THE METABOLISM OF ETHANOL

by

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INTRODUCTION

1. Historical Aspects

In the early eighteen hundreds, Liebig, a noted organic chemist, stated that alcohol was oxidized in the human and in the animal through aldehyde, lactic acid, oxalic acid and formic acid to carbonic acid, but so far as it is known, he had no experimental evidence for his statement.

By the mid-1800s experiments had been performed with intact animals that showed that ingested ethanol could be found in urine and in expired air. These observations led early investigators to conclude that alcohol was excreted from the body and was not metabolized. However, Dupre (1872) and Anstie (1874) showed that the portion of alcohol recovered from the lungs and urine over a long period of time was an insignificant part of the total amount of the administered alcohol.

Other workers, during this time interval were unable to identify any metabolic products after ethanol administration and therefore concluded that the alcohol was completely oxidized to carbon dioxide and water.

Ethanol metabolism was studied from a nutritional and metabolic point of view during the years 1880 through the early 1900s. In 1891 Strassman demonstrated that the bodies of animals given alcohol over a long period of time had an increased amount of body fat as compared to control animals. During this period of time it was noted that following the administration of alcohol in individuals not accustomed to it there was an increased nitrogen excretion. Later it was recognized that alcohol was able to spare

protein to the same extent as an isocaloric amount of carbohydrate or fat. Thus an important concept developed in this period of time was the recognition that alcohol could be considered as utilizable food material.

In the early 1900s the emphasis on research in the alcohol field changed from nutritional aspects of the metabolism of alcohol in the body to its rate of disappearance. Workers such as Grehant (31) in 1903 analyzed whole animals at various times after the injection of alcohol to obtain estimates of the rate of its disappearance. In addition, attempts were made to estimate the rate of alcohol utilization in man by the decrease in respiratory quotient after alcohol administration. The introduction of a micromethod for ethanol determination in 1922 by Windmark (96) combined with other advances in methodology, allowed investigators to examine more quantitatively problems relating to alcohol absorption, distribution and elimination.

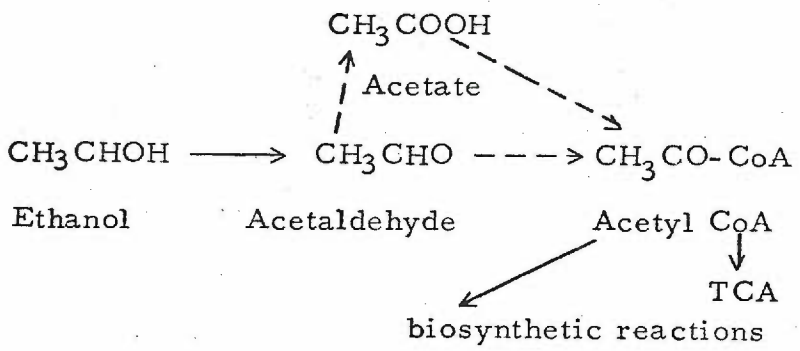
In recent years three main lines of research have been followed in the study of alcohol metabolism. These are: 1. the factors that influence the rate of alcohol metabolism and the relation of this rate to the metabolism of other compounds in the body; 2. the sites of alcohol metabolism in the organism; 3. the enzymes involved in alcohol metabolism.

It is now known that ethyl alcohol is readily utilized by many animals and the processes of this metabolism have been the subject of extensive investigations, not only for biochemical and physiological reasons, but also for sociological ones.

That alcohol is utilized by an animal is immediately apparent from its rapid disappearance following administration. A dose of 0.25 grams of ethanol per kilogram body weight in a cat is quickly distributed in the animal's body and disappears from its blood stream at a rate of 0.1 gram per kilogram per hour (44, 45). In agreement with earlier findings only a small portion of this was recovered in the urine and expired air during the experimental period. The chemical pathways followed by alcohol, the sites of chemical change within the tissues, and the ultimate fate of the alcohol carbon are questions of prime importance for the understanding of this subject and will be examined later in this section.

The main pathway of alcohol metabolism in the body, as shown in Figure 1, involves first the oxidation of alcohol by alcohol dehydrogenase which forms acetaldehyde (70, 71, 72, 73). Experimental evidence obtained by many workers suggests that the liver is the chief tissue involved in this conversion (3, 45, 55, 56, 63). The resulting acetaldehyde is then further metabolized by liver or it is carried by the blood stream to other tissues for further metabolism. It is quite likely that most of the acetaldehyde is converted to acetyl CoA, possibly by first conversion to acetic acid and then by reactions involving coenzyme A. Acetyl CoA, as a key metabolic intermediate in many metabolic pathways, enables radioactive ethanol to be incorporated into Krebs cycle intermediates, carbon dioxide, lipid, proteins and carbohydrate.

Figure 1.



Outline of Alcohol Metabolism

It is generally believed that the zinc-containing, NAD-requiring, alcohol dehydrogenase present in liver is responsible for the larger part of ethanol metabolism in man and animals (53, 56) although recently a microsomal alcohol dehydrogenase requiring NADPH and O_2 has been detected (6).

2. Enzymes Involved in Ethanol Metabolism

The enzyme alcohol dehydrogenase catalyzes the oxidation of ethanol to acetaldehyde. During this reaction the coenzyme NAD is reduced to NADH and a proton is released into the media. The reaction proceeds according to the formula:

$$CH_3CH_2OH + NAD \underset{\text{Alcohol Dehydrogenase}}{\rightleftharpoons} CH_3CHO + NADH + H^+$$

The properties of alcohol dehydrogenase isolated from yeast were first investigated by Negelein and Wulff (69). Later in 1938 Lutwak-Man isolated and purified this same enzyme from mammalian liver and studied its chemical and physical properties (59). About 10 years later Bonnichser and Wassen (4, 5) were able to prepare

the first crystalline samples of alcohol dehydrogenase from horse liver. Since this time the purification of this enzyme from crude tissue extracts has become a favorite procedure for teaching the principles of isolation and handling labile proteins.

Liver alcohol dehydrogenase has a molecular weight of 84,000 and contains 2 atoms of zinc per molecule of enzyme. The Michaelis constant for the enzyme varies with the pH and reaches a minimum value of 5×10^{-4} M at pH 8. The turnover number (the number of molecules of alcohol oxidized by one molecule of enzyme per minute at 20° C) has been found to be 140. Theorell and Bonnichen (84) made the discovery that two molecules of coenzyme combine with each molecule of enzyme. It is interesting that alcohol dehydrogenase can catalyze the reaction.

$$\text{Vitamin A} \rightleftharpoons \text{retinene},$$

retinene being the aldehyde corresponding to Vitamin A.

Leloir and Muñoz were the first to demonstrate that pyruvic acid can act as a hydrogen acceptor for the NADH formed in the oxidation of ethanol. Under certain conditions, in vitro, the addition of pyruvate to liver slices metabolizing alcohol results in a 2- or 3-fold increase in alcohol disappearance; under these circumstances lactic acid is formed (46). Forsander and others have noted that the ratios of the concentrations of lactate to pyruvate in the medium of rat livers perfused with ethanol increases five to seven times due primarily to a decrease in the pyruvate concentration (27).

A second possible mechanism for the oxidation of alcohol to

acetaldehyde is given by Keilin and Hartree (39, 40). They have observed that ethyl alcohol in the presence of peroxide and catalase reacts to form acetaldehyde and water. Chance (15) has studied the kinetics of this reaction and suggests the following reaction order.

$$\text{Catalase} + H_2O_2 \longrightarrow \text{Catalase}\ (H_2O_2)$$

$$\text{Catalase}\ (H_2O_2) + (CH_5OH) \longrightarrow \text{Catalase} + 2H_2O + CH_3CHO$$

Some alcohols other than ethanol react in a similar way. Catalase is found in liver and kidney tissues in fairly high concentrations and may be responsible for at least part of ethanol metabolism. In vitro experiments by Lundquist and others have shown that in a liver homogenate, catalase can oxidize ethanol provided that a hydrogen peroxide forming enzyme such as glucose oxidase is added (54). Lundquist noted that in his preparation the spontaneous formation of hydrogen peroxide was too slow to account for any measurable part of the ethanol metabolism.

Some recent work shows that other pathways of metabolism of ethanol exist. Kamil, Smith and Williams (38) have shown that small amounts of alcohol can be excreted in the urine as ethyl glucuronide and Bostrom and Vestermark (6) have recently identified ethyl sulfate as a urinary excretion product following the administration of large amounts of ethanol.

While it is generally accepted that the primary product of ethanol metabolism is acetaldehyde, there are a number of possible pathways that acetaldehyde can follow. The most obvious reaction is probably the oxidation of acetaldehyde to acetic acid.

3. Enzymes Involved in Acetaldehyde Metabolism

Westerfeld (95) lists six enzymes found in mammalian tissues which are capable of utilizing acetaldehyde as substrate. These enzymes, their cofactors and the reaction products are listed below.

(a) Aldolase:

$$CH_3CHO + H_2PO_3\text{-}O\text{-}CH_2\text{-}CO\text{-}CH_2OH \longrightarrow H_2PO_3\text{-}O\text{-}CH_2\text{-}CO\text{-}CHOH\text{-}CHOH\text{-}CH_3$$

(b) Glyceraldehyde-3-phosphate Dehydrogenase:

$$CH_3CHO \xrightarrow[\text{Glutathione}]{NAD} CH_3CO\text{-}S\ \text{Glut.} \xrightarrow{H_3PO_4} CH_3CO\text{-}OPO_3H_2$$

$$CH_3CO\text{-}OPO_3H_2 \xrightarrow{CoA} CH_3CO\text{-}CoA$$

(c) Carboxylase:

$$CH_3CHO + CH_3CO\ COOH \xrightarrow[\text{Thiamin, Lipoic Acid}]{NAD} CH_3\text{-}CHOH\text{-}CO\text{-}CH_3\ (\text{Acetoin}) + CO_2$$

(d) Xanthine Oxidase:

$$CH_3CHO \xrightarrow[O_2]{Mo\ B_2\ Fe} CH_3COOH$$

(e) Aldehyde Oxidase:

$$CH_3CHO \xrightarrow[O_2]{Mo,\ B_2} CH_3COOH$$

(f) Aldehyde Dehydrogenase:

$$CH_3CHO \xrightarrow{NAD} CH_3COOH$$

The problem of determining the quantitative importance of each of these reactions has been difficult. Aldolase, carboxylase, and glyceraldehyde-3-phosphate dehydrogenase are abundant in muscle whereas Lubin and Westerfeld state that the major part of acetaldehyde metabolism takes place in the liver (50). Lundquist and others (51) in studying the metabolism of acetaldehyde in rat liver preparations under anaerobic conditions have noted that all of the acetaldehyde that disappeared from their media could be accounted for by ethanol and acetic acid formation. In view of this, Lundquist concluded that condensation reactions such as acetoin synthesis are of little quantitative importance.

Tracer studies contained in this thesis show that a variety of tissues have the ability to convert acetaldehyde to carbon dioxide presumably by means of the Krebs cycle involving acetate or acetyl CoA as intermediates. Antabuse, a drug which inhibits Xanthine oxidase, aldehyde oxidase and aldehyde dehydrogenase causes acetaldehyde to accumulate after ethanol administration (92). At the present, it is difficult to assign the major metabolic function to any one aldehyde oxidizing system. The liver enzymes that are known to have quantitative significance in the metabolism of ethanol all convert in the test tube acetaldehyde to acetic acid rather than to acetyl CoA but whether or not this is true in vivo has not been established. During the metabolism of ethanol by various liver preparations, workers have noted the accumulation of acetic acid in the incubation media (17, 18, 51, 52, 54). In addition, Lundquist and others (53) have observed in humans an increase in the blood acetate concentration

after the ingestion of ethanol.

In contrast to mammalian systems, certain microorganisms are known to be capable of converting acetaldehyde directly to acetyl CoA without the intervention of acetic acid (11). The possibility of finding an enzyme system in liver that can convert acetaldehyde directly to acetyl CoA is enhanced by the findings of Brady and Gurin (7) that acetaldehyde is a better precursor of fatty acids than is acetate. In addition, it has been observed that ethanol-^{14}C is found to a greater extent in tissue glycogen, protein, cholesterol, fatty acids, and phospholipids than is acetate-^{14}C (73, 76). The possibility that these findings might possibly be due to or influenced by differences in permeability, pool sizes and other factors will be discussed later in light of the findings of this thesis.

4. Acetic Acid Metabolism

Mitochondria of rat liver, pigeon liver and rat kidney have been found to metabolize a variety of long and short chain aldehydes to the corresponding acids. Weinhouse (90) expressed the view that the aldehydes are oxidized directly to the corresponding acids without the formation of an active acyl derivative, since the cofactor requirements for this reaction were only ATP and NAD.

Forsander and Raiha (26) while studying alcohol metabolism by perfusion of isolated rat livers, isolated a number of carboxylic acids from the liver perfusate. While rat blood normally contains pyruvate, β-hydroxybutyrate, lactate and small amounts of acids from the tricarboxylic acid cycle, after intraperitoneal injection of alcohol,

acetate and acetoacetate were found in addition to increased amounts of the other acids cited. Perfusion of rat liver with blood containing radioactive ethanol resulted in the production of labeled acetate, acetoacetate, β-hydroxybutyrate as well as small amounts of other acids.

Lundquist determined the concentration of acetate in the blood of man and found a value of 2 μg./ml., an amount which was independent of the nutritional regime. He later observed that during the metabolism of ethanol the level of blood acetate increased to 19-57 μg./ml. in twenty subjects with the corresponding concentrations of ethanol in the blood ranging from 0.2 to 2.6 mg./ml. Lundquist, et al. (54) found that the formation of free acetate from ethanol took place primarily in the liver and that the acetate formed is utilized mainly in extrahepatic tissues. Acetate production can be inferred from the results of Forsander, Raiha and Suomalaenen (25), who showed that while very little radioactive carbon dioxide is formed by rat liver perfused with blood containing carbon-14 labeled ethanol, marked production of radioactive CO_2 occurred when the same effluent blood was allowed to perfuse through the hind quarters of the animal.

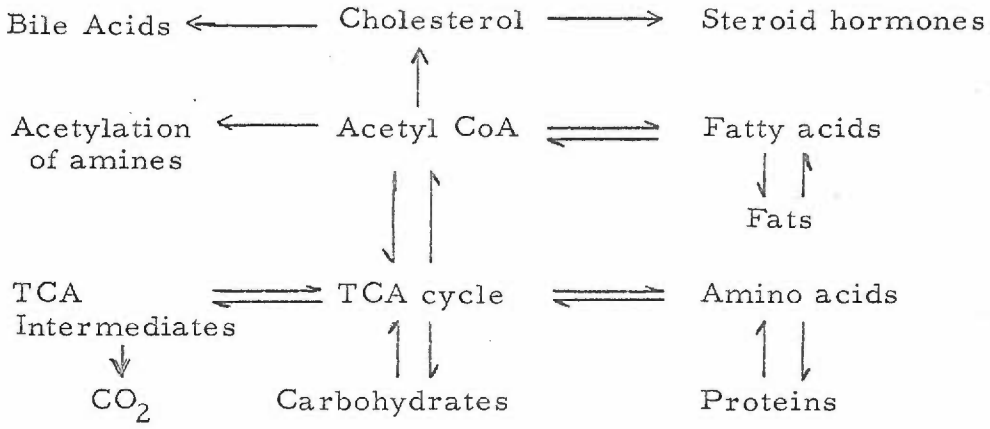
Free acetate requires activation before it can be metabolized. Acetyl CoA, the active form of acetate, was first isolated by Lynen and his associates in 1951. Acetyl CoA formation requires ATP according to the following reactions:

1. $CH_3C(=O)-OH + ATP \rightleftharpoons$ Acetyladenylate + PP

   Acetate

2. Acetyl adenylate + CoA $\rightleftharpoons$ Acetyl CoA + AMP

Once acetyl CoA has been formed it can reasily be metabolized by a variety of pathways. Evidence of this is found in experiments where labeled ethanol gives rise to radioactive CO_2, fatty acids, cholesterol, proteins, carbohydrates, and many other compounds as shown below:



5. Sites of Alcohol Metabolism

Quantitatively the liver is the most important organ for oxidizing alcohol. Liver slices and homogenates can oxidize ethanol in vitro (36), and liver preparations are used as raw material for the preparation of alcohol dehydrogenase and other alcohol oxidizing enzymes. Larsen (44) has recently examined the question of the importance of the liver in in vivo alcohol metabolism and claimed to find evidence for extrahepatic utilization of alcohol. He gave alcohol to normal subjects by slow intravenous infusion at a constant rate, until a stable low blood alcohol level was reached. The rate of infusion was then increased until a new stable blood level was reached.

At each stable blood level, the rate of infusion presumably equaled the rate of utilization. When Larsen plotted each blood level against its respective infusion rate and connected the points by a straight line, he found that it did not pass through the origin, but gave an intercept on the infusion rate axis, which he interpreted as extra-hepatic utilization. Kalant (37) points out that the conclusion is probably not justified, for at very low levels of blood alcohol, when the alcohol dehydrogenase is not saturated, there is not a straight line relationship between the alcohol concentration and the rate of utilization, but rather an exponential one, as was shown by Marshall and Fritz (60).

Liver perfusion experiments indicate that alcohol can be removed from the blood at a rate corresponding to one-half to five-sixths the rate expected in the whole organism (21, 34, 43, 44, 45).

Lundquist (54) in experiments with rat liver suspensions suggests that the oxidation of ethanol by liver suspensions proceeds at about the same rate as in the living animal. However, he points out that because of the high rate of energy metabolism of the rat the loss through the lungs, (provided equilibrium is attained between blood and alveolar gases), could be responsible for as much as one-third to one-half of the total elimination at a blood concentration of 2 mg./ml.

Some experiments suggest that muscles can also oxidize alcohol to a certain degree. Fleischman (21) found a lowering of the respiratory quotient when frog muscles were made to contract in

Ringer's solution containing alcohol. Hammill (35) and Fisher (22) found the isolated heart capable of utilizing alcohol. Bartlett and Barnet (3) have shown that heart slices and diaphragm preparations have very low alcohol-oxidative activities. This is in agreement with earlier work performed on isolated perfused limb preparations (56) and on eviscerated animals (64). These findings suggest that the musculature can play only a small role in the first step of alcohol metabolism.

The role of the kidneys in alcohol metabolism is quite uncertain as the literature is full of conflicting reports. Kidney damage studies with uranium nitrate have been cited to prove a lack of alcohol oxidation by this group. Bartlett (3) in contrast has found that kidney slices have a high rate of ethanol oxidation, in fact, several times greater than liver slices on a weight basis. They point out however, that in the rat there is approximately seven times as much liver tissue as kidney tissue. Wartburg and Eppenberger (88) have found that a combination of kidney slices with liver homogenate results in an increase of about 70% over the computed sum of the single oxidation rates. In addition, they observed that kidney slices could oxidize 49.2 ± 17.6 μmoles of ethanol/gm. of dry weight/hr. in contrast to the liver's ability to oxidize only 19.2 ± 6. Oxidation of ethanol has also been demonstrated in kidney slices by Masoro and Abramowitch (62).

It has been widely discussed for some years whether or not brain tissue is capable of oxidizing and utilizing alcohol. The findings of Sutherland and Burbridge suggest that ethanol can be meta-

bolized in vitro by brain tissue (10, 83). But Kalant (37) points out that their findings could also be explained by nothing more than the diffusion of alcohol into the brain slices. Results of other in vitro experiments vary considerably. Dewan (19) found that in vitro preparations of ox brain are capable of forming acetaldehyde and acetic acid from added alcohol. He later examined brains from dogs, cats, pigs, cows, guinea pigs and rabbits and found them able to oxidize alcohol in vitro (20). In contrast, Bartlett and Barnet found no formation of $^{14}CO_2$ by brain tissues from rats incubated with carbon-14 ethanol.

As to other tissues, Leloir and Muñoz have examined testes, spleen and intestinal tract in vitro and did not find any oxidizing ability. Masoro, Abramowitch and Birchard (63) found a slight oxidation of ethanol by spleen but could not demonstrate oxidation in striated muscle.

6. In Vivo Ethanol Metabolism

The complex nature of the intact animal necessarily makes the nature of in vivo ethanol metabolism very complicated. Certainly man has been metabolizing ethanol for many hundreds of years, but only recently has he had the tools with which to examine with precision the quantitative aspects of his in vivo ethanol metabolism. Much of our information about the rate as well as other aspects of in vivo alcohol metabolism has been obtained with the dog as the experimental animal. In this animal and in others an increased acetaldehyde concentration has been shown to occur after the administration of ethanol.

At first glance, it would seem quite likely that acetaldehyde should appear since it is the first oxidation product in ethanol metabolism; but this is not necessarily true. Negelein and Wulff (66) have shown that the equilibrium between ethanol and acetaldehyde in an in vitro situation is greatly in favor of the alcohol by a ratio of 3000:1. In certain animals such as the dog that accumulate acetaldehyde after receiving ethanol, the ratio is in the order of 300:1 (43). Why acetaldehyde should accumulate to the extent it does is a mystery since the rate of acetaldehyde metabolism in all species studied is faster than the rate of alcohol metabolism.

Schulman, Zurek and Westerfeld (73) compared ethanol-1-^{14}C and acetate-1-^{14}C in parallel metabolic experiments in vivo by measuring the incorporation of isotope into various tissue constituents one-half and six hours after the administration of 2-21 μmoles of substrate. The alcohol contributed two to three times as much isotope to tissue glycogen, protein, cholesterol and fatty acids as did acetate, but both substrates were otherwise parallel in the labeling effected in all tissues studied. They suggest that free acetate was not an intermediate in the metabolism of alcohol basing their conclusions on the idea that if ethanol is oxidized to acetate there should have been greater metabolic dilution of the ethanol, if they are both metabolized by the same pathway. It is possible that compartmentation and or greater permeability of the ethanol might explain these results.

Results with eviscerated animals have added to the confusion of in vivo studies on ethanol metabolism, for example, Mirshy and Nelson (63,64) found no elimination of ethanol at all in the eviscerated

rabbit, whereas Lundsgaard (56, 57) found a utilization in the eviscerated cat which was about 10 percent of that of the intact animal. Clark, et al. (16) found in the eviscerated dog a utilization rate of 19 and 31 percent of the control animals.

Russell and Van Bruggen (73), using a labeling ratio technique, were able to demonstrate in vivo that the labeling of precursor acetyl-CoA for cholesterol and fatty acid biosynthesis, as well as for the acetylation of amines was consistent with the conversion of ethanol to acetyl-CoA either by direct or indirect means. They noted the major portion of the administered ethanol is metabolized to carbon dioxide and that the ratios of incorporation for ethanol-2-^{14}C to ethanol-1-^{14}C (0.6 at two hours) were significantly different than the ratios for acetate-2-^{14}C to acetate-1-^{14}C (0.8). They found that the ethanol-1-^{14}C and acetate-1-^{14}C each label the carbon dioxide fraction to the same degree, but that the ethanol-2-^{14}C is less effective in labeling carbon dioxide than the corresponding acetate-2-^{14}C. They suggest that these differences might be due to differences in the ability of any one tissue to metabolize the compounds in question, differences in uptake of labeled ethanol or acetate by the tissues, or differences in the metabolic pathways of the tissues.

7. Statement of Purpose

The purpose of the studies presented in this thesis is to examine critically the abilities of different tissues to utilize ethanol, acetaldehyde and acetate as substrates of biosynthetic and oxidative reactions. While many experiments have been carried out on the

utilization of ethanol by preparations in vitro, many discrepancies appear in a comparison of the published results of different investigators. This could be related to differences in experimental conditions, procedures and analytical methods, and species or strain. Many investigators have examined independently different aspects of ethanol, acetaldehyde or acetate metabolism, but none has studied comparatively the metabolism of the three compounds in one or more tissues under standardized conditions.

Several important questions have been asked in the course of these studies:

(1) Is acetate an obligatory intermediate in the synthesis of acetyl CoA from ethanol in the major tissues of the body.

(2) If ethanol is quantitatively metabolized to acetate and subsequently converted to lipid, protein, carbohydrate and CO_2, why is more ethanol than acetate incorporated in vivo into these compounds in certain tissues?

(3) What tissues are capable of utilizing ethanol as a substrate in vitro and to what extent?

(4) What is the rate limiting step in the conversion of ethanol to acetate if differences exist in the rate of utilization and acetate can be shown to be an obligatory intermediate in the conversion of ethanol to acetyl CoA?

MATERIAL AND METHODS

The radioactive tracers used were acetaldehyde-1,2-^{14}C, acetate-1-^{14}C, acetate-2-^{14}C, acetate-2-^{3}H, ethanol-1-^{14}C, ethanol-2-^{14}C, and ethanol-1-^{3}H and were obtained primarily from the New England Nuclear Corporation.

Since isotope purity was of prime importance in this work, it was routinely checked by isotope dilution analysis (48, 73) and by gas chromatography. Small amounts of the tracer ethanol were found to be constantly converted to acetic acid and to carbon dioxide. This occurred even though the tracers were always stored in water solution at -16° C. Radioactive acetic acid and carbon dioxide were removed by distillation of the ethanol after first adding NaOH to the solution. The CO_2 and acetic acid as sodium salts remained in the distilling flasks. It was found that by flushing the distillation apparatus with nitrogen before the distillation and by storing the tracer ethanol in an atmosphere of nitrogen, less of the ethanol was converted to acetic acid.

Male albino rats of the Sprague Dawley strain of 190 and 250 grams weight were used for the major portion of this research and were obtained from Pacord Research Animals in Beaverton, Oregon. The rats were trained to eat 10 grams of Purina Rat Chow in one hour, once in the morning and again in the afternoon. At the end of the feeding period the animals were then fasted for an additional hour before they were used for experimental purposes. Drinking water

was available to them at all times. It has been shown that this type of nutritional control is necessary to minimize the variability seen in lipogenesis (85, 86, 91, 92). When the rats were to be used for in vitro experiments they were killed by decapitation immediately after the one-hour fasting period.

Tissue Slice Preparations

The tissue or tissues to be used for an experiment were removed from the animal and placed in chilled Krebs phosphate Ringer's solution at pH 7.4. Heart, lung, brain, liver and kidney slices were prepared on a Mickle Tissue Chopper, which was set to produce slices 0.5 mm. in thickness. Diaphragms were cut into 1.0 mm. strips with the same device. The sliced tissues were transferred to tared Warburg flasks containing chilled buffer in the main compartment and the substrate to be used in one of the sidearms.

In experiments where two tissues were to be incubated in the same flask, the procedure was the same as previously stated except that the flasks were weighed three times, once to obtain the tare weight, again after the first tissue had been added and finally after the addition of the second tissue. After the final weighing, the flasks were flushed with two liters of 100% oxygen during one minute, the removable centerwells containing 0.2 ml. of 6N NaOH absorbed on a folded filter paper were placed in the center compartment and the flasks were attached to the manometers and placed in the Warburg bath. After a 15-minute equilibration period in the 37° C bath the appropriate substrate was added and three minutes later the stopcock

on the manometers were closed. Manometer readings were then taken at either 30 or 50 minutes to determine the oxygen consumption. After a one-hour incubation period the tissues were "killed" and the total CO_2 released from the media by the addition of concentrated HCl. One hour later the centerwells were removed, the tissue was washed twice with distilled water. If two kinds of tissue were present, they were separated by means of small forceps.

In the in vitro ethanol time course experiments, 1 gram of liver slices were weighed and placed in flasks containing 27 ml. Krebs phosphate Ringer's solution at pH 7.4. Each flask was flushed with 100% oxygen at a rate of six liters/minute for one minute and shaken in a 37° C. water bath. At 15-minute time intervals 0.5 ml. samples of the fluid were removed from the flasks by means of a 7-inch, 18-gauge needle inserted through a rubber seal on the flask to a point just below the surface of the buffer solution. The sample from the flask was then added to a small test tube containing 0.5 ml. absolute ethanol and 0.5 ml. glacial acetic acid.

Separation of Lipids

The tissues were transferred to screw cap tubes (16 mm. x 130 mm.) containing 5 ml. of 11% alcoholic KOH. These tubes were then heated on the steam bath for two hours and the alcohol which had evaporated from the tubes during the saponification was replaced with distilled water. After the tubes had cooled the solution was extracted four times with equal volumes of petroleum ether and these extracts were then pooled and taken to dryness on a water bath, and then

TABLE I.

RECOVERY OF CHOLESTEROL-4-^{14}C

tissue or standard	cpm added	cpm recovered	% recovery
cholesterol *	2893	2797	96.7
cholesterol	2893	2847	98.4
cholesterol	2893	2807	97.0
liver **	1433	1375	96.0
liver	1433	1391	96.9
liver	1433	1404	97.8

* 0.3 mg. of unlabeled cholesterol added as carrier to 5 ml. of 4% KOH, no tissue was present.

** Tracer cholesterol was added to 5 ml. of an alcoholic KOH digest of normal liver.

TABLE II.

RECOVERY OF PALMITIC ACID-^{14}C

tissue or standard	cpm added	cpm recovered	% recovery
palmitic acid*	3426	3520	102.7
palmitic acid	3426	3390	98.9
palmitic acid	3426	3410	99.5
liver**	6638	6853	103.3
liver	6638	6362	95.7
liver	6638	6439	96.8

*2 mg. of unlabeled palmitic acid added as carrier to 5 ml. of an 11% solution of KOH, no tissue present.

** Tracer palmitic acid was added to 5 ml. of an alcoholic KOH digest of normal liver.

brought up to a final volume of 10 ml. with 95% ethanol. This first extract is referred to as the nonsaponifiable fraction. After the last extraction the alcohol-KOH solution was acidified with concentrated HCL to a pH of less than 3 using Congo red indicator paper. The solution was then extracted three more times with equal volumes of petroleum ether to obtain the saponifiable fraction, this being chiefly the fatty acid fraction. Table 1 indicates that tracer cholesterol is well recovered. Table 2 gives the recovery of palmitic acid-^{14}C from an alcoholic KOH tissue digest of normal liver.

Separation of Ethanol and Acetic Acid

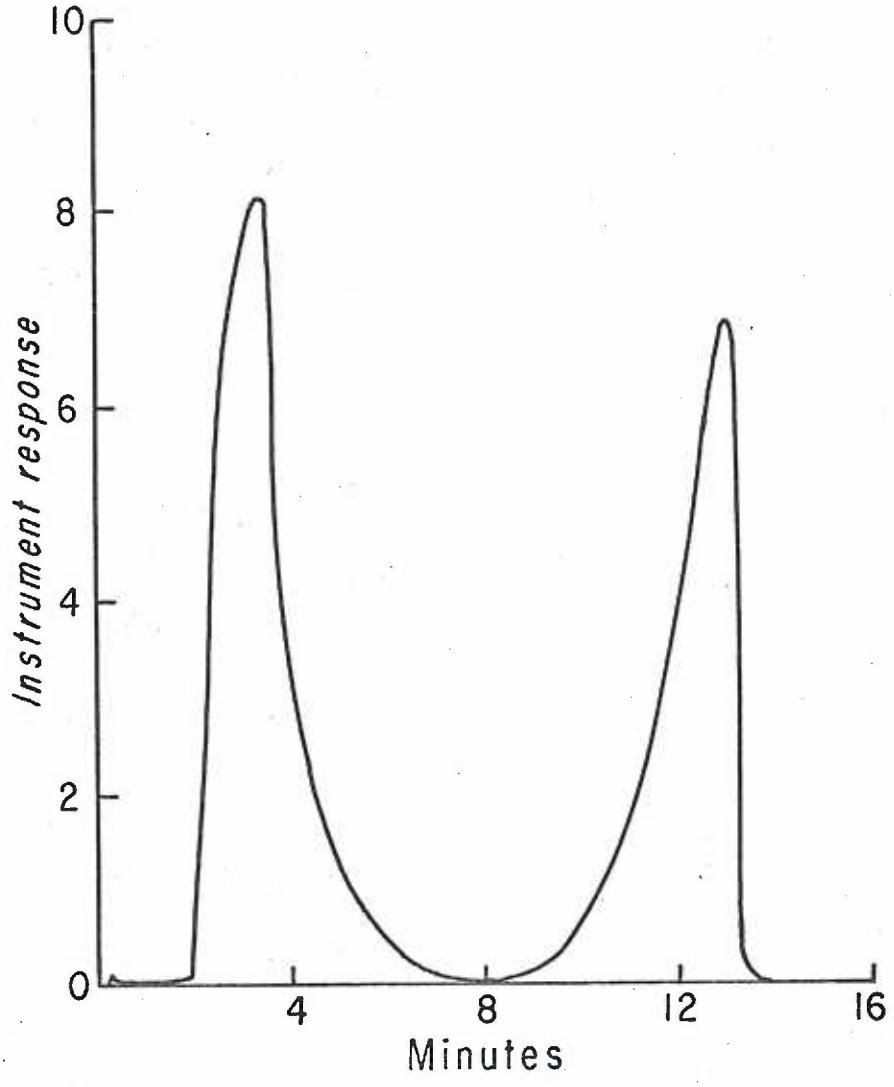
Aliquots of these solutions to be analyzed for ethanol and acetate were fractionated on an F and M model 720 gas chromatograph. A preparative column, 8 feet in length and 5/8 inch in outer diameter containing 45-50 mesh Chromosorb W and coated with 20% by weight silicone rubber, was used for the separation. The injection port and detector temperature were maintained at 295° and 300° C., respectively. The flow rate of the helium carrier gas was 100 cc./min. The column temperature was maintained at 50° C, until the maximum of the ethanol peak appeared, at which time the column oven temperature was programmed to increase at the rate of 4° C./minute to a maximum temperature of 250° C.

The ethanol fraction was collected by inserting a glass U-shaped tube fitted through a silicone rubber seal into the exit port of the gas chromatograph as soon as the ethanol started to emerge from the column. The lower portion of the glass tube was immersed

Figure II

Graphic tracing representing the separation of ethanol and acetic acid by gas chromatography under the following conditions:

flash heater temperature	300° C
detector temperature	320° C
initial column temperature	50° C
final column temperature	250° C
rate of temperature program	4° C/min.
carrier gas	helium
flow rate	100 cc /min.
detector	thermal conductivity
attenuation	8
sample size	300 µl.


10
8
6
4
2
0
Instrument response
4
8
12
16
Minutes

in a dry ice acetone bath. The efficiency of this collection system was measured by the distillation of appropriate standards and was found to be about 75%. See Table 3. As soon as all of the ethanol had passed through the column the first tube was removed. At the first indication of appearance of the slower, acetic acid fraction, a second tube was inserted and another fraction was then collected as before with an efficiency of 75%. Figure 2 shows the separation of ethanol and acetic acid obtained under the described conditions.

The fractions were removed from the collection tubes by rinsing the tubes several times with scintillation fluid (9) and bringing the total volume of the washings to 10 ml.

Cannulation and Blood Sampling

After a one-hour fasting period several rats were anesthetized with 2.0 mg. of Nembutal per kilogram body weight. The abdominal cavities were opened and tracer ethanol was injected into the hepatic-portal vein. Blood samples were then taken from the hepatic vein, inferior vena cava and in some cases from the heart (by external puncture) at specific time intervals using heparinized tuberculin syringes fitted with #20 needles. The blood samples were centrifuged and aliquots of the plasma fractions were added to small test tubes containing a mixture of absolute ethanol and glacial acetic acid. These tubes were then centrifuged to precipitate the denatured protein. Aliquots of these solutions were fractionated by gas chromatograph using the previously described conditions.

New Zealand White Rabbits weighing 4 kilograms were

used in several in vivo experiments. These animals were purchased from the University of Oregon Medical School Animal Care Departments and were used without special feedings. The rabbits were given 0.8 mg. of Nembutal by intraperitoneal injection and were maintained in a state of light anesthesia with diethyl ether applied to a nose cone.

After the rabbits were anesthetized, the abdominal cavities were opened and tracer ethanol was injected into the portal vein, or in one case into one of the large ear veins. Samples of blood were then taken at specific time intervals from the hepatic vein, inferior vena cava and the abdominal aorta. These blood samples were treated in the same manner as the samples obtained from the rats.

Tissue Uptake Studies

The movement of radioactive ethanol and acetic acid into rat muscle tissue was studied by incubating pieces of preweighed rat abdominal muscle in Krebs phosphate Ringer's solution at pH 7.4 containing radioactive ethanol and acetic acid at one or ten millimolar concentrations. The tissues were shaken in small Erlenmeyer flasks for various time intervals and the contents of the flasks were dumped quickly onto a small strainer and given several quick rinses with aqueous solutions containing nonradioactive ethanol and acetic acid. Then, after blotting, the tissues were placed in tubes containing a known amount of absolute ethanol. After several days' equilibration the supernatant fraction of the solution was analyzed for radioactivity.

Abdominal skins obtained from Rana pipiens purchased from the Carolina Biological Supply in Gladstone, Oregon, were mounted between glass chambers containing Krebs phosphate Ringer's solution at pH 8.0. These frog skins were used to compare the movement of radioactive ethanol and acetic acid through a series of biological membranes. One or ten millimolar solutions of ethanol and acetic acid were placed on the outside of the skin with plain Ringer's solution on the inside. After a suitable equilibration period, samples were taken at specific time intervals from the inside compartment and these samples were analyzed for radioactivity.

Radio Assay Techniques

The radioactive carbon dioxide, produced from carbon-14 substrates, except acetaldehyde in the in vitro experiments, was analyzed using a dry plate technique which was developed here. The centerwells, containing 0.2 ml. of 6N NaOH and filter paper from the incubation flasks were dropped into test tubes containing 9.8 ml. distilled water. After mixing, the contents of the tubes were allowed to stand overnight and the next day 0.5 ml. aliquots of the alkaline solution were removed from the tubes and placed on stainless steel planchets containing small circles of lens tissue soaked with 0.2 ml. of "glue" solution. The planchets were then dried in a 110° C oven.

It was found that small amounts of radioactive acetaldehyde from the bathing solution would condense on the filter paper in the centerwells during incubation. (See Table 4.) This con-

TABLE III.

RECOVERY OF ACETIC ACID-^{14}C AND ETHANOL-^{14}C BY GAS CHROMATOGRAPHY

compound	quantity injected	cpm	cpm recovered	% recovery
acetic acid*	300 µl.	50,410	36,370	72.2
acetic acid	" µl.	50,410	38,494	76.4
acetic acid	" µl.	50,410	38,581	76.5
ethanol	" µl.	33,700	25,950	77.0
ethanol	" µl.	33,700	24,549	72.8
ethanol	" µl.	33,700	25,423	75.4

* samples injected as a solution of acetic acid:ethanol:water 9:10:1

TABLE IV.

CONTAMINATION BY TRACER ACETALDEHYDE AND ETHANOL OF CENTERWELLS DURING WARBURG EXPERIMENTS

tracer compound	cpm added	cpm recovered	% recovery
acetaldehyde	37,822	1070	2.82
acetaldehyde	37,822	1315	3.47
acetaldehyde	37,822	1700	4.49
ethanol	30,700	244	0.85
ethanol	30,700	192	0.63
ethanol	30,700	266	0.87

TABLE V.

LOSS OF TRACER ACETALDEHYDE FROM THE BUFFER DURING WARBURG EXPERIMENTS

tracer	cpm added	cpm recovered	% loss
acetaldehyde	11184	10436	6.6
acetaldehyde	11184	10639	4.9

densed acetaldehyde would later polymerize and remain on the lens tissue if the dry plate method of analysis were used. To avoid this, the carbon dioxide fractions obtained from incubations using acetaldehyde as the substrate were analyzed using a $BaCO_3$ technique developed in this laboratory. The loss of substrate acetaldehyde was further substantiated by the collection of radioactive acetaldehyde in the centerwells of flasks containing only buffer and substrate. Table 5 shows the results of these experiments.

Barium carbonate and dry plate samples were counted on a Nuclear Chicago Automatic C115 Low Background Gas Flow System Counter. This counter automatically changes the samples and prints the numbers of the samples and the time required to count a predetermined number of counts. This particular machine employs both an anticoincidence circuit and extra-heavy shielding to achieve a low background count rate. The low background feature was of prime importance when counting samples contained only a small amount of radioactivity.

Lipid extracts and samples obtained from the gas chromatograph were radioassayed with a Packard Tricarb Liquid Scintillation Spectrometer, Model 3000. This instrument is designed to count automatically groups of samples and to print the number of each sample as it is radioassayed, the time it was counted and the number of counts registered in each channel.

The particular instrument used has two channels which can be used to measure two β-emitting isotopes simultaneously if the energies of their emissions differ sufficiently. This feature

made it possible to count samples containing both carbon-14 and tritium. When both isotopes were used the efficiency of carbon-14 was about 40% and that of tritium about 14%.

The discrimination ratio technique worked out by Okita, et al., was used to assay radioactive carbon and hydrogen simultaneously when both isotopes were used (67).

Labeling Ratio Technique

The labeling ratio approach used in this thesis has been adopted from the work of Russell and Van Bruggen (69). If ethanol is converted quantitatively to acetate or acetyl CoA, the ratio of the incorporations of ^{14}C into a receptor metabolite from ethanol-1-^{14}C (E_1) and ethanol-2-^{14}C (E_2) should equal the ratio of the incorporations from acetate-1-^{14}C (A_1) and acetate-2-^{14}C (A_2). If the two carbons retain their positional identities, and if the carbon-1 position of ethanol is converted to the carbon-1 position of acetate, then in reactions where A_1/A_2 equals 1.0, E_1/E_2 must also equal 1.0. In this ratio evaluation, it is not important to have $E_1 = A_1$ or $E_2 = A_2$; it is only necessary for E_1/E_2 to equal A_1/A_2. Comparisons of the ratios permits accurate evaluations even if known or unknown intermediates between ethanol and acetate are formed or membrane permeability differences to acetate and ethanol exist, and permit the flow of label through variably sized "pools" of metabolites.

III. RESULTS

Oxygen consumption by the preparations in vitro was measured and recorded in μmoles/gram/hr. The results of the oxygen consumption studies, expressed as the means ± one standard deviation, are shown in Table VI. As is readily apparent from the data, marked differences exist in the capacity of the individual tissues to consume oxygen; this presumably relates to their metabolic activity. The kidney has the highest rate of oxygen consumption (129.2 ± 11.1 μmoles/gram/hr.), whereas the diaphragm has the lowest (49.6 ± 5.1). Oxygen consumption was not always constant in the same tissue from different animals. The heart slice preparations show this by the magnitude of the figure for standard deviation.

The results relating to the ability of the different tissues examined to convert ethanol and acetate into CO_2 are reported in Table VII. Great differences exist in the relative capacities of the individual tissues to utilize these substrates in the Krebs cycle, which is presumably the major route which converts these compounds into carbon dioxide fractions.

Because the resultant radioactivity found in the carbon dioxide fraction was very low in brain tissue when ethanol was the substrate, a dry plate technique was required to make certain that radioactivity was not contamination. In contrast to the low conversion rate for ethanol metabolism by the brain tissue, the kidney and liver were capable of converting large amounts of both ethanol and acetate into carbon dioxide. Representative tissues from different

TABLE VI.

OXYGEN UTILIZATION IN TISSUE SLICE PREPARATIONS

Tissue	Number of Flasks	O_2 Consumption*
Brain	40	52.3 ± 2.7
Diaphragm	36	49.6 ± 5.1
Heart	36	100.7 ± 23.6
Liver	56	80.1 ± 8.2
Kidney	50	129.2 ± 11.1
Spleen	32	54.7 ± 1.7

*μmoles/gram/hr.

TABLE VII.

COMPARISON OF VARIOUS SLICE PREPARATIONS REGARDING THEIR CONVERSION OF ACETATE-^{14}C AND ETHANOL-^{14}C INTO $^{14}CO_2$

Tissue	Tracer Substrates *			
	A_1	A_2	E_1	E_2
Brain	3.6 ± 0.2** (9) ***	1.8 ± 0.2 (10)	< 0.04 (11)	< 0.02 (10)
Diaphragm	23.4 ± 2.3 (8)	13.7 ± 3.0 (8)	0.40 ± 0.2 (10)	0.24 ± 0.1 (10)
Heart	45.2 ± 2.2 (8)	38.6 ± 5.0 (8)	0.84 ± 0.2 (10)	0.68 ± 0.1 (10)
Kidney	50.3 ± 8.8 (10)	31.6 ± 3.8 (10)	19.1 ± 4.3 (10)	11.6 ± 2 (10)
Liver	10.1 ± 2.0 (14)	3.4 ± 0.7 (14)	16 ± 3.1 (14)	6.3 ± 0.9 (14)
Lung	14.3 ± 1.0 (7)	6.7 ± 0.7 (7)	1.5 ± 0.6 (8)	0.69 ± 0.3 (8)
Spleen	17.9 ± 1.1 (8)	7.2 ± 0.5 (8)	0.7 ± 0.1 (8)	0.30 ± 0.0 (8)

* A_1 = acetate-1-C^{14}; A_2 = acetate-2-C^{14}; E_1 = ethanol-1-C^{14}, ethanol-2-C^{14}.

** percent incorporation = $\frac{\text{serial cpm in } CO_2}{\text{initial cpm in substrate}}$ x 100, ± one standard deviation.

100 µg. tissue x hr.

*** denotes the number of experiments or individual flasks used.

TABLE VIII.

INCORPORATION OF ACETALDEHYDE 1-2-^{14}C INTO $^{14}CO_2$

Tissue	Flasks	Percent Incorporation*
Brain	4	2.30 ± 0.43*
Diaphragm	4	14.7 ± 3.9
Heart	4	29.5 ± 2.8
Kidney	6	31.2 ± 8.5
Liver	6	5.50 ± 1.80
Lung	5	9.16 ± 2.60
Spleen	4	7.72 ± 0.61

* percent incorporation/100 mg./hr., ± one standard deviation

TABLE IX.

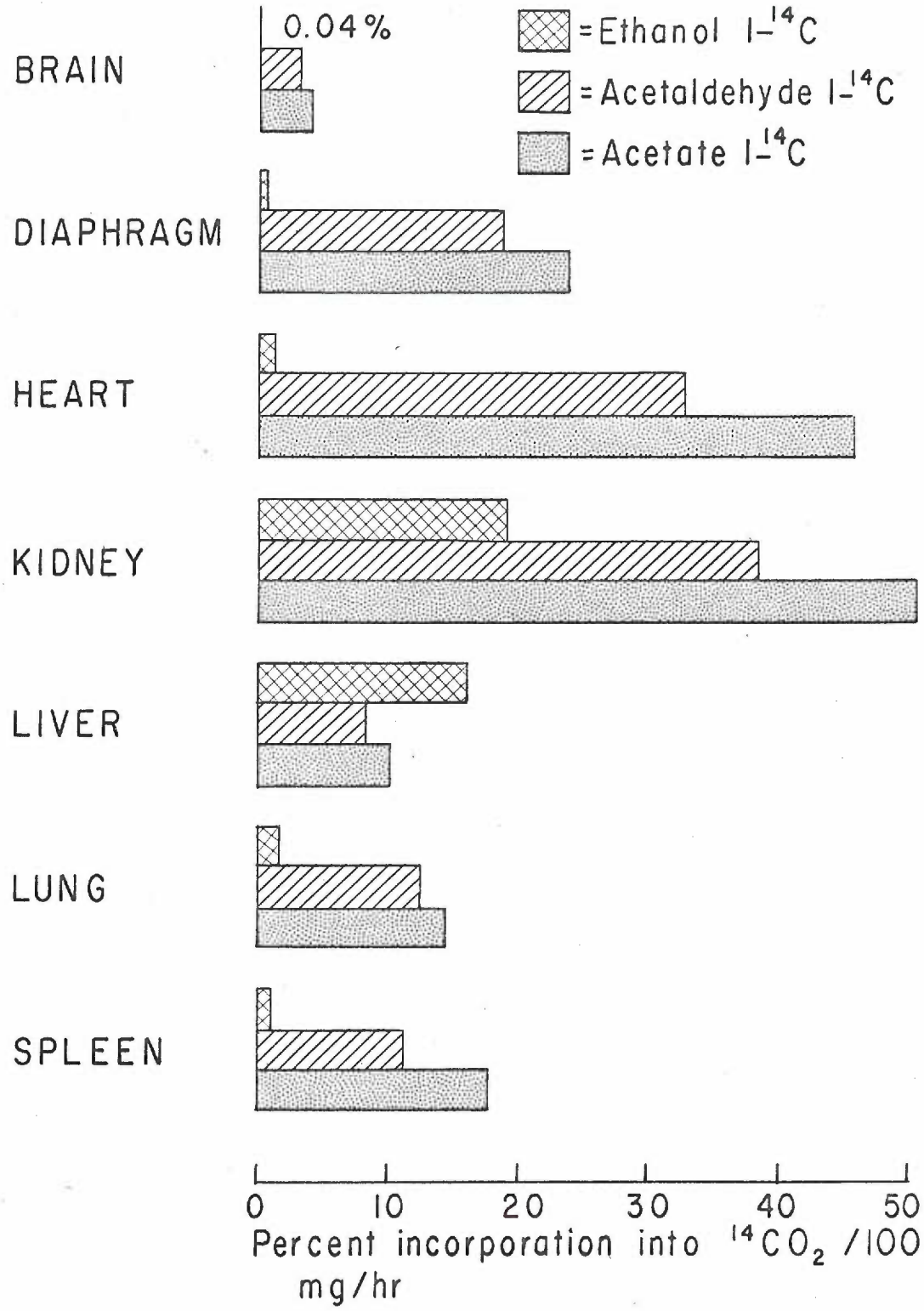
RATIOS OF PERCENT INCORPORATION OF C-1 AND C-2 OF RADIOACTIVE ACETATE AND ETHANOL OBTAINED FOR $^{14}CO_2$ IN VARIOUS TISSUE SLICE PREPARATIONS

Tissue	Substrates	
	A_1/A_2	E_1E_2
Brain	3.60/1.80 = 2.0	0.04/0.02 = 2.0
Diaphragm	23.4/13.7 = 1.7	0.40/0.24 = 1.7
Heart	45.2/38.6 = 1.2	0.84/0.68 = 1.2
Kidney	50.3/31.6 = 1.6	19.1/11.6 = 1.6
Liver	10.1/3.4 = 2.9	19/6.3 = 2.6
Lung	14.3/6.7 = 2.1	1.5/0.69 = 2.2
Spleen	17.8/7.2 = 2.5	0.77/0.30 = 2.6

Figure III

Bar graph representing the percent incorporation of ethanol-1-^{14}C, acetaldehyde-1-^{14}C and acetate-1-^{14}C into $^{14}CO_2$ by slice preparation

The incorporation values for acetaldehyde-1-^{14}C were estimated values as acetaldehyde-1-2-^{14}C was the actual substrate. The computations required in estimating these values are discussed in the text.


0.04%
= Ethanol 1-14C
= Acetaldehyde 1-14C
= Acetate 1-14C
BRAIN
DIAPHRAGM
HEART
KIDNEY
LIVER
LUNG
SPLEEN
0
10
20
30
40
50
Percent incorporation into 14CO2 /100 mg/hr

parts of the body were used so that this in vitro work might provide useful information when combined with in vivo data and provide insight as to how ethanol is metabolized in the intact animal.

The metabolism of acetaldehyde was studied in a number of slice preparations and the results of these experiments are represented in Table VIII. Acetaldehyde presented some difficulties owing to its low boiling point, 20° C, and its polymerization in the alkali contained within the centerwell of the Warburg flask. These difficulties were overcome by using the barium carbonate precipitation technique which specifically measured radioactive carbon dioxide, since radioactive organic contaminants are washed free of the precipitated barium carbonate during the washing with organic solvent. Even though acetaldehyde boils at less than 37° C (the temperature of incubation), because of the small amount of this compound present, the bulk of the acetaldehyde stayed in the buffer phase as was shown in Table III. It is interesting to note that acetaldehyde is definitely metabolized in all of the tissues examined.

The ratios of the percent incorporation into CO_2 of acetate-1-^{14}C/acetate-2-^{14}C and ethanol-1-^{14}C/ethanol-2-^{14}C are given in Table IX. The ratios provide useful information in assessing the biosynthetic capacity of each of these tissues. High ratios can be regarded as the result of increased synthesis by means of the Krebs cycle. This point will be further discussed below. As might be expected the lowest ratios were obtained with heart slices and the highest ratios with liver and spleen preparation.

Figure 3 was drawn from a part of the data represented in

Tables VII and VIII, and it has been constructed to give a quick idea of the relative ability of each of the tissues examined to metabolize ethanol-1-^{14}C, and acetate-1-^{14}C to $^{14}CO_2$. The incorporation of acetaldehyde-1-^{14}C into CO_2 has been calculated from the data obtained from the incorporation of acetaldehyde-1,2-^{14}C and from the ratios of incorporation of ethanol-1-^{14}C to ethanol-2-^{14}C and acetate-1-^{14}C to acetate-2-^{14}C into carbon dioxide. A sample calculation with the results obtained from diaphragm has been provided to illustrate how these values are determined. It is assumed that the actual ratios that would be obtained for the incorporation of acetaldehyde-1-^{14}C and acetaldehyde-2-^{14}C would be the same as those obtained for ethanol and acetate.

let x = the theoretical amount of $^{14}CO_2$ derived from the one position of acetaldehyde-1-2-^{14}C

y = the theoretical amount of $^{14}CO_2$ derived from the two positions of acetaldehyde-1-2-^{14}C

the value $x = 1.7\,y$ is obtained from ratios of incorporation of A_1/A_2 and ζ_1/ζ_2 into $^{14}CO_2$ in Table IX and $x + y = 14.7$ from Table VIII where 14.7 equals the present incorporation of acetaldehyde-1-2-^{14}C into carbon dioxide

$x + y = 14.7$ $\qquad$ $x = 1.7\,y$

$1.7\,y + y = 14.7$

$y = 5.4$ $\qquad$ substituting back for x

$x = 9.3$

TABLE X.

INCORPORATION OF VARIOUS SUBSTRATES INTO CO_2 BY SLICE PREPARATIONS, EXPRESSED AS BOTH PERCENTAGE AND RATIOS

Tissue	$\frac{\text{ethanol-1-}^{14}C}{\text{acetate-1-}^{14}C}$			$\frac{\text{ethanol-2-}^{14}C}{\text{acetate-2-}^{14}C}$		
Brain	$\frac{0.0400}{3.6}$	=	0.011	$\frac{0.02}{1.8}$	=	0.011
Diaphragm	$\frac{0.40}{23.4}$	=	0.017	$\frac{0.24}{13.7}$	=	0.018
Heart	$\frac{0.84}{45.2}$	=	0.0185	$\frac{0.68}{38.6}$	=	0.0176
Kidney	$\frac{19.1}{50.3}$	=	0.380	$\frac{11.6}{31.6}$	=	0.370
Liver	$\frac{16.0}{10.1}$	=	1.58	$\frac{6.30}{3.40}$	=	1.85
Lung	$\frac{1.5}{14.3}$	=	0.105	$\frac{0.69}{6.7}$	=	0.103
Spleen	$\frac{0.70}{17.9}$	=	0.039	$\frac{0.30}{7.2}$	=	0.042

TABLE XI.

INCORPORATION OF ^{14}C-ACETATE AND ^{14}C-ETHANOL INTO THE NONSAPONIFIABLE FRACTIONS IN TISSUE SLICE PREPARATIONS.

Tissue	Flasks	Incorporation* Substrates	
		Ethanol	Acetate
Brain	8	0.0034 ± 0.0016	0.128 ± 0.04
Diaphragm	8	0.0057 ± 0.0012	0.51 ± 0.10
Liver	8	2.97 ± 1.71	1.29 ± 0.59
Kidney	10	0.05 ± 0.028	0.124 ± 0.05

*percent incorporation/100 mg./hr.

TABLE XII.

INCORPORATION OF ^{14}C-ACETATE AND ^{14}C-ETHANOL INTO THE FATTY ACID FRACTION IN SLICE PREPARATIONS

Tissue	Flasks	Incorporation* Substrates	
		Ethanol	Acetate
Brain	8	0.0028 ± 0.0007	0.0145 ± 0.003
Diaphragm	8	0.00065 ± 0.0002	0.060 ± 0.018
Liver	8	3.63 ± 1.38	1.93 ± 0.77
Kidney	10	0.05 ± 0.02	0.15 ± 0.26

* percent incorporation/100 mgs. tissue/hr.

Then considering the hypothetical acetaldehyde-1-^{14}C to have the same initial specific activity as the actual uniformy labeled substrate, but now having all of the label in the one position one obtains a figure for the percent incorporation value of acetaldehyde-1-^{14}C into the CO_2 fraction of diaphragm 3 to be 18.6 (2x9.3 = 18.6).

The ratios of the ^{14}C incorporated into carbon dioxide from E-1, E-2, A-1 and A-2 are given in Table X. This table gives numerical values relating the differences in the abilities of these tissues to metabolize ethanol and acetate into carbon dioxide. These ratios show the vast differences that exist in the capacity of these tissues to metabolize ethanol and acetate into carbon dioxide. These ratios show the vast differences that exist in the capacity of these tissues to metabolize ethanol and acetate.

The percentage incorporation of ethanol-^{14}C and acetate-^{14}C into the nonsaponifiable fractions of brain, diaphragm, liver and kidney are given in Table XI. The liver demonstrates the greatest ability to incorporate tracer acetate and ethanol into this tissue fraction, which is generally considered to be largely cholesterol. Ethanol is incorporated better than acetate into the nonsaponifiable fraction in the case of the liver slices. In all other slice preparations acetate-^{14}C is observed to be a better precursor for the nonsaponifiable fractions than ethanol.

The incorporation of ^{14}C ethanol and acetate into the fatty acid fractions of brain, diaphragm, liver and kidney is given in Table XII. As observed in the last table, the liver is better able to convert acetate and ethanol into the lipid fractions than the other tis-

TABLE XIII.

THE METABOLISMS OF ACETATE AND ETHANOL BY DIAPHRAGM MUSCLE WITH OR WITHOUT THE ADDITION OF LIVER SLICES

Tissue	Fraction			
	Nonsaponifiable		Fatty Acid	
Label	^{14}C-E	^{3}H-A	^{14}C-E	^{3}H-A
Diaphragm	0.0017	0.008	0.0037	0.031
	0.0018	0.008	0.0043	0.021
	0.0011	0.008	0.0102	0.107
	0.0010	0.009	0.0097	0.080
$\bar{x}$	0.0014*	0.0082	0.0070	0.059
Diaphragm + Liver	0.0078	0.0056	0.034	0.025
	0.0166	0.0110	0.095	0.066
	0.0072	0.0025	0.054	0.043
	0.0093	0.0069	0.018	0.033
$\bar{x}$	0.0102	0.0065	0.050	0.043

*percent incorporation/100 mg./hr.

TABLE XIV.

APPARENT PERMEABILITY COEFFICIENTS FOR ETHANOL AND ACETATE WITH THE MOUNTED FROG-SKIN*

^{14}C-Ethanol (cm. hr.$^{-1}$)	^{14}C-Acetate (cm. hr.$^{-1}$)
0.1742	0.00226
0.1260	0.00234
0.1840	0.00099
0.1810	0.00098
0.1933	0.00106
0.1730	0.00159
$\bar{x}$ = 0.1719	$\bar{x}$ = 0.00153

*concentration 10 mMolar

sues, and this tissue utilizes ethanol better than acetate as a precursor for fatty acid biosynthesis.

The metabolism of ethanol and acetate by diaphragm muscle is represented in Table XIII. The amount of radioactive ethanol incorporated into both the nonsaponifiable and the fatty acid fractions of diaphragm is seen to increase when both diaphragm and liver slices were included in the same incubation flask. This was not found to be true for acetate. Acetate-^{14}C experiments were included to compare not only the ability of the diaphragm to utilize this substrate in the synthesis of lipid but also to rule out the possibility that the increased incorporation of ethanol into diaphragm lipid in the presence of liver slices was due to contamination of the diaphragm slices with small pieces of liver. Since there was no increase in the incorporation of acetate into diaphragm lipid in the presence of liver slices, it is assumed that the amount of contamination is negligible.

In considering the possibility that differences in the rate of permeability of acetate and ethanol in crossing biological membranes may exist, it was thought desirable to set up a model system to test this possibility. The "mounted" frog skin as previously described was chosen since this system was available in this laboratory and is a simple system to manipulate. To determine the permeability coefficients for ethanol and acetate, their rates of movement were measured over periods of several hours. The coefficients for ethanol and acetate are given in Table XIV. Ethanol is seen to move through the biological membrane at a rate of more than one hundred times that of acetate.

The uptake of acetate and ethanol has been studied in strips

FIGURE XV.

UPTAKE OF ^{14}C-ACETATE AND ^{14}C-ETHANOL BY RAT ABDOMINAL MUSCLE*

Compound	t (minutes)	Uptake µmoles/gram	Mean Uptake µmoles/gram
acetate	1	0.71	
		0.63	0.69
		0.73	
ethanol	1	1.35	
		1.37	1.48
		1.74	
acetate	2	0.66	
	2	0.72	0.69
	2	0.69	
ethanol	2	1.77	
	2	1.91	1.82
	2	1.77	
acetate	5	1.29	
	5	1.05	1.14
	5	1.08	
ethanol	5	3.11	
	5	2.86	3.16
	5	3.51	

*initial concentrations 10 mMolar

TABLE XVI.

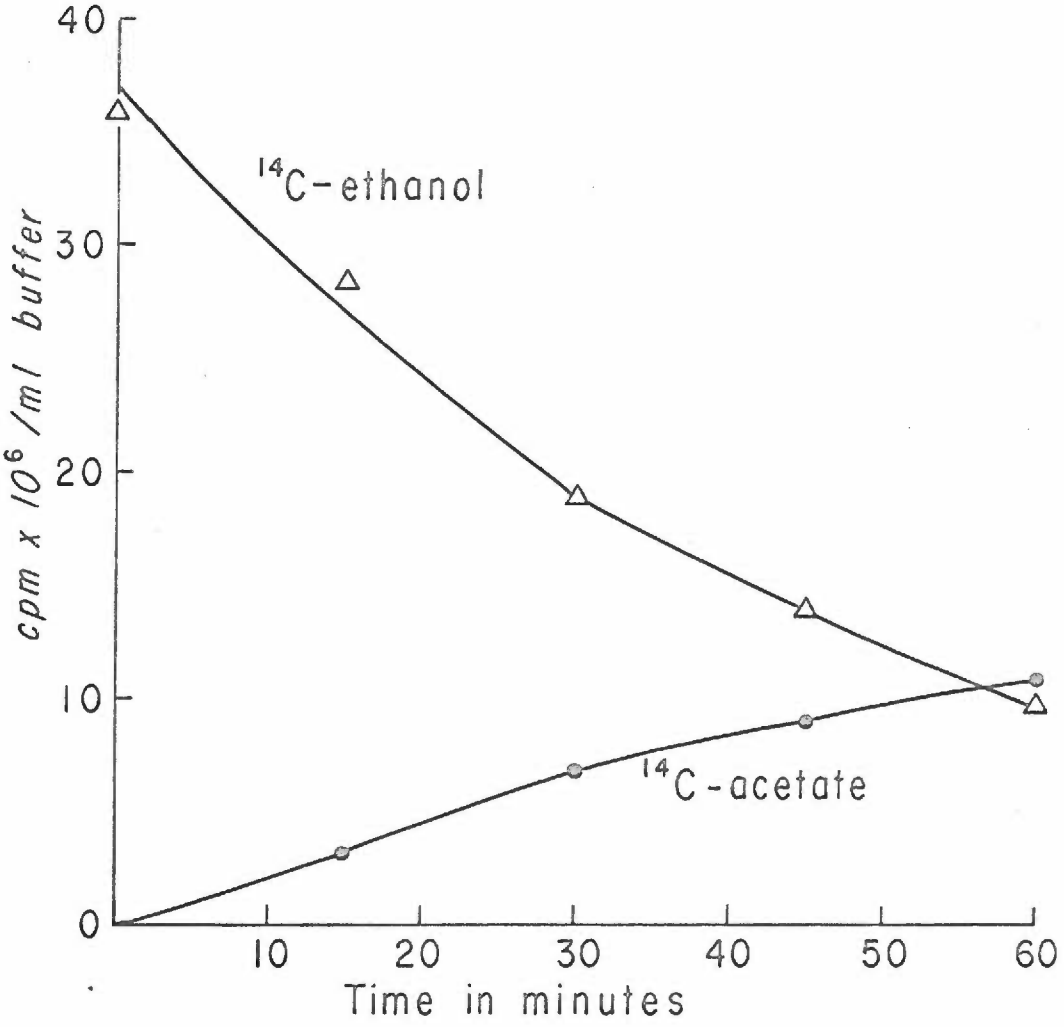
^{3}H-ETHANOL AND ^{14}C-ACETATE UPTAKE IN RAT LIVER SLICES*

Ethanol (μmoles/gram)	Acetate (μmoles/gram)
2.31	1.29
2.65	1.41
2.34	1.08
$\bar{x}$ = 2.43	$\bar{x}$ = 1.26

*incubation time 1 minute, concentrations 10 mMolar

Figure IV

Conversion of ^{14}C-ethanol to ^{14}C-acetate by rat liver slices


40
30
20
10
0
cpm x 10⁶ /ml buffer
¹⁴C-ethanol
¹⁴C-acetate
10
20
30
40
50
60
Time in minutes

of rat abdominal muscle. The results of these experiments are given in Figure XV. The uptake of ethanol is shown to be two to three times that of acetate in this preparation.

The uptake of tritium labeled ethanol and carbon-14 labeled acetate was studied simultaneously in rat liver slices. The results of these experiments are given in Figure XVI. Because of the very high permeability of ethanol and acetate into liver slices the incubation times were reduced to one minute. As in the case of the strips of muscle, ethanol moves into the tissue two times faster than acetate.

The rate of conversion of ethanol to acetate has been studied in liver slice preparations; the results of a representative experiment are presented in Figure IV. Samples were taken from the buffer solution at fifteen-minute intervals. The results are expressed as counts per minute per milliliter of buffer. During the first interval a substantial amount of ethanol was already removed from the media and a large portion of it could be accounted for by its conversion to radioactive acetic acid. After sixty minutes the amount of radioactive ethanol and acetate are approximately equal.

The in vivo metabolism of ethanol was studied in experiments where rats were given an intraportal injection of 20 μc. radioactive ethanol. At specified intervals after the injection of the tracer ethanol, blood samples were taken by means of heart puncture. The samples of blood were measured and immediately placed in tubes containing a known amount of ethanol and acetic acid. The samples were then centrifuged to precipitate the protein. Aliquots of the supernatant fraction were injected into the gas chromatograph for fractionation of

Figure VI

Time course of ^{14}C-ethanol and ^{14}C-acetate activity in the hepatic and the inferior vena cava veins of a rat following the intraportal administration of radioactive ethanol

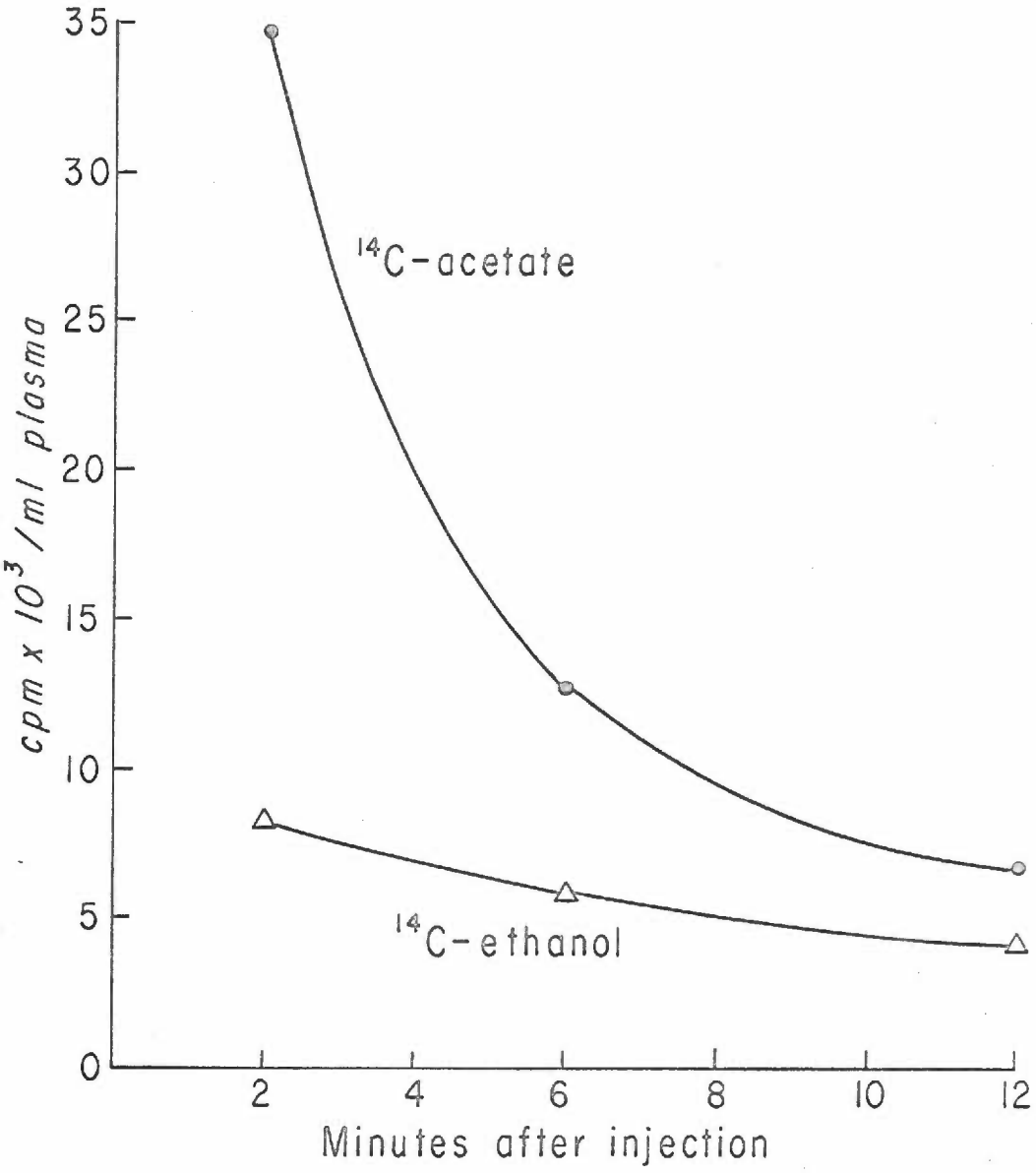

35
30
25
20
15
10
5
0
cpm x 10³/ml plasma
¹⁴C-acetate
¹⁴C-ethanol
2
4
6
8
10
12
Minutes after injection

Figure V

^{14}C-ethanol and ^{14}C-acetate time course curves in rat heart blood following the intraportal administration of ^{14}C-ethanol

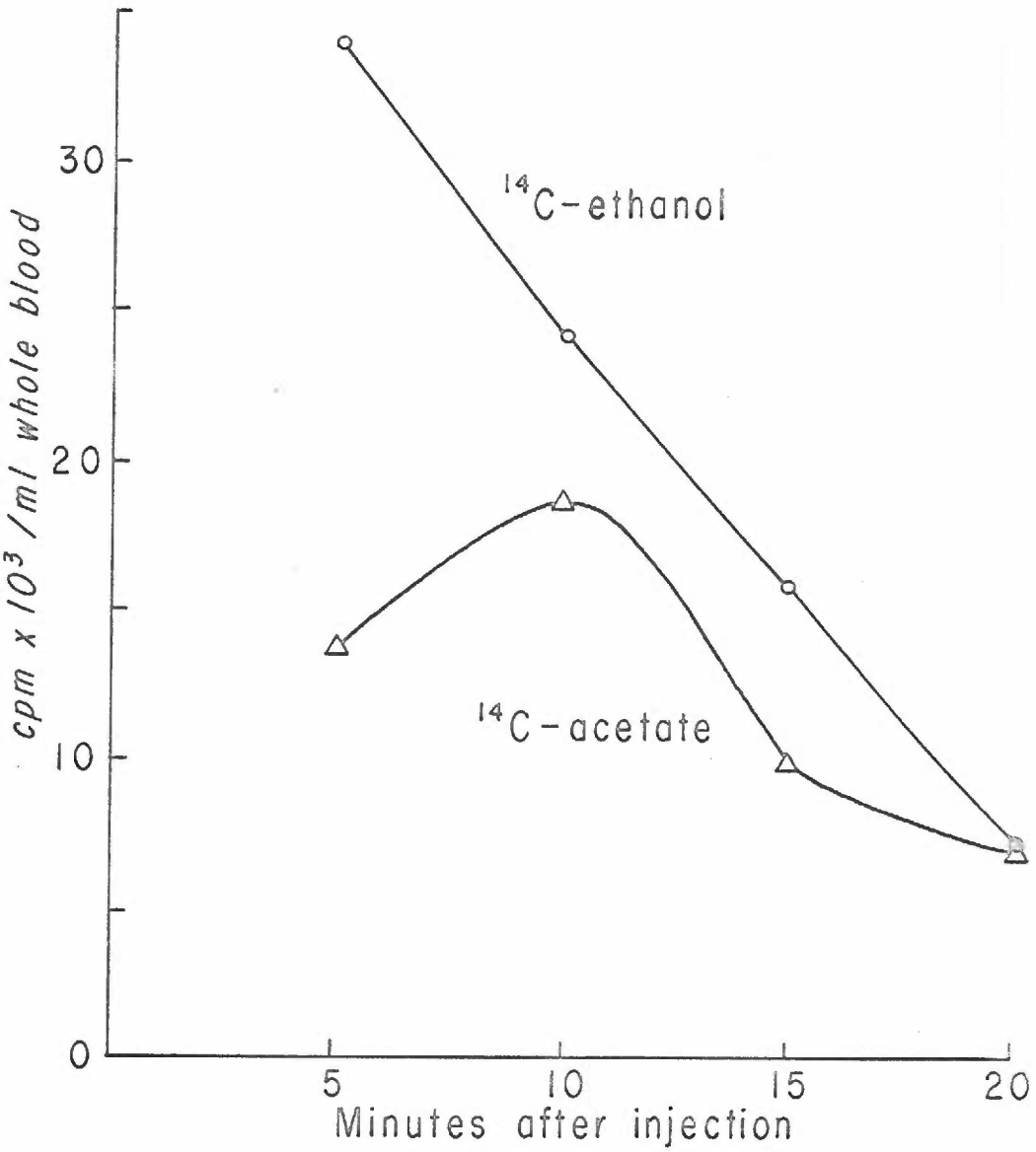

cpm x 10³ /ml whole blood
30
20
10
0
5
10
15
20
Minutes after injection
¹⁴C-ethanol
¹⁴C-acetate

Figure VII

^{14}C-acetate time course curve in the hepatic blood of a rat following ^{14}C-acetate administration via the portal system

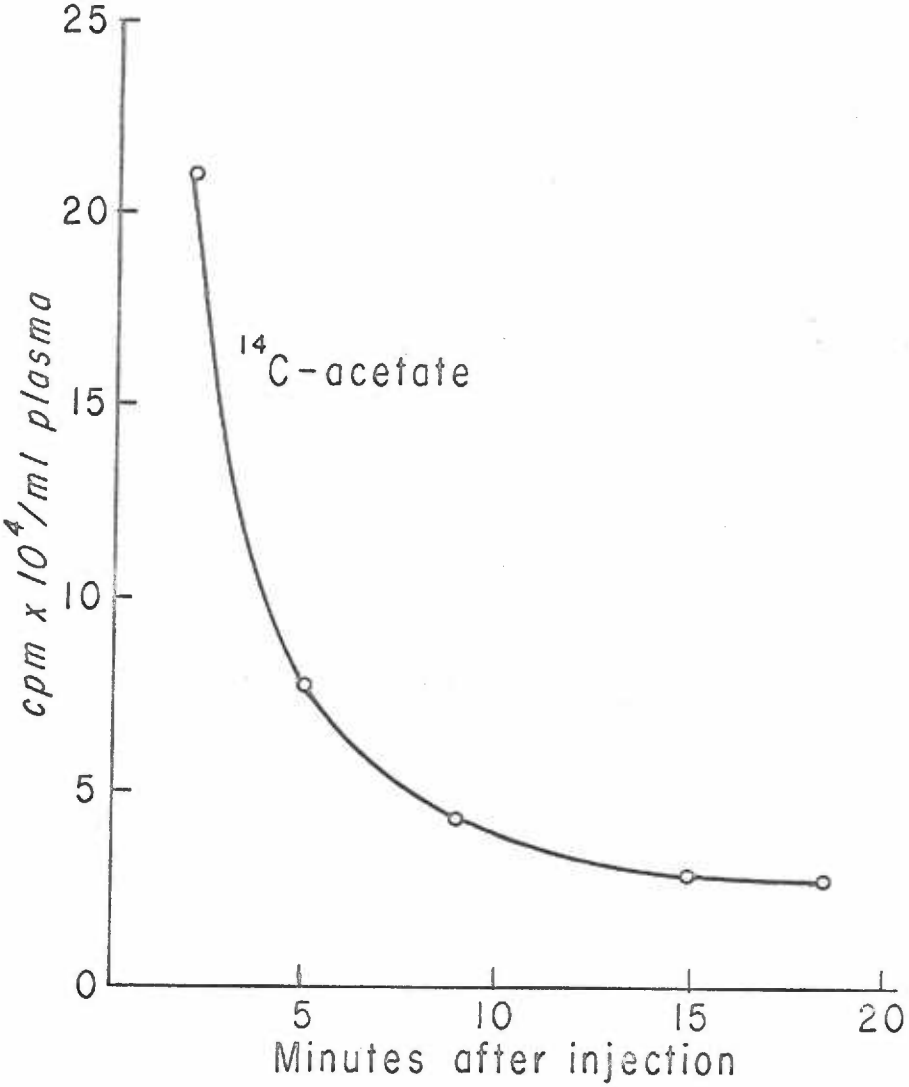

25
20
15
10
5
0
cpm x 10⁴/ml plasma
¹⁴C-acetate
5
10
15
20
Minutes after injection

Figure VIII

Time course of ^{14}C-ethanol and ^{14}C-acetate activities in the hepatic blood following the injection of ^{14}C-ethanol into the portal vein of a rabbit

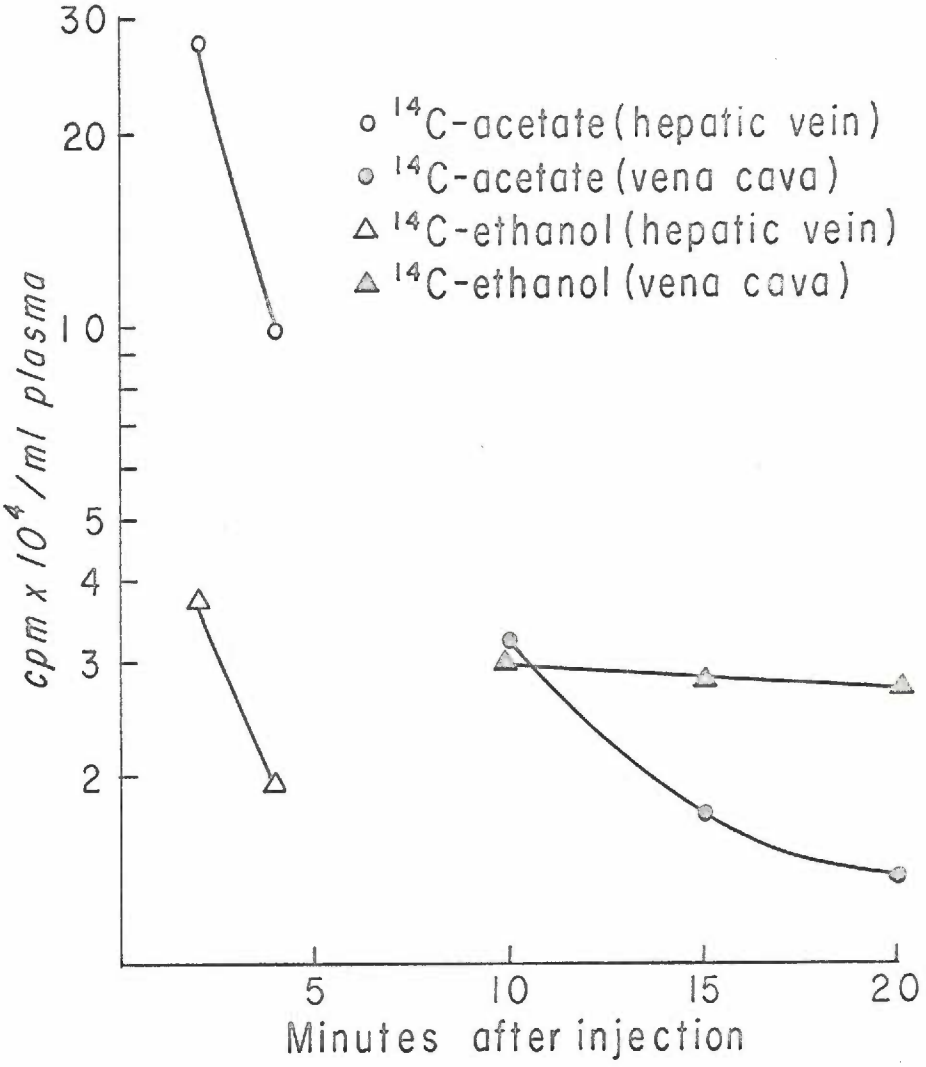

30
20
10
5
4
3
2
cpm x 10⁴/ml plasma
¹⁴C-acetate (hepatic vein)
¹⁴C-acetate (vena cava)
¹⁴C-ethanol (hepatic vein)
¹⁴C-ethanol (vena cava)
5
10
15
20
Minutes after injection

Figure IX

Time course of curves of ^{14}C-ethanol and ^{14}C-acetate in venous and arterial blood after the intraportal administration of ^{14}C-ethanol to the rabbit

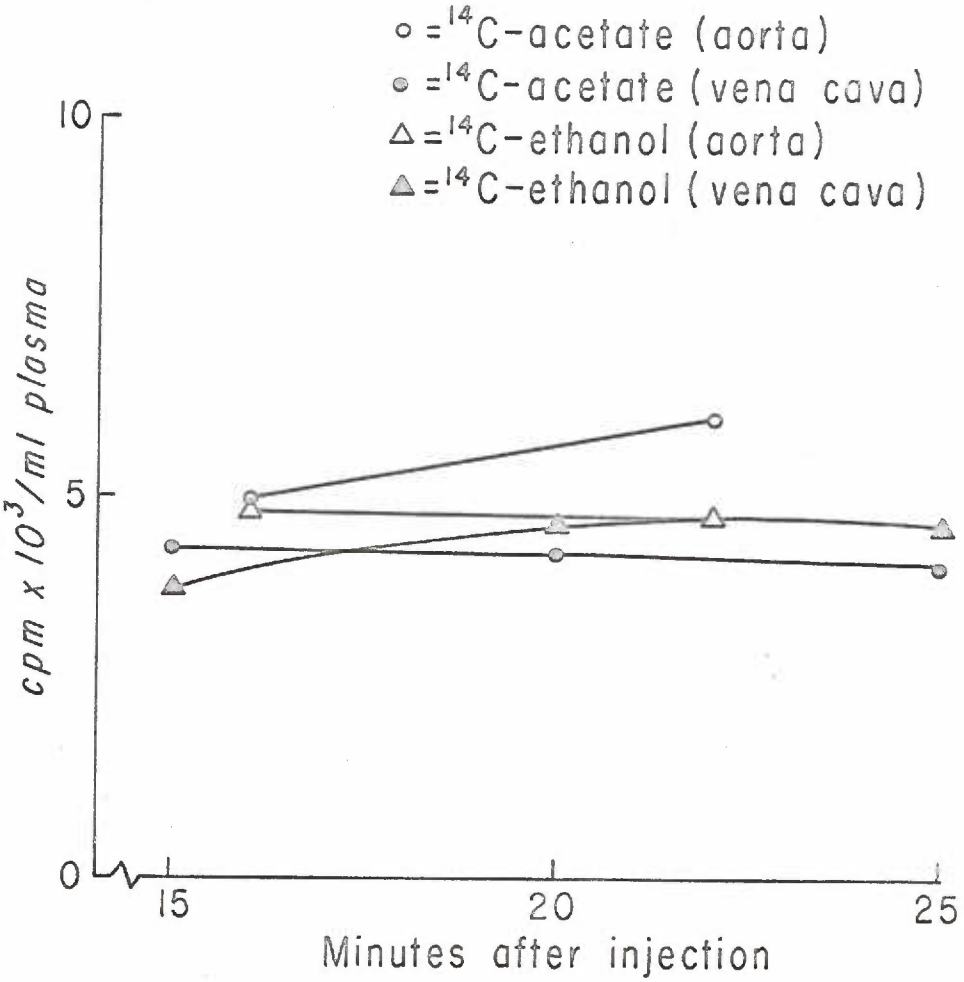
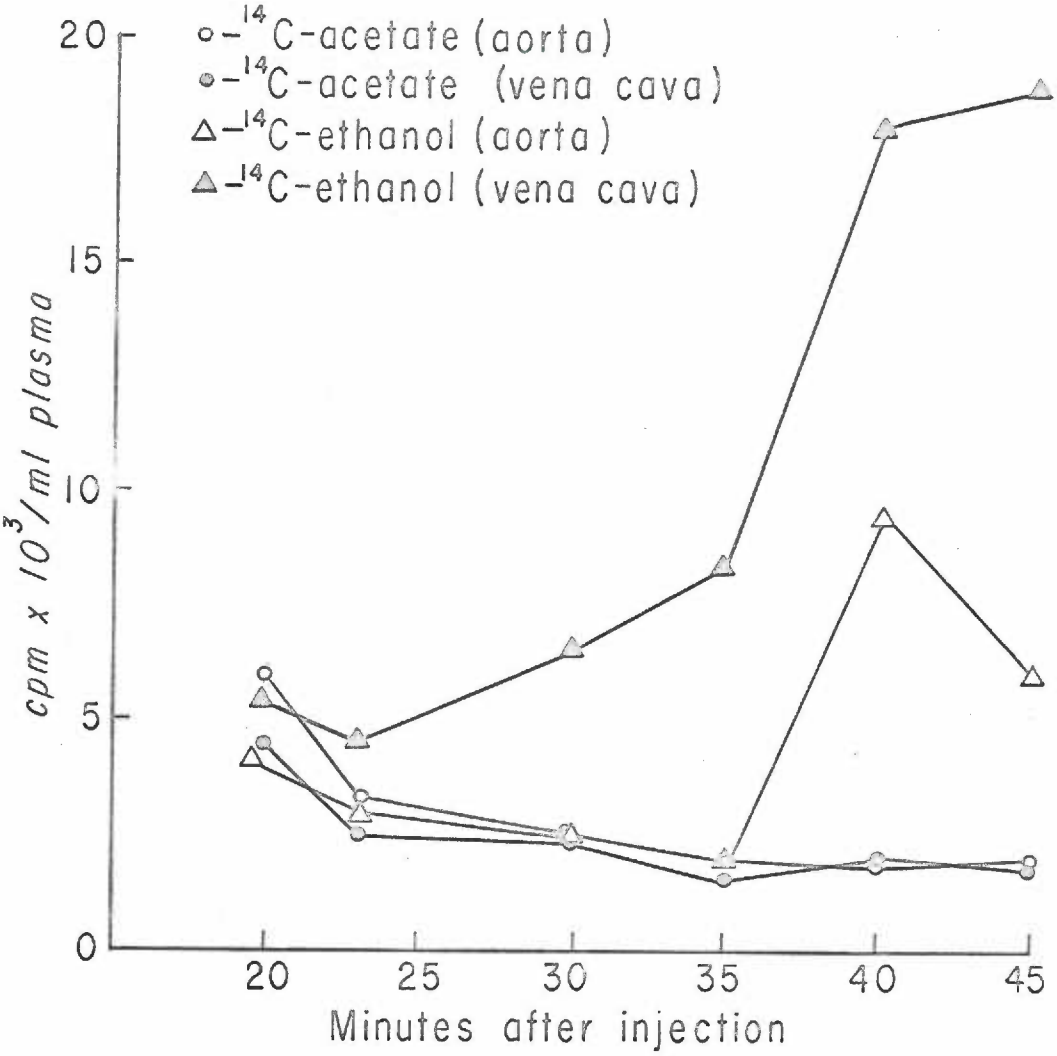

○ = 14C-acetate (aorta)
● = 14C-acetate (vena cava)
△ = 14C-ethanol (aorta)
▲ = 14C-ethanol (vena cava)
10
5
0
cpm x 10³/ml plasma
15
20
25
Minutes after injection

Figure X

Time course of ^{14}C-ethanol and ^{14}C-acetate activity in venous and arterial blood following injection of ^{14}C-ethanol into an ear vein of a rabbit


20
15
10
5
0
cpm x 10³/ml plasma
o–¹⁴C-acetate (aorta)
•–¹⁴C-acetate (vena cava)
△–¹⁴C-ethanol (aorta)
▲–¹⁴C-ethanol (vena cava)
20
25
30
35
40
45
Minutes after injection

the ethanol and acetic acid. The respective fractions were then collected and radioassayed as described in the methods section on page 23. The results of one such experiment are given in Figure V.

In an attempt to measure more directly the conversion of ethanol to acetate in the liver, blood samples were taken directly from the hepatic vein and inferior vena cava after the intraportal administration of 20 μc. of ethanol-^{14}C. Results from the experiments are given in Figure VI. It is apparent that 2.5 minutes, after injection, the amount of radioactive acetate appearing in the blood far exceeds the amount of radioactivity present as ethanol. Only a few samples were taken from the hepatic vein, the majority from the inferior vena cava.

The time course of a single injection of radioactive acetate is given in Figure VII. 20 μc. of acetate-^{14}C were injected into the portal vein of a rat weighing 300 grams. Samples were taken from the hepatic vein.

In several experiments rabbits were used to study the in vivo time course of radioactive ethanol and acetate. The rabbits provided several advantages: larger veins, and a larger volume of blood from which to sample. 2 minutes after intraportal administration of 20 μc. of ethanol-^{14}C, there is much more radioactivity in the form of acetate than ethanol in the hepatic vein.

Figure IX has been included to point out that there is very little extrahepatic utilization of ethanol and that almost a steady state concentration of radioactive ethanol and acetate is reached in the blood after 15 minutes of equilibration.

In one experiment an anesthetized rabbit received 20 μc. of

ethanol-^{14}C by injection into one of the larger ear veins. Several important things are evident from this experiment: one is the stepwise decrease of the amount of radioactivity present in the acetate fraction; secondly, that while taking the samples at 23 minutes after injection, massive hemorrhaging occurred. Immediately following this, there was a marked increase in the amount of radioactive ethanol in the venous blood and later a subsequent increase in arterial blood.

IV. DISCUSSION

1. Conversion of Ethanol, Acetaldehyde and Acetate to CO_2.

One of the contributions of this work is a comparative study of the ability of a number of tissues to metabolize ethanol, acetaldehyde and acetate.

In order to insure that differences found were not due to changes in incubation techniques, buffer systems or tracer concentrations, standard conditions were maintained throughout. Isotope purity was routinely checked. When new techniques of radioassay or extraction were employed they were checked out against techniques previously utilized in this laboratory.

(a) Brain slices.

A number of workers have studied the metabolism of ethanol by brain slices. The results of Table VII show that brain slices possess little or no ability to utilize ethanol as a substrate. The figures of 0.04 percent and 0.02 percent incorporation of ethanol-1-^{14}C and ethanol-2-^{14}C are approaching the limit of sensitivity of the analytical techniques used. It is even possible that some ethanol was nonenzymatically oxidized to acetate in the presence of oxygen and that this acetate was then converted to CO_2. This low utilization of ethanol is in accord with experiments done by Bartlett, et al. (3). The results of Burbridge and Sutherland (10, 85) suggest that brain can utilize ethanol for metabolic reactions but as pointed out previously they measured only the disappearance of ethanol from the medium which might

be accounted for by the uptake of ethanol by the slices.

Since acetaldehyde and acetate are metabolized at a rate of almost 100 times greater than ethanol, the rate limiting step in the conversion of ethanol to CO_2 appears to be oxidation of ethanol to acetaldehyde. Both the enzymes alcohol dehydrogenase and aldehyde dehyrogenase require NAD as a cofactor and thus it is reasonable to assume that the quantity of alcohol dehydrogenase in brain is extremely low. The low rate of acetate and acetaldehyde utilization is not surprising in view of the low oxygen consumption by this tissue and biological function of this tissue.

(b) Diaphragm and heart slices.

Diaphragm strips and heart slices were used to test the ability of muscle to utilize ethanol as a substrate. Diaphragm and heart may be considered representative of muscle in general as far as ethanol, acetaldehyde and acetate metabolism are concerned since similar results were obtained in one experiment abductor muscle.

The low rate of utilization of ethanol by diaphragm and heart preparations is in agreement with the findings of Bartlett, et al. (3). Studies on isolated perfused liver (24, 56, 57) and on eviscerated animals (65, 66) prove that the musculature could play only a very small role in the first step of the oxidation of ethanol.

No report of the rate of acetaldehyde utilization by muscle was found in the literature. It appears the amount of work reported on acetaldehyde metabolism is small in comparison to

the amount relating to ethanol and acetate.

The incorporation of acetate-1-^{14}C into respiratory CO_2 in diaphragm strips (23.4 ± 2.3) is in close agreement with the results obtained by Marcó and Van Bruggen (62) who using this same tissue found 26.4 ± 5.6 percent incorporation. The incorporations of acetate-1-^{14}C reported here for heart slices is substantially greater than the values obtained by Marcó and Van Bruggen, i.e. (45.2 ± 2.2) vs. (34.0 ± 9.4). This difference could be related to differences in the methods of preparation of the slices since their oxygen consumption values (59.8 ± 7.7 μmoles/g./hr.) are much smaller than the figure obtained in the present (100.7 ± 23.6). Muscle can incorporate far greater amounts of labeled acetaldehyde than ethanol into CO_2 indicating that in this tissue the rate limiting step in the conversion of ethanol to CO_2 is the formation of acetaldehyde (cf. brain slices).

(c) Liver slices.

The incorporation of a substantial amount of radioactivity from labeled ethanol into the CO_2 fraction was an expected result. This report is in complete agreement with the findings of others (3, 25, 26, 33, 52, 53, 65, 91) that the liver is the most important tissue in the initial step of the oxidation of ethanol. It is also substantiated by the finding that liver perfused with blood containing ethanol can remove alcohol from the blood at a rate 1/2 to 5/6 of that found in the whole organism. Such experiments have been made in dogs (21), cats (45, 46, 56) and rabbits (34).

The metabolism of acetaldehyde and acetate by liver slices is in agreement with the findings of Lundquist, et al. (53, 55). The percent incorporation of acetate-1-^{14}C into the CO_2 fraction of liver (10.1 $\pm$ 2.0) was similar to the results obtained by Van Bruggen and Emersen (11.7 $\pm$ 0.78) (88) and Marcó, et al. (11.9 $\pm$ 3.99) (62).

(d) Kidney slices.

At first glance the greater ability of the kidney to incorporate radioactivity from ethanol into CO_2 in comparison with liver (Table VII) seems quite remarkable and suggests an important role for the kidney in the oxidation of ethanol. Such a parameter is not, however, an adequate measure of the respective abilities of these organs to carry out alcohol oxidation. In the first place, in the rat there is approximately seven times as much liver tissue as there is kidney and thus the overall oxidizing capacity of the liver is greater. Experiments done by Wartburg and Eppenberger (91) show that acetate appears in the incubation media during the metabolism of ethanol by liver slices but not by kidney slices. They found that the combination of kidney slices with liver homogenate resulted in a 70 percent increase over the computed sum of the separate oxidation rates for ethanol. It appears from these data that in the liver unlike kidney the rate limiting step in the conversion of ethanol to CO_2 is the oxidation of acetate. This would imply that the initial rate of oxidation of ethanol by the liver is higher than shown by the $^{14}CO_2$ results.

(e) Lung slices.

Surprisingly slices of lung tissue were able to incorporate radioactivity from ^{14}C-ethanol into CO_2. The author is unaware of other experiments utilizing this tissue in the study of alcohol metabolism. The incorporation of ethanol-1-^{14}C and ethanol-1-2-^{14}C into the CO_2 fraction in this tissue was somewhat larger than the incorporation by muscle preparations but the quantitative significance of this tissue in the primary oxidation of ethanol is likely to be quite small because of its size in comparison to the muscle. Lundquist, et al. (56) have suggested that during the administration of appreciable amounts of ethanol to intact animals the loss of ethanol through the lungs can be quite sizeable especially in smaller animals whose respiration rate in relation to size is high.

Similar to muscle and brain preparations in lung tissue the rate of utilization of labeled acetaldehyde and acetate for the production of $^{14}CO_2$ is much greater than that of radioactive ethanol. This is in agreement with the proposal that the rate limiting step in the conversion of ethanol to CO_2 is its primary oxidation in the tissues cited.

(f) Spleen slices.

The spleen is another organ, in addition to the lung, which has received very little attention in regard to its ability to utilize ethanol as a substrate for oxidative metabolism. Results given in Table VI I show it has about the same capacity to incorporate radioactivity from ^{14}C-ethanol as most of the other

tissue preparations for ethanol-1-^{14}C and ethanol-2-^{14}C (0.7 ± 0.1, 0.30 ± 0.0, respectively). It can be assumed that like the brain and lung, the spleen can only play a small role in vivo in the oxidation of ethanol. The spleen shares with the muscle, lung and brain preparations an increased ability to utilize acetaldehyde and acetates as substrates in oxidative reactions. The incorporation of radioactivity into $^{14}CO_2$ from acetaldehyde-1-2-^{14}C (7.72 ± 0.61) is comparable to the values obtained for lung (9.16 ± 2.60) and liver (5.50 ± 1.80). Its incorporation is greater than with the brain preparation (2.30 ± 0.43) and less than with the diaphragm (14.7 ± 3.9), heart (29.5 ± 2.8) and the kidney (31.2 ± 8.5). The spleen shares in common with most of the tissues examined a high rate of utilization of radioactive acetate for the production of $^{14}CO_2$, (17.9 ± 1.1, 7.2 ± 0.5) for A-1 and A-2, respectively.

2. Ratios of Percent Incorporation of C-1 and C-2 of Radioactive Acetate and Ethanol into $^{14}CO_2$.

In vivo experiments of Russell and Van Bruggen (72) demonstrate that less than 18 percent of the administered acetate or ethanol is "fixed" into the fatty acid and cholesterol fractions. The major part of the acetate-1-^{14}C and ethanol-1-^{14}C carbon was converted into CO_2 (63.8% and 58.5%, respectively) during the two hour experimental period. The two hour time chosen by these workers gave the following ratios for the incorporation of radioactive acetate and ethanol into CO_2

$$(A_2/A_1) \quad \frac{51.1}{63.8} = 0.80 \qquad (E_2/E_1) \quad \frac{35.2}{58.5} = 0.60$$

Their data indicate that the C_1 of ethanol is converted into CO_2 in an amount similar to the amount from the C_1 of acetate (58.5% vs. 63.8%), but only 35 % of the C_2 of ethanol became CO_2 as compared to 51% of the C_2 of acetate. These ratios are shown to be significantly different. Russell and Van Bruggen (69) indicate that the differences seen in the A_2/A_1 and the E_2/E_1 ratios might reflect differences in the abilities of individual tissues (a) to metabolize these compounds, (b) differences in the uptake of labeled acetate or ethanol or (c) differences in metabolic pathways.

The ratios of incorporation of ethanol (E_1/E_2) and acetate (A_1/A_2) in in vitro slice preparations are given in Table IX. In the systems used, the concentrations of the isotope in the medium were found to remain relatively constant during the duration of the experiment, i.e., the system approached steady state conditions.

It has been pointed out by Weinman, et al. (92) that the CO_2 ratio for acetate (A_1/A_2) should not equal unity under steady state conditions unless the Krebs cycle is being used for oxidative purposes only.

The results in Table IX show that the ratios of incorporation (E_1/E_2) and (A_1/A_2) are equal for any given tissue examined. The widest variation between the (A_1/A_2) and (E_1/E_2) ratios (0.3) is not significant at the 95% confidence level. Hence this strongly suggests that the radioactivity from ethanol incorporated into CO_2 must involve an acetate or acetyl CoA intermediate if the assump-

tions of the labeling ratios approach are valid; it further suggests that the differences seen by Russell and Van Bruggen in the CO_2 ratios in vivo cannot be explained by differences in the metabolic pathways of ethanol and acetate. The involvement of acetate or acetyl CoA as an obligatory intermediate in the metabolism of ethanol is further substantiated by the ratios of incorporation of acetate (A_2/A_1) and ethanol (E_2/E_1) into fatty acids, cholesterol and acetylsulfanilamide obtained by the same workers.

3. In Vitro Incorporation of Acetate-^{14}C and Ethanol-^{14}C into the Fatty Acid and Nonsaponifiable Fractions.

It can be noted that in all of the tissues examined (see Tables XI and XII), that only the liver was capable of incorporating more radioactivity from ethanol into fatty acids and the nonsaponifiable fractions than acetate. The incorporation of ethanol-^{14}C into the nonsaponifiable fraction of liver was 2.97 $\pm$ 1.71 percent as compared to acetate 1.29 $\pm$ 0.59 percent. Incorporation of radioactivity into the fatty acid fraction amounted to 3.63 $\pm$ 1.38 for ethanol and 1.93 $\pm$ 0.77 for acetate. Approximately 100 times as much radioactivity was incorporated into diaphragm lipid from acetate than from ethanol but 30 to 50 times more in brain and kidney slice preparations.

The percent incorporation of acetate-^{14}C into the nonsaponifiable fraction reported here was in close agreement with the results of Marcő, et al. (62) 0.51 $\pm$ 0.10 vs. .57 $\pm$ 0.043. Similar values were also obtained for the conversion of acetate-1-^{14}C to fatty acids in this same tissue 0.065 $\pm$ 0.018 vs.

0.067 ± 0.043.

4. Conversion of Ethanol-^{14}C into Acetate-^{14}C by Rat Liver Slices.

It was originally felt that with the low levels of substrates used that complete metabolism of the compounds should take place providing the tissues could metabolize them. The finding that the incorporation ratios obtained for ethanol and acetate into CO_2 were the same in each of the individual tissues examined, suggests that biosynthetic intermediates from the metabolism of ethanol, such as acetate or Krebs cycle intermediates, might be released from one tissue and further metabolized in another tissue. The first experiments testing this hypothesis were performed by incubating liver slices in the presence of radioactive ethanol in special Erlenmeyer flasks. These flasks contained a centerwell partially filled with alkali for the collection of CO_2 and during the experiment were sealed with a rubber stopper. Both ethanol and acetate were isolated. Acetate was thought to be the prime product of ethanol metabolism under the conditions employed. Since, Lundquist and others (55) had previously found using much higher concentrations of ethanol that the disappearance of ethanol from rat liver suspensions could be accounted for by the formation of acetate. He also noted that acetaldehyde, the initial product of the oxidation of ethanol, did not accumulate in the reaction media under aerobic conditions.

Under the conditions in the experiments presently reported, ethanol is rapidly converted to acetate and at the end of the

50-minute experimental period, most of the ethanol that had disappeared from the media was recovered as acetate and CO_2.

It is possible that at least part of the radioactivity (20-30 percent) that could not be accounted for in the liver slice experiment, could be present as Krebs' cycle intermediates is strengthened by the findings of Katz and Chaikoff (39) and Weinman, et al. (92). These workers have isolated radioactive Krebs cycle intermediates from a liver slice system shortly after the addition of acetate-1-^{14}C.

It is well known that compounds other than acetate can give rise to Krebs cycle intermediates. For example, alpha-keto-glutarate is formed from glutamate, and oxaloacetate is formed from aspartate. Weinman, et al. (92) have made use of this concept of an influx of intermediates into the cycle to explain the greater yield of $^{14}CO_2$ obtained from acetate-1-^{14}C than from acetate-2-^{14}C under steady state conditions.

Additional evidence for the outflow of acetate label or other labeled intermediates from liver slices has been obtained from experiments in which liver slices and diaphragm strips have been incubated simultaneously and the incorporation of radioactivity into lipid measured. The results of these experiments are presented in Table XIII. In one group of control experiments ^{14}C-ethanol and ^{3}H-acetate were substrates for lipid synthesis by 100 mgs. of diaphragm. In the other group of experiments 100 mgs. of liver was also included in each flask. The results are expressed as the percent incorporation of radioactivity from the appropriate

substrates, into either the nonsaponifiable or fatty acid fractions, per 100 mgs. of diaphragm per hour. It is apparent that the addition of the liver slices markedly increases the incorporation of radioactivity into the lipid fractions from ethanol whereas the incorporation of tritium into both the fatty acid and nonsaponifiable fractions was unchanged.

5. In Vivo Conversion of Ethanol-^{14}C to Acetate-^{14}C.

To substantiate the hypothesis that the liver is the primary organ involved in the initial oxidation of ethanol, in vivo experiments were carried out in which radioactive ethanol was injected into the portal veins, and samples were then taken from other veins or arteries. It was felt that portal administration of labeled isotope would be similar to the intraperitoneal injection, since the vascular bed which perfuses the gut drains into the portal vein. The main difference of course was that in the case of a direct intravenous injection, the liver received a single "pulse" of activity and with intraperitoneal administration, the liver receives more of a perfusion.

The results are given in Figure IV and clearly show that when radioactive ethanol is injected into the portal vein of a rat, and blood samples are taken by heart puncture, a large amount of labeled acetate can be isolated as early as five minutes after injection. The amount of radioactivity in the acetate fraction reached a maximum value at 10 minutes and then decreased a rate comparable to the rate of ethanol disappearance.

Realizing the blood isolated by heart puncture represents "mixed" blood that has perfused the whole organism, a new approach was taken. This time ethanol-^{14}C was administered as previously described but blood samples were taken directly from the hepatic vein, thus a more direct measurement of the role of the liver in the primary oxidation steps of ethanol could be measured. In Figure V the results of such an experiment are given. It is immediately apparent that as soon as 2-1/2 minutes, after injection, the amount of radioactive acetate (279,000 cpm/ml. plasma) far exceeds the amount of radioactivity present as ethanol (37,600 cpm/ml. plasma). The amount of radioactivity in both of these fractions decreased markedly in the next few minutes. The concentration of radioactive acetate dropped to 98,300 cpm/ml. plasma and ethanol dropped to 19,560 cpm 4 minutes after the initial injection. After 4 minutes, the remaining samples were taken from the inferior vena cava and in this "mixed" blood the amount of radioactivity present as ethanol and acetate was approximately equal at 10 minutes 30,000 cpm/ml. plasma vs. 32,440 cpm.

Similar experiments were carried out with the rabbit as the test animal. The results were quite comparable to those obtained in the intact rat. At 2 minutes after the portal administration of ethanol, the amount of radioactivity in the acetate fraction, again as in the case of the rat, greatly exceeded the amount in the ethanol fraction, 35,000 cpm/mL plasma vs. 8,000 cpm. With this preparation it was possible to take a greater number

of samples than in the case of the rat. It was noted that as late as 12 minutes after injection the amount of acetate-^{14}C present in the hepatic blood was greater than the amount of ethanol-^{14}C, 7,000 cpm/ml. plasma vs. 4,000 cpm.

That the formation and release of acetate from ethanol by the liver observed in this work is in good agreement with the earlier observations of Lundsgaard (56, 57). This worker observed while performing perfusion experiments with isolated livers that the amount of oxygen used was too small to account for oxidation of all of the ethanol to carbon dioxide. He assumed, therefore that the oxidation of ethanol did not proceed past the acetic acid stage, and as proof for this hypothesis, he isolated a considerable amount of acetate from his perfusion medium during the experiment. Lundquist has also observed the in vivo formation of acetate from ethanol (54).

One experiment reported in Figure VI was done with an intact rat preparation in which radioactive acetate was administered by intraportal injection and samples were subsequently taken from the hepatic vein. The shape of the curve bears remarkable resemblance to the curve for the formation and disappearance curves for acetate given in Figure VIII.

The time course study of ethanol-^{14}C and acetate-^{14}C presented in Figure IX has been included to point out that there is very little extrahepatic utilization of ethanol in the rabbit. Ethanol was administered as previously described but blood samples were taken from only the abdominal aorta and from the

inferior vena cava at a site below the liver. It was noted that the concentration of ethanol-^{14}C was relatively constant between the time interval of 15 to 25 minutes in both the venous and arterial systems. This did not seem to be true for acetate however, for while the concentration of acetate-^{14}C remained constant in the venous blood its concentration increased in the arterial blood, indicating possibly extrahepatic utilization of acetate. This observation is well substantiated by the findings of Forsander, et al.(27).

One of the major premises of this thesis is that the liver initially receives most of the label when label is administered by intraperitoneal injection and converts the greatest proportion of the ethanol to acetate which is subsequently released. To verify this assumption ethanol-^{14}C was injected directly into an ear vein of a rabbit. In this experiment sufficient time (20 minutes) was given for at least partial equilibration, before the first samples were taken from the aorta and the vena cava. Several important facts were evident from this experiment. One is the steady decrease in the concentration of radioactive acetate during the experiment and while taking the 23-minute sample, massive hemorrhage occurred. Immediately following this, there was a marked increase in the amount of radioactive ethanol, firstly in the venous and secondly in arterial blood. These observations imply that a large amount of ethanol can be stored in the extracellular and possibly intracellular spaces of the tissue. This implies that under <u>in vivo</u> conditions where low concentrations of ethanol are used ethanol can be taken up by tissues and that the true rate of utilization then

would be a function of the rate of back diffusion of ethanol into the blood stream from the extra- and intracellular water. This assumes a very low rate of utilization of ethanol by the extrahepatic tissues as previously discussed in this thesis.

6. In Vitro Permeability Experiments.

Since the work of Snyder, et al. (80, 81) has shown that acetate is an obligatory intermediate in the conversion of ethanol to acetyl CoA, it became necessary to provide other explanations as to why ethanol incorporates into many tissue constituents better than acetate. The possibility that ethanol can move through biological membranes at a faster rate than acetate was studied in a number of experimental preparations.

One uncertainty in the experiments where the rate of uptake of ethanol and acetate were measured lies in the fact that one might be measuring only the equilibration of the substrate with extracellular water the slices and not the actual penetration of cell membranes. If only the "diffusion rates" were measured it becomes readily apparent that ethanol can diffuse at least 2-3 times faster into the extracellular water than does acetate. It is quite likely that what was actually measured was the rate of diffusion into the extracellular water and the rate of entry from the extracellular water into intracellular water. It might be possible to measure the rate of movement into the cell if inhibitors were used to block the metabolism of the substrates.

The apparent permeability coefficients for ethanol and

acetate in frog skin were measured and results given in Table XIV. With this preparation it is possible to get a direct measure of the rate of passage of both ethanol and acetate through a series of biological membranes. The rate of movement of ethanol through this system exceeds 100 times that of acetate strongly suggesting that the rate of entry of acetate might be the rate limiting step in the oxidation of acetate at low concentrations.

7. Summary of the Discussion Section.

It is apparent that any attempt to study the comparative metabolism of radioactive compounds in an in vivo system is very difficult to interpret because of the interrelation between the different tissues. Factors such as differences in the blood flow, permeability, and pool sizes in the different tissues also complicates the interpretation.

If the assumptions of the labeling ratios technique are valid the results obtained from the in vitro slice preparations can be regarded as evidence that ethanol follows the commonly accepted metabolic pathways as previously outlined in Figure I. The findings that only liver and kidney can utilize ethanol to any great extent was in agreement with the findings of other workers. The liver has been suggested as the primary organ involved in the initial steps of the oxidation of ethanol because of the large amount of alcohol dehydrogenase present and the relatively large blood flow to this organ. If ethanol is utilized primarily by the liver the question arises as to how ethanol-^{14}C can be incorporat-

ed into nonhepatic tissue fractions in vivo. The in vitro experiments performed with liver slices shed some light on this problem. When labeled ethanol was the substrate for liver it was found that in addition to the formation of radioactive CO_2 and lipid large quantities of radioactive acetate were present. The results of experiments performed by Forsander and others (26, 27) show that in addition to the formation of acetate the formation of radioactive Krebs cycle intermediates can be demonstrated. When radioactive ethanol was injected into the venous system of rats in vivo labeled acetate was isolated from the blood a few minutes after injection. The amount of radioactive acetate appearing in the hepatic blood after intraportal injection of ethanol, several minutes after injection was shown to far exceed the amount of radioactive ethanol in both the rabbit and the rat. If acetate can be shown to be present in the blood coming from the liver, it is reasonable to assume that other radioactive compounds will also be present. It is proposed that the high incorporation of radioactivity from ethanol into the lipids and proteins of the extrahepatic tissues is primarily due to the outflux of labeled acetate and other compounds from the liver which can be subsequently utilized by the extrahepatic tissues in biosynthetic and oxidative reactions.

The permeability experiments show that ethanol can move through biological membranes much faster than acetate. This could possibly explain why radioactivity from ethanol appears in respiratory CO_2 at an earlier time than does that of

acetate in the intact animal following the intraperitoneal injection of the tracer compounds. Also if the tracers were taken up by the portal system most of the label would go directly to the liver because liver metabolizes ethanol at a greater rate than it does acetate and the turnover time of the bicarbonate pool in this tissue is probably very short, due to its small size and its high blood flow, CO_2 produced in the liver should appear as respiratory CO_2 in a very short time, thus possibly accounting for the rapid appearance of radioactivity from ethanol into the respiratory CO_2. If the rate of production of radioactive CO_2 from ethanol is limited by its oxidation to acetic acid in the liver one would still expect high rates of incorporation in this fraction because of the very high outflux of acetate from the liver with subsequent catabolism of acetate in extrahepatic tissue.

V. SUMMARY AND CONCLUSIONS

1. Ethanol metabolism was studied in a number of tissue slice preparations using an experimental approach designed to determine if one or more pathways exist for the metabolism of ethanol to CO_2.
2. It appears that the assumptions of the labeling ratios technique are valid under the experimental conditions used and a direct comparison of acetate-^{14}C and ethanol-^{14}C label incorporations can be made.
3. The ratios of the $^{14}CO_2$ derived from acetate-1-^{14}C/acetate-2-^{14}C (A_1/A_2) and from ethanol-1-^{14}C/ethanol-2-^{14}C (E_1/E_2) were the same in all of the tissues examined.
4. In most of the tissues examined the rate limiting step in the conversion of ethanol to CO_2 was the primary oxidation step where acetaldehyde was formed.
5. The only tissue that could incorporate more radioactivity from ethanol-^{14}C into $^{14}CO_2$ than from acetate was the liver.
6. In all tissues examined with the exception of the liver acetate-^{14}C was incorporated to a greater extent than ethanol.
7. When liver slices were in cluded with diaphragm slices in the same incubation medium, the incorporation of radioactivity into diaphragm lipid from ethanol-^{14}C was increased. No effect on the rate of acetate-^{14}C incorporation was observed in this same system.
8. Under both <u>in vitro</u> and presumably, <u>in vivo</u> conditions, consider-

able amounts of ethanol-^{14}C could be converted in the liver into acetate-^{14}C which was subsequently released from the tissue.

9. Ethanol moved through the frog skin at a rate 100 times greater than that of acetate.

10. The uptake of ethanol was 2-3 times greater than that of acetate in both muscle and liver preparations.

11. It is concluded that the pathway of ethanol metabolism in vitro as well as in vivo is consistent with that shown in Figure I, i.e., ethanol to acetaldehyde to acetate to acetyl CoA.

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