carbohydrate oxidation. Thunberg(28) suggested that oxidative decarboxylation of pyruvate acid gave rise to acetic acid, two molecules of which were dehydrogenated to form succinic acid. Several amino acids, in the course of their metabolism, were found to form intermediates which were identical with products of either carbohydrate or fatty acid breakdown, pointing to protein as a potential source of acetic acid. Thus it was conceivable that the metabolic paths of the three major dietary and tissue components, fat, carbohydrate and protein, could converge at the two carbon stage and that, once this stage had been reached, only one common mechanism of oxidation would be required. From the foregoing it would seem that as an intermediate in the main chain of metabolic reactions, acetic acid should continually

arise in large quantities. An analysis of tissues and body fluids revealed that in the equilibrium state, acetic acid is present in quantities which are barely detectable(29). Specific methods for the determination of small quantities of acetic acid in biological mixtures are not available and it may also escape detection because it maybe firmly bound to protein in biological media(30, 31). Even if acetic acid were found to be practically absent, the suggested role of acetic acid as a major product of catabolism would not be contradicted. The condition which has to be satisfied, and which applies to all metabolites which lie on the main metabolic paths, is a capacity of the tissue to metabolize the intermediate as rapidly as it is formed. The concentration of the metabolite at any one time will then be less important. The significant concentration of acetic acid and other fatty acids of intermediate size in animal tissues was however cited as evidence against the theory of beta-oxidation (32).

Evidence for the occurrence of a metabolite which does not normally accumulate in sufficient amounts to permit analytical detection can be obtained with the aid of the isotope dilution method. The isotopic analogue of a suspected intermediate is administered to an animal, or added to an isolated system, and subsequently recovered either as such or in a combined form. A diminished isotope content of the isolated material indicates that the test substance had merged with identical unlabeled molecules formed in the

tissues. Bernhard, in an investigation of the acetylation of foreign amines with labeled test substances(23) found that the acetyl groups of excreted acetyl sulfanilamide contained 5 to 20 per cent as much isotope as the deuterio acetic acid added to the diet. He concluded that the fraction of acetyl groups contributed by exogenous acetate was related to the difference of isotope content in the test substance and excreted acetyl. From such experiments, Bloch and Rittenberg(13, 33) concluded that the isotopic dilution obtained in the acetylation process can be attributed to the merging of labeled dietary acetate with acetate arising in metabolism.

Formation of Acetoacetic Acid

Fatty acid oxidation has been investigated in a variety of systems; in the fasting or diabetic animal, in perfused livers, in various liver preparations, and in the intact animal with the aid of labeled test substances. Under all conditions except those of the intact animal studies, acetoacetic acid is the principal product of the oxidation process. Leloir and Munoz(34) detected acetic acid in the course of octanoate oxidation by liver, but the amount was insignificant compared with the quantities of ketone bodies which accumulated. Deuel and co-workers(35, 36) found a uniform increase of ketone body excretion with increasing chain length of the fatty acid administered. In vitro, fatty acids appeared to become increasingly resistant to oxidation as the length of the chain increased. The de-

pressing effect of higher fatty acids on the respiration of isolated tissue, noted by Quastel and Wheatley[37], was attributed to their surface activity. Lehninger showed, however, that oxygen uptake can be restored and that the higher fatty acids can be oxidized by addition of adenosine triphosphate to the liver system[38]. Under these experimental conditions[38], the carbon atoms of some even and odd numbered fatty acids could be accounted for as acetoacetate in nearly quantitative yeilds.

Since acetoacetic acid accumulated as the principal product of fatty acid oxidation, it is not surprising that acetoacetate rather than acetate was frequently emphasized as the primary oxidation product. However, experiments have furnished clear evidence for the correctness of the principle of beta-oxidation as proposed by Knoop[26]. Stepwise degradation of a biologically occurring fatty acid by elimination of two carbon atoms was first demonstrated by Schoenheimer and Rittenberg[11].

The two carbon fragment which is detached from the fatty acid chain was not identified as such, although its intermediate formation has been clearly established. Weinhouse, Medes and Floyd[39] investigated, in liver slices, the oxidation of octanoic acid which was labeled at the carboxyl group with C^{13}. The acetoacetic acid which accumulated as the main product, contained labeled carbon in nearly equal concentrations at the carbonyl and carboxyl positions. Acetoacetate with such isotope distribution

could not have resulted from the splitting of the C_8 acid into two four carbon compounds, nor could it have been formed exclusively from carbon atoms 5 to 8 of the octanoic acid. Acetoacetate must have been formed, at least in part, by random recondensation of liberated C_2 fragments.

The demonstration of the ketogenic effect of valeric acid[40, 41] had an important bearing on the development of more recent theories of fat oxidation. This finding could not be adequately explained by either classical beta-oxidation or by the hypothesis of multiple alternate oxidation. MacKay et al.[41] suggested that the five carbon chain of valerate was degraded to a three carbon and a two carbon unit and that two of the latter combined to yield acetoacetate. It became evident therefore that ketone bodies could be formed by recondensation of two carbon fragments split off from the fatty acid chain as well as by direct oxidation.

It is generally believed that amino acids are catabolized by oxidative deamination to alpha-keto acids with subsequent oxidation and decarboxylation to acids containing one carbon atom less. Amino acids which yield short chain fatty acids as intermediates should be sources of acetyl groups. This possibility has been studied with the amino acids leucine and valine which contained carbon chains labeled by deuterium[42]. Deuterioleucine yielded labeled acetyl groups to the same extent as did isovaleric acid indicating the acetic acid was formed as an intermediate in

the formation of acetoacetic acid. The ketogenic effect of leucine[43, 44] was explained on the same basis. Valine and isobutyric acid both are non ketogenic and are also ineffective as sources of acetyl groups.

Formation of acetate from pyruvate can be demonstrated to occur in animal tissues, either aerobically[45, 46, 47] or anaerobically by dismutation[45, 46, 48] but this reaction is not believed to represent an important pathway, because under the same conditions acetate itself is oxidized relatively slowly. It is considered more likely that the oxidation product of pyruvate is represented by a two carbon compound which is metabolically more reactive than acetic acid itself. A phosphoroclastic splitting of pyruvate into acetyl-phosphate and formate as it occure in bacteria[49] has not been observed in animal tissues.

Metabolism of Acetic Acid

The administration of labeled acetic acid to animals has been found to result in the incorporation of isotope into a variety of tissue sonstituents: glycogen[50, 51, 52, 53, 54], cholesterol[10, 12, 42, 55, 56, 57, 58, 59, 60], fatty acids[10, 55, 57, 61, 62], the dicarboxylic amino acids[63, 64], protoporphyrin[33, 65, 66], uric acid[67, 68], citrate[69] etc. It thus appears that in the biological formation of the cell constituents, acetic acid is of general importance as a source of carbon atoms. It has become increasingly evident in recent years that many body constituents of high molecular weight are synthesized by con-

densation of numerous small sized units rather than by the utilization and rearrangement of preformed large molecules.

The ability of animal tissues to utilize acetic acid efficiently is illustrated by the fact that when fed to animals, acetic acid does not appear in the urine in measurable amounts. In the experiments of Buchanan, Hastings and Nesbett[51] with acetate containing radioactive carbon in the carboxyl group, over fifty per cent of the acetate obsorbed by the rats was totally oxidized within two hours, as determined by the appearance of isotopic carbon in the expired carbon dioxide.

Since in isolated tissues, acetic acid showed less reactivity than in the intact animal the possibility had been widely discussed that acetic acid was metabolically converted to a more reactive form.

The Role of Acetyl Phosphate

It was the interest of Lipmann in the phenomenon of the Pasteur effect, namely the depression of fermentation in the respiring cell, that helped to explain the mechanism of acetate activation. By looking for a chemical explanation of this economy measure at the cellular level, he was diverted into a study of the mechanism of pyruvate oxidation, for it is at the pyruvic acid stage that respiration branches off from fermentation. For this study he chose a relatively simple system, that of a pyruvic acid oxidation enzyme in a certain strain of Lactobacillus delbrueckii.

An important event during this period was the accidental

observation that, in the L. delbrueckii system, pyruvic acid oxidation was completely dependent on the presence of inorganic phosphate. This observation was made in the course of attempts to replace oxygen by methylene blue. To measure the methylene blue reduction manometrically, Lipmann had to switch to a bicarbonate buffer instead of the otherwise routinely used phosphate buffer. In the bicarbonate buffer, pyruvate oxidation proved to be very slow, but the addition of a little phosphate caused a remarkable increase in rate.

In spite of such a phosphate dependence, the reaction did not at first indicate any phosphorylative step. Nevertheless, the suspicion remained that phosphate in some manner was entering into the reaction and that a phosphorylated intermediary was formed. As a first approximation, a coupling of pyruvate oxidation with adenylic acid phosphorylation was attempted. Addition of adenylic acid to the pyruvic oxidation system brought out a net disappearance of inorganic phosphate, which was accounted for as ATP. In parallel with the then just developing fermentation picture, Lipmann concluded that the missing link in the reaction chain was acetyl phosphate. In partial confirmation it was shown that a crude preparation of acetyl phosphate would transfer phosphate to adenylic acid. However, it still took quite some time to identify acetyl phosphate definitely as the product of pyruvic oxidation in this system[70, 71]. An important investigation during this and later work was the developement of analytical procedures[72] and in partic-

ular of the very useful hydroxamic acid method[73] for the determination of acyl phosphates and other reactive acyl derivatives.

There was a further unusual feature in this pyruvate oxidation system in that the product emerging from the process not only carried an energy-rich phosphoryl radical but the product was even more impressive through its energy-rich acetyl. It rather naturally became a contender for the role of "active" acetate, for the wide-spread existence of which the isotope experiments had already furnished extensive evidence. Lipmann was therefore, quite attracted by the possibility that acetyl phosphate could serve two rather different purposes, either to transfer its phosphoryl group into the phosphate pool, or to supply its active acetyl for the bio-synthesis of carbon structures, transferring, on each side of the oxygen center as indicated by Bentley's[74] early experiments on the cleavage of acetyl phosphate in H_2O^{18}.

Lipmann, realizing that a better understanding of the mechanism of group activation was a most urgent problem in biosynthesis, set out to find a suitable system on which to check the assumption that acetyl phosphate represented active acetate. After working out a relatively easy method to prepare the compound[72, 75], a first unexpected difficulty arose when it appeared that animal tissues rather generally contain a very active, specific, heat-stable, acetyl phosphatase[16, 76]. In crude preparations of

muscle, liver, and other tissues, the half-life of acetyl phosphate is only a few minutes. This phosphatase activity in animal tissues made tests with acetyl phosphate very difficult.

In looking for a sensitive method to study acetyl transfer, the acetylation of aromatic amines was chosen as a promising and technically easy procedure. Furthermore Lipmann was quite confident that any results obtained with this method could be generalized over the whole metabolic field concerning the transfer of active acetate, including such reactions as citrate, acetoacetate, and lipid synthesis. Acetylation of sulfonamide had been found to occur in rabbit liver slices(15, 77). Although acetylation occurred in rabbit liver homogenates, the reaction was rather weak. In search of a more active system, pigeon liver homogenate was tried and found to have an exceedingly potent acetylation system(16, 77). This finding of a particularly active acetylation reaction in cell-free pigeon liver preparations was most fortunate and played an important part in the development of the acetylation problem.

Although the acetyl phosphatase activity of the pigeon liver homogenate was considerable and, to some extent, obscured the test with acetyl phosphate, it became clear that acetyl phosphate did not furnish active acetate(16). Under anaerobic conditions with large concentrations of acetyl phosphate, no acetyl groups for the acetylation of sulfonamide could be derived under conditions where an easy

acetylation occurred with a respiring homogenate.

Using ATP and acetate as precursors, it was possible to set up a homogenous particle-free acetylation system obtained by the extraction of an acetone powder of pigeon liver. In this extract likewise, acetyl phosphate was unable to replace the ATP-acetate as acetyl precursor.

Role of Coenzyme A

In spite of the disappointment with acetyl phosphate, the decision to turn to a study of acetylation started then to be rewarding in another way. During these studies Lipmann became aware of the participation of a heat-stable factor which disappeared from the enzyme extracts on ageing or dialysis. This cofactor was present in boiled extracts of all organs as well as in microorganisms and yeast. It could not be replaced by any other known cofactor and he suspected that he was dealing with a new coenzyme. From then on, for a number of years, the isolation and identification of this coenzyme became the prominent task of his laboratory.

The pigeon liver acetylation system proved to be a convenient assay system for the new coenzyme[78], since on ageing for 4 hr. at room temperature, the cofactor was completely autolyzed. Fortunately, on the other hand, the enzyme responsible for the decomposition of this factor was quite unstable and faded out during the ageing, while the acetylation apoenzymes were unaffected.

Finding pig liver a good source of the coenzyme, a

reasonably large quantity of a highly purified preparation was collected allowing an extensive study on the chemistry of this material. In this analysis, particular attention was paid to the possibility of finding in this cofactor one of the vitamins, then not as yet identified with a specific metabolic function. Beverly Guirard, who worked with this preparation, did not seem to find any appreciable amounts of the known vitamins. However, she became aware of the fact that on prolonged enzymatic treatment, the value of pantothenic acid, as determined microbiologically, slightly increased. This gave the hint that the coenzyme did not release the pantothenic acid so easily, a fact known from experiments with pantothenic acid assay in tissue extracts. In confirmation, she found on acid hydrolysis of the coenzyme, considerable amounts of beta-alanine, corresponding to eleven per cent of the pantothenic acid in this preparation. It was later learned that this preparation was forty per cent pure.

The finding of a B-vitamin in the preparation gave the workers greater confidence that they were dealing with a key substance. Lipmann confirmed this finding by liberating the chemically rather unstable pantothenic acid from the factor, making use of observations on enzymatic cleavage of the coenzyme. Two enzyme preparations, intestinal phosphatase and an enzyme in pigeon liver extract, had caused indipendent inactivation. It then was found that through the combined action of these two enzymes, pantothenic acid was

liberated[79, 80].

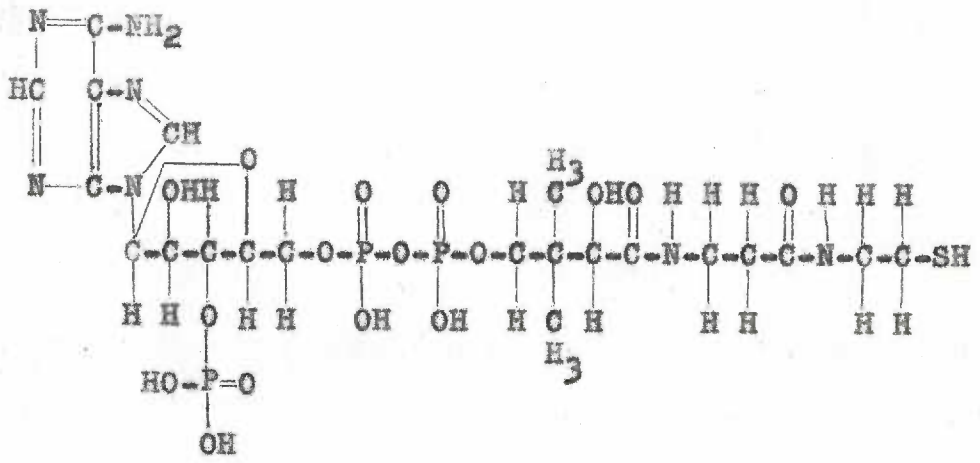
The two independent enzymatic cleavages indicated that in this new factor (coenzyme A) there existed two independent sites of attachment to the pantothenic acid molecule. One of these obviously was a phosphate link, presumably to one of the hydroxyl groups in pantothenic acid. The other moiety attached to pantothenic acid, which cleaved off by liver enzyme, remained unidentified for a long time. In addition to pantothenic acid, this sample of 40 per cent purity had been found to contain about two per cent sulfur by elementary analysis and this sulfur was identified by the cyanide-nitro-prusside test as a potential SH-grouping [81, 82]. Also, the coenzyme preparation contained large amounts of adenylic acid[82].

The phosphate link was soon identified as a pyrophosphate bridge[83]. 5-Adenylic acid was identified by Novelli[84] as an enzymatic split product and by Baddiley [85], through chemical cleavage. At the same time, Novelli made observations that indicated the presence of a third phosphate in addition to the pyrophosphate bridge. These indications were confirmed by analysis of a nearly pure preparation that was obtained by Gregory[86] from Streptomyces fradiae.

It was at this period that more attention was paid to the sulfur in the coenzyme. Lipmann and his group found that their purest preparation contained 4.3 per cent sulfur corresponding to one mole of sulfur per mole of panto-

thenate. They also found[87] that dephosphorylation of CoA yielded a compound containing pantothenic acid and the sulfur-carrying moiety, which they suspected as bound through the carboxyl. Through the work of Snell and his group[88], the sulfur containing moiety proved to be attached to pantothenic acid through a link broken by a liver enzyme. The moiety was identified as thioethanolamine by Snell, the moiety being linked peptidically to pantothenic acid.

Through analysis and synthesis, Baddiley[85] now identified the point of attachment of the phosphate bridge to pantothenic acid in the 4-position, and Novelli et al.[89] completed the structural analysis by enzymatic synthesis of "dephospho-CoA" from pantotheine-4'-phosphate and ATP. The third phosphate was shown by Kaplan[90] to attach in the 3-position on the ribose of the 5-adenylic acid. By these studies the structure of coenzyme A (CoA) was established.



Coenzyme A

During this slow but steady elaboration of the structure, Lipmann's group explored intensively, metabolic mechanisms in the acetylation field. By use of the enzymatic assay, CoA was found present in all living cells, animal, plants, and microorganisms[78]. Furthermore, the finding that all cellular pantothenic acid could be accounted for by CoA[78], made it clear that CoA represented the chief functional form of this vitamin.

The first attempts to explore further the function of CoA were made with pantothenic acid-deficient cells and tissues. A deficiency of pyruvate oxidation in pantothenic acid-deficient Proteus morganii, an early isolated observation by Dorfman[91, 92], now fitted rather well into the picture. Lipmann soon became interested in this effect, taking it as an indication for participation of CoA in pyruvate oxidation in P. morganii[93]. Using pantothenic acid-deficient yeast, Novelli et al.[94] demonstrated a CoA-dependence of acetate oxidation and Olson and Kaplan[95] found with duck liver a striking parallel between CoA content and pyruvic acid utilization.

Another significant step toward a generalization of CoA function for acetyl transfer was made by demonstrating its roll in the enzymatic synthesis of acetoacetate[96]. Soon afterwards Stern and Ochoa[97] showed a CoA-dependent citrate synthesis with a pigeon liver fraction similar to the one used by Soodak for acetoacetate synthesis. Novelli et al.[98] confirmed and extended this observation with ex-

tracts of E. Coli.

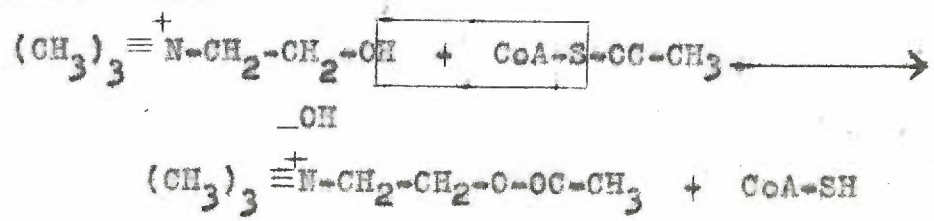
In the course of this work, which more and more clearly defined the acetyl transfer function of CoA, Novelli once more tried acetyl phosphate. It appeared that in E. Coli. extracts, in contrast to the animal tissue, acetyl phosphate as acetyl donor for citrate synthesis was more than twice as active as acetate plus ATP[98]. Acetyl phosphate, therefore, functioned as a potent microbial acetyl donor.

An important missing link in the picture was supplied through the work of Lynen[99] who chemically identified acetyl CoA as the thioester of CoA. Therewith the thioester link (CH_3-CO-S-CoA) was introduced as a new energy-rich bond, and this discovery added a very important facet to the understanding of the mechanisms of metabolic energy transformation.

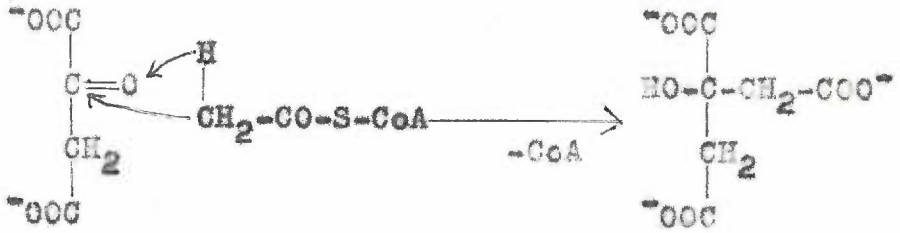
Mechanisms of Acetate Condensation

There is one feature in this picture that has attracted particular attention: that is the two fold type of activation involving (i) the carboxyl end, or the "head" end, of the acetyl and (ii) its methyl, or "tail" end.

The definition of the head reaction is relatively simple. Acetylation of arylamine or choline is a typical head reaction.



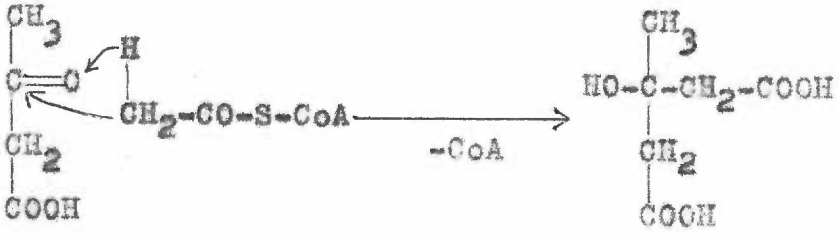
The second type, the methyl, or tail, activation, is not as well understood. In citric acid synthesis, the methyl end of the acetate engages in an aldol type of condensation with the carbonyl group of the oxalacetate as acceptor. This condensation requires an energy input that



Oxalacetate Citrate

must be derived from the thioester link. At the completion of the reaction, CoA appears to be liberated.

For a long time, the citrate reaction was the only known tail condensation. Within the last few years another interesting example has developed in the study of the precursors in steroid and isoprene synthesis. The initial condensation product in this series appears to be beta-methyl, beta-hydroxyglutarate (dicrotolic acid), formed

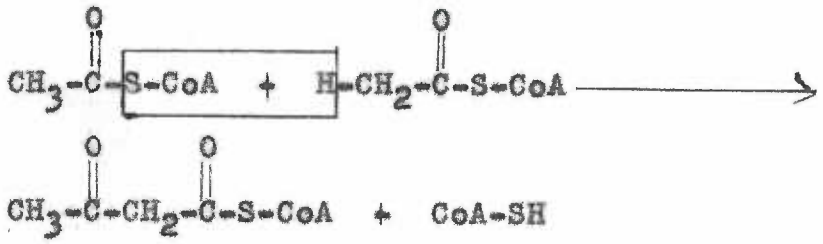


Acetoacetic acid Dicrotolic Acid

through condensation of acetoacetate with acetyl-CoA. The analogy between this and citrate condensation is apparent. This initial condensation seems to be followed by decarboxylation and dehydration to beta-methyl crotonic acid first

demonstrated by Bonner et al.[100] as intermediary in rubber synthesis.

In a third type, a combination of head and tail reaction takes place with two acetyl CoA's reacting with each other in a head-to-tail condensation. Calculation of the energy required yielded a figure of around 15 kilocalories which could be covered by one energy-rich bond[76]. By using as acetyl donor carboxyl-labeled acetyl phosphate, fed through transacetylase, the marker appeared in the carbonyl, as well as the carboxyl, part of acetoacetate. This demonstrated a head-tail character for the reaction. The



finer mechanism of this reaction between two acetyl CoA's has been elaborated in particular by Lynen's group[101], by Green's laboratory[102] and by Ochoa and his group[103]. Presumably in the building up of longer terpene chains, a head-tail condensation may occur between two beta-methyl crotonyl CoA's followed by hydrogenation and dehydration.

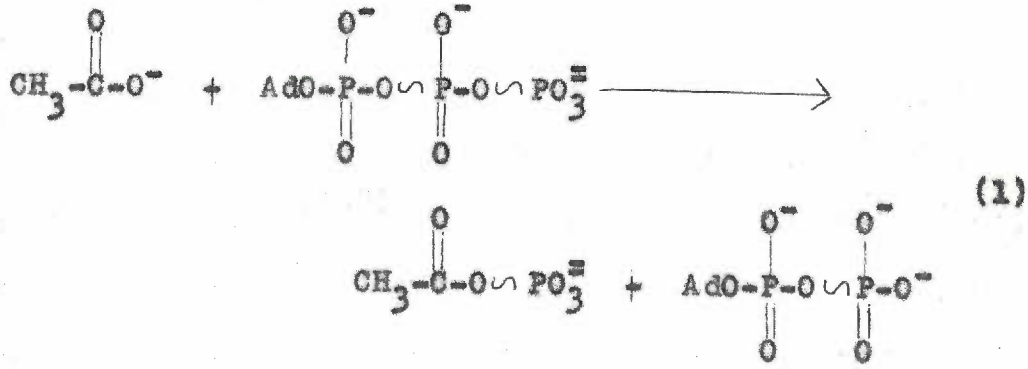
It may be well to mention here the role that succinyl CoA plays in this scheme. Succinyl CoA can be generated either from alpha-ketoglutarate by oxidation in the presence of the appropriate oxidase system or from succinate, CoA, and ATP in the presence of the succinyl CoA-phosphorylating enzyme. Succinyl-CoA in the presence of acetoacetic acid

forms acetoacetyl-CoA and succinic acid. Acetoacetyl-CoA in the presence of additional CoA, forms two molecules of acetyl-CoA providing another mechanism for the generation of acetyl-CoA.

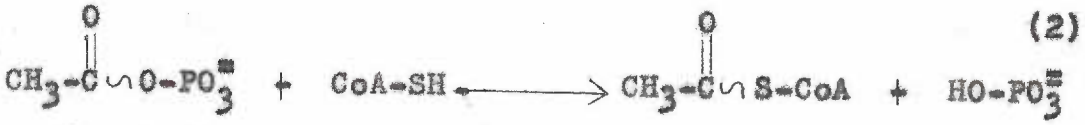
A new type of head-tail condensation, between succinyl-CoA and glycyl-CoA was suggested by Shemin's work on hemin synthesis[104]. The keto, amino dicarboxylic acid formed then appears to be decarboxylated to amino levulinic acid.

Current Theories for the Mechanism of Acetate Activation

The transformation of ATP bond energy is rather straightforward being a sequence of two independent enzymatic reactions, the first a transphosphorylation from ATP to acetate and the second, as discussed, a transacetylation from acetyl phosphate to CoA.



(1)



(2)

It should be noted that in the first transphosphorylation step the acetyl phosphate cleaves and condenses between O and P. In the second transacylation reaction, however, acetyl phosphate cleaves and condenses between the C and O. Thus the same molecule reacts on each side of the

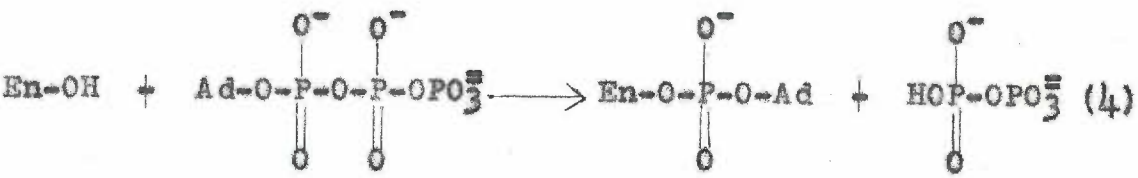
oxygen bridge between the carbon and the phosphorus. A shift from P-OC to PO-C chould actually be a feature of many condensations initiated by a phosphoryl split from ATP. These have become increasingly numerous, and are seen in glutamine, glutathione, pantothenate, and seemingly in protein synthesis.

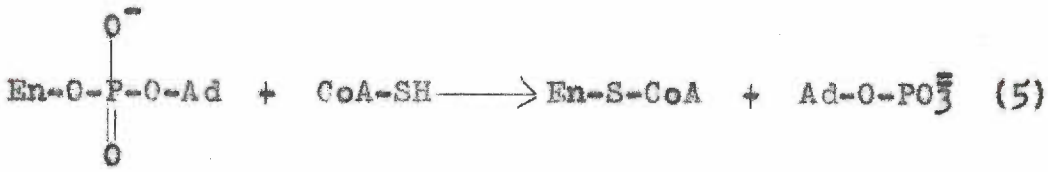
Jones et al.[105, 106, 107] have explored the reaction with liver and with yeast, and a surprising feature was uncovered, namely, that the initial phosphoryl split of ATP occured between the pyrophosphoryl group and AMP. The cleavage products of ATP were identified as inorganic pyrophosphate (PP) and adenylic acid (AMP).

Lynen[101] found some rather revealing information by the use of isotopes. It was found that ATP and radioactive inorganic pyrophosphate exchange in the absence of CoA or acetate. Such an exchange is compatible with an initial reaction between the enzyme and ATP, resulting in a covalent binding of AMP to the enzyme, En,

$$ATP + En \longleftrightarrow AMP\text{-}En + PP \qquad (3)$$

It furthermore was found that acetyl of acetyl-CoA exchanges with radioactive free acetate in the absence of ATP or pyrophosphate. This exchange would indicate an exchange of acetyl for enzyme in the final step. Therefore, an overall sequence (En standing for enzyme) was proposed as follows:



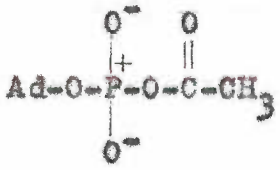


(5)

(6)

The middle step, that is, the substitution of enzyme-bound AMP by CoA is the most problematic but also the most interesting one, since it may foreshadow mechanisms implicating nucleotide activation for polynucleotide formation. No indication was found for the identity of the group on the enzyme through which the initial binding of AMP and further exchange with CoA might occur.

Since the existence of the postulated enzyme-bound substrates was inferred solely from isotope exchange experiments, Berg(108, 109) investigated this hypothesis once more to obtain further information on the nature of the intermediates and the mechanism of their formation. With a more highly purified enzyme than that previously employed, he found that the exchange of PP and ATP does not occur unless acetate is added. This, together with the absence of any exchange of Ad5P and ATP in the presence of CoA alone, as was predicted by Reactions 4 and 5, indicates that the proposed mechanism can not be maintained. Berg(108, 109) then presented evidence in support of another mechanism of acetyl CoA synthesis. This involves a primary reaction of ATP and acetate to form a previously undescribed compound, adenyl acetate



and its subsequent reaction with CoA to form acetyl CoA (Reactions 7 and 8).

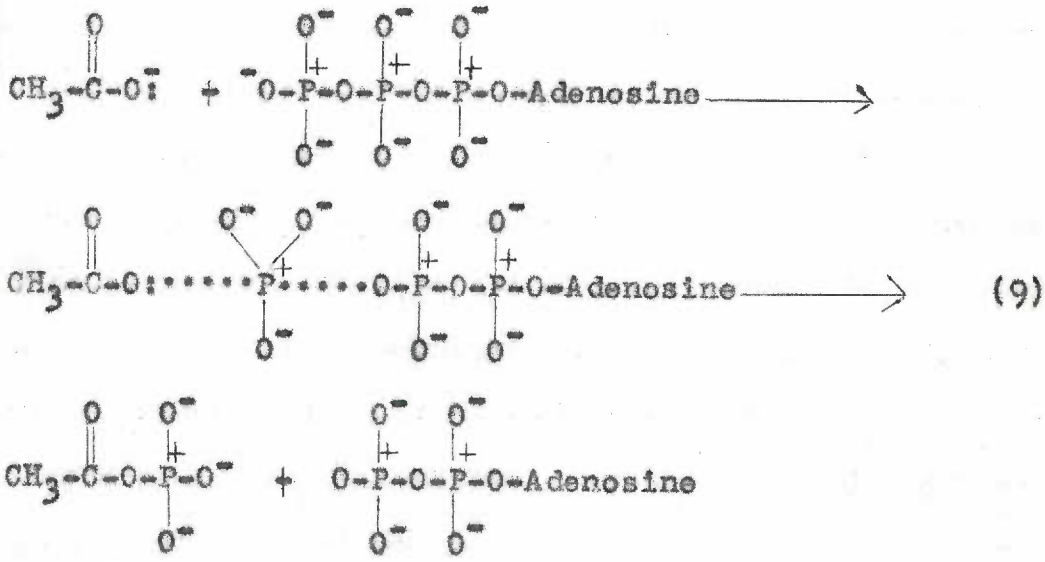
$$\text{ATP} + \text{acetate} \longleftrightarrow \text{adenyl acetate} + \text{PP} \qquad (7)$$

$$\text{Adenyl acetate} + \text{CoA} \longleftrightarrow \text{acetyl CoA} + \text{Ad5P} \qquad (8)$$

Adenyl acetate has been synthesized and demonstrated to react enzymatically in the manner shown above. However, attempts to achieve the net enzymatic synthesis and isolation of adenyl acetate from ATP and acetate have so far been unsuccessful.

The formation of adenyl acetate as an intermediate in acetyl CoA formation by yeast aceto-CoA-kinase is supported by the following observations. (1) The exchange of PP and ATP requires acetate; (2) synthetic adenyl acetate is readily converted to ATP in the presence of PP and to acetyl-CoA with CoA; (3) the CoA-independent accumulation of acethydroxamic acid, PP, and Ad5P in the presence of hydroxylamine; (4) the requirement of acetate and CoA for the exchange of Ad5P with ATP; (5) and the requirement of Ad5P and PP for the exchange of acetate with acetyl CoA. Further support for the proposed mechanism has been provided by the O^{18} exchange studies of Boyer et al.(110). It was found that O^{18}-carboxyl-labeled acetate gave rise to excess O^{18} in the phosphate group of Ad5P. These results could be predicted from reactions 7 and 8.

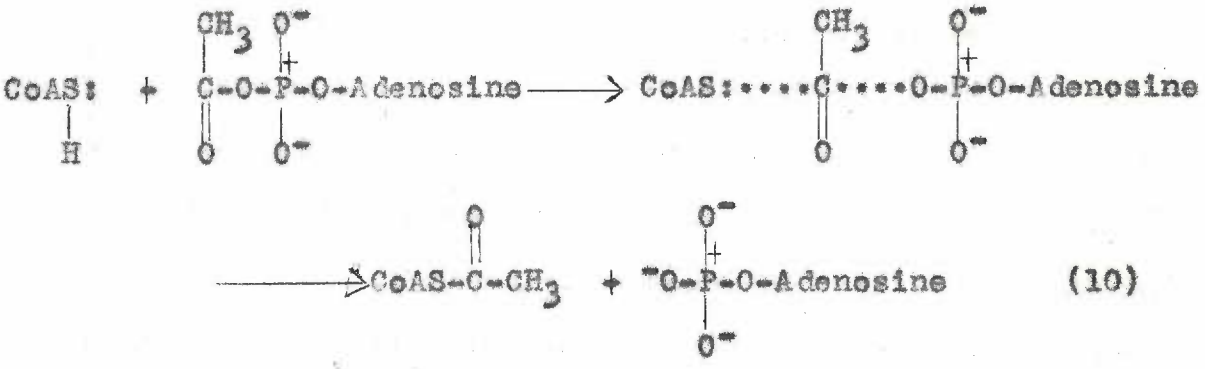
In considering the mechanism of the aceto-CoA-kinase reaction and its analogy with the formation and utilization of acetyl phosphate, certain similarities are apparent. Koshland[111], in a discussion of the mechanism of group transfer reactions, pointed out that many of the well known enzymatic group transfers may be considered as single displacement or substitution reactions. The reaction may be formulated as follows to illustrate the phosphorylation of acetate:



(9)

First, there is a nucleophilic attack on the terminal P atom by the unshared pair of electrons of the acyl oxygen atom, forming an activated complex which breaks down to ADP and acetyl phosphate. In the case of the aceto-CoA-kinase reaction, however, the nucleophilic attack by acetate may be considered to occur in a similar way, but on the adenylic acid phosphorus atom liberating PP and forming a derivative of acetyl phosphate; namely, adenyl acetate. The formation of acetyl CoA by phosphotransacetylase and by aceto-CoA-

kinase also appears to be analogous when considered in this way. Thus a nucleophilic attack by CoA on the acyl carbon atom displaces, in one case phosphate, and in the other, Ad5P (Reaction 10).



(10)

Although it is clear that the formation of acetyl CoA via acetyl phosphate is catalyzed by two separate enzymes, all attempts to show that the aceto-CoA-kinase reaction involves more than one enzyme have been negative. Also studies with the yeast enzyme have given no indication of a separation of the activities shown in Reactions 7 and 8.

It therefore seems possible that acyl adenylate formation may represent a general mechanism for the activation of fatty acids, amino acids, and a variety of compounds containing an acyl group.

It would appear that after nearly 100 years of investigation by many research groups, the activation of acetate may now be known and may be summarized in the following equation:

ATP + acetate + CoA $\longleftrightarrow$ acetyl-CoA + Ad5P + PP

Formation of the acetyl-CoA moiety may occur either by Lipmann's hypothesis, the summation of Reactions 4, 5, and

6, or the hypothesis of Berg, namely the summation of Reactions 7 and 8.

CHAPTER III

Materials, Methods, and Techniques

Animals

The animals used in the experimental work were young adult male rats of the Sprague-Dawley strain, obtained from the Northwest Rodent Farm, Pullman, Washington. Male rats were used to eliminate the variation of fat composition that is know to occur in female rats due to the cyclic effects of sex hormones. Female rats also have a different fat distribution pattern than do male rats, so only the male sex was used. The weights of the rats upon arrival, ranged from 150 gms. to 175 gms. The rats were sorted as to weight, marked by clipping a portion of the ear with a sharp scissors, and placed in wire cages housed in a cage-hood assembly(112).

Trained Feeding

The animals were fed Purina Laboratory Chow, ad libitum, and given adequate water for one or two days. Trained feeding followed and consisted of giving each rat approximately 10 gms. of Purina Laboratory Chow every 12 hours. The rat was allowed one hour to consume his food after which all uneaten food was removed from the cage. Care was taken to see that each rat maintained a steady weight gain. Those animals that did not gain weight or maintain their weight on such a regime were not used. About four to five days were necessary to train the rats to eat all of the allotted

food in the required time of one hour. Rats were weighed daily and only rats that had adjusted to the regime and maintained their weight for a minimum of one week after the initial training period and appeared healthy, were used in the experiments. It was necessary to incorporate a program such as this to insure a constant nutritional state for each animal.

Preparation of Sodium Acetate-1-C^{14}

The sodium acetate -1-C^{14} used in the following metabolic experiments was synthesized in our laboratory(113) from $BaCO_3$ obtained from Oak Ridge, Tennessee. One millicurie lots of the carboxyl labeled acetic acid were stored as dried sodium acetate in 40 ml. narrow mouth centrifuge tubes covered with rubber vial closures. Two of such lots were used in the present studies. To each lot was added 25 ml. of distilled water to disolve the acetate, thus each ml. contained approximately 40 microcuries. Each lot of labeled acetate was radioassayed(114) as infinitely thick $BaCO_3$ and counted under a thin end window G.M. tube (Tracer Lab. TGC-2). The observed counts were corrected for coincidence and thickness when necessary. Multiplication by 2.4 yielded the equivalent value of the sample as if it had been counted with Nuclear's D-47 counter fitted with the thin micro-mil window. One ml. of acetate lot #200 contained 3.72×10^6 counts per minute and 5.2 micromoles of sodium acetate-1-C^{14} and 1 ml. of lot #402 contained 3.93×10^6 counts per minute and 2.126 micromoles of sodium

acetate-1-C^{14}.

In order to keep contamination and loses of the acetate preparation to a minimum the lots were kept frozen between injections.

A Sample Experimental Procedure

A 200 gm. rat was selected and fed 10 gms. of Purina Laboratory Chow at 8:00 a.m. and any food that remained uneaten after one hour was removed. In the mean time the sodium acetate-1-C^{14} was removed from the freezer and warmed to room temperature after which it was thoroughly mixed. The rat was allowed to assimilate the chow for one hour and then a final weighing of the animal was made. About five minutes prior to injection, one or two ml. of labeled acetate was aspirated into a two ml. syringe. The aspiration was accomplished by fitting a one and one-half inch, 21 gauge needle to the syringe and inserting the needle through the rubber vial closure which capped the round bottom, heavy walled, 40 ml., centrifuge tubes containing the sodium acetate-1-C^{14}. A volume of air equal to the volume of acetate aspirated was injected into the tube to prevent the creation of a vacuum upon aspiration. In all cases a total volume of two ml. was injected into the rat. If only one ml. of acetate was used, the volume was adjusted to two ml. with distilled water. The rat was held, by an assistant, in the supine position in preparation for injection. The abdominal skin was raised gently by the thumb and forefinger and the needle inserted into the abdominal

cavity. Injection was made when it was assured that the needle was in the intraperitoneal space. Following the injection, the rat was immediately placed in a special chamber and allowed to metabolize the injected acetate for two hours.

Metabolism Assembly(115)

Since, when in use, the metabolizing chamber is constantly being swept free of expired $C^{12}O_2$ and $C^{14}O_2$, a quantitative recovery of $C^{14}O_2$ is possible. It is also possible to take periodic samples of the expired CO_2 and so follow its specific activity with time. For a more detailed discussion of the metabolism assembly the reader is referred to the above reference.

Termination of an Experiment

After two hours the rat was removed from the metabolism chamber and immediately sacrificed by decapitation. The abdomen was quickly incised and the liver removed and weighed. The gut was next removed and weighed. The skin was then stripped from the carcass and each of these tissues weighed separately. The head and spleen were added to the carcass fraction. The four tissue fractions were placed in boiling flasks, of appropriate size, containing 25% KOH in 95% ethanol. The total time required for removing the rat from the metabolism chamber, blunt dissection, weighing, and placing all of the tissue fractions in the digestion flasks, did not exceed four to five minutes. The digestion mixture was prepared 30 minutes prior to sacrificing and

the flasks were placed on electric heaters and brought to boiling temperature. All tissue fractions were added to the boiling digest mixture so that enzymatic activity would be immediately arrested. The digestion mixtures were prepared as indicated:

Fraction	Flask Size	ml. 95% Ethanol	Grams KOH
Liver	100 ml.	50	12.5
Gut	250 ml.	150	37.5
Carcass	500 ml.	200	50.0
Skin	250 ml.	100	25.0

Each fraction was digested under reflux for a total of two hours.

Lipid Extraction - Aliquot Technique

With the developement of more sensitive radioassay and weighing techniques, much less lipid was needed for assay purposes so that less than the total amount of each of the tissues could be used. However, by taking only a part of each tissue, the sample would not necessarily represent the total tissue.

A new lipid separation technique was developed which consisted of pipetting an appropriate aliquot of the liquid digest mixture, after it had been filtered into graduated cylinders, to a 1"x8" screw cap culture tube, therefore obtaining a sample that is representative of the total digest. The size of the aliquots used was 25 ml. for the liver fraction and 10 ml. each for the gut, carcass and skin fractions.

Since all extraction procedures were carried out in

the same tube into which the digest aliquot was pipetted, at least two transfers which would have been necessary, if the usual separatory funnel extraction technique had been used, were eliminated.

The significance of minimal transfers and solvent volumes can be appreciated in light of the small quantities of lipid present in one gram of liver, these being about 25 mg. of fatty acid and 2 mg. of cholesterol. The technique of carrying out all precedures in one vessel minimized losses which would be unavoidable by more bulky methods employing several transfers and has enabled us to obtain reproduceable recoveries.

In order to provide the gas-tight seal necessary for extraction with low-boiling solvents, the screw caps were supplied with a 1/16" thick Teflon liner.

Prior to extraction, boiling stones were added to the tissue digest and the tissue digest aliquot was concentrated to one-half volume over a steam bath with the aid of a jet of nitrogen. The digest was then restored to a volume of 25 ml. with water.

To extract the unsaponified lipid, cholesterol, from the tissue digest, 30 ml. of low boiling petroleum ether (B.P. 30°-60°C) were added to each tube containing digest. The caps were screwed on securely and tested for leaks by shaking vertically a few times by hand. The tubes were then placed in a horizontal position on a shaking machine and shaken for 5 minutes at a rate of 280, 1½" strokes per min-

ute. The boiling stones that were added during the concentration aided in the mixing of the petroleum ether and the digest. The tubes were then placed in a vertical position for about 5 minutes or until complete separation had occured. Any emulsion that formed was broken up by the addition of a few drops of 95% ethanol. The petroleum ether layer was then aspirated with a 30 ml. syringe equipped with a 6 inch, 18 gauge blunted needle. The aspirated petroleum ether was transferred to a 250 ml., wide mouth, erlenmeyer flask containing 5 ml. of 1N KOH.

Five such extractions were performed to remove the non-saponifiable lipids. The 5 ml. of 1N KOH were aspirated, after gentle shaking, and returned to the extraction tube. The petroleum ether was then washed with 5 ml. of distilled water, and this was again aspirated and returned to the extraction tube. Enough anhydrous sodium sulfate to cover the bottom of the petroleum ether flask was added to remove water.

The contents of the extraction tube were then acidified with concentrated HCl (to congo red paper). This was done in an ice bath to prevent excessive heating. Five extractions with petroleum ether were performed to remove the saponified lipids, the petroleum ether was again transferred to flasks containing 5 ml. of distilled water to wash out any substrate that might have been carried over. After the 5 ml. of water was aspirated from the flasks, anhydrous sodium sulfate was added as before.

The flasks containing the lipid fractions were capped with rubber caps and allowed to stand over the sodium sulfate over night and were then filtered into 500 ml. erlenmeyer flasks using five 50 ml. petroleum ether washes. It was important that a plug of glass wool, inserted into the funnel stem, was used instead of filter paper for filter off the sodium sulfate for if filter paper filtration is used, a considerable evaporation of the solvent occurs and appreciable amounts of lipid remain on the filter paper. The petroleum ether was then evaporated almost to dryness on a steam bath, removed from the bath, and taken to dryness with a stream of dry nitrogen while the flask was still warm.

Four ml. of alcohol were then added to the flasks containing the lipid fractions. The flasks were then returned to the steam bath and warmed until the refluxing column of solvent reached midway up the flask when they were removed from the bath and allowed to cool to room temperature.

The cooled solvent was then transferred to appropriate volumetric flasks with the aid of volumetric pipettes. The solvent was aspirated into the pipettes just to the bulb, thereby avoiding loss of lipid in the expanded portion of the pipettes. This process of refluxing, cooling, and transferring was repeated five times, using a total volume of 25 ml. of ethanol for each fraction.

The fatty acids were then determined gravimetrically by evaporation of a 5 ml. aliquot of the above solution. The evaporation took place in ½"x1 3/4" shell vials with the

aid of a stream of nitrogen and a 70°C water bath. The cholesterol was determined both by gravimetric and colorimetric procedures. The gravimetric analysis of the cholesterol was carried out in the same manner as described above for the fatty acids and the procedure used for the colorimetric determination was the Zlatkis method(116).

Radioassay

In the past, C^{14}-labeled cholesterol was radioassayed by prior combustion of the sterol to CO_2 and subsequent plating as $BaCO_3$. To eliminate the need for combustion and $BaCO_3$ plating, some cholesterol samples had been plated infinitely thin and so assayed. Cholesterol digitonides were upon occasion plated directly. Fatty acids were also often combusted to CO_2 and plated as $BaCO_3$ but were also radioassayed by the direct counting of "plates" of the fatty acids. A more convenient assay procedure is one using infinitely thin samples obviating the self absorption phenomena characteristic of weak beta emitters such as C^{14}.

It may be well to stop here and define what is meant by plating "infinitely thin" and "infinitely thick". Infinitely thin plating is defined as applying that quantity of lipid to a plate so as to render the counts per minute proportional to the quantity on the plate, that is to say, should the quantity plated be doubled, the counts per minute would be doubled. On the other hand plating infinitely thick requires the application of sufficient lipid to the plate so that if additional lipid is added there is no in-

crease in the counts per minute.

New radioassay equipment that became available during these studies made it necessary to evaluate existing radioassay techniques.

A re-evaluation of infinitely thin plating was accomplished by pipetting increasing amounts of a standard fatty acid solution into aluminum cups of 5 cm.^{2} in area and evaporating off the solvent by means of a heat lamp. The fatty acid used was C^{14} carboxyl labeled palmitic acid obtained from the Fisher Scientific Supply Co., and contained 40 mg. of palmitic acid containing 1 micro curie of C^{14}. A sample of the palmitic acid was dissolved in ethanol and diluted so that 1 ml. of the solution contained 0.476 mg. of the fatty acid, equivalent to 0.0119 micro curies or 26,418 disintegrations per minute. The amounts of fatty acid plated ranged from 0.0048 mg. per cm.^{2} to 0.19 mg. per cm.^{2}. The planchets were counted in an open window, Q gas, 3 place manual counter.

The data obtained from the above experiment is shown in Table I. It was first believed that the data revealed a definite break in the specific activity and the ratio of the counts per disintegrations at the level of 0.038 mg. per cm.^{2} which would indicate that the upper limit of "infinite thinness" had been reached or that the self absorption of the fatty acid had become of significant magnitude. It was felt that though this break was small, any break observed would be the result of absorption of the very weak beta

particles allowing all other particles of greater energy to escape. For this reason all "infinite thin" plating was done at the 0.038 mg. per cm.^{2} level or less. A closer statistical examination of the data revealed that the specific activity of the fatty acids for every plate weight used, 0.0048 mg. per cm.^{2} to 0.1523 mg. per cm.^{2}, was statistically the same and belonged to the same family of numbers. This finding, though invalidating our previously postulated maximum "infinitely thin" plate weight, indicated that all plate weights used in the metabolic experiments were within the "infinitely thin" region. Even though no maximum plate weight established, these statistics suggested that our radioassays of fatty acids were performed at an unnecessary low plate weight.

"Infinitely thin" plating of cholesterol was re-evaluated at this time. Table II reveals the data for cholesterol when treated in the same manner as was done for the fatty acids. When the cholesterol was plated between 0.0040 mg. per cm.^{2} and 0.1608 mg. per cm.^{2} all specific activities were statistically the same indicating that this range of plate weights was within the "infinitely thin" region.

It is necessary when plating "infinitely thin" that the concentration of the solid material be known. Thus gravimetric analysis is accomplished simply by pipetting a known volume of the alcohol containing the fatty acids or the cholesterol into tared shell vials and evaporating the alcohol off in a 70°C water bath with the aid of a jet of

TABLE I

Fatty Acids

Planchet Number	mg./cm.^{2}	cpm-bkg.	S.A. cpm/mg.x10^4	cnts./dis.
1	0.0048	834	3.50	0.6312
2	0.0095	1562	3.28	0.5911
3	0.0143	2371	3.32	0.5982
4	0.0190	3149	3.31	0.5959
5	0.0240	3999	3.36	0.6054
6	0.0286	4874	3.41	0.6149
7	0.0333	5474	3.29	0.5920
8	0.0381	6374	3.35	0.6031
9	0.0428	6874	3.21	0.5782
10	0.0476	7624	3.20	0.5771
11	0.0524	7826	2.99	0.5386
12	0.0571	8676	3.04	0.5473
13	0.0666	10,451	3.14	0.5651
14	0.0762	11,176	2.93	0.5288
15	0.0857	13,301	3.10	0.5594
16	0.0952	15,176	3.19	0.5744
17	0.1142	18,274	3.20	0.5764
18	0.1333	21,074	3.16	0.5698
19	0.1523	24,074	3.16	0.5695
Mean			3.22	0.5798
S.D.			0.144	0.0259
S.E.			0.033	0.0066

TABLE II

Cholesterol

Planchet Number	mg./cm.^{2}	cpm-bkg.	S.A. cpm/mg.x10^4	cnts./dis.
1	0.0040	599	2.98	0.5397
2	0.0080	1163	2.89	0.5237
3	0.0121	1743	2.89	0.5235
4	0.0161	2381	2.96	0.5363
5	0.0201	2871	2.86	0.5173
6	0.0241	3525	2.92	0.5293
7	0.0281	4125	2.93	0.5309
8	0.0322	4675	2.91	0.5265
9	0.0362	5175	2.86	0.5180
10	0.0402	5575	2.77	0.5023
11	0.0442	6100	2.76	0.4996
12	0.0482	6500	2.69	0.4880
13	0.0563	7775	2.76	0.5003
14	0.0643	8675	2.70	0.4885
15	0.0724	9375	2.59	0.4692
16	0.0804	10,275	2.56	0.4629
17	0.0965	13,875	2.88	0.5208
18	0.1126	14,825	2.63	0.4770
19	0.1286	16,795	2.61	0.4723
20	0.1608	20,450	2.54	0.4606
Mean			2.79	0.5043
S.D.			0.143	0.0257
S.E.			0.032	0.0057

nitrogen. The vials were desicated and then weighed to obtain the mg. of solid material per unit volume.

The specific activity of the fatty acids and cholesterol were determined by the use of the following equations:

$$\text{S.A.} = \text{cpm/mg. lipid} = \frac{\text{total cpm - background}}{\text{mg. of lipid in planchet}}$$

The $C^{14}O_2$ collected from the metabolism assembly was radioassayed as $BaCO_3$ (114). The specific activity of the $BaCO_3$ samples were corrected to infinite thickness. Determinations made earlier in this laboratory showed that 20 mg. of $BaCO_3$ per cm.^{2} was the minimum amount for infinite thick plating. The calculations for determining specific activity were made from the following equations:

(more than 20 mg. per cm.^{2})

$$\text{S.A.} = \frac{\text{observed counts-bkg. x plate weight}}{\text{20 mg. x 5 cm.}^2 \text{ x mg. of sample}}$$

(less than 20 mg. per cm.^{2})

$$\text{S.A.} = \frac{\text{observed counts-bkg. x plate weight}}{G_f \text{ x 20 mg. x 5 cm.}^2 \text{ x mg. of sample}}$$

where G_f is the factor which is the fraction of the maximum activity. 5 cm.^{2} represents the area of theplate used, and bkg., the background.

Fatty Acid Purification

Both saturated and unsaturated fatty acids form salts with metallic ions, whose solubility in water and organic solvents varies with the nature of the metallic ion and the chain length, degree of unsaturation, and other character-

istics of the acid radical. The solubility of most of the salts, including those of the alkaline metals (sodium, potassium, and lithium); the alkali earths (calcium, magnesium, barium, and strontium); as well as the heavy metals (lead, zinc, iron, cobalt, nickel, mercury, gold, and silver), have been investigated in a variety of organic solvents, including methanol, ethanol, amyl alcohol, ethyl ether, acetone, benzene, toluene, and in mixtures of these and other solvents. However, no method has been developed which leads to effective quantitative separation of each component acid of mixtures, but only to fractions which are relatively homogeneous with respect to certain types of acids.

Several methods requiring the use of calcium, copper, barium and lead salts were investigated. Using calcium, copper and barium salts of the fatty acids of biological origin in alcohol or acetone, proved unsatisfactory for the salts formed a gummy mixture making washings of this mixture very difficult.

Our attention next turned to probably the oldest and most widely used of the metallic salt separation methods which depends on the differential solubility of the lead soaps of a mixture of fatty acids in either ether or alcohol. It has been employed primarily for the separation of saturated from unsaturated acids and was introduced by Gusserow[117] in 1828. Since its introduction, it has been investigated many times and various modifications have been

adopted to improve its general application. Among the important modifications was one introduced by Twitchell[118] which provided for the use of ethanol in the place of ether for partitioning the lead salts.

The lead salt-ether method has been adopted by the Association of Official Agricultural Chemists (AOAC) and the lead salt-alcohol method by the American Oil Chemists' Society (AOCS).

The principal of this purification and separation method is that the lead salts of myristic, palmitic, stearic, and higher saturated acids are practically insoluble in chilled ethanol, whereas those of oleic, linoleic and linolinic acids are appreciably soluble. By converting an acid mixture into its lead salts and treating these salts with chilled ethanol, a separation of saturated from unsaturated acids can thus be accomplished.

Since this simple separation can be done with relative ease, this provides a convenient preparatory step to further separation of the saturated and unsaturated fatty acids. Since both palmitic acid and oleic acid have the same R_f values in chromatographic methods, the needed separation is usually accomplished by either bromination or mild oxidation of the fatty acid mixture. When the seperation of the saturated from the unsaturated fatty acids can be done with relative ease using the lead salt method, such steps as bromination and oxidation can be eliminated.

The method that became most suitable for our needs

resulted from a modification of the Official A.O.C.S. Lead Salt-Alcohol Method. Details of this method are as follows. A sample of C^{14} labeled biological fatty acids, from in vivo studies, containing 0.1 gram of fatty acids accurately weighed was dissolved in 10 ml. of 95% ethanol. 0.3 grams of powdered lead acetate was likewise dissolved in 10 ml. of 95% ethanol and both solutions were heated to boiling. The lead acetate solution was slowly added to the solution of fatty acids with continuous stirring. After first cooling to room temperature, the mixture was then maintained at 15°C. for two hours, following which it was filtered through a small Buchner funnel with the aid of suction. The collected lead soaps were washed with 20 ml. of 95% ethanol cooled to 15°C.

A sample of the filtrate which contained the lead salts of the liquid or unsaturated fatty acids and the excess lead acetate was tested with concentrated sulfuric acid to determine whether precipitation was complete, i.e., whether the filtrate actually contained an excess of lead acetate.

The collected lead soaps of the solid or saturated fatty acids were suspended in 25-30 ml. of hot 95% ethanol. To the alcoholic solution of lead soaps, 0.5 ml. of glacial acetic acid was added. The solution was then heated to boiling and stirred to dissolve the lead soaps after which the solution was held at 15°C. for two hours,

The solution was filtered through a Buchner funnel and washed as in the first filtration. After the separated

lead soaps of the saturated fatty acids were freed of ethanol, they were transferred to a beaker with the aid of approximately 25 ml. of ethyl ether and decomposed by the addition of 5-10 ml. of nitric acid (1-3). After transferring the solution to a separatory funnel with the aid of ethyl ether, the ethereal solution was washed with distilled water until the wash waters were neutral to methyl orange. The ethereal solution was then transferred to a tared erlenmeyer flask and the ether removed by evaporation under a current of nitrogen. The residue was then desiccated in vacuo for several hours and then weighed and the percentage of solid fatty acids present in the sample was calculated.

The filtrates containing the alcoholic solutions of unsaturated fatty acids were pooled. The ethanol was distilled off on a steam bath under an atmosphere of nitrogen to prevent oxidation. After the addition of 0.1 ml. glacial acetic acid and 0.1 ml. of hydrochloric acid, to decompose the soaps as before, the solution was transferred to a separatory. The unsaturated fatty acids were then extracted 5 times with 10 ml. portions of ethyl ether. The ethereal solution was washed with distilled water until the washings were neutral to methyl orange. The ether was distilled off by warming in a water bath and with a stream of nitrogen. The unsaturated fatty acids, which were in a tared flask, were desiccated in vacuo with a nitrogen atmosphere for several hours and then weighed and the amount of unsaturated

fatty acids determined.

There are limitations, however, to this method of separation. Unsatisfactory results are obtained when this method is applied to butterfat, coconut, palm kernel, and similar oils because they contain saturated acids (myristic and shorter chain length) whose lead salts remain in part or entirely with the unsaturated acid fraction. Also, this method cannot be used in separating the mixed fatty acids of fats and oils which contain erucic (rape and mustard oils), elaeostearic, chaulmoogric, hydnocarpic, or similar acids, because the lead salts of these acids are not very soluble in ether.

CHAPTER IV

Results and Discussion

Medes[119] states that, "if a tracer is being used to determine a reaction rate, such as the rate of fatty acid synthesis, it is necessary to evaluate the effect of the tracer itself on the system. It is conceivable that the tracer substance may have varying effects on different reactions; consequently one may find different patterns of metabolic conversions at different substrate concentrations". In order to eliminate erroneous interpretations of total synthesis and synthesis rates using incorporation data it is imperative that the substrate itself should have no influence on the reactions being evaluated. It therefore, becomes necessary to define the amount of substrate that is truly tracer in the system that is being studied.

The literature contains little information in regard to this important problem, that is, defining the size of a tracer dose of metabolite. In 1952 Wick and Drury[120], while studying acetate metabolism in extrahepatic tissues, observed that after injecting labeled sodium acetate intravenously into eviscerated rabbits, the rate of oxidation of acetate was proportional to the amount of acetate in the body fluids. Although there was considerable variation among rabbits given the same dose of acetate, the animals receiving the largest dose of acetate (14.2 millimoles per kilogram) oxidized the same percentage of the labeled

acetate to CO_2 as did those receiving the smallest dose (2.44 millimoles per kilogram). This indicated to them that a dose of 14.2 millimoles per kilogram was tracer in amount for the eviscerated rabbit when injected I. V.

Frantz and Bucher[121] studied the effect of increasing the amount of acetate upon the amount of acetate oxidized to CO_2 and upon the amount of acetate incorporated into cholesterol. The system used in their study was a rat liver homogenate preparation. The results of their experiments revealed that with increasing substrate amounts, the rate at which the acetate was being oxidized to CO_2 remained nearly constant over the range of 10 to 50 micromoles of acetate. The incorporation of label into cholesterol was however linear only at the lower concentrations. At the 20 micromole level a plateau was reached where no further increase in incorporation into cholesterol occurred. It was observed at the highest concentration level, 50 micromoles, that there was actually a decrease in the incorporation of the acetate into cholesterol. To summarize the results of this experiment, the investigators observed that as the substrate acetate was increased, the incorporation of acetate into CO_2 continued to rise proportionally to the size of the dose while the incorporation into cholesterol reached a plateau at a lower acetate level.

Medes, et al.[119], studied the effects of various concentrations of C^{14}-labeled lactate, glucose, and acetate upon their incorporation into fatty acids, cholesterol,

acetoacetate and CO_2 using liver slices from rats in various nutritional states. The dose of acetate (methyl-labeled) and DL-lactate (2-labeled) studied included four different amounts ranging from 2 micromoles to 2000 micromoles. The uniformly labeled glucose was studied at three different dose levels, namely 20, 200, and 1000 micromoles. The total volume of the substrate mixture was 40 ml. in every case to which was added 5 gm. of liver slices.

The results showed a constant ratio of the amount of substrate metabolites to the incorporation of isotope resulting in a proportional increase in the incorporation of labeled molecules into fatty acid, cholesterol, acetoacetate and CO_2 with increased amounts of substrate. Their curves showed the incorporation of the metabolites into each of the four products to be parallel. The curves are linear at the lower dose levels but tend to fall off or plateau at higher amounts of substrate. The conclusion that was drawn from the parallelism of the curves was that lipogenisis and ketogenesis, can occur at a constant rate which is independent of wide ranges in substrate amounts.

Emerson and Van Bruggen[122] performed a similar study with rat liver slices. The substrate used in their investigation was carboxyl labeled acetate. The range of substrate amounts, however, was extended in the lower regions, that is from 0.0025 micromoles to 2530 micromoles per 27 ml. substrate volume. The CO_2 forming system reflected a proportional increase in activity with increasing

amounts of substrate up to 25.3 micromoles. This proportionality was lost at the higher concentrations. Plotting the anticipated response, rather than the actual specific activities, against micromoles of substrate, revealed the sensitivity of the fatty acid, cholesterol and CO_2 compartments to varying amounts of substrate. The anticipated response was calculated as the percent of the specific activity which would have been found had the change in the product specific activity been proportional to the substrate specific activity increase. Thus, a ten-fold increase in product specific activity with a ten-fold increase in substrate activity would represent 100 percent of the anticipated response. The activity response obtained for the lipid fractions proved that these systems are much more sensitive to the amount of substrate present than is the CO_2 compartment. At the substrate level of 0.025 micromoles, the fatty acid fraction showed only 90 percent of the expected activity whereas the cholesterol fraction showed only 60 percent of the activity expected. This response continued to decrease until at the 2530 micromole level the activity responses were 5, 5, and 0 percent for CO_2, fatty acids and cholesterol respectively.

These earlier studies involved the use of liver slices in fixed volumes of substrate. Under such conditions it is generally considered that the substrate becomes available to the slices directly as the concentration of substrate is increased.

The greater part of the studies on lipid metabolism done to date in this laboratory have involved the use of intact animals. When it became clear that the in vitro experiments had demonstrated a potential tracer technique difficulty, it was felt desirable to study the same phenomena in the intact rat. It was realized from the beginning that useing an intact animal for experiments certainly had its disadvantages. Many factors or variables exists which are constantly operative and difficult to control. Variables such as the dynamic endocrine system and the neurovascular network have the ability to alter absorption rates, rates of metabolism, etc., making interpretation of data obtained from metabolic experiments difficult. It was felt, however, that by controling the nutritional state of the animals by "trained feeding", that these variables might be kept to a minimum.

In the in vivo studies, the same general plan was used as for the in vitro studies previously discussed. In general, the idea was to add increasing amounts of labeled acetate I.P. to the intact rat so that as the labeled acetate was distributed to the tissues it would merge with endogenous acetate and so label the acylating acetyl precursors to an extent depending upon the amount of label introduced. Ideally then one adds increasing amounts of acetate-1-C^{14} of constant specific activity, progressively increasing the specific activity of the "precursor pool". Practically there is a limit to which increasing amounts of

labeled acetate can be given, for both the extreme radioactivity involved as well as the great cost, limits the amount of label that can be used. To obviate this difficulty, graded amounts of unlabeled acetate were used to increase the size of the acetate "dose"----and with this "cold acetate" there was mixed enough of acetate-1-C^{14} to permit convenient radioassay techniques to be used. This variation in the specific activity of the tracer substrate together with the fact that different "batches" of acetate were used made it necessary to adjust or arithmetically correct the specific activity of the dose. This was accomplished by adjusting the final specific activity of the substrate using appropriate factors as seen in Tables IV and V. In the case where a different batch of acetate was used, as in the 15 and 14 series, the specific activity of the products measured were divided by the ratio of the specific activities of the acetate substrates, namely 2.57. In the second case, the specific activity of the products were multiplied by a factor proportional to the increase in unlabeled acetate added.

Following I.P. injection of labeled acetate it was observed that approximately 66.5% of the labeled acetate (Table III) was incorporated into the CO_2 expired in the 2 hours following injection. Since CO_2 is a natural metabolic product of protein, carbohydrate and lipid metabolism, it may be expected that any upset in the metabolic pathway being studied would be reflected either in the amount of

TABLE III

CO_2 Data

Animal	Moles of expired CO_2			% Incorporation		
	1 hr.	2 hr.	Total	1 hr.	2 hr.	Total
15	16.8	16.1	32.9	49.9	8.9	58.8
15B	16.5	14.9	31.4	47.6	7.4	55.0
8	16.3	16.0	32.3	75.1	12.4	87.5
8B	15.2	13.9	29.1	67.4	12.5	79.9
14	15.5	16.3	31.8	46.1	11.5	57.6
14B	17.7	16.5	34.2	50.6	8.4	59.0
14C	15.7	14.4	30.1	48.9	9.2	58.1
9	15.5	12.8	28.3	69.7	8.2	77.9
9B	13.7	12.8	26.5	68.6	13.1	81.7
10	11.1	10.9	22.0	55.0	10.8	65.8
10B	13.7	12.8	26.5	51.5	14.7	66.2
10C	18.0	15.1	33.1	55.9	9.1	65.0
11	9.8	11.1	20.9	53.8	10.4	64.2
11B	14.6	13.8	28.4	55.8	10.0	65.8
12	18.5	14.9	33.4	46.8	12.3	59.1
12B	14.7	15.4	30.1	53.7	11.8	65.5
12C	18.2	18.8	37.0	54.0	12.9	66.9
13	16.0	15.5	31.5	49.1	14.1	63.2
13B			27.6			68.7
13C	17.6	16.0	33.6	53.2	12.4	65.6
Mean			30.0			66.1
S.D.			4.01			8.78
S.E.			0.90			1.96

CO_2 expired or the percent of the labeled incorporated into the expired CO_2 or both.

A disadvantage that exists when using an intact animal, namely, that the size of the endogenous acetate "pool" is unknown. Suppose that the endogenous acetate "pool" was less than some "normal" value. If acetate-1-C^{14} is injected into the intact animal the total acetate "pool" would be comparatively rich in the labeled acetate, thus one would expect the expired CO_2 to have a greater specific activity. Conversely, if the endogenous acetate "pool" was greater than "normal", the injected acetate-1-C^{14} would be less concentrated, thus reducing the specific activity of the expired CO_2. This would explain the variation seen in the percent incorporation of the acetate-1-C^{14} into CO_2.

Another variable to be accounted for is the amount of CO_2 expired and the difference between the first hour and second hour collection. This may be readily explained by the fact that the rat is necessarily in an excited state during injection and its entering into the metabolism chamber. Such excitement usually results in hyperventilation for the first 20-30 minutes tending to cause more CO_2 to be expired during the first hour collection than during the second hour.

As seen in Table III, the moles of CO_2 expired in two hours by each animal are statistically the same. The same is true for the percent incorporation figures. This information convinced us that the higher dosages had no

effect on the enzyme systems necessary for the metabolism of the acetate, in other words, the rat was able to metabolize the highest dose in a normal manner. The amount of expired CO_2 and the percent incorporation seen in previous experiments performed in this laboratory were within the ranges seen in Table III, further relating this data with that of other workers.

The CO_2 specific activity data is recorded in Tables IV and V and these data are presented graphically in Fig. 1. The linear increase of $C^{14}O_2$ specific activity with dosage increases can now be considered in terms of the original hypothesis----namely that with increasing tracer dosage, the specific activity of the product will rise directly in proportion to the dosage.

The line representing the CO_2 response, in Fig. 1, approximates a 45° angle between the equally spaced coordinates. This is interpreted as showing a linear response to the dosage, i.e., as the dose was doubled, so was the specific activity doubled, a condition that must be satisfied if the dose is considered to be tracer in amount.

When the specific activity of the precursor "pool" is changed, one would expect this change to be reflected proportionately in the lipid products providing that similar tracer conditions exist as is seen in the CO_2 system. The following Tables (Table VI - Table X) present the data obtained on the fatty acids of liver, gut, carcass and skin.

TABLE IV

First Hour CO_2 Data

Animal	Dose uM of acetate	Dose Counts x10^5	Cnts. at constant S.A. x10^6	S.A. of CO_2 cpm/mM	f	Corr. S.A. of CO_2 cpm/mM
15	0.2126	3.93	0.153	11.7	÷2.57	4.6
15B				11.4		4.4
8	1.3	9.31	0.931	43.0	1.0	43.0
8B				41.3		41.3
14	2.126	39.3	1.53	116.7	÷2.57	45.4
14B				112.4		43.7
14C				123.0		47.9
9	2.6	18.6	1.86	84.1	1.0	84.1
9B				93.1		93.1
10	5.2	37.2	3.72	184.6	1.0	184.6
10B				140.6		140.6
10C				115.8		115.8
11	10.4	74.4	7.44	410.0	1.0	410.0
11B				284.0		284.0
12	57.11	37.2	41.0	98.3	x11.0	1081.0
12B				142.0		1562.0
12C				111.0		1221.0
13	519.1	37.2	371.0	120.1	x99.7	11,974.0
13B				112.8		11,246.0

TABLE V

Second Hour CO_2 Data

Animal	Dose uM of acetate	Dose Counts x10^5	Cnts. at constant S.A. x10^6	S.A. of CO_2 cpm/mM	f	Corr. S.A. of CO_2 cpm/mM
15	0.2126	3.93	0.153	2.2	*2.57	0.86
15B				2.0		0.78
8	1.3	9.31	0.931	7.3	1.0	7.3
8B				8.4		8.4
14	2.126	39.3	1.53	27.4	*2.57	10.7
14B				20.1		7.8
14C				25.3		9.8
9	2.6	18.6	1.86	11.9	1.0	11.9
9B				19.1		19.1
10	5.2	37.2	3.72	36.6	1.0	36.6
10B				43.0		43.0
10C				22.8		22.8
11	10.4	74.4	7.44	69.7	1.0	69.7
11B				53.7		53.7
12	57.11	37.2	41.0	31.5	x11.0	346.5
12B				29.8		327.8
12C				25.5		280.5
13	519.1	37.2	371.0	35.1	x99.7	3499.0
13B				29.0		2891.0

Figure 1

This figure represents the carbon dioxide response, for both the first and the second hour collections, to increasing doses of acetate. The specific activities of the carbon dioxide (cpm/millimole) are plotted against the dose of acetate injected/200 gram rat).

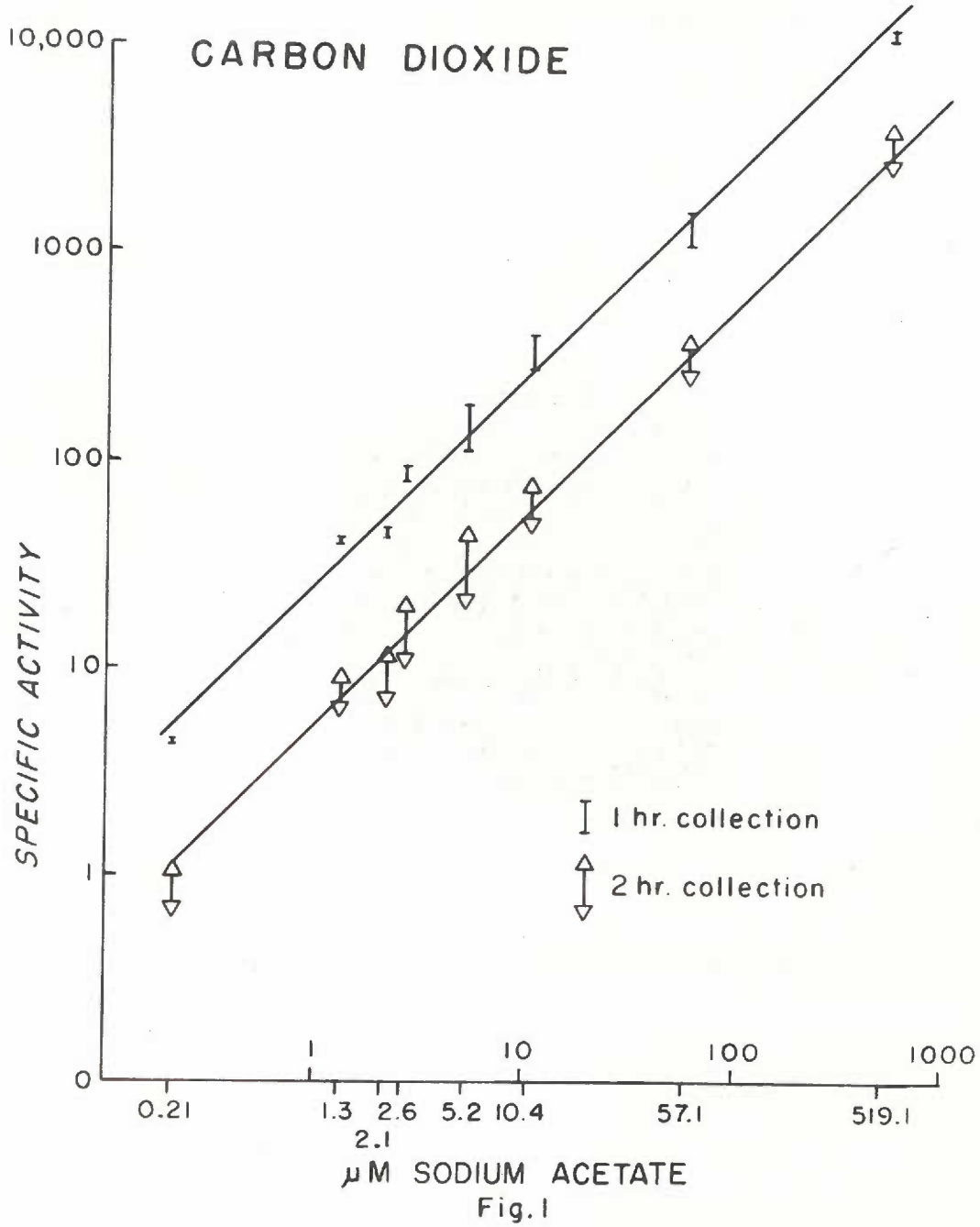

CARBON DIOXIDE
10,000
1000
100
10
1
0
SPECIFIC ACTIVITY
1 hr. collection
2 hr. collection
1
10
100
1000
0.21
1.3
2.1
2.6
5.2
10.4
57.1
519.1
μM SODIUM ACETATE

Fig. 1

The mg. of fatty acid per gm. of tissue for the four tissues studied as well as their respective percent incorporations are shown in Table VI. The average values of mg. of fatty acid per gm. of tissue were 33.2, 34.6, 55.9 and 131.9 for liver, gut, carcass and skin respectively. All values for the various animals are statistically within the limits of plus or minus two standard deviations. Also the respective percent incorporation of acetate into the fatty acids were 1.1, 4.1, 6.8 and 1.6 for the liver, gut, carcass and skin. Again none of the percent incorporation values for the various animals appeared to differ significantly from the mean. These mean values both for percent incorporation and mg. per gm. of tissue, closely approximated mean values obtained from previous experiments in this laboratory.

Figures 2 through 5 are the responses of the fatty acids of the various tissues to increasing acetate dosages. The data used for plotting those graphs is found in the corresponding Tables VII through X. It can be observed that the plotted lines of the figures are straight lines and approach the angle of 45° when the specific activity of the fatty acids are plotted against the dose on log-log paper. It is apparent that even when the acetate dose was increased 2600 fold, tracer conditions were satisfied. The anomally seen in Figure 2 at the lowest dose, that is, a higher specific activity than would be expected may be explained by the following. At the low dose of 0.2 micromoles, the liver

TABLE VI

Fatty Acid Data

	Liver		Gut		Carcass		Skin	
Animal	mg.* F.A.	% Inc.	mg.* F.A.	% Inc.	mg.* F.A.	% Inc.	mg.* F.A.	% Inc.
15	32.2	1.90	29.2	6.60	44.1	6.90	101.0	1.20
15B	35.4	2.20	39.2	7.00	70.9	10.1	152.3	0.98
8	31.8	0.87	36.5	7.50	54.2	10.8	141.5	2.00
8B	32.2	0.63	34.5	4.50	52.2	8.40	125.7	1.00
14	39.0	1.00	37.1	5.70	55.3	7.30	149.9	1.60
14B	34.2	0.85	30.0	5.90	46.4	6.90	128.9	1.00
14C	35.6	0.67	28.4	4.70	43.2	8.00	97.5	0.70
9	31.8	0.90	25.3	4.20	50.8	9.30	112.8	1.20
9B	29.5	1.00	30.1	3.80	55.1	7.50	145.4	0.80
10	32.8	0.68	34.8	4.40	52.8	6.70	135.9	1.20
10B	28.9	0.56	29.2	2.70	46.0	4.60	133.7	3.80
10C	31.5	1.50	37.3	4.00	57.7	7.50	115.6	1.30
11	33.8	0.57	38.5	4.10	60.0	5.00	134.1	1.10
11B	31.7	0.58	33.9	2.40	94.7	6.50	181.2	1.30
12	31.3	2.20	33.2	3.80	60.2	6.30	165.2	2.40
12B	32.4	0.48	42.9	2.00	58.4	5.30	112.8	1.50
12C	36.1	1.50	41.6	3.70	52.8	5.20	140.6	2.60
13	34.7	0.95	30.2	1.80	50.6	4.90	94.2	1.90
13B	33.3	1.20	45.0	1.90	60.3	5.10	197.0	2.60
13C	35.2	1.60	35.3	2.00	51.8	4.00	72.1	1.20
Mean	33.2	1.09	34.6	4.10	55.9	6.80	131.9	1.60
S.D.	2.38	0.55	5.27	1.73	11.2	1.86	29.97	0.77
S.E.	0.53	0.12	1.18	0.39	2.51	0.42	6.70	0.17

* mg. fatty acid/gm. tissue

TABLE VII

Liver Fatty Acid Data

Animal	Dose uM of acetate	Dose Counts x10^5	Cnts. at constant S.A.x10^6	S.A. of F.A. cpm/mg.	f	Corr. S.A. of F.A. cpm/mg.
15	0.2126	3.93	0.153	150	÷2.57	58.3
15B				189		73.5
8	1.3	9.31	0.931	160	1.0	160.0
8B				110		110.0
14	2.126	39.3	1.53	763	÷2.57	296.9
14B				720		280.2
14C				570		221.8
9	2.6	18.6	1.86	316	1.0	316.0
9B				394		394.0
10	5.2	37.2	3.72	487	1.0	487.0
10B				484		484.0
10C				1232		1232.0
11	10.4	74.4	7.44	825	1.0	825.0
11B				918		918.0
12	57.11	37.2	41.0	1620	x11.0	17,820.0
12B				428		4708.0
12C				1140		12,540.0
13	519.1	37.2	371.0	745	x99.7	74,277.0
13B				907		90,428.0
13C				1190		118,643.0

Figure 2

Figure 2 represents the response of the fatty acid of the liver to increasing amounts of acetate. The specific activity of the fatty acid (cpm/mg.) are plotted against the dose (micromoles of acetate/ 200 gram rat).

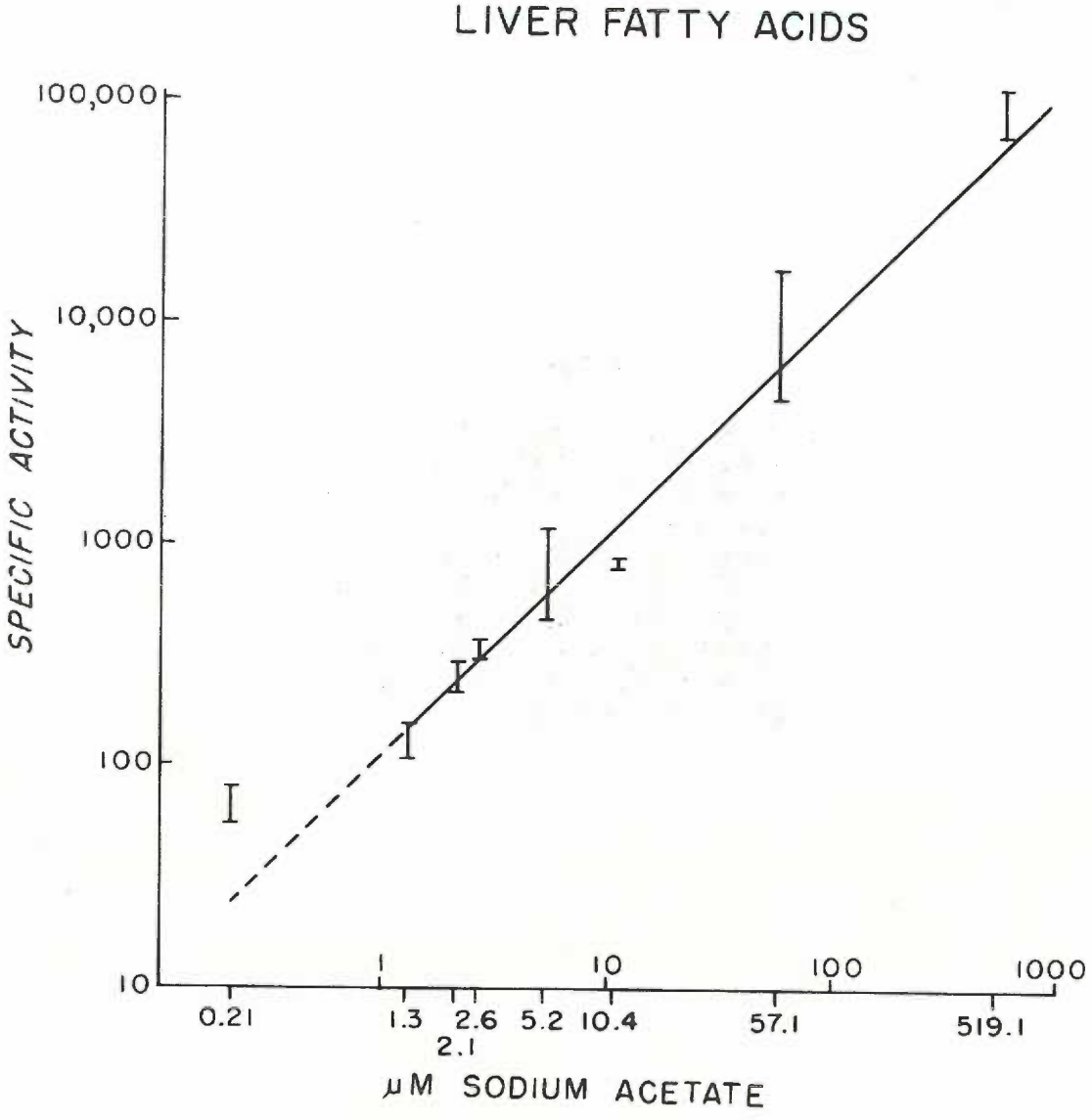

LIVER FATTY ACIDS
100,000
10,000
1000
100
10
SPECIFIC ACTIVITY
1
10
100
1000
0.21
1.3
2.1
2.6
5.2
10.4
57.1
519.1
μM SODIUM ACETATE

FIG. 2

TABLE VIII

Gut Fatty Acid Data

Animal	Dose uM of acetate	Dose Counts $x10^5$	Cnts. at constant S.A. $x10^6$	S.A. of F.A. cpm/mg.	f	Corr. S.A. of F.A. cpm/mg.
15	0.2126	3.93	0.153	136	•2.57	52.9
15B				115		44.7
8	1.3	9.31	0.931	338	1.0	338.0
8B				190		190.0
14	2.216	39.3	1.53	924	•2.57	395.5
14B				1272		494.9
14C				987		384.0
9	2.6	18.6	1.86	506	1.0	506.0
9B				451		451.0
10	5.2	37.2	3.72	771	1.0	771.0
10B				562		562.0
10C				673		673.0
11	10.4	74.4	7.44	1370	1.0	1370.0
11B				1072		1072.0
12	57.11	37.2	41.0	640	x11.0	7040.0
12B				329		3619.0
12C				503		5533.0
13	519.1	37.2	371.0	395	x99.7	39,382.0
13B				298		29,711.0
13C				367		36,590.0

Figure 3

Figure 3 represents the response of the fatty acid of the gut to increasing amounts of acetate. The specific activity of the fatty acid (cpm/mg.) are plotted against the dose (micromoles of acetate/ 200 gram rat).

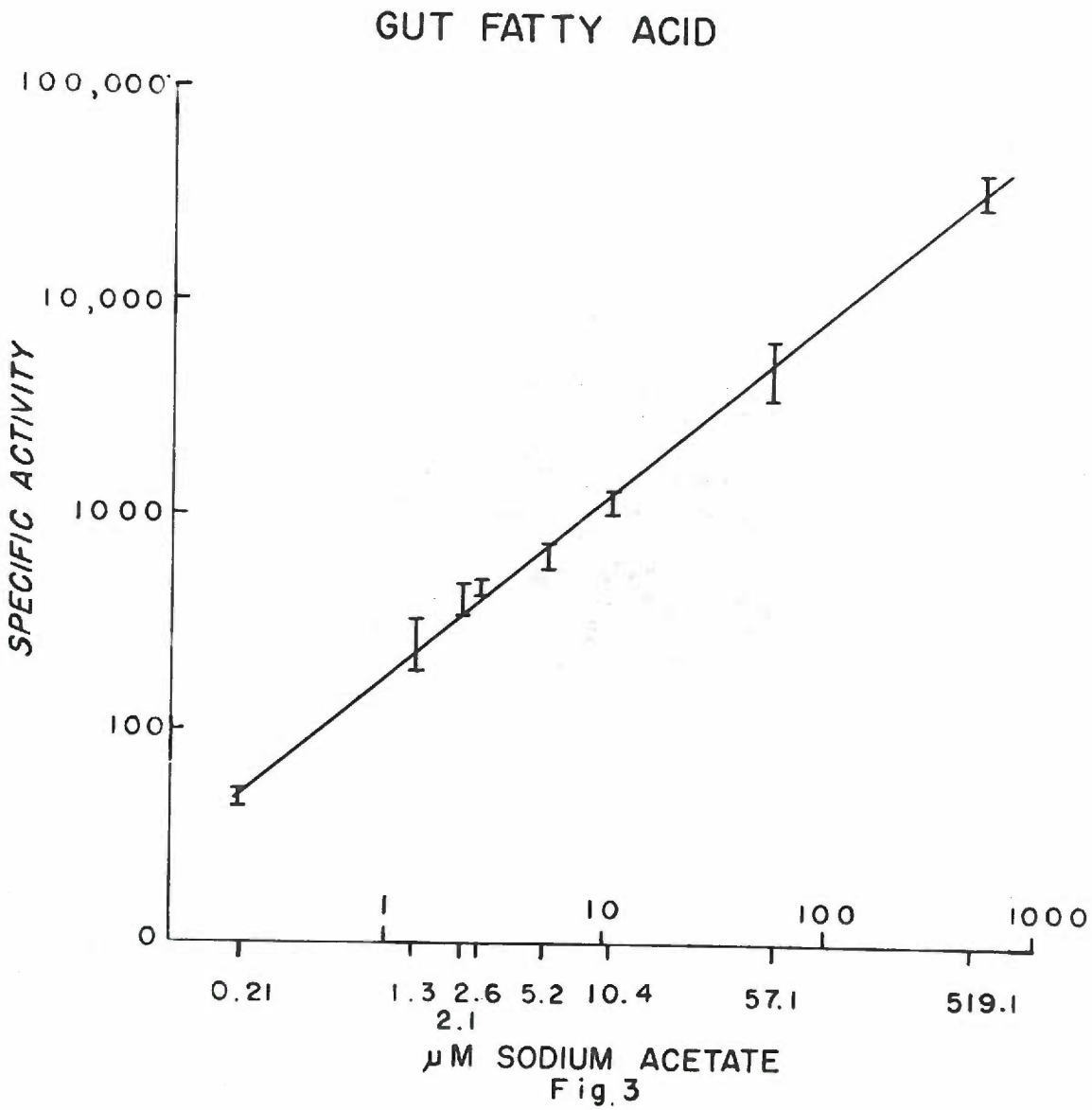

GUT FATTY ACID
100,000
10,000
1000
100
0
SPECIFIC ACTIVITY
1
10
100
1000
0.21
1.3
2.1
2.6
5.2
10.4
57.1
519.1
μM SODIUM ACETATE

Fig. 3

TABLE IX

Carcass Fatty Acid Data

Animal	Dose uM of acetate	Dose Counts x10^5	Cnts. at constant S.A.x10^6	S.A. of F.A. cpm/mg.	f	Corr. S.A. of F.A. cpm/mg.
15	0.2126	3.93	0.153	28.2	÷2.57	11.0
15B				27.0		10.5
8	1.3	9.31	0.931	84.0	1.0	84.0
8B				71.6		71.6
14	2.126	39.3	1.53	265.0	÷2.57	103.1
14B				280.0		108.9
14C				349.0		135.8
9	2.6	18.6	1.86	157.0	1.0	157.0
9B				121.0		121.0
10	5.2	37.2	3.72	209.0	1.0	209.0
10B				168.0		168.0
10C				230.0		230.0
11	10.4	74.4	7.44	284.0	1.0	284.0
11B				252.0		252.0
12	57.11	37.2	41.0	170.0	x11.0	1870.0
12B				137.0		1507.0
12C				182.0		2002.0
13	519.1	37.2	371.0	160.0	x99.7	15,952.0
13B				142.0		14,157.0
13C				130.0		12,961.0

Figure 4

Figure 4 represents the response of the fatty acid of the carcass to increasing amounts of acetate. The specific activity of the fatty acid (cpm/mg.) are plotted against the dose (micromoles of acetate/ 200 gram rat).

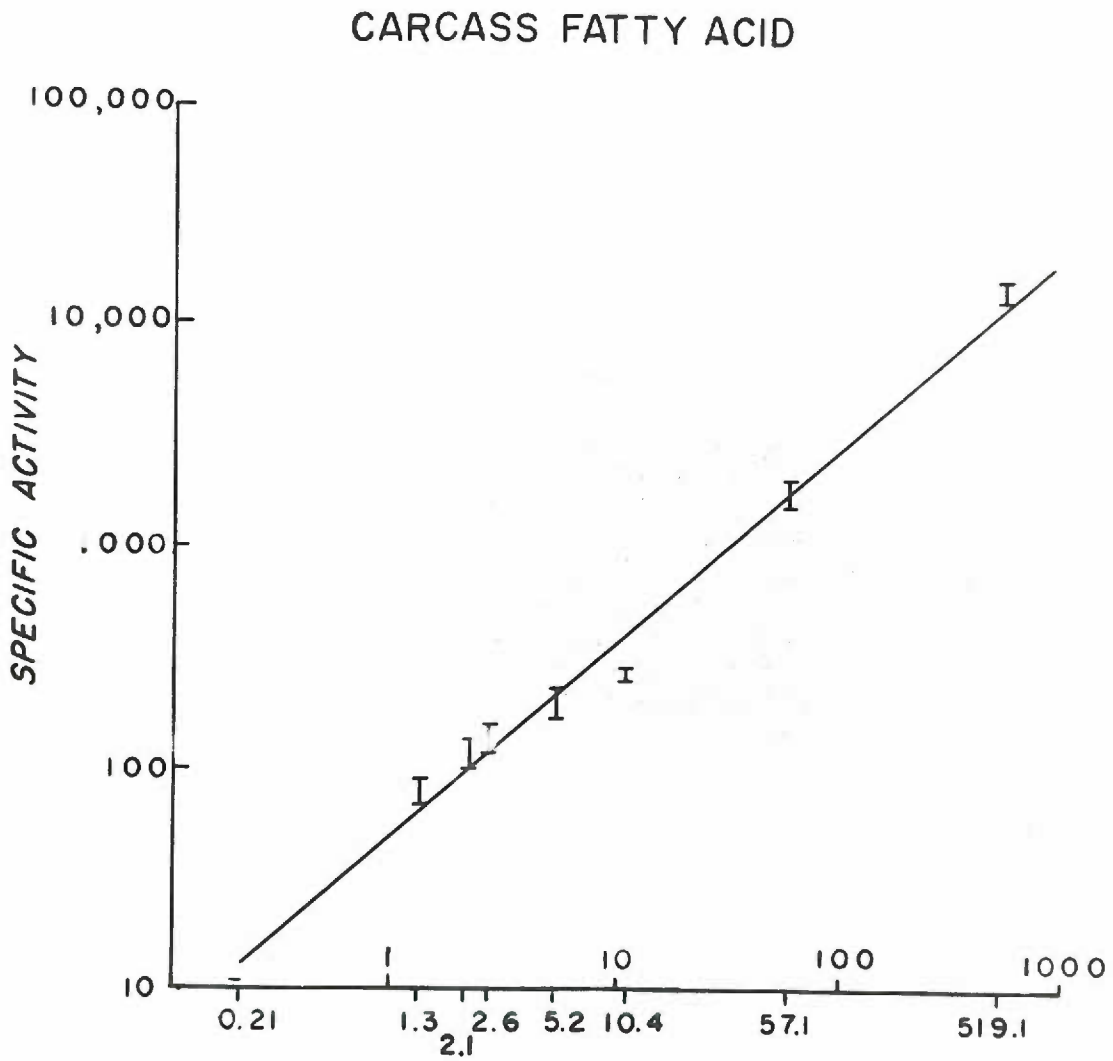

CARCASS FATTY ACID
100,000
10,000
.000
100
10
SPECIFIC ACTIVITY
1
10
100
1000
0.21
1.3
2.1
2.6
5.2
10.4
57.1
519.1
μM SODIUM ACETATE

Fig. 4

TABLE X

Skin Fatty Acid Data

Animal	Dose uM of acetate	Dose Counts x10^5	Cnts. at constant S.A.x10^6	S.A. of F.A. cpm/mg.	f	Corr. S.A. of F.A. cpm/mg.
15	0.2126	3.93	0.153	8.5	÷2.57	3.3
15B				5.4		2.1
8	1.3	9.31	0.931	22.5	1.0	22.5
8B				15.7		15.7
14	2.126	39.3	1.53	77.0	÷2.57	30.0
14B				63.0		24.5
14C				65.0		25.3
9	2.6	18.6	1.86	40.0	1.0	40.0
9B				20.0		20.0
10	5.2	37.2	3.72	62.0	1.0	62.0
10B				81.0		81.0
11	10.4	74.4	7.44	102.0	1.0	102.0
11B				109.0		109.0
12	57.11	37.2	41.0	90.0	x11.0	990.0
12B				90.0		990.0
12C				127.0		1397.0
13	519.1	37.2	371.0	146.0	x99.7	14,556.0
13B				81.0		8076.0
13C				140.0		13,958.0

Figure 5

Figure 5 represents the response of the fatty acid of the skin to increasing amounts of acetate. The specific activity of the fatty acid (cpm/mg.) are plotted against the dose (micromoles of acetate/ 200 gram rat).

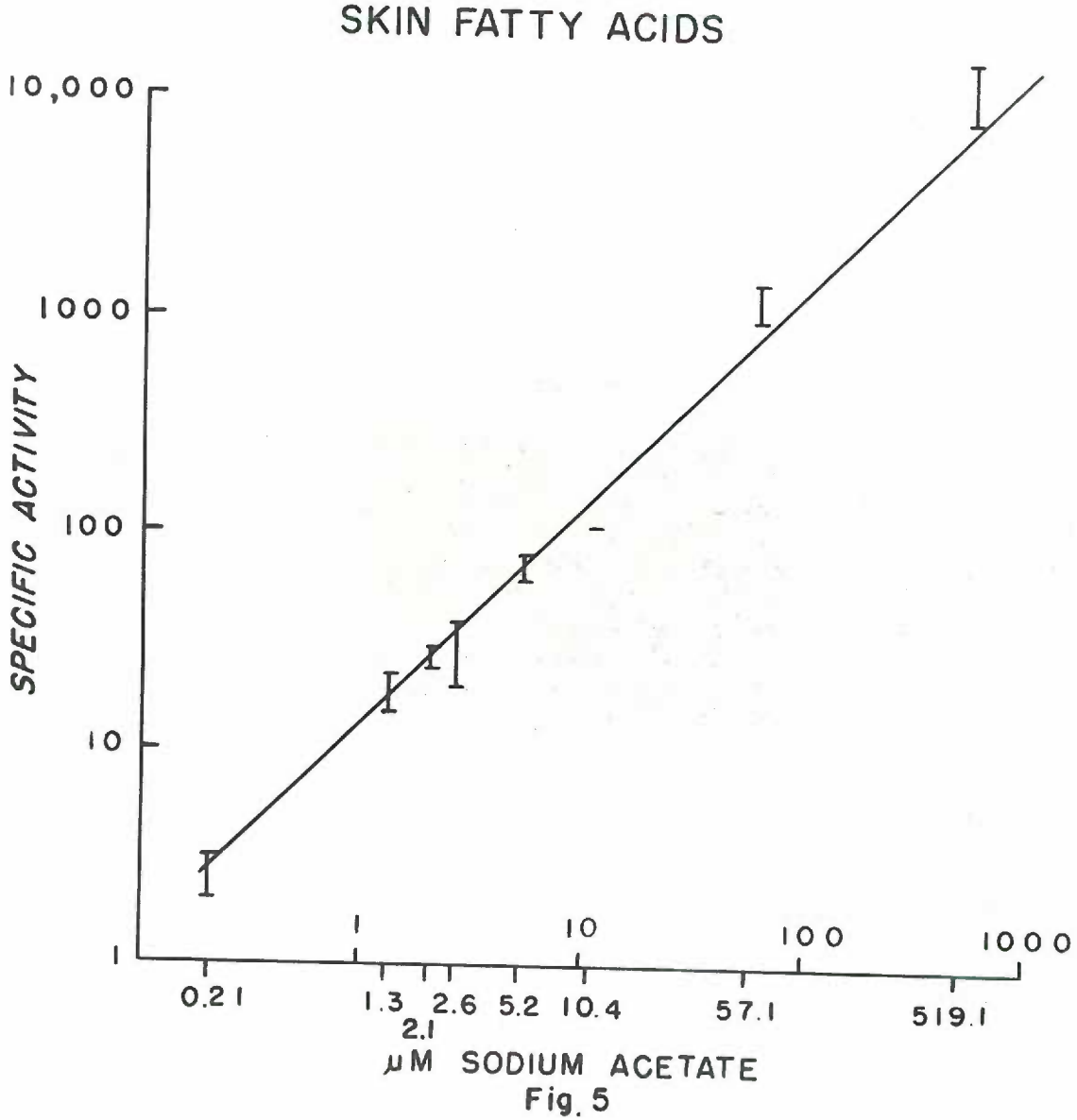

SKIN FATTY ACIDS
10,000
1000
100
10
1
SPECIFIC ACTIVITY
1
10
100
1000
0.21
1.3
2.1
2.6
5.2
10.4
57.1
519.1
μM SODIUM ACETATE

Fig. 5

tissue may selectively incorporate the acetate into its own fatty acid compartment. If this were true, this would also be reflected in the other tissues by a decrease in the specific activities of their respective fatty acids. In Figure 4, the carcass tissue reveals this very thing, a specific activity less than one would expect. Of course the other tissues, skin and gut may also share in the deprivation but perhaps due to the few animals used at this dose level, these tissues do not show this same reduced specific activity.

The data presented for the fatty acids shows that the incorporation of the labeled acetate into the fatty acids was independent of and not influenced by the wide range of dosages studied.

The response of cholesterol to increasing amounts of acetate in vivo is nearly the same as was seen for fatty acids and CO_2 but much different from that previously described in liver homogenates and liver slices. The mg. of cholesterol per gm. of liver, gut, carcass and skin remained essentially constant over the dosage range as seen in Table XI. These amounts being 2.21, 1.47, 1.33 and 2.52 for the respective tissues. 0.13, 0.19, 0.18 and 0.12 are the mean values for percent incorporation of acetate into cholesterol. Tables XII through XV show the cholesterol specific activity found for the different dosages. Linearity is again displayed when the cholesterol specific activity is plotted against dose on log-log paper. This linearity is such that

TABLE XI

Cholesterol Data

Animal	Liver mg.* Chol.	Liver % Inc.	Gut mg.* Chol.	Gut % Inc.	Carcass mg.* Chol.	Carcass % Inc.	Skin mg.* Chol.	Skin % Inc.
15	1.63	0.08	1.45	0.22	0.67	0.12	----	0.09
15B	1.13	0.11	1.48	0.18	1.48	0.20	----	0.11
8	2.55	0.12	1.53	0.20	1.73	0.29	2.79	0.14
8B	2.37	0.12	1.67	0.21	1.98	0.23	2.48	0.10
14	1.54	0.09	0.94	0.13	0.96	0.12	2.51	0.09
14B	2.15	0.03	1.32	0.10	1.65	0.11	2.62	0.06
14C	2.19	0.20	0.87	0.15	0.59	0.15	2.28	0.10
9	2.59	0.11	1.38	0.17	1.85	0.22	2.87	0.11
9B	2.29	0.14	1.87	0.19	1.50	0.20	2.13	0.11
10	2.73	0.15	1.55	0.18	1.91	0.23	2.38	0.11
10B	2.06	0.06	1.18	0.11	2.08	0.19	2.94	0.13
10C	1.78	0.09	1.50	0.15	0.45	0.07	1.37	0.11
11	3.19	0.19	1.67	0.24	1.21	0.16	2.87	0.13
11B	1.79	0.08	1.72	0.14	1.97	0.20	3.19	0.10
12	1.98	0.10	1.46	0.17	1.83	0.21	2.22	0.12
12B	2.06	0.07	1.53	0.20	1.65	0.20	2.68	0.12
12C	1.83	0.24	1.67	0.40	0.46	0.10	2.31	0.22
13	3.06	0.25	1.41	0.19	----	----	2.52	0.13
13B	2.96	0.24	1.78	0.20	1.99	0.30	2.38	0.15
13C	2.40	0.20	1.41	0.20	0.23	0.08	2.37	0.11
Mean	2.21	0.13	1.47	0.19	1.33	0.18	2.52	0.12
S.D.	0.58	0.06	0.25	0.06	0.62	0.07	0.40	0.03
S.E.	0.13	0.01	0.06	0.01	0.14	0.02	0.09	0.01

* mg. cholesterol/gm. tissue

TABLE XII

Liver Cholesterol Data

Animal	Dose uM of acetate	Dose Counts x10^5	Cnts. at constant S.A. x10^6	S.A. of Chol. cpm/mg.	f	Corr. S.A. of Chol. cpm/mg.
15	0.2126	3.93	0.153	133.0	+2.57	51.8
15B				308.0		120.0
8	1.3	9.31	0.931	299.0	1.0	299.0
8B				294.0		294.0
14	2.126	39.3	1.53	1725.0	+2.57	671.0
14B				465.0		181.0
14C				2858.0		1112.0
9	2.6	18.6	1.86	498.0	1.0	498.0
9B				740.0		740.0
10	5.2	37.2	3.72	1390.0	1.0	1390.0
10B				774.0		774.0
10C				1332.0		1332.0
11	10.4	74.4	7.44	3200.0	1.0	3200.0
11B				2123.0		2123.0
12	57.11	37.2	41.0	1410.0	x11.0	15,510.0
12B				1075.0		11,825.0
12C				3803.0		41,833.0
13	519.1	37.2	371.0	2435.0	x99.7	243,000.0
13B				2124.0		212,000.0
13C				2378.0		237,000.0

Figure 6

Figure 6 represents the response of the cholesterol of the liver to increasing amounts of acetate. The specific activity of the cholesterol (cpm/mg.) are plotted against the dose (micromoles of acetate/ 200 gram rat).

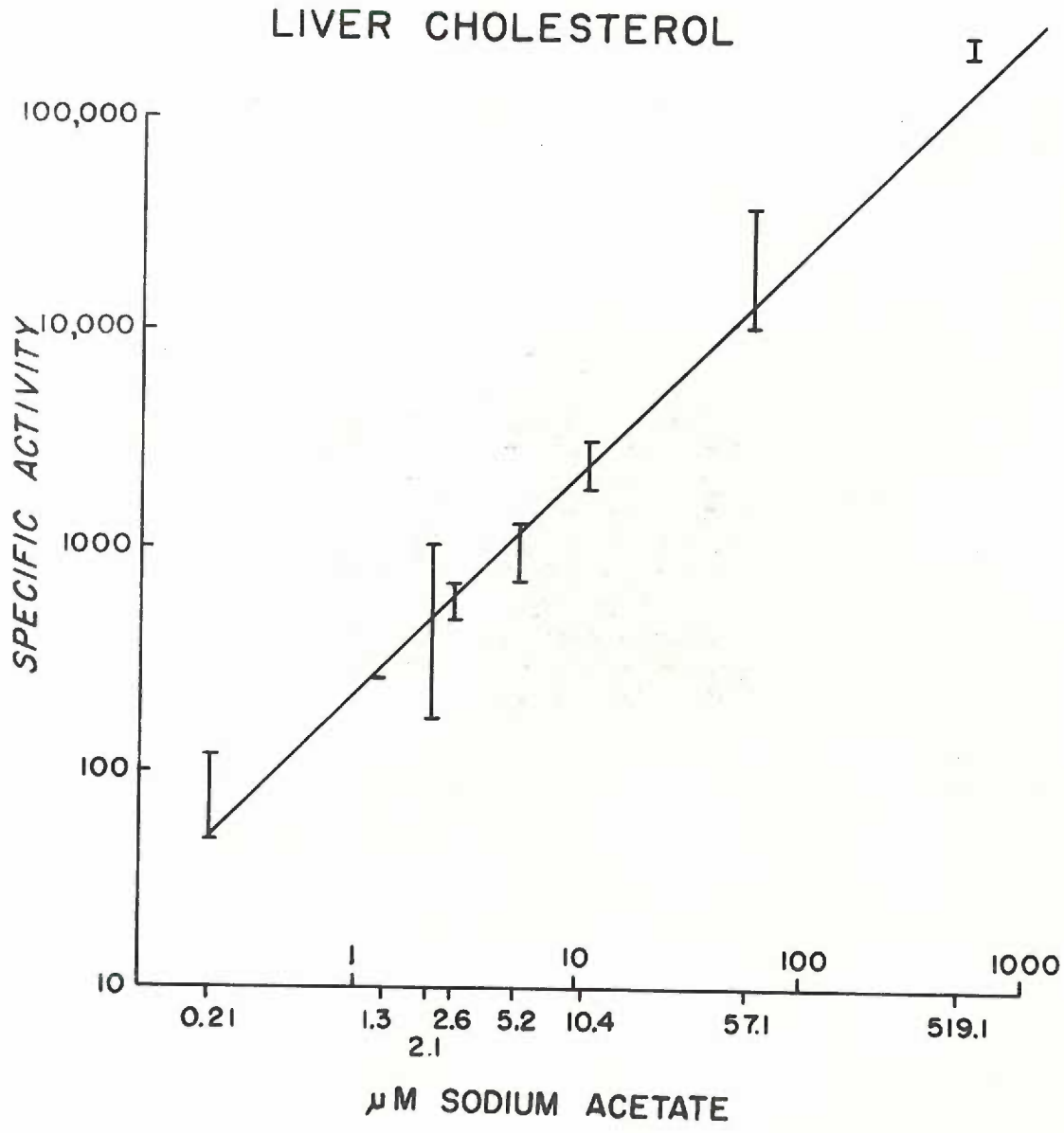

LIVER CHOLESTEROL
I
100,000
10,000
1000
100
10
SPECIFIC ACTIVITY
1
10
100
1000
0.21
1.3
2.1
2.6
5.2
10.4
57.1
519.1
μM SODIUM ACETATE

FIG. 6

TABLE XIII

Gut Cholesterol Data

Animal	Dose uM of acetate	Dose Counts x10^5	Cnts. at constant S.A.x10^6	S.A. of Chol. cpm/mg.	f	Corr. S.A. of Chol. cpm/mg.
15	0.2126	3.93	0.153	98.0	÷2.57	38.1
15B				81.0		31.5
8	1.3	9.31	0.931	226.0	1.0	226.0
8B				195.0		195.0
14	2.126	39.3	1.53	835.0	÷2.57	324.9
14B				531.0		206.6
14C				1066.0		414.8
9	2.6	18.6	1.86	404.0	1.0	404.0
9B				381.0		381.0
10	5.2	37.2	3.72	726.0	1.0	726.0
10B				587.0		587.0
10C				680.0		680.0
11	10.4	74.4	7.44	1968.0	1.0	1968.0
11B				1210.0		1210.0
12	57.11	37.2	41.0	669.0	x11.0	7359.0
12B				957.0		10,527.0
12C				1428.0		15,708.0
13	519.1	37.2	371.0	922.0	x99.7	91,923.0
13B				849.0		84,645.0
13C				1008.0		100,498.0

Figure 7

Figure 7 represents the response of the cholesterol of the gut to increasing amounts of acetate. The specific activity of the cholesterol (cpm/mg.) are plotted against the dose (micromoles of acetate/200 gram rat).

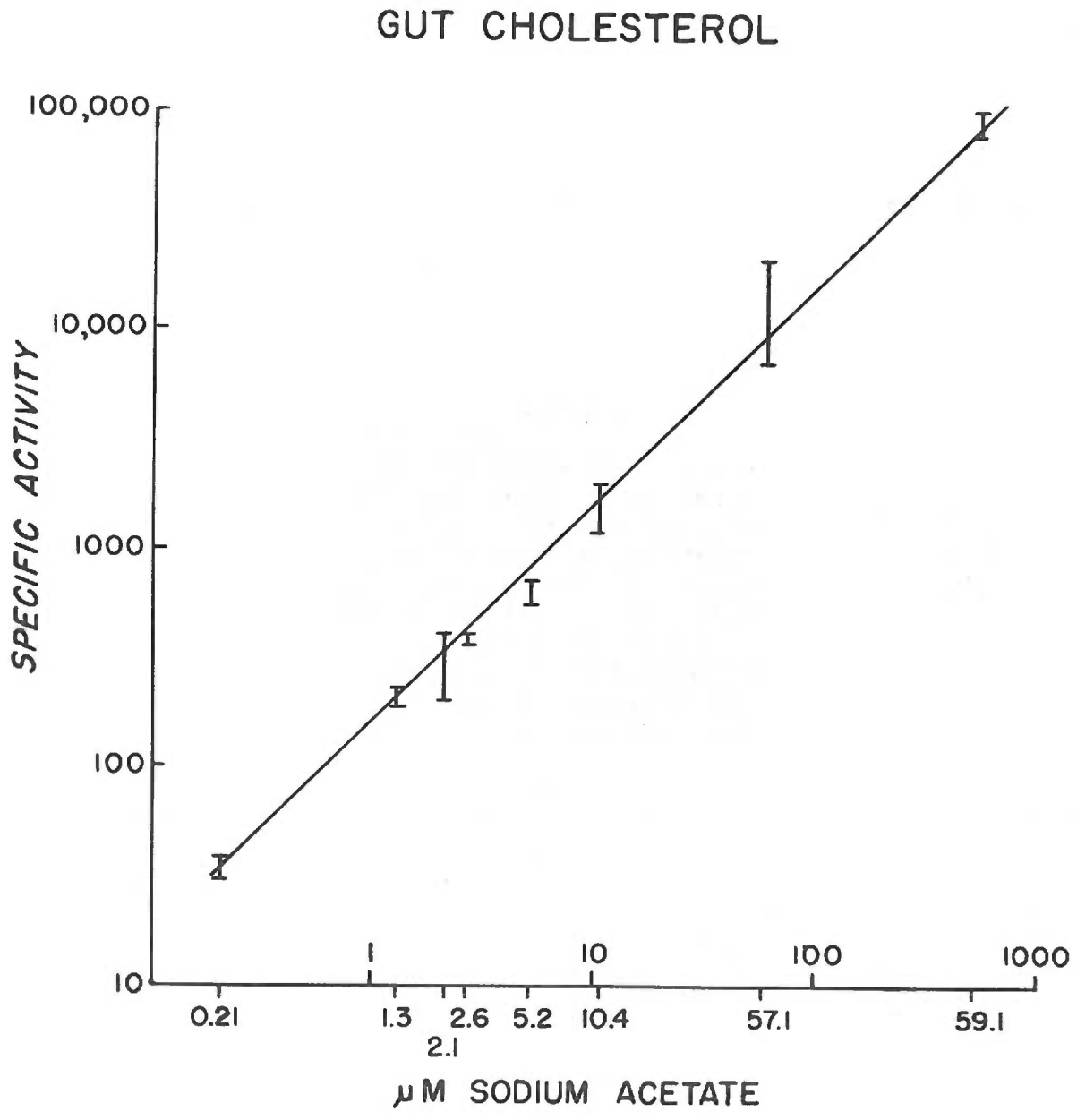

GUT CHOLESTEROL
100,000
10,000
1000
100
10
SPECIFIC ACTIVITY
1
10
100
1000
0.21
1.3
2.1
2.6
5.2
10.4
57.1
59.1
μM SODIUM ACETATE

FIG. 7

TABLE XIV

Carcass Cholesterol Data

Animal	Dose ul of acetate	Dose Counts x10^5	Cnts. at constant S.A.x10^6	S.A. of Chol. cpm/mg.	f	Corr. S.A. of Chol. cpm/mg.
15	0.2126	3.93	0.153	34.0	÷2.57	13.2
15B				28.0		10.9
8	1.3	9.31	0.931	74.0	1.0	74.0
8B				54.0		54.0
14	2.126	39.3	1.53	270.0	÷2.57	105.1
14B				128.0		49.8
14C				517.0		201.2
9	2.6	18.6	1.86	106.0	1.0	106.0
9B				124.0		124.0
10	5.2	37.2	3.72	208.0	1.0	208.0
10B				166.0		166.0
10C				303.0		303.0
11	10.4	74.4	7.44	462.0	1.0	462.0
11B				395.0		395.0
12	57.11	37.2	41.0	198.0	x11.0	2178.0
12B				464.0		5104.0
12C				193.0		2123.0
13B	519.1	37.2	371.0	271.0	x99.7	27,019.0
13C				580.0		57,826.0

Figure 8

Figure 8 represents the response of the cholesterol of the carcass to increasing amounts of acetate. The specific activity of the cholesterol (cpm/mg.) are plotted against the dose (micromoles of acetate/ 200 gram rat).

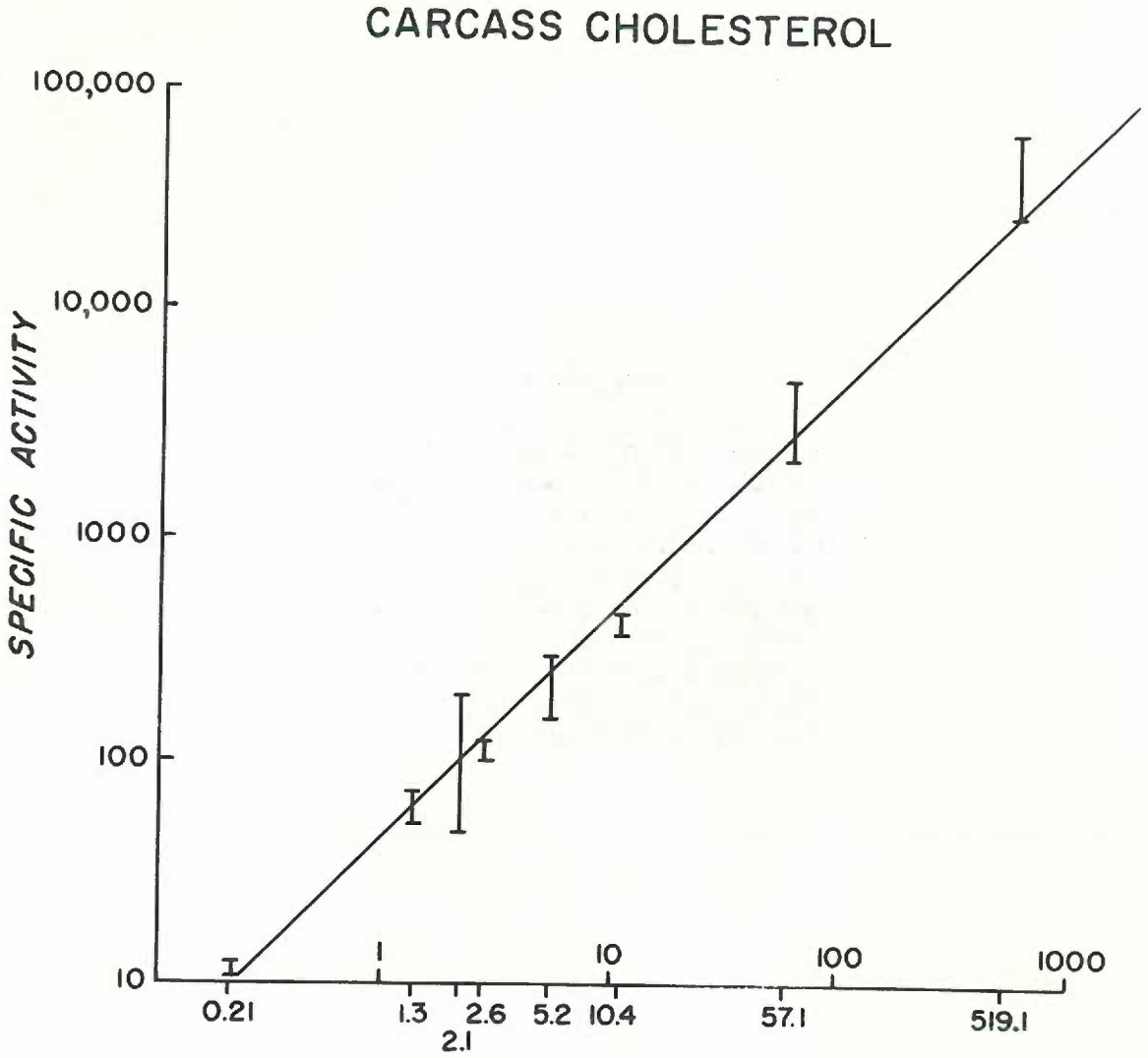

CARCASS CHOLESTEROL
100,000
10,000
1000
100
10
SPECIFIC ACTIVITY
1
10
100
1000
0.21
1.3
2.1
2.6
5.2
10.4
57.1
519.1
μM SODIUM ACETATE

FIG. 8

TABLE XV

Skin Cholesterol Data

Animal	Dose uM of acetate	Dose Counts x10⁵	Cnts. at constant S.A.x10⁵	S.A. of Chol. cpm/mg.	f	Corr. S.A. of Chol. cpm/mg.
15	0.2126	3.93	0.153	45.0	÷2.57	17.5
15B				47.0		18.3
8	1.3	9.31	0.931	83.0	1.0	83.0
8B				88.0		88.0
14	2.126	39.3	1.53	283.0	÷2.57	110.1
14B				178.0		69.3
14C				375.0		145.9
9	2.6	18.6	1.86	148.0	1.0	148.0
9B				200.0		200.0
10	5.2	37.2	3.72	352.0	1.0	352.0
10B				317.0		317.0
10C				618.0		618.0
11	10.4	74.4	7.44	611.0	1.0	611.0
11B				520.0		520.0
12	57.11	37.2	41.0	347.0	x11.0	3817.0
12B				317.0		3487.0
12C				693.0		7623.0
13	519.1	37.2	371.0	408.0	x99.7	40,678.0
13B				421.0		41,974.0
13C				389.0		38,783.0

Figure 9

Figure 9 represents the response of the cholesterol of the skin to increasing amounts of acetate. The specific activity of the cholesterol (cpm/mg.) are plotted against the dose (micromoles of acetate/ 200 gram rat).

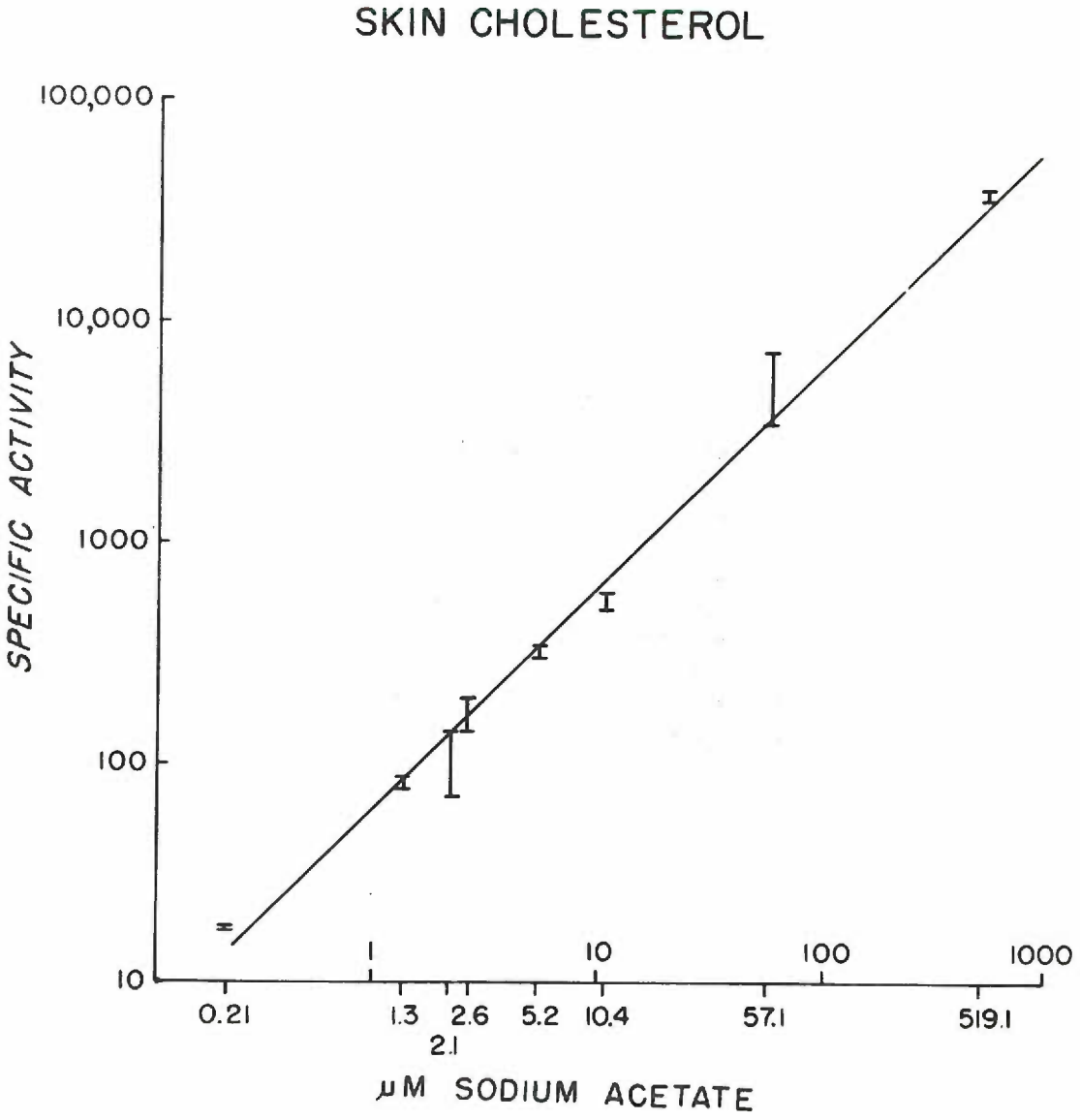

SKIN CHOLESTEROL
100,000
10,000
1000
100
10
SPECIFIC ACTIVITY
1
10
100
1000
0.21
1.3
2.6
2.1
5.2
10.4
57.1
519.1
μM SODIUM ACETATE

FIG. 9

it conforms to the previously defined tracer conditions. The conclusion that can be drawn from such a response is that the intact rat utilized all doses as if they were physiological in amount. Thus, again we can be assured that when acetate is injected within the limits studied into a normal rat, as is commonly done in our laboratory, we are well within tracer limits.

The highest dosage used in these studies was 519.1 micromoles per 200 gm. rat. This amount is considerably below the 2530 micromoles per gm. of tissue reported in earlier studies from this laboratory. The highest dosage of 519.1 mg. represented the greatest amount of our sodium acetate preparations that could be given to the animals without causing convulsions and death. When this dose was exceded, the rats displayed extreme thirst followed by convulsions which ended in death. Upon autopsy, it was observed that the visceral cavity was filled with intra-peritoneal fluid and secondly the surrounding tissues appeared inflamed as in acute peritonitis, suggesting that higher dosages caused a severe salt imbalance.

CHAPTER V

Summary

A method of purification of fatty acids from biological origins has been described. In this same study a biological mixture of fatty acids may be fractionated into saturated and unsaturated fatty acids --- an aid to further lipid separation.

A new practical method for plating fatty acids for radioassay has been described, permitting the use of small quantities of lipids eliminating correction factors for thick plates.

A new lipid extraction procedure is outlined making use of aliquots of the total tissue digests thus minimizing size and amount of glassware and errors due to stop-cocks, stop-cock grease, leaks, etc.

A study was conducted to determine what amount of acetate constituted a tracer dose. The CO_2, fatty acid and cholesterol compartments of liver, gut, carcass and skin revealed that all dosages, ranging from 0.2 micromoles to 519.1 micromoles, were tracer in amount. The use of higher dosages was prohibited by the death of the experimental animals.

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