ETHANOL METABOLISM

IN THE INTACT RAT

by

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INTRODUCTION

1. Historical Aspects

Even though mankind has been acquainted with alcohol for centuries, it has been only in recent times that the physiological effect of alcohol could be studied profitably by the scientific community. Certainly not until the concept of metabolism as a series of integrated chemical processes was developed, could serious thought be given to the problem of alcohol metabolism. As late as 1870, the fate of alcohol in the body was described as follows (100):

> "Once the alcohol having passed into the mouth and down the throat, it has to pass on down the whole line; or if it stops it burns a sore. The hand passes it into the mouth, and into the stomach it goes. The stomach calls out "Hot ball!" and throws it, as quick as possible, into the blood. The blood says, "Hot ball take it!" and swiftly throws it into the heart. The heart pumps it into the arteries, and the arteries rush it into the brain, and skin, and everywhere, trying to drive it out. The pores of the skin open, and say "Throw it out here!". The lungs open, and hurl some of it off with the breath. The liver takes a part of it, and the kidneys take some of it, and pass it through the urine. The whole system makes haste to get rid of it, and does get rid of it. It is thrown out of the system just as it went in, --undigested, --unchanged, alcohol. But part of it

remains in the system, --lodges in various organs of the body. What about that part?"

Nearly a century later, the question is still being asked, "What about that part?"

Early attempts to understand ethanol metabolism and to characterize metabolites were fraught with difficulties, biochemical methods at that time being crude and unavailable. Baron Von Liebig assumed that alcohol was oxidized in humans and in animals "through aldehyde, oxalic acid and formic acid to carbonic acid", but as far as is known, experimental evidence for his assumption was completely lacking (49).

With the development of chemical methods of analysis, Dupre' in 1872 (27) and Anstie in 1874 (3) observed that following alcohol administration, only an insignificant amount of that administered could be recovered from the excretions. As it was impossible to demonstrate the presence of any metabolic products of alcohol in the body or in the excretions, it was concluded that 95-98% of the ingested alcohol was totally oxidized in the organism to carbon dioxide and water (2, 11, 89).

It was realized quite early that alcohol could be considered as a food stuff and that some relationship existed between metabolism and ingested alcohol. The precise influence alcohol had on the body was not obvious, since Henrijean (42) and Bodländer (13) in 1883 found that the "general metabolism" was not increased by the ingestion of alcohol. Eight years later, Strassmann (103) demonstrated that animals given alcohol over a long period of time had more adipose tissue than control animals. The end of the nineteenth century came with little insight into the actual metabolism of alcohol, but held in general

acceptance was the notion that alcohol could be utilized by the animal body.

Around the turn of the century, data began to accumulate on the rate with which blood alcohol levels decreased following alcohol administration. In subsequent years a plethora of reports appeared describing the blood levels of alcohol following standard dosage. A plot of such values against time results in the well known "blood alcohol curves". The question of whether a hyperbolic or a rectilinear curve best describes the disappearance of blood alcohol, and the question of whether or not the slope of the curve is dependent upon the original concentration of blood alcohol remain aspects of this problem which have not, as yet, been entirely resolved. The contemporary opinion concerning this aspect of alcohol metabolism is that ethanol oxidation is somewhat increased with increasing blood concentrations of alcohol. Since the maximum concentration possible in the living organism is limited (less than 0.3 gm/100), the elimination curve for alcohol generally is represented by a straight line (49).

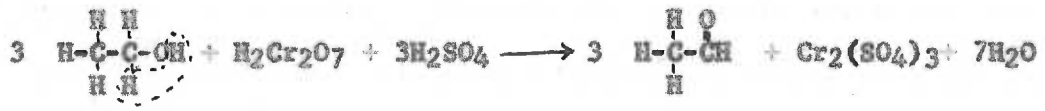
With the application of isotopic techniques to biochemistry, a new era began in the field of intermediary metabolism. Studies on the metabolism of alcohol directly benefited from these methodological advances, and aspects of the alcohol problem became vulnerable which, heretofore, had remained unapproachable. The ability of ethanol to participate in common pathways of metabolism became apparent, and the fact that radioactively tagged ethanol labeled nearly every class of compound in the animal body more than confirmed the opinion of the early workers who claimed an active role for ethanol in metabolism.

2. Chemical Considerations

From the chemical standpoint, an alcohol may be considered as derived from a water molecule by the replacement of a hydrogen atom by an alkyl group. Since both water and alcohols contain a hydroxyl group, alcohols would be expected to undergo reactions analogous to those of water, and in many respects this is true.

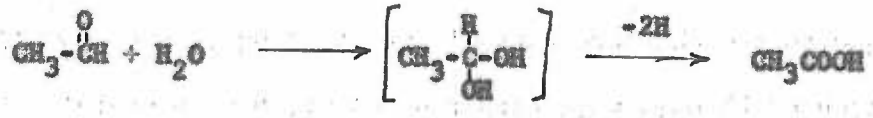
The family of primary alcohols are highly susceptible to oxidation by chromic acid, by potassium dichromate, or by potassium permanganate. Since methane and ethane are resistant to the same reagents, the presence of oxygen in a molecule confers susceptibility to further oxidation. In ethyl alcohol one of the two carbon atoms is linked to oxygen, and hence is already oxidized, while the other is joined to hydrogen and carbon, and corresponds to the carbon atoms of the inert ethane. Hence, oxidizing agents attack the molecule at the former, rather than the latter position. The initial oxidation product is acetaldehyde, which on further oxidation is attacked in the already oxidized part of the molecule and yields acetic acid as an end product resistant to further attack.

The first step in the overall process can be regarded as dehydrogenation rather than oxidation, involving elimination of a hydroxylic hydrogen together with a hydrogen of the hydroxylated carbon atom, with direct establishment of a carbon to oxygen double bond.



The reagent employed to effect the reaction is regarded not so much as an oxidizing agent but as a hydrogen acceptor. The second step leading to acetic acid also can be interpreted as a dehydrogenation if

it is supposed that the reaction proceeds through the transient formation of an unstable hydrate of acetaldehyde. This scheme can be shown as follows:



Confirmation that in some cases the hydrated aldehyde is involved is offered by the observation that aldehydes are resistant to oxidation by silver oxide in an anhydrous state but are capable of conversion into acids by this reagent in the presence of water.

Organic compounds containing carbon, hydrogen and oxygen can be combusted in oxygen to produce carbon dioxide and water as the sole products. By this procedure, it is possible to obtain some estimate of the heat content of the compounds. The energy change accompanying the complete combustion of one mole of a compound is referred to as its heat of combustion. The heats of combustion of ethanol, acetaldehyde and acetic acid are listed below (37, 118).

Heats of Combustion to Carbon Dioxide and Water

Compound	$-\Delta H$ (Kcal/mole at 25° C)
ethanol	327
acetaldehyde	279
acetic acid	209

It is quite readily seen that ethyl alcohol furnishes the largest amount of heat energy of these three compounds when completely oxidized. The step-wise oxidation of ethanol is associated with a decrease in the energy content of each subsequent compound.

In living systems, the complete oxidation of ethanol, acetaldehyde and acetic acid to carbon dioxide and water furnishes the amount of energy equivalent to the heat energy liberated in their chemical (as contrasted to biochemical) combustion. In the biological system, the energy is liberated in small increments, as the compounds undergo chemical transformations in which energy is liberated at definite steps in the reaction sequence. Chemical combustion is characterized by the liberation of energy all at once, a process which would make it impossible for the body to trap and use energy liberated, so the organism harnesses the energy by coupled energy storing reactions through intermediary metabolism.

3. Biochemical Considerations

Analogous to the chemical oxidation of ethyl alcohol, a plausible biochemical sequence can be considered in which ethanol metabolism occurs in two enzymatically controlled steps, (1) the oxidation of ethanol to acetaldehyde and (2) the subsequent conversion of acetaldehyde to acetic acid. Acetic acid can then undergo activation with ATP and coenzyme A, which can participate in metabolic reactions. This sequence can be shown as follows:

$$\underset{\text{ethanol}}{CH_3CH_2OH} \longrightarrow \underset{\text{acetaldehyde}}{CH_3CHO} \longrightarrow \underset{\text{acetic acid}}{CH_3COOH} \longrightarrow \underset{\text{acetyl-CoA}}{CH_3\overset{O}{\overset{\|}{C}}SCoA}$$

That this scheme is a metabolically feasible one is substantiated by a number of observations.

When deuterium labeled ethanol (CD_3CD_2OD) was administered simultaneously with sulfanilamide, Bernhard found 1/6 - 1/3 of the deuterium

as acetyl sulfanilamide excreted in the urine (8). Lundsgaard (66), while performing perfusion experiments with isolated livers observed that the amount of oxygen used was too small to account for the oxidation of all of the alcohol to carbon dioxide. He assumed, therefore, that the oxidation of ethanol did not proceed past the acetic acid stage, and as proof for the validity of this assumption, he found a considerable amount of acetic acid in the perfusion blood during the experiment. However, complete oxidation of ethanol can occur in the proper system since more than 90 per cent of the radioactive carbon of C^{14}-ethanol can be recovered as $C^{14}O_2$ from expired air within 10 hours of its administration (6).

4. Enzymes Involved in Alcohol Metabolism

a. Conversion of Ethanol to Acetaldehyde

The only enzymes known that are capable of metabolizing ethanol, convert it to acetaldehyde. These enzymes are alcohol dehydrogenase and catalase, but, in the animal body, the relative importance of each of these two enzymes in the conversion of ethanol to acetaldehyde is an area which needs clarification.

Keilen and Hartree (54) originally showed that catalase, in the presence of peroxide, could oxidize ethanol to acetaldehyde.

$$\underset{\text{ethanol}}{CH_3CH_2OH} + H_2O_2 \xrightleftharpoons{\text{catalase}} \underset{\text{acetaldehyde}}{CH_3CHO} + 2H_2O$$

Catalase is found in mammalian liver, kidney, and erythrocytes. The molecular weight of liver catalase is close to 250,000 and 4 atoms of iron per mole are thought to be present since the iron content is 0.09% (76). The pH optimum is between 6.3 - 9.5 and it has a Michaelis

constant of 0.025 M. Catalases have turnover rates higher than those found for any other enzymes. One mole of catalase is capable of decomposing more than 2,000,000 molecules of H_2O_2 per minute (118).

Alcohol dehydrogenase is also known to convert ethanol to acetaldehyde.

$$\underset{\text{ethanol}}{CH_3CH_2OH} + NAD^+ \xrightleftharpoons[\text{dehydrogenase}]{\text{Alcohol}} \underset{\text{acetaldehyde}}{CH_3CHO} + NADH + H^+$$

This enzyme is readily available from mammalian liver. The liver enzyme has a molecular weight of 84,000, and it contains 2 atoms of zinc per mole of enzyme. In addition to ethanol, the enzyme reacts with methanol and higher aliphatic alcohols. The Michaelis constant for alcohol dehydrogenase varies with pH, reaching a minimum at pH 8, 5×10^{-4} M.

Theorell and Bonnichsen (105) made the interesting discovery that the spectrum of reduced diphosphopyridine nucleotide, NADH, is altered in the presence of equimolar proportions of the crystalline liver alcohol dehydrogenase. The concentration of alcohol dehydrogenase giving maximum effect is 0.5 M., corresponding to a complex of the type ADH.$(NADH)_2$. The close association between enzyme and cofactor provides a complex relationship, the complexity increasing when the substrate interaction is considered as well. It has been found that the entropies of complex formation between enzyme and substrate are small but vary in sign depending upon the substrate and the pH (36).

Mahler et al. (71) recently reported that from theoretical considerations, at least one of the binding sites on the liver alcohol dehydrogenase enzyme is identical for reduced and oxidized diphosphopyridine nucleotide. This site is identified as the enzymebound zinc ion.

Inhibition experiments by these same workers suggested further that ethanol and acetaldehyde are not bound at the same site or in the same manner.

The oxidation of alcohol by the alcohol dehydrogenase - NAD system appears to be the rate limiting step for alcohol metabolism. Because of this, acetaldehyde should not accumulate. During the metabolism of alcohol, the corresponding acetaldehyde : alcohol ratio is about 1:40. It is evident that the oxidation of alcohol stops when relatively little acetaldehyde has been formed.

Liver normally contains a NAD : NADH ratio of 1.7 : 1. During alcohol metabolism the NAD : NADH ratio falls to 1.2 : 1 (34, 95). Another important factor in determining the rate of alcohol metabolism could be the rate at which NADH is reoxidized to NAD.

At the present time it is not possible to quantitatively evaluate alcohol metabolism in terms of the function of either catalase or alcohol dehydrogenase, or both of them. Since acetaldehyde is the primary reaction product of both enzymatic reactions, this product would result whether the alcohol was converted to acetaldehyde by the action of catalase or by means of alcohol dehydrogenase.

b. Acetaldehyde Metabolism

A number of enzymes capable of utilizing acetaldehyde as substrate have been identified in mammalian tissues (52, 84, 119). These enzymes are summarized as below:

Enzyme	Substrate(s) (acetaldehyde plus)	Product
aldolase	dihydroxyacetone phosphate	methyl tetrose phosphate
aldolase	glyceraldehyde-3-phosphate	deoxyribose-5-phosphate
aldolase	glycine	threonine (or allo-threonine)
glyceraldehyde-3-phosphate dehydrogenase	- - - - -	acetate
aldehyde oxidase	- - - - -	acetate
aldehyde dehydrogenase (mutase)	- - -	acetate (and ethanol)
xanthine oxidase	- - - - -	acetate
carboxylase	pyruvate	acetoin

Although these many enzyme systems have been identified in mammalian tissues and are known to utilize acetaldehyde as a substrate, the quantitative role of any one of them in alcohol metabolism is not known.

According to Richert and Westerfeld (85) molybdenum-containing enzymes are relatively unimportant in acetaldehyde metabolism in the intact animal. A protein-free diet fed to dogs for eight weeks did not alter the overall rate of acetaldehyde metabolism, but did decrease the liver xanthine oxidase activity (a molybdenum-containing enzyme) and decreased the liver molybdenum content. These studies may mean that xanthine oxidase and other similar molybdenum-containing enzymes are not major factors in acetaldehyde metabolism *in vivo*, but the possible presence of residual molybdenum-containing enzymatic activity in small, but adequate amounts to handle the acetaldehyde present, cannot be excluded.

Xanthine oxidase, aldehyde oxidase and aldehyde dehydrogenase are abundant in liver, and are all inhibited by the drug, Antabuse (bis(diethylthiocarbamyl)disulfide). Antabuse inhibition of aldehyde dehydrogenase has been shown to be of the competitive type (38). Aldehyde oxidase as well as xanthine oxidase cited above, are both molybdenum-containing enzymes, and over 2/3 of the molybdenum can be removed from the liver without affecting acetaldehyde metabolism. Even though xanthine oxidase and aldehyde oxidase might be excluded from the in vivo oxidation of acetaldehyde, still, no direct quantitative role has been assigned for the third enzyme, aldehyde dehydrogenase.

A mutase action involving two molecules of acetaldehyde can be a possibility for acetaldehyde metabolism since ethanol has been established as a natural component of the living cell (58, 72, 1). This dismutation reaction is shown below.

$$\underset{\text{acetaldehyde}}{2CH_3CHO} \xrightleftharpoons{\text{mutase}} \underset{\text{ethanol}}{CH_3CH_2OH} + \underset{\text{acetic acid}}{CH_3COOH}$$

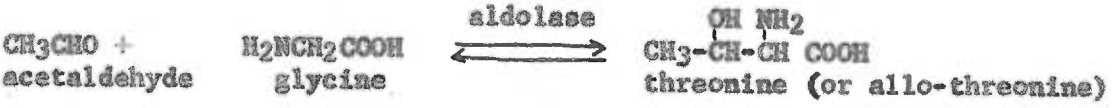
The following values are representative of the tissues in which ethanol has been identified and the concentrations measured (McManus):

Tissue	Concentration (umoles/100 gm. tissue)
rat liver	106
rat plasma	49
rabbit liver	67
human liver	145
rat kidney	23
rat skeletal muscle	38
rat heart	106

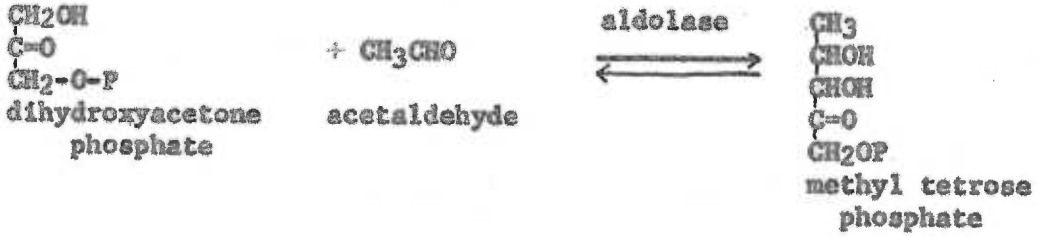
The presence of ethanol in the normal, non-alcohol treated animal tissue requires some explanation as to its origin. A reversal of any of the acetaldehyde pathways coupled with the reversal of the ethanol to acetaldehyde reaction could account for the presence of ethanol, as could a dismutation of acetaldehyde.

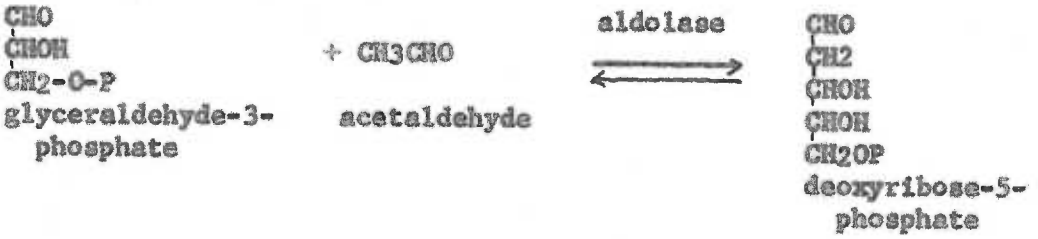
Four condensation reactions are reported to occur in mammalian tissues (119). The first condensation is mediated by a carboxylase enzyme and the reaction is as follows:

The second condensation reaction is reported to be facilitated by an aldolase enzyme and is as follows:



The third and fourth are also reactions catalyzed by aldolase enzymes and are as follows:





In an attempt to ascertain which of the possible pathways for acetaldehyde play a significant role, Lundquist et al. (64) studied

the reactions which take place in rat-liver suspensions under conditions where the number of potential pathways for acetaldehyde are limited by the absence of molecular oxygen. These workers found that under anaerobic conditions, acetaldehyde is transformed rapidly into ethanol and acetate. The part of the aldehyde that disappeared which was not due to ethanol formation was independent of the acetaldehyde concentration and it was found that the whole of the aldehyde removed could be accounted for by the formation of ethanol and acetate. Therefore, Lundquist concluded that during ethanol metabolism in the living organism, condensation reactions of acetaldehyde can be of no quantitative importance.

Studies with rat brain homgenates indicated that when acetaldehyde was metabolized in the presence of pyruvate, acetoin was a product of the reaction (102). It is entirely possible that individual tissues may perform different enzymatic reactions with acetaldehyde. The ability of brain tissue to carry out carboxylase action, whereas the liver is seemingly unable to, would tend to argue in favor of specialized enzymes in different tissues.

5. Ethanol Metabolism

a. Conversion of Ethanol to Acetaldehyde

The evidence supporting acetaldehyde as an intermediate in alcohol metabolism is quite sound. It has been known for at least a quarter of a century that acetaldehyde was a product of ethanol oxidation, since acetaldehyde was isolated as a reaction product following incubation of tissue slices and brei in the presence of ethanol (68). Acetaldehyde also can be demonstrated in the blood during alcohol

metabolism, and when alcohol oxidation is accelerated by means of insulin or pyruvic acid, the concentration of acetaldehyde is increased (102, 103). Although acetaldehyde is repeatedly found in the blood during alcohol metabolism in dogs (79, 123, 45), cats and rabbits (56), and in man (40), it does not seem to be an intermediate product of normal metabolism (48). In man, during the metabolism of alcohol, the concentration of blood acetaldehyde rarely exceeds 1 mg. per 100 ml. while alcohol concentrations are in the order of 200-300 mg. of alcohol per 100 ml. of blood. With low blood levels of alcohol, only negligible amounts of acetaldehyde are found.

Certain substances have been reported to increase the rate of alcohol oxidation. These substances include insulin plus glucose (80), fructose (65), pyruvate, and alanine (95). This area is still controversial. (21, 55). Increased oxidation rates of alcohol were best demonstrated when the initial rate of oxidation was relatively high. Since the oxidation of ethanol to acetaldehyde seems to be the rate-limiting reaction in alcohol metabolism, the effect of these substances is believed to be on this reaction. However, large daily variations in the rate of alcohol metabolism in the same animal (77), strongly suggest that some factor other than the concentration of alcohol dehydrogenase is involved in determining the rate of this reaction (120). The only mechanism which is apparent at present is through some influence on the reoxidation of NADH (95).

b. Acetaldehyde Metabolism

A portion of the discussion of acetaldehyde metabolism can be found in Section 4b of the Introduction.

In experiments performed by Casier and Polet (17), it was found that the metabolism of radioactive acetaldehyde and of radioactive ethyl alcohol had common traits. From the total amount of alcohol and acetaldehyde metabolized one-half hour after administration, 62% of the alcohol and 66% of the acetaldehyde were found in the tissues as fixed C^{14}, while 38% of the ethanol and 33% of the acetaldehyde were measured in the expired air as $C^{14}O_2$.

Alcohol given to animals premedicated with Antabuse resulted in higher (2-10 times) concentrations of acetaldehyde in the blood than was found with the same concentration of alcohol in non-treated animals (40, 41, 59, 70). Antabuse produces a block in acetaldehyde metabolism and, thus, exhibits an effect only when acetaldehyde is produced, as during the metabolism of alcohol. Both the alcohol and the acetaldehyde were metabolized to some extent in the presence of Antabuse, but higher concentrations of blood acetaldehyde were found following administration of the drug (81).

Mitochondria of rat liver, pigeon liver and rat kidney have been observed to metabolize a variety of long and short chain aldehydes to the corresponding acids. The view was expressed that the aldehydes are oxidized directly to the corresponding acid without the formation of an active acyl derivative, since the cofactor requirements for this oxidation were only ATP or NAD (114).

Heart muscle proteins have been found to catalyze the decarboxylation of pyruvic acid with the formation of acetoin. Acetaldehyde, added to the pyruvate system, was utilized in the formation of acetoin (39). This acetoin-forming system has been reported to be present in brain tissue as well.

Small amounts of acetaldehyde apparently can arise from the β-alanine produced as an intermediate in pyrimidine degradation (82) as shown in the following sequence of reactions:

$$\underset{\beta\text{-alanine}}{H_2NCH_2CH_2COOH} \xrightarrow[\text{reaction}]{\text{transamination}} \underset{\text{malonic semialdehyde}}{CHO\text{-}CH_2\text{-}COOH} \longrightarrow \underset{\text{acetaldehyde}}{CH_3CHO} + CO_2$$

Although this reaction may produce small amounts of acetaldehyde, from a quantitative standpoint, acetaldehyde is relatively unique to alcohol metabolism.

c. Acetate (Acetyl-CoA Metabolism)

For some years, it has been known that acetate required activation before it could become metabolized. Lynen and his associates in 1951 isolated "active acetate" from baker's yeast and identified it as acetyl-coenzyme A (69). Free acetate, as a result of its administration or as a result of endogenous production, requires that activation precede any further metabolism. Acetate is activated by the following series of reactions:

(1) acetate + ATP $\rightleftharpoons$ acetyl adenylate + PP_i

(2) acetyl adenylate + CoA $\rightleftharpoons$ acetyl-CoA + AMP

After activation, acetyl-CoA can undergo various reactions. The largest proportion of acetyl-CoA is metabolized via the tricarboxylic acid cycle, with lesser amounts becoming incorporated into fatty acids and cholesterol. It is also through intermediates of the tricarboxylic acid cycle that acetyl-CoA carbon can be incorporated into proteins and carbohydrates.

Ethanol and acetaldehyde are precursors of citric acid cycle compounds, carbohydrates, proteins and lipids. Presumably, this is

because ethanol and acetaldehyde are metabolized to acetyl-CoA. It is interesting to note that ethanol and acetaldehyde appear to be better precursors of fatty acids, cholesterol and proteins than is acetate. Such conclusions are based on greater labeling of lipids and proteins by tracer ethanol.

In an attempt to clarify the observation that ethanol can label some products better than acetate, Westerfeld's group (97) reported on experiments in which sulfanilamide was used as a trapping agent for acetyl-CoA in pigeon liver slices. It was shown that C^{14} - ethanol apparently was converted to C^{14}-acetyl-CoA without dilution by endogenous substrates, whereas C^{14}-acetate was diluted by endogenous acetate. This observation affords one explanation for better incorporation of C^{14}-ethanol into lipids and proteins than C^{14}-acetate.

6. Products of Ethanol Metabolism

A number of studies have been initiated in order to obtain specific information about the products of the metabolism of ethanol. It was hoped that the identification and study of these metabolic products would lead to definite conclusions concerning the fate of ethanol in the body.

Forsander and Raiha (33, 34) were able to identify and measure pyruvate, β-hydroxybutyrate, lactate, and small amounts of tricarboxylic acid cycle acids in normal rat blood. After the intraperitoneal administration of ethanol, acetate and small amounts of acetoacetate were found in the blood too. <u>In vitro</u> studies on perfused rat liver, without added ethanol, resulted in the presence of lactate in the effluent blood, β-hydroxybutyrate, acetoacetate and pyruvate also being

present but in lesser amounts. With the addition of ethanol to this system, besides these acids a considerable amount of acetate was found. Other perfusion experiments with ethanol-1-C^{14} and ethanol-2-C^{14}, showed similar results with the exception that an unidentified acid was found.

Lipid materials can be demonstrated to originate from products of ethanol metabolism. Reports have appeared (96, 90, 7) indicating that the label of C^{14}-ethanol can be incorporated into fatty acids, cholesterol and phospholipids. These tissue components were labeled in liver, gut, brain, skin and adipose tissues. Smith and Newman (96) showed that the specific activities of mouse lipid fractions from several tissues were approximately the same following ethanol-C^{14} and acetate-C^{14}.

While studying the possible synethesis of long-chain fatty acids from ethanol-1-C^{14}, Schiller et al. (88) discovered that the hydrolysis of fats to glycerol and fatty acids resulted in a reduction in the specific activity of the fatty acids with respect to the unhydrolyzed fat. To pursue this observation, they studied the mechanism by which glycerol becomes labeled from ethanol-C^{14}.

Upon the degradation of glycerol, after repeated injections of C^{14}-ethanol, the pattern of labeling was such that a pathway for the labeling of glycerol could be explained on the basis that alcohol was converted to acetyl-CoA, was metabolized through the tricarboxylic acid cycle to pyruvate, and then was converted into glycerol. This conclusion was deduced from the observation that the end carbons of the glycerol molecule contained all of the label from ethanol-1-C^{14}. When ethanol-2-C^{14} was employed, equal labeling was found in all three carbons of the glycerol molecule. This observation is again consistent

with the hypothesis that ethanol proceeds via the route just outlined.

In 1957 a report appeared (90) in which the incorporations of alcohol and acetate carbons into acetoacetate and β-hydroxybutyrate by malonate-inhibited rat liver homogenates were compared. Chemical degradation of the acetoacetate permitted a differential determination of the radioactivity in the C-1 position of acetoacetate and in the derived acetone (C-2, 3 and 4 of acetoacetate). Since no radioactivity was present in the iodoform prepared from the acetone, all of the activity found in the acetone molecule was assigned to the carbonyl carbon (C-3 of acetoacetate).

When this experiment was tried with acetate-1-C^{14}, the carboxyl and carbonyl groups of the acetoacetate were about equally labeled (C-1 and C-3 respectively). However, when ethanol-1-C^{14} was incubated with the liver homogenate, the acetone obtained from the degradation of the acetoacetate contained much more radioactivity than did the CO_2; these results indicated that the C-3 of the acetoacetate derived from ethanol contained from 10 to 25 times as much isotope as the carboxyl carbon (C-1)!

The β-hydroxybutyrate present in these filtrates was also oxidized to acetoacetate and degraded similarly. Essentially no radioactivity was found in the β-hydroxybutyrate derived from the acetate-1-C^{14}, but the β-hydroxybutyrate derived from ethanol-1-C^{14} seemed to contain appreciable amounts of radioactivity. These results led to the suggestion that the ethanol-1-C^{14} or some product of it, such as acetaldehyde, was being metabolized by a condensation with unlabeled acetyl-CoA to give 4-carbon units predominantly labeled in the 3-position.

Studies were carried out with rat kidney homogenates and similar results were obtained. This was unexpected because rat kidney, unlike the liver, contains enzymes which can effect a randomization of the label between C-1 and C-3 of acetoacetate (16). Even if the acetoacetate formed from ethanol-1-C^{14} contained a large predominance of isotope in the C-3 position, a subsequent splitting of the 4-carbon unit and recombination of the 2-carbon units by rat kidney should have tended to equalize the distribution of isotope between C-1 and C-3. Since this did not occur, the methods used to obtain these results were questioned and subject to more rigorous evaluations. Reevaluations showed that the high activity previously credited to the carbonyl group of the acetone (C-3 of acetoacetate) was, in reality, due to an impurity. When the effect of this impurity was eliminated from the data, the actual distribution of isotope in the acetoacetate derived from ethanol-1-C^{14} was approximately equal in the C-1 and C-3 positions. Consequently, there was no longer any basis for believing that the isotope distribution in the acetoacetate derived from ethanol-1-C^{14} was essentially different from that obtained with acetate-1-C^{14}. A complete description of this series of experiments can be found in the review by Westerfeld and Schulman (122).

The formation of acetoacetate from ethanol has also been investigated in another laboratory (34). When alcohol was perfused, through the intact rat liver, acetoacetate was formed. By following the rate of oxidation of the alcohol and measuring the blood volumes in the liver, it was concluded that alcohol is totally oxidized to ketone bodies by the rat liver. The ketone bodies were able to be detected in the hepatic venous blood.

Finally, one of the products resulting from the presence of alcohol in the body is the sulfate ester of ethanol. Primary alcohols have been shown to be detoxified by sulfation (124, 14). In the rat these detoxication products of the aliphatic alcohols, the mono-alkyl sulfates, are found to be excreted in the urine.

7. Tissues Responsible for Alcohol Metabolism

The liver is generally considered to be the most important organ for the oxidation of ethanol (49). Liver slices and brei oxidize alcohol readily (5, 6, 57, 68) and liver contains appreciable amounts of the dehydrogenase enzyme. Surviving livers artificially perfused with blood containing alcohol, removed alcohol at rates corresponding to one-half to five-sixths of the rate observed in intact dogs (30), cats (68), and rabbits (41). In partially hepatectomized animals, the rate of alcohol disappearance from the blood decreased as more liver tissue was removed, until the alcohol disappearance became almost nil when the animals were totally hepatectomized (74, 75). Liver damage caused by arsenic, chloroform, phosphorus and antimony resulted in a lowered capacity of the organism to oxidize alcohol. Eviscerated animals have been shown to metabolize alcohol very slowly (19, 20, 62).

Muscle and kidney tissues have been found capable of ethyl alcohol metabolism. In vitro preparations of rat diaphragm, heart muscle and kidney were observed to convert C^{14}-labeled ethanol to $C^{14}O_2$ (6, 31, 57). It was found that the alcohol oxidation capacity of the kidneys exceeds that of the liver (4). However, the quantitative role that the kidneys play in the overall metabolism of ethanol in the intact animal is not known. Testes, spleen, adipose tissue and intestinal tract have been found to have a limited capacity for alcohol oxidation (32, 60).

Reports concerning nervous tissue, in particular the brain, are interesting. Dwan (26) found in vitro preparations of ox brain to be capable of forming acetaldehyde and acetic acid from added alcohol and he chemically identified both metabolites. He also found brains of dog, cat, pig, cow, guinea pig and rabbit (in fact, all species examined) to have the capacity for alcohol oxidation (25). Bartlett and Barnet (6), however, using C^{14}-labeled ethanol found no formation of $C^{14}O_2$ from rat brain tissue.

Also in rat brain homogenates (10), acetaldehyde increased the synthesis of acetyl choline and acetoin, and decreased the formation of citric acid from pyruvate or acetate. In these studies, alcohol had no effect on acetoin or citrate synthesis, but increased acetyl choline formation. Evidence was also forthcoming from the same authors that the conversion of acetaldehyde to acetyl-CoA, by a coenzyme A dependent reaction not requiring ATP or DPN, could be carried out using brain acetone powder. Acetoin production was also noted in a system containing acetyl-CoA and acetaldehyde. The two major questions concerning alcohol metabolism in brain tissue remain unanswered; is alcohol metabolized by brain tissue and if so, are there pathways unique unto the brain? Present evidence on the pathways of ethanol in brain tissue is inconclusive and many experiments need to be done.

8. Perspective

The aim of the experiments presented in this thesis is to determine, to some degree of finality, the quantitative importance of the pathways for the metabolism of ethyl alcohol in the intact rat. The contributions

of this work to the field of ethanol metabolism are essentially three, (1) to report on studies that have been performed on the *in vivo* system, thereby providing information not heretofore demonstrated, (2) to describe and demonstrate a new experimental approach used to compare the relative fates of the carbons of the acetate and ethanol molecules, and (3) to report evidence and to present discussion pertinent to defining the complete metabolic pathway for ethanol metabolism.

A survey of the literature concerned with ethanol metabolism will verify the fact that a considerable volume of work has accumulated over the years. A closer scrutiny, however, will make apparent the fact that even with this large amount of work, it cannot be firmly and unequivocally stated that there is only one pathway for the metabolism of ethanol in the normal mammalian body. The reason why alternate routes for the metabolism of ethanol have not been identified and/or eliminated entirely from consideration is due to the experimental designs that have been applied.

MATERIAL AND METHODS

Animals

The animals used in this study were male, albino rats of the Sprague-Dawley strain. They were procured from Pacord Research Animals in Beaverton, Oregon. At the time the experiments were performed, the rats weighed between 200 and 250 grams. All animals were housed in cage-hood assemblies developed in this laboratory (106).

Feeding

A strict feeding regimen was necessary in order to insure that the nutritional states of the animals at the time of each experiment were comparable. Nutritional control has been shown to be necessary in order to minimize the variability of lipogenesis and cholesterologenesis which are dependent upon fasting and feeding periods (18, 104, 108, 109, 116). Consequently, the animals were trained to a feeding program. Trained feeding consists of allowing each rat to eat 10 grams of Purina Rat Chow for one hour, twice daily. Within a day or two, all animals eat their entire 10 grams of ration during the one-hour feeding period. Fasting periods were considered to be initiated at the end of the one-hour feeding period and after any uneaten food was removed from the cage. Drinking water was available to the animals at all times.

In special feeding experiments, finely powdered Purina Rat Chow was supplemented with either sulfanilamide or benzoic acid. Complete details for these experiments are furnished in subsequent sections. However, in these special instances, the feeding regimen was conducted as described above.

Radioisotopes

Ethanol-C^{14} and Acetate-C^{14} . . . . Criteria of Purity

For this study, it was imperative that the radioactive compounds used be of the highest chemical purity and that the positions of the radioactive carbons be known with certainty.

The acetate and ethanol tracer compounds were purchased from the New England Nuclear Corporation. This company provided information that the ethanol was chemically pure by processing the ethanol through a preparatory gas chromatography column and collecting the fraction which corresponded to ethanol.

Isotopic dilution analyses were conducted in our laboratory on these compounds to verify the chemical purity. The principle of this analysis is as follows: The compound whose purity is to be established is added and mixed well with a relatively large amount of pure, but unlabeled, carrier. The amount of the carrier is known. Radioassay of a portion of this mixture is performed and a specific activity value (cpm/mole of material) is determined. A derivative which is as specific as possible for the compound being tested is then synthesized. The derivative is purified and a known amount is radioassayed; a specific activity value is determined for the derivative.

Since the specific activity values for the starting materials (i.e., the acetate and the ethanol) are determined on a mole basis, the specific activity values of a starting compound and the respective derivative should be equal if the starting compound was chemically pure. A higher specific activity value for the derivative would indicate chemical contamination of the starting material.

Presented in Table 1 are the results of the isotope dilution analyses that were performed on the ethanol-1-C^{14}, ethanol-2-C^{14}, acetate-1-C^{14} and acetate-2-C^{14} used in the experiments reported in this thesis. The derivatives were 3, 5-dinitrobenzoates of the ethanols (61) and the p-nitrobenzyl esters of the acetates (93).

Table 1

Compounds	Starting material Specific Activity (μc/mmole)	Derivative Specific Activity (μc/mmole)
Acetate-1-C^{14}	1.42	1.40
Acetate-2-C^{14}	1.49	1.47
Ethanol-1-C^{14}	2.00	1.89
Ethanol-2-C^{14}	1.96	2.04

Sodium formate-C^{14}

Sodium formate-C^{14} was purchased from the New England Nuclear Corporation. The specific activity of the formate when purchased was 4.7 μc/mg. After dilution, the aqueous sodium formate solution contained 16.5 μc/ml. This solution was used in the "formate" excretion experiments.

L-Serine-3-C^{14}

L-Serine-3-C^{14} was purchased from the Nuclear-Chicago Corporation. The specific activity of the original material was 2.0 mc/mmole. Total activity of the serine was 50 μc. After dilution the concentration of activity was 10.5 μc/ml. This solution was used also in the "formate" excretion experiments.

Dosage

Besides ascertaining the purity of the compounds being employed in studies such as these, it is important to obtain information as to

what influence the compound being injected might have on the system under study. The amount of substrate that would alter the normal metabolism of the endogenous substrate is unique for each system as well as each set of experimental conditions. The flooding of a "pool" by a labeled material is in violation of the principles of isotopic tracer methodology. An experimental criteria must therefore be established for the desired conditions of the experiment in order to state with assurity that the tracer compound was used at a tracer concentration for the experiment. The tracer level for acetate and ethanol as used under the conditions of this experiment is shown in Table 2 (109).

Table 2

Tracer Amounts of Acetate and Ethanol Employed

Compound	Activity Injected (μc)	Amount Injected (mg)
Acetate-1-C^{14}	18-20	2.2
Ethanol-1-C^{14}	18-20	4.2
Acetate-2-C^{14}	18-20	2.2
Ethanol-2-C^{14}	18-20	4.2

Chemical Methods

General Experimental Procedure

After the animals were trained to feeding, an animal was fed his morning's ration for an hour, fasted for an hour and then injected with a tracer dose of either acetate or ethanol via the intraperitioneal route. Immediately following the injection, the animals were placed into the intact animal apparatus (See Figure 3) and respiratory carbon

dioxide collected for the subsequent two hours. At the end of the two hours, the animals were decapitated and separated into four tissue fractions, viz., liver, gut, carcass and skin. These fractions were weighed, dissolved in 25% (w/v) alcoholic KOH and refluxed for two hours. At the end of the reflux period, quantitative recoveries of cholesterol were made with petroleum ether extractions. The digest was then acidified, and the fatty acids were removed quantitatively with petroleum ether. (87).

Chemical Determinations and Procedures

Cholesterol

Digitonin was added to an aliquot of the non-saponifiable fraction and the digitonides precipitated (43). The digitonides were processed by a modification of the method of Sperry and Webb (99) and the washed sterol digitonide was dissolved in anhydrous methanol. An aliquot of this methanolic solution was removed and taken to dryness. The amounts of cholesterol were determined by the method of Zlatkis (126).

Color densities were measured with a modified Bausch and Lamb Spectronic 20 colorimeter (22) at a wave length setting of 560 mu. Standard curves were prepared and standards were run with each set of determinations. Dilutions of the cholesterol samples were made so that the concentration did not exceed 0.10 mg. or fall below 0.025 mg/5 ml. of color reagent which gives opticaldensity readings between 0.20 and 0.76 on our instrument. All samples were run in duplicate.

Fatty Acids - Gravimetric Determinations

The extracts containing the fatty acids were transferred to tared shell vials and the solvent evaporated at 60° C. (water bath) under nitrogen. The vials were then dried in a dessicator and weighed. The amounts of fatty acid were obtained by difference. Having obtained these values, and knowing the dilution factors, the amounts of fatty acids in the total fractions were calculated.

Fatty Acids - Chromatographic Separation

In order to refine the gross fatty acid fraction, reversed-phase column chromatography was employed. The column technique was a modification of that used by Silk and Hahn (94) while the solvent systems for elution of the fatty acid fractions were adapted after Popjak and Tietz (83).

The column packing was prepared by mixing silanized Hyflo Super-Cel with paraffin oil in ether and evaporating the ether. The paraffin oil-coated diatomacious earth was then dried in a vacuum oven (about 15 mm Hg pressure) with heating to 60° C. for one hour. The dried packing was stored in a closed bottle until used.

Preparation of the column was carried out by making a slurry of the column packing with 83% aqueous acetone. This slurry was poured into the column, previously heated to 35° C. by water circulating in the jacket of the column, and packed as evenly as possible by a glass plunger. The final column dimentions were 39cm x 0.9cm. Solvent flow through the column was regulated by applying a positive pressure to the column with a Simplex Minipump #65117, the system from the pump cylinder to the bottom of the column being closed with capillary tubing

and sealed pressure fittings. The delivery from the column was approximately 30ml/hr.

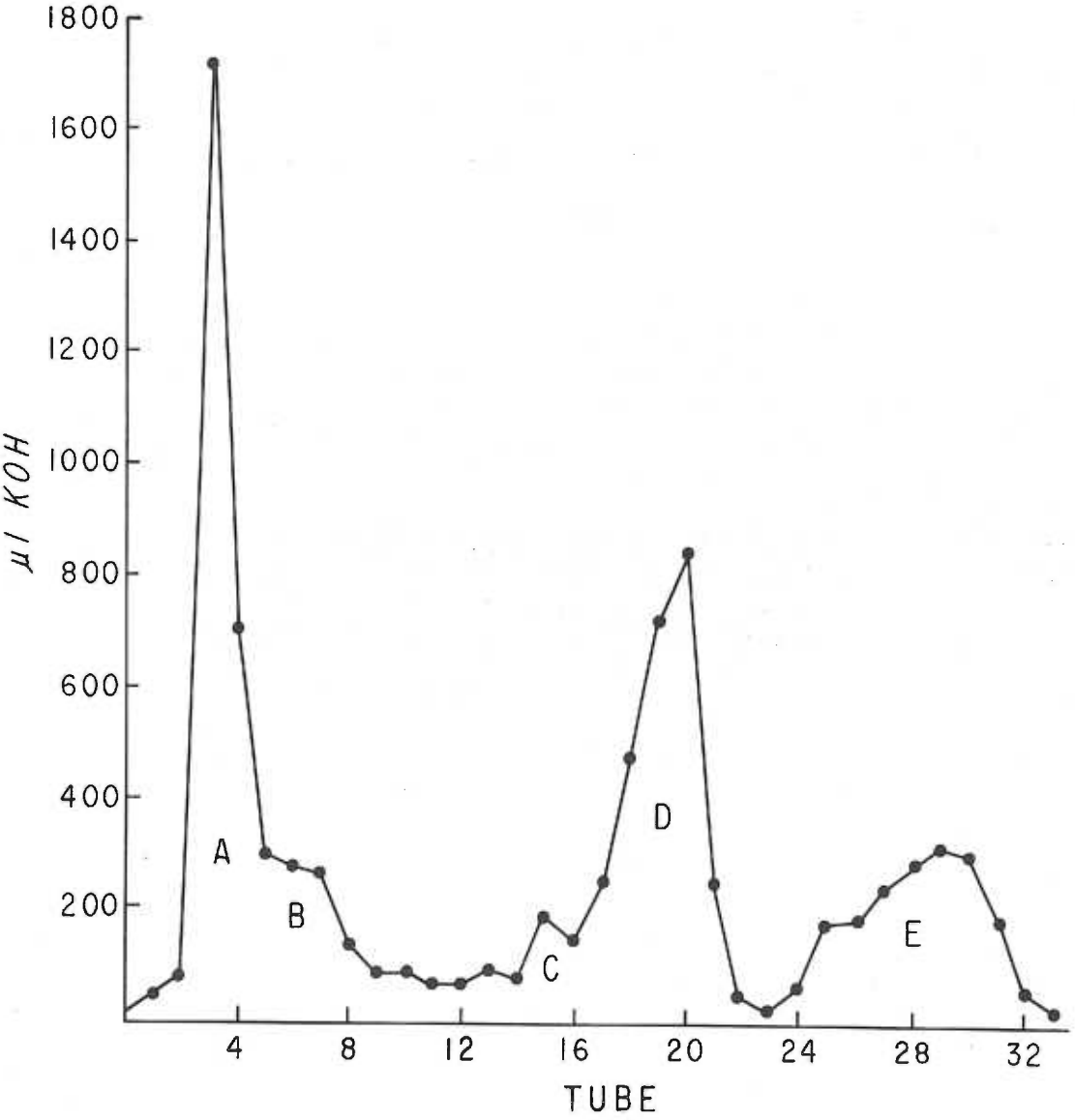
Elution of the fatty acids from the column was effected with aqueous acetone mixtures, the acetone concentration being increased to 45, 55, 65, 75, and 83% as the elution continued. The eluate was collected under nitrogen, the fraction collector being controlled on a time basis and the fractions being approximately 10ml. These fractions were transferred to a 40ml cuvette and titrated with 0.0115N methanolic KOH using a Manostat microburette, No. MB 1300. The titration was monitored and the end-point determined with a Coleman Junior Spectrophotometer, Model 6A, which detected the change in wavelength of the light absorbed by the indicator, bromothymol blue, as the titration progressed. The end-point was considered as the point at which further addition of alkali produced no further change in the absorption at 650 mμ.

Recovery of the fatty acids from the titrated fractions was accomplished by acidifying with a few drops of concentrated HCl and extracting repeatedly with petroleum ether (b.p. 40-60° C.). Evaporation of the petroleum ether under nitrogen yielded the fatty acids which were stored in a desiccator <u>in vacuo</u>.

Figure 1 presents the elution titration curve of the fractions obtained from the separation of the fatty acid fraction from liver tissue, five distinct peaks being apparent. For the isolations of the fatty acids in each peak, the tube(s) corresponding to peak concentrations of fatty acids were acidified and extracted.

Figure 1

Elution titration curve of fractions obtained from the separation of the fatty acid fraction from liver tissue by reversed-phase column chromatography


1800
1600
1400
1200
1000
800
600
400
200
µl KOH
A
B
C
D
E
4
8
12
16
20
24
28
32
TUBE

Carbon Dioxide Titration

The total quantity of carbon dioxide expired during the two-hour experimental period was collected in 200 ml of 1N NaOH as sodium carbonate. The carbonate was titrated with standardized hydrochloric acid. Two indicators were used. A 1% phenolphthalein in alcohol was used at the beginning of the titration and 0.1% methyl orange in alcohol was added when the pH was reduced to about 8.

At the start of the titration the pH of the solution was around 11 or above and the color was phenolphthalein red. When enough acid was added to complete the reactions

(1) $NaOH + HCl = NaCl + H_2O$ and

(2) $Na_2CO_3 + HCl = NaHCO_3 + NaCl$

the pH of the solution was about 8.4, due to the sodium bicarbonate. The red color had disappeared, and at this point 2 drops of methyl orange indicator were added to impart an orange color to the solution. The further addition of acid gives the red color of methyl orange and marked the completion of the reaction.

(3) $NaHCO_3 + HCl = H_2CO_3 + NaCl$

The pH of the solution at the end of the titration was about 3.0.

Acyl Derivatives

The reactive intermediates of lipid metabolism can be detected and isolated by the derivatives that they form with hydroxylamine (35).

Hydroxylamine can react with most substances of the general struction, R-COX, as follows:

$$R\text{-}COX + NH_2OH \longrightarrow \underset{\text{acyl hydroxamic acid}}{R\text{-}\overset{\overset{O}{\|}}{C}\ NHOH} + HX$$

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

the kind of grouping attached to the acyl.

The formation of hydroxamic acids by a non-enzymatic reaction of hydroxylamine with a tissue homogenate containing the expected acyl intermediates is of interest since it offers a good method for detection and separation of the different acyls. At any one time, the biological system will contain many acyl compounds so that it is of value to be able to separate the various hydroxamic acids. The separation can be accomplished by chromatographic techniques.

The hydroxamate method used followed the procedures previously established here for reaction conditions, such as optimum concentration of hydroxylamine, length of time the reaction should run for quantitative product formation, and the temperature at which the enzymes which influence this system are inactivated (28, 113).

Hydroxamic Acids

Hydroxamic acids were formed from thioacyls present in liver tissue by allowing a homogenate of liver tissue to react with hydroxylamine. The method used was that developed in this laboratory (28, 113). The method used was as follows: A freshly excised sample of liver was minced and a portion, weighing approximately one gram, was accurately weighed and transferred to a 55 ml. homogenizer tube, and the tube was immediately immersed in a water bath at 70° C. for 10 minutes. Two ml of 0.3 M hydroxylamine (pH 7.4) was added for each gram of liver, and the tissue was homogenized with a Teflon homogenizer. The homogenate was allowed to react at room temperature for 30 minutes.

The homogenate was then transferred to a 50 ml. screw cap culture tube using 15 ml. of butanol as the transferring fluid. The tube was capped and shaken for 30 minutes at full speed on a horisontal

shaker. The tube was then centrifuged at 1200 rpm for 10 minutes, and decanted into a 50 ml graduated centrifuge tube. The homogenate residue was extracted twice more with 10 ml. portions of butanol as above, and the butanol extracts pooled. The volume of the butanol was reduced to 10 ml with nitrogen on a 60° C. water bath.

To the concentrated butanol extract, 5 ml. of water were added and the solution made alkaline to phenolphthalein. The water layer was drawn off with a 10 ml. syringe fitted with a 7 inch needle. The butanol was reextracted with 2 ml of water until the pink color of the phenolphthalein in the butanol was removed (3-5 times). The water extracts were pooled, neutralized and reduced to a convenient volume for spotting on chromatographic paper.

An aliquot of the water extract (butanol also, if desired) was chromatographed on Whatman #3 filter paper that was previously rinsed with glacial acetic acid and dried. The solvent system was a butanol: acetic acid: water (4:1:5) system which requires about 16 hours for good separations. The chromatographic tank was at room temperature for these experiments. Following chromatography, the strips were air dried and radioassayed.

Acetylation of Sulfanilamide

Sulfanilamide was fed for three days to four rats at the level of 50mg of drug/rat/day. At the end of the three day feeding period, a single rat was injected with one of the following:

(a) 20uc acetate-1-C^{14}

(b) 20uc acetate-2-C^{14}

(c) 20uc ethanol-1-C^{14}

(d) 20uc ethanol-2-C^{14}

The animals were placed in individual glass "cages" for urine collection and kept there for 12 hours. At this time 5 ml. of distilled water was injected intraperitoneally and the animals returned to the collection cages for another 12 hours. The acetylated sulfanilamide was collected according to the method of Bernhard (8).

The acetyl sulfanilamide was crystallized once, dried and weighed. The recoveries were as follows:

A-1	24.6mg
A-2	12.4mg
E-1	17.2mg
E-2	12.7mg

The melting point of these materials was 205-210° C. The value reported in the literature is 216°.

Formate Excretion

This procedure was employed in order to test for the presence of C^{14}-formate in the urines of rats having received C^{14}-acetate and ethanol.

The method that was used was modified from that of Weinhouse and Friedmann (115). In brief, the method consists in the administration of a C^{14}-labeled compound to rats by intraperitoneal injection, followed immediately with another injection of unlabeled formate. Formate is excreted into the urine under these conditions, and the presence of radioactivity in the excreted formate indicates that formate is a metabolic product of the injected, labeled compound.

These experiments were performed on 230 gram rats. 2.0 millimoles of sodium formate (1 ml. of a 2.0 M solution of sodium formate) were

injected intraperitoneally followed immediately by an injection of the C^{14}-acetate or ethanol. Immediately following the injections the animals were placed for 6 hours in chambers for urine collection. The urine samples were filtered quantitatively into a distillation flask and acidified with 2 ml of 50% sulfuric acid, 0.5 grams of silver sulfate were added to prevent volatilization of chloride as HCl. Repeated distillations (up to six) were carried out until a quantitative transfer of the volatile acids was effected.

The distillate was passed through a Dowex-1 ion exchange column (acetate form) and formate and acetate were retained on the column. Ethanol was not retained and passes through. Acetate and formate were then selectively eluted with 0.1N NaOH and 3M NH_4NO_3. Figure 2 demonstrates the type of elution curves that were obtained.

The acetate fraction was combusted in a persulfate apparatus and the CO_2 was trapped in sodium hydroxide. Radioassay of this alkali-carbonate solution was done by the liquid sample assay technique. The formate fraction was radioassayed as a liquid sample in 3M ammonium nitrate.

Hippuric Acid

Benzoic acid was fed for three days to four rats. On the fourth day the rats were injected with C^{14}-acetate or C^{14}-ethanol and the urine was collected for the subsequent 12 hours. Hippuric acid was recovered and purified from these urine samples and then known amounts were radioassayed.

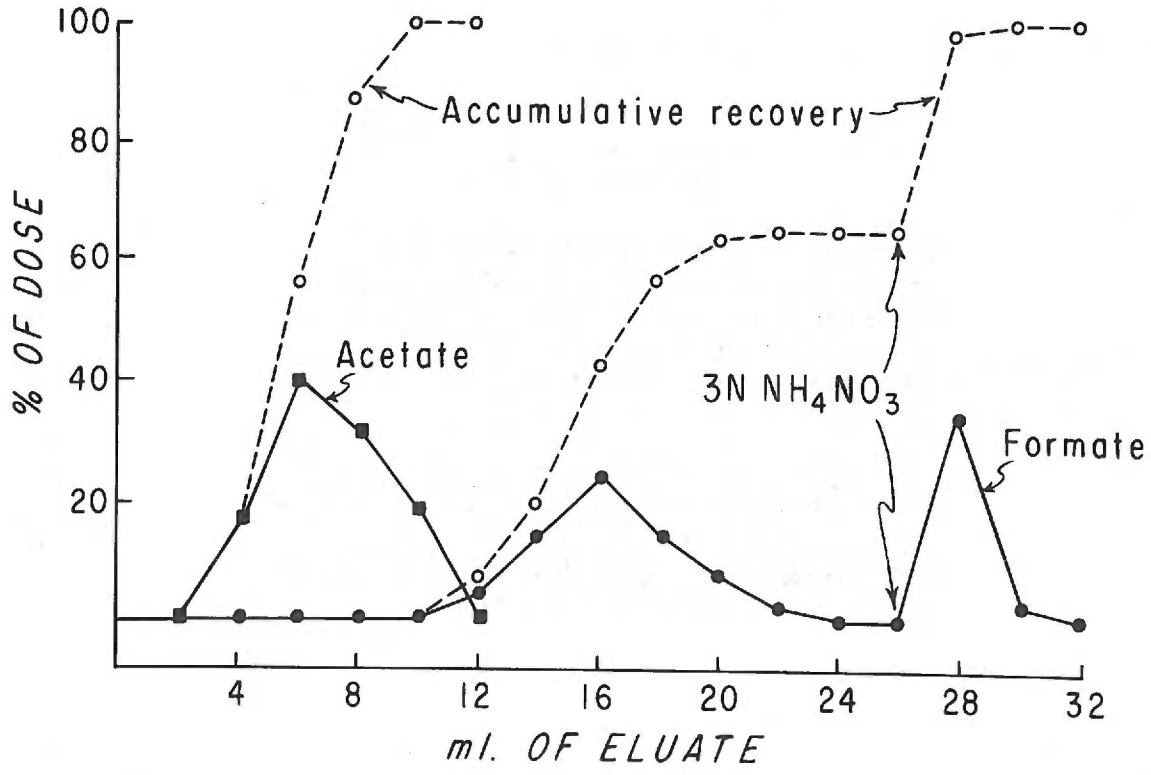
The benzoic acid that was fed was crystallized twice from water at which time the melting point was 122° C. (literature value 122° C.).

Figure 2

Elution of acetate and formate from ion exchange column

A Dowex-1 (acetate form) ion exchange column was loaded with C^{14}-acetate and C^{14}-formate. These acids were eluted with 0.1N NaOH until the acids had been separated. The remainder of the formate was then eluted with 3N NH_4NO_3.

The solid line represents the percentage of the radioactivity as acetate or formate recovered in each milliliter of eluate. The broken line represents the accumulated recovery of acetate or formate.


100
80
60
40
20
% OF DOSE
Accumulative recovery
Acetate
3N NH4NO3
Formate
4
8
12
16
20
24
28
32
ml. OF ELUATE

Each rat consumed approximately 125 mg of benzoic acid per feeding (i.e., 250 mg/day). By the third day of feeding, the rats were all consuming comparable amounts of benzoic acid supplemented diet.

Hippuric acid was extracted from the urine as follows: The volumen of the urine was measured and solid ammonium sulfate (5gms/10ml. urine) was added. After the ammonium sulfate was dissolved, 10 drops of thymol blue indicator was added, and concentrated HCl added until the solution was definitely "pink". The solution was mixed and placed in the refrigerator for 30 minutes for crystalization.

The crystals of hippuric acid were filtered off and washed 2-3 times with ice cold water. The filter paper and the precipitate were quantitatively transferred to a beaker, a little water was added and the mixture heated to dissolve the crystals. The hot mixture was filtered, cooled, and allowed to crystallize. The crystals were filtered and redissolved in hot water, decolorizing charcoal was added and the mixture filtered again while hot. The recrystallized hippuric acid was in needle form and gave a sharp melting point at 189.5° C. (literature value 189-190° C.).

Equipment used for Radioassays

Intact Animal Assembly

The equipment used for the continuous monitoring of radioactive respiratory carbon dioxide is pictured in Figure 3. An animal was injected intraperitoneally with the appropriate tracer and placed in the animal chamber. The animal remained in the chamber for two hours. During this time, the metabolism assembly was constantly flushed with dry, CO_2-free air. The expired air was passed through concentrated

Figure 3

Intact animal assembly for the measurement of respiratory C^{14} activities (110)

The components are as follows: (from left to right)

a. Animal chamber
b. Sulfuric acid bubbler to remove excess moisture from the expired air
c. Geiger-Mueller tube
d. Scaler (on top)
e. Counting rate computer (on bottom)
f. Milliammeter recorder
g. Large carbon dioxide absorber (containing excess of 1N NaOH)
h. Cartesian manostat


COUNTING RATE
COMPUTER
ON

sulfuric acid for drying, and then conducted to the Geiger-Mueller tube which is housed in a lead shield. The Geiger-Mueller tube impulses were scaled on a Berkeley Decimal Scaler Model 100 and a Berkeley counting rate computer, Model 1600, which activated a millimeter recorder. The gas stream was then passed through a container of 1N NaOH, which trapped all the CO_2 for further quantitative titration and radioassay. For a complete description of the intact animal assembly see Van Bruggen and Hutchens (110).

Other Radioactivity Analyses

All other radioactivity analyses were done using four instrument assemblies, three of the instruments were Geiger Mueller tube instruments and the fourth was a scintillation counter. For the most part, the samples were counted for at least 1600 total counts. In a few cases the samples were counted for only 800 counts.

End Window Instrument

The Geiger-Mueller end window system was a Nuclear Scaler, Model 163, attached to a Traced Lab 1.8 mg/cm^2 end window tube housed in an aluminum-lead shield. This instrument was used exclusively for liquid sample counting.

Automatic C115 Low Background Gas Flow System (Nuclear Chicago)

This low background counting system was designed to provide automatic detection with a low background of less than 2.0 cpm. The low background is obtained because of an abundance of shielding, and a "guard" detector tube that acts as a cosmic ray shield, operating through an anticoincidence circuit. An automatic sample

changer, Model C-110A, a Nuclear scaler, Model 183B, were used with this detector.

Vanguard Strip Counter

The hydroxamate paper chromatograms were assayed on a Vanguard Autoscanner, Model 880. This instrument has a 4π detector for maximal counting efficiency. The detectors are without windows. The counting efficiency for C^{14} is approximately 15%. All strips were run at 12 inches/hour, with a collimator slit opening of 0.5 inch and maximum scale deflection equivalent to 300 cpm.

Liquid Scintillation Counting

The hippuric acid samples were dissolved in the appropriate scintillator solvent system (See below - "Sample Preperation") and counted on a Packard Tricarb Liquid Scintillation Spectrometer, Model 314 Ex-2. By using internal standards it was established that the efficiency of counting was 56%.

Sample Preparation

Liquid Samples

The radioassay technique of aqueous samples containing carbon-14 was developed in and reported from our laboratory (112). The liquid sample is pipetted into a stainless steel planchet. A small drop of 1 per cent aerosol solution is added which reduces surface tension and allows the sample to cover the bottom of the planchet in an even film. A thin Mylar film (DuPont) is then placed, as covering, over the planchet and held in place by a rubber band. "Counting" is then done under the end window Geiger tube with an efficiency of 0.18%.

Lipid Samples

The lipids were counted as infinitely thin samples on concentric ring copper planchets. A sample described as infinitely thin implies a condition of negligible self-absorption. This amount can be determined experimentally by adding radioactive material in small increments to the planchet until further addition no longer gives a linear increase in activity. This deviation from linearity is the point at which the infinitely thin criterium has been exceeded with the beginning of a measurable amount of self-absorption. This amount has been determined previously in this laboratory and found to be less than 0.8mg per cm^2 in copper concentric ring planchets for both cholesterol and fatty acids. The sample was placed in the planchet as a solution and the solvent was evaporated under an infra-red ray lamp.

Paper Chromatographic Strips

An aliquot of the aqueous fraction of the hydroxamate extraction procedure (described earlier) was spotted on acetic acid washed Whatman #3 filter paper strips. Following chromatography in the butanol-acetic acid-water solvent system, the strips were air dried at room temperature. The strips were scanned for radioactivity without further preparation.

Solid Samples

The solid samples, viz. acetyl-sulfanilamide and hippuric acid were each counted after dissolving weighed amounts of each material into an appropriate organic solvent. Acetyl sulfanilamide was dissolved in dioxane and counted by the liquid sample technique under the end-window Geiger tube.

The hippuric acid samples, previously weighed, were dissolved in 15 ml of a scintillator solution comprised of the following ingredients:

7.00 grams P P O (2,5-diphenyloxazole)

0.05 grams POPOP (2,2-p-phenylene bis(5-phenyloxazole))

100 grams naphthalene

These amounts were dissolved in 1 liter of 1,2-dimethoxyethane (ethylene glycol dimethyl ether) which had a boiling point of 83-35^0 C. This solvent was treated before use by perculating it through an alumina column to remove peroxides. The scintillator solutions were counted with the Packard Tri-carb Scintillator Spectrophotometer.

Statistical Methods

The t-test was the statistical test employed for all comparisons. This test is a small sample test and is used to test for statistical differences between pairs of means. In this thesis, any p-value equal to or less than 0.05 is considered to indicate statistical significance.

The assumptions upon which this test is based are that the samples are from normal populations and that the variances of any two populations being compared are equal. The advantage of using the t-test lies in the test's insensitivity to violations of its two assumptions. The sampling behavior of "t" depends solely upon the number of samples and is independent of the population mean and population standard deviation.

See also Section A of the Appendix for a discussion of the method used to evaluate the ratios with the t-test.

RESULTS AND DISCUSSION

So as to present the results of these studies clearly and without undue repetition, the section of Results will be presented concurrently with the Discussion. The Appendix contains much of the data from which the ratio values were obtained as well as the statistical treatment that was used.

1. Experimental Design

The aim of the experiments presented in this thesis is to determine, to some degree of finality, whether or not there is more than one pathway for the metabolism of ethyl alcohol in the intact mammalian system. The conventional approach to this type of problem would be to administer ethanol or acetaldehyde to an animal and to follow the time course of blood and urine levels. This early type of approach yielded a great deal of information, but much of it is still controversial. Certainly, from this early work, definitive information as to the quantitative or even the qualitative metabolic fate of ethanol was not obtained.

With the application of tracer techniques to problems in intermediary metabolism, the amount of qualitative information, in regard to the fate of ethanol, increased enormously. Tracer techniques usually give definite answers to certain questions. For example, it is quite easy to show that some part of a specific precursor is incorporated or fixed into a particular product. This kind of experiment affords gross answers to precursor-product relationships. However, this type of experiment lacks profundity, for it does not answer the following questions:

(a) Did the precursor molecule (with its "tagged" atom) go as an intact unit into the product?

(b) Did the precursor molecule go to the product by a direct route ?

(c) Did some of the precursor become diverted to a side-product off of the direct route?

(d) Did the precursor go via several alternate pathways to a common intermediate precursor?

(e) Does the pathway of product labeling represent the only pathway for product formation?

(f) Are there intermediates involved in the formation of the product that may arise from parallel pathways of metabolism?

(g) Does the amount of tracer appearing in the product have quantitative significance in terms of the rates of reactions being studied?

(h) What are the rates of turnover of the metabolic pools involved?

(i) Do the membranes of the biological system discriminate between precursors being studied?

These questions are impossible, or at best difficult, to answer by experimental approaches in which ethanol, labeled or not, is used and its subsequent metabolism studied. In addition, in the interpretation of "tracer" experiments, these questions are often overlooked; but these same questions come very much to the front when attempts are made to use C^{14}-labeled ethanol to assess the metabolic fate of that molecule. Point (i), of course, is only applicable in comparative

studies between two different labeled compounds, e.g., acetate and ethanol.

Since ethanol is supposed to be converted to acetate or acetyl-CoA, it would appear that a logical approach would be to compare the utilization of acetate-1-C^{14} with that of ethanol-1-C^{14}. However, the series of questions cited above raise doubt that such an approach could yield definitive information.

Schulman et al. (92), followed this approach and compared the metabolic fates of tracer acetate and tracer ethanol. They chose to evaluate the labeling response at two different time intervals in an attempt to resolve a portion of the problem concerned with permeability and activation. But in so doing, these workers obviously created new problems in terms of the "turnover" of tissue constituents. In all fractions and tissues studied, ethanol label was incorporated to a greater extent than was the acetate. Schulman concluded "some divergence in metabolic pathways for at least a part of the alcohol". Westerfeld and his collaborators have continued to study ethanol metabolism (91, 122, 97, 90), but as yet have not established an alternate pathway for ethanol.

Brady and Gurin (15) while pioneering much of the early work on cholesterologenesis, cited evidence that acetaldehyde label was found in cholesterol, biosynthesized by liver slices, to a greater extent than was acetate label. These authors were aware that factors of permeability, activation and toxicity could easily influence the comparisons of label incorporation. They concluded by saying, "The considerations, of course, apply equally well to comparable experiments carried out with the intact animal, in which the mode of administration, the

rate of diffusion, permeability, toxicity, and complicating action of numerous organs and tissues make quantitative comparisons of different precursors very difficult".

None of the current studies have suggested that ethanol is not metabolized by the sequence, ethanol to acetaldehyde to acetate (acetyl-CoA), nor have the isotopic studies shown that a significant part of the ethanol is metabolized by pathways other than this sequence. However, none of the reported studies have been designed to establish unequivocally the presence of another pathway of ethanol metabolism and/or to determine the quantitative significance of this pathway.

In the present studies, the following considerations led to the adoption of "labeling ratios" as an approach. If acetate is known to go intact into a biosynthesized metabolite, then acetate-1-C^{14} should label this metabolite in an identical manner as does acetate-2-C^{14}. In this situation the incorporation of acetate-2-C^{14}/acetate-1-C^{14} (A_2/A_1) should equal 1. If acetate is a prime product of ethanol metabolism, then the carbon-1 of the acetate formed should follow the carbon-2 of acetate in a quantitative manner. Now if the two carbons retain their positional identities, and if the carbon-1 position of ethanol is converted to the carbon-1 of acetate, then in those reactions where A_2/A_1 equals 1.0, E_2/E_1 should equal 1.0. In this ratio evaluation, it is not important to have $E_2=A_2$ or $E_1=A_1$; it is only necessary for E_2/E_1 to equal A_2/A_1. This comparison then allows for the formation of known and unknown intermediates between ethanol and acetate, it allows membrane permeability to acetate and ethanol to differ, and permits

the flow of label through variable sized "pools" of metabolites.

In contrast to the reactions in which $A_2/A_1=1.0$ as would be the case in fatty acid synthesis, there are metabolic pathways in which the carbons of the precursor are distinguished from each other. In one of these, cholesterol biosynthesis, the carbon-1 of acetate is in part lost from the final product yielding a ratio of A_2/A_1 of 1.25.

2. Biosyntheses Involving Acetyl-CoA

The approach to the problem of evaluating the similarities and differences between acetate and ethanol metabolism was first to determine the labeling patterns from C^{14}-acetate and C^{14}-ethanol in common metabolic products. For this purpose, lipid components were chosen, since lipid techniques are common to this laboratory. The cholesterol and fatty acids that were chosen to be examined should reflect the labeling of the acetyl-CoA "pool", since acetyl-CoA is known to be the metabolic precursor of both of these materials.

Before the data are presented and discussed, one point should be re-emphasized. There is good evidence that a proportion of ethanol that is metabolized proceeds by way of the pathway-ethanol to acetaldehyde to acetate. The purpose of these studies is to determine whether or not this sequence is the only sequence by which alcohol metabolism occurs.

a. Cholesterol

The first metabolic criterion that is to be examined is cholesterol. The complete "raw" data and standard deviations can be found in Section II of the Appendix. The ratios of the incorporations of C^{14}-acetate and C^{14}-ethanol into digitonin-precipitable sterols are presented in Table 3.

Table 3

The ratios obtained from the Digitonin-Precipitable Sterols.

Tissue	A_2/A_1	E_2/E_1	p-value
Liver	$\frac{0.29}{0.18} = 1.61$	$\frac{0.68}{0.45} = 1.51$	>.05
Gut	$\frac{0.28}{0.21} = 1.33$	$\frac{0.65}{0.37} = 1.76$	>.05
Carcass	$\frac{0.25}{0.25} = 1.00$	$\frac{0.42}{0.33} = 1.27$	>.05
Skin	$\frac{0.14}{0.08} = 1.75$	$\frac{0.17}{0.13} = 1.31$	>.05
Mean Ratio	1.42	1.46	

Statistical evaluations (t-test) of the ratios show that no difference at the 95% confidence level is demonstrated for liver, gut, carcass or skin tissue fractions (See Section I of the Appendix for method of evaluating ratios).

The fact that the labeling ratios between acetate and ethanol do not differ implies that the acetyl-CoA "pool" for cholesterol biosynthesis is labeled similarly from C^{14}-acetate and C^{14}-ethanol. It can be concluded from this observation that if an alternate pathway for ethanol does exist, the intermediates of the pathway have not entered into cholesterol biosynthesis, or if they have, the positional identities of the carbinol and methyl carbons are maintained.

It can also be seen that the incorporation of label into liver and gut cholesterol is consistently higher from ethanol than from acetate. This is demonstrated in Table 4 in which the incorporation of label from ethanol-1-C^{14} into liver cholesterol differs significantly from the incorporation from acetate-1-C^{14} at the 0.01 level. Acetate-

2-C^{14} and ethanol-2-C^{14} incorporation into liver cholesterol differs significantly at the 0.02 level. Statistical differences between acetate and ethanol incorporation are found in gut tissue as well. Though cholesterol fractions from carcass and skin tissues consistently demonstrate ethanol incorporations that exceed acetate incorporations, no statistical differences are found.

Table 4

Comparison of C^{14}-Acetate and C^{14}-Ethanol Incorporations into Digitonin-Precipitable Sterols.

Tissue	A_1	E_1	A_2	E_2	p-value
Liver	0.18	0.45			$< .01$
Liver			0.29	0.68	$< .02$
Gut	0.21	0.37			$< .01$
Gut			0.28	0.65	$< .05$
Carcass	0.25	0.42			$> .05$
Carcass			0.25	0.33	$> .05$
Skin	0.08	0.13			$> .05$
Skin			0.14	0.17	$> .05$

The observation that the incorporation of label into cholesterol occurs to a greater extent from ethanol than from acetate is not a new one. Westerfeld was one of the first investigators to make this observation (92). The meaning of it is not clear, but a recent report by Westerfeld's group has presented experimental support for one possibility (97). These workers found that the conversion of C^{14}-ethanol to C^{14}-acetyl-CoA occurred without dilution by endogenous substrates,

whereas C^{14}-acetate was diluted endogenously. A smaller pool size for ethanol would result in less dilution of the labeled ethanol. Consequently, there would be a greater probability that a labeled ethanol would enter into a reaction from the ethanol "pool" than a respectively labeled acetate would from the acetate "pool". If this is the true explanation for the phenomena, then one further conclusion can be formulated. From these considerations it should be possible to minimize the formation of free acetate as an intermediate in ethanol metabolism of liver and gut tissues. If acetaldehyde is converted exclusively to free acetate, no preferential labeling on the part of ethanol should be apparent. It seems very probable, then, that some acetaldehyde is converted directly to acetyl-CoA without first becoming mixed with the free acetate "pool".

The ratios of the C-2/C-1 positions of the acetate and ethanol approximate the theoretical value of 1.25, calculated from considerations of the cholesterol biosynthetic pathway. This comparison is presented in Table 5.

Table 5

Comparison of Ethanol and Acetate Ratios with the Theoretical Ratio for Cholesterol Biosynthesis.

Tracer	Ratio Theoretical	Observed*	p-Value
Acetate	1.25	1.43	= 0.10
Ethanol	1.25	1.37	>0.10

*The observed values represent the means of the summation of ratios for all four tissues.

The similarity in labeling of the acetyl-CoA pool for cholesterol biosynthesis from acetate and ethanol is demonstrated by the closeness

with which the acetate and ethanol ratios approximate the theoretical value of 1.25. The value of 1.25 is arrived at in the following way:

Cholesterol is formed by the condensation of

18 units of acetate, i.e.,

18 acetate (36C) ⟶ 6 mevalonic acid (36C)

6 mevalonic acid (36C) ⟶ 6 isopentenyl-PP (30C) + $6CO_2$

The 6 CO_2 formed in this decarboxylation arise from the C_1 position, the carboxyl of acetate.

6 isopentenyl-PP (30C) ⟶ 1 squalene (C_{30})

1 squalene (C_{30}) ⟶ 1 cholesterol (C_{27}) + $3CO_2$

The 3 CO_2 formed here arise from carbons that were originally the methyl carbon, (C_2) of acetate.

18 acetate ⟶ 1 cholesterol + $6C_1$ as CO_2 + $3C_2$ as CO_2

so that of the 27 carbons in cholesterol, 12 originated from C_1 and 15 from the C_2 positions of acetate. This gives a ratio of C_2/C_1 =

$$15/12 = 1.25$$

The comparison of the acetate and ethanol ratios with the theoretical value of 1.25 was done using the summated values from the entire animal. It can be concluded, then, that the labeling of cholesterol from ethanol-1-C^{14} and ethanol-2-C^{14} for the entire animal does not differ from the labeling pattern expected if ethanol were converted quantitatively to acetyl-CoA before participating in cholesterologenesis. The acetyl-CoA for cholesterol synthesis appears to be labeled in qualitatively the same fashion from ethanol-C^{14} as from acetate-C^{14}.

b. Fatty Acids

The saponifiable fraction was isolated from liver, gut, carcass and skin tissues and used to determine the pattern of labeling from acetate-C^{14} and from ethanol-C^{14}. The complete "raw" data and the standard deviations can be found in Section III of the Appendix. In Table 6 the ratios of the incorporations of acetate and ethanol into the saponifiable fraction are presented.

Table 6

Ratios of the Incorporation of C^{14}-Acetate and C^{14}-Ethanol into the Saponifiable Fraction

Tissue	A_2/A_1	E_2/E_1	p-Value
Liver	$\frac{1.00}{0.73} = 1.37$	$\frac{1.59}{1.51} = 1.05$	$> .05$
Gut	$\frac{5.39}{6.13} = 0.89$	$\frac{2.34}{1.92} = 1.22$	$< .02$
Carcass	$\frac{5.76}{5.50} = 1.05$	$\frac{4.03}{3.27} = 1.23$	$> .05$
Skin	$\frac{1.31}{0.93} = 1.41$	$\frac{1.61}{1.70} = 0.95$	$> .05$
Mean Ratio	1.18	1.11	

No statistical difference at the 95% confidence level is found for the ratios of acetate and ethanol in the saponifiable fraction of liver, carcass and skin tissues. Gut tissue, however, shows a significant difference at the 98% confidence level.

Table 7

Comparison of C^{14}-Acetate and C^{14}-Ethanol into Fatty Acids

Tissue	A_1	E_1	A_2	E_2	p-Value
Liver	0.73	1.51			< .01
Liver			1.00	1.59	> .05
Gut	6.13	1.29			< .01
Gut			5.39	2.34	< .01
Carcass	5.50	3.27			< .01
Carcass			5.76	4.03	> .05
Skin	0.93	1.70			< .01
Skin			1.31	1.61	> .05

A greater incorporation of label from ethanol-C^{14} than from acetate-C^{14} could be the resultant of "pool" sizes that are not the same. The greater incorporation of label from ethanol would indicate that the endogenous ethanol pool is smaller than the endogenous free acetate "pool" in liver and skin tissues.

The observation that acetate-C^{14} is incorporated to a greater extent than ethanol-C^{14} in carcass tissue could be caused by two factors. Acetate and ethanol "pool" sizes could be different with the ethanol "pool" being the larger of the two or else, some tissue(s) of the carcass fraction could be incapable of metabolizing ethanol as readily as acetate. The tissues and organs contained in the carcass fraction includes the following: adrenals, brain, spleen, kidneys, testicular tissue, adipose tissue and lungs, i.e., everything that is left after the liver, gastrointenstinal tract and skin have been removed from the rat.

The possibility that ethanol is not as easily metabolized by some tissue components of the carcass fraction seems a likely explanation. If certain tissues of the carcass fraction are unable to metabolize ethanol, either due to the fact that alcohol dehydrogenase is not present and/or because ethanol is not permeable to those tissues, low label incorporations from ethanol-C^{14} would be expected. This is particularly true since tracer levels of acetate and ethanol were used in these studies. At those low concentrations of acetate and ethanol, the animal tissues were not flooded with the labeled compounds, and complete metabolism of the compounds should have taken place if the tissues were capable of metabolizing them. However, there are certain tissues that are unable to metabolize ethanol. Adipose tissue, for instance, is one tissue that is unable to metabolize ethanol since no $C^{14}O_2$ could be found following incubation of adipose tissue with ethanol-1, 2-C^{14}. With either acetate-1, 2-C^{14} or glucose-U-C^{14} as the substrate, $C^{14}O_2$ was detected as a product of the tissue metabolism (60).

The meaning of the significant difference that is noted between the acetate and the ethanol ratios in gut tissue is not clear. Some insight into this problem might be obtained if the label incorporations from acetate and ethanol are compared. Acetate appears to label gut fatty acids 2 to 5 times better than does ethanol. There are two conditions that could give these results. If the gut ethanol "pool" were larger than the acetate "pool", a greater dilution of

the labeled ethanol would take place with unlabeled ethanol, and less label would be incorporated into the fatty acid fraction in a finite period of time if it is assumed that similar reaction rates govern acetate and ethanol metabolism. The fact that cholesterol is not labeled better from acetate than from ethanol in gut tissue would mean that either separate acetate and ethanol "pools" exist for cholesterol and fatty acid synthesis, or that ethanol is not readily metabolized by gut tissue. These considerations do not explain the differences in A_2/A_1 and E_2/E_1 ratios, but they stress the point that wide differences could exist in individual tissues with respect to acetate and ethanol utilization.

Since ethanol-C^{14} labels liver, carcass and skin in a way which is indistinguishable from acetate-C^{14}, support is given to the concept that the acetyl-CoA "pool" for fatty acid biosynthesis is labeled by ethanol-C^{14} in a manner similar to that done by acetate-C^{14}. The similarity of the ratios of acetate-C^{14} (A_2/A_1) and ethanol-C^{14} (E_2/E_1) rules out the possibility of label discrimination by means of a pathway for ethanol that is not available to acetate. A pathway such as the formation of acetoacetate from acetaldehyde cannot be verified or rejected on the basis of these data. Therefore, it is possible to say only that the labeling of fatty acids of liver, carcass and skin from acetate-C^{14} and ethanol-C^{14} is indistinguishable as judged from this work.

The ratios for ethanol and acetate are near the theoretical value of 1.00 calculated for fatty acids. The statistical evaluation of the comparison of the observed ratios with the theoretical ratios is presented in Table 8.

Table 8

Comparison of Ethanol and Acetate Ratios with the Theoretical Ratio for C^{14}-Fatty Acids

Tracer	Theoretical	Observed*	p-Values
Acetate	1.00	1.18	$> .05$
Ethanol	1.00	1.11	$> .10$

*The observed values represent the means of the summation of ratios for all four tissues.

These results indicate that the acetyl-CoA pool for the biosynthesis of fatty acids is similarly labeled by acetate and ethanol.

The theoretical value (1.00) for fatty acids results from the fact that in the sequence of biosynthetic reactions, there are as many C_2 carbons from acetate incorporated into the acids as there are carbons from the C_1 position of acetate, i.e., acetate is incorporated as a unit into the fatty acid product.

Since the observed ratios approximate the theoretical ratios, it can be concluded that the labeling of fatty acids from ethanol-1-C^{14} and ethanol-2-C^{14}, considering the entire animal, does not differ from the pattern expected if ethanol were converted directly to acetyl-CoA before participating in this synthesis scheme. The acetyl-CoA "pool" for fatty acid synthesis appears to be labeled, qualitatively, the same from ethanol-C^{14} as from acetate-C^{14}.

Certain specific fatty acids of the sponifiable fraction were isolated in order to determine more precisely their specific activities and ratios. For this purpose, reverse phase column chromatography was employed. Fatty acid fractions were eluted from a Super Cel and paraffin oil column with increasing proportions of acetone in an aqueous acetone mixture (See Methods and Materials for complete details).

Table 9 presents the ratios of the specific activities of a particular fraction (Peak D of Figure 1 - Methods and Materials) from liver tissue that has been shown by gas-liquid chromatography, to be approximately 80% stearic acid and 20% palmitic acid.

Table 9

Ratios of Specific Activities of Fraction from Super Cel Column

cpm/mg

$$\frac{A_2}{A_1} \quad \frac{158.7}{160.3} = 0.99$$

$$\frac{E_2}{E_1} \quad \frac{168.7}{151.1} = 1.12$$

The ratios of the specific activities are similar for acetate and ethanol injected animals. In both cases, the ratios approximate the theoretical ratio of 1.00 very closely. This finding confirms the data obtained from the less refined saponifiable fraction and reaffirms the conclusion that both ethanol and acetate label the acetyl-CoA "pool" in qualitatively the same way. That is to say, though the acetyl-CoA "pool" might have a higher specific activity after ethanol than after acetate, the relative proportion of the label ending up in the 2-position of acetyl-CoA from acetate or ethanol-2-C^{14} is the same as that ending up in the 1-position of acetyl-CoA from acetate-or

ethanol-1-C^{14}.

c. Hydroxamic Acid Study

In an attempt to gain a more precise understanding of the labeling of the acetyl-CoA "pool" in liver tissue by acetate-C^{14} and ethanol-C^{14}, this hydroxamate study was undertaken.

The formation of the acyl hydroxamic acid derivatives is effected by the reaction of thioacyls with hydroxylamine.

$$\underset{\text{acyl-CoA}}{R\text{-}\overset{O}{\overset{\|}{C}}SCoA} + \underset{\text{hydroxylamine}}{H_2NOH} = \underset{\text{acyl hydroxamate}}{R\text{-}\overset{O}{\overset{\|}{C}}NHOH} + \underset{\text{coenzyme A}}{CoASH}$$

Free coenzyme A is liberated as a product of the reaction. The hydroxamates can be extracted with butanol and partitioned against water to separate the short and long chained compounds (See Methods and Materials). Due to the ease with which the hydroxamates can be identified and their concentrations estimated (hydroxamates react with ferric chloride to form a magenta-colored complex), it was anticipated that a value for the specific activity could be determined. This proved to be impossible, however, since the hydroxamates were present in amounts too small to estimate colorimetrically with any precision.

It is possible, though, to compare radioactive labeling of the reactive acyls by observing the pattern by which the reactive acyls are labeled from acetate-C^{14} and ethanol-C^{14}. The relative pattern of radioactivity at some particular time following tracer administration could indicate whether or not reactive acyl labeling was grossly similar or dissimilar for acetate and ethanol.

The point in time which was chosen to examine the labeling pattern of the reactive acyls was 15 minutes following the injection. By

observing the pattern of labeling at this short time period, it was thought that if some alternate pathway for ethanol did exist, a different labeling pattern might be present at this short time before isotopic equilibrium took place.

Acetate-2-C^{14} and ethanol-2-C^{14} were tracers compared in this study for two reasons. First, the incorporation of label from acetate and ethanol in the expired carbon dioxide demonstrated a larger difference when the label was in the C-2 positions of acetate and ethanol than when in the C-1 positions (See Results and Discussion 3c). Secondly, preliminary studies with hydroxamate labeling indicated that a difference in the relative labeling of the reactive acyls occurred only after acetate and ethanol-2-C^{14} were administered. Figure 4 presents the chromatographic tracings representing areas of radioactivity obtained from the water soluble hydroxamic acids following the labeling of the reactive acyl pools with acetate-2-C^{14} and ethanol-2-C^{14}.

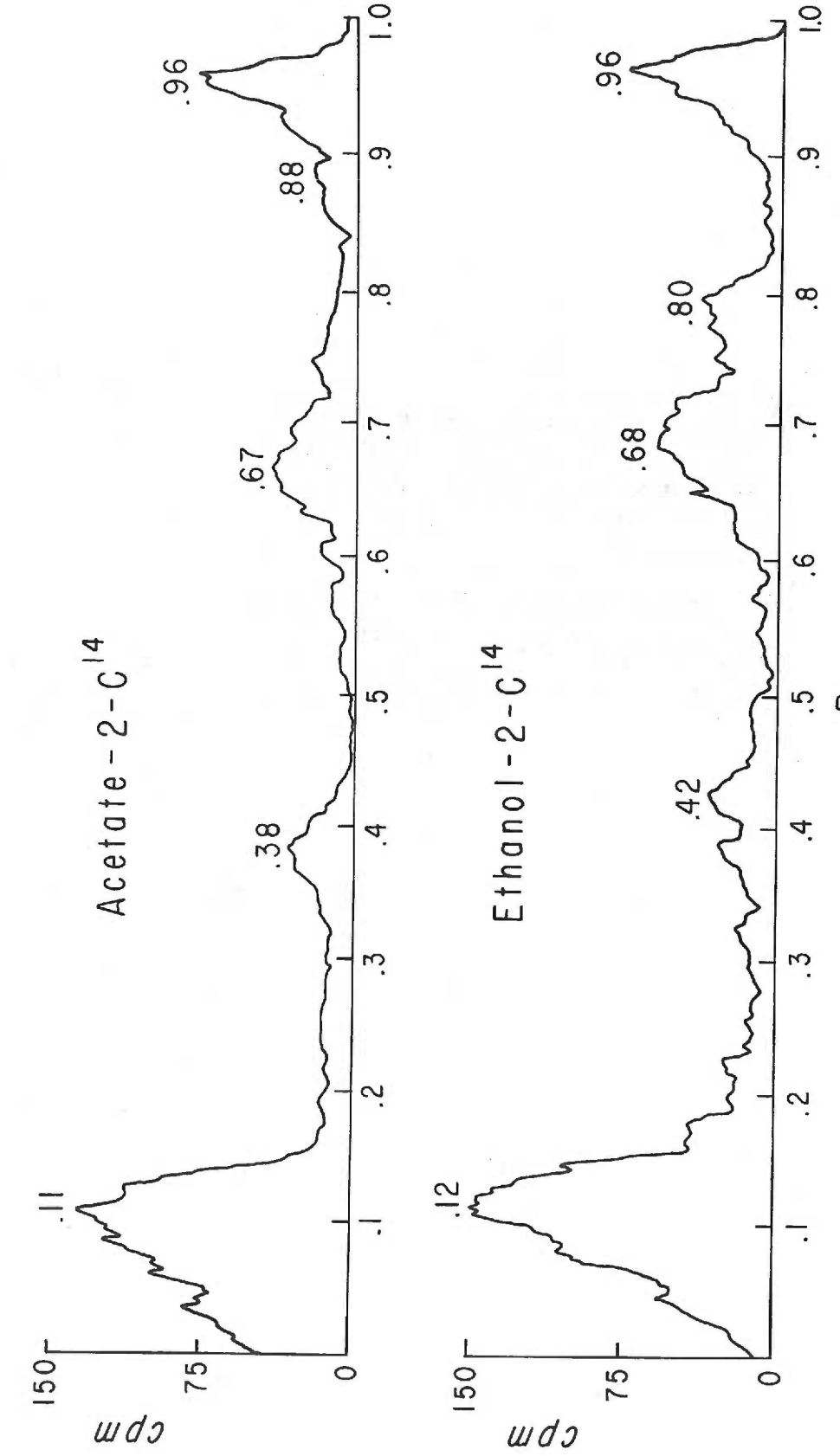
The prominent peaks indicating areas of radioactivity are seen at R_f values of 0.11-0.12, 0.38-0.42, 0.67-0.68, and 0.96. In addition to these peaks, two others are noted, a small peak appearing at R_f 0.88 on the acetate-2-C^{14} chromatogram and the other one corresponding to R_f 0.80 on the ethanol-2-C^{14} chromatogram.

The gross similarity of the two chromatograms is evident, particularly with regard to the radioactivity appearing at R_f values of less than 0.70. The significance of the peaks at 0.88 on the acetate chromatogram and 0.80 on the ethanol chromatogram is not known, since in some cases, radioactivity appearing at R_f values greater than 0.70 might be attributed to contamination of the water soluble (short chain)

Figure 4

Chromatographic tracing representing areas of radioactivity obtained from water soluble hydroxamic acids following injections of acetate-2-C^{14} and ethanol-2-C^{14}

The animals were sacrificed 15 minutes after the injections. The numbers above the tracings represent the R_f values for the particular peak.


Acetate-2-C14
Ethanol-2-C14
cpm
150
75
0
.11
.38
.67
.88
.96
.12
.42
.68
.80
.96
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Rf

hydroxamates with small amounts of the water insoluble compounds.

It is concluded, then, that the labeling pattern of the reactive acyls from acetate-and ethanol-2-C^{14} does not differ. This conclusion is consistent also with the data reported for fatty acid and cholesterol labeling from acetate-and ethanol-C^{14}.

d. Acetylation of Sulfanilamide

It is well known that certain amines are detoxified in the mammal by acetylation. These acetylated amines are excreted into the urine. The acetyl group for this acetylation process originates from acetyl-CoA, and, as a result, compounds like sulfanilamide can be employed as "trapping agents" for the active acetyl group. The product of this enzymatic acetylation is shown below.

N^4 - Acetylsulfanilamide

As much as 60% of ingested sulfanilamide is acetylated by the liver and a smaller amount by some extra-hepatic tissues (73).

Sulfanilamide was fed to four rats for three days to establish a steady state level of sulfanilamide in each rat. On the fourth day acetate-C^{14} and ethanol-C^{14} were injected and the acetyl sulfanilamide excreted in the urine during the subsequent 24 hours was collected. The specific activity of the acetyl sulfanilamide was determined and the results are shown in Table 10.

Table 10

Acetylation of Sulfanilamide from C^{14}-Acetate and C^{14}-Ethanol

	Acetylsulfanilamide (mg)	Activity (cpm)	Specific Activity (cpm/mg)
A_2	12.4	97.3	7.8
A_1	24.6	202.7	8.2
E_2	12.7	97.1	7.6
E_1	17.2	128.5	7.5

$$\frac{A_2}{A_1} = 0.95 \qquad \frac{E_2}{E_1} = 1.01$$

The specific activity ratios of 0.95 and 1.01 show that no apparent differences exist in the acetylating acetyl-CoA pool labeling following equal activity doses of acetate and ethanol. The results from this experiment give no indication for an alternate pathway for alcohol metabolism.

3. Appearance of Respiratory Carbon Dioxide

Since carbon dioxide is a major, terminal metabolite of oxidative processes, differences in pathways between ethanol and acetate could result in different incorporations of label into the expired carbon dioxide in a two-hour experimental period.

The C^{14}-carbon dioxide time course curves for the two-hour period following C^{14}-acetate and C^{14}-ethanol are shown in Figures 5 and 6.

It can be seen from these curves that the appearance of radioactive carbon dioxide follows a similar time course when acetate-1-C^{14} and ethanol-1-C^{14} are the radioactive tracers injected. The $C^{14}O_2$ from ethanol-1-C^{14} appears in a slightly shorter time than does the

Figure 5

C^{14}-carbon dioxide time course curves
for the two-hour period following
C^{14}-acetate and C^{14}-ethanol

The $C^{14}O_2$ activity is expressed
as counts/minute

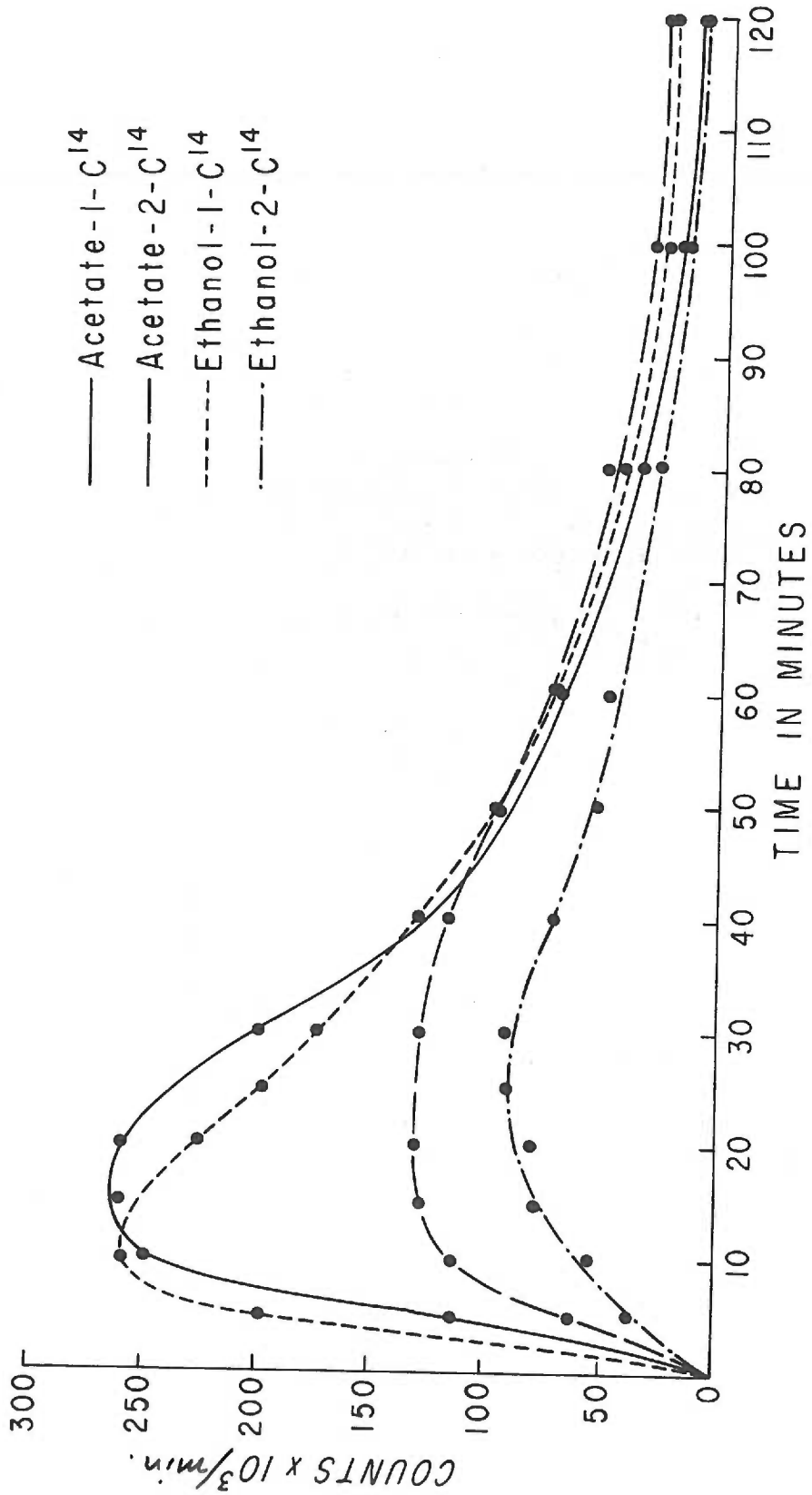
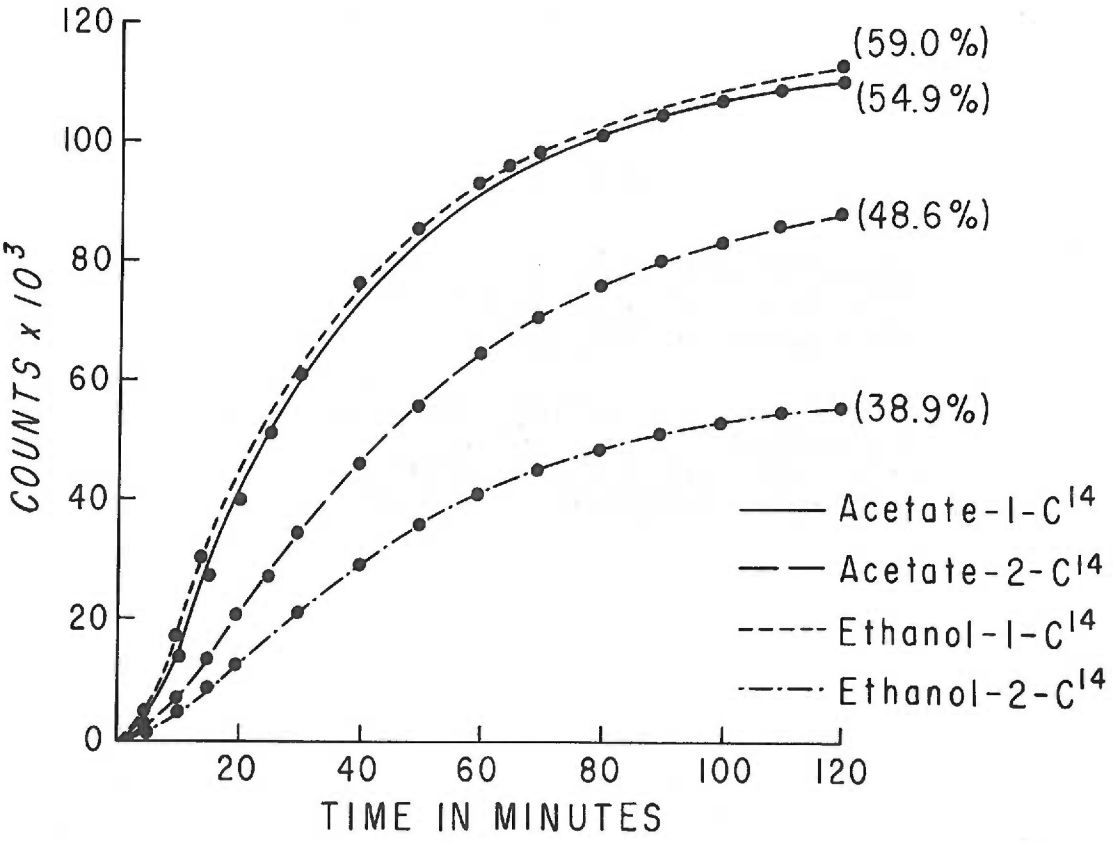

Acetate-1-C14
Acetate-2-C14
Ethanol-1-C14
Ethanol-2-C14
COUNTS x 10³/min.
300
250
200
150
100
50
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME IN MINUTES

Figure 6

Cumulative C^{14}-carbon dioxide curves for the two-hour period following C^{14}-acetate and C^{14}-ethanol

The numbers in parentheses represent the percentage of the dose of C^{14}-label incorporated into the respiratory carbon dioxide in two hours.


120
100
80
60
40
20
0
COUNTS x 10³
20
40
60
80
100
120
TIME IN MINUTES
(59.0 %)
(54.9 %)
(48.6 %)
(38.9 %)
Acetate-1-C¹⁴
Acetate-2-C¹⁴
Ethanol-1-C¹⁴
Ethanol-2-C¹⁴

$C^{14}O_2$ from acetate-1-C^{14} and this observation can be attributed to differences in absorption and/or to differences in endogenous pool sizes for acetate and ethanol.

The time course curves for $C^{14}O_2$ appearance following acetate-2-C^{14} and ethanol-2-C^{14} differ considerably from the time course of $C^{14}O_2$ following acetate-1-C^{14} and ethanol-1-C^{14}. It is apparent that the C-1 label of both acetate and of ethanol appears sooner in expired CO_2 than does the methyl label from these compounds.

Prior to the appearance of C^{14} activity in the respiratory CO_2, the administered compound must be absorbed from the peritoneal cavity, be transported to the organ(s) in which it is to be metabolized, cross the cellular membrane barrier and be converted to a form able to enter the oxidative pathways. The CO_2 formed in the subsequent decarboxylation reactions then enters the bicarbonate pool of the body, the greater part of which is cleared from the blood via the respiratory system.

This overall process is an extremely rapid one, as is verified by the fact that C^{14} activity appears in respiratory CO_2 within one minute after intraperitoneal injection of each of the tracer compounds.

The slower appearance of the methyl carbons of acetate-2-C^{14} and ethanol-2-C^{14} as $C^{14}O_2$ is in keeping with the fact that the C-2 carbon of the acetyl-CoA entering the tricarboxylic acid cycle is "buried" in the intermediates of the TCA cycle and is not as readily available to participate in the decarboxylation reactions. A more complete discussion of these considerations will be presented later.

As shown by the statistical evaluations of Section V of the Appendix, no differences exist between the four groups of animals in terms of the total amounts of CO_2 produced in two hours. The meaning of the label incorporation figures and the significance of the labeling ratios may thus be evaluated. If ratios are established by relating the $C^{14}O_2$ incorporations in two hours from acetate-2-C^{14} and acetate-1-C^{14} and, likewise, the $C^{14}O_2$ incorporation from ethanol-2-C^{14} and ethanol-1-C^{14} in two hours, a comparison can be made of the gross production of CO_2 from decarboxylation reactions originating from the C-1 and C-2 positions of acetate and ethanol respectively. Table 11 presents the incorporations of C^{14} into CO_2 from labeled acetate and ethanol as related by ratios.

Table 11

Ratios of the Incorporation of C^{14}-Acetate and C^{14}-Ethanol into $C^{14}O_2$

$$\frac{\text{Acetate-2-C}^{14}}{\text{Acetate-1-C}^{14}} = \frac{51.1}{63.8} = 0.80$$

$$\frac{\text{Ethanol-2-C}^{14}}{\text{Ethanol-1-C}^{14}} = \frac{35.2}{58.5} = 0.60 \qquad p < .01$$

In considering the acetate ratio, a value of less than 1.0 is expected, since the distribution of label in the intermediates of the TCA cycle would favor the retention of the 2-position of acetate. The comparative metabolism of the two carbons of acetate is described by the ratio of 0.80 and is consistent with this consideration.

The experimentally found ratio of 0.60 for ethanol differs significantly from the 0.80 ratio established by the acetate tracers. The incorporations of label into $C^{14}O_2$ from acetate-1-C^{14} and ethanol-1-C^{14} are 63.8 and 58.5% respectively. These values do not

differ statistically from each other. However, the incorporation of only 35.2% of the C^{14} from ethanol-2-C^{14} into $C^{14}O_2$ in two hours is statistically different from the 51.1% of the C^{14} from acetate-2-C^{14} incorporated into $C^{14}O_2$ in the same period of time. The lower ratio for ethanol appears to be due to the retention of the 2-position of ethanol in the animal, and not to an enhanced release of the 1-position as CO_2. It seems that the carbon atoms of acetate and ethanol do not appear in expired carbon dioxide to the same extent in the 2-hour experimental period. This pattern of CO_2 labeling, as seen by the two ratios, might strongly suggest a metabolic conversion of ethanol carbon to CO_2 by a pathway other than by oxidation of acetyl-CoA.

To summarize the data thus far, there appears to be evidence from the carbon dioxide data that a pathway for the metabolism of ethanol exists that is different from the conventional pathway through acetaldehyde to acetate. However, the data from studies on the incorporation of label from ethanol and acetate into fatty acids, cholesterol and acetyl sulfanilamide do not substantiate the claim for an alternate pathway of ethanol metabolism. One other point is important. The lower ratio for the ethanol incorporations into CO_2 (.60 compared to 0.80 ratio from acetate) is due to the retention of the 2-position of ethanol and not to an enhanced release of the 1-position. As a consequence, it would seem that an alternate pathway for the metabolism of ethanol would involve the cleavage of the ethanol molecule. Also, it would seem that the cleavage should occur in such a way that the 1-carbon of ethanol would appear as CO_2 and the 2-carbon of ethanol

would remain in the animal body, presumably as a participant in some "1-carbon" metabolic pathway.

4. Urinary Formate Studies

A metabolic scheme that might explain observations such as these, would be some pathway unique to ethanol that would have glyoxylic acid as an intermediate. Glyoxylic acid can undergo the following decarboxylation reaction (118):

$$\underset{\text{glyoxylic acid}}{OHC\text{-}COOH} \longrightarrow \underset{\text{formic acid}}{HCOOH} + \underset{\text{carbon dioxide}}{CO_2}$$

The carboxyl carbon of glyoxylic acid becomes CO_2 by this reaction while the oxo-carbon could remain in the body as a participant in "one-carbon" metabolism.

Since the metabolism of glyoxylate provides for the loss of the 1-carbon of ethanol as CO_2 and the incorporation of the methyl carbon as a "one carbon" entity, viz., formate, an experiment was devised to test for the preferential formation of formic acid (perhaps by way of glyoxylic acid) as an intermediate in ethanol metabolism.

Weinhouse and Friedmann (115) developed a method (in which a simple "trapping" procedure for the formate was employed) to test to what extent certain compounds were metabolized via formate in the intact rat. In brief, the method consists of administering a C^{14}-labeled compound to rats by intraperitoneal injection, together with unlabeled sodium formate, and assaying the radioactivity of the formate excreted in the urine during the subsequent six hours.

Table 12 represents the urinary radioactivity found following injections of C^{14}-acetate and C^{14}-ethanol using the formate flooding and trapping technique.

Table 12

Urinary Radioactivity

Tracer	No. Exp.	Dose (μc)	Label Inc. into Urine (% of dose)
Acetate-1-C^{14}	2	17	3.5
Acetate-2-C^{14}	2	18	3.2
Ethanol-1-C^{14}	2	18	3.4
Ethanol-2-C^{14}	2	18	3.2

$$\frac{A_2}{A_1} = 0.91 \qquad \frac{E_2}{E_1} = 0.93$$

The striking similarity in ratios following acetate and ethanol injections suggests that no significant difference exists in total products excreted into the urine under the conditions prescribed for these experiments.

In order to assess the amount of labeled formate in the urinary radioactivity, acetate and formate fractions were obtained by separation on an ion exchange column (See Material and Methods).

Table 13 presents the determination of radioactivity in acetate and formate from the urines of rats injected with acetate-C^{14} and ethanol-C^{14}.

Table 13

Urinary Excretion of Labeled Acetate and Formate

Tracer	Dose (μc)	% Dose Excreted as: Acetate	Formate
Acetate-1	17	0.3	< 0.01
Acetate-2	18	0.2	< 0.01
Ethanol-1	18	< 0.01	< 0.01
Ethanol-2	28	< 0.01	< 0.01
Serine-3	11	< 0.01	1.9
Formate	16	< 0.01	2.9

From these results it can be concluded that no significant amount of radioactive formate can be detected in the urines of rats following either C^{14}-ethanol or C^{14}-acetate injections. The fact that 1.9% of the radioactivity injected as serine-3-C^{14}, a known C_1 donor, can be detected by this procedure as well as 2.9% of the radioactivity given as C^{14}-formate, suggests that a formate intermediate could be detected in the intact animal by this method if it were formed in significant enough amounts and if the labeled formate was exchangeable with the formate "pool". It appears then, that the cleavage of ethanol to a formate moiety and carbon dioxide during ethanol metabolism, via some compound such as glyoxylic acid, does not take place to an extent sufficient for its detection by this procedure.

5. Hippuric Acid Study

Since glyoxylate is an intermediate in glycine metabolism, the sampling of the free glycine "pool" should give some indication as to the relative labeling of glyoxylate from C^{14}-acetate and C^{14}-ethanol. A convenient means whereby the free glycine "pool" can be sampled is

by the conjugation reaction of glycine with benzoic acid. A rat fed benzoic acid will excrete into the urine the conjugation product of benzoic acid and glycine, namely hippuric acid. This material is easily isolated from urine and purified. The crystallized, pure hippuric acid was weighed and dissolved into an appropriate scintillator solution and assayed by liquid scintillation counting.

Table 14 presents the specific activities of the hippuric acid excreted following injections of C^{14}-acetate and C^{14}-ethanol.

Table 14

Specific Activities of Hippuric Acid Following C^{14}-Acetate and C^{14}-Ethanol

Tracer	Hippuric Acid (mg)	Radioactivity (cpm)	S.A.* (cpm/mg)
Acetate-1	4.6	807.8	175.6
Acetate-2	5.0	1035.0	207.2
Ethanol-1	1.3	181.2	139.4
Ethanol-2	5.0	1278.5	255.7

*corrected to a dose of 20uc.

$$\frac{A_2}{A_1} = 1.2 \qquad \frac{E_2}{E_1} = 1.8$$

The ratios of 1.8 and 1.2 indicate that glycine is labeled from C^{14}-ethanol in a manner similar to the labeling from C^{14}-acetate.

Likewise, if the specific activity of the hippuric acid labeled by ethanol-2-C^{14} is related by ratio, to acid labeled from acetate-2-C^{14} it is seen that the methyl carbon of ethanol does not label the free glycine "pool" in any way dissimilar to the way that the glycine "pool"

is labeled by the methyl carbon from acetate.

$$\frac{E_2}{A_2} = 1.2$$

A similar conclusion can be drawn concerning the C-1 position of both ethanol and acetate.

$$\frac{E_1}{A_1} = 0.8$$

From this experiment it can be concluded that acetate and ethanol are equally good precursors of glycine, and that if some of the glycine is formed via glyoxylic acid, then ethanol does not selectively label the glyoxylate "pool" in the intact rat.

The data from the last two experiments presented do not confirm the fact that a cleavage of the ethanol molecule takes place. By the use of the formate flooding technique no formate was preferentially formed from ethanol. On this basis glyoxylic acid and other two-carbon compounds which are capable of producing formate in the course of their metabolism, can be eliminated as a possibility in ethanol metabolism. Glyoxylic acid, in particular, can be eliminated as a metabolic intermediate more common to ethanol metabolism than to acetate metabolism since the free glycine pool is not labeled to a greater extent by ethanol than by acetate.

Since an enzymatic cleavage of the ethanol molecule does not appear to take place by a route unique to ethanol, some explanation is required to reconcile respiratory carbon dioxide data. It will be recalled that the lower ratio (E_2/E_1) of 0.60 was attributed to the

retention in the animal body of the methyl carbon of ethanol. Since the methyl carbon does not appear to become a "one-carbon" intermediate, viz., formate, then some other explanation must be proposed.

Another pathway which can selectively retain the methyl carbon of a two carbon compound such as acetate, or ethanol that is converted to acetate, is the tricarboxylic acid cycle. The interconversions of the tricarboxylic acid cycle, at early time periods after C^{14}-acetate is given tend to retain the methyl carbon of acetate and allow the carboxyl carbon to appear to greater extent into carbon dioxide. If the two carbons of acetate enter the TCA cycle as acetyl-CoA and if two carbons are evolved as CO_2 with each turn of the cycle, assuming that no carbons are made available via the cycle for biosynthetic reactions, then a series of calculations can be made to estimate the $C^{14}O_2$ produced by the cycle if acetate-1-C^{14} and acetate-2-C^{14} are the initial substrates.

For these calculations it is assumed that 100 cpm enter as C^{14}-acetate per turn of the TCA cycle and that the specific activity of the precursor pool is constant. A constant isotope pattern in the TCA cycle compounds and in the evolved $C^{14}O_2$ is then approached with successive turns of the cycle. Two turns of the cycle are necessary before any label appears as $C^{14}O_2$ from acetate-2-C^{14}. If, for each turn of the cycle, 100 cpm of C^{14}-acetate enters, then 100 cpm will be evolved commensing with the second turn when acetate-1-C^{14} is the labeled precursor. An increasing number of "counts" will be evolved commensing with the third turn of the cycle starting with 50 and approach-

ing 100 asymptotically when acetate-2-C^{14} is the precursor. By the twelfth turn of the cycle, the carbon dioxide ratio (A_2/A_1) for C^{14} from acetate-2-C^{14} and acetate-1-C^{14} approaches 1.00 very closely. These values can be seen in Table 15.

Table 15

Theoretical Carbon Dioxide Ratios for Each Turn of the TCA Cycle with Acetate-1-C^{14} and Acetate-2-C^{14} as the Precursors (117)

No. Turns of Cycle Completed	C^{14}-cpm in CO_2 Released in Turn of Cycle		CO_2 Ratio A_2/A_1 for each turn
	Acetate-1-C^{14}	Acetate-2-C^{14}	
1	0	0.0	-
2	100	0.0	0
3	100	50.0	0.50
4	100	75.0	0.75
5	100	87.5	0.88
6	100	93.8	0.94
7	100	96.9	0.97
8	100	98.4	0.98
9	100	99.2	0.992
10	100	99.6	0.996
11	100	99.8	0.998
12	100	99.9	0.999
Subsequent turns	100	Approaches 100	1.00

From a qualitative standpoint, it is apparent, by following the carbon atoms of the TCA cycle, that the methyl carbon of acetate should not appear as readily as CO_2 as would the carboxyl carbon of acetate. However, the number of turns of the cycle that are required before the $C^{14}O_2$ ratio from acetate-2-C^{14} and acetate-1-C^{14} approaches 1.00 is a function of the synthetic activity and TCA cycle-intermediate "pool" dilution that is occurring.

The validity of the absolute values as expressed in Table 15 can be tested experimentally by measuring the carbon dioxide formation from acetate-1-C^{14} and acetate-2-C^{14} in an *in vitro* system. An advantage of the *in vitro* system is that the specific activity of the precursor "pool" is constant. Under these conditions, with a variety of tissues and under a variety of experimental circumstances, it was noted that the $C^{14}O_2$ ratio was not unity. It was found that more $C^{14}O_2$ was derived from the carboxyl than from the methyl carbon of acetate (53, 117). It would appear, then, that the synthetic activity of the TCA cycle pathways makes it impossible to determine precisely the number of turns that the cycle must undergo in order for all of the C^{14} activity of the cycle intermediates from acetate-1-C^{14} or acetate-2-C^{14} to appear as $C^{14}O_2$.

This concept is consistent with present day knowledge. Even though the tricarboxylic acid cycle is regarded primarily as an oxidative cycle for the production of energy, the biosynthetic functions of the cycle cannot be overlooked. The amino acids, glutamic acid and aspartic acid, are readily synthesized from α-oxoglutarate and oxaloacetate, respectively, by transamination reactions. Citric acid

and isocitric acid have recently been shown to undergo enzymatic cleavages to provide acetyl-CoA units for fatty acid biosynthesis (98). Each of these reactions would tend to dissipate carbons from the cycle and, because of this, the specific activities of the TCA cycle intermediates would decrease as radioactive carbons were "lost" via these synthetic pathways.

The outflux of the carbons from the TCA cycle during synthesis of amino acids and fatty acids is not the only way that the specific activities of the cycle intermediates can be reduced. Dilution of the ∝-oxoglutarate and the oxaloacetate "pools" can arise from the deamination of the unlabeled amino acids, glutamate and aspartate. The interconversion reactions of amino acids and possibly other compounds common to carbohydrate metabolism, with intermediates of the tricarboxylic acid cycle provide means by which decreases in the specific activities of the cycle intermediates can occur.

However, as in Table 15, the carboxyl carbon from acetate-1-C^{14} would still appear as $C^{14}O_2$ in fewer turns of the TCA cycle than would the methyl carbon from acetate-2-C^{14}. The methyl carbon of acetate, being retained in members of the tricarboxylic acid cycle for a longer period of time, would have a greater chance of participating in side reactions from the cycle, and thereby becoming "lost" as far as $C^{14}O_2$ appearance is concerned. The extent to which the A_2/A_1 ratio or the E_2/E_1 ratio deviates from 1.0, according to this argument, is a function of the dynamic reactions taking place between members of the tricarboxylic acid cycle and compounds not in the TCA cycle. If this is indeed the case, then it becomes apparent that to study path-

ways of ethanol metabolism, an acetate ratio must be established for comparison with the ethanol ratio, and the validity of this comparison is dependent upon the assumption that "tracer levels" of acetate and ethanol have negligible influences upon either the "pool" sizes of the TCA cycle intermediates or the equilibrium constants of the cycle intermediates with the compounds not directly in the cycle. With liver homogenate preparations Dajani and Orten (24) found that the substrates, acetate and ethanol, increased "pool" sizes of the TCA cycle intermediates as follows:

Table

Acids	Acetate (4.5 mg) (75.0 μmoles) (μmoles increase/ 10 gm liver)	Ethanol (2 mg) (43.5 μmoles) (μmoles increase/ 10 gm liver)
Citric	1.9	1.2
Aconitic	0.6	0.6
Isocitric	0.5	0.5
α-Oxoglutaric	3.8	1.7
Succinic	4.0	1.8
Fumaric	3.6	1.8
Malic	3.0	1.7
Oxalacetic	1.1	0.6

Since these doeses of acetate and ethanol 4.5 mg and 2 mg, respectively, administered to the liver homogenates are comparable to the doses of acetate (4.2 mg) and ethanol (2.2 mg) given to the intact animals in this study, a reasonable guess can be made that the acetate and ethanol

given under the conditions reported in this thesis effect increases in the cycle intermediates that do not exceed those reported in Table 16, even if the total administered tracer was metabolized by the 10 gram liver of the animal. From this it seems reasonable to say that acetate and ethanol ratios can be compared for $C^{14}O_2$ excretion with fair assurance that the differences observed are probably not caused by Krebs cycle "pool" size differences under the conditions of these experiments.

In this discussion it has been assumed that the specific activity of the precursor, acetyl-CoA, "pool" remains constant. However, it can be shown mathematically that the expressions obtained for the CO_2 ratios from acetate (as in Table 15), but assuming side reactions, are valid even if the specific activity of the inflowing acetate declines exponentially or in any other manner expressed as a function of time (Section E and F of Appendix-117).

In the description of the fate of the intraperitoneally injected acetate or ethanol a number of processes are seen to participate, any one or combination of which could be rate limiting and, therefore, discriminatory for the over all metabolism of the acetate or ethanol. The main processes are the following: (a) the absorption of the tracers from the intraperitoneal cavity, (b) the transport of the tracers to the metabolic tissue(s), (c) the permeability of the tracers to the membrane(s) of the metabolic tissue(s), (d) the mixing of the tracers with their respective, endogenous "pools" and finally, (e) the enzymatic activation of these molecules for subsequent metabolism.

Three observations would tend to eliminate intraperitoneal absorption, transport, metabolic tissue permeability and compound activation as being discriminatory to the extent that significant influences in the final ratios could be effected. These observations are as follows: (1) the similarity in the time of appearance of radioactive carbon dioxide following intraperitoneal injections of labeled acetate and ethanol, (2) the similarity in the shapes of the carbon dioxide time course curves from acetate-1-C^{14} and ethanol-1-C^{14}, and (3) the magnitudes of the activities of the radioactive carbon dioxide along the time course of its appearance.

If acetate or ethanol was absorbed more selectively by the membranes lining the peritoneal cavity allowing a significant discrimination between the two compounds, we might expect to observe marked differences in the time at which radioactive carbon dioxide would appear. It is observed, however, that the appearance of radioactive carbon dioxide from both acetate and ethanol occurs within the first minute following the injection. Assuming that diffusion principles govern the absorption of the acetate and ethanol, rather than some enzymatic process or carrier mechnism, the conclusion can be drawn that absorption of acetate and ethanol is not a discriminatory process due to the similarities in time of appearance of $C^{14}O_2$ following C^{14}-acetate and C^{14}-ethanol injections.

Likewise, by the same reasoning, transport to the metabolic tissue(s) and the subsequent penetration into these cells of acetate and ethanol can be disregarded as processes which discriminate between these two compounds.

Finally the activation of acetate and ethanol can be dismissed as a discriminatory process since the shapes of the time course curves for expiratory $C^{14}O_2$ appearance are similar, in fact nearly superimposable, for acetate-1-C^{14} and ethanol-1-C^{14}. Also the magnitude of activity at any one time is comparable and the initial appearance of $C^{14}O_2$ from the injected acetate and ethanol is rapid.

In this discussion of rate limiting variables, it has been assumed that only one variable was operative. Simultaneous shifting of a series of variables will lead to unpredictable results and undue confusion if presented in detail here.

If these events can be considered to occur in such a way as to provide non-discriminatory handling of acetate and ethanol, then any differences in the labeling ratios from these two precursors can be attributed to a metabolic pathway common to one that is not common to the other, to different abilities of tissues to metabolize ethanol in comparison with acetate, or possibly to both of these alternatives occuring simultaneously.

The abilities of the various tissues of the mammalian body to metabolize acetate and ethanol is known to be different. For example, adipose tissue is unaffected by the addition of ethanol while ethanol stimulates fatty acid synthesis from acetate in liver slices. The inability of adipose tissue to respond, metabolically, to ethanol is apparently due to the inability of adipose tissue to oxidize ethanol (60). Different tissues, then, would seem to possess the ability of metabolizing acetate and ethanol, just acetate and not ethanol, or to metabolize acetate but ethanol only poorly.

Katz (53) in fact, claims that in the intact animal, "administered acetate is oxidized mainly in extra hepatic tissues". Ethanol on the other hand is considered to be metabolized primarily in hepatic tissue (See Introduction). Discrimination, caused by selective tissue metabolism, could be a major problem since it cannot be assumed that all of the various metabolic pathways operate to the same extent in all tissues, nor can it be assumed that "pool" sizes of corresponding pathways in different tissues are the same. The carbon dioxide data would be affected more than the acetyl-CoA "pool" studies in regard to comparative tissue metabolism, since the cholesterol and the fatty acid data describe the incorporation of label into a particular tissue for both ethanol and acetate, whereas the $C^{14}O_2$ collected is the net production from the whole animal, the exact origin of the carbon dioxide not being known.

It seems reasonable to conclude, then, that the significant difference obtained for the C^{14} ratios of the expired CO_2 can be attributed to a selective metabolism of ethanol by the different tissues of the body. It also appears reasonable to assume that the pool sizes of the intermediates in the TCA cycle vary from tissue to tissue as does the synthetic pathway activity of the TCA cycle intermediates. It is entirely possible that the difference in $C^{14}O_2$ ratios between acetate and ethanol indicate a selective metabolism in the tissues of the body for ethanol and, consequently, the tissue distribution for ethanol metabolism differs from that of acetate.

Since it has been shown that the acetyl-CoA "pool" is labeled by ethanol-C^{14} in a manner indistinguishable from acetate-C^{14}, and since the most plausible pathway to explain the discrepancy in labeling

ratios obtained for the expired $C^{14}O_2$ has been eliminated as a possibility, viz. a pathway through glyoxylic acid or some other compounds which gives a C-1 intermediate when metabolized, it appears possible to conclude that ethanol is metabolized *in toto* to acetyl-CoA before participating further in metabolic sequences.

SUMMARY AND CONCLUSIONS

1. Ethanol metabolism in the intact rat was studied with an experimental approach designed to determine if one or if multiple pathways exist for ethanol.
2. With this experimental approach, ethanol metabolism is compared to acetate metabolism by the use of labeling ratios to ascertain if ethanol is converted quantitatively to acetate before being further metabolized. If, indeed, acetate is the sole product of ethanol metabolism, then the ratio of radioactivity incorporated into any metabolic product when ethanol-1-C^{14} and ethanol-2-C^{14} are the labeled precursors should be the same as when acetate-1-C^{14} and acetate-2-C^{14} are used.
3. The use of the labeling ratios helps to but does not completely eliminate tissue permeability differences, endogenous pool size differences, and other unknown physical factors which invalidate a direct comparison of acetate-C^{14} and ethanol-C^{14} label incorporations.
4. Ratios of the incorporated label from acetate-2-C^{14}/acetate-1-C^{14} (A_2/A_1) and from ethanol-2-C^{14}/ethanol-1-C^{14} (E_2/E_1) in cholesterol, fatty acids and acetyl sulfanilamide indicate that acetyl-CoA is labeled indistinguishably by acetate-C^{14} and ethanol-C^{14}. It appears from these experiments that ethanol is metabolized to acetate (acetyl-CoA) before participating further in metabolic reactions.

5. Ethanol-C^{14} labeled cholesterol and fatty acids to a greater extent than did acetate-C^{14} in liver tissue. This observation can be explained by different endogenous pool sizes for these two compounds. Because of this fact, it is proposed that acetaldehyde is converted directly to acetyl-CoA without first becoming free acetate.

6. Ratios of the incorporated label from acetate-2-C^{14}/acetate-1-C^{14} (A_2/A_1) and ethanol-2-C^{14}/ethanol-1-C^{14} (E_2/E_1) into respiratory carbon dioxide are significantly different. This finding suggests a pathway unique to ethanol.

7. The significantly lower ratio, E_2/E_1, for respiratory carbon dioxide is attributed to the retention of the 2-position of ethanol in the rat body.

8. Formate-C^{14} has been investigated as an intermediate in ethanol metabolism. By a special formate flooding technique for the detection of formate as a metabolic intermediate in the intact animal metabolism, formate was found not to be preferentially formed from ethanol as compared to acetate.

9. Glyoxylic acid labeling from acetate-C^{14} and ethanol-C^{14} was studied indirectly by determining the labeling of glycine from these labeled precursors as the benzoic acid conjugate, hippuric acid. It was found that ethanol-C^{14} labels glycine in a fashion similar to acetate-C^{14}.

10. Discussion is presented on the validity of interpreting the carbon dioxide labeling ratio difference as an indication for an alternate pathway of ethanol metabolism. It is concluded that the difference in ratio found for respiratory carbon dioxide can

be attributed to a combination of the inabilities of certain tissues to metabolize ethanol and to the dissimilarities of the tricarboxylic acid cycles activities in a variety of tissues.

11. It is concluded that in the intact rat, ethanol metabolism takes place via the pathway, ethanol to acetaldehyde to acetyl-CoA.

APPENDIX

Section I

The statistical method used to evaluate the ratios was the t-test (See Methods and Materials). To do this, it was necessary to process the raw data as follows:

Given these samples

	Group 1		Group 2	
	a_1	b_1	c_1	d_1
	a_2	b_2	c_2	d_2
	a_3	b_3	c_3	d_3
mean:	a	b	c	d

To compare the means of these samples expressed as ratios, e.g. a/b and c/d, first one set of samples from each group is chosen and the means are determined, i.e.,

$$\frac{a_1 + a_2 + a_3}{3} = a \quad \text{and} \quad \frac{c_1 + c_2 + c_3}{3} = c$$

The samples of the second set in each group are then individually divided into the respective means calculated for the first set in that group, thus establishing "individual ratios", e.g.

$$\frac{b_1}{a}, \frac{b_2}{a} \& \frac{b_3}{a} \quad \text{and} \quad \frac{d_1}{c}, \frac{d_2}{c} \& \frac{d_3}{c}$$

These "individual ratios" can then be handled as individual samples and the procedure for the t-test can be applied directly to them. A check on the results can be performed by reversing the procedure within each group, i.e.

$$\frac{a_1}{b}, \frac{a_2}{b} \& \frac{a_3}{b} \quad \text{and} \quad \frac{c_1}{d}, \frac{c_2}{d} \& \frac{c_3}{d}$$

The t-test is then applied. The t-values calculated by both procedures should be identical, for all practical purposes, with each other.

Section II

Cholesterol Digitonide Data

Each value represents the percentage of the dose that was incorporated into cholesterol of the indicated tissues following an injection of ethanol-1-C^{14} (E_1), ethanol-2-C^{14} (E_2), acetate-1-C^{14} (A_1), or acetate-2-C^{14} (A_2).

Liver

Sample	E_2	E_1	A_2	A_1
1	0.43	0.35	0.28	0.13
2	0.60	0.43	0.27	0.23
3	1.15	0.42	0.31	0.16
4	0.79	0.50	0.46	0.17
5	0.41	0.56	0.27	0.19
6			0.17	-
Mean*	0.68 ± .28	0.45 ± .08	0.29 ± .08	0.18 ± .05

$$\frac{E_2}{E_1} = 1.51 \qquad \frac{A_2}{A_1} = 1.61$$

Gut

Sample	E_2	E_1	A_2	A_1
1	0.44	0.35	0.23	0.14
2	0.62	0.36	0.25	0.23
3	1.20	0.31	0.31	0.19
4	0.64	0.39	0.36	0.19
5	0.33	0.44	0.31	0.22
6			0.23	0.28
Mean*	0.65 $\pm$.30	0.37 $\pm$.04	0.28 $\pm$.04	0.21 $\pm$ .04

$$\frac{E_2}{E_1} = 1.76 \qquad \frac{A_2}{A_1} = 1.33$$

Carcass

Sample	E_2	E_1	A_2	A_1
1	0.33	0.20	0.19	0.11
2	0.39	0.24	0.25	0.18
3	0.67	0.40	0.19	0.23
4	0.44	0.31	0.28	0.14
5	0.27	0.49	0.44	0.22
6			0.13	0.61
Mean*	0.42 $\pm$.14	0.33 $\pm$.11	0.25 $\pm$.09	0.25 $\pm$ .19

$$\frac{E_2}{E_1} = 1.27 \qquad \frac{A_2}{A_1} = 1.00$$

Skin

Sample	E_2	E_1	A_2	A_1
1	0.14	0.09	0.11	0.04
2	0.17	0.10	0.16	0.09
3	0.24	0.11	0.11	0.10
4	0.17	0.18	0.23	0.06
5	0.14	0.17	0.13	-
6			0.10	0.12
Mean*	0.17 $\pm$.04	0.13 $\pm$.04	0.14 $\pm$.04	0.08 $\pm$ .03

$$\frac{E_2}{E_1} = 1.31 \qquad \frac{A_2}{A_1} = 1.75$$

*Mean $\pm$ standard deviation.

Section III

Fatty Acid Data

Each value represents the percentage of the dose that was incorporated into fatty acids of the indicated tissues following an injection of ethanol-1-C^{14} (E_1), ethanol-2-C^{14} (E_2), acetate-1-C^{14} (A_1), or acetate-2-C^{14} (A_2).

Liver

Sample	E_2	E_1	A_2	A_1
1	2.26	2.02	0.51	0.61
2	1.29	1.25	0.63	0.94
3	1.20	1.87	0.93	0.58
4	2.36	1.21	1.19	0.62
5	0.83	1.22	1.16	0.85
6			1.55	0.75
Mean*	1.59 $\pm$.61	1.51 $\pm$.35	1.00 $\pm$.35	0.73 $\pm$ .13

$$\frac{E_2}{E_1} = 1.05 \qquad \frac{A_2}{A_1} = 1.37$$

Gut

Sample	E_2	E_1	A_2	A_1
1	2.68	2.20	3.92	4.50
2	2.69	1.54	4.25	5.10
3	2.36	2.04	5.37	8.34
4	2.27	2.10	6.22	4.88
5	1.70	1.72	6.59	6.68
6			6.01	7.29
Mean*q	2.34 ±.36	1.92 ±.35	5.39 ±.99	6.13 ± 1.40

$$\frac{E_2}{E_1} = 1.22 \qquad \frac{A_2}{A_1} = 0.89$$

Carcass

Sample	E_2	E_1	A_2	A_1
1	4.83	3.93	3.62	5.55
2	5.09	2.87	4.25	5.30
3	2.53	3.40	5.55	5.75
4	3.99	3.05	5.35	4.30
5	3.73	3.08	7.03	7.41
6			8.77	4.67
Mean*	4.03 ±.91	3.27 ±.37	5.76 ±1.72	5.50 ±.99

$$\frac{E_2}{E_1} = 1.23 \qquad \frac{A_2}{A_1} = 1.05$$

Skin

Sample	E_2	E_1	A_2	A_1
1	2.52	1.38	0.87	0.56
2	1.16	1.56	1.23	0.97
3	1.74	1.63	1.23	0.87
4	1.37	2.22	1.42	0.96
5	1.27	1.73	1.81	1.17
6			-	1.03
Mean*	1.61 $\pm$.49	1.70 $\pm$.28	1.31 $\pm$.31	0.93 $\pm$.19

$$\frac{E_2}{E_1} = 0.95 \qquad \frac{A_2}{A_1} = 1.41$$

*Mean $\pm$ standard deviation.

Section IV

Carbon Dioxide Data

Each value represents the percentage of the dose that was incorporated into expired carbon dioxide following an injection of ethanol-1-C^{14} (E_1), ethanol-2-C^{14} (E_2), acetate-1-C^{14} (A_1) and acetate-2-C^{14} (A_2).

Sample	E_2	E_1	A_2	A_1
1	38.9	50.9	48.6	54.9
2	31.9	59.7	54.8	64.5
3	34.0	59.0	49.0	60.8
4	35.5	60.6	51.1	66.7
5	35.8	62.2	52.0	65.9
6			51.1	69.8
Mean*	35.2 $\pm$2.3	58.5$\pm$3.9	51.1$\pm$2.0	63.8$\pm$4.8

*Mean $\pm$ standard deviation.

Section V

Amount of Carbon Dioxide Exhaled in Two Hours by Rats Injected with Labeled Acetate and Ethanol

(in umoles)

Sample	E_1	E_2	A_1	A_2
1	32.0	27.6	30.8	27.2
2	30.4	31.2	30.8	28.8
3	30.4	29.2	29.6	36.1
4	30.4	33.0	26.8	29.6
5	31.4	26.0	30.8	35.8
6			28.8	33.2
Mean*	30.9 ± 0.7	29.4 ± 2.5	29.6 ± 1.5	31.8 ± 3.4

Statistical Comparisons

Comparison between:	p-Value
E_1 & E_2	$p > .05$
E_1 & A_1	$p > .05$
E_1 & A_2	$p > .05$
E_2 & A_1	$p > .05$
E_2 & A_2	$p > .05$
A_1 & A_2	$p > .05$

*Mean $\pm$ standard deviation.

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