STUDIES ON THE PATHWAY OF ETHANOL METABOLISM

by

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CHAPTER I

INTRODUCTION

Preliminary remarks

Peoples in many parts of the world learned, in antiquity, that fruits or grains which were stored in the heat, under certain conditions, produced unusual effects when eaten. Thus, from prehistoric times, humans have had available cumulative experiences with alcoholic beverages, but have conspicuously failed to use them. Certainly, from the time of the sons of Noah, alcoholic drinks have been a problem which has taxed social and individual control.

The abuse of the use of alcoholic beverages in the United States led to the prohibition movement. This reached a climax in the prohibition amendment to the United States Constitution. The subsequent repeal of this legislative device for control did not remove the problem any more than did the original passage. It is rather interesting to note how little effort was made to obtain scientific information about the physiological effects of alcohol and their relation to alcohol addiction or to social problems deriving from them. It is also interesting to observe how little people wanted to learn about alcohol in a scientific way. They seemed much to prefer their violently differing emotional speculations about it. Until recently, the excessive, uncontrollable use of alcohol (alcoholism) was regarded as a manifestation of spiritual weakness, a deficiency of moral fiber, an insufficiency of will power; it carried more the label of sin than of disease.

Gradually a change has come, and alcoholism is becoming looked upon as a disease, a disease still with social, moral and religious

ramifications to be sure, but nevertheless a disease. As a result, considerable systematic scientific study on all aspects of the alcohol problem has been undertaken in this country and abroad in the past thirty years. Not the least of these fields of investigation is that concerned with the intermediary pathway of alcohol metabolism. The past twenty years has seen the elucidation of the major metabolic pathways of acetate, i.e., the activation of acetate to acetyl CoA, followed by the use of acetyl CoA in the synthesis of fatty acids and sterols or its oxidation to CO_2 and H_2O via the Krebs' cycle. The major question involved in this aspect of the alcohol problem is whether alcohol follows a unique metabolic pathway, or whether it simply enters the two-carbon pool of acetyl CoA units which are normally derived from the breakdown of fatty acids and other foodstuffs. As will become clear in the following brief review, this question has not yet been satisfactorily answered.

Review of ethanol metabolism

It was not until the turn of the century that there was general acceptance of the very important fact that ethanol could be utilized as a foodstuff by the mammalian organism. Following this, attention was directed to the rates of metabolic use of alcohol and then, following the development of micromethods for the determination of alcohol, the general facts about alcohol absorption, distribution and elimination in the human and animal body were cleared up.

The information available on alcohol metabolism in 1935 was summarized as follows (33): alcohol is readily absorbed in the intestinal tract, but certain foodstuffs, especially protein and fat, are

able to delay the absorption considerably. The distribution of alcohol in the body follows the equation:

$$A = p \times c \times r$$

A being the total amount of alcohol in the organism in grams, p the body weight in kilograms, and c the alcohol concentration in milligrams per gram of blood. The factor r is the proportion of the body in which the alcohol is distributed; on the average it is 67% ± 20%, but it is relatively constant in the same individual from time to time. From this formula it is possible to calculate that an average individual is able to oxidize about one gram of alcohol per hour per 10 kilograms of body weight.

These early general conclusions are beyond dispute and are accepted as fact by workers in the field. As we turn to look at some specific aspects of this problem, namely that of the slope and shape of the blood alcohol curve in the post absorptive period, we find many points of disagreement. Jacobsen (33) has extensively reviewed this work, which spans three decades, and it is from his article that the following information was obtained.

Mellanby, in 1919, first reported that the decrease of alcohol content in the organism, as measured by the concentration in blood, follows a straight line from the point where the absorption is ended and equilibrium is established between the tissues and the surrounding fluid until all measurable remnants of alcohol have disappeared from the blood. The most common expression of this slope is found in Widmark's factor β, which indicates how many milligrams of alcohol disappear from each milliliter of blood per unit time. The β factor

was found to be independent of the concentration of alcohol in the blood. These observations have been confirmed in many species by numerous investigators.

In spite of this, the conclusion that the metabolic rate of alcohol is independent of the concentration of alcohol in the organism has been repeatedly challenged, and convincing experimental evidence has been brought against it. Some investigators have found a hyperbolic rather than a rectilinear curve, and others who report a rectilinear curve, claim a definite dependence of the slope of the curve on the original concentration of alcohol; i.e., they say that the metabolic rate of alcohol is higher with a higher initial concentration of alcohol, but the rate remains constant until all of the alcohol has disappeared.

Jacobsen (33) summarizes our present knowledge in the following way: "The rate of oxidation of ethyl alcohol in the organism is somewhat increased with increasing concentrations of alcohol. Within the concentrations possible in the living organism this increase is so small that the elimination curve of alcohol generally follows a straight line, and for forensic purposes no error is made if we assume a rectilinear curve for alcohol." The importance of this rather unusual elimination curve will become apparent later as we discuss the enzymic processes involved.

Attention during the last two decades has been directed toward elucidation of the possible enzymes involved in the oxidation of ethanol and, especially since the introduction of radioisotopes, to

the identification of intermediary products.

Again this is an area of disagreement between workers, and at the time of this writing there is no universally accepted pathway (s) of ethanol metabolism. We might logically approach this subject by outlining the proposed pathway as is presented in a modern textbook (27):

(A) ethanol + DPN $\xrightarrow[\text{dehydrogenase}]{\text{alcohol}}$ acetaldehyde + DPNH + H^+

(B) acetaldehyde + DPN + H_2O $\xrightarrow[\text{dehydrogenase}]{\text{aldehyde}}$ acetic acid + DPNH + H^+

An alternate pathway for reaction B above is the conversion of acetaldehyde directly to acetyl CoA without the intervention of acetic acid:

(C) acetaldehyde + DPN + HS-CoA $\xrightarrow[\text{dehydrogenase}]{\text{aldehyde}}$ acetyl CoA + DPNH + H^+

The enzymes capable of catalyzing this reaction have been prepared from Clostridium kluyveri (51).

Although the evidence for this general pathway is fairly substantial, it is by no means conclusive. The literature is filled with conflicting reports, and there are a number of findings that are not easily explained by the above postulated pathway.

In the following pages we will look at these two steps independently and present the evidence for and against them.

(A) ethanol + DPN $\xrightarrow[\text{dehydrogenase}]{\text{alcohol}}$ acetaldehyde + DPNH + H^+

In the past thirty years there has accumulated a considerable volume of evidence to support the theory that the first step in the metabolism of ethanol is its conversion to acetaldehyde. In, <u>in vitro</u> experiments with tissue slices or tissue brei incubated with ethanol,

acetaldehyde can be isolated as a reaction product (41). The level of acetaldehyde in the blood of normal humans has been reported by Stotz (52) to range from 0.002 to 0.037 mg. %. This same author reports levels of 0.7 to 1.4 mg. % acetaldehyde following ingestion of sufficient alcohol to reach a blood alcohol level of 100 to 180 mg. %. Hald et al. (34) report an increase of 0.105 mg. % acetaldehyde in the blood of humans following ingestion of 40 milliliters of absolute alcohol. Hulpieu et al. (32) report similar findings in rabbits, their reported values for serum acetaldehyde concentration before and after administration of 1 gram of alcohol per kilogram of body weight were 0.001 mg. % and 0.063 mg. % respectively. Similar results have been reported for other species.

The evidence that acetaldehyde is a product of ethanol metabolism was strengthened by Hald, Jacobsen and Larsen following their discovery of a compound which has the effect of sensitizing an animal to alcohol (35). This compound, tetraethylthiuram disulfide, better known under its proprietary name antabuse, has been extensively investigated by these workers. They found that antabuse does not delay the disappearance of alcohol from the blood, but that the blood levels of acetaldehyde are five to ten times higher following alcohol ingestion in humans and animals pretreated with antabuse than in subjects not given antabuse (34, 35, 39). In animals treated with antabuse and not given alcohol, they found no accumulation of acetaldehyde. From these observations they concluded that acetaldehyde plays an insignificant role as an intermediate in metabolic processes, except during the oxidation of ethanol.

The Danish workers also reported that the physiological effects of infused acetaldehyde, notably the increased pulse and respiratory rate, the increased alveolar dead space and the decreased alveolar CO_2, were qualitatively and quantitatively identical to those following administration of antabuse and alcohol, suggesting that the toxic effects of antabuse and alcohol are due to elevated acetaldehyde levels. This latter finding has been denied by several investigators who have failed to find any correlation between the level of acetaldehyde and the presence or absence of symptoms (42), or any true qualitative or quantitative similarity of symptoms (23).

To date, two purified enzyme systems have been prepared from animal tissues which are capable of oxidizing alcohol in vitro. These are the alcohol dehydrogenase and catalase systems.

Alcohol dehydrogenase

Animal alcohol dehydrogenase was first studied by Lehman (33) who showed its dependence on DPN. In 1938 Lutwak-Mann purified alcohol dehydrogenase from horse liver and studied its properties more closely (41). Her semi-pure preparation of alcohol dehydrogenase required cozymase (DPN), "cozymase factor" and an electron acceptor (methylene blue or pyocyanine). When the "cozymase factor" was obtained from skeletal muscle or heart muscle, the preparation oxidized alcohol to acetaldehyde, the latter being recovered quantitatively. The results varied when the "cozymase factor" was obtained from liver tissue, and the importance of this will be mentioned later.

Bonnichsen and Wassen (4) crystallized horse liver alcohol dehydrogenase in 1948, and using this preparation Theorell and Bonnichsen

(3) in 1951 extensively studied the kinetics of the reaction:

$$\text{ethanol} + \text{DPN} \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} \text{acetaldehyde} + \text{DPNH} + H^+$$

Their work has been very ably reviewed by Jacobsen (33). Two of their results are of extreme practical interest. The turnover number (the number of molecules of alcohol oxidized by one molecule of enzyme per minute) is found to be 140 at 20°. It is probably somewhat higher at 37°, but on the other hand this figure is determined under optimal conditions which may will not be found in the organism. Using the turnover number 150 it can be shown that 1 millimole, i.e., 73 grams, of the enzyme (M.W. 73,000) is able to oxidize 150 x 46 milligrams of alcohol which is equal to 6.9 grams of alcohol per minute, or 414 grams per hour. In order to oxidize 10 grams of alcohol per hour, 1.7 grams of alcohol dehydrogenase must be present in the organism. This is not an astonishingly high figure. Bonnichsen obtained a yield of 1 gram of crystalline dehydrogenase per kilogram of horse liver, and thus it does not seen incredible that a human liver weighing about 1.5 kilograms should contain 1.5 grams of alcohol dehydrogenase, enough to oxidize the amount of alcohol actually oxidized by the human organism per hour. Another interesting point is that the Michaëlis constant (an expression of the affinity between enzyme and substrate) is so low that the rectilinear course of the physiological alcohol elimination found in human experiments is to be expected from the kinetic data of the in vitro experiments with the pure enzyme.

Catalase

A possible second mechanism for the oxidation of alcohol to

acetaldehyde was demonstrated by Keilin and Hartree (28, 29). When alcohol and catalase are coupled with an oxidation system which reacts directly with molecular oxygen with the formation of H_2O_2, alcohol is oxidized to acetaldehyde with the simultaneous reduction of H_2O_2 to H_2O. The oxidation systems they studied included uricase and uric acid, xanthine oxidase and acetaldehyde, d-amino-acid oxidase and alanine, xanthine oxidase and hypoxanthine. The general scheme of the reaction is as follows:

$$\text{ethanol} + H_2O_2 \xrightarrow{\text{catalase}} \text{acetaldehyde} + 2H_2O$$

$$\text{acetaldehyde} + H_2O + O_2 \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{acetic acid} + H_2O_2$$

Unlike the alcohol dehydrogenase enzyme, catalase will oxidize methyl as well as ethyl alcohol, and at an equal rate.

There have been many educated guesses about the relative importance of these two pathways of ethanol metabolism in vivo. The majority opinion holds that catalase plays a minor role at best. This is based on the following observations. First, the kinetics of the DPN-alcohol dehydrogenase process would suggest a rectilinear shape for the alcohol disappearance curve, while the kinetics of the catalase-hydrogen peroxide process would suggest a hyperboloidal shape for the alcohol curve (9). Most workers in the field, as mentioned above, have found the curve to be rectilinear in all but extremely dilute concentrations. Still more convincing is that in an in vitro system of catalase and H_2O_2, methanol and ethanol are oxidized at the same rate (29), while in vivo ethanol is oxidized at least four to five times as rapidly as methanol. While the problem is not completely

resolved, the overall result would be qualitatively the same by either process, ethanol being oxidized to acetaldehyde.

Thus, much of the knowledge gained in the past quarter of a century points toward acetaldehyde as the immediate oxidation product of ethanol. There are, however, certain observations not easily explained by this hypothesis.

First, as alluded to earlier, the semi-pure, cell free, alcohol dehydrogenase preparation of Lutwak-Mann, when composed of horse liver alcohol dehydrogenase, DPN, and so called "cozymase factor" obtained also from horse liver, metabolized alcohol by some pathway other than via acetaldehyde. That is, when alcohol was added, it disappeared from the system and acetaldehyde was not recoverable. When acetaldehyde was added to this same system, it was recovered quantitatively, unaltered in structure.

A second point that has never been explained is why acetaldehyde accumulates at all during alcohol metabolism. Westerfeld (71) makes the statement: "At first glance it would seem obvious that if alcohol were converted to acetaldehyde, the latter should appear in body fluids; but this is not necessarily true. The equilibrium between alcohol and acetaldehyde in an <u>in vitro</u> system is greatly in favor of the alcohol by a ratio in the order of 1300:1. In those species which accumulate acetaldehyde after receiving alcohol, the blood alcohol: acetaldehyde ratio is of the order of 130:1. In other words, there is ten times as much blood acetaldehyde present as would be expected from the <u>in vitro</u> equilibrium figures. This point alone might not be too

significant since the ratio could be influenced by the formation of complexes between the enzyme and reduced DPN. However, the rate of acetaldehyde metabolism is much faster than the rate of alcohol metabolism in all species studied, and all of the acetaldehyde formed by the oxidation of alcohol should theoretically be destroyed as rapidly as it is formed. How and why it accumulates to the extent that it does is not at all clear."

Newman (45) found that dogs metabolized acetaldehyde more rapidly than they do ethanol. He administered 1 gram of alcohol per kilogram of body weight intravenously to normal dogs over a ten minute period and found them able to metabolize alcohol at the rate of 100 milligrams of alcohol per kilogram per hour, a figure in close agreement with that found by others. The blood concentration of acetaldehyde slowly increased over the six hour period of the experiment. Yet these same dogs when infused with acetaldehyde at the rate of 185 milligrams per kilogram per hour, nearly twice the quantity that could be formed from the alcohol of the previous experiment, after an initial rapid rise to nearly 1.5 mg. %, showed a steady decline in serum concentration for the remainder of the experiment, despite a continued infusion at the same rapid rate. Westerfeld et al. (72) injected acetaldehyde into a dog four hours after a large dose of alcohol. At the time of injection the blood level of alcohol was 125 mg. % and that of acetaldehyde 0.1 mg. %. The amount of acetaldehyde administered was sufficient to raise the blood level to at least 15 mg. % if it were completely distributed throughout the body unaltered. Yet, three minutes after the injection was completed, the blood level was less

than 1 mg. %, and had practically returned to the starting value within 30 minutes. Throughout this time the blood alcohol curve continued to decline at its previously established rate. The rate of acetaldehyde metabolism was the same, whether or not the animal was metabolizing alcohol at the time of its administration.

There is a great deal of evidence that the metabolism of alcohol is favored by the simultaneous metabolism of carbohydrate. During alcohol metabolism the blood glucose level is increased with a simultaneous decrease in liver glycogen (47, 49, 53). When liver glycogen is depleted by fasting, the utilization of alcohol is depressed (40, 43). Feeding large doses of carbohydrate increases the rate of alcohol oxidation (8). Insulin, and more especially insulin plus glucose, increases the rate of alcohol metabolism (10, 11, 20). One intermediate of glucose metabolism, i.e., pyruvate, has been shown by numerous investigators to accelerate the oxidation of alcohol in vivo and in vitro (22, 40, 76-79), however, this effect of pyruvate does not seem to be found under all circumstances, for Hulpieu et al. (31) found no accelerating effect in dogs, nor did Gregory (24) or Kinard (37). The apparent discrepancy between these results seems to be answered by the fact that the latter authors worked with animals already oxidizing alcohol at a maximal rate, which for dogs lies between 21 and 25 mg. % decrease in blood alcohol per hour. Once this rate is reached, it can not be exceeded by giving doses of pyruvate (76).

It has been postulated that the accelerating effect of pyruvate is due to a coupled oxidation-reduction reaction with alcohol, in which

alcohol is oxidized to acetaldehyde, and pyruvate is reduced to lactate (77). In agreement with this proposal several investigators have found an increased blood lactic acid concentration during alcohol metabolism, especially after administration of pyruvate (30, 40, 77). Others have reported opposite results, i.e., a simultaneous fall in both pyruvate and lactate following the administration of alcohol (78). Westerfeld et al. (77) and Leloir and Munoz (40) have both reported a stimulation of alcohol oxidation by lactate as well as by pyruvate, Westerfeld working with intact dogs and Leloir with rat liver slices. This latter finding, the stimulation of alcohol metabolism by lactate, is difficult to reconcile with the theory that the accelerating effect of carbohydrate on alcohol oxidation is due to a coupled oxidation-reduction reaction involving the oxidation of alcohol to acetaldehyde and the reduction of pyruvate to lactate.

For the sake of completeness, the work of Vitale et al. (65) should be mentioned. These investigators, working with intact rats, reported a depression of alcohol metabolism by both lactate and pyruvate. Working with humans they found that administration of sucrose also caused a decrease in the rate of disappearance of alcohol from the blood. An explanation of the apparently completely contradictory results of these authors and other work mentioned above is not apparent at this time.

Briefly, then, to sum up an extensive and sometimes contradictory literature on this subject, simultaneous carbohydrate metabolism seems to have a stimulatory effect on the oxidation of alcohol. The reason

for this, to date, has not been clearly shown, but any acceptable theory of the pathway of alcohol metabolism must take this stimulatory effect of carbohydrate into account.

DPN is a necessary constituent of the proposed pathway of alcohol oxidation via the alcohol dehydrogenase enzyme system. DPN is a niacin containing coenzyme, and as such one might anticipate a decrease in the rate of alcohol oxidation in a niacin deficient animal. Nelson and Abbenhaus (44) found the opposite results, i.e., niacin deficiency in rats actually caused an increase in the rate of alcohol metabolism. Also, recent work by Smith et al. (46) indicated that injection of nicotinamide into mice six hours prior to the injection of alcohol had no effect on the rate of alcohol metabolism, although the DPN concentration in the liver was raised tenfold. This latter finding could be explained if DPN is normally present in excess of that needed in the alcohol dehydrogenase reaction, and that the latter reaction is the rate limiting factor. However, the reason for the increase in the rate of alcohol metabolism in the face of a niacin deficiency is not so easily explained.

Thus, while the process,

$$\text{ethanol} + \text{DPN} \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} \text{acetaldehyde} + \text{DPNH} + \text{H}^+$$

is widely accepted as the pathway of alcohol metabolism and has much evidence to substantiate it, there are a number of observations which are not readily explained by this hypothesis. The strongest evidence in support of this pathway perhaps is the finding of acetaldehyde in the blood in significant quantities only after alcohol intake. The

method of determination of blood acetaldehyde in most general use since 1943 is that of Stotz (52). This method has proven to be very sensitive, but is not entirely specific. Of the compounds tested by Stotz, he found interference by such volatile substances as diacetyl, formaldehyde and paraldehyde; and if distillation was done from an acid solution, by pyruvate and lactate as well. In addition to the lack of complete specificity, the procedure requires the use of concentrated acids and high temperatures. It is not entirely illogical to question the possibility of either an as yet unidentified "active aldehyde" intermediate in alcohol metabolism being determined as acetaldehyde, or the decomposition of a complex formed between a normal tissue component and the first breakdown product of alcohol, this chemical or thermal decomposition yielding acetaldehyde as one of its products. This latter possibility seems not unlikely in view of the work of Schulman and Westerfeld (73) in which they found that the C-3 position of acetoacetate formed by malonate inhibited rat liver homogenates incubated with ethanol-1-C^{14} contained ten to twenty-five times as much label as did the C-1 position. Using acetate-1-C^{14} in this same system the C-1 and C-3 positions of acetoacetate were labeled equally. They postulated as a result of this work, the condensation of acetaldehyde-1-C^{14} formed from ethanol-1-C^{14} with endogenous acetyl CoA to form B-hydroxybutyrate-3-C^{14} or its CoA derivative. Since that time they have reported the presence of a highly radioactive impurity in their filtrate prepared from the rat liver homogenate incubated with ethanol-1-C^{14} (74). This radio-contaminant precipitated with the acetoacetic

acid fraction. The compound accounted for most of the radioactivity in the filtrate, was stable when dry, has been separated by paper chromatography, eluted and shown to form acetaldehyde-C^{14} when subjected to a Hg-acetone procedure. Since this compound did not accumulate in the absence of malonate, it is not known whether it is a normal metabolite of ethanol or an artifact arising from a reaction between acetaldehyde and malonyl CoA. Though we can draw no final conclusions from their work at this time, it does make us acutely aware that the conversion of ethanol to acetaldehyde and then the latter to acetate, as the pathway or at least the only pathway of ethanol metabolism, has not been conclusively shown.

(B) metabolism of acetaldehyde

The pathway of acetaldehyde metabolism is not yet established. There is no doubt that alcohol can be converted to acetyl CoA. Bernhard (2) as early as 1940, using deuterio acetic acid, showed the in vivo acetylation of foreign amines by acetic acid. Using deuterio ethanol he again observed the excretion of the deuterio acetyl derivative of a foreign amine which was co-administered. In fact, Bernhard found that ethanol was a better precursor of the acetylating agent than was acetic acid. This work was confirmed by Block and Rittenberg (2) who, however, found that acetate and ethanol were equal in their effectiveness as sources of acetyl. The acetylating agent in these reactions has been well established to be acetyl CoA. More recently, Forsander et al. (19) have isolated the carboxylic acids formed during the oxidation of alcohol by intact rats and by perfused rat livers. They found that rat blood normally contains

pyruvate, β-hydroxybutyrate, lactate, and small amounts of acids from the tricarboxylic acid cycle. After intraperitoneal alcohol administration to intact animals, acetate and acetoacetate were found in addition. After perfusion experiments with ethanol-1-C^{14} and ethanol-2-C^{14}, acetate, acetoacetate, β-hydroxybutyrate, pyruvate and an unidentified acid contained C^{14} activity. In view of these experiments, the overall conversion of at least some of the ethanol to acetate and acetyl CoA can be accepted as fact. If acetaldehyde is indeed an obligatory intermediate of alcohol metabolism, then it in turn must be converted to acetyl CoA. This conversion to acetyl CoA need not, however, be a direct one.

Six enzyme systems have been isolated which *in vitro* catalyze the oxidation of acetaldehyde. Westerfeld (71) has reviewed the literature on this subject, and inasmuch as none of these enzymes has been shown to be of major importance *in vivo*, they will not be further discussed here.

Schulman, Zurek and Westerfeld (75) compared the ability of various rat tissues *in vivo* to incorporate the label of ethanol-1-C^{14} or acetate-1-C^{14} into glycogen, protein, cholesterol or fatty acids. They found that all tissues studied utilized both ethanol and acetate, but that ethanol consistently contributed two to three times as much isotope to the tissue constituents studied as did acetate. This was true for both large and small doses of label, and in short and long-term experiments. They concluded that acetate could not be an obligatory intermediate of ethanol metabolism. Their reasoning was that

if both components are metabolized by the same tissues, as indicated by their results, that the pool size of any common intermediates would be the same for both compounds and would be expected to dilute both labels equally. Thus an increased dilution of administered acetate-1-C^{14} could not explain its decreased incorporation into tissue components. The fact that ethanol-1-C^{14} proved a better precursor of tissue constituents in long term as well as short term experiments, precluded the idea that a more rapid metabolism of the ethanol could explain its greater incorporation. Free acetate therefore can not be an obligatory intermediate of ethanol oxidation, for it does not seem likely that there is a way that tracer doses of ethanol could be metabolized via acetate and at the same time contribute two to three times as much isotope to the tissue constituents as does the acetate itself. If acetyl CoA is an obligatory intermediate of acetate metabolism, and all known pathways of acetate metabolism involve its conversion to acetyl CoA, then a similar argument can be used against acetyl CoA being an obligatory intermediate of alcohol metabolism. That is, if the size of the acetyl CoA pool is identical for both acetate and ethanol, and both compounds are metabolized to the same degree in a given unit of time, then it would seem that there was little chance that ethanol-1-C^{14} could contribute two to three times as much label to the products formed from the acetyl CoA than acetate-1-C^{14} does, and at the same time acetyl CoA be an obligatory intermediate of both compounds. The above reasoning has two weaknesses. First, the matter of the relative rates of

metabolism of ethanol and acetate. Schulman et al. in the above quoted article do not report any CO_2 data to substantiate a similar rate of metabolism for ethanol and acetate, but they do make the statement that "on the basis of CO_2 production, small doses of alcohol and acetate were oxidized at the same rate." The second point open to argument is whether the acetate and acetyl CoA pools available to these two substrates are indeed identical. The only direct evidence to support this is the fact that all tissues studied utilized both compounds. The possibility that different parts of the cell oxidize each of the two substrates along identical pathways has not been excluded.

There has been little isotopic work done with acetaldehyde itself. Brady and Gurin (26) did incubate rat liver slices with either double labeled acetate or double labeled acetaldehyde and compared the effectiveness of these compounds in the labeling of cholesterol and fatty acids. They found that acetaldehyde incorporated about 1½ times as much label into both cholesterol and fatty acids as did acetate. These findings have not been confirmed, but if true would seem to indicate a greater similarity between the metabolism of acetaldehyde and alcohol than between acetaldehyde and acetate.

Rationale of present approach

Thus, while the tentative pathway of alcohol metabolism is that as outlined above, there certainly is no universal acceptance of this scheme, which appears to be at variance with several reported findings as has been indicated.

Some of the apparent discrepancies may be due to differences of procedure of one investigator as compared to another, or to differences in animal preparation, strain, or species employed. Thus, while several groups have compared one or more aspects of acetate, ethanol or acetaldehyde metabolism, no one group has done a truly comprehensive comparative study of the metabolic fates of all three compounds under standardized conditions. If acetate or acetyl CoA serves as a common obligatory intermediate of the metabolism of these three compounds, such a comparative study should reveal parallel incorporations of these compounds into all tissue constituents. If the metabolic pathways of one or more of these compounds is divergent, however, it might result in gross differences in the incorporation of that compound into tissue components.

We have developed in this laboratory in the past few years methods for the study of the incorporation of tracer quantities of C^{14} labeled precursors into CO_2, cholesterol and fatty acids, both in intact animals and in liver slice preparations. Ethanol, acetaldehyde and acetate are all known to serve as precursors of these tissue constituents. It was felt then that parallel experiments employing C^{14} labeled acetate, ethanol and acetaldehyde might be expected to furnish information as to the similarity or divergence of the metabolic pathways of these three compounds.

CHAPTER II

METHODS

Animal preparation

Source of animals

The animals used in these studies were male albino rats of the Sprague-Dawley strain obtained from the Northwest Rodent Co. at Pullman, Washington. The rats weighed between 125 and 175 grams when obtained, and were maintained in a cagehood assembly developed in this laboratory (55). Animals were trained to feeding and weighed between 200 and 240 grams at the time of sacrifice.

Trained feeding

Previous communications from this laboratory (56, 60, 69, 70) have dealt with the effect of feeding and fasting upon lipid labeling in the intact rat and in the liver slice preparation. Lipid labeling from tracer acetate has been shown in both preparations to be directly related to the immediate past food consumption to such an extent that the lack of control of feeding and fasting periods might well obscure lipogenic variations being studied. Others have reported, largely from *in vitro* studies, the effect of various dietary constituents upon lipogenesis (12, 15, 16, 25, 50, 68). Thus, it becomes of paramount importance to control the variables of both the time and quality of feeding, if other effects upon lipogenesis are to be studied. Our method of training to feeding was similar to that previously reported from this laboratory (56). The animals, upon arrival, were placed in the cagehood assembly and given water and Purina chow *ad libitum* for

several days to allow them to become accustomed to their new environment, to regain any weight lost during shipment, and to allow them to approach the desirable experimental weight. The controlled feeding regimen was then begun, and consisted of two daily feedings of ten grams of Purina laboratory chow per animal. The chow was placed in the cage at twelve hour intervals and, at the end of one hour, any food remaining in the cage was removed but water was allowed ad libitum. Four to six days of this regimen were sufficient to train the rats to eat the entire ten grams almost immediately, and rarely was it necessary to remove any food from a cage after the first week of train-feeding. The rats showed a consistent daily weight gain of four to six grams. All rats were on this regimen for at least five days prior to use.

Preparation of diabetic rats

Inducement of diabetes

The diabetic animals being reported in this work were made diabetic with alloxan, using a technique developed in this laboratory by Rose K. Wong (64). All animals used had blood sugar levels in excess of 200 milligrams percent, and were used three to six weeks after alloxan injection.

Feeding regimen

The feeding regimen of the diabetic animals varied from that of the normal animals. It was found that the diabetic animals deteriorated rapidly when allowed only two one-hour feeding periods per day. It became necessary to allow them food and water ad lib until just prior to the experiment. At that time a twelve hour fast was

instituted followed by a one-hour feeding period. The twelve hour fasting period left the diabetic animals hungry enough to guarantee us that they would eat during their one-hour feeding period, even though they had not previously been trained to feeding. Thus the normal and diabetic animals were comparable as to their immediate past food intake, i.e., each had a terminal one-hour feeding period of Purina chow preceded by a twelve hour fast.

Radioisotopes

Source of radioisotopes

The acetate-1-C^{14} used in these experiments was synthesized in our laboratory by a procedure previously described (57). The remainder of the radioisotopes employed were purchased from commercial sources, ethanol-1-C^{14} and ethanol-2-C^{14} from Nuclear Chicago Corp., acetaldehyde-1-2-C^{14} from Volk Radio Chemical Co., and acetate-2-C^{14} from Tracerlab Inc.

Doses of radiotracers employed

In vitro

If a labeled molecule such as acetate-C^{14} is used in amounts exceeding tracer concentration, i.e., that amount that will not influence the reaction being studied, the incorporation of the acetate label will no longer quantitatively reflect the rates of reactions prevailing prior to its introduction. Emerson and VanBruggen (59) have presented data on the changes seen in CO_2, fatty acid, and cholesterol radioactivities when standardized liver slice preparations were studied over a wide range of tracer acetate-1-C^{14} concentration. The changes they reported in the specific activity responses indicate that CO_2-,

fatty acid-, and cholesterol-forming systems each have a critical level of "tracer concentration" above which tracer conditions are violated. They found these tracer levels to be surprisingly low for the two lipid systems, the fatty acid specific activity response at 0.025 μmoles was only 90% of that expected on the basis of the response at 0.0025 μmoles, and the cholesterol response at 0.025 μmoles only 60% of that expected on the basis of the response at 0.0025 μmoles. In view of these findings, it becomes important to standardize the dosage of "tracers" employed if rates of reactions are to be compared.

In practice, the minimum size of a dose of radioisotope that can be employed is determined by the specific activity of that compound, the percentage incorporation of that compound into the product to be assayed, the degree of dilution that the label will undergo, i.e., the amount of unlabeled product present, and the sensitivity of available assay methods. The concentrations of tracers that we employed and that were found to fulfill these requirements in our in vitro experiments are given in table I.

Table I

Concentrations* of tracers employed in the in vitro experiments

tracer	minimum conc. in micromoles	maximum conc. in micromoles
acetate-1-C^{14}	0.21	0.89
ethanol-1-C^{14}	0.25	0.77
acetate-2-C^{14}	1.10	1.20
ethanol-2-C^{14}	1.10	1.16
acetate-1-2-C^{14}	0.63	1.26
acetaldehyde-1-2-C^{14}	0.46	1.70

* per gram of liver slice

In vivo

A study of the role of tracer acetate concentration in CO_2-, fatty acid-, and cholesterol-forming systems of the intact animal has also been done in our laboratory (16). The specific activity responses of these systems were found to be linear in doses ranging from 0.2 to 519 μmoles. The doses we employed were well within this range, and are given in table II.

Table II

Dosages of tracers employed in the in vivo experiments

tracer	minimum dose in micromoles	maximum dose in micromoles
acetate-1-C^{14}	0.94	2.34
ethanol-1-C^{14}	2.48	7.70
acetate-2-C^{14}	14.5	33.7
ethanol-2-C^{14}	4.30	11.0
acetate-1-2-C^{14}	6.30	7.50
acetaldehyde-1-2-C^{14}	2.28	26.4

In vitro experiments

Liver slice technique

The liver slice procedure used was that perfected in this laboratory by R. J. Emerson (18). As the procedure is outlined in the above work in detail, only a brief outline will be presented here. The technique for lipid separation and assay has been altered considerably from that described by Emerson (18), and will be given in detail.

Following the fasting period, which was one hour for the normal animal and two hours for the diabetic, the animal was killed by decapitation, the liver rapidly dissected free, and immersed in 4° buffer.

The buffer used was Krebs-Ringer phosphate buffer, pH 7.4, and was prepared according to the method of Umbreit, Burris and Stauffer (54).

A block of tissue of convenient size, generally 1.5 x 1.5 centimeters, was cut from the liver and placed on a disc of moistened filter paper on a Stadie-Riggs microtome. Slices of quite uniform thickness, averaging 0.5 millimeter, were easily obtained and randomly distributed among previously weighed vials containing 5 milliliters of chilled buffer. Approximately one gram of tissue slices was added to each vial (torsion balance) and then the final total weight was accurately determined on a Gram-Atic balance. The slices were then added to 125 milliliter Warburg flasks with the use of a funnel and the funnel rinsed with 20 milliliters of chilled buffer. The flasks were then gassed with 100 % O_2 for one minute at a flow rate of six liters per minute while the medium was agitated. Previously prepared CO_2 absorption tubes containing 0.4 milliliter of 9N NaOH on #50 Whatman filter paper were placed in the center wells. The flasks were then attached to the manometers and placed in the constant temperature water bath at 37° C. One half hour was allowed for temperature equilibration before the radio-tracer was tipped in from the side arm. Because the flasks had to be removed from the water bath for approximately fifteen seconds to tip in the substrate, another two minutes were allowed to regain constant temperature and the manometers were then closed and pressure readings taken at fifteen minute intervals throughout the experiment.

At the end of the 60 minute incubation period the manometers were opened and 0.5 milliliter of 12N H_2SO_4 was added to the incubation

mixture to stop metabolic activity and to release any CO_2 present in the substrate. An additional half hour was allowed for collection of CO_2 in the center well.

Tissue fractionation and assay

CO_2 transfer and plating

At the end of the CO_2 collection period, the flask was removed from the bath, quickly removed from the manometer, and stoppered briefly until the CO_2 absorber was removed from the flask. After removal of the absorber tube, the filter paper roll was removed from the tube with a pair of rat-tooth thumb forceps and transfered to a 50 milliliter thick-walled centrifuge tube. The absorber tube was then washed five times with hot, CO_2-free, distilled water; the washings being added to the centrifuge tube containing the paper. The tube was then sealed with a tight fitting rubber vial closure and allowed to stand. At a convenient time, usually the following day, the tube was opened and the filter paper strip was grasped at one corner with a small hemostat and held over the tube while hot, CO_2-free distilled water was allowed to drip on it until the total volume in the centrifuge tube was about 40 milliliters. About 10 milliliters of this volume were contributed by the five washings from the CO_2 absorber tube and the remaining 30 milliliters was the quantity of water used to elute the NaOH and Na_2CO_3 from the filter paper strip. A known amount of carbonate, as standard Na_2CO_3 solution, was added to the tube to serve as carrier. The carbonate was precipitated as $BaCO_3$ and plated by our usual technique (62).

Control flasks containing buffer, CO_2 absorber, and acetate-C^{14}

were run repeatedly and the center well never contained more than two micromoles of CO_2 after the incubation, transfer and plating procedures described above. This amount of CO_2 represents only about 2% of the total CO_2 generally found at the end of one hour of incubation. These control samples never counted more than seven counts per minute above background.

Slice transfer and saponification

After removal of the CO_2 absorber, the substrate was decanted, the slices rinsed three times with ten milliliter portions of distilled water, and transfered with a small spatula to a 75 milliliter screwcap culture tube. Twenty milliliters of 2 molar KOH in 95% alcohol were added to the tube containing the washed slices and the tube was covered with a large glass marble. It was then placed on a steam bath and allowed to reflux slowly for two hours. Additional alcohol was added as necessary to maintain the volume.

At the end of two hours, the marble was removed and the alcohol blown off. Five milliliters of alcohol were then added to the tube and the volune taken to 20 milliliters with water, thus giving a 25% solution of alcohol.

Lipid extraction

All extraction procedures were carried out in the 75 milliliter screw-cap culture tubes into which the slices were initially transfered and in which they were saponified. Twenty milliliters of low boiling petroleum ether (B.P. 30°-60°) were added to the tube containing the saponified slices dissolved in 20 milliliters of the water-alcohol solution. The cap was screwed on securely and tested for

leaks by shaking a few times by hand. Several such tubes were then placed in a horozontal position on a shaking machine and shaken for five minutes at a rate of 180 strokes per minute, the horozontal excursion being 1½ inches. The tubes were removed and allowed to stand in a vertical position for about five minutes or until complete separation occured. If an emulsion persisted it was easily broken by the addition of a little 95% alcohol delivered as a fine jet from a polyethylene "wash bottle". The petroleum ether layer was then removed using a 30 milliliter syringe fitted with an 18 gauge 8 inch needle and was added directly to a 50 milliliter volumetric flask containing a boiling chip. This extraction procedure was repeated three more times, making a total of four extractions, each with 20 milliliters of petroleum ether. The 50 milliliter volumetric flask to which the petroleum ether layers were added was cautiously placed on a warm area of the cover of the steam bath and evaporated to a volume of 5-10 milliliters so as to make room for the next extract. After the last extract had been added to the volumetric flask, the contents were evaporated to 2-5 milliliters, then several milliliters of alcohol were added and the remainder of the petroleum ether evaporated. After cooling, the flask was made to volume with 95% alcohol. Radioassay and cholesterol colorimetric determinations were done on aliquots of this solution without further fractionation.

Following extraction of the nonsaponifiable lipid, the contents of the extraction tube were acidified with HCl to a congo red endpoint. During the acidification procedure the tubes were shaken while suspended in an ice bath to prevent excessive warming. The fatty acids

were then extracted with petroleum ether, the procedure being essentially the same as for the nonsaponifiable lipid as outlined above except that only three extractions were found to be necessary.

Lipid recoveries

The above extraction procedure removed many tedious steps from the lipid extraction procedure previously used in this laboratory, and gave equally good recoveries. Recoveries were determined by adding a known amount of labeled palmitic acid (palmitic acid-1-C^{14}, Fisher Scientific Co.) or labeled cholesterol (cholesterol-4-C^{14}, Radioactive Products Inc.) to aliquots of rat tissue digest and fractionating the lipids as described above. The tissues used were liver, gut, carcass and skin. The distribution of cholesterol and fatty acid between

Table III

Percent recovery of cholesterol-4-C^{14} from tissue digest

tissue fraction	cpm added	cpm recovered one extract	% recovery	theoretical recoveries two extracts	three extracts	four extracts	five extracts
liver	1150	930	81	96.4	99.3	99.9	99.9
gut	1150	760	66	88.4	96.1	98.7	99.6
carcass	1150	685	60	84.0	93.6	97.4	99.0
skin	1150	705	61	84.0	93.6	97.4	99.0

petroleum ether and the tissue digest in 25% alcoholic aqueous solution was determined by measuring the percent of the added label removed by the ether in one extraction. The results for cholesterol are shown in table III. Also shown are the theoretical yields for a total of two to five extractions based on the determined distribution. Even with the carcass fraction, which gave the smallest coefficient of distribu-

tion, four extractions would remove 97.4% of the total labeled cholesterol added, and presumably the same percentage of the unlabeled cholesterol present. An additional extraction of this fraction could be expected to yield only an additional 1.6%, and we did not feel that this yield warrented the time expenditure involved in an additional extraction.

When known amounts of palmitic acid-1-C^{14} were added to tissue digests, the least favorable distribution for the fatty acid in the ether phase was 80%. This gives a theoretical recovery of 96% after two extractions and a total of 99.2% after a third extraction.

Table IV

Lipid recovery by the modified extraction procedure

tissue fraction	cpm recovered in cholesterol fraction	cpm recovered in fatty acid fraction	% recovery of label	% contamination
	2500 cpm of cholesterol-4-C^{14} added			
liver	2500	0	100	0
gut	2482	0	99.3	0
carcass	2350	0	94.0	0
skin	2360	0	94.4	0
	24300 cpm of palmitic acid-1-C^{14} added			
liver	370	24600	101	1.5
gut	170	24850	102	0.7
carcass	60	24000	98.8	0.2
skin	0	24700	102	0

The total extraction procedure was carried out on eight tissue digests, labeled cholesterol being added to four and labeled palmitic acid to the other four. The recovery of the label and the cross contamination between the two classes of lipids are shown in table IV.

It is seen that the recovery of the added label is essentially complete, that an average of less than 1% of the added radio-palmitic acid is removed during extraction of the nonsaponifiable fraction, and that no contamination of the fatty acid fraction by the labeled cholesterol was found.

Quantitative determination of lipids

Color reacting sterols in the nonsaponifiable fraction (mainly cholesterol) were determined as cholesterol by the method of Zlatkis (80). Aliquots of the saponifiable fraction were taken to dryness with a N_2 jet in previously desiccated, tared vials. The net weight was presumed to be due to fatty acids, and is so reported in the experiments to be described.

Radioassay of lipids

Lipids were radioassayed directly at infinite thinness (<0.15 mg. per cm.^{2}), using a D-47 gas flow counter with a micromil window (Nuclear Chicago Corp.). The infinite thinness procedure was developed in this laboratory and has been described elsewhere (36). The efficiency of counting by this procedure was 28.7%.

In vivo experiments

Metabolism chamber

All animals in the in vivo series were used after a "one hour fast" following their last one hour feeding period. After being weighed, the animals were injected with the appropriate tracer intraperitoneally using a 1½ inch, 21 gauge needle on a Luer Lok syringe. They were immediately placed in the metabolism chamber (61) where they remained for two hours. During this time the metabolism assembly was

constantly flushed with dry, CO_2-free air, the expired gas being passed first through concentrated sulfuric acid for drying, then across the window of a thin end window G. M. tube contained in a lead shield. The scaler to which the G. M. tube was attached activated a count rate computer, which in turn activated a milliammeter recorder, thus giving a continuous graph of the relative specific activity of the expired CO_2. The air stream was then passed through a container of 1N NaOH, thus trapping all of the expired $C^{12}O_2$ and $C^{14}O_2$ for further quantitative titration and radioassay (see below). For a more detailed description of the assembly see VanBruggen and Hutchens (61).

Tissue fractionation and assay

CO_2 titration

The total quantity of CO_2 expired during the two hour period in the metabolism chamber was determined by titration with standard HCl. The reactions involved in this titration are as follows:

CO_2 collection in NaOH yields

$$CO_2 + 2NaOH \longrightarrow Na_2CO_3 + H_2O \qquad (A)$$

Titration with HCl leads to the following reactions

$$NaOH + HCl \longrightarrow NaCl + H_2O \qquad (B)$$

$$Na_2CO_3 + HCl \longrightarrow NaHCO_3 + NaCl \qquad (C)$$

$$NaHCO_3 + HCl \longrightarrow NaCl + H_2CO_3 \rightleftharpoons H_2O + CO_2 \qquad (D)$$

At the phenolphthalein endpoint, pH 8.4, reactions (B) and (C) are complete, and the original Na_2CO_3 is present as $NaHCO_3$. As the titration is continued to a methyl orange endpoint, the $NaHCO_3$ is converted quantitatively to H_2CO_3 which decomposes into water and CO_2, and the

latter is released from the titration flask. When the phenolphthalein endpoint is reached (reaction C above), each millimole of $NaHCO_3$ present is equivalent to one millimole of CO_2 reacting in equation (A). Since one milliequivalent of HCl is needed for each milliequivalent of $NaHCO_3$ to complete reaction (D), the milliequivalents of HCl required to titrate from a phenolphthalein endpoint to a methyl orange endpoint are equivalent to the millimoles of CO_2 originally absorbed in the NaOH solution. The equation for calculating total millimoles of expired CO_2 then is:

$$\text{mM of } CO_2 = \frac{(M-P)(\text{normality of HCl})(\text{total vol. of collected } CO_2)}{\text{volume of aliquot titrated}}$$

where

P = burette reading at the phenolphthalein endpoint

M = burette reading at the methyl orange endpoint

The actual technique used was as follows:

All titration flasks to be used were pre-flushed with N_2 before addition of the samples to be titrated. If a series of samples were to be determined at one time, they were all measured in advance, but kept tightly stoppered except during the actual titration to prevent accumulation of atmospheric CO_2. Ten to fifteen milliliter aliquots were used, and for this volume a 50 milliliter Erlenmeyer flask with a small magnetic stirring bar was found to be very satisfactory. A ten milliliter burette, graduated in 0.02 milliliter, was used for addition of the HCl. When nearing endpoints, quantities of HCl less than a drop were removed from the burette tip with a fine stream of water from a polyethylene "wash bottle". The titration was carried out under a continuous flow of N_2.

A drop of 1% phenolphthalein in alcohol was added at the beginning of the titration and HCl run in until the red color of phenolphthalein just cleared. The burette reading was recorded (P), and two drops of 0.1% methyl orange were added, giving a yellow color. Additional HCl was added until the pink color of methyl orange developed, marking the end of the titration. The final burette reading (M) was taken at this time.

In as much as the CO_2 liberated in this reaction was radioactive, the titration was carried out under the hood.

CO_2 radioassay

The radioactivity of the expired CO_2 was determined by liquid assay by a procedure developed in this laboratory for volatile aqueous samples (63).

Tissue saponification

Upon completion of the two hours in the metabolism assembly, the animal was immediately sacrificed by decapitation and separated by blunt dissection into fractions of liver, gut, carcass and skin, the head and spleen being added to the carcass fraction. These fractions were weighed and immediately added to flasks containing a previously prepared digest mixture of 25% KOH in 95% ethanol. This digest mixture was prepared as shown in table V.

The flasks containing the tissues were then placed on electric heaters and each tissue digested under reflux for two hours. At the end of this time the flasks were removed and the contents filtered while hot through glass wool into graduated cylinders. The flasks were then rinsed twice with small volumes (5-10 milliliters) of hot

Table V

Preparation of digest mixture

tissue fraction	digestion flask size	ml. 95% ethanol	grams of KOH
liver	100 ml	50	12.5
gut	500 ml	150	37.5
carcass	1000 ml	200	50.0
skin	500 ml	100	25.0

alcohol and twice with small volumes of hot distilled water. These washings were also filtered through the glass wool into the graduated cylinders. The digests were then allowed to cool and were taken up to convenient volumes and the volumes recorded. The digests were then well shaken to insure homogeneity and aliquots taken for lipid extraction. The customary final volumes of the digest mixtures and the aliquot sizes taken were:

tissue	total volume	aliquot taken
liver	100 ml	25 ml
gut	175 ml	10 ml
carcass	400 ml	10 ml
skin	175 ml	10 ml

The aliquots were transfered to 75 milliliter screw-top culture tubes, the tubes placed in a boiling water bath, and the entire alcohol phase removed with the aid of a N_2 jet. The digest was then reconstituted to approximately 25% alcohol by adding six milliliters of 95% ethanol and taking up to 25 milliliters with distilled water.

Lipid extraction, quantitization, and radioassay

The procedures for lipid recovery and assay were identical to those already described for the *in vitro* animals.

CHAPTER III

IN VITRO NORMAL ANIMALS

Results

Quantitative recoveries

One would not anticipate that tracer doses of substrate would have any influence upon the quantity of CO_2 produced or upon the O_2 utilized, or upon the concentration of lipids in the liver slice. As can be seen from tables VI and VII, no large differences were found in the amounts of CO_2, O_2, fatty acids and cholesterol isolated, regardless of the label used.

Table VI

CO_2 produced and O_2 utilized by liver slices

substrate	CO_2 number of animals	CO_2 recovery*	O_2 number of animals	O_2 recovery*
acetate-1-C^{14}	6	63.3 ±9.67	5	61.8 ±8.33
acetate-2-C^{14}	7	61.6 ±9.88	6	60.2 ±7.10
acetate-1-2-C^{14}	7	59.3 ±12.4	6	62.4 ±6.19
ethanol-1-C^{14}	4	56.0 ±7.55	3	62.6 ±5.56
ethanol-2-C^{14}	7	60.4 ±9.49	6	59.5 ±9.00
acetaldehyde-1-2-C^{14}	6	58.6 ±7.94	6	56.6 ±10.8
total	37	60.1 ±9.40	32	60.3 ±7.94

* Mean value in micromoles per gram, ± standard deviation

These results are in agreement with results previously reported from this laboratory (18), and tend to confirm the belief that the labeled substances used were indeed tracers, compared with each other, in that any one compound did not shift the parameters of comparison.

Percent incorporation

The percent incorporation of the various labeled compounds into

Table VII

Concentration of lipids in the liver slice

substrate	number of animals	cholesterol*	fatty acids*
acetate-1-C^{14}	6	2.26 ± 0.196	22.4 ± 5.05
acetate-2-C^{14}	7	2.25 ± 0.287	23.7 ± 2.79
acetate-1-2-C^{14}	7	2.15 ± 0.198	23.5 ± 3.74
ethanol-1-C^{14}	4	2.28 ± 0.147	23.2 ± 4.34
ethanol-2-C^{14}	7	2.14 ± 0.289	21.5 ± 4.55
acetaldehyde-1-2-C^{14}	7	2.09 ± 0.183	23.9 ± 2.35
total	38	2.19 ± 0.229	23.0 ± 3.71

* Mean value in milligrams per gram of liver, ± standard deviation

CO_2, cholesterol and fatty acids by the liver slices from the normal animals are shown in tables VIII, IX and X. It will be noted from the values in these tables, that even with control of the diet, as regards composition, quantity and time of feeding prior to an experiment, using an inbred strain of rats of uniform weight and age, and using nearly identical doses of substrate, that there are wide fluctuations of the percent incorporation of any given label into the lipid fractions. Although the $C^{14}O_2$ values have a maximum of a 1.5 fold range or spread of values, C-14 cholesterol values have as much as a fourfold range and fatty acid values an even greater range. Although it is likely that certain of the values could be shown statistically to not belong to the particular group, all values have been included in the present tables and comparisons made on a "horizontal basis" as is described below. This variability of the fatty acid labeling response of the slice preparation is due to factors beyond our knowledge and control, but suffices to conceal differences between the metabolism of the substrates if the means and standard deviations of the groups are

Table VIII

% incorp. of C^{14} into CO_2 by liver slices of normal animals ≠

	substrates					
animal	*A-1-C^{14}	*A-2-C^{14}	*A-1-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	***Ald-1-2-C^{14}
C-1a	11.1			12.0		
C-1b	8.9			12.8		
D-1	10.6	5.39	10.6		8.23	
D-3	10.6	4.79	7.02	15.8	7.25	5.74
D-4	7.6	4.56	7.40	16.0	4.09	6.42
D-5	11.5	4.61	7.92	14.8	4.61	4.87
D-6	13.3	5.98	7.82		6.15	5.27
D-7	14.0	5.81	8.17	14.3	7.91	5.43
ave.	10.9	5.19	8.16	14.3	6.37	5.55

≠ expressed as percent incorporation per gram of tissue per hour

*A = acetate **E = ethanol ***Ald = acetaldehyde

Table IX

% incorp. of C^{14} into cholesterol by liver slices of normal animals ≠

	substrates					
animal	*A-1-C^{14}	*A-2-C^{14}	*A-1-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	***Ald-1-2-C^{14}
C-1a	1.53			2.14		
C-1b	1.47			2.15		
D-1	1.74	2.77	2.80		3.87	
D-3	4.48	6.46	4.63	5.88	8.02	5.04
D-4	3.43	6.40	4.43	7.02	9.12	4.23
D-5	4.71	6.31	5.34	6.18	6.41	3.40
D-6	7.20	10.3	8.11		12.1	5.97
D-7	4.81	5.87	4.06	5.44	6.52	2.47
ave.	3.67	6.35	4.90	4.80	7.67	4.22

≠ expressed as percent incorporation per gram of tissue per hour

*A = acetate **E = ethanol ***Ald = acetaldehyde

Table X

% incorp. of C^{14} into fatty acids by liver slices of normal animals ≠

	substrates					
animal	*A-1-C^{14}	*A-2-C^{14}	*A-1-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	***Ald-1-2-C^{14}
C-1a	0.102			0.163		
C-1b	0.098			0.115		
D-1	0.040	0.121	0.067		0.085	
D-3	0.720	1.00	0.790	1.03	1.12	0.990
D-4	0.660	1.14	0.820	1.49	2.07	1.18
D-5	5.98	6.68	5.94	9.17	7.98	3.77
D-6	2.40	2.62	2.39		3.53	1.74
D-7	1.02	0.957	0.708	1.18	1.16	0.448
ave.	1.38	2.09	1.79	2.19	2.66	1.63

≠ expressed as percent incorporation per gram of tissue per hour

*A = acetate **E = ethanol ***Ald = acetaldehyde

used for comparisons. The liver slice preparation, however, offers an ideal means of minimizing the variability between animals, for all substrates can be used or tested on liver slices obtained from the same animal. With such an "internal control", the ratio of the responses of two substrates can then be determined, and it becomes possible to establish statistical differences between substrates when that difference is smaller than the difference between two animals with one substrate. In this thesis the data has been treated in this manner, and the ratio of the utilizations of substrates are shown in tables XI, XII, and XIII, and are graphically illustrated in figure 1. The statistical evaluation of the data was done by means of the t distribution, the equation being:

$$t = \frac{\bar{x} - 1}{s/\sqrt{n}}$$

Table XI

Ratios of the percent incorporations of various substrates into CO_2 by liver slices of normal animals

	substrates				
animal	acetate-1-C^{14} / acetate-2-C^{14}	ethanol-1-C^{14} / acetate-1-C^{14}	ethanol-1-C^{14} / ethanol-2-C^{14}	ethanol-2-C^{14} / acetate-2-C^{14}	acetate-1-2-C^{14} / acetaldehyde-1-2-C^{14}
C-1a		1.287			
C-1b		1.021			
D-1	1.967			1.527	
D-3	2.213	1.491	2.179	1.514	1.222
D-4	1.671	2.100	3.912	0.897	1.153
D-5	2.495	1.080	3.210	1.000	1.626
D-6	2.224			1.028	1.484
D-7	2.410	1.438	1.808	1.361	1.505
ave.	2.163	1.403	2.777	1.221	1.398

Table XII

Ratios of the percent incorporations of various substrates into cholesterol by liver slices of normal animals

	substrates				
animal	acetate-2-C^{14} / acetate-1-C^{14}	ethanol-1-C^{14} / acetate-1-C^{14}	ethanol-2-C^{14} / ethanol-1-C^{14}	ethanol-2-C^{14} / acetate-2-C^{14}	acetate-1-2-C^{14} / acetaldehyde-1-2-C^{14}
C-1a		1.399			
C-1b		1.463			
D-1				1.397	
D-3	1.442	1.313	1.364	1.241	0.919
D-4	1.867	2.047	1.299	1.425	1.047
D-5	1.340	1.482	1.037	1.016	1.571
D-6	1.431			1.175	1.358
D-7	1.246	1.131	1.199	1.111	1.644
ave.	1.465	1.473	1.225	1.228	1.308

Table XIII

Ratios of the percent incorporations of various substrates into fatty acids by liver slices of normal animals

	substrates				
animal	acetate-2-C^{14} / acetate-1-C^{14}	ethanol-1-C^{14} / acetate-1-C^{14}	ethanol-2-C^{14} / ethanol-1-C^{14}	ethanol-2-C^{14} / acetate-2-C^{14}	acetate-1-2-C^{14} / acetaldehyde-1-2-C^{14}
C-1a		1.598			
C-1b		1.173			
D-3	1.389	1.431	1.087	1.120	0.798
D-4	1.727	2.258	1.389	1.816	0.695
D-5	1.117	1.533	0.870	1.195	1.576
D-6	1.092			1.347	1.374
D-7	0.938	1.157	0.983	1.212	1.580
ave.	1.253	1.525	1.082	1.338	1.203

Figure 1

Ratios of the percent incorporation of various labels into CO_2 and lipids by the normal rat liver slice

In the row of columns at the bottom of the figure the percent incorporation of acetate-1-C^{14} into each of the fractions is given the value of 100 and the percent incorporation of the other labels plotted as a percentage of the acetate-1-C^{14} response. The p values listed refer to the significance of the difference between the percent incorporation of the respective label and the percent incorporation of acetate-1-C^{14}.

The other rows are handled similarly except that for the middle row the percent incorporation of ethanol-2-C^{14} into these tissue fractions is given the value of 100 and the other labels compared to it, while for the upper row acetate-1-2-C^{14} serves as the basis for comparison.

For ease in reading, the p values that are significant at a 0.95 confidence level have been placed in parentheses.

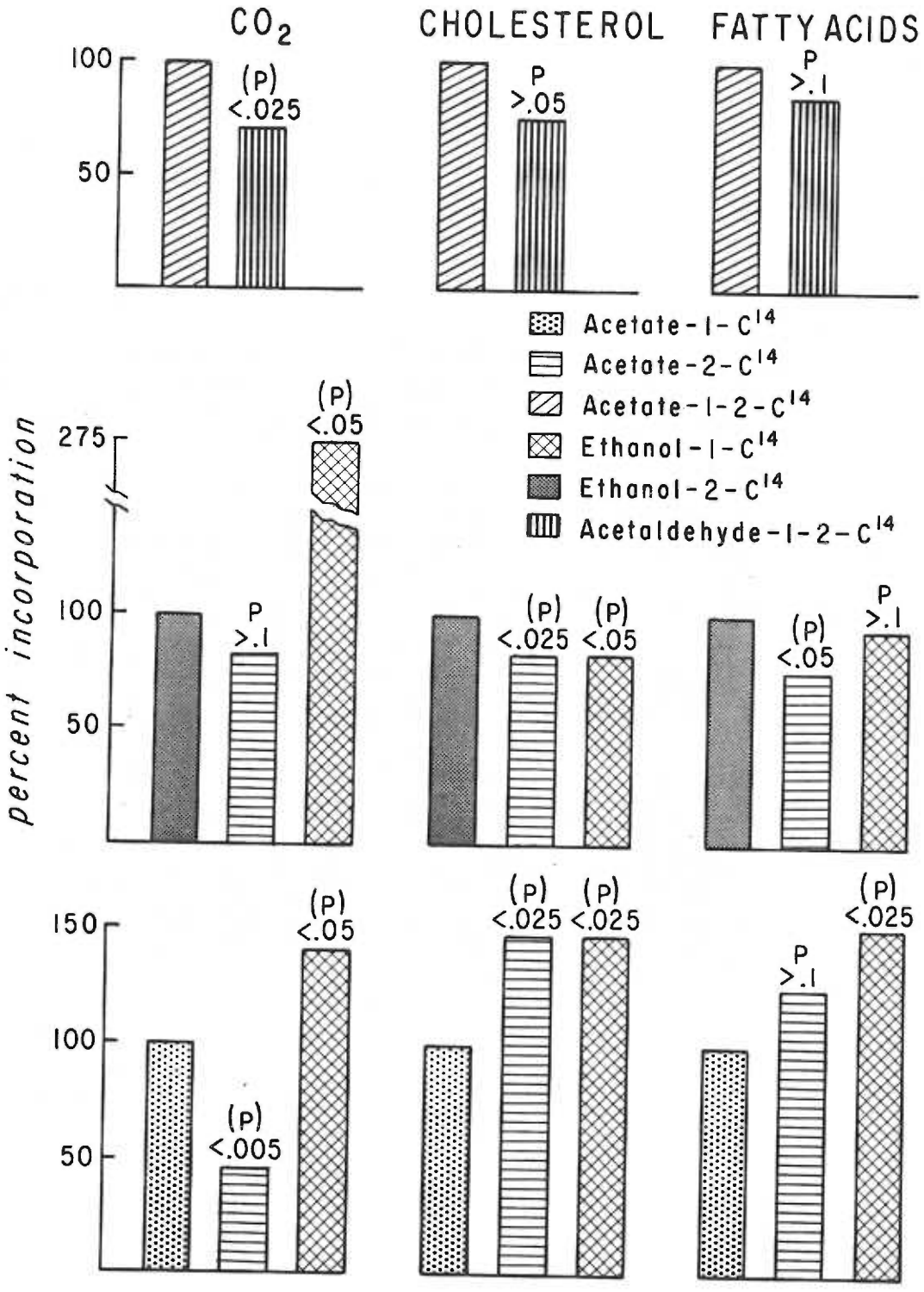
CO2
CHOLESTEROL
FATTY ACIDS
100
50
(P) <.025
P >.05
P >.1
Acetate-1-C14
Acetate-2-C14
Acetate-1-2-C14
Ethanol-1-C14
Ethanol-2-C14
Acetaldehyde-1-2-C14
percent incorporation
275
100
50
(P) <.05
P >.1
(P) <.025
(P) <.05
(P) <.05
P >.1
150
100
50
(P) <.05
(P) <.005
(P) <.025
(P) <.025
P >.1
(P) <.025

Fig. 1

where $\bar{R}$ is equal to the mean of the ratios of the percent incorporations of the two substrates, n is equal to the number of such ratios, and s^2 is equal to $\frac{R^2 - n(\bar{R}^2)}{n-1}$. The p values so calculated are shown on figure 1, and for ease in reading, those that are significant at a 0.95 confidence level are circled. For ease in graphing, the value for the percent incorporation of one of the labels in each group of three is arbitrarily taken to be 100, and some other response compared with it on a percentage basis.

CO_2

As seen in figure 1, the C-1 carbons of both ethanol and acetate are better sources of respiratory CO_2 than are the C-2 carbons by ratios of 2.8:1 for ethanol and 2.2:1 for acetate.

From the same figure it is seen that the C-1 position of ethanol is a better source of $C^{14}O_2$ than is the same position of acetate, by a ratio of 1.4:1. It is also seen that the mean percent incorporation of ethanol-2-C^{14} into respiratory CO_2 compared to the mean percent incorporation of acetate-2-C^{14} is not significantly higher when tested at a 0.95 confidence level.

Acetate-1-2-C^{14} was a significantly better precursor of respiratory CO_2 than was double labeled acetaldehyde-C^{14}, and since either position of ethanol was an equal or better precursor of CO_2 than similarly labeled acetate, it can safely be concluded that ethanol-1-2-C^{14} is likewise a better precursor of CO_2 in this preparation than is acetaldehyde-1-2-C^{14}.

Cholesterol

The methyl carbons of both acetate and ethanol were significantly

better precursors of cholesterol than were the C-1 carbons, by ratios of 1.47:1 and 1.22:1 respectively. Ethanol labeled in either position was a significantly better precursor of cholesterol than was acetate, the ratio of the percent incorporation of ethanol-1-C^{14}:acetate-1-C^{14} being 1.47:1 and that of ethanol-2-C^{14}:acetate-2-C^{14} being 1.23:1.

At a 0.95 confidence level, there was no difference in the magnitude of acetate-1-2-C^{14} and acetaldehyde-1-2-C^{14} incorporation into cholesterol.

Fatty acids

Again, ethanol labeled in either position was a significantly better precursor of fatty acids than was similarly labeled acetate, the ratio ethanol-1-C^{14}:acetate-1-C^{14} being 1.5:1, and ethanol-2-C^{14}: acetate-2-C^{14} being 1.3:1.

There was no significant difference between acetate and acetaldehyde in fatty acid labeling.

The C-1 and C-2 carbons of either ethanol or acetate were equally efficient precursors of liver fatty acids.

Discussion

1. On the increased incorporation of C-1 carbon into CO_2

Working on the basis that the largest amount of metabolic CO_2 arises from oxidative decarboxylations of the tricarboxylic acid (TCA) cycle it is to be expected that the C-1 carbons of both acetate and ethanol would make greater contributions to the CO_2 in short term experiments than would the respective methyl carbons. As one follows the carbon of acetyl CoA units through multiple turns of the TCA cycle

it becomes obvious that the decarboxylation steps of the cycle more readily remove the C-1 carbon of the original acetyl CoA than they do the C-2 carbon. Strisower, Kohler and Chaikoff (17) have calculated the percentage distribution of the methyl and carboxyl labels of acetate into the four carbon positions of oxaloacetic acid after successive turns of the TCA cycle. They have shown that the carboxyl label is found only in the C-1 and C-4 positions of oxaloacetic acid, the carbons that will be removed in the next turn of the cycle. The methyl label, however, becomes distributed throughout all four carbon positions of oxaloacetic acid, with the greatest amount being found in the C-2 and C-3 positions. The CO_2 arising from the cycle then would be expected to contain more of the original carboxyl carbons, while compounds derived as intermediates from components of the cycle would be expected to contain a greater percentage of the methyl carbons of the original acetate. Katz and Chaikoff (14) working with rat liver slices found that the ratio of $C^{14}O_2$ derived from acetate-1-C^{14}:$C^{14}O_2$ derived from acetate-2-C^{14} ranged in their experiments from 2.4:1 to 3.8:1, and the range as reported from the literature varied from 2:1 to 5:1. Our results presented above, giving a ratio of 2.2:1 for acetate and 2.8:1 for ethanol are in agreement with the results of these authors and reflect this somewhat selective release of the C-1 position as CO_2.

2. On the increased incorporation of methyl label into cholesterol

Konrad Bloch (1) studied the distribution of labeled acetate carbon in cholesterol and found that the methyl carbon contributed 1.27 times as much activity to cholesterol as did the carboxyl carbon.

Popjak (48) in 1954 succeeded in biosynthesizing squalene from radio acetate. The squalene was sufficiently labeled to allow chemical degradation and identification of the acetate carbon positions. The formation of cholesterol by the cyclization of this squalene would result in the incorporation of 15 methyl carbons of acetate into cholesterol and 12 carboxyl carbons, giving a ratio of 15/12 or 1.25. The present findings of ratios of 1.47 for incorporation into cholesterol of C^{14} derived from $\frac{\text{acetate-2-}C^{14}}{\text{acetate-1-}C^{14}}$ and of 1.22 for incorporation into cholesterol of C^{14} derived from $\frac{\text{ethanol-2-}C^{14}}{\text{ethanol-1-}C^{14}}$ are in close agreement with the work of Bloch and Popjak.

3. On the equal incorporation of C-1 and C-2 label into fatty acids

The theory that fatty acid synthesis is a simple reversal of the beta oxidation degradative pathway has been rendered unlikely by the recent work of Salih Wakil (66) and Roscoe Brady (5). They have shown by means of purified enzyme systems that acetyl CoA is converted to malonyl CoA by the addition of CO_2. By the hypothesis of Wakil, this malonyl CoA then condenses with acetyl CoA to form α-carboxy-β-keto butyryl CoA. By successive steps of reduction, removal of H_2O, and further reduction, this is converted to α-carboxy butyryl CoA which is then decarboxylated to form butyryl CoA. The butyryl CoA then condenses with another molecule of malonyl CoA and the equivalent reactions of the previous steps repeated. The process of addition of malonyl CoA followed by decarboxylation is presumed to continue until fatty acids of appropriate chain length are formed. The hypothesis

of Brady is similar, except that he would condense the malonyl CoA with an aldehyde rather than with the analogous acyl CoA derivative, in an aldol condensation of the Knoevenagel type. Decarboxylation would occur in this same step giving rise to β-hydroxy butyryl CoA. This would then be converted successively to butyryl CoA and butyraldehyde. The butyraldehyde would then condense with a second molecule of malonyl CoA and the process repeated until fatty acids of sufficient chain length were formed.

The hypothesis of Brady is more acceptable to some from a chemical standpoint, but has less experimental evidence to support it than does the hypothesis of Wakil.

By either mechanism, however, the CO_2 that is removed from the malonyl CoA unit contains the same carbon that was added to the original acetyl CoA to form malonyl CoA; for while label from exogenous radio-CO_2 appeared in the malonyl CoA, the fatty acid ultimately formed was without activity. The net overall process also remains the same, long chain fatty acids being formed from the union of multiple two carbon units. Since this is the case, there should be no difference in the incorporation of the C-1 or C-2 carbons of acetate or ethanol into fatty acids, and indeed we have found no significant differences in this work.

4. On the increased incorporation of ethanol-C^{14} into CO_2, cholesterol and fatty acids

As mentioned above, ethanol seemed to be a better precursor of CO_2, cholesterol and fatty acids than was acetate. This was true for the C-1 position, while the difference was not statistically signifi-

cant at a 0.95 confidence level for the incorporation of the C-2 carbon. The relative contributions of these compounds to the labeling of the three tissue components were:

	$\frac{\text{ethanol-1-C}^{14}}{\text{acetate-1-C}^{14}}$	$\frac{\text{ethanol-2-C}^{14}}{\text{acetate-2-C}^{14}}$
CO_2	1.4	1.2
cholesterol	1.5	1.2
fatty acids	1.5	1.3

When the data is viewed in this manner, it becomes readily apparent that the differences between the two components can be explained by a greater absorption and/or by a more rapid metabolism of the ethanol, and one need not postulate a pathway of ethanol metabolism different from that described above. Thus, one can let the relative percent incorporation into CO_2 indicate the relative amounts of the two compounds that have entered the tissues and become available to the metabolic pathways located there. From the above data it can be seen that 1.2 to 1.4 times as much ethanol as acetate became available to the liver slice oxidative system during the duration of the experiment. Likewise, 1.2 to 1.5 times as much ethanol as acetate was incorporated into tissue lipids. Thus, the same percentage of that part of the dose that actually becomes available to the tissues for metabolic processes is incorporated into tissue lipids from both acetate and ethanol.

There is no problem in concluding that these results are compatible with the thesis that acetate is an obligatory intermediate of ethanol metabolism, particularly if one assumes that the greater incorporation of ethanol is due to a more rapid transport of this

molecule across the cellular membrane. If the difference is due to a more rapid rate of ethanol metabolism rather than a greater flux across the cellular membrane — and we have no way of telling which effect we are seeing here — the findings are still compatable with the presently proposed pathway of ethanol metabolism which postulates that ethanol is converted to acetyl CoA rather than to acetate.

Another possibility exists to explain the increased incorporation of ethanol into CO_2, cholesterol and fatty acids without postulating different pathways, and it may be of profit to explore it briefly. For ease in discussion, we will assume that acetate is the common intermediate, but the same reasoning would hold if acetyl CoA were the intermediate.

If ethanol were metabolized in a different geometric position of the cell than was acetate, and the acetate intermediate common to the metabolic pathway of the two compounds did not become dispersed in a common acetate pool, then ethanol might indeed be absorbed and metabolized at the same rate as acetate and still put more label into CO_2, cholesterol and fatty acids than the acetate itself does. One might have to postulate, in this case, that the acetate pool available to ethanol was smaller than that which diluted the administered acetate. One would further have to postulate that acetate derived from ethanol was oxidized to CO_2, and also used for the synthesis of fatty acids and cholesterol, without entering a part of the cell that exogenously administered acetate enters, for increased incorporation of ethanol was found in all three components.

That such a complete separation and duplication of metabolic path-

ways exists can not be absolutely denied on the basis of our present knowledge, but it seems highly unlikely for the following reasons: First, alcohol dehydrogenase is found in the water soluble fraction of the cytoplasm of the liver cell (4), and thus the metabolism of ethanol probably begins here, and not in one of the sub-cellular particles that have been identified. Secondly, as far as we know, the synthesis of cholesterol and fatty acids, and the oxidation of fatty acids, take place only in specific areas of the cell. Thus, the synthesis of cholesterol appears to be limited to the microsome (7), the oxidation of fatty acids to the mitochondria (21), and while the exact site of fatty acid synthesis is not known, it does not occur in the mitochondria (21) but appears to occur in the cytoplasm. At least, complete enzyme systems able to convert acetyl CoA to long chain fatty acids are found in particle-free soluble extracts of liver and other tissues (6, 38, 67). These various sites, then, would seem to be common meeting grounds for acetate destined to become cholesterol or fatty acid, whether it be from exogenous acetate or acetate derived from the oxidation of ethanol. An increased incorporation of ethanol into cholesterol and fatty acids then does not seem to be logically explained on the basis of dilution by acetate pools of different size.

A different degree of dilution does not seem likely for the reasons mentioned. An increased rate of metabolism of ethanol over acetate also seems unlikely in view of the rather slow decline of blood alcohol following its ingestion, and the low turnover number and low Michaëlis constant (33) for the reaction

$$\text{ethanol} + \text{DPN} \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} \text{acetaldehyde} + \text{DPNH} + H^+$$

An increased rate of absorption of alcohol, on the other hand, is in keeping with our knowledge of the rapid rate of ethanol absorption by the gastric mucosa, and the great solubility of ethanol in both aqueous and in lipid systems. Of the various possibilities mentioned, the most likely explanation of the increased incorporation of ethanol into the tissue components would seem to be an increased rate of absorption of ethanol.

5. On the comparison of acetate and acetaldehyde

Acetate proved to be a significantly better precursor of respiratory CO_2 than was acetaldehyde, and the mean percent incorporation of acetate into cholesterol and fatty acids is higher than the mean for acetaldehyde. Thus, while ethanol proved to be a better precursor of these tissue constituents than acetate, acetaldehyde is a poorer precursor. These results are contrary to what one might expect if acetaldehyde is a metabolic intermediate between ethanol and acetate. However, if one again assumes that the percent of the dose that is incorporated into CO_2 is indicative of the percent absorbed by the tissue, then the tissue put an equal amount of the absorbed dose of both acetate and acetaldehyde into cholesterol, and a slightly greater percent of the acetaldehyde into fatty acids. When compared in this same manner, we saw that acetate and ethanol were equally efficient precursors of cholesterol and fatty acids. Assuming then that the percent of the dose incorporated into CO_2 is indicative of the percent of the dose absorbed and thus available to the tissues, then these results are consistent with the hypothesis that ethanol is oxidized to

acetaldehyde and the latter to acetate.

There is another possible explanation for the observed lower incorporation of acetaldehyde into CO_2, cholesterol and fatty acids, that would also be consistent with the view that acetaldehyde is metabolized via acetate. It may be that acetate and acetaldehyde are absorbed in the liver slice preparation at essentially the same rate, and that the decreased incorporation of the latter is a reflection of its slower rate of activation and metabolism. If this were the case, then ethanol could not be metabolized via acetaldehyde, as the rate of ethanol metabolism is equal to or greater than that of acetate, as we have seen above. One could postulate in this case that ethanol is converted directly to an "active aldehyde", a process more readily carried out in this preparation than is the activation of exogenous acetaldehyde.

In this regard it will be noted that Newman (45) and also Westerfeld (72) have both reported a rapid rate of metabolism of injected acetaldehyde. This would make it more likely that the decreased incorporation of acetaldehyde in the liver slice preparation is due to a decreased absorption and not to a decreased rate of metabolism. However, it must be pointed out that these other investigators were working with intact animals and administered pharmacological rather than tracer doses of acetaldehyde.

Our results for incorporation of acetaldehyde into fatty acids differ from those of Brady and Gurin (26) who reported acetaldehyde to be a better precursor of liver fatty acids than acetate, by a ratio of 1.5:1. It must be pointed out, however, that the conditions of the

two experiments varied in many respects. Of particular note is the difference in the amount of labeled substrate used, Brady and Gurin used 75 micromoles of acetaldehyde or acetate per flask, while we employed approximately 1 micromole. Emerson and VanBruggen (59) have pointed out the marked effect that increasing doses of tracer acetate have upon lipogenesis in the rat liver slice, the percent incorporation of acetate into fatty acids at a dose of 75 micromoles being approximately 1/3 of the anticipated response on the basis of the percent incorporation from a dose of 1 micromole. A similar study has not been done on acetaldehyde and there is no way of predicting the response to increasing doses of this compound. A different response of liver slices toward increasing doses of these two substrates could, however, explain the difference between our results and those of Brady and Gurin.

It is interesting to speculate on the possible meaning of the increased incorporation of acetaldehyde as compared to acetate into liver fatty acids (assuming that for both tracers the percent of the dose incorporated into CO_2 is indicative of the percent of the dose metabolized). If acetaldehyde must be oxidized to acetate prior to being converted to fatty acids, then one would expect either the same or a smaller percent of a dose of acetaldehyde to be incorporated into fatty acids, as compared to a dose of acetate. If, on the other hand, the synthesis of fatty acids occurs by the reaction of aldehydes with malonyl CoA, as postulated by Brady (5), then acetaldehyde would enter this synthetic pathway directly and would be expected to be incorporated to the same or to a greater extent into fatty acids than

would acetate, which would require prior conversion to acetaldehyde. While these results are not offered as strong support for Brady's hypothesis, it is certainly one way of interpreting the data.

Summary statements

1. Acetate-1-C^{14}, acetate-2-C^{14}, ethanol-1-C^{14}, ethanol-2-C^{14}, acetate-1-2-C^{14} and acetaldehyde-1-2-C^{14} have been compared as precursors of the CO_2, cholesterol and fatty acid fractions of surviving liver slices of normal rats in vitro.
2. The C-1 carbons of both ethanol and acetate were found to be better precursors of respiratory CO_2 than the methyl carbons. This is explained by the discriminative decarboxylations of the TCA cycle.
3. The methyl carbons of both ethanol and acetate were found to be better precursors of cholesterol than the C-1 carbons. This is explained by the known ratio of C-1 to C-2 carbons found in cholesterol which is biologically synthesized from acetate.
4. There was no significant difference in the percent incorporation of the carboxyl and methyl carbons of acetate or of the C-1 and C-2 carbons of ethanol into fatty acids. That this is to be anticipated from the proposed pathway of fatty acid synthesis is discussed.
5. Ethanol labeled in either position incorporated more carbon atoms into CO_2, cholesterol and fatty acids than did similarly labeled acetate. It is proposed that this is due to a greater absorption of ethanol by the liver slice. Other possible mechanisms to

explain this increased incorporation are discussed.

6. Acetate-1-2-C^{14} was found to be a significantly better precursor of CO_2 than acetaldehyde-1-2-C^{14}, and tended to be a better precursor of cholesterol and fatty acid than acetaldehyde-1-2-C^{14}, though the differences were not significant at a 0.95 confidence level. It is proposed that this again is due to a difference in the rate of absorption of these two compounds, though other possibilities are discussed.

7. The greater percent of the "available" dose of acetaldehyde incorporated into fatty acids, as compared with the "available" dose of acetate incorporated into this same fraction, is suggested to be consistent with the hypothesis that fatty acid synthesis occurs via an aldol condensation of the Knoevenagel type, as postulated by Roscoe Brady (5).

8. It is concluded that the data presented here for the normal rat liver slice is consistent with though not proof of the presently proposed pathway of ethanol metabolism, the oxidation of ethanol to acetaldehyde and the oxidation of the latter to acetate of acetyl CoA.

CHAPTER IV

IN VITRO DIABETIC ANIMALS

Results

The diabetic rat liver slice has been shown to have a decreased rate of lipogenesis from acetate (58). Elwood and VanBruggen have shown that a substantial part of this block of lipogenesis occurs prior to the formation of β-OH butyrate, perhaps on the basis of a decrease in the availability of acetate for CO_2 and lipid formation and a decreased acetate pool size.

If ethanol and acetaldehyde are metabolized solely via acetate, this depression of lipogenesis in the diabetic should be similar for all three labels. If these compounds are metabolized via alternate pathways not requiring prior conversion to acetate, then significant differences might be found in the diabetic preparation in the conversion of these compounds to lipids. It is for this reason that the diabetic preparation was studied.

Quantitative recoveries

As was also previously noted for the normal animals, there was little difference found in the amounts of CO_2, O_2, cholesterol or fatty acids isolated from the diabetic animals, regardless of the tracer employed. These results are summarized in tables XIV & XV.

Percent incorporations

The comparison of the results of the various substrates was handled statistically in the same manner as the comparison in the normal animals, and are plotted in a similar manner in figure 2. The actual values of the percent incorporations are given in tables

Table XIV

CO_2 produced and O_2 utilized by liver slices of diabetic animals

substrate	CO_2 number of animals	CO_2 recovery*	O_2 number of animals	O_2 recovery*
acetate-1-C^{14}	6	57.7 ± 9.41	6	65.6 ± 6.65
acetate-2-C^{14}	6	58.0 ± 5.08	6	63.0 ± 8.16
acetate-1-2-C^{14}	6	55.2 ± 7.50	6	61.3 ± 5.97
ethanol-1-C^{14}	6	54.6 ± 12.1	6	63.7 ± 7.10
ethanol-2-C^{14}	6	57.4 ± 5.58	6	63.0 ± 9.52
acetaldehyde-1-2-C^{14}	6	49.7 ± 9.17	5	66.2 ± 4.15
total	36	55.4 ± 8.33	35	63.8 ± 6.66

* Mean value in micromoles per gram per hour, ± standard deviation

Table XV

Concentration of lipids in the diabetic liver slice

substrate	number of animals	cholesterol*	number of animals	fatty acids*
acetate-1-C^{14}	4	2.29 ± 0.146	6	27.6 ± 2.28
acetate-2-C^{14}	4	2.12 ± 0.240	6	28.7 ± 2.16
acetate-1-2-C^{14}	4	2.28 ± 0.240	6	27.0 ± 1.37
ethanol-1-C^{14}	4	2.31 ± 0.305	6	26.5 ± 2.37
ethanol-2-C^{14}	4	2.48 ± 0.312	6	26.4 ± 2.55
acetaldehyde-1-2-C^{14}	4	2.35 ± 0.418	6	26.1 ± 1.81
total	24	2.30 ± 0.278	36	27.1 ± 2.15

* Mean value in milligrams per gram of liver, ± standard deviation

XVI, XVII, and XVIII, and the ratios of the responses of the substrates that we are interested in comparing are given in tables XIX, XX, and XXI.

CO_2

Comparing similar portions of figures 1 and 2, we find that the relative percent incorporation of the various labels into CO_2 in the

Table XVI

% incorp. of C^{14} into CO_2 by liver slices of diabetic animals ≠

	substrates					
animal	*A-1-C^{14}	*A-2-C^{14}	*A-1-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	***Ald-1-2-C^{14}
F-1	16.7	6.44	9.27		8.91	5.62
F-2	19.9	9.94	14.1	29.2	13.4	6.45
F-3	16.1	6.76	10.7	19.7	8.77	2.58
F-4	17.5	5.57	10.6	20.4	9.48	5.00
F-5	17.4	7.46	11.9	21.8	11.7	5.72
F-6	16.7	6.93	10.4	26.7	11.6	5.94
ave.	17.4	7.18	11.2	23.6	10.6	5.14

≠ expressed as percent incorporation per gram of tissue per hour

*A = acetate **E = ethanol ***Ald = acetaldehyde

Table XVII

% incorp. of C^{14} into cholesterol by liver slices of diabetic animals ≠

	substrates					
animal	*A-1-C^{14}	*A-2-C^{14}	*A-1-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	***Ald-1-2-C^{14}
F-1	0.939	1.35	1.05	1.03	1.51	0.670
F-2	0.855	1.10	0.781	1.35	1.90	0.541
F-3	1.32	1.34	1.26	1.59	1.76	0.517
F-4	1.20	1.37	1.23	1.51	1.93	0.525
F-5	0.445	0.948	0.591	0.933	1.36	0.264
F-6	0.032	0.058	0.038	0.043	0.046	0.029
ave.	0.799	1.03	0.825	1.08	1.42	0.375

≠ expressed as percent incorporation per gram of tissue per hour

*A = acetate **E = ethanol ***Ald = acetaldehyde

diabetic preparation are similar to those of the normal liver slice in vitro. That is, the C-1 carbons of both ethanol and acetate are

Table XVIII

% incorp. of C^{14} into fatty acids by liver slices of diabetic animals ≠

	substrates					
animal	*A-1-C^{14}	*A-2-C^{14}	*A-1-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	***Ald-1-2-C^{14}
F-1	0.051	0.070	0.065	0.075	0.082	0.061
F-2	0.168	0.228	0.137	0.310	0.313	0.129
F-3	0.753	0.566	0.457	0.561	0.610	0.248
F-4	0.287	0.241	0.237	0.272	0.283	0.108
F-5	0.038	0.131	0.082	0.087	0.111	0.058
F-6	0.029	0.068	0.044	0.040	0.040	0.037
ave.	0.221	0.217	0.170	0.224	0.240	0.116

≠ expressed as percent incorporation per gram of tissue per hour

*A = acetate **E = ethanol ***Ald = acetaldehyde

better precursors of CO_2 than the C-2 carbons. Either carbon of ethanol is a better precursor of CO_2 than is the same carbon of acetate. Lastly, acetate-1-2-C^{14} is a better labeler of CO_2 than is double labeled acetaldehyde.

Cholesterol

Again, we find that the relationship of the percent incorporations of ethanol and acetate into cholesterol are identical in the diabetic and in the normal animal, i.e., ethanol is a better precursor of cholesterol than is acetate, and the C-2 carbon of either compound is a better precursor than the C-1 carbon.

We find a statistically significant difference in the amount of label incorporated into cholesterol from acetate-1-2-C^{14} and acetaldehyde-1-2-C^{14}, the former being more readily incorporated. We did not find this difference to be statistically significant in the normal

Table XIX

Ratios of the percent incorporations of various substrates into CO_2 by liver slices of diabetic animals

	substrates				
animal	acetate-1-C^{14} / acetate-2-C^{14}	ethanol-1-C^{14} / acetate-1-C^{14}	ethanol-1-C^{14} / ethanol-2-C^{14}	ethanol-2-C^{14} / acetate-2-C^{14}	acetate-1-2-C^{14} / acetaldehyde-1-2-C^{14}
F-1	2.593			1.384	1.649
F-2	2.002	1.467	2.179	1.348	2.186
F-3	2.382	1.224	2.246	1.297	4.147
F-4	3.142	1.166	2.152	1.702	2.120
F-5	2.332	1.256	1.858	1.572	2.080
F-6	2.410	1.599	2.302	1.674	1.751
ave.	2.477	1.342	2.147	1.496	2.322

Table XX

Ratios of the percent incorporations of various substrates into cholesterol by liver slices of diabetic animals

animal	substrates: acetate-2-C^{14} / acetate-1-C^{14}	ethanol-1-C^{14} / acetate-1-C^{14}	ethanol-2-C^{14} / ethanol-1-C^{14}	ethanol-2-C^{14} / acetate-2-C^{14}	acetate-1-2-C^{14} / acetaldehyde-1-2-C^{14}
F-1	1.438	1.097	1.466	1.119	1.567
F-2	1.287	1.579	1.407	1.727	1.444
F-3	1.015	1.205	1.107	1.313	2.437
F-4	1.142	1.258	1.278	1.409	2.343
F-5	2.130	2.097	1.458	1.435	2.239
F-6	1.813	1.344	1.070	0.793	1.310
ave.	1.471	1.430	1.298	1.299	1.890

Table XXI

Ratios of the percent incorporations of various substrates into fatty acids by liver slices of diabetic animals

	substrates				
animal	acetate-2-C^{14} / acetate-1-C^{14}	ethanol-1-C^{14} / acetate-1-C^{14}	ethanol-2-C^{14} / ethanol-1-C^{14}	ethanol-2-C^{14} / acetate-2-C^{14}	acetate-1-2-C^{14} / acetaldehyde-1-2-C^{14}
F-1	1.373	1.471	1.093	1.171	1.066
F-2	1.357	1.845	1.010	1.373	1.062
F-3	0.752	0.745	1.087	1.078	1.843
F-4	0.840	0.948	1.040	1.174	2.194
F-5	3.447	2.289	1.276	0.847	1.414
F-6	2.345	1.397	1.000	0.588	1.189
ave	1.686	1.446	1.084	1.039	1.461

Figure 2

Ratios of the percent incorporation of various labels into CO_2 and lipids by the diabetic rat liver slice

In the row of columes at the bottom of the figure the percent incorporation of acetate-1-C^{14} into each of the three fractions is given the value of 100 and the percent incorporation of the other labels plotted as a percentage of the acetate-1-C^{14} response. The p values listed refer to the significance of the difference between the percent incorporation of the respective label and the percent incorporation of acetate-1-C^{14}.

The other rows are handled similarly except that for the middle row the percent incorporation of ethanol-2-C^{14} into these fractions is given the value of 100 and the other labels compared to it, while for the upper row acetate-1-2-C^{14} serves as the basis for comparison.

For ease in reading, the p values that are significant at a 0.95 confidence level have been placed in parentheses.

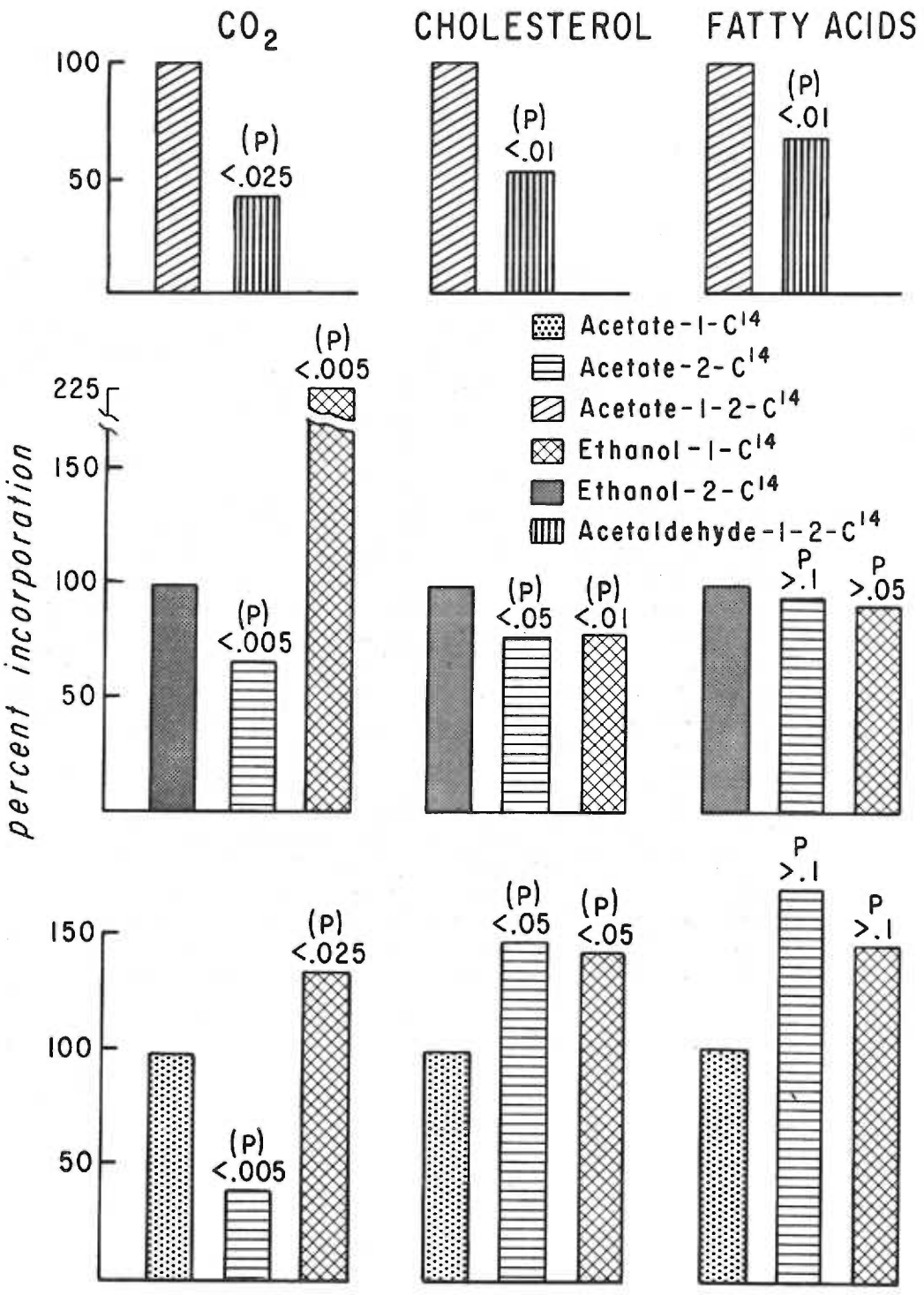
CO2
CHOLESTEROL
FATTY ACIDS
100
50
(P) <.025
(P) <.01
(P) <.01
Acetate-1-C14
Acetate-2-C14
Acetate-1-2-C14
Ethanol-1-C14
Ethanol-2-C14
Acetaldehyde-1-2-C14
percent incorporation
225
150
100
50
(P) <.005
(P) <.005
(P) <.05
(P) <.01
P >.1
P >.05
150
100
50
(P) <.025
(P) <.005
(P) <.05
(P) <.05
P >.1
P >.1

Fig. 2

animal, and it may be related here to the more markedly decreased incorporation of acetaldehyde into CO_2, as we shall discuss presently.

Fatty acids

As in the normal preparations, we find here that there is no significant difference between the C-1 and C-2 carbons in their labeling of fatty acids. Contrary to the finding in the normal, in the diabetic animal we find no significant difference between ethanol and acetate in the amount of activity getting into fatty acids.

Again, as with cholesterol, we find a significant difference between the amount of label incorporated into fatty acids from acetate and acetaldehyde, the former being a better precursor.

Discussion

1. On the increased incorporation of C-1 carbon into CO_2

Again, this is in keeping with the selective oxidative decarboxylations of the TCA cycle as discussed in the previous section.

2. On the increased incorporation of the methyl label into cholesterol

Again, our findings are the same in these diabetic preparations as in the normal animals, the methyl carbons of acetate and ethanol contributing 1.4 and 1.3 times as much label to cholesterol as the C-1 carbons. This is in keeping with the fact that, as discussed in the previous section, of the 27 carbons of cholesterol, 15 are derived from the methyl carbon of acetate and 12 from the carboxly carbon.

3. On the equal incorporation of the C-1 and C-2 label into fatty acids

Our findings in these diabetic animals, as in the normals, are in agreement with the hypothesis that the net synthesis of fatty acids

occurs by union of multiple two carbon units.

4. On the relative incorporations of ethanol and acetate into CO_2, cholesterol and fatty acids

The relative incorporations of these compounds into the three tissue components studied were:

	$\frac{\text{ethanol-1-C}^{14}}{\text{acetate-1-C}^{14}}$	$\frac{\text{ethanol-2-C}^{14}}{\text{acetate-2-C}^{14}}$
CO_2	1.34	1.50
cholesterol	1.43	1.30
fatty acids	1.45	1.04

Again, as in the normal animals, if the percent incorporation into CO_2 is assumed to represent the relative amount of the substrate actually made available to the tissues, the diabetic is handling both compounds similarly in the synthesis of cholesterol, within the limits of experimental error. This holds true for fatty acid synthesis from the C-1 carbons, but not from the methyl carbons. On the basis of the percent incorporation into CO_2, the methyl carbon of acetate is a better precursor of liver fatty acids in the diabetic liver slice than is the methyl carbon of ethanol. We offer no explanation for this finding, and can not reconcile it with the presently known schemes of metabolism. It may be that it is an artifact of small sampling of a large population, or due simply to experimental error.

5. On the comparison of acetate and acetaldehyde

In the diabetic preparation, acetate proved to be a significantly better precursor of CO_2, cholesterol and fatty acids than acetaldehyde. If calculated on the assumption that the percent incorporation into CO_2 indicates the amount of the label absorbed, then the tissue handled

both compounds in a quantitatively similar manner in the synthesis of cholesterol, and incorporated slightly more acetaldehyde than acetate into fatty acids, a relationship similar to that found in the normal animals. Again, this increased incorporation of acetaldehyde into fatty acids, when compared in this manner, would seem to be in keeping with the hypothesis of Brady (5), that fatty acid synthesis occurs by means of repeated aldol condensations.

The differences in the percent incorporation of the label of the two compounds into lipids is significant in these diabetic animals for both cholesterol and fatty acids. In the normal animals, though the difference is in the same direction, it is not significant for either of the lipid fractions. The explanation for this probably lies in the fact that the relative incorporation of acetaldehyde into CO_2 is further depressed in the diabetic preparation than in the normal. If, as we have assumed, the percent incorporation into CO_2 indicates the amount of substrate made available to the metabolic pathways of the tissue, then one would expect that a depression in the percent incorporation of label into CO_2 would be accompanied by an equal depression in the percent incorporation of that label into cholesterol and fatty acids. This indeed is what was found.

The significance of this decreased utilization of acetaldehyde by the diabetic in the formation of CO_2 will be discussed in the following pages where there is compared the diabetic and normal <u>in vitro</u> preparation in respect to formation of CO_2, cholesterol and fatty acids from various substrates.

Summary statements

1. Acetate-1-C^{14}, acetate-2-C^{14}, ethanol-1-C^{14}, ethanol-2-C^{14}, acetate-1-2-C^{14} and acetaldehyde-1-2-C^{14} have been compared as precursors of the CO_2, cholesterol and fatty acid fractions of surviving liver slices of diabetic rats *in vitro*.
2. The C-1 carbons of both ethanol and acetate were found to be better precursors of respiratory CO_2 than the methyl carbons. This is explained by the selective oxidative decarboxylations of the TCA cycle.
3. The methyl carbons of both ethanol and acetate were found to be better precursors of cholesterol than the C-1 carbons. This is explained by the known ratio of methyl to carboxyl carbons found in cholesterol which is biologically synthesized from acetate.
4. There was no significant difference in the percent incorporation of the C-1 and C-2 carbons of acetate or ethanol into fatty acids. That this is to be anticipated from the proposed pathway of fatty acid synthesis has been previously discussed.
5. Ethanol labeled in either position incorporated more carbon atoms into CO_2 and cholesterol than did similarly labeled acetate. It is proposed that this is due to a greater absorption of ethanol by the liver slice.
6. Acetate was found to be a significantly better precursor of CO_2, cholesterol and fatty acids than acetaldehyde. It is proposed that this is due to a greater absorption of the acetate.
7. The ratio of $\frac{C^{14}O_2 \text{ derived from acetate-1-2-}C^{14}}{C^{14}O_2 \text{ derived from acetaldehyde-1-2-}C^{14}}$ was greater in the diabetic preparation than in the normal. The significance

of this will be discussed in the following section.

8. When compared on the basis of the percent of the dose that actually was metabolized, as indicated by CO_2 percent incorporation figures, acetaldehyde was a better precursor of fatty acids than was acetate. This would seem to be in keeping with the hypothesis of Brady, that fatty acid synthesis occurs by means of repeated aldol condensations.

9. It is concluded that the data presented here for the diabetic rat liver slice is consistent with, but not proof of, the presently proposed pathway of ethanol metabolism, the oxidation of ethanol to acetaldehyde and the oxidation of the latter to acetate or acetyl CoA.

CHAPTER V

DIABETIC vs NORMAL IN VITRO

Results

While there was found an almost identical pattern when comparing the relative incorporations of the various labels into CO_2 and lipids by both the normal and diabetic slice preparation, when the percent incorporation of any one label into these fractions is compared there are found marked quantitative differences. These differences will be pointed out in the present section.

Percent incorporation

These results have been previously presented, and are given in tables VIII, IX and X for the normal animals, and in tables XVI, XVII, and XVIII for the diabetics. The results, however, are also presented graphically in figure 3. In this graph, the percent incorporation of the label by the normal animal is arbitrarily given the value of 100, and that of the diabetic animal is given as a percentage thereof.

CO_2

Though the diabetic preparation shows no significant difference in the amount of CO_2 respired during the course of the experiment, the CO_2 formed contained a significantly greater amount of the tracer carbon from acetate and ethanol than did the CO_2 of the normal preparation.

Different results are seen when acetaldehyde is used as tracer. Here we find the same percent of the dose incorporated into CO_2 by both the normal and diabetic preparations.

Cholesterol

The incorporation of labeled acetate into cholesterol was reduced

Figure 3

Comparison of the normal and diabetic in the incorporation of label into CO_2, cholesterol and fatty acids

The percent incorporation of each tracer compound into CO_2 and lipids by the normal animal is given the value of 100, and the percent incorporated by the diabetic animal is plotted as a percentage of the normal response. The p values for the differences between the diabetic and normal preparations in the percent incorporation into fatty acids and cholesterol are .005. The p values for the difference in percent incorporation into CO_2 are .05 for all labels except acetaldehyde-1-2-C^{14}, in which the p value is .1.

A-1 = acetate-1-C^{14} A-2 = acetate-2-C^{14}
E-1 = ethanol-1-C^{14} E-2 = ethanol-2-C^{14}
A-1-2 = acetate-1-2-C^{14}
Ald-1-2 = acetaldehyde-1-2-C^{14}

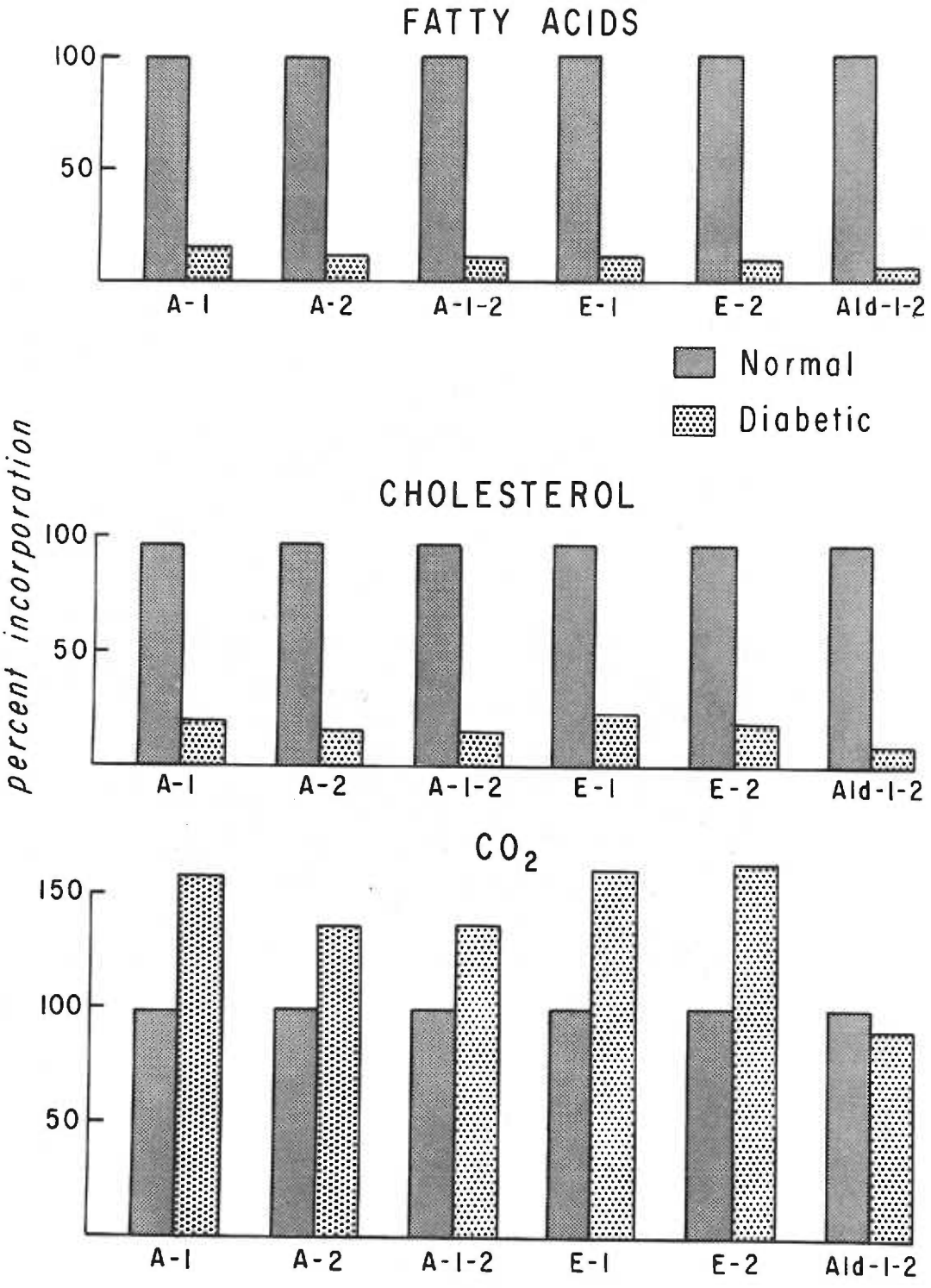
FATTY ACIDS
100
50
A-1
A-2
A-1-2
E-1
E-2
Ald-1-2
Normal
Diabetic
percent incorporation
CHOLESTEROL
100
50
A-1
A-2
A-1-2
E-1
E-2
Ald-1-2
CO_2
150
100
50
A-1
A-2
A-1-2
E-1
E-2
Ald-1-2

Fig. 3

82% in the diabetic liver slice, the incorporation of labeled ethanol was reduced 80%, and the incorporation of labeled acetaldehyde was reduced 91%. These values on acetate are in close agreement with those reported by Elwood and VanBruggen (58), who found the incorporation of acetate-1-C^{14} into cholesterol reduced by 83% in the diabetic liver slice. The p value for the difference is significant at a 0.995 confidence level.

Fatty acids

The incorporation of labeled acetate into fatty acids was reduced 88% in the diabetic liver slice, the incorporation of labeled ethanol was reduced 90%, and the incorporation of labeled acetaldehyde was reduced 93%. Again, these figures are in close agreement with the 92% reduction in the percent incorporation of acetate-1-C^{14} into fatty acids by diabetic liver slices reported by Elwood and VanBruggen (58). The p value for the difference is significant at a 0.995 confidence level.

Discussion

1. On the percent incorporation of C^{14} label of acetate and ethanol into CO_2 in the diabetic liver slice

As has been pointed out, the diabetic preparation produced a slightly decreased amount of CO_2 in the experimental period, but the CO_2 formed contained a significantly greater amount of tracer carbon from acetate and ethanol than did the CO_2 formed by the normal animals. This finding is not in accord with the observation of Hotta and Chaikoff (13) who concluded, on the basis of the amount of $C^{14}O_2$

recovered, that the diabetic slice oxidizes acetate to a slightly greater degree than does the normal slice. Our data on the amount of CO_2 produced and on $C^{14}O_2$ activity indicates that the diabetic rat forms the same amount or less CO_2 but converts more of the administered labeled acetate or ethanol to $C^{14}O_2$ than does the normal rat. This observation on the increased incorporation of labeled ethanol and acetate into CO_2 is in agreement with the observation of Elwood and VanBruggen (58) for acetate-1-C^{14}, though they found, in addition, a statistically significant decrease in the total amount of CO_2. They suggested on the basis of their findings that the supplies of acetate (the size of the acetyl CoA pool?) in the diabetic are decreased, with a resultant slowing of the acetate flux through the oxidative cycle, but with a concurrent decreased dilution of the acetate tracer dose. While the present findings do not demonstrate a significant decrease in the total amount of CO_2 formed in the diabetic as was found by these other workers, their suggestion of a decreased availability of acetate and a decreased acetate pool in the diabetic would explain the results equally well.

2. On the percent incorporation of C^{14} label of acetaldehyde into CO_2 in the diabetic vs normal liver slice

The diabetic liver slice incorporated the same percent of the dose of acetaldehyde into CO_2 as did the normal liver slice. This is contrary to the results found for acetate and ethanol, and suggests a dissimilarity in the metabolic pathways of ethanol and acetaldehyde.

Thus, if ethanol and acetaldehyde are metabolized via identical pathways, i.e., acetaldehyde is an obligatory intermediate of ethanol

metabolism, then any block in the oxidation of acetaldehyde caused by alloxan diabetes should affect ethanol metabolism as well. However, we find that the diabetic is able to put 65% more of a dose of labeled ethanol into CO_2 than is the normal, while putting 7% less of a dose of acetaldehyde into CO_2 than the normal. If the suggestion made above is correct, that the explanation for the increased incorporation of administered acetate and ethanol carbon into CO_2 in the diabetic is due to a decreased acetyl CoA pool, then the rate of metabolism of acetaldehyde in the diabetic rat must be depressed over that of the normal to result in CO_2 of similar specific activity in conjunction with a lesser dilution of the acetyl CoA.

The argument may be presented that the decreased formation of CO_2 from acetaldehyde in the diabetic is not due to a specific block in acetaldehyde metabolism in this preparation, one that does not exist for ethanol, but that it reflects a different cellular site of metabolism, a site where the acetate pool is not decreased. However, as we have seen, lipid synthesis from acetaldehyde is also further depressed in the diabetic preparation than is lipid synthesis from ethanol, and as these synthetic pathways seem to occur in specific areas of the cell that form common meeting grounds for acetate from any source, this argument would not seem valid. Also, if the water soluble cytoplasmic enzyme alcohol dehydrogenase is concerned with the first step of alcohol oxidation, then ethanol must be converted to its first intermediate in the cytoplasm of the cell. This would also be the area into which acetaldehyde, absorbed from the surrounding medium, would be first introduced. Thus acetaldehyde from either source would find

itself in the same cellular environment, and would be expected to follow the same metabolic pathway.

It is difficult to reconcile the postulate that ethanol is oxidized to acetaldehyde as an obligatory intermediate with this finding of a specific difference in the diabetic preparation in the metabolism of acetaldehyde to CO_2, cholesterol and fatty acids; a block that does not affect ethanol metabolism. There is one additional possibility that we are unable to exclude on the basis of these experiments, that could explain these differences in the effect of alloxan diabetes on the metabolism of acetaldehyde and ethanol. That is, that the condition of alloxan diabetes causes a partial block in the absorption of acetaldehyde, i.e., some change in membrane permeability or transport that affects acetaldehyde but does not affect ethanol or acetate.

3. On the decreased incorporation of all labels into both lipid fractions

The finding of decreased lipid labeling in the diabetic preparation is in keeping with the results previously reported from this laboratory (58).

The percent incorporation into cholesterol by the diabetic slice was reduced 82% and 80% for acetate and ethanol respectively, and into fatty acids 88% for acetate and 90% for ethanol. These results, in conjunction with similar increases in percent incorporation into CO_2 of 45% and 64% for acetate and ethanol respectively, would indicate that there was no specific block in the diabetic animal in the metabolism of ethanol, but that both acetate and ethanol were being influenced in this preparation by a common metabolic aberration (s).

The greater depression of lipogenesis from acetaldehyde, 91% reduction in percent incorporation into cholesterol and 93% reduction in percent incorporation into fatty acids, is in keeping with the finding of relatively lesser quantities of acetaldehyde incorporated into CO_2 in the diabetic liver slice. These results, for reasons discussed above, suggest a specific block in the alloxan diabetic liver slice in the metabolism of acetaldehyde. This block must occur prior to the formation of acetyl CoA.

Summary statements

1. Diabetic and normal rat liver slices were compared in their ability to incorporate acetate-1-C^{14}, acetate-2-C^{14}, acetate-1-2-C^{14}, ethanol-1-C^{14}, ethanol-2-C^{14} and acetaldehyde-1-2-C^{14} label into CO_2, cholesterol and fatty acids.
2. There was no significant difference between the normal and diabetic preparations in the amount of CO_2 produced or O_2 utilized: or in the amount of lipid per gram of liver.
3. The incorporation of acetate-C^{14} label into CO_2 was increased 45% in the diabetic preparation. The incorporation of ethanol-C^{14} label into CO_2 was increased 64% in the diabetic preparation. There was no significant difference between the normal and diabetic slice in the incorporation of acetaldehyde-C^{14} label into CO_2.
4. The incorporation of all labels into cholesterol was decreased in the diabetic slices, the percentage reduction being 82% for acetate, 80% for ethanol and 91% for acetaldehyde.
5. The incorporation of all labels into fatty acids was decreased in

the diabetic slices, the percentage reduction being 88% for acetate, 90% for ethanol and 93% for acetaldehyde.

6. The increased incorporation of acetate and ethanol carbons into CO_2, and their decreased incorporation into lipids in the diabetic preparation is suggested to be on the basis of a decreased availability of acetate for CO_2 and lipid formation and a decreased acetate pool size. The similar responses of these two compounds to the metabolic aberration (s) of alloxan diabetes indicates that there is no specific block in the diabetic animal to the metabolism of ethanol.

7. The dissimilarity in the response of acetaldehyde and ethanol metabolism to diabetes suggests that acetaldehyde is not an obligatory intermediate of ethanol metabolism.

CHAPTER VI

IN VIVO NORMAL ANIMALS

Results

It is well known that the presence of certain metabolic pathways may be predicted on the basis of *in vitro* evidence, but it is not generally recognized that this same evidence does not necessarily yield information on the quantitative importance of such pathways in the intact animal. In view of the potential hazards of application of *in vitro* data, it seemed necessary to compare the incorporation of acetate, ethanol, and acetaldehyde carbon into CO_2, cholesterol and fatty acids in the intact animal as was done in the liver slice.

Quantitative recoveries

The means and standard deviations of the amounts of CO_2 expired by the test animals during the two hour stay in the metabolism chamber are recorded in table XXII. The values range from 26.4 to 28.0 millimoles, there being no significant difference between any of the animals, regardless of the label employed.

Table XXII

Respiratory CO_2 of test animals*

label	number of animals	mean	standard deviation
acetate-1-C^{14}	6	27.3	0.67
acetate-2-C^{14}	4	28.0	2.87
acetate-1-2-C^{14}	4	27.8	2.63
ethanol-1-C^{14}	8	27.4	2.17
ethanol-2-C^{14}	4	26.4	1.31
acetaldehyde-1-2-C^{14}	4	28.5	0.57
total	30	27.5	1.83

* As millimoles of CO_2 per two hours

Table XXIII

Lipid concentrations* of pooled animals

	cholesterol			fatty acids		
tissue	number of samples	mean	standard deviation	number of samples	mean	standard deviation
liver	21	2.48	0.39	21	32.1	5.39
gut	23	1.50	0.25	21	38.0	10.3
carcass	23	1.58	0.20	21	56.4	14.4
skin	23	2.52	0.26	21	125.0	33.7

* As milligrams per gram of tissue

The concentration of lipids in the various fractions studied, i.e., liver, gut, carcass and skin, are shown in table XXIII. In as much as the concentration of lipid did not vary with the substrate administered, the results were pooled, and the means and standard deviations of the lipid concentrations calculated for the pooled samples.

CO_2 specific activity

The results of the continuous assay of the C^{14} activity of respiratory CO_2 are plotted for each tracer and illustrated in figure 4. While the total administered counts varied somewhat with each label, all specific activity figures were calculated and plotted on the basis of unitized doses of 1.5×10^6 counts per minute for each tracer.

Radioactivity from ethanol-1-C^{14} (see fig. 4) finds its way into CO_2 more rapidly than does radioactivity from acetate-1-C^{14}, the peak activity being reached in 10 minutes following administration of ethanol-1-C^{14} and in 16 minutes following administration of acetate-1-C^{14}. The peak specific activity of expired CO_2 following ethanol-1-C^{14} injection is 12% higher than that following acetate-1-C^{14} injection.

These relationships hold for the specific activities following

Figure 4

Respiratory $C^{14}O_2$ activities following intraperitoneal injection of various C^{14} labeled tracers

The curves plotted are the averages of seven experiments with ethanol-1-C^{14}, eight experiments with acetate-1-C^{14}, four experiments with ethanol-2-C^{14}, four experiments with acetate-2-C^{14}, and three experiments with acetaldehyde-1-2-C^{14}. The $C^{14}O_2$ activity is expressed as counts per minute per minute. All dosages have been unitized to 1.5×10^6 counts per minute assayed as infinitely thick $BaCO_3$.

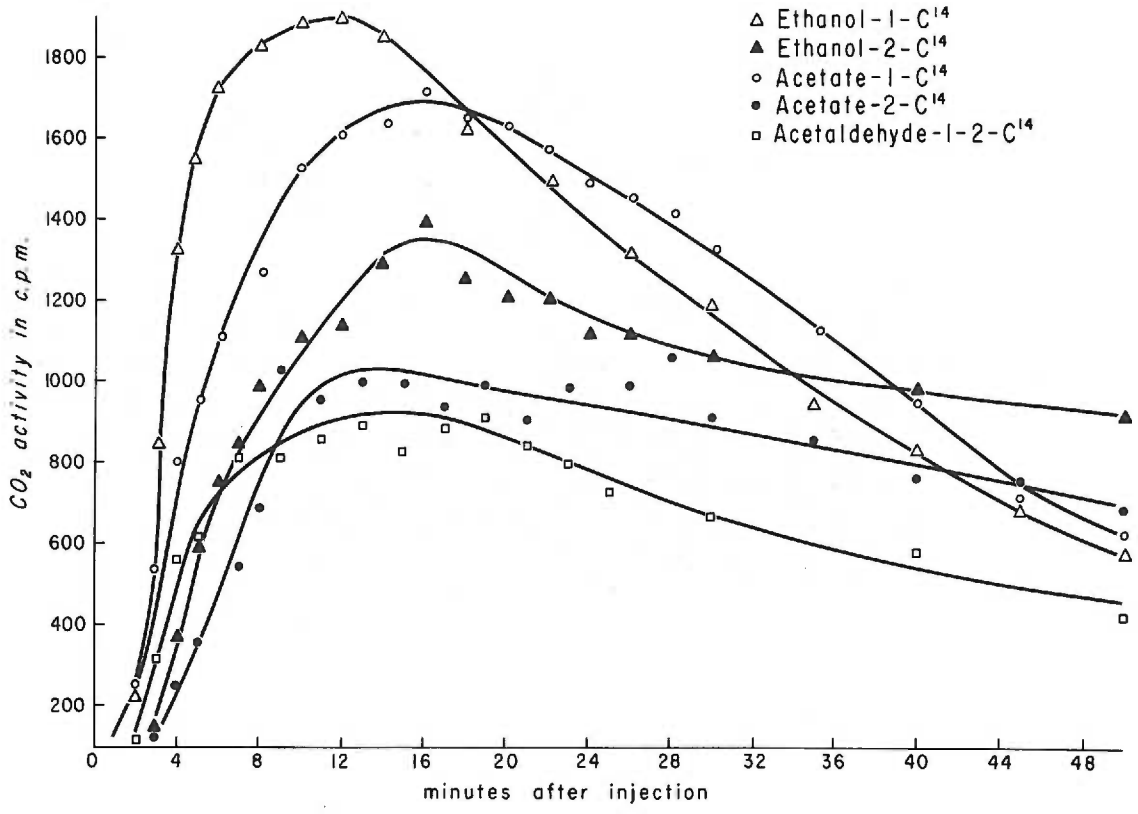
△ Ethanol-1-C14
▲ Ethanol-2-C14
○ Acetate-1-C14
● Acetate-2-C14
□ Acetaldehyde-1-2-C14
CO2 activity in c.p.m.
200
400
600
800
1000
1200
1400
1600
1800
0
4
8
12
16
20
24
28
32
36
40
44
48
minutes after injection

Fig. 4

Table XXIV

Percent incorporation into CO_2 in vivo

	substrates					
	*A-1-C^{14}	*A-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	*A-1-2-C^{14}	***Ald-1-2-C^{14}
No. of animals	8	4	7	4	4	4
	57.1[f] ± 4.85	51.6 ± 4.40	60.4 ± 3.42	51.1 ± 3.42	48.1 ± 1.88	32.0 ± 4.47
p values		>.05		<.005		<.005
			>.1			
				>.1		

*A = acetate **E = ethanol ***Ald = acetaldehyde

[f] mean ± standard deviation

administration of ethanol-2-C^{14} and acetate-2-C^{14}, i.e., the rise in specific activity is faster and continues to a higher level following ethanol-2-C^{14} administration than after acetate-2-C^{14} administration.

The C-1 label of either ethanol or of acetate appears sooner in expired CO_2 than does the methyl label, and also the $C^{14}O_2$ reaches a higher specific activity.

Percent incorporation

CO_2

The values for the incorporation of the six labels into CO_2 are shown in table XXIV.

There is no significant difference in the percent of the dose of acetate incorporated into CO_2 as compared to ethanol during the duration

of the experiment. This is true for both the C-1 and C-2 carbons, for the p value of both comparisons was >0.1. There is a difference in the incorporation of the C-1 and C-2 carbons into CO_2, the methyl carbon of both compounds showing a decreased incorporation. This difference is significant only at a 0.90 confidence level for acetate but at a 0.995 confidence level for ethanol.

Only 67% as much acetaldehyde-1-2-C^{14} label is incorporated into expired CO_2 as acetate-1-2-C^{14} label. This difference is significant at a 0.995 confidence level.

Cholesterol

These results are given in table XXV and shown graphically in figure 5. Another method of comparison, i.e., the use of the ratios of the percent incorporations into cholesterol for the various labels, is given in table XXVII.

As can be seen in figure 5, C^{14} carbon of ethanol, of either the C-1 or C-2 position, contributed more radioactivity to the nonsaponifiable lipid fraction than did C^{14} carbon of similarly labeled acetate. This difference is significant at the 0.95 confidence level for the skin and liver fractions in the comparison of C-1 label, and for all four fractions in the comparison of the methyl label.

The methyl label of ethanol was incorporated into cholesterol to a greater degree than was the C-1 label. The difference was statistically significant for all four fractions. The mean percent incorporation of acetate-2-C^{14} into cholesterol seemed to be higher than the mean percent incorporation of acetate-1-C^{14} for all four fractions, but the differences were much smaller than those seen in comparing the

Table XXV

Percent incorporation into cholesterol *in vivo*

	substrates					
	*A-1-C^{14}	*A-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	*A-1-2-C^{14}	***Ald-1-2-C^{14}
No. of animals	4	4	4	4	3	4
			liver			
	0.130[1] ± .026	0.221 ± .091	0.286 ± .056	0.678 ± .191	0.172 ± .074	0.206 ± .144
			gut			
	0.249 ± .032	0.329 ± .093	0.312 ± .045	0.791 ± .219	0.244 ± .061	0.305 ± .077
			carcass			
	0.186 ± .041	0.325 ± .153	0.361 ± .129	0.764 ± .183	0.205 ± .061	0.239 ± .091
			skin			
	0.066 ± .008	0.201 ± .050	0.149 ± .026	0.378 ± .112	0.122 ± .042	0.107 ± .044

*A = acetate **E = ethanol ***Ald = acetaldehyde

[1] mean ± standard deviation

two carbon positions of ethanol. As is indicated by the p values, a significant difference is seen only for the skin fraction.

There was no significant difference in the percent of the dose of acetaldehyde incorporated into cholesterol by any of the animal tissues when compared to acetate.

Figure 5

Percent incorporation of various labels into cholesterol by tissues of normal intact rats

The p values for the significance of the difference between each pair of labels are listed for each tissue. For ease in reading, those that are significant at a 0.95 confidence level have been placed in parentheses.

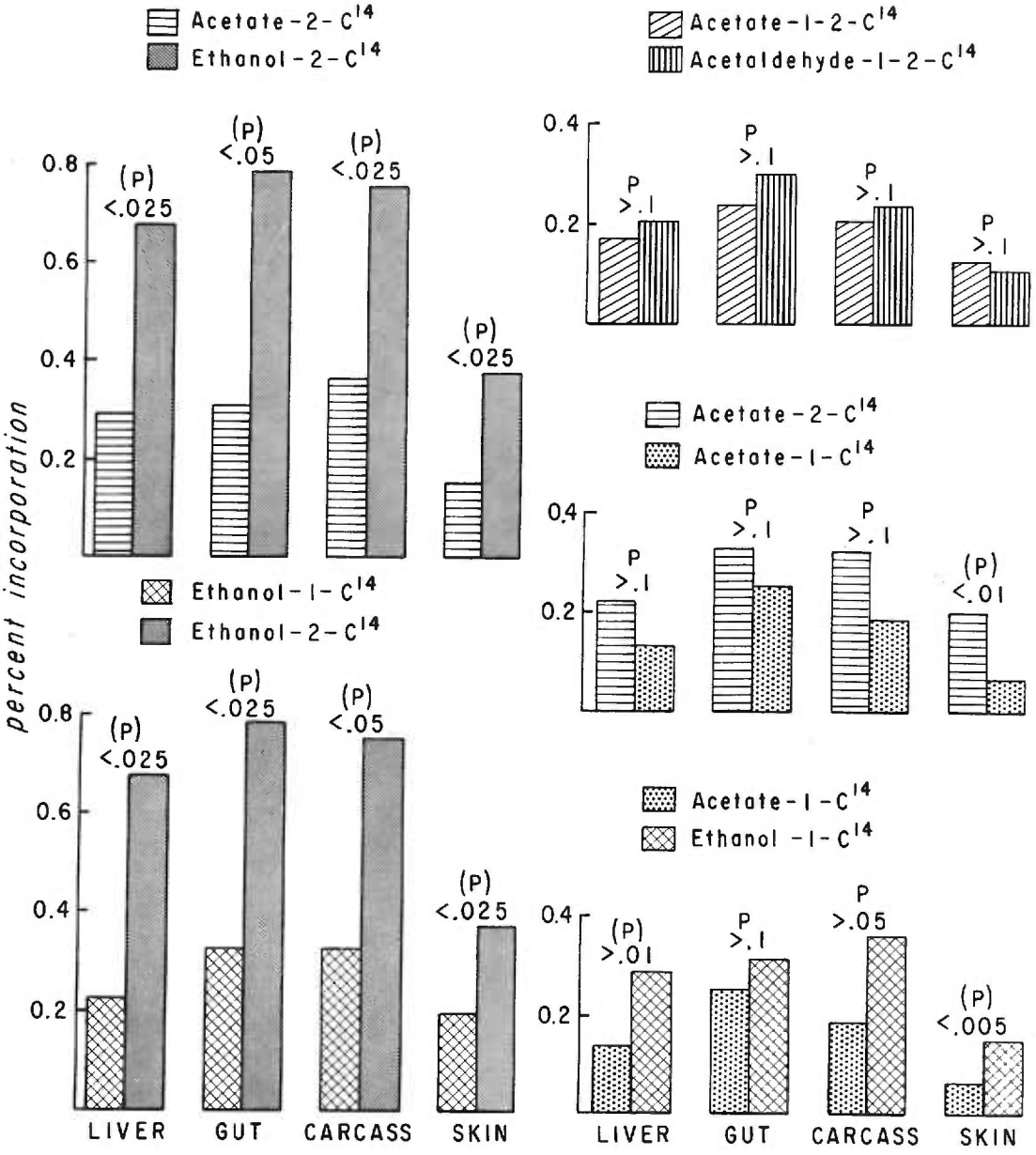
Acetate-2-C14
Ethanol-2-C14
Acetate-1-2-C14
Acetaldehyde-1-2-C14
percent incorporation
0.8
0.6
0.4
0.2
(P) <.025
(P) <.05
(P) <.025
(P) <.025
P >.1
P >.1
P >.1
P >.1
Acetate-2-C14
Acetate-1-C14
P >.1
P >.1
P >.1
(P) <.01
Ethanol-1-C14
Ethanol-2-C14
(P) <.025
(P) <.025
(P) <.05
(P) <.025
Acetate-1-C14
Ethanol-1-C14
(P) >.01
P >.1
P >.05
(P) <.005
LIVER
GUT
CARCASS
SKIN
LIVER
GUT
CARCASS
SKIN

Fig. 5

Table XXVI

Percent incorporation into fatty acids in vivo

	substrates					
	*A-1-C^{14}	*A-2-C^{14}	**E-1-C^{14}	**E-2-C^{14}	*A-1-2-C^{14}	***Ald-1-2-C^{14}
No. of animals	4	4	4	4	3	4
			liver			
	0.962≠ ± .47	0.454 ± .12	1.542 ± .81	2.701 ± 1.44	0.477 ± .23	0.759 ± .26
			gut			
	7.230 ± 1.23	3.108 ± 1.05	2.438 ± .27	3.763 ± .06	4.277 ± 1.83	3.453 ± .27
			carcass			
	7.465 ± 1.34	4.613 ± 1.64	5.348 ± 1.13	5.945 ± .55	4.917 ± .87	4.553 ± 1.15
			skin			
	0.858 ± .16	1.375 ± .23	1.790 ± .59	2.525 ± .65	1.713 ± .26	1.200 ± .46

*A = acetate **E = ethanol ***Ald = acetaldehyde

≠ mean ± standard deviation

Fatty acids

The means and standard deviations of the percent incorporations into fatty acids are given in table XXVI, and the ratios of the percent incorporations of the various labels are given in table XXVII. The data is illustrated graphically in figure 6.

There is no significant difference in the incorporation of the

Table XXVII

Ratios of the percent incorporations into CO_2 and lipids in vivo

	ethanol-1-C^{14} / acetate-1-C^{14}	ethanol-2-C^{14} / acetate-2-C^{14}	acetate-2-C^{14} / acetate-1-C^{14}	ethanol-2-C^{14} / ethanol-1-C^{14}	acetaldehyde-1-2-C^{14} / acetate-1-2-C^{14}
			CO_2		
	1.06	0.99	0.90	0.85	0.67
			Cholesterol		
liver	2.20	3.07	1.70	2.38	1.20
gut	1.25	2.40	1.32	2.53	1.25
carcass	1.94	2.35	1.75	2.12	1.17
skin	2.25	1.88	3.02	2.53	0.88
			Fatty acids		
liver	1.60	5.96	0.47	1.75	1.59
gut	0.34	1.21	0.43	1.54	0.81
carcass	0.72	1.29	0.62	1.11	0.93
skin	2.09	1.84	1.60	1.41	0.70

Figure 6

Percent incorporation of various labels into fatty acids by tissues of normal intact rats

The p values for the significance of the difference between each pair of labels are listed for each tissue. For ease in reading, those that are significant at a 0.95 confidence level have been placed in parentheses.

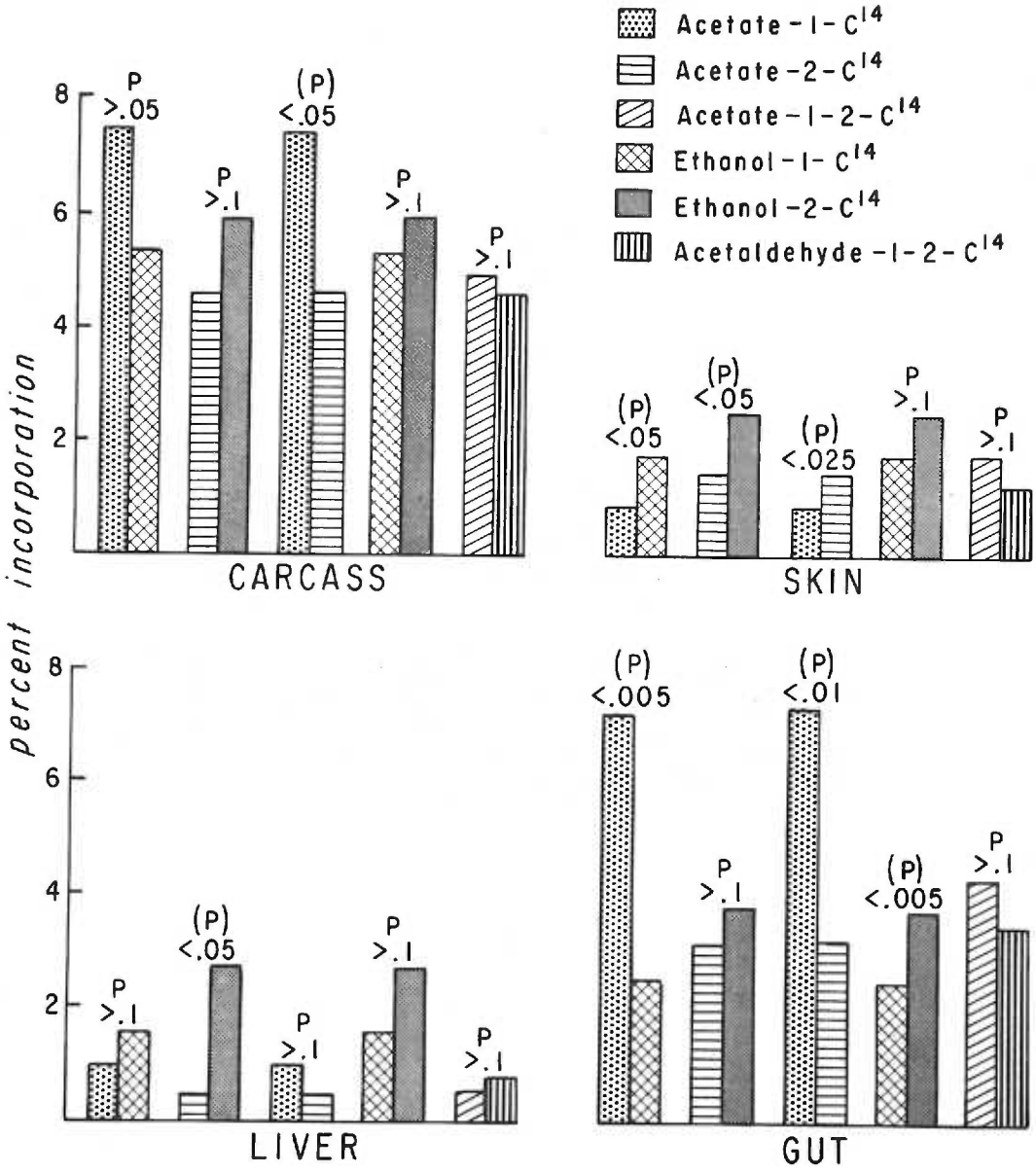
Acetate-1-C14
Acetate-2-C14
Acetate-1-2-C14
Ethanol-1-C14
Ethanol-2-C14
Acetaldehyde-1-2-C14
percent incorporation
8
6
4
2
P >.05
P >.1
(P) <.05
P >.1
P >.1
CARCASS
(P) <.05
(P) <.05
(P) <.025
P >.1
P >.1
SKIN
P >.1
(P) <.05
P >.1
P >.1
LIVER
(P) <.005
P >.1
(P) <.01
(P) <.005
P >.1
GUT

Fig. 6

C-1 as compared with the C-2 carbon of either ethanol or acetate into liver fatty acids. Likewise, we find no significant difference in the percent incorporation of the C-1 carbon of ethanol as compared to the C-1 carbon of acetate into liver fatty acids. The incorporation of the C-2 carbon of ethanol into liver fatty acids, however, is sixfold higher than is the incorporation of the C-2 carbon of acetate, a difference which is significant at a 0.95 confidence level.

The incorporation of these various labels into gut fatty acids follows a different pattern. Here we find that the C-1 carbon of acetate is a significantly better precursor of fatty acids than is the C-2 carbon of acetate, while the C-2 carbon of ethanol is a significantly better precursor of gut fatty acids than is the C-1 carbon of ethanol. The C-1 carbon of acetate is a significantly better precursor of gut fatty acids than is the C-1 carbon of ethanol, while there is no significant difference in the percent of the dose of labeled C-2 carbons of these two compounds found in gut fatty acids.

We find in the carcass fraction, as well as in the gut fraction, that the C-1 carbon of acetate is a significantly better precursor of fatty acids than is the C-2 carbon. The differences between the other labels are qualitatively the same in the carcass and gut fractions, though the remainder of those in the carcass fraction fail to show a statistical difference at our accepted confidence level.

In the skin fraction, both carbons of ethanol proved to be significantly better precursors of fatty acids than the same carbons of acetate. The C-2 carbon of acetate was a better precursor of skin fatty acids than was the C-1 carbon, these two carbons of ethanol showing no

significant difference in the percent incorporation into skin fatty acids.

There was no significant difference in the percent of the dose of acetate or acetaldehyde incorporated into fatty acids of any of the fractions, in spite of the fact that acetate incorporated 33% more carbon into expiratory CO_2 than did acetaldehyde.

Discussion

1. On CO_2 specific activity curves

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

That the overall process is an extremely rapid one is indicated by the fact that C^{14} activity appeared in respiratory CO_2 within one minute after intraperitoneal injection of each of the tracers. The steeper slope and higher peak of the specific activity curve of the ethanol $C^{14}O_2$ curve as compared to acetate would indicate that ethanol is handled more readily in one or more of the steps in this sequence. Acetaldehyde, on the other hand, would appear to be less readily oxidized by the intact rat than is acetate.

That the C-2 carbon of these compounds appears less quickly in respiratory CO_2 is in keeping with the fact that the C-2 carbon of acetate

becomes "buried" in the C-2 and C-3 positions of oxaloacetate, positions from which they are not as readily removed by the oxidative decaroxylations of the TCA cycle.

The finding of a more rapid in vivo oxidation of ethanol as compared to acetate agrees with our in vitro studies in which the increased incorporation of ethanol into CO_2, cholesterol and fatty acid was suggested to be accounted for on the basis of a more rapid absorption and/or activation of ethanol.

Although ethanol showed a higher initial incorporation into respiratory CO_2, the total percent of ethanol and acetate incorporated into CO_2 were identical at the end of the two-hour period. This is in keeping with the finding that the CO_2 specific activity curve following ethanol-1-C^{14} administration showed an earlier and steeper decline which followed the initial steeper incline.

The total incorporation of methyl label into CO_2 after the two-hour period was less than that for the C-1 label, there being a reduction of 10% for acetate and of 15% for ethanol. This reduction is likely due to the fact that the TCA cycle serves as a source of precursors for synthetic pathways, as well as acting as an oxidative pathway. Compounds such as glycogen, aspartic acid and glutamic acid that are formed from intermediates of the TCA cycle should contain a larger percentage of the C-2 carbons of the original acetyl CoA entering the cycle than of the C-1 carbons. These C-2 carbons will be shunted away from the decarboxylations of the cycle and will not appear as $C^{14}O_2$ until the compounds formed from them are themselves oxidized.

The total incorporation of acetaldehyde-1-2-C^{14} label into CO_2 was reduced 33% as compared to the total incorporation of acetate-1-2-C^{14} label. This suggests that either less acetaldehyde was metabolized during the two-hour period, or that acetaldehyde preferentially entered anabolic pathways without prior conversion to acetate. The fact that there was no increased incorporation of acetaldehyde into cholesterol or into fatty acids, the two synthetic pathways examined, would suggest that the decreased incorporation into CO_2 is on the basis of a decreased metabolism. A postulated decreased rate of metabolism would not seem to be in agreement with the results of Westerfeld (72) and of Newman (45), who reported a rapid rate of metabolism for acetaldehyde. These workers, however, measured only the rate of disappearance of acetaldehyde from the blood, which need not be synonymous with its rate of metabolism. In addition, the metabolism of acetaldehyde, although rapid, may well be exceeded by the processes that metabolize alcohol and acetate. It is likely that the present data is more pertinent to this problem than are the studies of Westerfeld and Newman.

2. On the incorporation of acetate vs ethanol carbon into cholesterol

Our results showing ethanol to be a significantly better precursor of cholesterol than is acetate are similar to those of Schulman and Westerfeld (75) who reported values of 1.3 to 1.9 for various rat tissues for the ratio of $\frac{\text{the \% inc. of ethanol-1-C}^{14}}{\text{the \% inc. of acetate-1-C}^{14}}$ into cholesterol one-half hour after intraperitoneal injection of acetate or ethanol tracer. Our values for this ratio in the various tissues studied ranged from 1.25 to 2.25. Our values for the ratio of $\frac{\text{the \% inc. of ethanol-2-C}^{14}}{\text{the \% inc. of acetate-2-C}^{14}}$ into

cholesterol are even larger, ranging from 1.88 to 3.07 for the various tissues (table XXVII).

In the liver slice preparation we also found that ethanol was a better precursor of cholesterol than was acetate. It was pointed out in a preceding section that ethanol in the slice prepratain was an equally better precursor of CO_2 as compared to acetate, the data suggesting then that the increased incorporation of ethanol into cholesterol was on the basis of an increased absorption or metabolism of the ethanol and not necessarily indicative of different metabolic pathways for ethanol and acetate.

The significantly increased incorporation of ethanol into cholesterol as compared to acetate in vivo, can not be so easily explained, as the CO_2 data would suggest that these two compounds were metabolized to the same extent in vivo during the time of this experiment.

It is also difficult to explain this difference on the basis of a greater metabolic dilution of the acetate, if ethanol and acetate are metabolized via the same pathway and via a common intermediate such as acetyl CoA. Unlabeled acetyl CoA should then dilute the alcohol and acetate equally, as each labeled substrate is converted to acetyl CoA.

If alcohol metabolism were largely confined to the liver and acetate were metabolized by other tissues as well, it could be postulated that acetate would be diluted more than alcohol even though they both followed the same metabolic pathway. However, the results clearly indicate that alcohol and acetate were metabolized by the same tissues.

If alcohol and acetate metabolism were confined to specific and different areas of the cell, and the acetyl CoA of these areas were not

in equilibrium, it could be postulated that the acetyl CoA pool available to acetate was the larger, and thus the acetate would be diluted more than the alcohol, even though they both followed the same metabolic pathway. This seems an unlikely explanation for two reasons. First, one would have to postulate that the acetyl CoA of these two areas did not equilibrate or mix prior to their incorporation into cholesterol. This, then, would necessitate that the complete enzyme systems needed for cholesterol synthesis would be found in at least two areas of the cell. On the basis of our present knowledge, however, cholesterol synthesis appears to occur almost solely in the microsomes. Thus Nancy Bucher (7) found that over 80% of the total cytoplasmic cholesterol was contained in the microsomes, and that over 90% of newly formed cholesterol obtained at short time intervals after the injection of labeled acetate was in the microsomal fraction. The microsome then would become a common meeting area for acetyl CoA being converted to cholesterol, regardless of the origin of the acetyl CoA. The mixing at this step, of acetyl CoA derived from acetate and from ethanol, would make it difficult to explain the greater incorporation of ethanol into cholesterol on the basis of a greater metabolic dilution of the acetate.

A secondapproach can be used to arrive at a similar conclusion. Thus, if the greater incorporation of ethanol into cholesterol, as compared to acetate, were due to a greater metabolic dilution of the acetate and not to alternate pathways, whether this be due to metabolism within different organs or to different intracellular sites of metabolism, then this greater metabolic dilution of acetate must result in a smaller total percent of the dose of acetate being metabolized within the time of the

experiment. That is, a difference in the dilution of the label would account for a difference in the specific activity of the cholesterol formed from that label, but only a difference in the total amount of the dose of acetate and ethanol metabolized during the time of the experiment could account for a difference in percent incorporation figures, if the pathways are identical and proceed through a common intermediate. Therefore, one must postulate in addition to a larger acetyl CoA pool, a longer half time for the turnover of that pool, a turnover time so prolonged, in fact, that during the two hours of the experiment 1.25 to 3.07 times as much acetyl CoA formed from ethanol has been cleared as has acetyl CoA formed from acetate. If this were true, one should see a proportionate decrease in the amount of C^{14} incorporated into CO_2 and fatty acids from acetate-C^{14}. However, we find that the same percentage of the dose of acetate and ethanol is incorporated into CO_2 during the two hours of the experiment, and that the total recoveries of C^{14} label in CO_2 and lipids are nearly identical, being 74.3% for acetate-1-C^{14} and 72.6% for ethanol-1-C^{14}.

Since the greater utilization of isotopic alcohol as compared with acetate for the synthesis of cholesterol can not be readily explained as due to either a greater rate of alcohol metabolism or to a greater metabolic dilution of the acetate, we must agree with the conclusion of Schulman and Westerfeld (75) that it possibly reflects some divergence in metabolic pathways for at least part of the alcohol.

3. On the incorporation of C-1 vs C-2 carbon into cholesterol

As has been discussed previously, of the 27 carbon atoms of

cholesterol biologically synthesized from acetate, 15 are derived from the C-2 carbon and 12 from the C-1 carbon. Thus, one would anticipate that any C^{14} labeled compound which serves as a precursor of cholesterol and undergoes conversion to acetyl CoA prior to its incorporation into cholesterol, would incorporate 1.25 times as much C-2 as C-1 label into the sterol. As we have seen in the liver slice preparation, the ratios for the percent incorporations into cholesterol of the labels of $\frac{\text{acetate-2-C}^{14}}{\text{acetate-1-C}^{14}}$ and $\frac{\text{ethanol-2-C}^{14}}{\text{ethanol-1-C}^{14}}$ were both close to this value. The *in vivo* results for the comparison of the C-1 and C-2 labels of acetate are also in agreement, within the range of experimental error, for all fractions except skin. The cholesterol fraction contains other non-saponifiable lipids besides cholesterol, and the known heterogeneity of skin sterols may explain the difference noted in that fraction.

The findings are quite different for ethanol, the methyl label contributing about 2.4 times as many carbons to cholesterol as does the C-1 label. A ratio of this magnitude is not easily explained if ethanol is converted to acetyl CoA prior to its incorporation into cholesterol. This difference between ethanol and acetate in the ratios of C-1 and C-2 carbons incorporated into cholesterol offers additional evidence to suggest that ethanol is not solely metabolized via acetyl CoA as an obligatory intermediate.

4. On the incorporation of acetate and ethanol into fatty acids

As has been previously discussed, evidence gained from *in vitro* studies with isolated enzyme systems suggests that fatty acid synthesis occurs by the union (net) of multiple acetyl CoA units. Our results

with the *in vitro* liver slice preparation were consistent with this hypothesis, i.e., we found no significant difference between the percent incorporation of label of acetate-1-C^{14} and acetate-2-C^{14} into liver fatty acids. The results with the C-1 and C-2 labels of ethanol were similar, suggesting a similarity in the metabolic pathways of these two compounds in the liver slice.

The *in vivo* results for the liver fraction are similar to the *in vitro*, i.e., there is no significant difference in theincorporation of the C-1 as compared to the C-2 label of either acetate or ethanol into liver fatty acids.

The fatty acid percent incorporation figures for the other three tissues, gut, carcass and skin, show significant differences between the incorporations of C-1 and C-2 carbons. The carboxyl carbon of acetate is a significantly better precursor of gut and carcass fatty acids than is the methyl carbon, the methyl carbon of acetate is a significantly better precursor of skin fatty acids than is the carboxyl carbon, and the methyl carbon of ethanol is a significantly better percursor of gut fatty acids than is the C-1 carbon of ethanol. These results suggest that fatty acid synthesis in these three tissues *in vivo* does not occur solely by the net union of multiple acetyl CoA units, for if such were the case, there should be no difference in the percent incorporation into fatty acids of the C-1 and C-2 label of acetyl CoA precursors.

The gross dissimilarities found in the incorporation of ethanol and acetate into tissue fatty acids also suggest that acetate may not be an obligatory intermediate of ethanol metabolism in each of the tissues of the intact rat. Thus, more ethanol-1-C^{14} is incorporated into liver

and skin fatty acids, while gut and carcass fatty acids show a greater incorporation of acetate-1-C^{14}. The increased incorporation of ethanol-1-C^{14} as compared to acetate-1-C^{14} into liver fatty acid is in agreement with the results of Schulman and Westerfeld (75) who found that ethanol-1-C^{14} was also a better precursor of kidney, plasma and brain fatty acids. The only tissue that they examined that incorporated more acetate C-1 as compared to ethanol C-1 label into fatty acids was muscle, the ratio for the percent incorporation of ethanol-1-C^{14}:acetate-1-C^{14} in this tissue being 0.67:1.0. This ratio is almost identical to the ratio of 0.72:1.0 that we found for this same comparison in the carcass fraction, a fraction which is composed, on a weight basis, primarily of muscle.

The same reasoning can be used here, as was used previously in the discussion of cholesterol synthesis, to conclude that this difference between ethanol and acetate is not due to a difference in the rate of metabolism or to a difference in metabolic dilution. Additional evidence is presented here to support this conclusion, for if one of these factors were having an influence here, resulting in a decreased incorporation of one of the labels into fatty acids, then this label should show a decreased incorporation into the fatty acids of all tissues. We find no such consistency.

If the concentration of enzymes necessary for the conversion of ethanol to acetate were different in the different tissues, this might explain how one tissue could incorporate more, while another incorporates less ethanol than acetate into fatty acids; even though acetate or acetyl CoA were a common intermediate for both tracers. If this were the case, however, we should find the same differences for the incorporations of

these tracers into cholesterol, for this would also require prior conversion of ethanol to acetate or acetyl CoA. These tissue differences, however, were not seen in cholesterol synthesis.

The comparison of the percent incorporation figures of the C-1 and C-2 positions of ethanol and acetate appears to offer additional evidence in support of the hypothesis that ethanol and acetate are not metabolized solely via a common acetyl CoA intermediate. Thus, if the differences noted between the incorporation of acetate and ethanol into fatty acids were due to differences in the rate or magnitude of conversion of these compounds to their common acetyl CoA intermediate, and not to divergent metabolic pathways, then the ratio for both compounds for the percent incorporation of the C-1 vs the C-2 carbon into fatty acids should be the same. That is, the organism should not be able to differentiate between the acetyl CoA formed from acetate and that formed from ethanol, and therefore the relationship of the percent incorporation of the C-1 carbon of acetyl CoA to the percent incorporation of the C-2 carbon of acetyl CoA into fatty acids should be the same, regardless of the source of the acetyl CoA. As will be seen from table XXVII, these ratios are quite different for the two compounds; ethanol-2-C^{14} contributing more label to the fatty acids of all four fractions than does ethanol-1-C^{14}, while acetate-2-C^{14} contributes less label to the fatty acids of the liver, gut and carcass fractions than does acetate-1-C^{14}.

Again, we must conclude from this data that at least part of the ethanol may be metabolized via some pathway other than via conversion to acetyl CoA.

5. On the incorporation of acetaldehyde into lipids

The lack of any significant difference in the incorporation of acetaldehyde as compared to acetate into both cholesterol and fatty acids, by all tissues studied, indicates a great similarity in their metabolic pathways. It must be concluded that the results are consistent with, though not proof of, the hypothesis that acetaldehyde and acetate are metabolized via a common intermediate, such as acetyl CoA.

This parallelism between the metabolism of acetate and acetaldehyde, and the lack of such similarity in the metabolism of acetate and ethanol, would indicate that acetaldehyde may also not be an obligatory intermediate of ethanol metabolism.

Summary statements

1. Acetate-1-C^{14}, acetate-2-C^{14}, ethanol-1-C^{14}, ethanol-2-C^{14}, acetate-1-2-C^{14} and acetaldehyde-1-2-C^{14} have been compared in their abilities to serve as precursors of CO_2, cholesterol and fatty acids in intact normal rats.
2. The time course of CO_2 specific activity following intraperitoneal injection of these substrates is presented
3. No significant difference was found in the percent incorporation of ethanol or acetate label into expired CO_2 during a two-hour period following injection. The C-1 carbon of both compounds was incorporated to a greater extent into CO_2 than was the C-2 carbon during this same time interval.
4. The percent of the dose of acetate-1-2-C^{14} incorporated into CO_2 was 50% higher than that of acetaldehyde-1-2-C^{14}.

5. Ethanol labeled in either position was found to be a better precursor of cholesterol than was similarly labeled acetate, the difference for the C-2 labels being larger than that for the C-1 labels.
6. The C-2 label of both ethanol and acetate was a better precursor of cholesterol than was the C-1 label.
7. There was no correlation found in the percent incorporation of ethanol and acetate into fatty acids by the various tissues, some finding ethanol a better precursor and others incorporating a larger percent of the dose of acetate into fatty acids.
8. Significant differences were found in the incorporation of the C-1 as compared to the C-2 label of both ethanol and acetate into gut, carcass and skin fatty acids. Again no correlation was found between the incorporation of the two carbons of ethanol and acetate, gut for instance incorporating more of the C-1 than C-2 carbon of acetate into fatty acid, while incorporating more of the C-2 than C-1 carbon of ethanol into the same fraction.
9. There were no significant differences in the percent incorporations of acetate and acetaldehyde into either lipid fraction by any of the tissues studied.
10. It is concluded from these results that fatty acid synthesis in gut, carcass and skin tissue may not occur solely by the union of multiple acetyl CoA units; that neither acetaldehyde nor acetyl CoA are obligatory intermediates of ethanol metabolism in the intact rat; and that the great similarity between the incorporations of

acetate and acetaldehyde into lipids of all four fractions studied suggests that these two compounds may be metabolized via a common intermediate.

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