LIPOGENESIS FROM C^{14} ACETATE

by

CECIL K. CLAYCOMB

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APPROVED:

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

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Introduction

The work embodied in this thesis is a direct outgrowth of an Atomic Energy Commission contract awarded to Dr. E. S. West, University of Oregon Medical School, in the fall of 1948. Briefly, the contract proposes the following: to study, by use of acetic acid and ethanol labeled with C^{14}, various factors relating to the simultaneous synthesis and degradation of cholesterol, fatty acids, and ketone bodies in the animal. The specific phases of this general program being investigated under this contract are outlined, with some explanation, in Sections A, B, and C which follow; this thesis is primarily concerned with Section B.

A. To study, by use of acetic acid labeled with C^{14}, the relation of ketone body formation to the hypercholesterolemia and fatty liver condition of the diabetic state.

It is well established that hypercholesterolemia, along with fatty livers, is in general associated with a state of ketosis in the animal body. It has also been shown that the ketone bodies are formed in the liver from acetic acid derived from the breakdown of fatty acids in metabolism. That cholesterol and fatty acids are also synthesized in the liver from acetic acid has been well established. In view of the fact that, without insulin, there is a greatly increased rate of fatty acid breakdown, the production of hepatic acetic acid by the diabetic animal is excessive, since the initial stages of fatty acid metabolism occur in the liver with the formation of acetic acid.

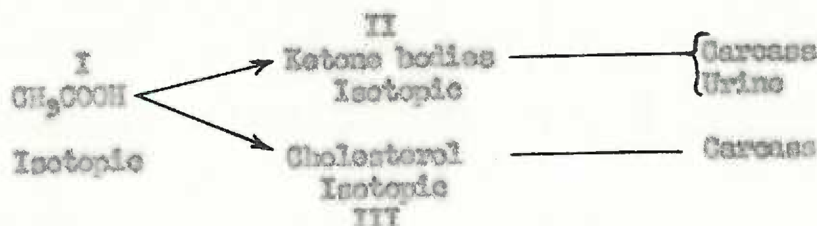
This excessive production of acetic acid leads to excess ketone body synthesis and resulting ketonemia and ketonuria. It is logical to assume

that in this condition there will also be increased cholesterol synthesis from the acetic acid.

Our theory then postulates that the hypercholesterolemia and ketosis of the diabetic state are related through simultaneous synthesis of cholesterol and ketone bodies from a common precursor, namely, the acetic acid from fatty acid oxidation.

Our experimental plan is to give isotopic acetic acid by intra-peritoneal injection to normal and diabetic rats and to follow the rates of appearance of C^{14} in the urinary and carcass ketone bodies and in the carcass cholesterol and fatty acids. The rate of disappearance of C^{14} incorporated by the animal into cholesterol and ketone bodies is also to be followed. Data from such experiments should indicate any relation between ketone body and cholesterol syntheses, and also whether degradation products of cholesterol may be used in ketone body synthesis.

After such experiments have provided adequate information, attempts are to be made to alter the distribution of acetic acid between ketone body and cholesterol formation through the action of hormones and other agents.



I, II, and III are increased in the diabetic state. Our object is to prove that increased III is related to synthesis from I just as is increased II.

The problem of hypercholesterolemia and fatty livers in the uncontrolled diabetic state is of much clinical importance, and the experiments

outlined should provide fundamental information relative to this condition.

B. To study, by the use of acetic acid labeled with C^{14}, the simultaneous syntheses and degradations of fatty acids and cholesterol in the body, and factors which modify these syntheses. This problem is also related to hypercholesterolemia and fatty livers, and is of broad medical importance.

The liver synthesizes both fatty acids and cholesterol from acetic acid, but little is known relative to fatty infiltration of the liver caused by vitamin and hormone deficiencies and excesses, poisons, starvation, etc.

The experimental procedure being followed in the development of this portion of the program is injection of isotopic, carboxyl-labeled sodium acetate into experimental animals (rats and mice) and observation of the rates of incorporation of this C^{14} into cholesterol and fatty acid molecules. Changes in these rates are observed under variable conditions of two types: a series of experiments involving animals under a controlled program of fasting conditions prior to the injection of labeled acetate; and a second series in which the controlled factor is the time the animal is allowed to metabolize a given injection. (This time will hereinafter be referred to as time of maximum utilization.)

Isotopic acetic acid is given to animals, and the simultaneous rates of appearance and disappearance of C^{14} in the fatty acids and cholesterol of the body are followed. This has been done with normal animals on a standard diet and, subsequently, with animals under conditions which produce hypercholesterolemia and fatty livers. Studies with agents which

prevent and relieve these conditions are now in progress by workers in this laboratory. It is felt that such experiments will provide a better understanding of normal and pathological cholesterol and fatty acid metabolism than is possessed at the present time.

In the experiments concerned with time of maximum utilization it is desirable to determine the rates of incorporation of administered radioactive acetate into fatty acids and cholesterol of different tissues. Consequently, the animals are "fractionated" into brain and spinal cord, liver, skin, gut, and carcass.

C. To study the relative rate of isotopic ethanol ($CH_3C^{14}H_2OH$) incorporation into cholesterol, fatty acids, and ketone bodies in both the normal and the alloxan-diabetic animal. This phase of the program is now being investigated by Dr. E. S. West and associates in the Radioisotopic Laboratory of the University of Oregon Medical School.

As has been pointed out, one of the main phases of the Atomic Energy Commission contract administered by Dr. West is the evaluation of acetic acid metabolism in the normal and in the fasted animal. It is also desirable in this connection to study the rates of synthesis of lipids in the normal animal in relation to the total time of maximum utilization. This thesis is primarily concerned with the rates of lipid synthesis in the normal animal.

CHAPTER I

HISTORY

Contrary to common belief, the use of isotopes in biochemical studies is approaching the quarter century mark. While isotopes such as C^{11}, C^{13}, C^{14}, P^{32}, D, S^{35}, N^{15}, have become part of the everyday metabolic research tools since the advent of the "A" bomb, some were used before that time. However, only with artificially produced radioactive elements has their use become widespread. Because of the great current interest in isotopes as biological tracers, a brief review may be of value.

In 1923 Hevesy[1] used naturally occurring radioactive thorium B (Pb^{212}) to study "The Absorption and Translocation of Lead by Plants". The roots of bean plants were placed in solutions of labeled lead nitrate, and the uptake of the lead by the various portions of the plant was determined by the radioactivity subsequently found when these different parts were ashed. He concluded that the element did not combine with carbon in the plant but remained in ionizable form.

Although this pioneer experiment established the pattern for isotope use in biochemical research, it was not until isotopes of other elements more closely related to tissue composition became available that use of isotopes in the field of intermediate metabolism became promising as a research procedure.

One of the first of these newer isotopes to be used in metabolic studies was deuterium, D, or heavy hydrogen, H^2, discovered in 1932 by Urey and his colleagues, who soon developed practical methods for production of D_2O in high concentration. Deuterium is unique among the isotopes in that the ratio of its mass to that of its common isotope, H^1, is approximately two. In high concentrations it (D) is toxic to lower forms of life[2].

G. N. Lewis "ascribed this effect to physicochemical causes, primarily to the lower mobility of the deuterium ion as compared with that of the hydrogen ion, and pointed out that the physiological effects of this difference should be observable only in relatively high concentrations of it.[‡]"

Subsequent investigators who have used this heavy isotope of hydrogen have kept its concentration at safe levels, as did Hevesy and Hofer[3], who immersed a small fish in water containing 10% D and observed that the carbon bound hydrogen did not exchange with the D of the D_2O. However, the hydrogen that was of labile nature, i.e., that attached to O and/or N, did enter into very rapid equilibrium with the D of deuterium oxide. Hevesy and Hofer[4], one year later, were the first to use "tagged" atoms to trace an administered compound in humans. One of these authors ingested two liters of heavy water (450 p.p.m.) and at frequent intervals thereafter determined the deuterium content of his urine. They stated that a water molecule remains in the human body 13 ± 1.5 days and that, during warm weather, about 60% of the H_2O molecules leave the body by evaporation.

It was not until Urey made available large amounts of D in 1934 that the study of the fate of metabolites in the body could be instituted on a promising scale by Rittenberg and Schoenheimer. These workers used deuterium as a tracer atom component of substances undergoing metabolism in the body. The deuterium, possessing chemical properties similar to ordinary hydrogen, did not qualitatively change the chemical properties of substances containing it.

Previous to this, investigators used compounds with labels which gave rise to unphysiological substrates. Therefore, the results obtained were

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‡ Isotopes in Biology and Medicine, p. 4, Hans T. Clarke, The University of Wisconsin Press, Madison, 1949.

based upon abnormal conditions. This limitation does not apply to the study of compounds in which carbon-bound hydrogen atoms are replaced by deuterium. Rittenberg and Schoenheimer found that the deuterium-containing molecules are biochemically indistinguishable from those containing ordinary hydrogen. Therefore, with enrichment of the deuterium content of a metabolite*, a label can be introduced into a molecule which allows an investigator to trace the fate of the metabolite in an organism. These men were able to demonstrate with a deutero-labeled fat[5] that all the fats of an animal are in equilibrium with the exception of the essential, highly unsaturated lipids, such as linoleic and linolenic.

This finding was contrary to the prevailing concept at that time, *viz.*, that once fats are deposited in the depots they remain as relatively inert substances. These experiments would not have been possible without the aid of an isotopic "tagged" compound.

While Schoenheimer and Rittenberg were evaluating the possibilities of deuterium as a label in the study of intermediate lipid metabolism, Urey and his group developed a practical process for the enrichment of ammonia with the naturally occurring stable isotope N^{15}. This element became another tool for biochemical investigations.

Keston and associates[6] fed low protein diets containing isotopic ammonium citrate to growing rats and reported that the animals synthesized glycine from dietary isotopic ammonia. They isolated isotopic glycine from the tissues of the animals. To prove that any labeled amino acid isolated was synthesized from the isotopic N^{15} and not formed by an

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* "In all natural metabolites the hydrogen atoms include only one deuterium atom for every five thousand protium atoms." (*Isotopes in Biology and Medicine*, p. 6, Hans T. Clarke, The University of Wisconsin Press, Madison, 1949.)

exchange reaction, they treated N^{15}-containing amino acids with boiling HCl and natural amino acids or NH_4Cl and failed to demonstrate any exchange of amino acid N^{15} with the ordinary N of the natural amino acids or the NH_4Cl.

An experiment of especial interest performed by Schoenheimer and associates[7] involved the feeding of the amino acid, leucine, labeled with N^{15} and also with deuterium (attached to the carbon atoms), to rats. Leucine isolated from the tissue proteins was found to have the isotopes in an altered ratio. This ratio indicated that more than one-third of the labeled N in the original leucine had been replaced by non-isotopic N. Only with the use of this type of isotope technique could it have been possible to demonstrate the rapid opening and reformation of peptide linkages. However, whether the process involves complete rupture of a protein into all of its constituent amino acids or whether the peptide linkages are opened and closed one at a time is a matter of conjecture with the information presently available. Schoenheimer, Ratner, Rittenberg, and Heidelberger[8] also demonstrated that, by administration of isotopic amino acids to rats, the plasma proteins, like other tissue proteins, take part in metabolic reactions involving the incorporation of dietary nitrogen.

Radioactive sulfur (S^{35}) was employed by Tarver and Schmidt[9] for studying biological problems. They showed that in normal metabolism the sulfur of cystine is derived, at least in part, from methionine, but not from elementary sulfur nor from sulfate. Radioactive isotopes of iron have been used by Whipple and his associates[10] in biochemical and physiological studies of erythrocytes. Quimby and Smith[11] used radio-sodium in the study of peripheral vascular disease. Hylin[12] used P^{32} to determine blood volume and circulation time. Various investigators have employed many radioactive elements in studies concerning the metabolism of inorganic substances.

Radioactive Carbon with a mass of 14 was used exclusively in the A.E.C. contract work of which this thesis is a part. The reasons for this are:

(1) It is safe to handle because of the very low energies associated with its beta emission (0.15 Mev maximum).

(2) The carbon isotope is obtainable in the form of $BaC^{14}O_3 + BaCO_3$ in any amounts and at reasonable prices (Oak Ridge, Tennessee).

(3) Since its half life is greater than 5,000 years, there is ample time to synthesize compounds containing the isotope, use them as substrates, and determine their fate in metabolic studies.

(4) It can be detected with the use of suitable electronic equipment, whereas N^{15}, H^{2}, and C^{13} detection all require elaborate equipment of which an expensive apparatus, the Mass Spectrograph, is the chief instrument.

The use of C^{14} in tracer studies of lipogenesis is covered in pages 12 through 22 of this thesis. Therefore, a few comments will be made about the naturally occurring isotope of mass 13 and the radioactive, short-lived C^{11} isotope (half life 21 minutes). It is obvious that an isotope with a half life of only 21 minutes can be used only at or very near its site of production; and, as previously mentioned, C^{13} requires a mass spectrograph for evaluation.

Isotopes of carbon were first employed as laboratory tools about eleven years ago when the naturally occurring isotope C^{13} and the radioactive isotope of mass 11 became available. The first biochemical use of these substances was as CO_2 or bicarbonate. It was discovered very early that CO_2 is not merely an end product of metabolism, but is incorporated into other compounds of the body, either in whole or in part.

Wood, Werkmann, Hemingway and Nier[13] found that suspensions of bacteria in the presence of $NaHCO_3$ enriched with C^{13} incorporate the isotope into the carboxyl position of succinic acid.

Evans and Slotin[14] incubated pigeon liver slices in radioactive $NaHC^{13}O_3$ and subsequently were able to isolate radioactive alpha-keto-glutaric acid. The utilization by liver slices of CO_2 in urea formation was demonstrated by Rittenberg and Waelsch[15] who used C^{13}, and this CO_2 utilization in urea formation was also demonstrated by Evans and Slotin[16] using C^{11}. Both groups used the isotope in the form of $NaHCO_3$.

C^{11} as $C^{11}O_2$ or as $NaHC^{11}O_3$ has been used by many researchers to investigate the role of carbon dioxide in the metabolism of yeast[17], protozoa[18], bacteria[19], molds[20], and in growing plants[21]. However, only those experiments in which experimental animals or tissues therefrom were used will be considered in this thesis.

The participation of carbon dioxide in the mammalian carbohydrate cycle was demonstrated by Hastings and associates[22]. This group administered radioactive bicarbonate to fasted rats. They were able to isolate liver glycogen that exhibited radioactivity indicating that approximately one in every eight carbon atoms of glycogen had been derived from the isotopic bicarbonate. These same investigators obtained a similar result when glucose and radioactive bicarbonate were fed simultaneously[23].

It is apparent that most of the experiments with radioactive carbon, C^{11}, were performed with the carbon as inorganic $C^{11}O_2$ or $NaHC^{11}O_3$. This was necessary because its short half life, 21 minutes, poses a difficult barrier for the synthesis of compounds containing it.

Chemists at Harvard undertook the difficult task of synthesizing lactic acid containing C^{11}. Two types of labeled lactic acid were prepared:

one in which the isotope resided solely in the carboxyl position, and the other in which both the alpha and beta positions were labeled but the carboxyl position was not. Conant and associates[24] observed that the feeding of carboxyl-labeled lactic acid to rats resulted in 20% of the ingested C^{11} appearing in the CO_2 expired during two and one-half hours. Liver glycogen had incorporated 1.6% of the administered isotope. On the other hand, when lactic acid with the carbon isotope in the alpha and beta positions was fed to rats under identical conditions[25], 10% of the administered C^{11} was recovered in the expired CO_2. The liver glycogen contained 3.2% of the initially administered activity.

The differences observed between these experiments indicate that at least two metabolic pathways can be followed by the carbon atoms of lactic acid. These can be explained if we assume that initially, in both experiments, lactic acid is oxidized to pyruvic acid, i.e., $CH_3CHOHCOOH \xrightarrow{(O)} CH_3COCOOH$. Then part of the pyruvic acid may be incorporated into glycogen and other compounds and part may be decarboxylated to CO_2 and "Active Acetyl". "Active Acetyl" then combines with oxalacetate to enter the tricarboxylic acid cycle. From this metabolic cycle the carbon atoms again may be incorporated into glycogen, etc., or oxidized to CO_2. It is necessary to bear in mind that, for every two carbon atoms entering the tricarboxylic acid cycle, two must appear as CO_2. Therefore, although the initial alpha and beta labeled carbon atoms of the lactic acid can be incorporated into the glycogen molecule after they reach the acetate stage, no net increase in the total amount of glycogen is obtained; only an exchange of carbon atoms occurs. Accordingly, carboxyl labeled lactic acid would be expected to give greater incorporation of C^{11} into CO_2 and less

into glycogen than the alpha and beta labeled acid. Such was the case. Since in both experiments the per cent of isotope incorporated into glycogen was small, it appears that lactic acid is largely converted into other metabolites.

Cholesterol

A brief review of cholesterol anabolism and catabolism is necessary at this point. A number of sterol interrelationships in metabolism have been worked out in the last ten years by men such as Schoenheimer, Rittenberg, Bloch, Chaikoff, Little, and others, who used D, C^{13}, and C^{14} as tracers. These men with isotopic tools have contributed enormously to our knowledge of sterol chemistry.

Rittenberg and Schoenheimer[26] were pioneers in the study of lipogenesis with the aid of tracer techniques. These workers found that deuterium is incorporated into tissue lipids such as cholesterol and fatty acids when the drinking water of experimental animals contains D_2O. This incorporation of deuterium into cholesterol and fatty acids is the result of many chemical processes. Since the diet fed by these workers was cholesterol free, the isolated isotopic sterol was the result of endogenous synthesis.

Bloch and Rittenberg[27] in 1944 incubated deuterium-labeled acetic acid with liver slices and found D in the cholesterol formed. They also found[28] that any compound giving rise to acetic acid in metabolism, which includes such substances as leucine, alanine, ethanol, and isovaleric, butyric, n-valeric and myristic acids, also forms cholesterol.

Bloch, Borek and Rittenberg[29] observed that the synthesis of cholesterol requires the action of various suitably organized enzymes. This

reaction takes place readily in slices of surviving liver, but stops completely when the organization of the tissue is destroyed by grinding. They reported the rate of cholesterol synthesis in liver homogenates to be less than one ten thousandth of the rate in slices. Another liver slice experiment was reported by Little and Bloch[30] who incubated the slices with C^{13} methyl-labeled acetic acid and also with C^{14} carboxyl-labeled acetate. From the results obtained they concluded that acetic acid is the principal if not the sole precursor of cholesterol. In this same experiment it was observed that the methyl group of acetic acid is the source of Carbon atoms 18, 19, 26, 27, and possibly 17 of the cholesterol molecule. Carbon atoms 10 and 25 are derived from the carboxyl part of the acetic acid. These experiments also led them to believe that the half life (i.e., time required to lose half of the incorporated activity) of liver cholesterol is six days, and for carcass cholesterol 31 to 32 days.

While early in-vitro experiments indicated the liver to be the main site of cholesterol synthesis, it is not the only place of formation. Chaikoff and associates[31] observed the synthesis of labeled cholesterol from "tagged" acetate by surviving beef adrenal slices. They also found that other tissues are capable of incorporating isotopic acetate into the cholesterol molecule. These include kidney, testis, small intestine, brain, and skin. The rat tissues most active in cholesterol synthesis are liver and skin of the adult rat, and brain and skin of the day old rat[32].

It is interesting that only in the new born rat experiments of Chaikoff and associates was cholesterol synthesized by brain tissue. This observation was supported by that of Sperry and Waelsch[33], who gave

D_2O to rats and found the highest concentration of D in the unsaponifiable fraction of brain and spinal cord four days after birth. If the two carbon fragment is accepted as being the principal source or sole precursor of cholesterol, then it is probable that at first when the central nervous system of the rat is proliferating most rapidly, it uses this acetate-like entity most readily. As the animal's rate of growth increases, the rest of the metabolic processes utilize more of the two carbon fragments and thus may reduce the availability of acetic acid for central nervous system utilization and eventually bring myelination to a standstill with consequent slowing of lipid replacement in the adult rat brain.

This slow replacement of brain lipids supports the previous observation of Bloch and associates[34], who injected dogs intravenously with deuterium-labeled cholesterol and found it in all tissues except brain and spinal cord. They were also unable to isolate any radioactive cholesterol from the adult brain of rats after administration of C^{14}-labeled acetate. In contrast to this, workers in our Radioisotopic Laboratory have been able to demonstrate small but significant activity in the cholesterol and fatty acids isolated from brain and spinal cord of rats injected with C^{14} acetate. These experiments are described in detail in Chapter III of this thesis.

It has been shown that an animal can synthesize cholesterol from a variety of substances. It is of interest to know the effect of dietary cholesterol upon this synthesis and something of the body's capacity to degrade cholesterol. These questions are answered by the early experiments of Schoenheimer and Breusch[35], who performed dietary balance studies upon mice. It was observed that mice on diets low in cholesterol synthesize

the equivalent of the body content in a month and when fed high cholesterol diets destroy five times this amount. They stated that tissue cholesterol is continually being formed and destroyed and either a positive or negative balance may be found depending upon experimental conditions at the time of the determination. Bloch[36] states that dietary effects can be neglected, since a cholesterol equilibrium exists in the animal so that the daily excretion of fecal sterols corresponds roughly to the quantity of cholesterol synthesized during the same period.

Gould and Taylor[37] used an isotopic approach to evaluate the effects of dietary cholesterol upon the ability of subsequently isolated tissues to synthesize this sterol. They observed that when dogs and rabbits which had been maintained on high cholesterol diets for six weeks were sacrificed and the tissues incubated with isotopic (C^{14}) acetate, the cholesterol synthesis was suppressed to a few per cent of that synthesized by animals on standard diets. Good agreement between in-vitro and in-vivo experiments was noted when isotopic (C^{14}) acetate was administered by stomach tube to animals fed high cholesterol diets for six weeks prior to giving the radioactive substance.

Synthesis rates of cholesterol were studied by Alfin-Stater and associates[38], who found the tissues of rats to incorporate D of D_2O into cholesterol at rates arranged in the following order: adrenals lungs liver kidney brain. They also observed that brain tissues have a very low rate of cholesterol turnover.

Conn[39] investigated the rate of serum cholesterol turnover and reported that persistent ACTH stimulation of the adrenal cortex results in a significant decrease of total serum cholesterol. The ester fraction exhibited

the greatest decrease, requiring four to five days to return to normal values. In patients with Addison's disease no decrease was observed with ACTH stimulation. Conn postulated from these findings that accelerated adrenal activity results in decreased serum cholesterol. The negative response in Addison's disease eliminates the possibility that a thyrotrophic contaminant of ACTH might have been responsible for the decrease in serum cholesterol.

Factors which may favor removal of cholesterol from the blood are: increased adrenal cortical activity (from stress, ACTH, or epinephrine), formation of or conversion to other sterols, synthesis of cholic acid, and integration into the general metabolic processes. In support of these possible paths of cholesterol metabolism is a pertinent observation of Bloch[40] in which he gave deuterium-labeled cholesterol to a pregnant woman and isolated pregnanediol containing deuterium from the urine. Since the isotope concentration of the isolated sterol was of the same order of magnitude as that of her blood cholesterol, he considers the direct conversion of cholesterol to progesterone to be a normal process and that the progesterone so formed is then reduced to pregnanediol and excreted. Later Anker and Bloch[41] reported evidence suggesting that cholesterol is converted to cholestenone and excreted as its reduction product, coprosterol, in the feces. When deutero-tagged cholestenone was fed to rats, a high concentration of the isotope was found in dihydrocholesterol and in feces coprosterol, but little in the isolated cholesterol. They proposed the following reaction:

Bloch and Rittenberg[42] found cholesterol to form cholic acid through oxidation of the side chain and slight modification of the nucleus. This conversion was demonstrated by intravenous injections of deuterium-labeled cholesterol into a dog with an anastomosis between the gall bladder and the kidney pelvis. From the urine, which contained biliary secretions, they isolated cholic acid containing deuterium.

Gould[43] showed that cholesterol is involved in general animal metabolism. He administered biosynthesized C^{14}-labeled cholesterol to rats and mice and found an appreciable fraction of the C^{14} incorporated in respiratory CO_2, fecal fatty acids, and liver. Only a very small fraction of the initial radioactive carbon was found in the blood. Van Bruggen, Hutchens, and West[44] were also able to demonstrate the incorporation of C^{14} into CO_2 and tissue fatty acids when biosynthesized C^{14}-labeled cholesterol was administered to young rats. In six hours 1% of the cholesterol C^{14} activity, given in a single stomach tubing, was expired as $C^{14}O_2$. From the initial specific activity of the administered cholesterol as compared to that of the isolated liver cholesterol they concluded that at least one-fourth of the total liver cholesterol was replaced in six hours.

In order to be as certain as possible that the radioactivity found in the isolated fatty acids was from incorporation of C^{14} of administered cholesterol, and not from contamination by C^{14} cholesterol _per se_, these lipids were subjected to extensive purification. Fatty acids which contained 10 or more carbon atoms were precipitated as Cu-Ca salts, separated and regenerated as free acids. These acids were treated with digitonin to remove any cholesterol that might have been carried through the copper lime salt procedure. The purified fatty acids contained 5% of the administered

isotopic carbon. Brain and spinal cord fatty acid specific activity was 2.75 c.p.m./mg., a value only exceeded by that of the acids from the gut (3.3 c.p.m./mg.). This was unexpected because previous investigators have reported only slight isotopic incorporation into central nervous system lipids.

Fatty Acids

The field of fat metabolism is very extensive, and many capable experimenters have contributed to our knowledge of it. However, it is beyond the scope of this thesis to review lipogenesis thoroughly; therefore, only the experiments in which isotopes were used as biochemical aids will be reviewed, and then only to a limited extent.

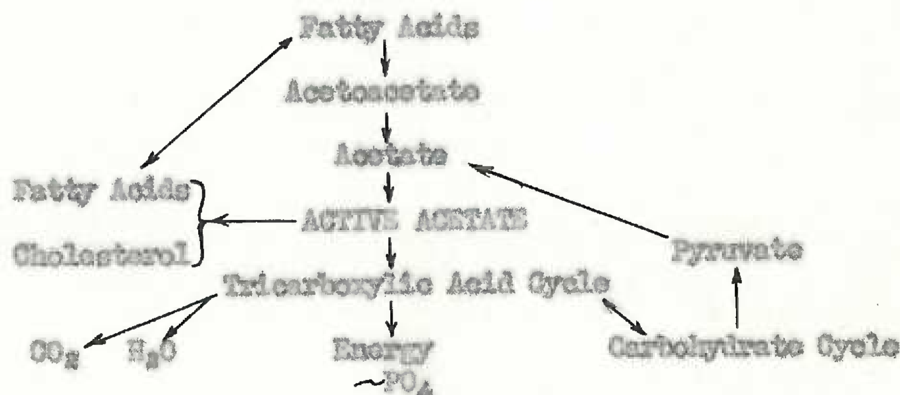
Schoenheimer and Rittenberg[45] are again foremost as pioneers in the use of isotopic technique in studies of fat metabolism. They observed that the isotopic concentration in the fats of animals fed deutero-labeled fat decreased exponentially when natural diet feeding was resumed. In the short time of three days, one-half of the deuterium of the body fats had disappeared, indicating the fats of an animal to be in a highly mobile state. Fat deposits of animals fed a normal diet and water containing deuterium oxide, which resulted in a constant level of deuterium intake, were found to acquire deuterium at almost the same rate as deuterium disappeared from the labeled depots in the previous experiment. Fatty acids, both saturated and singly unsaturated, were found to be synthesized in-vivo. No deuterium was found in the essential fatty acids. This failure of an animal to synthesize essential fatty acids has been substantiated

by later experimenters, using, in addition to deuterium, the isotopes of carbon, C^{11}, C^{13}, and C^{14}.

Fatty acids, according to the present concept, are broken down by beta oxidation with successive removal of a two carbon entity. These acetic-like fragments can be further oxidized to CO_2, HOH, and energy via the tricarboxylic cicle, or they may undergo condensation reactions, giving rise to acetoacetic acid and fats, as well as cholesterol. It is reasonable, from the information that we now possess, to postulate that lipid, carbohydrate, and protein metabolisms are all interrelated through this two carbon compound, which for lack of a better name we term "active acetyl" or "active acetate".

As pointed out above, both fatty acids and cholesterol are formed from acetate. Bloch[46] has stated that fatty acids need not be intermediates in the conversion of acetate to cholesterol; in other words, this two carbon fragment can be incorporated directly into fats or into cholesterol. Gurin and Brady[47] were able to isolate labeled long-chained fatty acids when C^{14} carboxyl-labeled acetate, or acetic acid precursor such as pyruvate, butyrate, hexanoate, and octanoate were incubated with rat liver slices.

Below is a brief sketch of the probable path of the acetate fragment:



Bloch and associates[48] fed rats diets containing C^{14} carboxyl-labeled acetate and were able to establish the following relations: saturated acids of liver reach half of their maximal isotope concentration in less than one day; the unsaturated acids require two days to reach this level. Saturated carcass lipids require 16 to 17 days, and unsaturated ones need 19 to 20 days to reach half of their maximum isotopic C^{14} incorporation.

Most workers in lipid metabolism believe that the liver is the most active site of cholesterol and fatty acid synthesis. However, Chaikoff's group[49] found that the conversion of carbohydrate to fatty acid proceeds significantly in extra-hepatic and extra-intestinal tissues. When this group injected C^{14}-labeled acetate into hepatectomized rats[50], they found that skin, testis, small intestine, and kidney all synthesized "tagged" lipids.

It is appropriate at this time to discuss briefly abnormal lipid metabolism. The object of the work embodied in this thesis was to throw additional light upon the processes of normal lipid metabolism to serve as a basis for future work on lipid metabolism in pathological states.

In the disease of diabetes, the animal cannot oxidize carbohydrate normally, which leads to an elevation in blood sugar. Accompanying this is a subsequent rise in the circulating ketone bodies resulting from an increase in fatty acid breakdown from which the animal must obtain most of its energy. For example, Chaikoff and associates[51] found that the ability of diabetic rat liver slices to utilize carbohydrate for fatty acid formation is drastically reduced. This is in agreement with the findings of Stetten and Boxer[52], that failure to utilize glucose for fatty acid synthesis is a major metabolic defect in diabetes. An in-vivo experiment

of Chaikoff and associates[33] unexpectedly showed that the rate of conversion of plasma glucose to CO_2 by the alloxan-diabetic rat does not differ significantly from that of the normal rat. These workers considered this difference to be reconciliable by assuming that glucose oxidation remains normal or near normal in some extra-hepatic tissue or tissues of sufficient bulk to mask the depression observed for liver. Their interpretation of their work is open to question, however, since the blood glucose levels of their experimental animals were very much above any level observed for the average alloxan-diabetic rat. This apparently normal plasma glucose oxidation by the diabetic rat could well be due to a mass action effect, since Stadie and associates[34] found a marked increase in glycogen synthesis with liver slices of normal rats when the incubating medium contained a high concentration of glucose.

Another abnormal type of metabolism is found in the fasting animal. In this condition the utilization of stored food somewhat resembles that of the diabetic in that energy is largely derived from fats since carbohydrate reserves are depleted. In both states, the animal shows increased blood ketone bodies due to increased fat metabolism.

An in-vitro experiment using liver slices, performed by Chaikoff and associates[35], indicates that the previous nutritional state of an animal influences the conversion of glucose to fatty acids. These workers reported that 1% of the C^{14} of glucose uniformly "tagged" with C^{14} added to liver slices was recovered in fatty acids when the diet of the animal had contained 60% dextrose, 22% casein, cellu flour, and mineral salts. Fasting of 24 hours reduced the C^{14} incorporation into fatty acids to one tenth that of the non-fasted animals. However, the recovery of the radioisotope

in the expired CO_2 did not differ appreciably from that of the non-fasted animals. In rats on diets devoid of carbohydrate, hepatic lipogenesis fell off rapidly. A three day diet composed entirely of protein or of protein and fat resulted in an extraordinary reduction in hepatic lipogenesis. Three day feeding of only carbohydrate showed no impairment in lipogenic capacity. A ten day diet consisting of only carbohydrate resulted in a moderate reduction in fatty acid synthesis. Chaikoff and associates therefore concluded that dietary carbohydrate is essential to maintenance of the capacity of hepatic tissue to convert glucose into fatty acids.

Carbon Dioxide

Respiratory CO_2 is an indicator of the overall metabolism of an animal and must be taken into account when evaluating any experiment designed to study the utilization of metabolites. The rate of excretion of $C^{14}O_2$ has been followed after the intraperitoneal injection into rats of:

(1) isotopic C^{14} bicarbonate[56] ($Na_2C^{14}O_3$)
(2) isotopic C^{14} acetate[57] ($CH_3C^{14}OONa$)
(3) isotopic C^{14} succinate[58] ($NaOOCH_2CH_2C^{14}OONa$)

Data obtained from these experiments yielded information as to the rates of isotopic carbon dioxide elimination, as well as the rate at which CO_2 participates in the metabolic processes of the body. Gould and associates[59] designed an experiment to study these rates, because previous measurements of excretion of isotopic CO_2 reported in the literature had been few, and in these the rate following a single injection of isotopic material had not been determined. These men followed the excretion of

$C^{14}O_2$ and total $C^{14}O_2$ and CO_2 formation at frequent intervals up to four hours. They observed the rates of excretion by normal rats following the intra-peritoneal injection of isotopic $NaHCO_3$, carboxyl-labeled sodium acetate, and carboxyl-labeled sodium succinate. It was found that the cumulative excretion of radioactive carbon in four hours was 95% after bicarbonate injection, 87% after acetate, and 86% after succinate administration. The rates of change of the specific activity of the CO_2 were interpreted in terms of the metabolic reactions of the substances injected. The data obtained are presented in the graph on page 24.[60]

The preponderance of the experiments concerning nutritional effects upon animals have been of the in-vitro type; and, while these are important, they are not physiological. Therefore, experiments were designed to evaluate the rates at which rats incorporate the C^{14} of $CH_3C^{14}OONa$ into carbon dioxide, fatty acids, and cholesterol in a period of one hour, and also to investigate the in-vivo effects of varying the time of maximal utilization (the interval of time that an animal is permitted to metabolize an injected intra-peritoneal dose of C^{14} carboxyl-labeled sodium acetate). These experiments and their results are explained in detail in Chapter III.

EXPLANATION OF FIGURE 1

Total C^{14} excreted: The accumulated percentage of C^{14} incorporated into respiratory CO_2.

Present as inorganic C^{14}: Calculated from a similar $NaHC^{14}O_3$ injection experiment in which it was found that a 100 gm. rat has an inorganic carbonate pool of 2.00 millimoles. Therefore, from the weight of the animals used and the total amount of CO_2 expired at any one time, the inorganic C^{14} in the animal can be calculated if one assumes this carbonate will have the same specific activity as that of the C^{14} in the respiratory CO_2.

Present as organic C^{14}: C^{14} as acetate remaining in the animal at any period of time, i.e., the initial amount injected less the sum of expired C^{14} as CO_2, and C^{14} as inorganic carbonate.

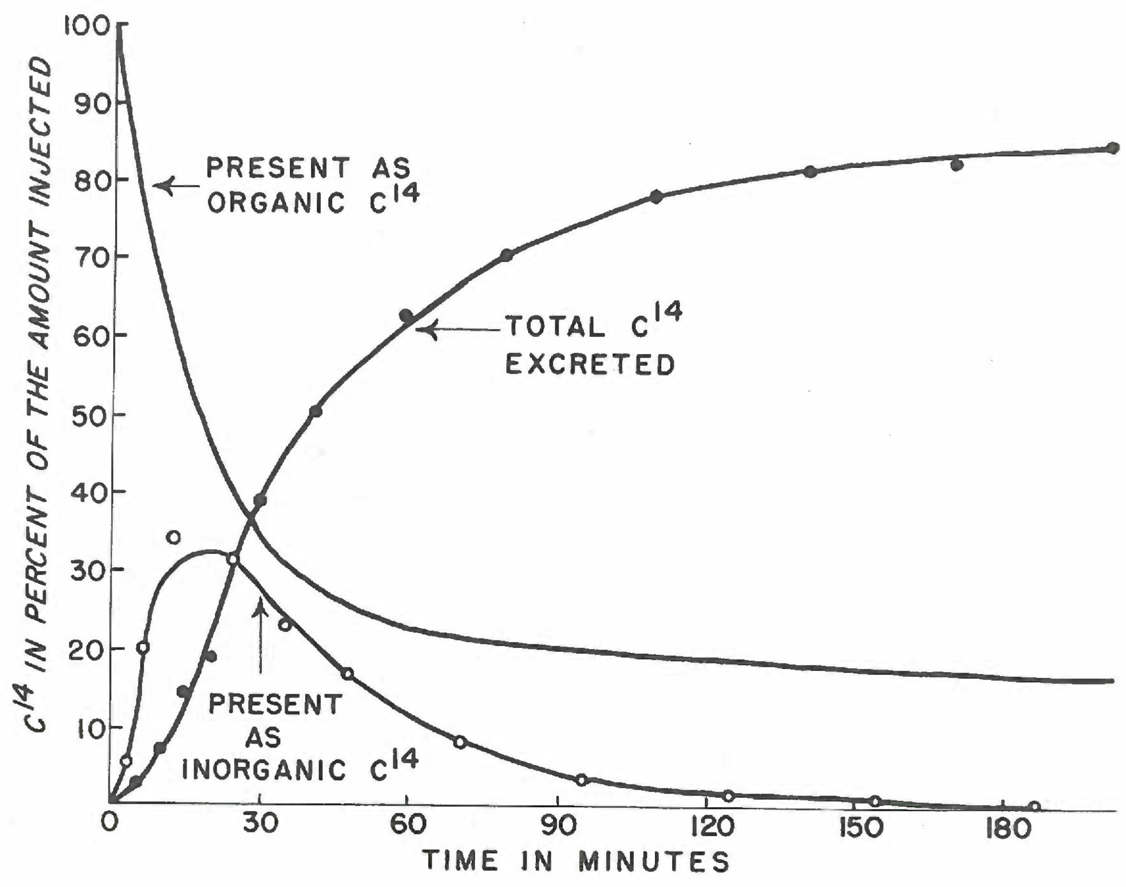


Fig. 1
100
90
80
70
60
50
40
30
20
10
0
30
60
90
120
150
180
C14 IN PERCENT OF THE AMOUNT INJECTED
TIME IN MINUTES
PRESENT AS
ORGANIC C14
TOTAL C14
EXCRETED
PRESENT
AS
INORGANIC C14

CHAPTER II

EXPERIMENTAL TECHNIQUES

A. Plating of $BaC^{14}O_3$

B. Combustion of Non-Volatile Samples

C. Preparation of $CH_3C^{14}OONa$ from $BaC^{14}O_3$

D. Treatment and Injection of Animals

E. Tissue Lipid Fractionation

1. Fasting Experiments

2. Time of Maximum Utilization Experiments

F. Carbon Dioxide Collection

G. Validity of Fractionation Technique

1. Isotope Dilution Experiment

2. Purity of Cholesterol

3. Purity of Fatty Acids

H. Counting of C^{14} and Calculation of Results

In this chapter a brief review of experimental methods will be presented and data given to indicate the validity of the methods used.

A. Plating of $BaCO_3$

Since the only isotope used in these studies was C^{14}, and since the biological materials to be studied were readily converted to CO_2, a convenient radioassay method for CO_2 or its derivatives was required. A solid, insoluble derivative of CO_2, $BaCO_3$, was selected as the material for the assay procedures.

A number of techniques have been described for the preparation of $BaCO_3$ samples which are suitable for counting. These include filtration[61], centrifugation[62], and evaporation[63]. Filtration and evaporation methods were found to require extensive, time-consuming manipulations. A centrifugation technique, which minimizes handling but gives reproducible plates, was developed. Although Calvin and associates[64] have previously suggested this type of sample preparation, the details of the method were not given.

The apparatus and methods developed and described below give high recovery of $C^{14}O_2$, consistently reproducible specific activities, and sample surfaces that are smooth, uniform, and free from cracks. Eight samples can be prepared in one to two hours, a small portion of this time being used for manipulation, and the remainder for the several periods of centrifugation. The details of this work have been published in _Nucleonics_[65].

To determine the radioactivity of a sample of $C^{14}O_2$, the solution containing $C^{14}O_3^{=}$ was precipitated with $BaCl_2$ at 50°‡, washed by suspension in ether-alcohol, centrifuged in round bottom glass tubes, and the supernatant liquid removed. The $BaCO_3$ was resuspended in ether-alcohol, stirred, and transferred to a special apparatus, using a modified medicine dropper. The apparatus, designed by the Radioisotope Laboratory, is shown in Figure 2. This precipitation assembly containing the suspended $BaCO_3$ was centrifuged, the ether-alcohol removed by aspiration, and the cups again centrifuged until the precipitate, which had collected upon the tared aluminum plate, appeared dry. The $BaCO_3$ discs were weighed and counted under thin end window

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‡ It has been shown by Regier[66] that adequate control of particle size is essential. When the precipitation is done at 50°, the most desirable particle size is obtained.

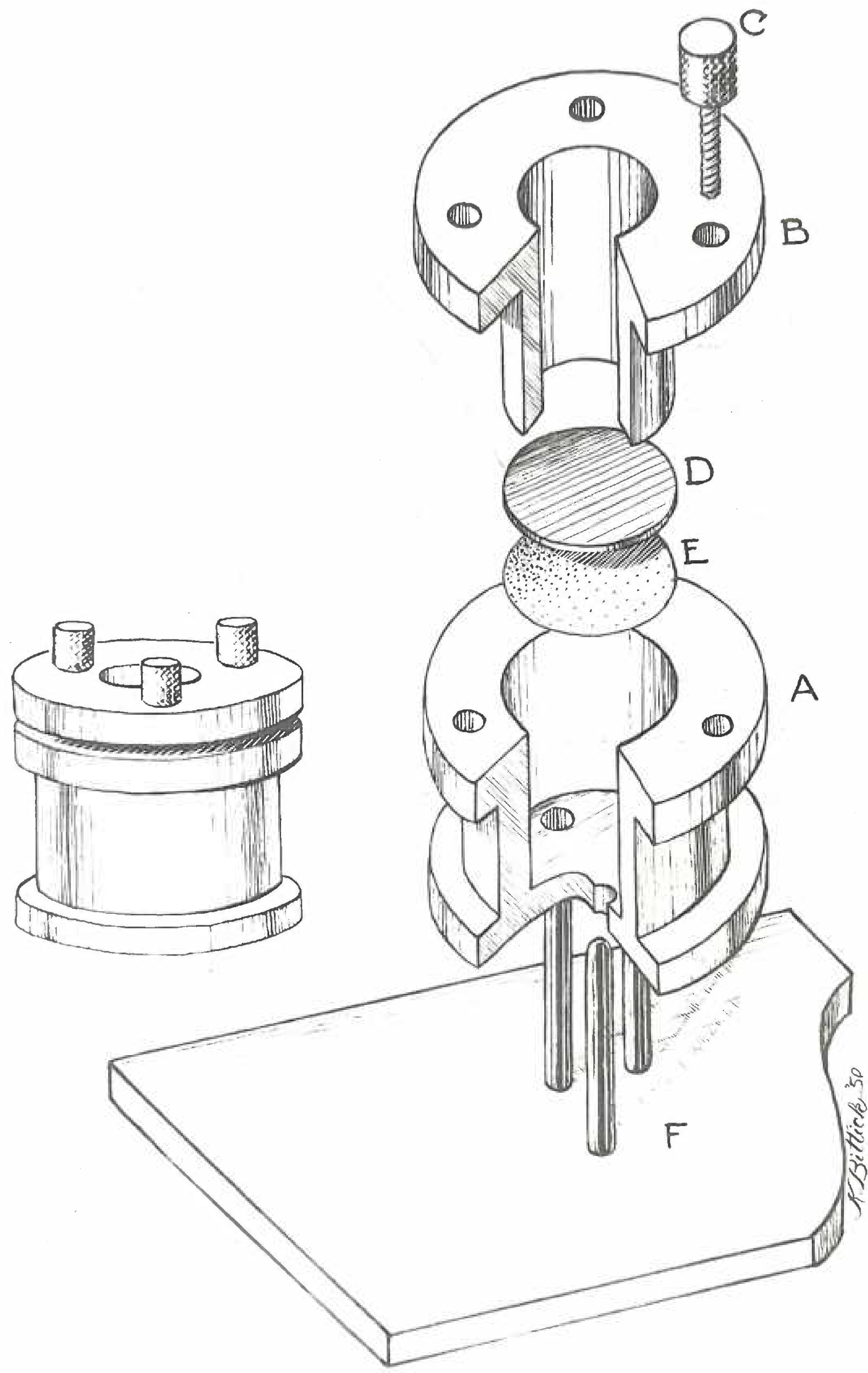
EXPLANATION OF FIGURE 2

The apparatus consists of a Duralumin cup A and sleeve B held in place by three thumb screws C.

The internal diameter of A is 1-1/4 in., walls are 3/8 in. thick, cup depth is 1-1/4 in., and bottom and top flanges are of 2-15/32 in. diameter, so that the unit fits snugly into the 250 ml. trunion cup of International Centrifuge Size 1. The bottom of A has three holes to admit the prongs of adaptor F.

Sleeve B fits with close tolerance in A and has an internal diameter of one inch. The bottom edge of B is carefully machined to a tight fit between B and an aluminum plate D. In operation, plate D is held firmly between the bottom of A and sleeve B by means of thumb screws projecting through the flange of B into matching threaded holes in the upper flange of A.

Plate D is punched from 1 mm. polished aluminum sheets. Care must be taken to remove all burrs or projections from the edges. Washing with organic solvents to remove any greasy film assures better adherence of the $BaCO_3$ to the plate.



PLATING ASSEMBLY
Fig. 2

counters or in an internal gas counter. In order to obtain constant, reproducible results, the radioactivity of the $BaCO_3$ was determined at infinite sample thickness or adjusted to this thickness.

The weight of $BaCO_3$ necessary for infinitely thick samples was found by preparing a series of samples at different weights but with constant specific activity. From these plates self-absorption curves were plotted. Data for these curves were obtained from a series of ten consecutive preparations made by precipitating the $C^{14}O_3^=$ from known $Na_2C^{14}O_3$ solutions, the weight of the precipitated $BaCO_3$ ranging from 31.7 mg. to 414.2 mg.

The recovery of $CO_3^=$ from the standard solution used to prepare the plates is indicated in Table 1. Tables 2 and 3 show the reproducibility of the plating procedure. Table 2 lists the results of eight consecutive preparations (two series of four each) designed to test the range of activity of identical samples. As the probable error indicates, good reproducibility is assured by the procedure. Table 3 shows that a constant amount of C^{14} activity can be diluted by increasing amounts of inert carbonate to yield plates of varying activities. Calculation of total activities by the equation with Table 3 provides a method of evaluating reproducibility over a wide range of sample weights.

B. <u>Combustion of Non-Volatile Samples</u>

Once the basic methods of treatment of C^{14} as $BaCO_3$ had been established, a modification of the Van Slyke-Folch "wet combustion" method was developed to oxidize fatty acids and cholesterol to CO_2 and H_2O. The apparatus used is shown in Figure 3.

TABLE 1

RECOVERY OF CARBONATE BY THE PLATING PROCEDURE

$BaCO_3$ mg/plate	$BaCO_3$ mg/cm^2	% Recovery $BaCO_3$*
31.7	6.3	107
43.5	8.7	105
59.1	11.8	100
85.7	17.1	96.7
115.5	23.1	97.7
156.0	31.2	97.7
204.1	40.8	97.3
263.1	52.6	98.4
312.5	62.5	95.7
414.2	91.2	99.06

Mean recovery of 6 protected samples = 97.65%

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* The first four preparations were made without complete protection from atmospheric CO_2 and thus illustrate the degree of contamination that may occur.

TABLE 2

REPRODUCIBILITY OF THE PLATING PROCEDURE
AT CONSTANT SPECIFIC ACTIVITY

(Identical Samples)

$BaCO_3$ + mg/plate	$BaCO_3$ mg/cm²	Activity † cpm/plate	Specific ‡ Activity
151.9	30.6	1,792	17.92
155.1	31.7	1,780	17.80
153.0	31.0	1,780	17.80
152.5	30.8	1,780	17.80
153.0	31.0	1,818	18.18
154.2	31.4	1,798	17.98
151.0	30.3	1,767	17.67
154.6	31.5	1,773	17.73

Standard deviation = 0.161

Relative probable error = 0.60%

+ $BaCO_3$ plates were prepared by precipitating a mixture of 1 ml. of $Na_2C^{14}O_3$, 1.5 ml. of 0.5M Na_2CO_3, and 4 ml. of 0.5M NaOH with 5.0 ml. of 0.5M $BaCl_2$-NH_4Cl.

† Counted immediately under the window of an RCL mk 1, md 3, 1.5 mg/cm² end-window GM tube.

‡ At infinite thickness or above, only the top 20 mg/cm² contributes to the count, so that one may calculate the specific activity as

$$\text{Specific activity} = \frac{\text{total counts (cpm/plate)}}{20 \times \text{sample area (cm}^2\text{)}}$$

TABLE 3

REPRODUCIBILITY OF THE PLATING PROCEDURE
AT VARYING SPECIFIC ACTIVITIES

(Constant Activity with Varying Amounts of Carrier)

ml 0.5M Na_2CO_3 carrier	Calc. $BaCO_3$ mg/plate	Calc. $BaCO_3$ mg/cm²	cpm/ sample	Calculated activity*
2.4	118.4	23.6	2092.2	2477.2
3.0	148.0	29.8	1776.0	2628.5
3.6	177.6	35.5	1415.0	2513.0
4.2	207.2	41.8	1268.5	2628.3
4.8	236.8	47.3	1105.9	2618.8

Standard deviation = 72.48

Relative probable error = 1.89%

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* Total calculated activity is determined from the following equation:

$$\text{Calculated activity} = \frac{\text{cpm/sample} \times \text{calc. mg } BaCO_3}{20 \times \text{area of sample } (cm^2)}$$

EXPLANATION OF FIGURE 3

The combustion apparatus has five components: an oxidation flask A with oxidant reservoir B, a delivery or adapter tube C, an absorption flask F, and a mercury manometer E. An evaporation sleeve G supplements the basic equipment. In the following descriptions, exact dimensions are not given, as they are not critical. Comparison of unit parts with the ₸* joints as shown will serve to approximate all actual dimensions.

1. Combustion flask A is made from a male ₸ 19/38 joint sealed to a 10 ml. micro-Kjeldahl flask. The 6 to 10 ml. oxidant receiver, tube B, is joined to flask A at about 130 degrees to the long axis of flask A.

2. The adapter, or delivery tube C is made from two female ₸ 19/38 joints, shortened and united at an angle of approximately 100 degrees. A two-way oblique-bore stopcock, D, is sealed midway and at the top of tube C. A ₸ 10/30 male joint is sealed midway and on the side of C.

3. A capillary mercury manometer E having a female ₸ 10/30 joint is connected to the 10/30 joint on C and is easily removed for cleaning.

4. Absorption flask F is made from a male ₸ 19/38 joint and a 30 ml. micro-Kjeldahl flask.

5. Evaporation adapter or sleeve G is made from a female ₸ 19/38 joint, and a length of capillary tubing H runs coaxially within the joint and extends beyond it. Tubing H is held in place by being passed through a short piece of rubber tubing that also passes over the end of G. Side arm I connects with an aspirator.

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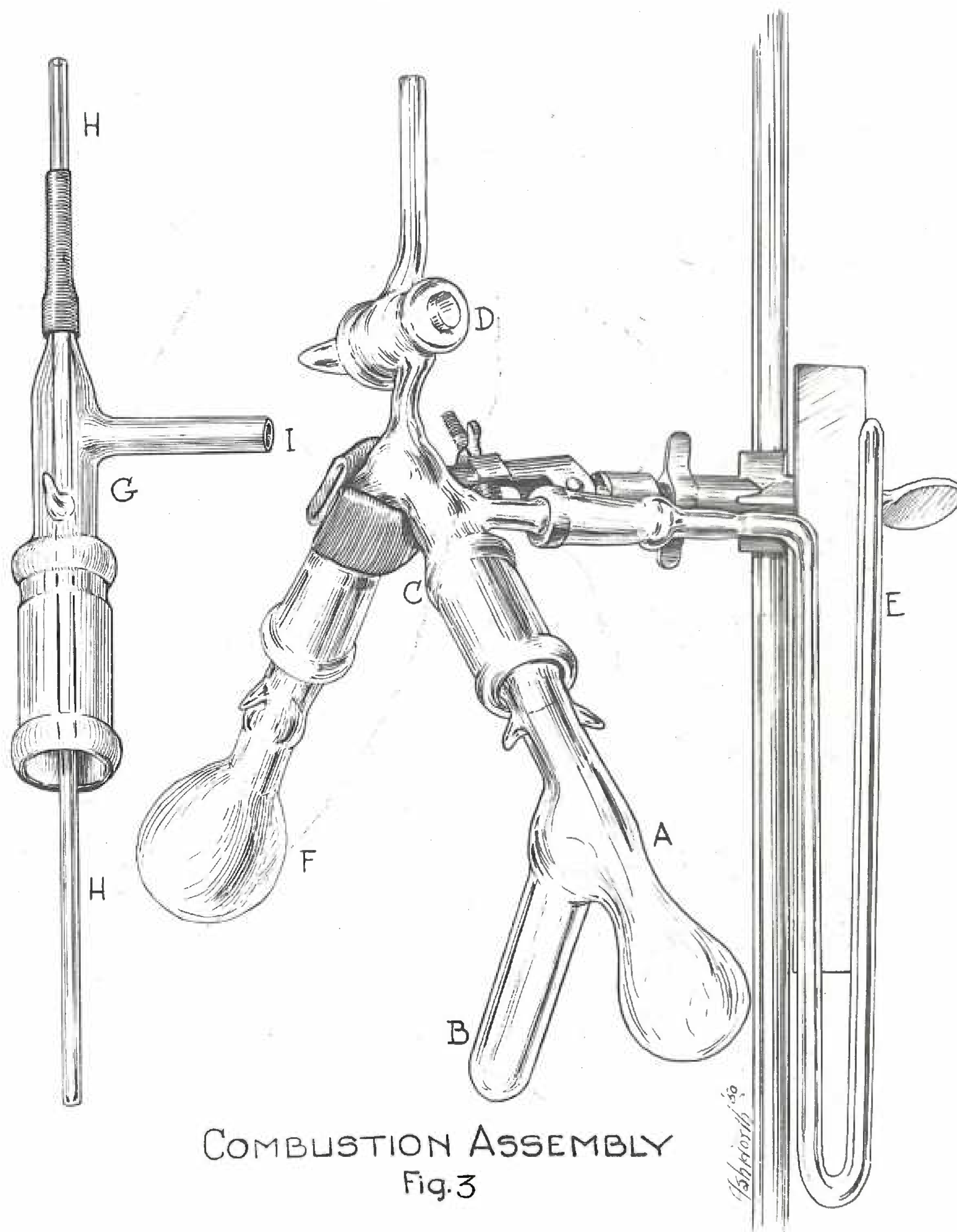
* National Bureau of Standards symbol for "standard taper". Numerator of following fraction gives large diameter in mm., denominator gives length of ground surface.



COMBUSTION ASSEMBLY
Fig. 3

The sample to be combusted was put into tube A, and the Van Slyke-Folch wet oxidation mixture, prepared according to Calvin[67] (less potassium iodate), was placed in side arm B. A known amount of standard sodium hydroxide, about 0.75N, was pipetted into Flask F. The assembled apparatus was then evacuated to approximately 20 to 30 mm. Hg through stopcock D, and the oxidizing solution added to the sample in tube A by rotating the flask 180°. The bulb of flask A was heated in a 160° bath, while absorption flask F was cooled in an ice bath. The heating was continued for 10 to 15 minutes, the flask removed from the bath and allowed to cool. The amount of CO_2 produced was determined by titration with standard HCl to the phenolphthalein end point. The entire alkaline carbonate solution or any aliquot thereof was used for $BaCO_3$ plating as previously described.

The data of Table 4 illustrate the range of recovery of carbon by this procedure. Since these data were obtained on relatively large samples, the reproducibility and reliability of the technique in recovering graded amounts of C^{14} activity in the presence of varying amounts of carrier was studied. Table 5 presents the data obtained by the "combustion" of two 100 lambda samples of $Na_2C^{14}O_3$ and by the direct precipitation and plating of two equivalent samples without combustion. These data show the efficiency of CO_2 distillation and absorption in terms of recovered activity.

C. Preparation of $CH_3C^{14}OONa$ from $BaC^{14}O_3$

It was apparent that large amounts of $CH_3C^{14}OONa$ would be needed to pursue the contemplated research program. Carboxyl-labeled acetate can be purchased but is expensive. Therefore, it was desirable to synthesize it from low cost $BaC^{14}O_3$. The activity of the synthesized radio-acetate can be

TABLE 4

CARBON RECOVERY BY WET COMBUSTION

Material combusted*	Sample mg.	Mmole carbon Theo.	Found	% Recovery
Potassium acid phthalate	22.9	0.898	0.882	98.2
	22.1	0.866	0.816	94.2
	20.5	0.804	0.731	90.9
	24.5	0.960	0.907	94.5
	16.9	0.662	0.633	95.6
	24.9	0.976	0.899	92.1
			Mean =	94.3
Cholesterol	10.1	0.705	0.648	91.9
	12.9	0.900	0.901	100.0
	7.6	0.530	0.507	95.7
	8.6	0.600	0.578	96.3
	7.1	0.500	0.487	97.4
	10.6	0.740	0.717	96.9
	10.2	0.712	0.686	96.3
			Mean =	95.6
Digitonin	13.1	0.593	0.543	91.6
	13.2	0.598	0.532	89.0
	16.4	0.743	0.651	87.6

* Samples combusted with 5 ml. of combustion reagent. CO_2 was absorbed in 3 ml. of 0.752N NaOH. Blank of 0.03 mmoles CO_2 subtracted. The phthalate and cholesterol were of purified reagent quality, but the digitonin was of questionable purity.

TABLE 5

RECOVERY OF ACTIVITY FROM MICRO AMOUNTS OF SAMPLE

Sample no.	Procedure	λ C^{14} sample	Mmoles $CO_3^=$ absorbed in combustion	Mmoles $CO_3^=$ added as carrier	Total Mmoles $CO_3^=$/sample	Calc. mg. $BaCO_3$/sample	cpm/sample	Calc. total activity[‡]	Average total activity
1	Combusted[≠]	100	0.086[≠]	0.72	0.806	159.0	312.4	496.8	
2	Combusted[≠]	100	0.036[≠]	0.72	0.756	149.2	309.6	461.8	479.3
3	Not Combusted[≠]	100	---	0.72	0.72	142.1	343.4	488.0	
4	Not Combusted[≠]	100	---	0.72	0.72	142.1	327.3	465.1	476.6

[≠] Active samples 1 and 2 were dried in flask A, combusted, titrated, and 0.72 mmoles carrier carbonate added before precipitation. Active samples 3 and 4 were not combusted but added to the carrier $CO_3^=$ and precipitated for plating.

[≠] The variations in these values actually represent variations in the blank values, since the carbonate from the samples combusted is too small to titrate.

[‡] Calculated total activity =

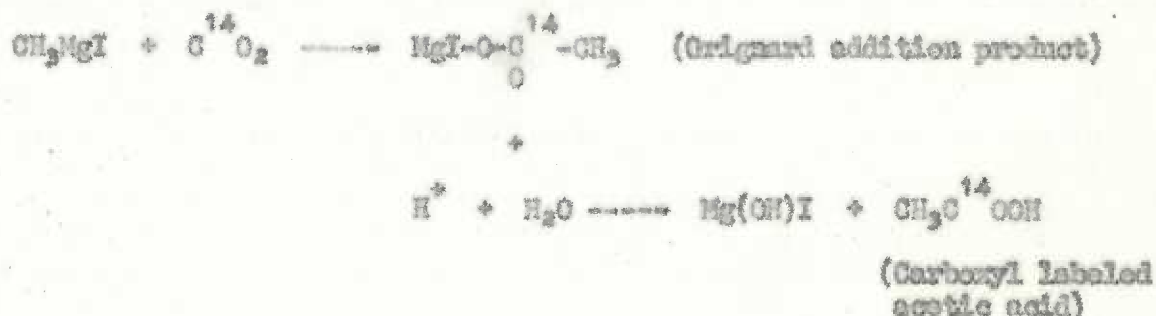
$$\frac{\text{cpm/sample x calc. mg } BaCO_3}{20 \text{ x sample area } (cm^2)}$$

estimated from the amount of $BaC^{14}O_3$ used, if the efficiency of the reaction is known. By comparison of the radioactivity of the synthesized product with the activity of acetate obtained from commercial sources, it is possible also to confirm the activity of the carboxyl-labeled product prepared.

Many published articles give procedures for the synthesis of C^{14}-labeled acetate, both by chemical and microbiological methods. A good review of these methods has been given by Calvin in Isotopic Carbon[68], but all involve relatively macro quantities of $BaC^{14}O_3$ and complex and expensive equipment. Initially an attempt was made to scale down some of these methods and to synthesize carboxyl-labeled acetate in semi-micro quantities by the carbonation of a methyl Grignard reagent. However, all attempts were unsuccessful until an apparatus designed by this laboratory was employed. It is small, compact, inexpensive, and easily assembled. However, if used under the proper conditions, good yields of carboxyl-labeled acetate are obtained. With the technique employed, reproducible yields of 80 to 90 per cent were realized when 0.25 to 1.0 millimoles of acetate were synthesized.

The synthesis of acetate using this equipment (see Figure 4) was as follows. The radioactive barium carbonate was placed in flask C and the assembly evacuated. $C^{14}O_2$ was generated from $BaC^{14}O_3$ when 40% perchloric acid, stored in vial A, was added to the carbonate. The liberated CO_2 was dried by passing through tube D containing anhydrone, and then frozen in the bottom of flask E, cooled by a liquid nitrogen bath. Methyl Grignard reagent in vial G, prepared according to the procedure of Gilman[69] was added to the layer of solid carbon dioxide by opening stopcock F. Highest yields

of carboxyl-marked acetate were obtained when the CO_2 and methyl Grignard reagent were added in alternate small increments to flask E. When this flask was removed from the liquid N_2 bath and warmed to 0° in an ice bath, carbonation of the Grignard reagent occurred, and the resulting compound was decomposed by addition of water and acid. The overall reactions for the synthesis may be represented as follows:



$$CH_3MgI + C^{14}O_2 \longrightarrow MgI\text{-}O\text{-}\underset{O}{C^{14}}\text{-}CH_3 \quad \text{(Grignard addition product)}$$

$$+$$

$$H^+ + H_2O \longrightarrow Mg(OH)I + CH_3C^{14}OOH \quad \text{(Carboxyl labeled acetic acid)}$$

The acetic acid was recovered from the reaction mixture by distillation. It was found advisable to add Ag_2SO_4 prior to distilling to prevent any HI contamination from Mg(OH)I decomposition during this operation.

The design of the reaction vessel made it possible to carry out the acetic acid removal directly by connecting it to the condensor H shown in Figure 5, thereby eliminating exposure of the operator to radioactivity from any unreacted $C^{14}O_2$. During the distillation, reservoir G was kept filled with water which was added to the distilling mixture in a continuous trickle through stopcock F. This not only furnished the water required in the acetic acid separation but also allowed a higher boiling mixture to be maintained. The receiving vessel I, equipped with a burette, permitted continuous titration of the distillate with standard base. This enabled the operator to determine the acetic acid distillation rate and yield. A CO_2 trap

EXPLANATION OF FIGURE 4

The apparatus has three components: a CO_2 generator C, a delivery tube D, and a carbonation and distillation flask E, all of Pyrex glass.

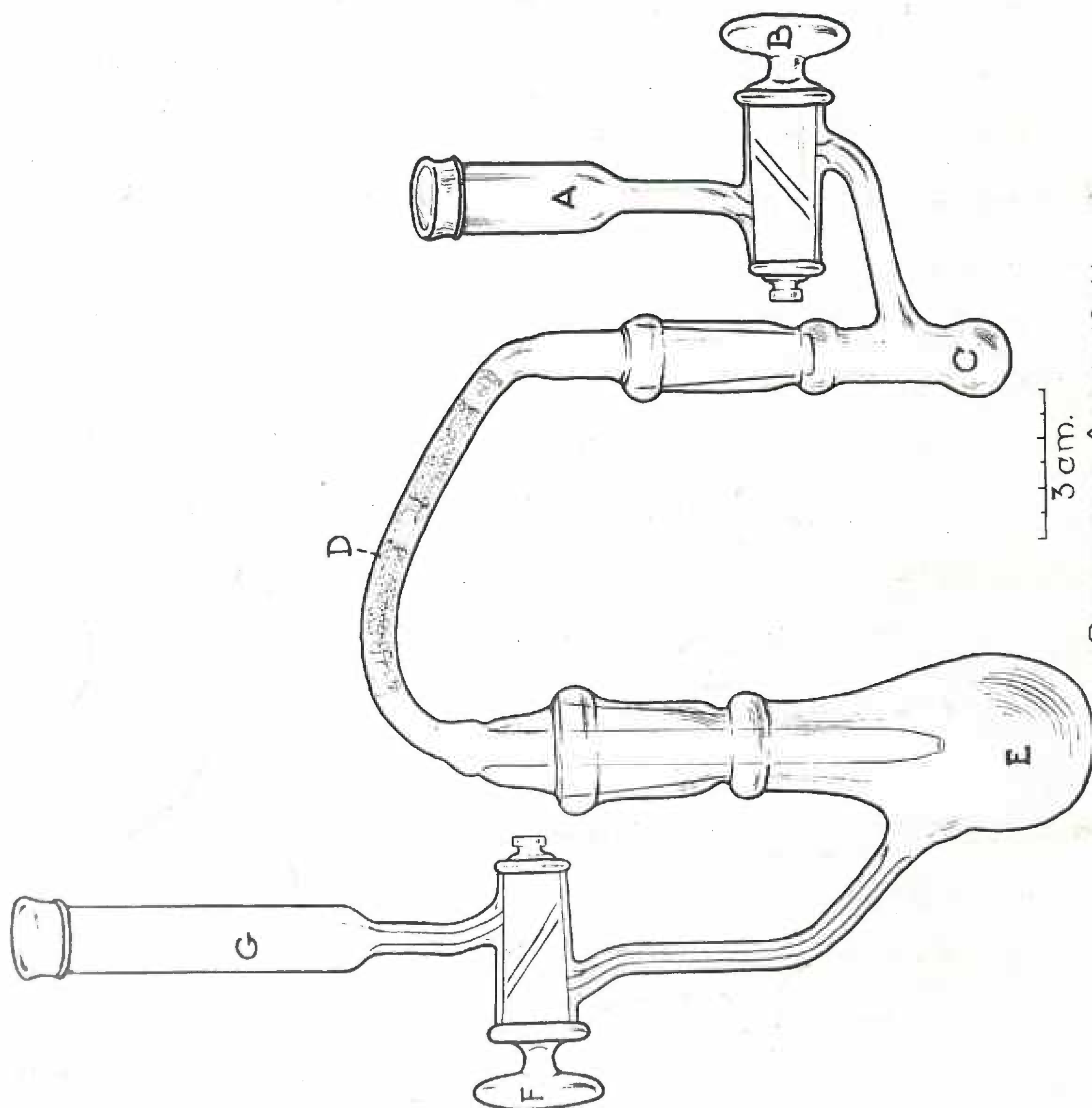
1. Flask C is made from a female ₮ 14/35 joint and has a small bulb at the end. It is fitted with a side arm having a two-way oblique-bore stopcock between flask C and reservoir A above. The capacity of reservoir A is approximately 5 ml. It is closed during the reaction with a tightly fitting rubber vial closure.

2. Reaction flask E is made from a 30 ml. Kjeldahl flask and a female ₮ 19/38 joint. The flask is joined to the 10 ml. reservoir G by a 2 mm. bore capillary tube through a two-way oblique-bore stopcock. Since the final product is distilled from the same flask, it is well to have the stopcock above the ₮ joint on E. Reservoir G is also fitted with a rubber closure. The capillary tube from stopcock F to flask E should enter flask E at such an angle as to direct entering reagent to the bottom of the flask.

3. Flasks C and E are connected by tube D containing 60 to 80 mesh anhydrone held in place with glass-wool plugs. The tube is fitted with ₮ joints to fit C and E.

4. A liquid nitrogen bath is used to cool flask E during the reaction.

5. Distillation of the acetic acid is accomplished with the aid of the assembly shown in Figure 5. The ₮ 19/38 joint of the condenser H serves to attach the flask E.

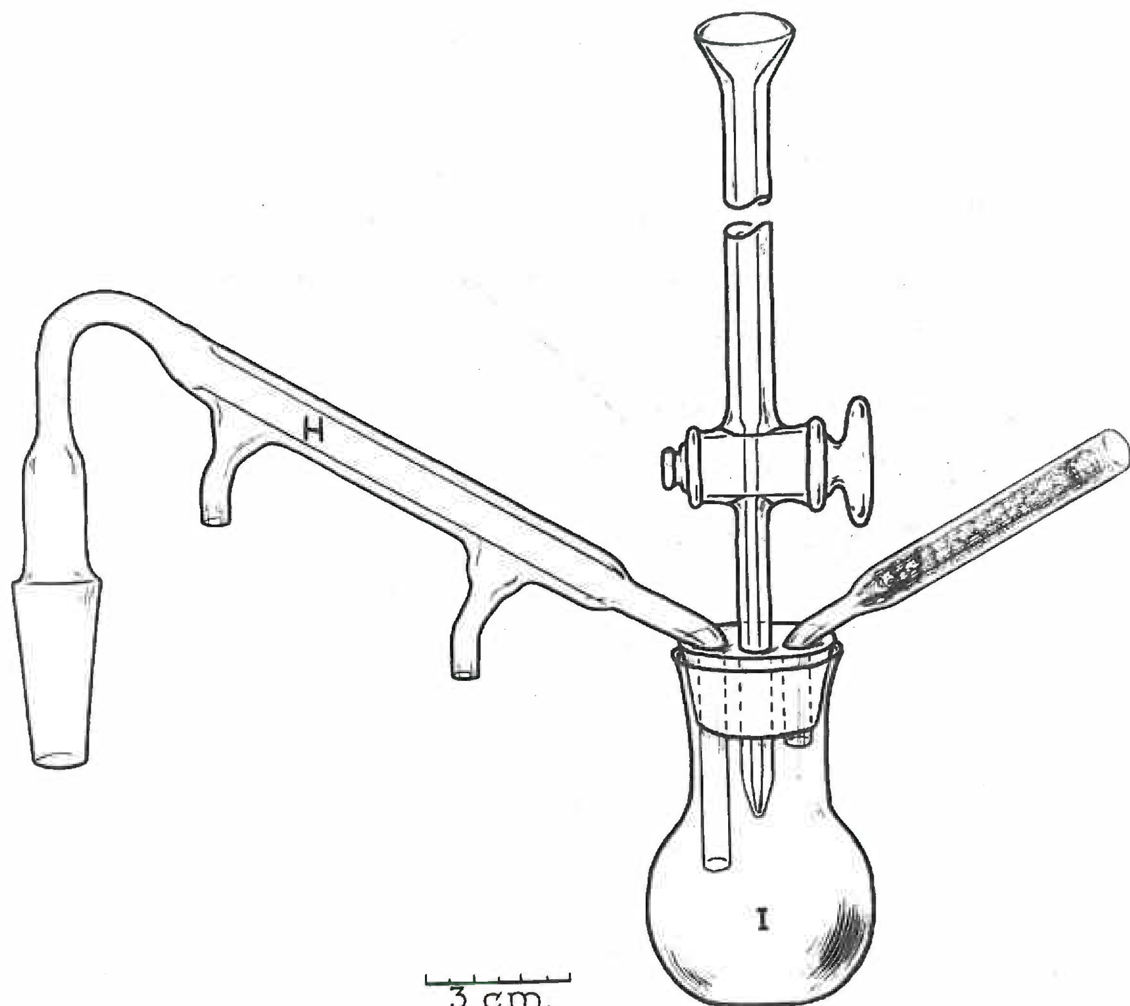


SYNTHESIS ASSEMBLY
Fig. 4

EXPLANATION OF FIGURE 5

The apparatus has two main components: a condenser H, with a ₮ 19/38 joint for attachment of reaction flask E (Figure 4), and flask I, which serves to collect the distillate.

In addition to these, there is also a three-hole rubber stopper, which is used to close flask I and provides for the entrance of the end of condenser H, the tip of a microburette, and a soda-lime absorption tube.



DISTILLATION ASSEMBLY
Fig. 5

served as a vent for the closed system. The results of this procedure are tabulated in Table 6.

In addition to the reported isotopic experiments, four consecutive runs were made with inactive $BaCO_3$ to check the efficiency of the apparatus. The first determination in this series consisted of 0.503 millimole of $BaCO_3$ and 2.0 milliequivalents Grignard reagent and gave a 72.8% yield. The next three involved 2.7 milliequivalents of Grignard reagent and gave yields of 95.0, 90.4, and 88.4% respectively.

Table 6 lists the results of five reactions using $BaC^{14}O_3$ which had a reported activity of 0.0136 mc./mg. It is readily seen that, under controlled conditions, the yield of acetic acid, as determined by NaOH titration, is essentially the same as that observed in the non-isotopic reactions above.

Sodium acetate thus obtained was diluted to a convenient volume, aliquots evaporated and combusted to CO_2 and plated as $BaCO_3$. These $BaCO_3$ samples were counted at infinite thickness (carrier Na_2CO_3 added) at a distance of 2 mm. from a thin window G.M. tube.

The ratio between the number of counts per sample and the absolute number of disintegrations per sample can be approximated for any standardized counting procedure if aliquots of a standardized radiochemical, such as Oak Ridge-assayed $BaC^{14}O_3$, are treated in an analogous manner, and also by determination of and correction for procedural losses of combustion, plating, and counting. This ratio indicated a counting efficiency of approximately one and one-half to two percent.

TABLE 6

SYNTHESIS OF C^{14} CARBOXYL-LABELED ACETATE

Synthesis number	$BaCO_3$ used mmoles	$BaCO_3$ used mc	Grignard used meq	Base required meq	Percent[*] yield	Calc. yield as mg NaAc	Counts[/] per yield
1[≠]	0.373	0.998	0.85	1.52	68.0	20.8	5.6×10^6
2[§]	0.381	1.02	1.674	2.79	73.0	22.8	8.4×10^6
3	0.307	0.822	1.953	2.56	83.4	21.0	5.9×10^6
4	0.413	1.111	2.092	3.63	87.7	29.7	1.11×10^7
5	0.812	2.17	4.185	7.10	87.3	58.2	2.76×10^7

[*] Calculated from milliequivalents base required.

[/] Reaction product diluted to 100 ml. Aliquots combusted and plated as previously described.

[≠] Loss of an undetermined amount of $BaC^{14}O_3$ during transfer of the weighed $BaCO_3$ to reaction flask C (Figure 4)[70].

[§] Vacuum loss during run (pinhole discovered in apparatus after synthesis). Apparatus was repaired and a subsequent "cold run" showed 87% efficiency.

D. Treatment and Injection of Animals

The animals were injected intraperitoneally with one ml. of sodium acetate (1.04×10^6 c.p.m.) per 100 grams of body weight and placed singly or in pairs into the metabolism vessel A (Figure 6). A stream of CO_2-free air was pulled through the vessel and then through an absorption column, B, containing standard NaOH. The respiratory carbon dioxide of the rat was thus carried into the basic solution where it reacted with the NaOH, forming Na_2CO_3. The apparatus was designed to collect CO_2 for intervals ranging from a few minutes up to one hour.

At the desired time the air flow was discontinued and the animals killed by insertion of a $CHCl_3$ saturated cotton plug into the neck of the metabolism vessel.

E. Tissue Lipid Fractionation

1. Fasting Experiments

After the animal was sacrificed, it was put *in toto* into a flask containing alcoholic KOH and digested under reflux for four to five hours. At the end of this time any undissolved material was removed by filtration. Evaporation of the solution to one-half volume removed practically all of the alcohol, and water was then added to the syrupy material to bring the total volume to 500 ml. This aqueous solution was then transferred to a 2000 ml. separatory funnel. The non-saponifiable fraction was removed by repeated extractions with low boiling petroleum ether; the exact volumes and procedure used are listed in the flow sheet shown in Table 7. These ether extracts were combined, washed with 1N KOH and then H_2O to remove any saponifiable matter, dried with Na_2SO_4, and concentrated by distillation to

EXPLANATION OF FIGURE 6

The apparatus is composed of three component parts: metabolism jar A, CO_2 absorbers B, and $Ba(OH)_2$ tube D.

1. Two sizes of metabolism jar A are used; one has a capacity of one rat, the other, of two. Each is equipped with a rubber stopper containing an air inlet and outlet. These tubes are of sufficient size to permit an adequate flow of air into chamber A.

2. The absorbers B each contain 100 ml. NaOH, and a sintered glass disc is sealed into each so that the entering CO_2-carrying air from jar A is dispersed into fine bubbles. This arrangement will absorb 25 millimoles of CO_2 in 10 minutes.

3. $Ba(OH)_2$ tube D traps any expired $C^{14}O_2$ not absorbed in B.

4. Bypass tube C permits flushing of A without CO_2 contamination of B.

5. Line E extends to a constant vacuum source.

6. CO_2-free air is conducted into the metabolism chamber through tube F.

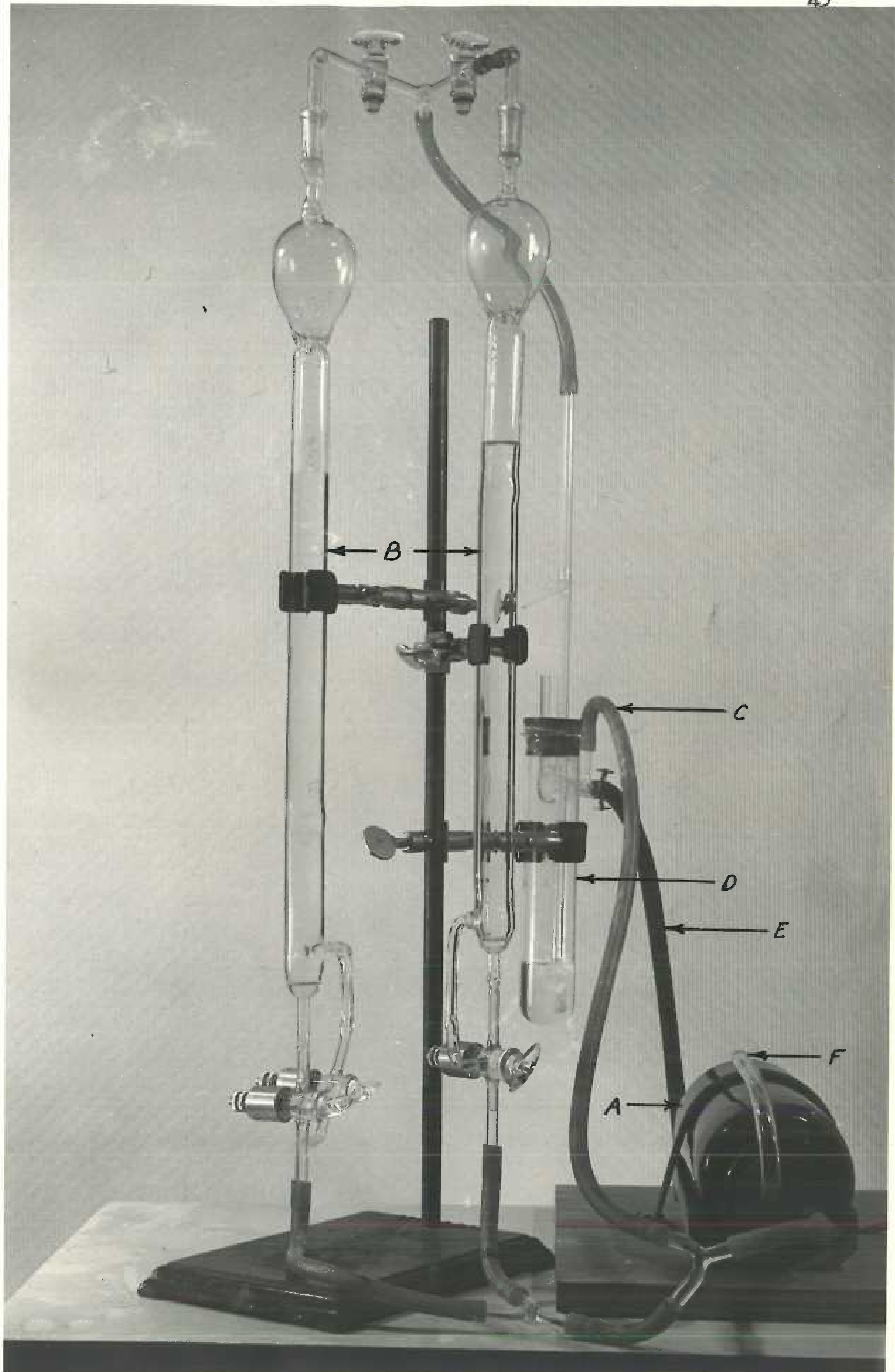


Fig. 6

TABLE 7

200 grams tissue

Digest in 400 ml. alcohol containing 100 grams KOH

with reflux for 4 hours, cool

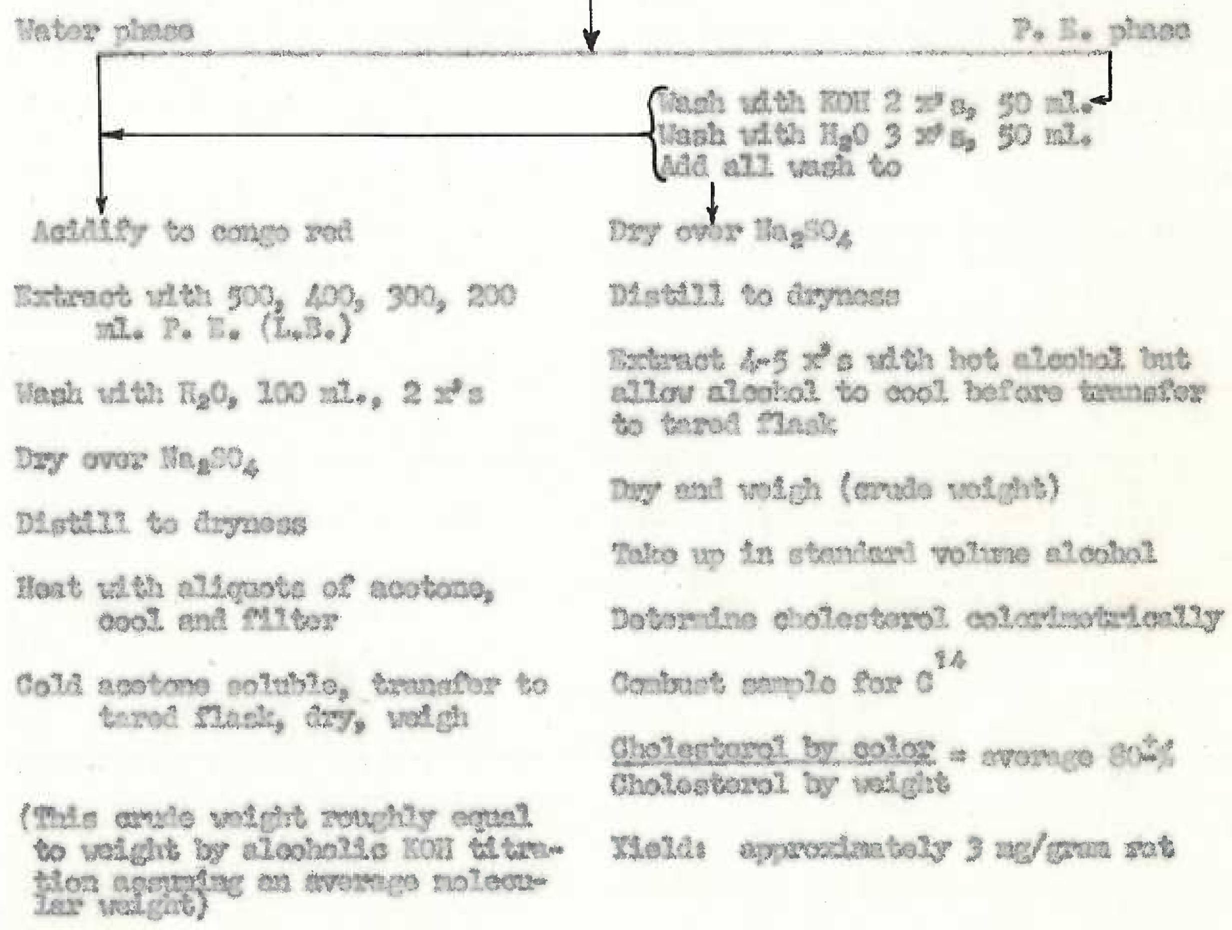
Filter on coarse sintered glass, wash residue with hot alcohol, hot petroleum ether, hot H_2O, each two times with 100 ml. each

Evaporate to 1/2 volume

Add H_2O to 500 ml. total volume

Add 150 ml. alcohol

Extract with 500, 400, 300, 200 ml. petroleum ether (L.B.)



dryness. The resulting solid was dissolved in hot alcohol, cooled, and filtered through fine filter paper into a tared volumetric flask. The alcohol was removed by evaporation and the dry weight of this unsaponifiable fraction determined.

For the determination of cholesterol the contents were dissolved in alcohol and diluted to a known volume. Aliquots of the solution were evaporated and developed colorimetrically by a modified Schoenheimer-Sperry method. Comparison of the tared weight with the amount found by colorimetric determination indicated that approximately 80% by weight of the crude unsaponifiable fraction was cholesterol. The amount of incorporation of radioactive carbon into the fraction studied was found from counting $BaCO_3$ samples which had been prepared by evaporating aliquots to dryness, "wet oxidising", and plating.

The saponifiable fraction of the digest was then separated from the aqueous phase by acidifying to congo red with concentrated HCl and extracting the liberated fatty acids with low boiling petroleum ether. Table 7 gives the exact volumes used. The extracts were combined, washed with H_2O, dried over anhydrous Na_2SO_4, and the solvent removed by distillation. The solid fatty acids were dissolved in hot acetone, cooled, and filtered into a tared Erlenmeyer flask. The purified lipids were recovered by evaporation of the acetone in a stream of N_2 or air and then vacuum desiccated over H_2SO_4. After weighing, portions were removed for direct combustion or were dissolved in a known volume of acetone, from which aliquots were transferred to the combustion flasks, dried and combusted by the previously described technique. The amount of C^{14} incorporation into the fatty acids was found by counting $BaCO_3$ samples subsequently prepared.

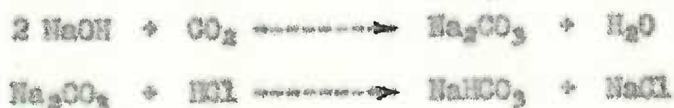
2. Time of Maximum Utilization Experiments

In this study the methods employed were essentially the same, except that the animals were treated in pairs with their individual tissues being pooled prior to digestion. The rats were decapitated immediately upon removal from the metabolism vessel, and the livers excised as rapidly as possible. The animals were further separated into skin, gut, brain and spinal cord, and carcass; blood from the decapitation was added to the carcass fraction. Tissues were weighed and immediately placed in alcoholic KOH. These operations were performed as rapidly as possible to arrest any further metabolic activity. Because of the smaller amount of tissue involved, as compared to the whole animal work of the previous experiment, the volumes of solvents and reagents were reduced proportionately. However, the least volume of digestant mixture used for any tissue was 100 ml. of alcohol and 25 grams of KOH.

The digestion, isolation, and treatment for evaluation of the degree of C^{14} incorporation into the tissue lipids and respiratory carbon dioxide were carried out similarly for both series of experiments. In the latter series it was also observed that the weight ratio of cholesterol to that of the unsaponifiable fraction was 0.8.

F. CO_2 Collection

The expired CO_2 of both series of experiments, at the termination of the collection time, were transferred by washing into volumetric flasks and diluted to 100 ml., and aliquots were titrated to the phenolphthalein end point for total CO_2 determination. From the following reactions



$$2\ NaOH + CO_2 \longrightarrow Na_2CO_3 + H_2O$$

$$Na_2CO_3 + HCl \longrightarrow NaHCO_3 + NaCl$$

the milliequivalents of CO_2 collected can be calculated. To determine the radioactivity, one-half to one ml. aliquots of the basic carbonate solution were treated with a NH_4Cl-$BaCl_2$ reagent which precipitated the C^{14} as $BaC^{14}O_3$. Using such small amounts necessitated addition of inert Na_2CO_3 to give infinite thick samples for counting.

G. Validity of Fractionation Technique

1. Isotope Dilution

As will subsequently be shown, the per cent incorporation of the C^{14} into the lipid fractions of each experiment was calculated from the specific activity (c.p.m./mg. of compound) multiplied by the total amount of the compound. Therefore, it is readily apparent that the total amount of fatty acid or cholesterol isolated was of extreme importance. In many biochemical procedures, techniques of solvent partitionings, etc., are used that are felt to be adequate; but often no positive information is available regarding the validity of this assumption. To test the efficiency of the extraction procedure, two investigators in the Radioisotope Laboratory (Drs. J. T. Van Bruggen and T. T. Hutchens), using an isotope dilution method, separately digested two normal mice, one 10 grams, the other 30 grams, in alcoholic KOH. Before refluxing was started, weighed amounts of a biosynthesized cholesterol (89.7 c.p.m./mg., as $BaCO_3$ at infinite thickness) were added to the digestion mixture. The volume of digestant and all subsequent solvent and reagent volumes were adjusted to the proportions of 10/200 on the basis of the previous 200 gram weight as described in Table 7. The 30 gram animal was treated as if it were a 10 gram mouse; therefore, a three-fold excess of tissue was present.

From the 10 gram mouse, to which had been added 2.1 mg. of labeled cholesterol, there were isolated 33 mg. of cholesterol that had a specific activity of 5.54 c.p.m./mg., or a total of 182.8 c.p.m. The total counts that could have been isolated were 2.1 mg. x 89.7 c.p.m./mg. = 188.4 c.p.m. Thus 188.4 c.p.m. ÷ 5.54 c.p.m./mg. = 34.0 mg. of cholesterol which should have been recovered, or 33.0 mg./34.0 mg. = 97% efficiency of recovery was achieved. With the 30 gram animal the specific activity of the isolated cholesterol, using the same reasoning as for the 10 gram mouse, indicated that 88.0 mg. should have been obtained, whereas only 50.5 mg. were isolated, a recovery efficiency of 57%. Thus when the ratio of reagents to tissue was low, poor recovery resulted. Consequently, all the work reported herein is based upon the proper amounts of reagents as indicated in Table 7.

2. Purity of Cholesterol

Whereas the necessity of total isolation of all the cholesterol and fatty acids is important, it is even more important that they be pure. That is, if the isolated cholesterol were to contain any contaminating fatty acid, related sterols, or other biological substance that contained appreciable radioactivity, the observations noted would not be valid. Therefore, a number of experiments were done to determine the degree of purity of lipids isolated by the aforementioned technique.

In the first, the cholesterol from eight mice which had been injected intraperitoneally with $CH_3C^{14}OONa$ was isolated and both the digitonide and acetate prepared. The latter was made by the pyridine cleavage method of Bergmann[71] and acetylated after the procedure of West[72]. Both compounds were combusted and plated as $BaCO_3$, and counted. As can be seen from

Table 8, this extensive purification caused only a 5.1% difference, an amount not significant when one realizes that the counting efficiency of the electronic equipment and the statistical variation of radio decay have a combined error of this magnitude.

In the second experiment, two investigators in the Radioisotope Laboratory (Van Bruggen and Hutchens) combusted biosynthesized labeled cholesterol as the crude compound, as the digitonide, as the cholesterol bromide, and finally again as a cholesterol regenerated from the dibromide. As shown in Table 9, little is to be gained by extensive purifications that involve many days of work, for activities in terms of counts per minute per milligram did not significantly change.

3. Purity of Fatty Acids

Since the fatty acids were isolated from the aqueous portion of the saponification mixture, it is possible that contaminating unsaponifiable substances, viz., cholesterol, might contribute to the apparent radioactivity. Acetic acid might also be a contaminant, since it is both the first member of the fatty acids series and the usual material injected. To check the purity of the isolated fatty acids, a series of integrated experiments was performed; these were: purification of the acids with Cu-Ca salt formation and regeneration of the free fatty acids, removal of any contaminating cholesterol as the digitonide, and digestion of non-isotopic animals in the presence of radioactive sodium acetate.

Cu-Ca salts of two labeled fatty acid preparations were made by dissolving 300 mg. of each sample in 30 ml. of 1N KOH and adding 2 ml. each of $CuSO_4$ and $Ca(OH)_2$ solutions. The heavy flocculent precipitate formed

TABLE 8

SUCCESSIVE PURIFICATION OF TISSUE CHOLESTEROL

VERSUS SPECIFIC ACTIVITY

Specific Activity (c.p.m./mg.) as Cholesterol when combusted as:	Animal No.								
	1	2	3	4	5	6	7	8	Avge.
Cholesterol acetate	21.6	24.3	25.8	17.3	20.1	15.7	23.0	15.6	
Cholesterol digitonide	24.0	29.6	23.7	21.2	19.2	23.6	22.5	17.6	
Difference CA/CD	+1.1	+1.2	-1.1	+1.2	-1.1	+1.5	+1.0	+1.1	
% difference CA/CD	4.5	4.0	4.6	5.6	5.2	6.3	4.4	6.2	5.1

TABLE 9

Stage of Purification	Specific Activity
1. Crude cholesterol (non-saponifiable fraction)	98
2. As digitonide precipitate	93
3. Cholesterol regenerated from digitonide	100
4. Cholesterol as the dibromide	85+
5. Cholesterol regenerated from the dibromide	87+

contained only the acids of ten or more carbon atoms, the lower members of the series remaining as soluble soaps in the supernatant solution. These Cu-Ca salts were separated by centrifugation, washed by suspending in H_2O, and again centrifuged. Acidification with concentrated HCl liberated the lipids as free fatty acids, which were then recovered from the aqueous phase by repeated extractions with petroleum ether. (Total volume used was 500 ml.) These extracts were combined, washed with H_2O and dried over anhydrous Na_2SO_4. The organic solvent was removed by distillation and the purified acids dried in vacuum over H_2SO_4. The results obtained from combustion, plating, and counting are shown below.

Fatty Acid	Initial Sp. Ac.	Final Sp. Ac.	Difference Sp. $Ac._f$ - Sp. $Ac._i$	% Difference Dif. ÷ Sp. $Ac._f$
A	15.3	16.4	+1.1	+7
B	85.0	79.8	-7.2	-8.4

Other samples of these fatty acids were dissolved in alcohol, 10% acetic acid added, and an alcoholic solution of digitonin added. Although no visible precipitate was discernible after standing twenty-four hours at room temperature, the solution was filtered through very fine filter paper discs. When these were dry they were counted for one hour under a G.M. tube and also for an hour in a gas flow instrument (which has a counting efficiency some four times that of the G.M. apparatus). In neither instance could radioactivity be demonstrated. Therefore, it is believed that the fractionation techniques used in this thesis result in separation of the pure fatty acids which are uncontaminated with cholesterol. Van

Bruggen and associates[73] obtained similar results in their labeled biosynthesized cholesterol feeding experiments.

The possibility that in the isolation of fatty acids and cholesterol acetic acid would be carried as a contaminant was considered. Such acetic acid contamination appeared unlikely, but experiments were designed to furnish specific information on this matter. Three male mice were killed by sharp cranial blows and placed in an alcoholic KOH digestion mixture, and 1/2 ml. of 1.07×10^6 c.p.m./ml. C^{14} carboxyl-labeled acetate was added. (This amount is based upon one ml. of acetate per 100 grams of tissue.) The usual refluxing, isolation, and evaluation techniques were followed. When duplicate $BaCO_3$ samples of the cholesterol and fatty acids were prepared and counted, no significant activity over background was found. Therefore, it does not appear that any of the radioactive sodium acetate had been isolated with either the saponifiable or non-saponifiable biological material.

H. Counting of C^{14} and Calculation of Results

In the relatively new field of isotopic tracer work, there is at present little uniformity in the manner in which data is presented. To aid in understanding the data presented and to establish clearly the methods used in obtaining the conclusions reported, the following definitions and calculations of activities were developed. With these calculations it is possible to interrelate experimental data from numbers of experiments.

All samples were combusted and plated in duplicate and counting methods chosen to minimize errors due to low counts, as well as those due to coincidence losses of very active samples.

To count $BaCO_3$ at infinite thickness, it is necessary to have 20 mg. of carbonate per square centimeter, since at this weight or above only the top 20 mg./cm². layer contributes to the activity observed because of self absorption phenomena. The activity of this layer is calculated as shown in I below.

I. Activity of top layer = total c.p.m./plate - background

II. C.p.m./mg. $BaCO_3$ = $\frac{I}{20 \text{ mg./cm}^2. \times \text{area (cm}^2)}$

Since the area of samples prepared = 5 cm², then

$$\frac{I}{20 \text{ mg./cm}^2. \times 5 \text{ (cm}^2)} = \frac{I}{100} \text{ c.p.m./mg. } BaCO_3$$

III. Total plate counts = II x weight $BaCO_3$ (mg.) = c.p.m./plate

IV. Specific Activity of substances studied =

$$\frac{III}{\text{mg. substance contributing to plate}} = \text{c.p.m./mg. substance}$$

V. C.p.m. of radioactivity incorporated in substance

= IV x mg. substance isolated

= c.p.m./total substance

VI. Per cent incorporation into substance

$$= \frac{V \times 100}{\text{c.p.m. injected}} = \text{per cent}$$

As has been previously stated, the activity of a sample measured in the laboratory is reported in terms of counts per unit time as $BaCO_3$ at infinite thickness. Since the counts obtained represent only a fraction of actual disintegrations, and since counting rates of any one sample may vary from day to day, it is necessary to have some absolute method for comparison.

This is particularly true for the evaluation of the degree of incorporation of a tracer into biological material. As reported in VI above, this per cent incorporation represents a ratio between recovered and administered activity. Within practical limits, then, the absolute nature of activities is less important than the proper evaluation of both members of the ratio. It is therefore only necessary to know the apparent activity of the compound isolated as compared to that calculated for the administered metabolite at that time. With this in mind, the ratio of c.p.m. from a C^{14} "tagged" polystyrene standard and from barium carbonate samples prepared from one ml. of the carboxyl-labeled acetate was determined. To find the counts which could be expected at any time from a given amount of labeled acetate (with C^{14}), it is only necessary to multiply the c.p.m. of the polystyrene standard with this predetermined ratio. Therefore, it is necessary to administer only enough of the radioisotopic material for statistical counting data after it has undergone biological dilution in the experiments.

A sample calculation of the activity to be expected from the isotopic sodium acetate given is:

Counts recorded from counting the standard . . . .541.7 c.p.m.

Ratio of standard counts to those of $BaCO_3$ samples prepared from one ml. aliquots of carboxyl labeled acetate . . . . 1897/ml.

Milliliters of radioactive acetate injected . . 3.75 ml.

Therefore the total number of counts injected can be calculated as follows:

541.7 c.p.m. x 1897/ml. x 3.75 ml. =

3.82×10^6 c.p.m.

Obviously the tables presented can not contain all of the arithmetical computations necessary to obtain the per cent of C^{14} incorporation into the various lipid and respiratory carbon dioxide. Since only the essential data are given, it is appropriate at this time to present a sample calculation in its entirety. The following information was obtained from rat No. 17, which had been injected intraperitoneally one hour post absorptive with labeled acetate and the tissues treated as previously discussed. Duplicate $BaCO_3$ plates were prepared.

Weight of rat	185 grams
Milliliters of acetate injected (1 ml./100 gm. of tissue	1.85 ml.
Total counts of C^{14} as $BaCO_3$ at infinite thickness, injected: 1.85 ml. x 1.07×10^6 c.p.m.	2.0×10^6 c.p.m.
Weight of fatty acids isolated	4.10 grams
Milligrams of fatty acid combusted (1)	10.3 mg.
Milligrams of fatty acid combusted (2)	10.5 mg.
Net mg. of $BaCO_3$ from CO_2 precipitation (1)	175.4 mg.
Net mg. of $BaCO_3$ from CO_2 precipitation (2)	169.1 mg.
Activity recorded from top 100 mg. of carbonate (less background) (1)	140.1 c.p.m.
Activity recorded from top 100 mg. of carbonate (less background) (2)	140.7 c.p.m.

C.p.m./mg. of $BaCO_3$ equation II

(1) 140.1/100	1.40 c.p.m.
(2) 140.7/100	1.41 c.p.m.

Total plate counts, equation III

(1) 1.40 c.p.m. x 175.4 mg.	245.6 c.p.m.
(2) 1.41 c.p.m. x 169.1 mg.	238.4 c.p.m.

Specific activity of fatty acids, equation IV

(1) 245.6 c.p.m. / 10.3 mg.	23.8 c.p.m./mg.
(2) 238.4 c.p.m. / 10.5 mg.	22.7 c.p.m./mg.
average specific activity of two samples	23.3 c.p.m./mg.

Total amount of C^{14} incorporated into fatty acids, equation V

23.3 c.p.m./mg. x 4,100 mg.	9.5×10^4 c.p.m.

Per cent of injected C^{14} recovered in fatty acids, equation VI

$$\frac{9.5 \times 10^4 \text{ c.p.m.}}{2.0 \times 10^6 \text{ c.p.m.}} \times 100 \qquad 4.8\ \%$$

Similar calculation were carried out upon all the lipid fractions and on the respiratory CO_2 obtained from the Fasting and Time of Maximum Utilization experiments.

CHAPTER III

EXPERIMENTAL RESULTS AND INTERPRETATIONS

A. Fasting Experiments

The literature contains a few reports on the effects of fasting upon lipogenesis. In several of these studies[74,75] isolated tissue slices from starved animals were used, and the labeled tracer was added to the substrate media. In others[76,77], using intact animals, the label (D_2O) was continuously administered in the drinking water over a prolonged period of time. Both of these approaches are limited in the type of information obtained. In the case of the isolated tissue deprived of its normal circulation and thus of its "exogenous" energy supply, the information gained is difficult of interpretation. Lipogenic processes operative in the intact animal constantly supplied with a "tagged" compound may be continuously variable during the prolonged fast, although only an average of these effects is seen at the termination of the experiment.

In the experiments described below, an attempt has been made to define more precisely the physiological state, after fasting, by the injection of a sufficient amount of radioactive material which would allow adequate labeling of fatty acids and cholesterol fractions in the short period of one hour. It was believed that the concurrent collection of CO_2 would permit an evaluation of metabolic oxidations during this time.

A total of sixteen young Sprague-Dawley male rats seven weeks old was used in these experiments. For five to seven days before fasting was begun, these rodents had been hand-fed twice a day an amount of standard diet that was completely eaten in a short time, and which caused a normal gain as

shown by daily weight records. This controlled feeding permitted the choice of similar animals and allowed close control over any fasting period imposed. The close supervision also made it possible to eliminate any animals with obvious organic disturbances, diarrhea, etc. At the end of the desired time of starvation the animal was injected intraperitoneally with one ml. of $CH_3C^{14}OONa$ (1×10^6 c.p.m./ml.) per 100 grams of body weight and immediately placed in the metabolism chamber. The respiratory CO_2 was carried by a stream of CO_2-free air from the chamber into an absorber containing standard NaOH. After one hour the rat was killed with $CHCl_3$ and digested _in toto_ in alcoholic KOH. The imposed fasting periods were so arranged that two animals could be injected and digested on the same day, with subsequent tissue fractionation commencing on the next day. Four rats were used after one hour and four after twenty-four hours of fasting. The other four pairs were used at increasing intervals of twenty-four hours, _i.e._, after 48, 72, 96, and 120 hours of fasting. During the starvation period there was free access to water at all times.

Table 10 lists the pertinent data regarding the body weight and the body fatty acid losses of the fasting period. It can be observed that body fat decreases more rapidly than does body weight during fasting. This is in agreement with the results reported by Bible[78], who studied the changes in body weight and fat of fasted rats. It is interesting to note that these results are similar to those found for humans on semi-starvation diets, in which a 25% decrease in body weight was accompanied by a 70% loss of body fat over a period of 24 weeks[79].

TABLE 10

HISTORY OF ANIMAL PRIOR TO $CH_3C^{14}OONa$ INJECTION

Animal number	Hours of Fast	Initial Body Weight	Body Weight Loss	Percent Loss	Grams of Fatty Acid Isolated	% Loss* Average
5	1	200	0	0	7.5	
6		200	0	0	8.6	
17		185	0	0	4.1	
18		210	0	0	8.9	
average		198.8	0	0	7.2	0
7	24	197	0	0	8.2	
8		203	0	0	6.2	
19		198	6.0	3.0	4.1	
20		196	12.0	6.1	3.1	
average		198.5	4.2	2.2	5.4	25
9	48	190	37.0	19.5	4.3	
10		192	32.0	16.6	3.6	
average		191.0	34.5	18.1	4.0	44
11	72	185	42.0	22.7	1.6	
12		198	53.0	26.8	1.8	
average		191.5	47.5	24.7	1.7	76
13	96	192	47.0	24.5	1.2	
14		187	39.0	20.8	1.4	
average		189.5	43.0	22.7	1.3	82
15	120	191	36.0	18.8	3.0	
16		189	44.0	23.1	1.0	
average		190.0	40.0	21.0	2.0	72

* Assumption that one hour animals represent normal average amount of fat.

The influence of the nutritional state of an animal upon its subsequent ability to form fatty acids is shown in Table 11. The data indicate that the greatest decrease of total fatty acids occurred after 72 to 96 hours of fasting, whereas the greatest decrease in the rate of C^{14} incorporation into fatty acids was found at 24 hours. That is, only 2.32% of the injected acetate dose was recovered in the isolated lipids, or only 54% of that found for the one hour rats. An additional fasting period of 96 hours resulted in an additional decrease of 0.97%. At 120 hours there was 1.35% incorporation of C^{14} into the body fats, this being 31% that of the one hour animals. That this decrease is not due primarily to a slowing of the lipogenic rate but to a decrease of total animal fatty acids isolated is indicated in Table 11 by the specific activities of these lipids. It is also surprising to note that, even though the total body fat at the end of 120 hours was reduced to 70% of the normal values (Table 10), considerable lipogenic ability is still evident from the 1.35% incorporation of the C^{14}.

These results are at variance with those of Masoro[80] and associates, who used liver slices from rats starved 24 hours, and found that the ability of these tissues to incorporate C^{14} of labeled glucose into fat had decreased to 1/10 that observed for the normal rat. This apparent discrepancy may be partially explained by the different experimental techniques used (in-vitro and in-vivo) and by the possibility that glucose yields upon catabolism a type of two carbon fragment[81], which differs from that formed by acetic acid or sodium acetate.

As mentioned previously, these experiments were designed to study the effects of fasting upon cholesterol synthesis as well as the synthesis of fatty acids. As can be observed from Table 12, the total amount of body

TABLE 11

C^{14} INCORPORATION INTO FATTY ACID

(Fasting Experiments)

Animal Number	Hours Fast	Grams iso-lated	Sp. Ac. c.p.m./mg.	Total Activity * c.p.m. x 10^4	% C^{14} Inc.	% of 1 hr. value (avg)
5	1	7.5	12.0	9.0	4.2	
6		8.6	8.2	7.0	3.3	
17		4.1	23.3	9.5	4.8	
18		8.9	12.4	11.1	4.9	
average		7.2	13.9	9.15	4.30	100
7	24	8.2	5.6	4.6	2.1	
8		6.2	6.3	3.9	1.8	
19		4.1	16.0	6.5	3.1	
20		3.1	15.0	4.8	2.3	
average		5.4	10.7	4.95	2.32	54
9	48	4.3	7.4	3.2	2.0	
10		3.6	12.0	4.2	2.5	
average		4.0	9.7	3.70	2.25	52
11	72	1.6	15.0	2.4	1.6	
12		1.8	17.0	3.0	1.9	
average		1.7	16.0	2.70	1.75	41
13	96	1.2	17.3	2.1	1.4	
14		1.4	22.4	3.2	2.0	
average		1.3	19.8	2.65	1.70	40
15	120	3.0	9.2	2.8	1.6	
16		1.0	17.3	1.9	1.1	
average		2.0	13.5	2.35	1.35	31

* Basis of 1 x 10^6 c.p.m./ml. of $CH_3C^{14}OONa$ per 100 grams rat tissue

TABLE 12

C^{14} INCORPORATION INTO CHOLESTEROL

(Fasting Experiments)

Animal Number	Hours Fast	Mg. isolated	Sp. Ac. c.p.m./mg.	Total Activity* c.p.m. x 10^{-4}	% C^{14} Inc.	% of 1 hr. value (avg)
5	1	411	51.8	2.17	1.01	
6		414	74.5	3.08	1.40	
17		445	26.0	1.20	0.59	
18		415	40.3	1.70	0.74	
average		421	48.2	2.04	0.94	100
7	24	437	29.0	1.20	0.56	
8		450	27.0	3.50	1.62	
19		449	24.6	1.10	0.52	
20		462	17.0	0.80	0.38	
average		450	24.4	1.65	0.77	84
9	48	420	20.0	0.84	0.51	
10		475	22.0	1.03	0.60	
average		448	21.0	0.94	0.56	59
11	72	480	7.6	3.70	0.24	
12		420	12.4	5.20	0.33	
average		450	10.0	4.45	0.29	30
13	96	495	13.4	0.50	0.32	
14		452	14.2	0.51	0.32	
average		473	13.8	0.51	0.32	34
15	120	473	18.3	0.73	0.41	
16		509	16.9	0.62	0.37	
average		491	17.6	0.68	0.39	41

* Basis of 1 x 10^{6} c.p.m./ml. of $CH_3C^{14}OONa$ per 100 grams rat tissue

cholesterol remained relatively constant, even showing a slight increase as the fast progressed. This elevation might be due, in part, to an increase in endogenous acetate being made available for synthesis by the increased catabolism of fatty acids (from concept of beta-oxidation of fat giving rise to acetyl-like fragments). Such increased catabolism of fat becomes necessary since the fasting animals' carbohydrate reserves are quickly depleted, and therefore a greater proportion of its energy requirements must come from oxidation of body fat. However, this concept is difficult to reconcile in view of Bloch's work[62], in which he showed that dietary acetate was incapable of quantitatively modifying foreign amine acetylation reactions. Also, if an increased acetate "pool" did stimulate cholesterol anabolism, a similar effect might be expected for fatty acid synthesis. Examination of the cholesterol and fatty acid specific activities shows a striking difference: the specific activities of fatty acid remained relatively constant, while those of the cholesterol decreased as the fast progressed. A possible explanation of this difference is that both cholesterol formation and breakdown were decreased with total body cholesterol remaining constant, while anabolism of the fatty acids was unchanged, but increased catabolism occurred.

The decrease observed in the total amount of C^{14} incorporation into cholesterol after 72 hours (being less than one-half that of the one hour rats) is due to appreciable decrease in specific activities rather than to a lessening in the amount isolated, as was true for fatty acids.

The CO_2 data of Table 13 indicate that the total amount of CO_2 expired decreased as the fast progressed, with the exception of the 24 hour animals. This drop would be expected from the generally lowered metabolic reactions

TABLE 13

C^{14} INCORPORATION INTO RESPIRATORY CO_2

(Fasting Experiments)

Animal Number	Hours Fast	mM. Collected	Sp. Ac. c.p.m./mM. x 10^{-5}	Total Activity‡ c.p.m. x 10^{-5}	% C^{14} Inc.	% of 1 hour value (avg.)
5	1	9.2	1.3	12.0	55.8	
6		12.3	0.7	8.9	41.5	
17		8.1	1.1	8.5	43.1	
18		13.5	0.9	11.5	51.1	
average		10.7	0.9	10.2	47.9	100
7	24	9.9	1.5	14.2	66.3	
8		7.2	1.6	11.3	52.6	
19		9.8	1.1	11.2	52.8	
20		8.5	1.2	10.1	47.8	
average		8.8	1.4	11.7	54.9	115
9	48	8.5	0.6	5.2	31.7	
10		7.2	1.1	8.3	48.4	
average		7.9	0.86	6.8	40.1	83
11	72	4.5	1.4	6.2	40.0	
12		8.1	1.1	8.7	56.1	
average		6.3	1.3	8.0	48.0	100
13	96	6.1	1.1	7.1	45.5	
14		5.4	1.2	6.3	40.0	
average		5.8	1.2	6.7	42.8	88
15	120	4.0	1.4	5.6	31.8	
16		5.4	1.0	5.6	33.3	
average		4.7	1.2	5.6	32.6	68

‡ Basis of 1 x 10^6 c.p.m./ml. of $CH_3C^{14}OONa$ per 100 grams rat tissue

associated with starvation[63]. The per cent of radioactive carbon incorporation into the expired CO_2 did not decrease proportionally; however, a marked change can be seen at 120 hours. At that time only 33% of the injected isotope was recovered in the expired air, or 68% of that amount incorporated by the one hour animals. Interpretation of these results is difficult because the animals were not in a basal state during the period of study, and no measures were used to minimize the hormonal effects (epinephrine, etc.) of fear or slight traumatic shock from handling at the time of the injection. The variation of physical activity of the different rats while in the metabolism chamber might have also contributed to the diversified results obtained. It must be kept in mind that two additional factors could also result in a lowering of the CO_2 collected. One is a decrease in the metabolic rate, and the other is a decrease in the respiratory quotient[64] found in an animal burning primarily carbohydrate to that of one using mostly fatty acids for its source of energy.

A summary of the essential portions of tables 10 through 13 is given in Table 14.

B. Time of Maximum Utilization Experiments

In the preceding experiments, animals starved 1 to 120 hours were allowed to metabolize a standard amount of C^{14} carboxyl-labeled acetate for one hour and then were digested. After the biological products were isolated, the per cent of C^{14} incorporated into each was determined.

The experiments described above furnished information in regard to the lipogenic mechanisms of starvation, the time of fat synthesis remaining fixed at one hour.

TABLE 14

NUTRITIONAL EFFECTS UPON ACETATE INCORPORATED

INTO FATTY ACIDS, CHOLESTEROL, AND CARBON DIOXIDE

			% of Injected Dose Incorporated into:			% of One Hour Experiment		
No. of Animals	Hours Fast	Body Wt. Loss grams	Fatty Acids	Choles-terol	CO_2	Fatty Acids	Choles-terol	CO_2
4	1	0	4.30	0.94	47.9	100	100	100
4	24	6 - 12	2.32	0.77	54.9	54	84	115
2	48	32 - 37	2.25	0.56	40.1	52	59	83
2	72	42 - 53	1.75	0.29	48.0	41	30	100
2	96	39 - 47	1.70	0.32	42.8	40	34	88
2	120	36 - 44	1.35	0.39	32.6	31	41	68

The first animal work done in the Radioisotope Laboratory involved the use of mice injected with labeled acetate in which the time of sacrifice varied from two to eight hours. From this work, much to our surprise, it was found that apparently maximal acetate utilization occurred in an hour or two. This early work, together with information gained in the experiments reported above, prompted us to investigate the dynamics of the early phases of lipogenesis in the non-fasted animal. Since apparently a two-hour period covers the most active phase of acetate metabolism, animals were sacrificed at 0, 7, 15, 30, 60, 90, and 120 minutes, after receiving a standard tracer dose of labeled acetate. The per cent of C^{14} incorporation into fatty acids and cholesterol of brain and spinal cord, liver, skin, carcass and gut were studied. The lipid components were isolated from the tissues by the previously described techniques. Respiratory CO_2 was collected during the indicated times for all animals except those of the zero and seven minute periods. In the latter experiment the rats were sacrificed immediately after injection of the labeled acetate, while in the zero time experiment the animals were chloroformed just prior to injection; hence in neither instance was there an opportunity to collect any expired carbon dioxide.

To assure uniformity of nutritional state, young male rats of the Sprague-Dawley strain were brought to the laboratory approximately one week prior to use and fed an amount of food which resulted in a daily weight gain. Here, again, only animals that appeared healthy were used for experimental purposes. Each rat was injected intraperitoneally with one ml. of labeled acetate per 100 grams of body weight and placed in the metabolism chamber, (A, in Figure 6) with the exception of the zero time animal, in

which the rats were chloroformed, injected with acetate, and immediately decapitated. The other rats were sacrificed at the termination of the metabolism period. Tissues were rapidly excised and placed for digestion in separate flasks of alcoholic KOH, with the corresponding tissues from two animals being pooled in each flask. The biological components were fractionated as previously described and the incorporation of C^{14} calculated as shown in Chapter II. Results of individual experiments are given in Tables 15 through 21.

Since even at 15 minutes the rats had shown a remarkable incorporation (see Table 17) of C^{14} into fatty acids and cholesterol, it was felt desirable to find a period of minimal incorporation. Two animals were injected with radio-acetate and placed together in the metabolism apparatus. Instead of passing a stream of air through the chamber, the rats were immediately killed with chloroform (respiration had ceased in two minutes). The rats were then decapitated and the different tissues excised. The liver, which is known to have a rapid turnover of lipids, was removed first, weighed, and put into alcoholic KOH. The total time required from injection to hepatectomy was five to seven minutes, hence the designation "seven minute determination".

From table 22 (a summary of the fatty acid data from Tables 15 through 21) it can be observed that C^{14} was incorporated into the fatty acids of all fractions studied. The variability of specific activities and percentage of C^{14} incorporations observed makes one hesitant to present any definite conclusions. However, from inspection of Table 22, it may be seen that the carcass gave greatest incorporation, with gut, liver, and skin following in

TABLE 15

TIME OF MAXIMUM UTILIZATION - 0 MINUTES

All values are the sum of two animals except that of per cent incorporation which is an average of two rats.

Specific Activity is reported at counts per minute per milligram.

Rat No.	Wt. grams	Dose
97	233	4.5 ml. (1.04 x 10^6 c.p.m./ml. of $CH_3C^{14}OONa$)
98	197	

	Tissue	Isolated Weight Grams	Specific Activity	Total Activity (x 10^{-3})	Per Cent Incorporation
FATTY ACID	Brain & Spinal Cord	0.1058	0.3	0.0	0.00
	Liver	0.4594	1.6	0.7	0.02
	Skin	4.0958	0.4	1.4	0.03
	Carcass	14.0411	2.0	28.0	0.63
	Gut	1.1785	9.2	11.0	0.24
				(x 10^{-4})	
CHOLESTEROL	Brain & Spinal Cord	0.0548	0.00	0.0	0.00
	Liver	0.0321	0.18	0.2	0.00
	Skin	0.2680	0.04	1.1	0.00
	Carcass	0.1050	0.10	0.4	0.00
	Gut	0.0756	0.02	0.0	0.00

TABLE 16

TIME OF MAXIMUM UTILIZATION - 7 MINUTES

All values are the sum of two animals except that of per cent incorporation which is an average of two rats.

Specific Activity is reported at counts per minute per milligram.

Rat No.	Wt. grams	Dose
35	185	4.0 (1.04 x 10^6 c.p.m./ml. of $CH_3C^{14}OONa$)
36	190	

	Tissue	Isolated Weight Grams	Specific Activity	Total Activity (x 10^{-3})	Per Cent Incorporation
FATTY ACID	Brain & Spinal Cord	0.0862	1.0	0.09	$>10^{-2}$
	Liver	0.3725	12.1	0.66	0.20
	Skin	5.0986	2.2	11.00	0.29
	Carcass	7.7453	15.3	116.00	3.00
	Gut	0.8233	85.0	70.00	1.83
				(x 10^{-3})	
CHOLESTEROL	Brain & Spinal Cord	0.0483	0.4	0.02	0.00
	Liver	0.0543	77.5	4.21	0.11
	Skin	0.2405	0.8	0.20	$>10^{-2}$
	Carcass	0.2260	2.7	0.60	0.02
	Gut	0.0787	48.8	3.64	0.09

TABLE 17

TIME OF MAXIMUM UTILIZATION - 15 MINUTES

All values are the sum of two animals except that of per cent incorporation which is an average of two rats.

Specific Activity is reported at counts per minute per milligram.

Rat No.	Wt. grams	Dose
33	205	4.12 ml. (1.04×10^6 c.p.m./ml. of $CH_3C^{14}OONa$)
34	207	

	Tissue	Isolated Weight Grams	Specific Activity	Total Activity ($\times 10^{-3}$)	Per Cent Incorporation
FATTY ACID	Brain & Spinal Cord	0.1786	3.7	0.66	0.017
	Liver	0.3674	126.5	46.5	1.08
	Skin	5.5289	3.1	17.1	0.40
	Carcass	9.7307	8.1	78.8	1.85
	Gut	1.9887	36.4	72.4	1.70
				($\times 10^{-3}$)	
CHOLESTEROL	Brain & Spinal Cord	0.0232	5.9	0.137	0.003
	Liver	0.0232	597.3	13.90	0.32
	Skin	0.2340	13.2	3.09	0.07
	Carcass	0.3000	19.6	5.88	0.14
	Gut	0.0700	85.7	6.00	0.14
CO_2	Collection Period (Minutes)	(ml.)	($\times 10^{-4}$)	($\times 10^{-4}$)	
	0 - 5	2.13	2.32	4.94	1.15
	5 - 10	1.69	7.98	13.5	3.15
	10 - 15	2.97	11.2	33.3	7.78
	Total	6.79	21.5	51.74	12.08

TABLE 18

TIME OF MAXIMUM UTILIZATION - 30 MINUTES

All values are the sum of two animals except that of per cent incorporation which is an average of two rats.

Specific Activity is reported at counts per minute per milligram.

Rat No.	Wt. grams	Dose
25	203	2.1 ml. (1.04 x 10^6 c.p.m./ml. of $CH_3C^{14}OONa$)
26	203	

	Tissue	Isolated Weight Grams	Specific Activity	Total Activity (x 10^{-4})	Per Cent Incorporation
FATTY ACID	Brain & Spinal Cord	0.2287	1.5	0.03	0.008
	Liver	0.5388	111.3	6.0	1.44
	Skin	2.8836	7.6	2.2	0.53
	Carcass	6.1147	13.0	7.9	1.90
	Gut	1.6583	47.1	7.8	1.88
				(x 10^{-3})	
CHOLESTEROL	Brain & Spinal Cord	0.0603	0.2	0.009	0.002
	Liver	0.0325	312.0	10.0	0.24
	Skin	0.2768	4.2	1.2	0.03
	Carcass	0.2736	11.3	3.1	0.07
	Gut	0.1050	83.9	0.95	0.02
	Collection Period (Minutes)	(mM.)	(x 10^{-4})	(x 10^{-5})	
CO_2	0 - 15	6.8	4.1	2.8	6.7
	15 - 30	5.8	5.4	3.1	7.5
	Total	12.6	9.5	5.9	14.2

TABLE 19

TIME OF MAXIMUM UTILIZATION - 60 MINUTES

All values are the sum of two animals except that of per cent incorporation which is an average of two rats.

Specific Activity is reported at counts per minute per milligram.

Rat No.	Wt. grams	Dose
27	197	3.9 ml. (1.04×10^6 c.p.m./ml. of $CH_3C^{14}OONa$)
28	195	

		Isolated Weight Grams	Specific Activity	Total Activity ($\times 10^{-4}$)	Per Cent Incorporation
FATTY ACID	Brain & Spinal Cord	0.1957	1.8	0.03	0.008
	Liver	0.4385	81.1	3.55	0.92
	Skin	2.4602	4.95	1.22	0.32
	Carcass	5.5050	10.9	6.00	1.55
	Gut	1.0172	3.7	0.38	0.10
				($\times 10^{-3}$)	
CHOLESTEROL	Brain & Spinal Cord	0.0983	0.48	0.047	0.001
	Liver	0.0195	217.0	4.2	0.11
	Skin	0.2290	7.7	1.7	0.04
	Carcass	0.2811	11.8	3.3	0.08
	Gut	0.1062	76.9	8.2	0.21
	Collection Period (Minutes)	(mM.)	($\times 10^{-4}$)	($\times 10^{-5}$)	
CO_2	0 - 15	3.2	5.8	1.86	4.73
	15 - 30	4.5	8.5	3.79	9.64
	30 - 45	4.8	8.8	4.23	10.80
	45 - 60	3.5	7.7	2.67	6.80
	Total	16.0	30.8	12.55	31.97

TABLE 20

TIME OF MAXIMUM UTILIZATION - 90 MINUTES

All values are the sum of two animals except that of per cent incorporation which is an average of two rats.

Specific Activity is reported at counts per minute per milligram.

Rat No.	Wt. grams	Dose
29	195	3.85 ml. (1.04×10^6 c.p.m./ml. of $CH_3C^{14}OONa$)
30	175	

	Tissue	Isolated Weight Grams	Specific Activity	Total Activity ($\times 10^{-4}$)	Per Cent Incorporation
FATTY ACID	Brain & Spinal Cord	0.1234	3.05	0.038	0.01
	Liver	0.4072	112.8	4.59	1.19
	Skin	3.6700	13.3	4.88	1.26
	Carcass	8.0284	22.7	18.2	4.72
	Gut	0.9715	105.5	10.2	2.69
CHOLESTEROL				($\times 10^{-4}$)	
	Brain & Spinal Cord	0.0812	2.1	0.017	0.005
	Liver	0.0362	431.0	1.56	0.42
	Skin	0.2330	14.8	0.03	0.09
	Carcass	0.3590	31.2	1.12	0.30
	Gut	0.1100	112.0	1.23	0.33
	Collection Period (Minutes)	(mM.)	($\times 10^{-4}$)	($\times 10^{-5}$)	
CO_2	0 - 15	2.7	9.1	2.5	6.4
	15 - 30	8.3	11.6	9.7	25.0
	30 - 45	6.9	9.3	5.7	15.0
	45 - 60	7.2	4.8	3.4	8.8
	60 - 75	6.5	3.0	2.0	5.1
	75 - 90	6.4	2.2	1.4	3.6
	Total	38.0	40.0	24.7	63.9

TABLE 21

TIME OF MAXIMUM UTILIZATION - 120 MINUTES

All values are the sum of two animals except that of per cent incorporation which is an average of two rats.

Specific Activity is reported at counts per minute per milligram.

Rat No.	Wt. Grams	Dose
31	230	
32	240	4.8 ml. (1.04×10^6 c.p.m./ml. of $CH_3C^{14}OONa$)

	Tissue	Isolated Weight Grams	Specific Activity	Total Activity ($\times 10^{-3}$)	Per Cent Incorporation
FATTY ACID	Brain & Spinal Cord	0.1572	4.10	0.64	0.013
	Liver	0.3612	218.5	78.9	1.61
	Skin	6.0764	8.0	48.4	0.99
	Carcass	9.9356	9.2	91.2	1.89
	Gut	1.8679	65.2	122.2	2.53
				($\times 10^{-3}$)	
CHOLESTEROL	Brain & Spinal Cord	0.0451	3.9	0.18	0.004
	Liver	0.0255	704.0	17.9	0.37
	Skin	0.2500	22.1	5.5	0.11
	Carcass	0.3610	44.9	16.2	0.34
	Gut	0.1325	174.7	23.1	0.48
	Collection Period (Minutes)	(mM.)	($\times 10^{-4}$)	($\times 10^{-5}$)	
CO_2	0 - 15	6.5	4.4	2.8	5.7
	15 - 30	8.1	6.0	4.8	9.7
	30 - 45	6.1	5.9	3.6	7.3
	45 - 60	8.0	4.9	3.9	8.0
	60 - 75	5.4	3.8	2.1	4.3
	75 - 90	6.8	3.2	2.2	4.4
	90 - 105	5.8	2.6	1.5	3.1
	105 - 120	6.8	2.1	1.5	3.0
	Total	53.5	32.9	22.4	45.5

TABLE 22

INCORPORATION OF C^{14} OF $CH_3C^{14}OONa$ INTO FATTY ACIDS

All values are the sum of two animals except that of per cent incorporation which is an average of two rats.

Specific Activity is reported as counts per minute per milligram of fatty acids.

Sacrifice Time (Min.)		Brain and Spinal Cord	Liver	Skin	Carcass	Gut
0	Wt. grams	0.1058	0.4594	4.0958	14.0411	1.1785
	Sp. Ac.	0.00	1.6	0.35	2.0	9.2
	% Inc.	0.00	0.02	0.03	0.63	0.24
7	Wt. grams	0.0862	0.3725	5.0986	7.7453	0.8233
	Sp. Ac.	1.0	12.1	2.15	15.3	85.0
	% Inc.	0.002	0.017	0.29	3.0	1.83
15	Wt. grams	0.1786	0.3674	5.5289	9.7307	1.9887
	Sp. Ac.	3.7	126.5	3.1	8.1	36.4
	% Inc.	0.017	1.08	0.40	1.85	1.70
30	Wt. grams	0.2287	0.5398	2.8836	6.1147	1.6583
	Sp. Ac.	1.5	111.3	7.6	13.0	47.1
	% Inc.	0.008	1.44	0.53	1.90	1.88
60	Wt. grams	0.1957	0.4365	2.4602	5.5050	1.0172
	Sp. Ac.	1.8	81.1	4.95	10.9	37.0
	% Inc.	0.008	0.92	0.32	1.55	0.97
90	Wt. grams	0.1234	0.4072	3.6700	8.0284	0.9715
	Sp. Ac.	3.05	112.8	13.3	22.7	105.5
	% Inc.	0.01	1.19	1.26	4.72	2.69
120	Wt. grams	0.1572	0.3612	6.0764	9.9356	1.8679
	Sp. Ac.	4.10	218.5	7.97	9.18	65.2
	% Inc.	0.013	1.61	0.99	1.89	2.53

decreasing order. However, the specific activity figures show the liver to be the organ with the greatest lipogenic activity, followed by gut, carcass, and skin in that sequence. At 90 minutes all tissues except liver had reached their maximum incorporation.

In order to be certain that the very high values found in the carcass and gut fractions, at seven minutes, were due to true fatty acid activity and not to contaminating sterols, the acids were subjected to extensive purification. Only slight changes in values were noted. (See page 51 of this thesis, where lipid fractions A and B are respectively the carcass and gut fatty acids described above.)

It is apparent that, even at 15 minutes, considerable fatty acid anabolism occurred in all tissues but was most pronounced in the liver fraction. The latter observation might lead one to feel that fatty acids were synthesized in the liver and transported to the other organs. Although this transport of hepatic lipids may be a contributing factor to the values found in the extra-hepatic tissues, it seems improbable that all incorporation of the radioactive carbon occurred in the liver. This is borne out by the experiments of Masoro and associates[85], who demonstrated appreciable synthesis of fatty acids and cholesterol by tissues of intact hepatectomized rats. Further supporting evidence of extra-hepatic lipid synthesis is seen from Table 22, for in the zero and seven minute experiments the specific activity of the liver was less than found for other tissues.

That the per cent of C^{14} incorporated into carcass lipids was greatest is not surprising when the total tissue fatty acids are compared, since the weight of the carcass fatty acids represented from two to fourteen times the total amount of acids recovered from all of the other tissues. In

general, when the animals were allowed to metabolize the $CH_3C^{14}OONa$ for a period of 90 minutes, the greatest percentage of incorporation of the isotope was found.

Because considerable activity was present in all of the tissue fractions at seven minutes, an experiment was performed to establish the time of minimum lipid anabolism. In this experiment the rats were chloroformed and injected with "tagged" acetate, then immediately decapitated. The tissues were fractionated and the lipids isolated in the usual manner. The results obtained were unexpected in that appreciable activity was found in liver, carcass, and gut. The data of Table 15 show that the gut fraction had a specific activity of 9.2 c.p.m./mg. of fatty acids with an overall incorporation of C^{14} of 0.24%, and carcass fatty acids had a specific activity of 2.0 c.p.m./mg. with 0.63% incorporation having occurred. It is true that these observations are of a tentative nature since only two animals were used, but one valid conclusion can be drawn. This is that the absorption, transportation, and incorporation of the injected acetate C^{14} into biological materials appears astonishingly rapid. It is desired to repeat this experiment in the future, using a larger number of animals.

The brain and spinal cord data are unique in that a small but definite incorporation of C^{14} into the fatty acids was found (Table 22). Since these values show a slight increase with increasing metabolism periods, it is believed that the per cent incorporation figures have a significant meaning. This slight fatty acid lipogenesis of the central nervous system tissues is in accord with the observations of Sperry and Waelsch[66], who administered D_2O to young and adult rats and determined the amount of D that had been incorporated into the cerebral tissues. They found that young rats showed a

greater rate of lipogenesis than adults, in which the turnover of fatty acids was slow.

Sperry and Waelsch were not able to demonstrate any cholesterol anabolism of the central nervous system of the adult rat. The relative inertness of this sterol was substantiated by Bloch, Berg, and Rittenberg[87], who injected deutero-labeled cholesterol intravenously into dogs and were unable to find any activity in the cholesterol of the brain. From these experiments they concluded, ". . . the relatively large masses of cholesterol in the central nervous system are metabolically inert and here the function of cholesterol does not seem to be associated with any chemical reactions but evidently must reside in some physical or structural properties of the molecule."[88]

In opposition to this, the data in Table 23 (a summary of cholesterol values from Tables 15 through 21) show a small but definite incorporation of C^{14} into brain and spinal cord cholesterol. Critical examination of the specific activity found in the different experiments indicates an increasing incorporation of radio-carbon into the unsaponifiable fraction as the utilization time was increased. This is also reflected by the increase in per cent of incorporation. These differences may be due to different isotope techniques, or to the different biochemistry associated with the metabolism of deutero-labeled compounds. The experiments being reported are short term and involve relatively greater amounts of label than work previously reported.

The C^{14} found in the cholesterol fractions (Table 23) indicates a relatively lower incorporation than observed for the fatty acids. This is

TABLE 23

INCORPORATION OF C^{14} OF $CH_3C^{14}OONa$ INTO CHOLESTEROL

All values are the sum of two animals except that of per cent incorporation into cholesterol, which is an average of two rats.

Specific Activity is reported at counts per minute per milligram of cholesterol.

Sacrifice Time (Min.)		Brain and Spinal Cord	Liver	Skin	Carcass	Gut
0	Wt. grams	0.0548	0.0322	0.2690	0.1050	0.0756
	Sp. Ac.	0.0	0.18	0.0	0.1	0.0
	% Inc.	0.0	0.0	0.0	0.0	0.0
7	Wt. grams	0.0433	0.0543	0.2405	0.2260	0.0787
	Sp. Ac.	0.41	77.5	0.24	2.67	48.8
	% Inc.	0.0005	0.11	0.005	0.015	0.09
15	Wt. grams	0.0232	0.0232	0.2340	0.3000	0.0700
	Sp. Ac.	5.9	397.3	13.2	19.6	85.7
	% Inc.	0.003	0.32	0.07	0.14	0.14
30	Wt. grams	0.0603	0.0325	0.2768	0.2736	0.1050
	Sp. Ac.	0.15	312.0	4.2	11.3	83.9
	% Inc.	0.002	0.24	0.03	0.07	0.21
60	Wt. grams	0.0983	0.0195	0.2250	0.2811	0.1062
	Sp. Ac.	0.48	217.0	7.7	11.8	76.9
	% Inc.	0.001	0.11	0.04	0.08	0.21
90	Wt. grams	0.0812	0.0362	0.2330	0.3590	0.1100
	Sp. Ac.	2.12	431.4	14.5	31.2	111.8
	% Inc.	0.005	0.42	0.09	0.30	0.33
120	Wt. grams	0.0451	0.0255	0.2500	0.3610	0.1325
	Sp. Ac.	3.88	704.0	22.1	44.9	174.7
	% Inc.	0.004	0.37	0.11	0.34	0.48

in accord with the experiments of Rittenberg and Schoenheimer[89], who reported that cholesterol synthesis is a slow process. The results are also in agreement with Bloch's findings[90] that fatty acids need not be intermediates in the conversion of acetate to cholesterol. This can be seen from comparison of the specific activities of the liver fatty acids and cholesterol, as shown below.

Time of Experiment	Specific Activities (c.p.m./mg.) Liver Fatty Acids	Liver Cholesterol
0	1.6	0.18
7	12.6	77.5
15	126.5	597.3
30	111.3	312.0
60	81.3	217.0
90	112.8	431.4
120	218.5	704.0

From the above data it can be seen that the cholesterol specific activities are higher than the specific activities of the fatty acids, indicating that acetic acid carbon atoms are, in all probability, directly built into the sterol molecule rather than going through an intermediary synthesis into fatty acids. In support of this hypothesis is the observation of Little and Bloch[91], who demonstrated that acetic acid is the principal, if not the sole source of carbon atoms for cholesterol.

These experiments indicate that cholesterol synthesis in all tissues had not reached a maximum value in the periods studied, as shown by the continual increase of incorporation (Table 23). This is in agreement with

the findings of Bloch and associates[92], who found that the liver cholesterol does not reach half of its maximum isotope concentration (from $CH_3C^{14}OONa$) until six days, with carcass cholesterol requiring 31 to 32 days.

The increased incorporation of C^{14} into gut cholesterol is a composite of two physiological processes: one an actual formation of labeled sterol by the gut, the other a biliary secretion of "C^{14}-tagged" cholesterol synthesized by the liver. Consequently, the greater C^{14} found in the gut fraction would be expected to rise as the experiment period increased. These overall increases of C^{14} incorporation found in the unsaponifiable fractions as the time periods of the experiments were lengthened is surprising since maximum oxidation of acetate C^{14} to $C^{14}O_2$ occurs between 30 and 45 minutes, as can be seen from Figure 7. These values were found graphically by the method of Feller, Strisower and Chaikoff[93]. This method is based upon the observation of these investigators that the time of maximum value for specific activity of expired CO_2 corresponds with the time at which maximum labeling of the CO_2 in the body pool is found. At that time the specific activity of this pool has attained a value equal to that of the organic precursors of the CO_2. The top curve in Figure 7 is a graph of an average value for these activities as plotted on a semilog scale. It can be observed that a maximum is reached between 30 and 45 minutes.

The numerical values of this graph were obtained from the data of Table 24, which is a summary of the CO_2 findings shown in Tables 15 through 21. From figures shown elsewhere (page 87) the amount of $CH_3C^{14}OONa$ remaining in the rat at each period of CO_2 collection was calculated and is graphically represented as the bottom curve of Figure 7. Extrapolation of the

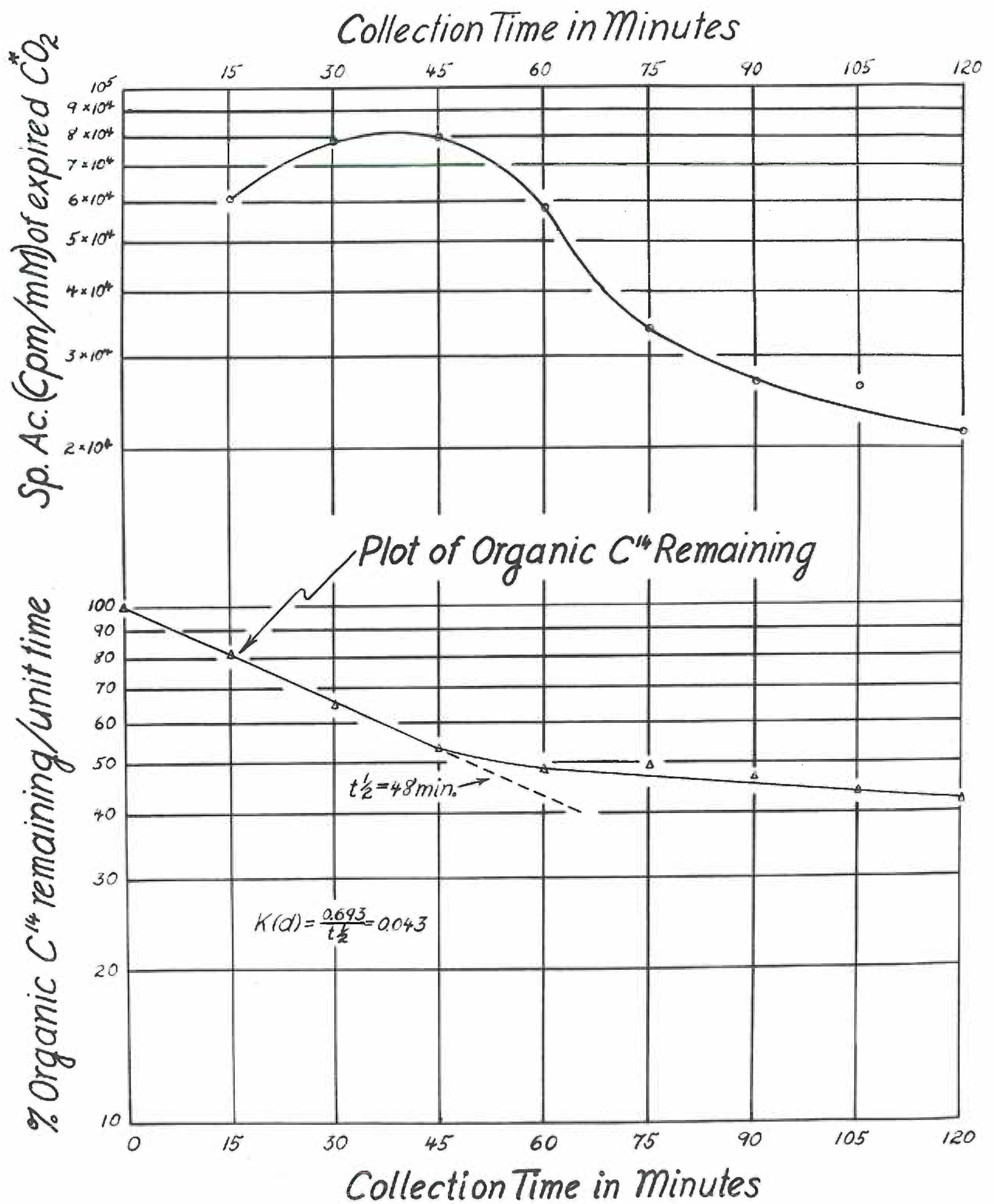


Fig. 7
Collection Time in Minutes
15
30
45
60
75
90
105
120
10^5
9×10^4
8×10^4
7×10^4
6×10^4
5×10^4
4×10^4
3×10^4
2×10^4
Sp. Ac. (Cpm/mM) of expired $\overset{*}{C}O_2$
Plot of Organic C^{14} Remaining
100
90
80
70
60
50
40
30
20
10
$t\frac{1}{2}$=48 min.
$K(d) = \frac{0.693}{t\frac{1}{2}} = 0.043$
% Organic C^{14} remaining/unit time
0
15
30
45
60
75
90
105
120
Collection Time in Minutes

TABLE 24

EXPIRED $C^{14}O_2$ AT TIME OF MAXIMUM UTILIZATION OF ACETATE BY RATS

($CH_3C^{14}OONa$ injected: 1 ml./100 gm. of body weight)

Collection Period (Min.)	Animal Nos.	Sacrifice Time* (Min.)	Millimoles	Sp. Ac. c.p.m./mMole x 10^{-4}	Total Activity x 10^{-3}	% Injected C^{14} Incorp. into $C^{14}O_2$	Accumulative Totals of: mM.	% Inc.
0-15	33-34	15	7.04	7.35	5.2	12.1	7.04	12.1
	25-26	30	6.82	4.1	2.8	6.7	6.82	6.7
	27-28	60	3.20	5.8	1.7	4.7	3.20	4.7
	29-30	90	2.74	9.1	2.5	6.4	2.74	6.4
	31-32	120	6.45	4.4	2.8	5.7	6.45	5.7
	average		5.25	6.1		7.1		7.1
15-30	25-26	30	5.79	5.4	3.1	7.5	12.61	14.2
	27-28	60	4.47	8.5	3.8	9.6	7.67	14.4
	29-30	90	8.31	11.6	9.7	25.9	11.05	31.3
	31-32	120	8.02	6.0	4.8	9.7	14.47	15.4
	average		6.65	7.9		13.0		19.8
30-45	27-28	60	4.82	8.8	4.2	10.8	12.49	25.2
	29-30	90	6.88	9.3	5.7	14.7	17.93	46.0
	31-32	120	6.12	5.9	3.6	7.3	20.59	22.7
	average		5.94	8.0		10.9		31.3
45-60	27-28	60	3.45	7.7	2.7	6.8	15.94	31.9
	29-30	90	7.15	4.8	3.4	8.8	25.08	54.9
	31-32	120	8.02	4.9	3.9	8.0	28.61	30.7
	average		6.21	5.8		7.9		39.2
60-75	29-30	90	6.53	3.0	2.0	5.1	31.61	59.9
	31-32	120	5.38	3.7	2.1	4.3	33.99	39.5
	average		5.96	3.4		4.7		49.7
75-90	29-30	90	6.35	2.2	1.4	3.6	37.96	63.5
	31-32	120	6.81	3.2	2.2	4.4	40.80	39.4
	average		6.58	2.7		4.0		51.5
90-105	31-32	120	5.77	2.6	1.5	3.1	46.57	42.5
105-120	31-32	120	6.79	2.1	1.5	3.0	53.36	45.5

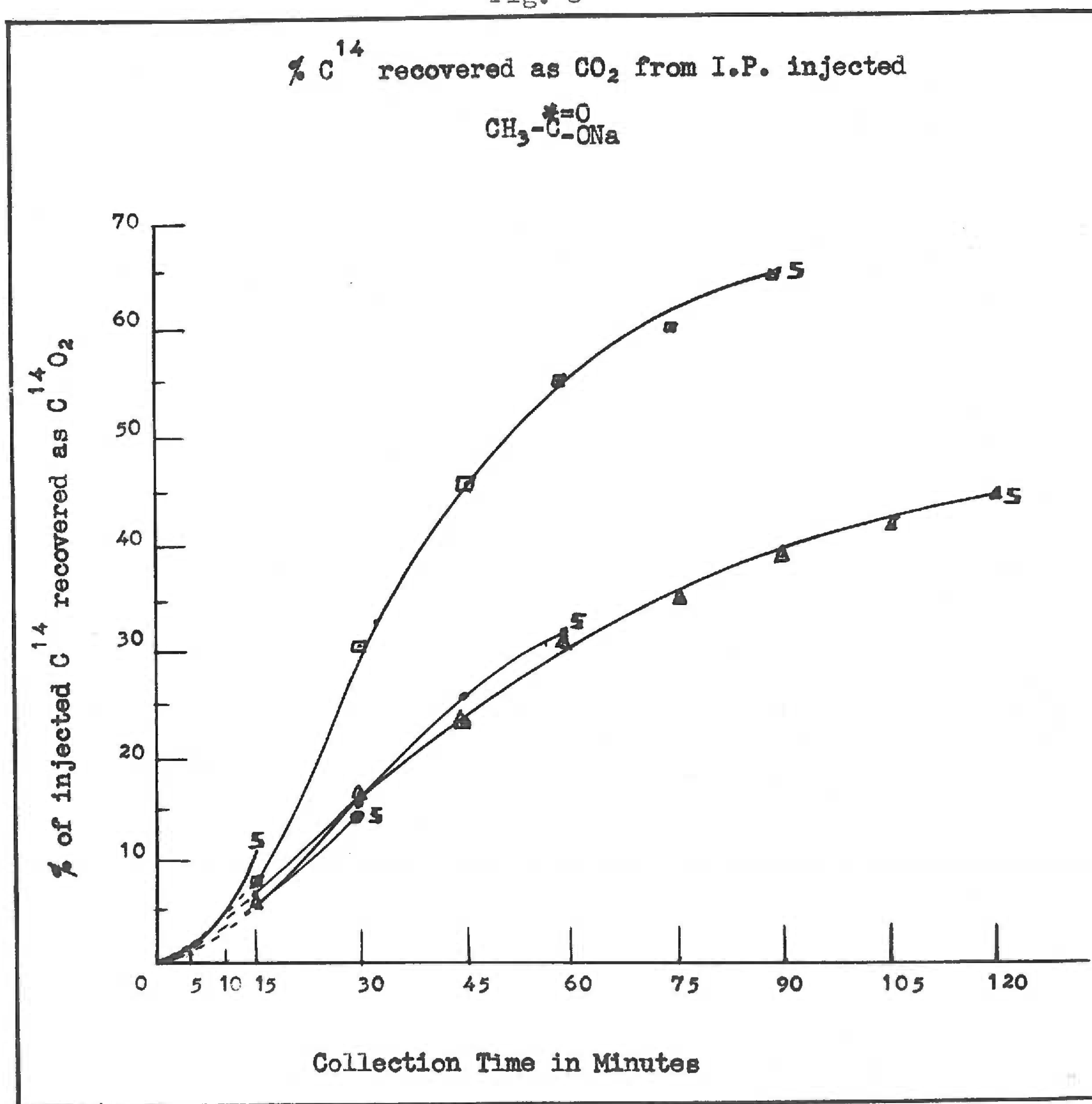
* Length of time between injection and sacrifice

linear portion of this curve gives the biological half life (T 1/2) of labeled acetate as 48 minutes and a K value for the disappearance of acetate of 0.043, i.e., K (decrease) = 0.693/48 = 0.043.

Because the average millimoles of expired CO_2, per unit time, remained relatively constant, as shown by Table 24, the total per cent of C^{14} detected in the respiratory CO_2 parallels the graph of the specific activity of the CO_2. That is, the greatest incorporation was found to be at about 30 minutes. That these figures are only approximate is readily apparent since the periods of CO_2 sampling are really a summation of fifteen minute intervals. If a maximum value is shown graphically as having been attained at 30 minutes, actually a peak may have been reached between sampling times. However, with short intervals, the general shape of the curve should remain approximately the same.

The incorporation of C^{14} into $C^{14}O_2$ is presented graphically in Figure 8. In this figure the termination of each experiment is denoted by the letter "S". Two points of inflections can be observed. The shape of the 0 to 15 minute portion of the curves was determined by dividing the collection periods of the 15 minute experiment into three five minute intervals. The values found for the 5, 10, and 15 minute periods were respectively 1.15, 3.15, and 7.78 per cent. The total per cent observed for 15 minutes was higher than for the other periods. This may be due to normal variances between rats or to mechanical and physical limitations in the separation of five minute aliquots of the respiratory air. This curve, however, gives the general form that would be expected from the extension of the other curves to zero time (dotted lines). The average of these curves has been plotted in Figure 8 as total C^{14} excreted.

Fig. 8

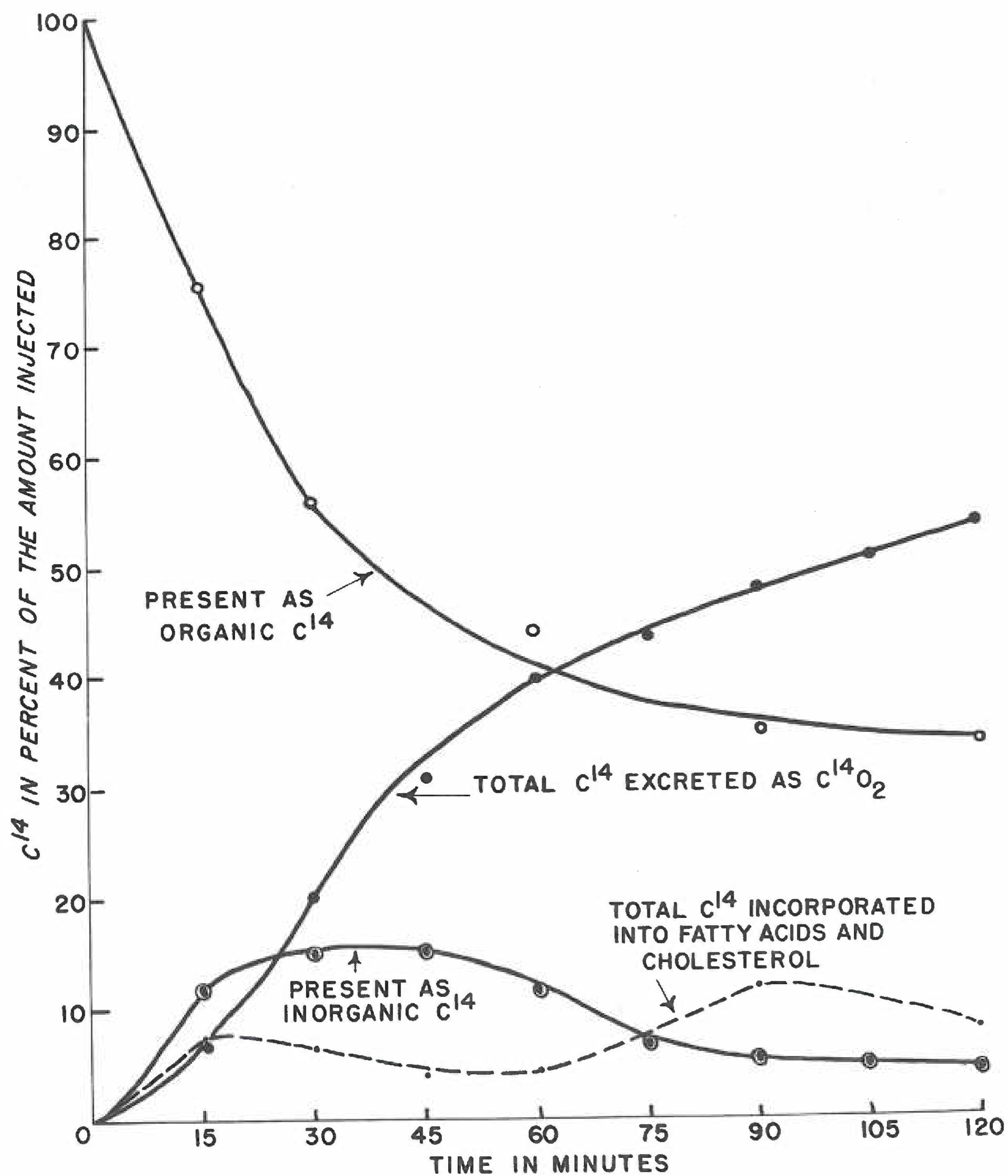


In order to calculate the amount of C^{14} present in inorganic form as $HC^{14}O_3^-$, bone $C^{14}O_3^=$, etc., a procedure of Gould and associates[94] was used. They determined the inorganic pool of carbonate by injecting $NaHC^{14}O_3$ into rats and calculating from the dilution of the isotope the total millimoles of inorganic CO_2. This value was found to be 2.00 mM/100 gram rat. Therefore, from the average weight of the animals used and the average specific activity of the expired CO_2, the inorganic C^{14} in an animal at any specific time can be calculated. One premise necessary for this calculation is that the specific activity of this inorganic carbon is the same as that of the expired carbon dioxide. With this information, the amount of C^{14} remaining as organic carbon ($CH_3C^{14}OONa$) at any specific time can be calculated, considering the administered acetate to represent 100% and subtracting from this the sum of the per cent incorporation values found from the curves of $C^{14}O_2$, inorganic C^{14}, and C^{14} incorporated into fatty acids and cholesterol. Graphic representation of this data is shown in Figure 9. The family of curves shown agree remarkably well with those of Gould and associates (page 25 of this thesis). These workers found that 87% of the isotopic carbon of $CH_3C^{14}OONa$ was expired as $C^{14}O_2$ in four hours, but they were unable to find activity in tissue lipids. Their observations are in opposition to those found in this series of experiments, in which considerable activity was demonstrable in all tissue fractions studied. However, a ten-fold increase in radioactivity over that used by Gould and associates was employed in this work.

From the slope of the expired carbon dioxide curve it appears that about 80% of the total injected radioactive carbon could be expected to be found in

Fig. 9

TIME OF MAXIMUM UTILIZATION



the respiratory CO_2 in four hours, which is the same order of magnitude as found by Gould and associates.

CHAPTER IV

SUMMARY

1. Methods, equipment, and data have been described for the preparation of $BaCO_3$ samples containing C^{14} which are suitable for the assay of C^{14} activity by the use of standard counting equipment.

Methods, equipment, and data have been presented for combustion of organic compounds containing C^{14} to $C^{14}O_2$. Data indicating the degree of reproducibility has been given.

Methods, equipment, and data have been presented for the semimicrosynthesis of $CH_3C^{14}OOH$ (C^{14} carboxyl-labeled acetic acid). Good yields are obtained when one-fourth to one millimole of labeled acetate is prepared from $BaC^{14}O_3$.

2. Techniques for the isolation of tissue fatty acids and cholesterol have been given, and the validity of these methods has been established.

Calculation and evaluation of C^{14} incorporation into the biological products, CO_2, cholesterol, and fatty acids, have been presented.

3. The effects of fasting upon subsequent lipogenesis have been studied by injection into rats of $CH_3C^{14}OONa$ after 1, 24, 48, 72, 96, and 120 hours of starvation. It was found that rats fasted up to 120 hours lose 25% of body weight and 70% of body fat during that time and that total cholesterol remains relatively constant. Appreciable lipogenesis of both cholesterol and fatty acid was observed during all degrees of fast. At 120 hours animals incorporated C^{14} into fatty acids at 31% of the rate found in the one

hour experiment. Cholesterol synthesis at that time was 41% of the values found for one hour animals. Respiratory CO_2 was depressed to 69% of the one hour group, and extremely variable results were found in the per cent of C^{14} incorporated into expired CO_2.

4. The time of maximum utilization of injected C^{14}-labeled acetate was determined. In these experiments, young male rats of a Sprague-Dawley strain were permitted to metabolize, for varying intervals of time, an intraperitoneal injection of C^{14} carboxyl-labeled acetate. These times were 0, 7, 15, 30, 60, 90, and 120 minutes. Fatty acids and cholesterol were isolated from brain and spinal cord, liver, skin, carcass, and gut. The percentage of radioactive carbon incorporated into each was determined. Fatty acids were observed to have reached a maximum incorporation value at 90 minutes after injection; cholesterol values did not reach a maximum during the interval (0-120 minutes) studied. The specific activity of isolated lipids was found to be a maximum for hepatic tissue. Evidence was presented that extra-hepatic lipid synthesis occurs. Specific activity of cholesterol from liver was greater than that found for the cholesterol of any other tissue. The activity of this cholesterol was higher than that of any other substance investigated. Brain and spinal cord were found to have incorporated significant C^{14} of the $CH_3C^{14}OONa$. This is in opposition to the concepts of most workers in the lipogenic field who believe no lipogenesis occurs in the adult brain.

Carbon dioxide was collected for all experiments from 0 to time of sacrifice with the exception of the zero and seven minute determinations. Reasons for this have been presented. Calculations are shown for finding

the time at which acetate oxidation was at a maximum. This value was found to be about 30 minutes.

The half life (T 1/2) of metabolic disappearance for sodium acetate was determined for these experiments. The half life of acetate was found to be 48 minutes with a K of 0.043. Curves are presented for expired $C^{14}O_2$, inorganic $C^{14}O_2$ (in bone, as bicarbonate, etc.) and C^{14} incorporation into fatty acid and cholesterol. The amount of $CH_3C^{14}OONa$ remaining at any time during the experiments was found by subtracting the sum of these curves from 100% at the time in question.

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