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Colorado State University, Ph.D., 1973
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THESIS

METABOLIC FATE OF ^{15}N- AND ^{14}C-BETAINE

Submitted by

Alonzo Chappell

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

March, 1973

COLORADO STATE UNIVERSITY

March, 1973

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY ALONZO CHAPPELL ENTITLED METABOLIC FATE OF ^{15}N- AND ^{14}C-BETAINE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Coadviser

Coadviser

Head of Department

ABSTRACT OF THESIS

METABOLIC FATE OF ^{15}N- AND ^{14}C-BETAINE

A study was conducted to determine if betaine was demethylated by rumen bacteria to methane and glycine, with the glycine being metabolized to ammonia and acetic acid, and to determine the effects of the energy level of the ration on betaine metabolism.

Four types of betaine were used in this study: (1) unlabeled betaine hydrochloride, (2) betaine hydrochloride with the carboxyl carbon labeled with ^{14}C, (3) betaine hydrochloride with at least one of the methyl-group labeled with ^{14}C, and betaine hydrochloride with ^{15}N.

The data showed that betaine metabolism was reduced as the energy level of the ration increased. On the low-energy ration, 42.13 per cent of the added betaine was metabolized as compared to 21.83 for the high-energy ration.

The two-dimensional paper chromatography failed to detect any radioactivity in glycine by scintillation counting and failed to detect any amino acids by scanning the whole sheet on a radiochromatogram scanner. A possible explanation could be that the glycine being synthesized was the only metabolized compound present and it was quickly metabolized to ammonia and acetic acid.

The data showed that ammonia was being synthesized from the ^{15}N-betaine. This ^{15}N-ammonia could be formed from glycine as hypothesized.

The labeled carboxyl group of betaine was found only in acetic acid. The per cent of ^{14}C-activity that disappeared from the carboxyl labeled betaine that was recovered as acetic acid was 24.37 and 68.34 for low-energy and high-energy rations, respectively.

The radioactivity from the ^{14}C labeled methyl groups of betaine was found in trimethylamine, carbon dioxide, and methane. These compounds accounted for 15.3, 4.19, and 19.67 per cent of the ^{14}C-activity that disappeared from the methyl-labeled betaine. The synthesis of methane indicated that demethylation of betaine was occurring. The pathway by which the carbon dioxide was formed was not determined.

This study failed to prove conclusively that betaine was demethylated to glycine which was in turn metabolized to ammonia and acetic acid, but added support to the probability of this pathway.

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CHAPTER I

INTRODUCTION

Liquid Protein Concentrate (LPC) is a by-product of the manufacture of beet sugar and contains about 13 per cent betaine. It is produced from the manufacture of monosodium glutamate and is composed only of condensed beet solubles product neutralized with ammonia. For many years, it was considered a waste and was discharged into the rivers. As the public became more ecologically conscious, health authorities ordered the sugar beet processing companies to stop this river pollution and a search was initiated to find ways to utilize this waste product. Since the end-product was high in non-protein nitrogen (NPN) which ruminants can utilize, the processing companies began adding this material to their sugar beet pulps. Linder (1967) showed that LPC could be utilized as an economical source of protein for ruminants.

Betaine is found in practically all plants and animals, but occurs abundantly in the sugar beet (Beta vulgaris) from which it derives its name. Davies (1936) reported that there is about 1.5 per cent of betaine nitrogen in beet molasses, 0.5 per cent in molasses beet pulp and 0.03 per cent in fresh beet tops (0.2 per cent of the dry matter). The ration of domestic animals normally does not contain

any betaine unless they are consuming sugar beet or by-products from sugar beets.

Since a large amount of betaine is found in LPC, research was initiated to determine if betaine was metabolized in the rumen. Previous results (Mitchell, 1968) showed that betaine was metabolized by rumen bacteria to trimethylamine, methane, carbon dioxide and acetate. Mitchell further showed that betaine in very large quantities was detrimental to the rumen bacteria. From his research, Mitchell (1968) concluded that rumen microorganism cleaved the betaine molecule into trimethylamine and acetic acid.

$$(CH_3)_3NCH_2COOH \xrightarrow[H]{} (CH_3)_3N + CH_3COOH$$

This pathway does not account for the synthesis of methane and carbon dioxide. One purpose of this study was to test the hypothesis that betaine is demethylated by rumen bacteria in the following manner.

Betaine ⟶ Dimethylglycine ⟶ Methylglycine ⟶
Glycine ⟶ Ammonia + Acetic Acid.

This pathway could account for the synthesis of methane and carbon dioxide.

A second purpose of this study was to determine if the energy level of the ration had any effect on betaine metabolism.

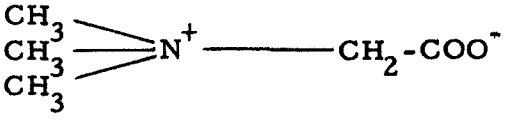
If the above hypothesis is true, betaine would serve several important functions in rumen metabolism because acetic acid is a

source of energy and ammonia can be used as a source of nitrogen for the syntheis of amino acids. The methyl groups may be used by rumen microorganism for the synthesis of methionine, creatine, and other compounds. When the metabolic fate of betaine is known, it may prove to have both economical and physiological importance.

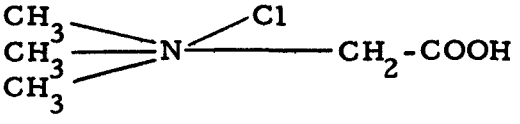
CHAPTER II

REVIEW OF LITERATURE

Betaine is a quaternary ammonium compound with the following structure:



It is a water soluble solid that decomposes at 300° C and is unaffected by bases, but reacts with hydrochloric acid to form a crystalline product shown by the following structure (Morrison and Boyd, 1967):



Synonyms of betaine are lycine, oxyneurine, glycine betaine, and trimethylglycine.

Feeding sugar beets or sugar beet processing by-products is not a new practice. Velich and Stanek (1904-5) reported that ruminants could cope effectively with betaine and showed a marked degree of accommodation to the feeding of regular amounts. A cow accustomed to beet molasses excreted no betaine, while sheep excreted the base only during the first few days. When betaine was fed to

dogs, one-quarter of it was absorbed and excreted in the urine, but when it was injected intravenously, all of it could be found in the urine.

Voltz (1907) conducted experiments to determine the nutritive value of betaine and its interrelationship to other nitrogen-containing complexes, as it was known that beet sugar molasses and beet sugar fodder may contain a relatively high proportion of betaine. He conducted experiments with sheep and found that a part of the nitrogen was retained by ruminants for some time and this nitrogen eventually appeared in the urine, especially when the animal was supplied with insufficient nitrogen, i.e., had negative N balanace. Calorimetric estimations showed that betaine was metabolized by ruminants. The nitrogen-free metabolities of betaine did not appear in the urine. He concluded that there was no reason to believe that betaine serves as a source of nutrient for the organism. Furthermore, it was probable that the compound in relatively large amounts was easily absorbable and, in fact, harmful to the ruminant. Voltz (1907) also fed betaine to dogs and reported that the total betaine nitrogen appears in the urine of the dog on the first day of feeding.

Kohlrausch (1911, 1912) reported that at least a part of the betaine was excreted unchanged in the urine by cats, dogs, and rabbits when it was fed (0.5 gm) or injected subcutaneously (0.1 gm). With rabbits, the betaine was partly destroyed and trimethylamine

appeared in the urine. Herbivorous animals metabolized more betaine than carnivorous animals when it was administered orally (Kohlrausch, 1911). When larger doses were fed, it produced diarrhea and increased salivary secretion, while the injection of even larger amounts caused necrosis at the site of injection (Kohlrausch, 1911).

Brigl and Benedict (1933) conducted research in which asparagine and betaine, supplemented with starch, were substituted for a part of the ration of sheep. In addition to the nitrogen balance, the distribution of nitrogen in the urine relative to the different groups was observed. They found that not more than 10 per cent of the ration can be supplemented by amides. The added amides were metabolized and secreted in the form of urea. When betaine was given, no unchanged betaine, methylamine or methylated urea was found in the urine.

Davies (1936) became interested in betaine because of the sporadic occurrence of a fishy flavor in the milk of cows fed sugar beet by-products. The tertiary bases, especially betaine, were regarded as the source of the fishy flavor in milk. Davies found that in the ruminant, the main tertiary nitrogenous metabolite of all the tertiary nitrogenous bases examined was trimethylamine oxide (TMAO). There were also small traces of trimethylamine (TMA) and insignificant traces of mono- and dimethylamine which occurred in

cow's urine. The nitrogen of simple bases (TMA and TMAO) is almost quantitatively and rapidly excreted as trimethylamine oxide. With betaine, choline and the methylester of dimethylaminoacetic acid, only 14 to 43 per cent of the nitrogen was excreted as trimethylamine oxide. The amount so excreted depended on the level of nitrogen intake, the nature of the base fed and the degree of accommodation in the animal to which the base was fed. Davies (1936) reported that excretion rates of tertiary metabolites show well-defined peaks depending on the nature and amount of the base fed. The feeding of 100 gm of betaine to a dairy cow showed a peak of excretion at about four and a half hours after feeding. No unchanged betaine was detected in the urine.

Biosynthesis of Betaine

1. Synthesis in Plants

The biosynthesis of betaine in the sugar beet has been determined. Cromwell and Rennie (1953a, 1953b) stated that betaine was formed in the root tissue from choline because they could not find choline oxidase in the leaf tissues. Betaine was translocated to the leaves where it accumulated. Cromwell and Rennie (1954), in later work, found their 1953 translocation statement to be false. They infiltrated solutions of choline and betaine aldehyde and found significant increases in the betaine content of the leaves. When the leaves infiltrated with solutions of choline were kept in an atmosphere of

nitrogen during the experimental period, insignificant increases in the content of betaine were observed. Leaves infiltrated with solutions of choline and exposed to light during the experimental period showed a greater increase in betaine content than leaves kept in darkness during the experimental period. Cromwell and Rennie (1954) concluded that the synthesis of betaine occurred in all tissues of higher plants as the results of oxidation of choline.

Bregoff and Delwiche (1955) infiltrated leaves of sugar beets with solution of acetate-2-^{14}C, glycine-2-^{14}C, formate-^{14}C, or bicarbonate-^{14}C. Appreciable activity in choline and betaine arising from alpha-labeled glycine or from formate was noted. Bicarbonate was incorporated to a lesser extent, and the alpha-carbon of acetate was not incorporated into choline or betaine. The specific activity of choline isolated, depending upon the experiment, was ten to thirty times that of the betaine when formate or glycine-2-^{14}C was infiltrated. Later work (Delwiche and Bregoff, 1958) added support to the theory that betaine was derived not by the direct methylation of glycine, but more probably via the methylation of aminoethanol and subsequent oxidation of choline.

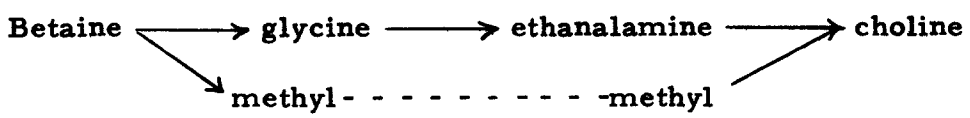
Bowman and Rohinger (1970) found that in the healthy and rust-infected wheat plant, betaine was synthesized from glycine via serine, ethanolamine, and choline with methionine as the methyl donor, and not by direct N-methylation of glycine.

2. Synthesis in Marine Animals

In the lobster (Homarus americanas), Bilinski (1960) found that the biosynthesis of betaine takes place by the oxidation of choline rather than by methylation of glycine. He found that choline-methyl-^{14}C was a very good precursor, whereas, glycine-2-^{14}C was not converted to betaine. This agrees with the work of Mann and Quastel (1937), who found that in the rat liver, choline was oxidized into betaine aldehyde which was then oxidized to betaine.

Metabolism of Betaine

Stetten (1941) reported that in the rat, dietary betaine was rapidly demethylated to glycine, little or none of it being directly reduced to choline. The schematic results of his observations were:



None of the above steps were reversible. The chief source of the methyl groups in the last steps, when the animals are kept on a normal diet, was probably methionine.

Norris and Benoit (1945) fed and injected both choline and betaine into rats. When choline was fed, there was a noticeable increase in urinary trimethylamine oxide. They could not find any evidence that injected choline was converted to trimethylamine oxide. There was practically no difference in trimethylamine oxide excretion

following the feeding or injection of betaine. Their conclusion was that bacterial action in the intestine was responsible for the formation of trimethylamine or trimethylamine oxide, which was excreted as trimethylamine oxide. Bilinski (1961), working with the lobster, could not find support for the possibility that the oxidation of choline to betaine was an intermediate step in the formation of trimethylamine oxide from choline.

Koch and Oelsner (1919), working with a fertilizer prepared from molasses waste, found an organism which attacked betaine and produced the following compounds: trimethylamine, ammonia, carbon dioxide, methanol, formaldehyde, and ethanol. These compounds appeared to be intermediate products in the production of carbon dioxide. Shieh (1964) found in the marine bacterium (Achromobacter cholinaphaoum) that choline was metabolized as follows:

Choline ⟶ betaine ⟶ dimethylglycine + formaldehyde ⟶ sacrosine (monomethylglycine) + formaldehyde ⟶ serine ⟶ pyruvate + ammonia. Transmethylation was not involved. This pathway had not previously been reported for any other bacteria or for animal tissue. Shieh (1968) found added support for this pathway. When betaine-1-^{14}C was incubated with cell-free extracts from his marine bacterium, dimethylglycine was found to be labelled and no radioactivity was present in formaldehyde. When betaine-methyl-^{14}C

was used as the substrate, both dimethyglycine and formaldehyde contained radio-activity. He concluded that the formaldehyde was derived from a methyl carbon of betaine.

Function of Betaine

The structure of betaine is very similar to that of choline, and most of the research has been concerned with the ability of an animal to use betaine as a replacement for choline. One of the functions of choline is to serve as a methyl group donor in body reactions. In 1939, du Vigneaud et al. (1939), demonstrated that the administration of choline enables the rat to utilize homocystine for growth purposes in lieu of methionine. Choline made possible the in vivo methylation of homocysteine to methionine. Betaine had a similar behavior to choline in permitting growth on a diet with homocysteine as the sole sulfur-containing amino acid. Later research (Chandler and du Vigneaud, 1940) demonstrated that betaine was less effective than choline in supporting growth of rats on a diet containing homocystine, but free of cystine and methionine. The difference was manifested principally in a several day delay in the growth response following the administration of betaine to deficient animals. When the rats were only three to four weeks old, this delay in action was often fatal. The mechanism by which betaine can bring about the transformation of homocystine to methionine appears to be absent or at least inefficient at first. It takes a period of several days after the compound

was provided dietarily for this mechanism to develop in rats; this was true even when betaine was injected. It was concluded that the compound choline was actually used by the body for the methylation of homocystine, and that betaine could be used after the organism acquired the ability to convert sufficient quantities of betaine to choline. The bacterial flora of the intestine was not necessary to the process because the result occurred when betaine was injected. Betaine and choline labeled with deuteriomethyl groups and ^{15}N was synthesized by du Vigneaud et al. (1946), and fed to growing rats. Isotopic analyses of the choline and creatine isolated from the rat tissues showed betaine to be an extremely effective methyl donor. Methyl groups from dietary betaine appeared in tissue choline almost as rapidly as they appeared from dietary deuteriocholine. The disparity in amount of ^{15}N and of deuterium found in the tissue demonstrated conclusively that betaine molecule is not converted as a whole to choline. du Vigneaud et al. (1946), fed dimethyglycine containing deuterium in the methyl groups to young rats. They found that transmethylation from this dimethylglycine to choline and to creatine occurred to only a very small degree.

Borsook and Dubnoff (1947) found that rat liver slices methylate homocystine, homocysteine, and homocysteine-thiolactone to methionine. Choline or betaine could serve as the methyl donor for the reaction. The reaction was faster with betaine than with choline

as the methyl donor. The transmethylations were independent of oxygen and were not inhibited by oxidation inhibitors. Dubnoff (1949) reported that the methylation of homocysteine in the presence of betaine was relatively independent of the oxygen tension, and that conditions of pH, anaerobiosis and inhibitors which decrease the oxidation of choline to betaine decrease methionine formation from choline.

Ferger and du Vigneaud (1950) reported that when betaine was administered, at levels providing equivalent amounts of methyl groups, the percentage of methyl groups oxidized to carbon dioxide in the expired air was greater for betaine than for choline. Methyl groups supplied in the form of choline were converted to carbon dioxide more slowly than betaine. Ferger and du Vigneaud speculated that this may be due to the dilution of the radioactive choline by the body choline into reactions other than those involving oxidation of the methyl groups. They also observed that betaine need not be converted to choline prior to oxidation.

Muntz and Hurwitz (1950) incubated rat liver homogenates with choline and homocysteine to form methionine. Dimethylaminoethanol, the expected end-product of the demethylation of choline, was not produced. Instead, dimethylglycine, the expected product of the demethylation of betaine, was formed. They concluded that in this system, choline does not lose a methyl group directly, but must first

be converted to betaine before methyl transfer can occur. This could be an explanation for the more rapid oxidation of betaine than choline.

Stekol et al. (1957), showed that the transmethylation action of betaine was not affected by Vitamin B_{12} or folic acid. The transfer of radiomethyl groups of betaine to tissue methionine in Vitamin B_{12} or folic acid deficient rats, mice, or chicks was the same as that in the normal control animals. Liver homogenates of rats deficient in Vitamin B_{12} or folic acid synthesized the same amount of methionine from radiobetaine and homocysteine as the liver homogenates of normal rats. Radiomethyl groups of betaine were incorporated into tissue methionine of normal mice, rats, and chicks to a greater extent than the radiomethyl groups of choline.

Luecke and Pearson (1945) added support to the fact that betaine was not converted directly to choline. They wanted to see if the feeding of betaine might conceivably be accompanied by an increase in the excretion of urinary choline. One sheep was fed twenty grams of betaine hydrochloride for two successive days. The feeding of betaine hydrochloride was not followed by any increase in choline excretion nor was any betaine excreted in the urine.

In 1955, Ericson (1955) reported a method for the partial purification of an enzyme "betaine-homocysteine-transmethylase," from pig and rat livers. Ericson (1960) homogenized and incubated various types of living materials with betaine and homocysteine in order to

test their ability to form methionine by means of methyl group transfer. The enzymes were found in the liver of all the vertebrates investigated, ranging from human (Homo sapiens) to lamprey (Petromyzon fluviatilis). These enzymes were not found in any appreciable amounts in any other vertebrate organ except in the kidney of the guinea pig. To date, the only non-vertebrate in which a betaine-homocysteine-methyl-transferase has been detected is the pond mussel (Anadonta cygnea). Methyl transferases were not found in plants or microorganisms. Dimethylglycine, but not choline, markedly inhibited the transfer reaction.

Sribney and Kirkwood (1945) found that betaine served as a source of labile methyl groups in the barley plant, as judged by the transfer of its methyls to the alkaloids N-methyltyramine and hordenine. Betaine failed to serve as a source of labile methyl groups in the young castor bean. A sample of choline isolated from barley plants to which methyl-labeled betaine was fed, proved to have label only in its methyl groups. They concluded that these plants can synthesize the methyl groups of choline from the methyl groups of betaine. Whether this was done by transmethylation or by direct reduction of betaine to choline could not be determined from their experiments. The comparatively low activity in the isolated choline was perhaps more consistent with a transmethylation than with a direct reduction. Byerrum et al. (1956), reported that in tobacco

plant, methyl groups of betaine may be incorporated into the N-methyl group of nicotine. The extent of incorporation of betaine methyl groups was about the same as the methyl group of methionine and the methyl groups of choline. When choline-methyl-^{14}C was fed to tobacco plants, radioactive betaine and dimethylglycine appeared. This result was interpreted to mean that a choline oxide system was present in these plants and that some choline was oxidized to betaine before the methylation of nicotine occurred.

Another function of choline is the prevention of perosis and the promotion of growth in birds and animals. Jukes and Welch (1942) fed choline-deficient diets to chicks and found that betaine and betaine aldehyde had a slight growth-promoting activity, but did not prevent perosis. Jukes (1940) reported that in turkeys, betaine was neither growth promoting nor antiperotic. Moyer and du Vigneaud (1942) reported that betaine was incapable of preventing perosis or of acting, to any large extent, as a growth essential in the chick. They concluded that the chick could not synthesize choline directly or indirectly from betaine.

Choline also serves to prevent abnormal accumulation of fat in the liver (fatty livers). The ability of betaine to prevent fat accumulation in the liver has been reported by several researchers (Best and Huntsman, 1932; Platt, 1939; and Moyer and du Vigneaud, 1942). An explanation for this lipotropic action may be the work of

Ridout et al. (1954). They found that choline and betaine maintained the serum cholesterol within normal range of rats on a 0.2 per cent cholesterol diet.

Webster (1942) reported that when rats were on diets poor in protein and rich in fats, betaine hydrochloride fed at the rate of fifty milligrams per rat daily diminished the severity of the liver lesions. Betaine was not as effective as increased protein in reducing the severity of liver lesions, but it was more effective than reducing the dietary fat.

Best et al. (1969, article reviewed by Hegsted, 1969), added 0.63 per cent betaine to a basal diet designed to produce cirrhosis of the liver in rats. For the ten rats on the trial, there was marked regression of cirrhosis in four and slight improvement in four others. Increasing the protein content to twenty per cent with peanut or soy protein, both of which are low in methionine, caused cirrhosis to increase in severity although all ten of the animals survived. With this higher dietary protein, betaine produced improvement equal to that obtained with choline.

Hemorrhagic degeneration of the kidney is the result of a dietary deficiency of choline and labile methyl groups. Griffith and Mulford (1941) reported that betaine, like methionine, contributes to the labile methyl supply of the body and may be substituted for choline. The effectiveness of betaine corresponds to the utilization

of only one of the three methyl groups. Supplee et al. (1945), reported that betaine supplementation of low-fat diets containing casein, lactalbumin or soy protein was substantially as effective as the same amount of choline in promoting growth in young rats, in the prevention of hemorrhagic degeneration of the kidneys and in maintaining normal kidney functions. Betaine exerted less lipotropic action. The amount of choline in the liver lipids of animals receiving choline or betaine supplements was substantially the same. Choline or betaine supplements are seemingly less essential for mature rats than for young animals.

Baumann and Hines (1918) tried to determine the origin of creatine. They perfused betaine, and betaine with arginine, in the hind leg of a dog. They could not find any increase in creatine elimination.

Strack et al. (1964), found twenty-five strains of Pseudomonas aeruginosa and two strains of Pseudomonas ovalis that were able to grow on (-) - carnitine, glycine betaine, or choline as the only carbon and nitrogen sources.

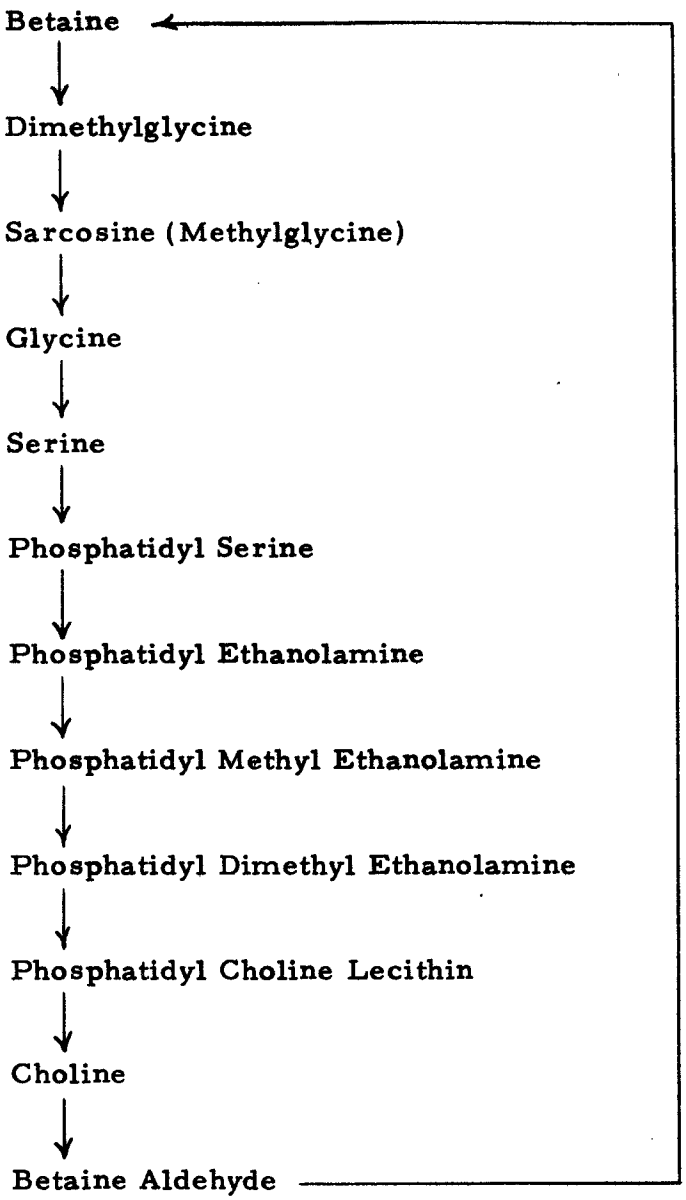
The review of the literature indicated that when betaine was fed to ruminants, most of the betaine was metabolized by the ruminants after they became accustomed to it. When betaine was fed to non-ruminants, most of the betaine was absorbed by the body and secreted in the urine unchanged. In ruminants, the nitrogen of betaine is

secreted in the urine as trimethylamine oxide or trimethylamine, depending on the level of nitrogen intake, and the accommodation of the animal.

The biosynthesis and metabolism of betaine in animal tissues are summarized in Figure 1 (abbreviated from Dagley and Nicholson, 1970). Note that none of the steps are reversible. In plants, choline can be synthesized from betaine.

Since betaine can be described as an oxidized form of choline, they have several functions in common. Both are methyl group donors. Betaine is a better methyl group donor than choline because choline must first be converted to betaine. Both can methylate homocystine or homocysteine to methionine. The transmethylation action of betaine is inhibited by dimethylglycine. The reaction takes place in the liver because this is the only organ which has the enzyme "betaine-homocysteine-transmethylase." Both choline and betaine can prevent fatty livers, liver lesions, and hemorrhagic degeneration of the kidney.

Figure 1. Interrelationships of Methylated Glycine and Choline Derivatives.


Betaine
Dimethylglycine
Sarcosine (Methylglycine)
Glycine
Serine
Phosphatidyl Serine
Phosphatidyl Ethanolamine
Phosphatidyl Methyl Ethanolamine
Phosphatidyl Dimethyl Ethanolamine
Phosphatidyl Choline Lecithin
Choline
Betaine Aldehyde

CHAPTER III

EXPERIMENTAL PROCEDURES

A series of *in vitro* rumen fermentations using betaine and isotopically labeled betaine as a substrate were conducted to determine the metabolic fate of this compound. A total of twelve separate fermentations were completed, using three types of isotopically labeled betaine, two dietary levels of energy, and four sheep as sources of rumen contents.

Experimental

This study was divided into two parts, a series of six *in vitro* fermentations using the rumen contents from sheep fed a low-energy ration and a series of six *in vitro* fermentations using the rumen contents from sheep fed a high-energy ration. Rumen contents were collected from four mature wethers with a mean weight of 65.3 kilograms which had been fitted with rumen fistulas. The sheep were maintained outdoors throughout the experiment. They were trained to enter stalls and were fed individually. The low-energy ration was one-fourth inch pelleted dehydrated alfalfa hay with ten grams of Liquid Protein Concentrate (LPC)[1] per kilogram of feed, and the

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[1]Supplied by The Great Western Sugar Company, Denver, Colorado.

high-energy ration was the Metabolic Laboratory Sheep Ration (Table 1) with ten grams of LPC per kilogram of feed. The LPC was poured on to the pellets prior to feeding.

Table 1. Ingredients of the Metabolic Laboratory Sheep Ration.

Suncured dehydrated alfalfa hay	47.1%
Whole corn	23.5%
Barley	23.5%
Iodized salt	0.9%
Molasses	5.0%
Vitamin A	140.8 IU/kg

The wethers were fed at the rate of 70 grams of feed/$kg^{3/4}$/day They were fed twice a day, 40 grams in the morning and 30 grams in the afternoon. Wethers received the appropriate ration for at least three weeks prior to collecting rumen contents for the in vitro fermentations.

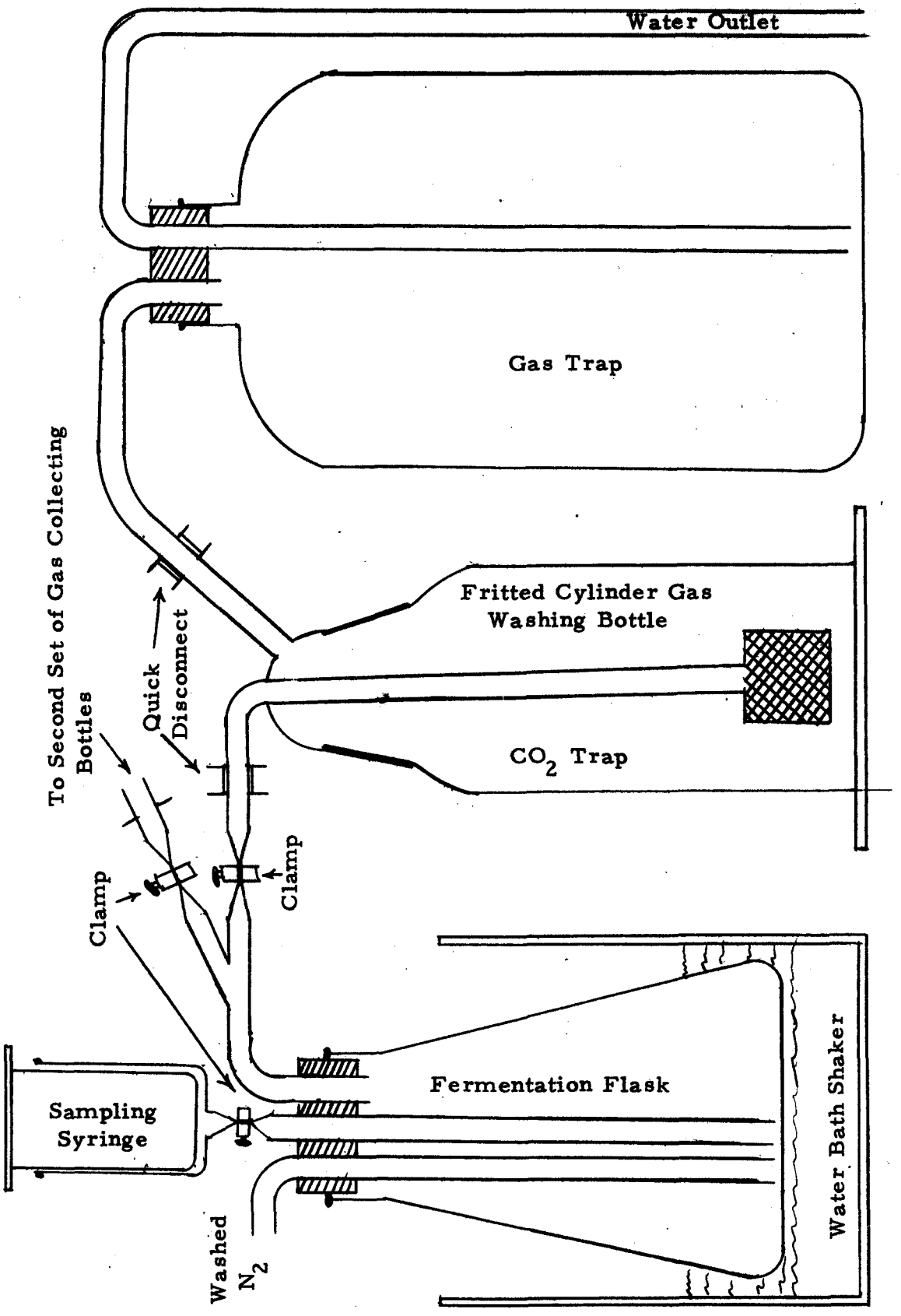
Rumen content collections were made approximately 12 hours after the wethers were fed, just prior to the particular in vitro fermentation.

General Procedure for Incubations

1. Apparatus

The fermentation flasks were 500 ml, large mouth Erlenmeyer flasks (Figure 2). Glass tubing (7 mm O.D.) and 12.7 millimeters O.D.

Figure 2. Fermentation Flask and Gas Collecting Apparatus.


Water Outlet
Gas Trap
To Second Set of Gas Collecting Bottles
Quick Disconnect
Fritted Cylinder Gas Washing Bottle
CO_2 Trap
Clamp
Clamp
Sampling Syringe
Fermentation Flask
Water Bath Shaker
Washed N_2

Tygon tubing were used to make fittings. Samples were collected with a 50 cc plastic disposal syringe. Carbon dioxide was trapped with 250 ml fritted gas washing bottles filled with low carbonate sodium hydroxide.

Methane and other gases were collected in two and one-half liter reagent bottles by the displacement of distilled water.

Fermentation flasks were incubated in a water-bath with shaker[2] where the temperature was maintained at 39.5 ± 0.5°C and reciprocated at the rate of 45 ± 5 cycles per minute.

2. Incubation Materials

The nitrogen gas used to flush the fermentation flask was washed with boric acid solution (40 grams H_3BO_3 per liter) and 2N sodium hydroxide in a fritted cylinder gas washing bottle to remove ammonia and carbon dioxide, respectively.

The phosphate buffer (pH 6.8) was prepared from 0.067M vacuum oven dried sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$) and 0.067M potassium phosphate (KH_2PO_4) (Oser, 1965) and adjusted to pH 6.8, by mixing the two solutions. The sodium and potassium buffer was used because it was free of carbon and nitrogen which reduced the exogenous carbon and nitrogen. Johnson (1966) reported that many researchers have arrived at a standard buffered medium for their

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[2]Manufactured by Precision Scientific Company, Chicago, Illinois.

in vitro fermentation techniques through experimental studies. Most of the media are based on so-called "McDougall's solution" which is an artificial saliva or buffer medium based on the analysis of sheep saliva. McDougall's solution contains several carbon and nitrogen compounds. It required approximately equal amounts of the two phosphate solutions to make the 6.8 pH phosphate buffer. The phosphate buffer contained 0.066 meq Na/ml and 0.033 meq K/ml. These values are below the values of 0.1 meq Na/ml and 0.037 meq K/ml for bovine rumen fluid reported by Emery *et al.* (1960).

Three types of isotopically labeled betaine were used--betaine hydrochloride-^{15}N,[3] betaine-hydrochloride-1-^{14}C,[4] and betaine-hydrochloride-methyl-^{14}C.[5] The manufacturer's analysis was accepted for the three isotopically labeled compounds. The total activity of the ^{14}C betaines was checked by counting a 10 µl sample in scintillation fluid. The betaine-hydrochloride-^{15}N was 99 per cent ^{15}N. The methyl-labeled betaine had at least one of its three methyl groups labeled and a specific activity of 21.67 µCi/mg. The carboxyl-labeled betaine had a specific activity of 10.73 µCi/mg. The amount of betaine added to each flask is given in Table 2.

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[3] Purchased from Nuclear Equipment Chemical Corporation, 165 Marine Street, Farmingdale, New York.

[4] Purchased from International Chemical and Nuclear Corporation, 13332 E. Amar Road, City of Industry, California.

[5] *Ibid.*, No. 4, p. 27.

Table 2. Material Added to the Fermentation Flasks Used in All *in vitro* Fermentation Trials.

Ingredients	Flask No. 1 Betaine-$^{14}CH_3$	Flask No. 2 Betaine-1-^{14}C
Phosphate Buffer	120.0 ml	120.0 ml
Strained Rumen Contents	180.0 ml	180.0 ml
Betaine-HCl	350.0 mg	350.0 mg
Betaine-^{15}N-HCl	50.0 mg	50.0 mg
Betaine-1-^{14}C-HCl	0.0 mg	0.3 mg
Betaine-$^{14}CH_3$-HCl	0.15 mg	0.0 mg

The fermentation flask (Table 2), except strained rumen content, and gas collecting bottle were prepared and placed in the water-bath (Figure 2). Approximately 500 milliliters of rumen contents were collected from each wether and pooled to reduce animal variation. The pooled rumen contents were immediately strained through four layers of cheesecloth and added to the fermentation flask. The flask was shaken and returned to the water-bath. No attempt was made to determine the number of bacteria or protozoa added or to add a standard microbial population to each flask.

3. Samples Taken and Time of Sampling

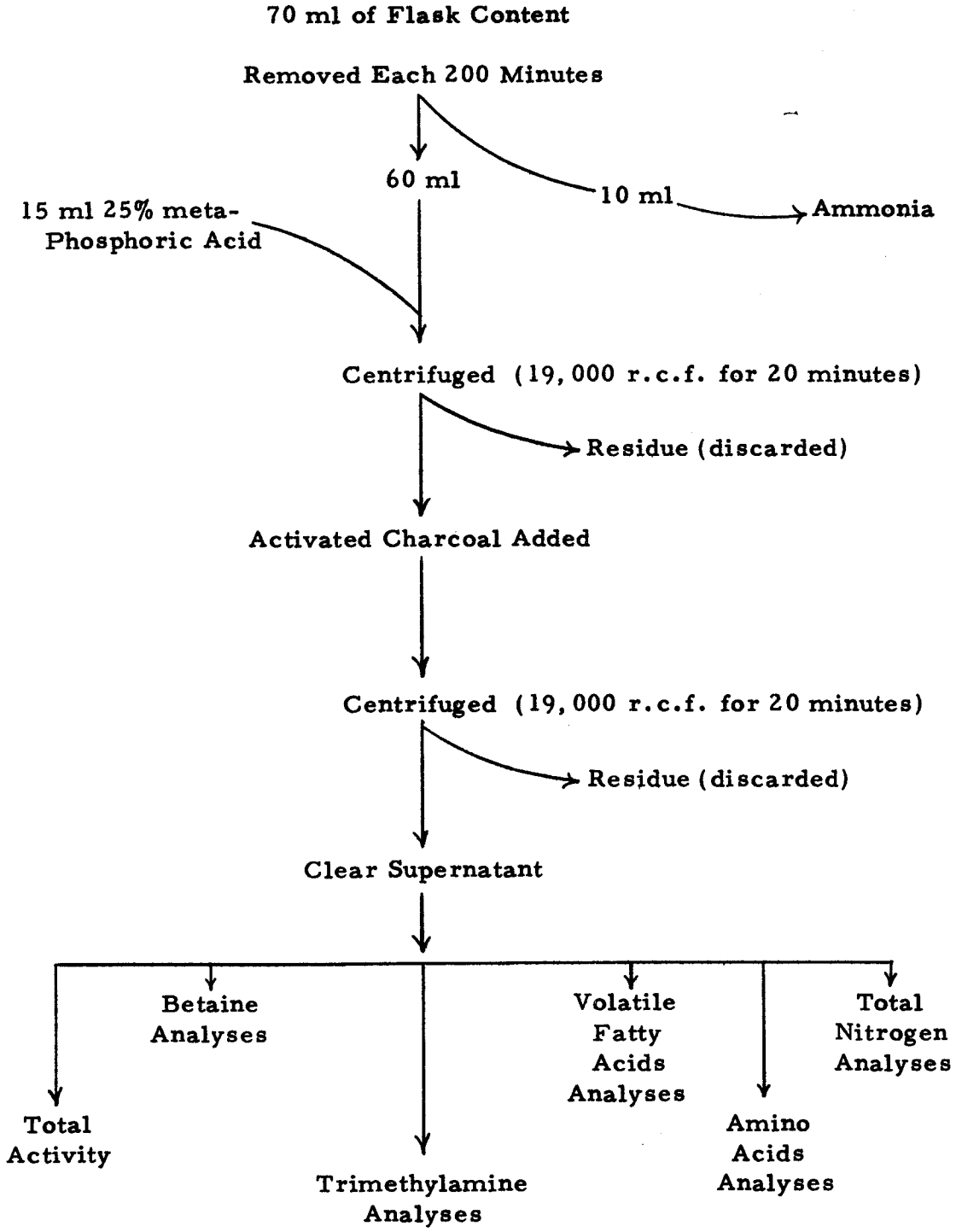
After the inoculum was added and the incubation started, a 70-ml sample was removed to establish initial values. Additional 70-ml samples were removed at 200 and 400 minutes after starting the

incubation. The remaining volume in the flask was removed at 600 minutes (Figure 3).

For each 70-ml sample removed, 10 ml were used to determine ammonia. To the remaining 60 ml of sample, 15 ml of 25 per cent meta-phosphoric acid was added to stop fermentation and the sample centrifuged at 19,000 relative centrifugal force (r.c.f.) for 20 minutes to remove forage particles, protozoa, and bacteria (Meger et al., 1967). Approximately one gm of activated charcoal was added to the supernate and the solution heated until it began to boil. The solution was centrifuged at 19,000 r.c.f. for 20 minutes and the clear supernate used for all analyses except ammonia (Figure 3). The charcoal residue was not analyzed for ^{14}C.

Before each 200 minute collection, the fermentation flasks were flushed by flowing approximately one liter of washed nitrogen gas through the system. The carbon dioxide produced during the incubation period was collected in 100 milliliters of 2N low-carbonate sodium hydroxide in a fritted cylinder gas washing bottle (Figure 2). The low-carbonate sodium hydroxide was prepared by adding 800 grams of low-carbonate sodium hydroxide pellets to a liter of carbon dioxide-free distilled water. The carbon dioxide was removed from the distilled water by allowing the water to boil vigorously for at least ten minutes. The flask of 20N low-carbonate sodium hydroxide was stopped and set aside for about three days in order to settle any

Figure 3. Flow Diagram for *in vitro* Fermentation.


70 ml of Flask Content
Removed Each 200 Minutes
60 ml
10 ml
Ammonia
15 ml 25% meta-
Phosphoric Acid
Centrifuged (19,000 r.c.f. for 20 minutes)
Residue (discarded)
Activated Charcoal Added
Centrifuged (19,000 r.c.f. for 20 minutes)
Residue (discarded)
Clear Supernatant
Betaine
Analyses
Volatile
Fatty
Acids
Analyses
Total
Nitrogen
Analyses
Total
Activity
Trimethylamine
Analyses
Amino
Acids
Analyses

extraneous matter. The 2N low-carbonate sodium hydroxide was prepared by adding the upper portion of the 20N low-carbonate sodium hydroxide to carbon dioxide-free distilled water.

The remaining gas was trapped in large bottles by the displacement of distilled water (Figure 2) and used to determine methane. The gas collecting bottles were changed each 200-minutes by opening one clamp and closing the other clamp at the Y leaving the fermentation flask.

Chemical and Radioactivity Analyses

1. Betaine

The amount of betaine present in the clear supernate was determined by the colorimetric method of Focht et al. (1956). This method is based upon measuring the color of the reineckate ion in 70 per cent acetone at 626 mu after the betaine as the reineckate has been separated from an acid solution of the sample. Trimethylamine forms a reineckate precipitate; therefore, a standard curve was prepared for trimethylamine using the same procedure. The betaine determinations were corrected for trimethylamine. The efficiency of the colorimetric method was determined from a betaine standard 1.3 gram of betaine hydrochloride diluted to a liter.

The radioactivity in ^{14}C-labeled betaine was determined by pipetting 0.5 milliliters of the betaine reineckate dissolved in 70 per cent acetone into a scintillation vial containing 20 ml of scintillation

fluid (Table 3) and counting.[6] Corrections were made for quenching, background, dilution, and radioactivity in trimethylamine.

Table 3. Composition of the Scintillation Fluid.

Ingredients	Amount
2,5 Diphenyloxazole	5 gm
1,4-bis 2-(5-phenyloxazolyl)-benzene	0.1 gm
1,4-Dioxane	380 ml
Naphthalene	50 gm
Toluene	380 ml
Ethanol	250 ml

2. Trimethylamine

Trimethylamine was determined by the method of Dyer (1959). The efficiency of the method was determined from a trimethylamine standard 0.692 gm of trimethylamine hydrochloride diluted to a liter.

The radioactivity of trimethylamine was determined by pipetting one mililiter of the toluene layer into a scintillation vial containing 20 ml of aqueous scintillation fluid as described for betaine. Corrections were made for background, quenching, and dilution.

[6] The scintillation vials were assayed on a Beckman Liquid Scintillation System, Model 1650, Beckman Instruments, Inc., LaJolla, California.

3. Volatile Fatty Acids

Acetic, butyric, and propionic acids were separated and collected for ^{14}C analyses by the use of the Silicic Acid Column using a modification of Ramsey's Method (Ramsy, 1963). Ten ml of clear supernatant was adjusted to pH of 9 to 10 with 20N sodium hydroxide and checked with 9.0 to 10.0 hydrion pH-short range test paper. The basic clear supernatant was dried at 80° C in a forced air oven.[7] Immediately preceding analyses on the column, several drops of distilled water were added to the dried sample to make a paste. The paste was acidified to pH of 2.5 to 3.5 with concentrated sulfuric acid and checked with 2.5 to 3.5 hydrion pH-short range test paper. Enough washed and dried silicic acid was added to the acidified paste to make a free flowing powder.

Silicic acid for use on the column was prepared by first removing the smaller particles, to insure a desired solvent flow rate without excess pressure. Small particles were removed by suspending 250 grams of silicic acid in one liter of water, allowing it to settle for four minutes and discarding the supernate. The remaining acid was filtered in a Buchner funnel and dried in a vacuum oven[8] at 110° C.

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[7]Model OV-490A-2, manufactured by Blue M Electric Company, 138th and Chatham Streets, Blue Island, Illinois.

[8]Model 5830, manufactured by National Appliance Company, Portland, Oregon.

The column (Figure 4) was prepared by acidifying 15 gms of the washed and dried silicic acid with ten ml of 0.5N sulfuric acid and adding sufficient benzene to make a slurry. A glass wool plug was placed in the bottom of the column to hold the silicic acid. The bottom of the column was connected to a vacuum. The slurry was transferred to the column and packed; a 6.35 millimeters glass rod was used to remove air pockets. The sample, in the form of an acidified paste, was added to the top of the column and kept under benzene until placed on the fraction collector.

The two eluting solvents were chloroform and a 35 per cent (v/v) mixture of tertiary butanol in acidified chloroform. The chloroform was acidified by adding 10 ml of 0.5N sulfuric acid to 500 ml of chloroform in a separatory funnel, shaking, allowing to stand for at least ten minutes, and then slowly drawing the chloroform off the bottom. After the prepared column was placed on the automatic Fraction Collector,[9] acidified chloroform was added to the lower portion of the solvent system and to the top of the prepared column. To the upper portion of the solvent system 35 per cent tertiary butanol was added. Approximately 3.6 kilograms per square 2.54 centimeters of nitrogen gas was applied to the system. The stopcock at the bottom of the column was adjusted to a flow rate of approximately 3 ml/min (Figure 4).

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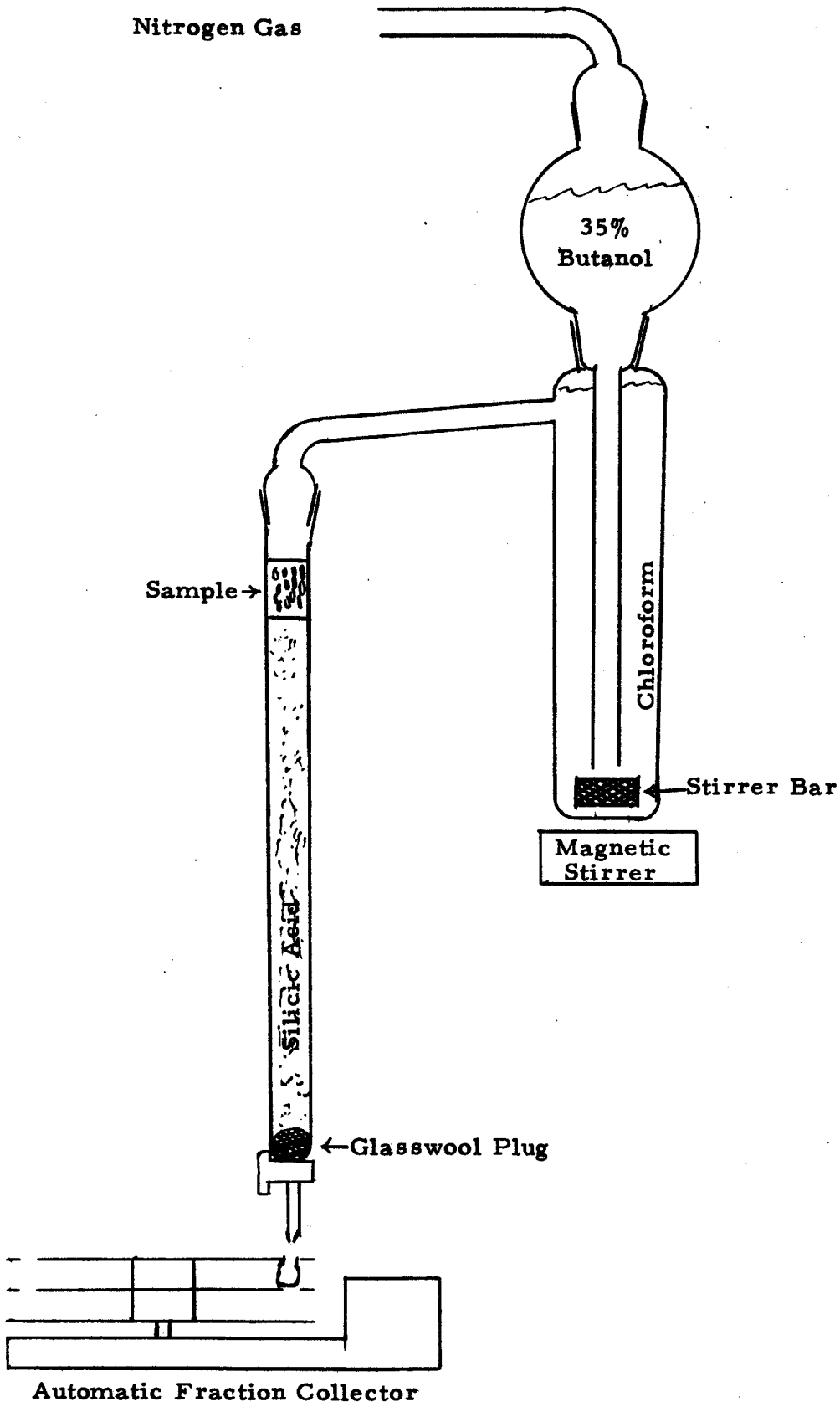
[9]Manufactured by Instrument Division, Warner Chilcott Laboratories, Richmond, Virginia.

Figure 4. A Schematic Drawing of the Apparatus for the Separation and Collection of Organic Acids.


Nitrogen Gas
35%
Butanol
Sample→
Chloroform
Stirrer Bar
Magnetic
Stirrer
Silicic Acid
←Glasswool Plug
Automatic Fraction Collector

Approximately 3 ml of solute were collected per minute in scintillation vials and each vial titrated with 0.01N ethanolic sodium hydroxide using ethanolic phenolpthalein (1 gm of phenolphthalein per 100 ml of absolute ethanol) as an indicator. Additional 0.01N ethanolic sodium hydroxide was added to all vials with discernible peaks, and these vials were dried at 80° C in a forced air oven. After drying, 20 ml of aqueous scintillation fluid were added to each vial, and counted as described for betaine. A standard solution of acetic, propionic, and butyric acids were prepared to determine the location of peaks and the percentage of recovery of the acids. The vials were corrected for milli-equivalents of ethanolic sodium hydroxide used to titrate the background acid and for endogenous radioactivity.

4. Amino Acids

Amino acids were separated and identified by two-dimensional paper chromatograms using a modification of Oser's Method (Oser, 1965). Five μl of clear supernatant and 5 μl of unlabeled glycine standard (106 mg of glycine diluted to 100 ml with distilled water) were used for each analysis. Glycine was added to all sheets for reference and ease in identifying the glycine spot. All sheets were allowed to air dry over night before they were placed in the next solvent or sprayed with ninhydrin. Paper chromatographic analyses were conducted using standard solutions of the following amino

acids--alanine, arginine, aspartic acid, glutamic acid, glycine, methionine, serine, and threonine, and ^{14}C-betaine and ^{14}C-acetic acids.

From one set of paper chromatograms, the glycine spot was removed and radioactivity determined by placing the spot in a scintillation vial and adding 20 ml of aqueous scintillation fluid and counting as described for betaine. The counts were corrected for background and quenching.

From another set of paper chromatograms, the 17.5 by 17.5 centimeters sheets were cut into 3.5 centimeter strips. The strips were taped together and the whole sheet analyzed for radioactivity using a Packard Radiochromatogram scanner.[10]

5. Total Nitrogen and Ammonia

Three ml of the 10 ml of fermentation flask content (Figure 2) collected each 200-minutes were placed in the distillation chamber of a LABCONCO Micro-Distillation apparatus.[11] Ten ml of 10N sodium hydroxide were added to the sample, and the volatile compounds distilled into 5 ml of boric acid-indicator solution. The boric acid-indicator solution contained 40 gm of boric acid in one liter of distilled water plus 25 ml of stock indicator solution (1.25 gm of methyl

[10] Model 7200A, manufactured by Packard Instrument Company, Inc., 2200 Warrenville Road, Downer Grove, Illinois.

[11] Purchased from LABCONCO Corporation, 8811 South Prospect, Kansas City, Missouri.

red and 0.825 gm of methylene blue in 1 liter of 90.0% ethanol). This fraction was called the "Basic Volatile Fraction" (BVF) because it may contain, in addition to ammonia, trimethylamine and other volatile compounds. The boric acid-indicator solution was titrated with 0.02N HCl to determine the approximate amount of nitrogen. Ammonia was determined by correcting the total N for the N in trimethylamine.

A comment should be made concerning the determination of ammonia. The basic volatile fraction values were determined on flask contents without any processing. The samples were capped and cooled immediately after collection to prevent the evaporation of ammonia, and analyzed as soon as possible there after. The flask contents contained small particles of feed and debris which made the solution very difficult to pipet resulting in variable values.

For ^{15}N determination, the corresponding BVF's from the 3 low-energy ration trials and the corresponding BVF's from the 3 high-energy ration trials were pooled, acidified with 0.02N HCl, and condensed to approximately 10 ml. These samples were pooled because the same amount of ^{15}N-betaine was added to all flask. This condensed solution was placed in the distillation chamber of the microdistillation apparatus, and approximately 10 ml of 10N sodium hydroxide were added. The volatile compounds were distilled into 15 ml of boric acid solution without indicator. The boric acid solution

was condensed to approximately 5 ml for ^{15}N determination. The percentage of ^{15}N was determined on an Isotope Ratio Mass Spectrometer[12] by Colorado State University Chemistry Department personnel.[13]

Total nitrogen was determined by micro-kjeldahl analyses. One gm of clear supernatant was digested with 3 ml of concentrated sulfuric acid and about 1.5 gm of catalyst (100 gm of K_2SO_4, 1 gm of powdered selenium, and 10 gm of $CuSO_4$). This mixture was digested until clear, e.g., about 45 minutes. The digested mixture was cooled and distilled into boric acid-indicator solution as described previously for the basic volatile fraction. The percentage of ^{15}N was determined by Colorado State University Chemistry Department personnel.

6. Gas Analyses

The carbon dioxide was precipitated from the low-carbonate NaOH in the fritted cylinder gas washing bottles as barium carbonate ($BaCO_3$), using a solution containing 61.0 gm of $BaCl_2 \cdot 2H_2O$ and 267.5 gms of NH_4Cl diluted to a liter with carbon dioxide-free distilled water. The NH_4Cl was added to maintain the alkaline pH for better precipitation.

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[12] Model MS 20, manufactured by Associated Electronic Industries, Scientific Apparatus Division, Boston Doct Road, Urmstom, Manchester, England.

[13] Dr. C. Gerald Warren and Mr. Clyde Webster.

The precipitate was heated to boiling, cooled, and was collected on a washed and tared 2.4 centimeter diameter glass fiber paper in a Tracerlab E-8B precipitation apparatus.[14] The precipitate was washed with distilled water and dried with acetone. The approximate amount of carbon dioxide produced was determined by the weight of $BaCO_3$ precipitate.

The radioactivity of the carbon dioxide was determined by counting planchettes of $BaCO_3$ as infinite thickness in a nuclear-Chicago Gas Flow Automatic, Low Background, Planchett Counter.[15] Corrections were made for background and efficiency.

The methane was collected from the large bottles by refilling the bottles with distilled water forcing the gas through a Haskins Model 303 Electric Furnace,[16] oxidizing the methane to carbon dioxide and collecting the carbon dioxide in 2N low carbonate NaOH. The efficiency of the system was determined by oxidizing a liter of 99% methane gas.[17] The amount and radioactivity in the methane was

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[14]Purchased from TRACERLAB, 1601 Trapelo Road, Waltham, Massachusetts.

[15]Model 4334, purchased from Nuclear-Chicago Corporation, Des Plaines, Illinois.

[16]Manufactured by Haskins, Detroit, Michigan.

[17]Purchased from Matheson Gas Products, P. O. Box 96, Joliet, Illinois.

determined from the amount and radioactivity in the carbon dioxide produced as described previously.

7. Total Activity in the Fermentation Flask

The total activity in the fermentation flask was determined by pipetting 0.5 ml of the clear supernatant into a scintillation vial. Twenty ml of aqueous scintillation fluid were added and the vial counted as described for betaine.

Expressions of Radioactivity

The efficiency (E) of each batch of aqueous scintillation fluid (ASF) was determined by the use of a quench curve prepared with a standard amount of a known radioactive source (^{14}C-toluene with 9800 CPM per 0.05 ml) in a standard volume of fluid. The only variables being the amount of ASF and the quenching substance, i.e., ASF was replaced with toluene and 70 per cent acetone for the trimethylamine and betaine analysis, respectively, and with distilled water for all other analyses.

Disintegrations per minute (DPM) was determined by the following formula:

$$DPM = \frac{CPM - Background\ CPM}{E}$$

CPM = counts per minute as read from the radioactive counting instrument.

Microcuries (μCi) were calculated by multiplying the DPM by 4.55×10^{-7}.

Gram atom carbon (gaC) is equal to the weight of $BaCO_3$ precipitate divided by the molecular weight of $BaCO_3$ because there is one gaC of carbon in one mole of $BaCO_3$.

Microcuries per gram atom carbon were determined by the following formula: BgCPM = Background CPM

$$\mu Ci/gaC = (CPM - BgCPM)\ [0.035\ \mu Ci/(gaC)(CPM)]$$

Microcuries of radioactivity recovered were determined by the following formula:

1. For CO_2 and CH_4

$$\mu Ci\ ^{14}C \text{ recovered} = (\mu Ci/gaC)(gaC)$$

2. For VFA

$$\mu Ci\ ^{14}C \text{ recovered} = (\mu Ci/meq)(meq)$$

Specific activity of CO_2 and CH_4 was expressed as μCi/gaC. For VFA, specific activity was expressed as μCi/meq.

Per cent ^{15}N was determined using the following formula:

$$\%^{15}N = 100\ \left[{}^{15}N/({}^{15}N + {}^{14}N)\right]$$

The ratio of ^{14}N to ^{15}N can be approximated by the following formula:

$$1/(\%^{15}N/100) \approx \text{atoms } {}^{14}N/\text{atoms } {}^{15}N.$$

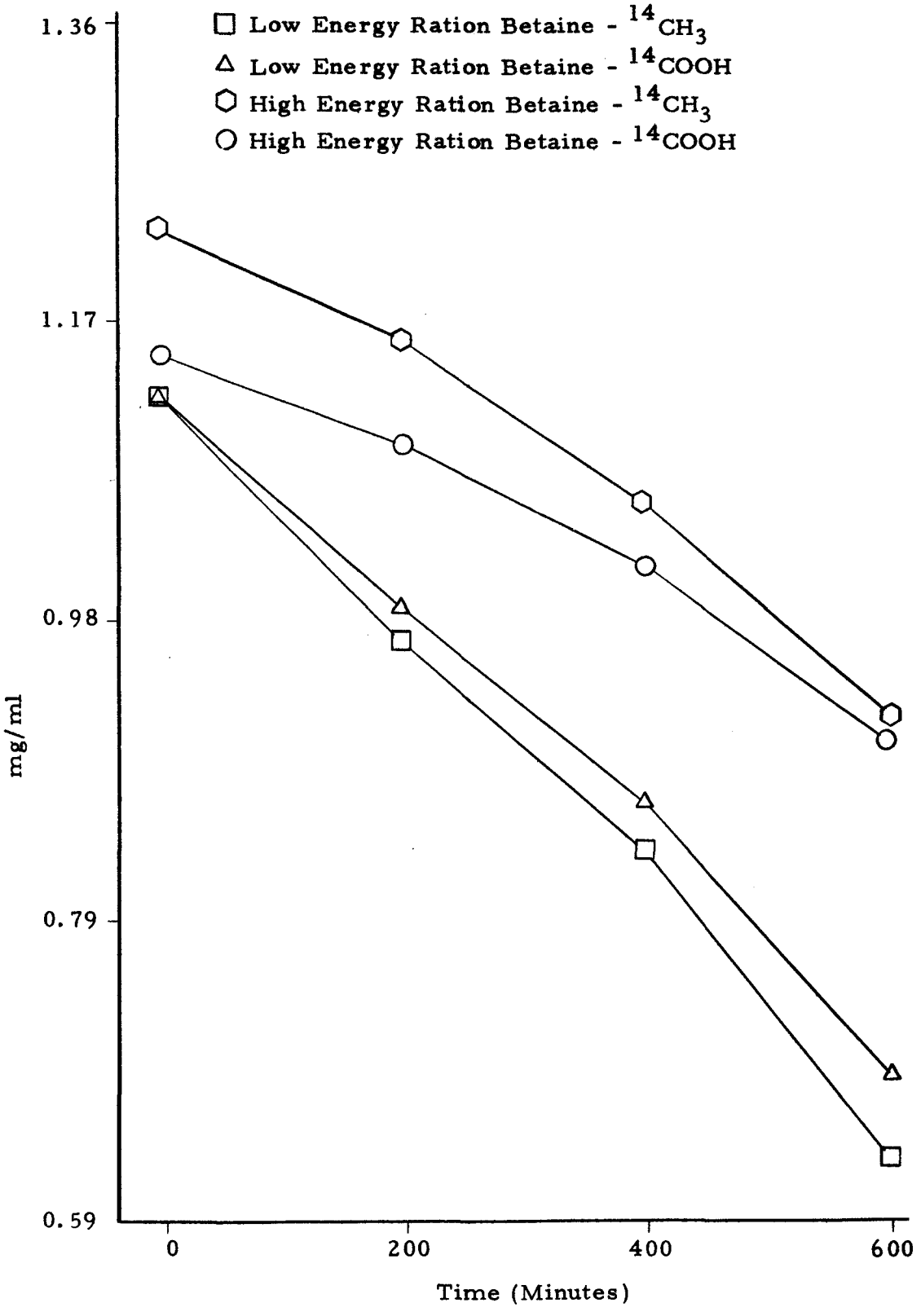
CHAPTER IV

RESULTS

Metabolism of Betaine

The concentrations of betaine with respect to time are shown in Figure 5 (Appendix Table 2). The initial concentrations of betaine were 1.12 ± 0.03 mg/ml and 1.19 ± 0.14 mg/ml for low-energy and high-energy rations, respectively. An analysis of variance showed that there were no significant differences ($P < 0.05$) between the initial betaine concentration for the two rations. The final betaine concentrations were 0.66 ± 0.13 mg/ml and 0.91 ± 0.08 mg/ml for the low-energy and high-energy rations, respectively. An analysis of variance showed that there were highly significant differences ($P < 0.005$) between the final betaine concentrations for the two rations. As one would expect, initial concentrations were essentially the same. However, if the betaine was metabolized at different rates, depending upon the concentration of available energy, then one would expect different final betaine concentrations. Because the interest was not only in the qualitative metabolism of betaine, but also the quantitative aspects, the results of the high and low energy rations (substrates) were analyzed separately.

Figure 5. Average Concentration of Betaine in the Flask at each Collection.


Low Energy Ration Betaine - $^{14}CH_3$
Low Energy Ration Betaine - $^{14}COOH$
High Energy Ration Betaine - $^{14}CH_3$
High Energy Ration Betaine - $^{14}COOH$
1.36
1.17
0.98
0.79
0.59
mg/ml
0
200
400
600
Time (Minutes)

A linear regression analysis of the data was performed. The results showed that the disappearance rate for betaine was 0.05 mg/ml/hour and 0.03 mg/ml/hour for the low-energy and high-energy ration, respectively.

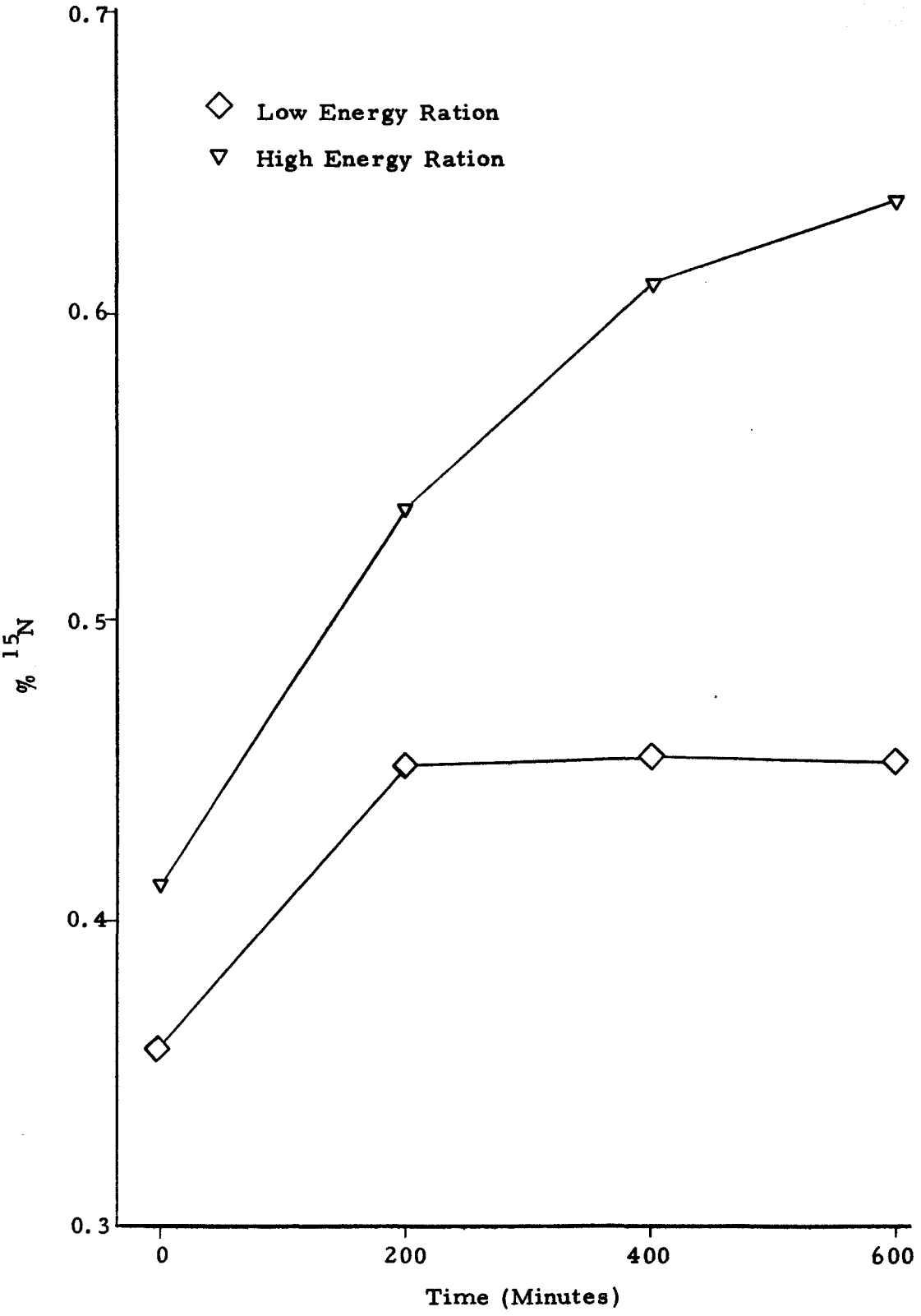
The ^{14}C-activity of betaine with respect to time is given in Appendix Table 3. The ^{14}C-activity data of betaine agreed with the betaine concentrations above. An analysis of variance on the per cent of ^{14}C-betaine that disappeared was conducted to determine if the position of the labeled ^{14}C had any effect on betaine metabolism. The results showed a non-significant difference ($P < 0.05$) in the per cent of methyl-labeled and carboxyl-labeled betaine that disappeared for both rations.

Pathways for Betaine Metabolism

Ammonia Analysis

The average per cent ^{15}N ammonia in the flask at each collection is shown in Figure 6 (Appendix Table 6). The per cent ^{15}N in ammonia increased throughout the 600-minute incubation period on the high-energy ration, i.e., increased from 0.41 to 0.64 per cent. The per cent ^{15}N in the ammonia on the low-energy ration increased only during the first 200-minute incubation period (0.39% to 0.45%). The data (Figure 6) suggests that ammonia was produced from ^{15}N-betaine throughout the 600-minute incubation period on the high-energy ration, but only during the first 200-minutes on the low-energy ration.

Figure 6. Per Cent ^{15}N in Ammonia in the Flask at Each Collection.


0.7
0.6
0.5
0.4
0.3
% ^{15}N
Low Energy Ration
High Energy Ration
0
200
400
600
Time (Minutes)

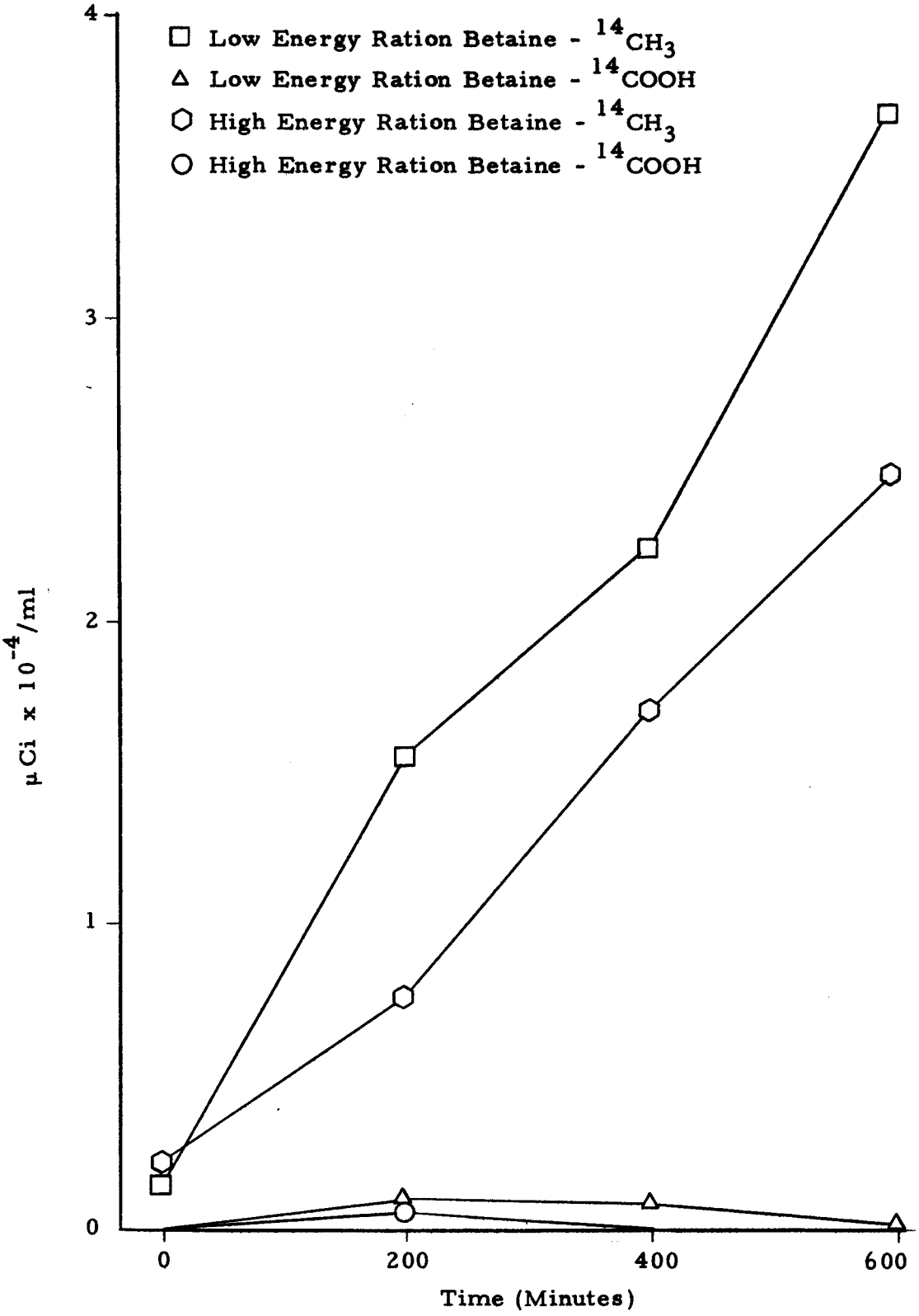
Amino Acid Analysis

The amino acids were separated by two-dimensional paper chromatography. Added unlabeled glycine made it possible to identify the glycine spot. Chromatographic sheets were analyzed by removing the glycine spot and counting in the scintillation counter as described for betaine. After the counts were corrected for background, they failed to show any ^{14}C labeled glycine. The two-dimensional paper chromatographic sheets that were analyzed on the radiochromatogram scanner also failed to show radioactivity in the glycine spot on any other area.

Trimethylamine Analysis

The ^{14}C-activity in trimethylamine is given in Figure 7 (Appendix Table 9). Very little, if any, ^{14}C-labeled trimethylamine was recovered from the ^{14}C-carboxyl labeled betaine; therefore, only the ^{14}C-methyl-labeled betaine data was considered. The initial ^{14}C-activity in trimethylamine was 1.4 ± 1.2 $\mu Ci \times 10^{-5}$/ml and 1.9 ± 1.6 $\mu Ci \times 10^{-5}$/ml for low-energy-high-energy rations, respectively. The final ^{14}C-activity in trimethylamine was 36.5 ± 34.7 $\mu Ci \times 10^{-5}$/ml and 24.9 ± 13.5 $\mu Ci \times 10^{-5}$/ml for low-energy and high-energy rations, respectively. There is no explanation for the large standard deviation observed for the final ^{14}C-activity in trimethylamine. This large variation was also observed in the actual trimethylamine concentrations (Appendix Table 8).

Figure 7. Average Amount of Activity in Trimethylamine in the Flask at Each Collection.


Low Energy Ration Betaine - $^{14}CH_3$
Low Energy Ration Betaine - $^{14}COOH$
High Energy Ration Betaine - $^{14}CH_3$
High Energy Ration Betaine - $^{14}COOH$
μCi x 10^{-4}/ml
0
1
2
3
4
0
200
400
600
Time (Minutes)

The per cent ^{14}C that disappeared from the methyl-labeled betaine recovered in trimethylamine was 16.6 and 14.0 for the low-energy and high-energy ration, respectively, for the 600-minute incubation period (Table 4).

Volatile Fatty Acids Analysis

The concentration of acetic, propionic and butyric acids in the flask at each collection are shown in Appendix Tables 11 and 12. The data indicated that the concentration of these volatile fatty acids increased with time. More volatile fatty acids were produced on the high-energy ration than on the low-energy ration.

Very little, if any, labeled volatile fatty acids were synthesized from the methyl-labeled betaine. Appendix Table 13 gives the specific activity and ^{14}C recovered in the volatile fatty acids from the carboxyl-labeled betaine. Practically all of the radioactivity was found in the acetic acid. Figure 8 shows that the average concentration of acetic acid on the high-energy ration increased throughout the 600-minute incubation period, while on the low-energy ration acetic acid increased only during the first 200-minute incubation period. The synthesis of acetic acid coincided with the synthesis of ^{15}N-ammonia (Figure 6).

The amount of radioactivity recovered in acetic acid (Figure 9) increased throughout the incubation period. The per cent radioactivity, that disappeared from the carboxyl-labeled betaine, recovered in

Table 4. Summary of the Initial ^{14}C Radioactivity Recovered.

Time (Minutes)	Compound	Low Energy Ration				High Energy Ration			
		Betaine-$^{14}CH_3$		Betaine-$^{14}COOH$		Betaine-$^{14}CH_3$		Betaine-$^{14}COOH$	
		μCi^1	%	μCi^1	%	μCi^1	%	μCi^1	%
0	Betaine	69.09	100.00	78.23	100.00	82.04	100.00	85.54	100.00
	Trimethylamine	0.14	-----	----	-----	0.19	------	-----	------
	Acetic Acid	-----	------	0.01	------	-----	------	0.14	------
200	Betaine	62.92	91.06	68.75	87.88	80.13	97.67	81.84	95.67
	Trimethylamine	1.54	2.23	-----	------	0.76	0.93	-----	------
	Acetic Acid	-----	------	1.65	2.11	-----	------	3.58	4.19
	Carbon Dioxide	0.12	0.18	0.05	0.06	0.03	0.03	0.05	0.06
	Methane	1.92	2.77	0.11	0.14	0.05	0.06	0.04	0.05
400	Betaine	55.79	80.75	53.46	68.34	72.72	88.64	76.88	89.88
	Trimethylamine	2.24	3.24	-----	------	1.70	2.07	-----	------
	Acetic Acid	-----	------	5.04	6.44	-----	------	7.15	8.36
	Carbon Dioxide	1.12	1.62	0.12	0.16	0.35	0.43	0.05	0.06
	Methane	4.11	5.94	0.23	0.30	2.84	3.46	0.03	0.04
600	Betaine	47.17	59.59	38.09	49.69	64.20	78.36	66.71	77.99
	Trimethylamine	3.65	5.28	-----	------	2.49	3.04	-----	------
	Acetic Acid	-----	------	9.54	12.19	-----	------	12.87	15.05
	Carbon Dioxide	1.16	1.67	0.02	0.11	0.55	0.68	0.13	0.15
	Methane	3.87	5.60	0.02	0.03	3.87	4.72	0.12	0.14

[1] $\mu Ci \times 10^{-4}/ml$

Figure 8. Average Concentration of Acetic Acid in the Flask at Each Collection.

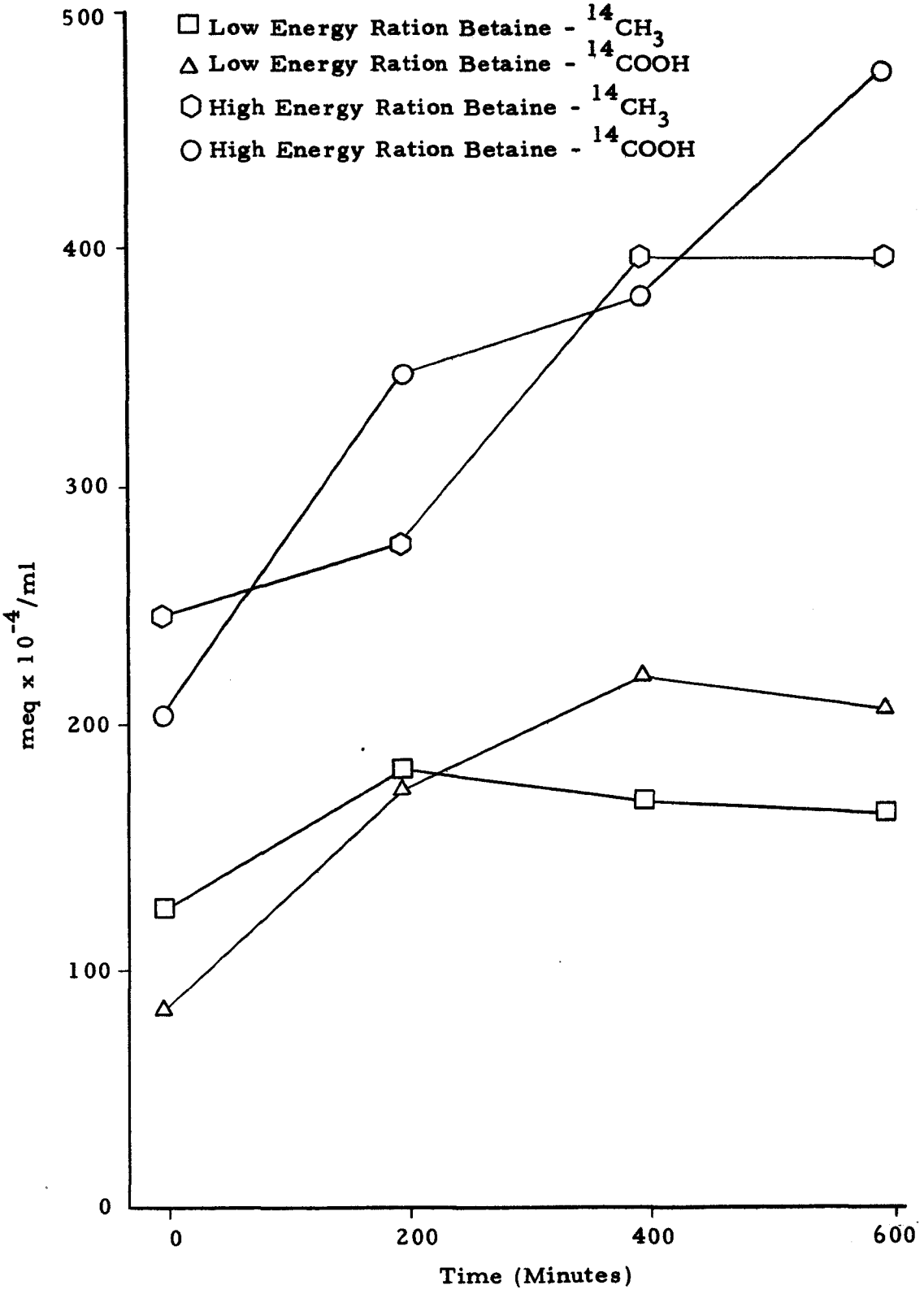
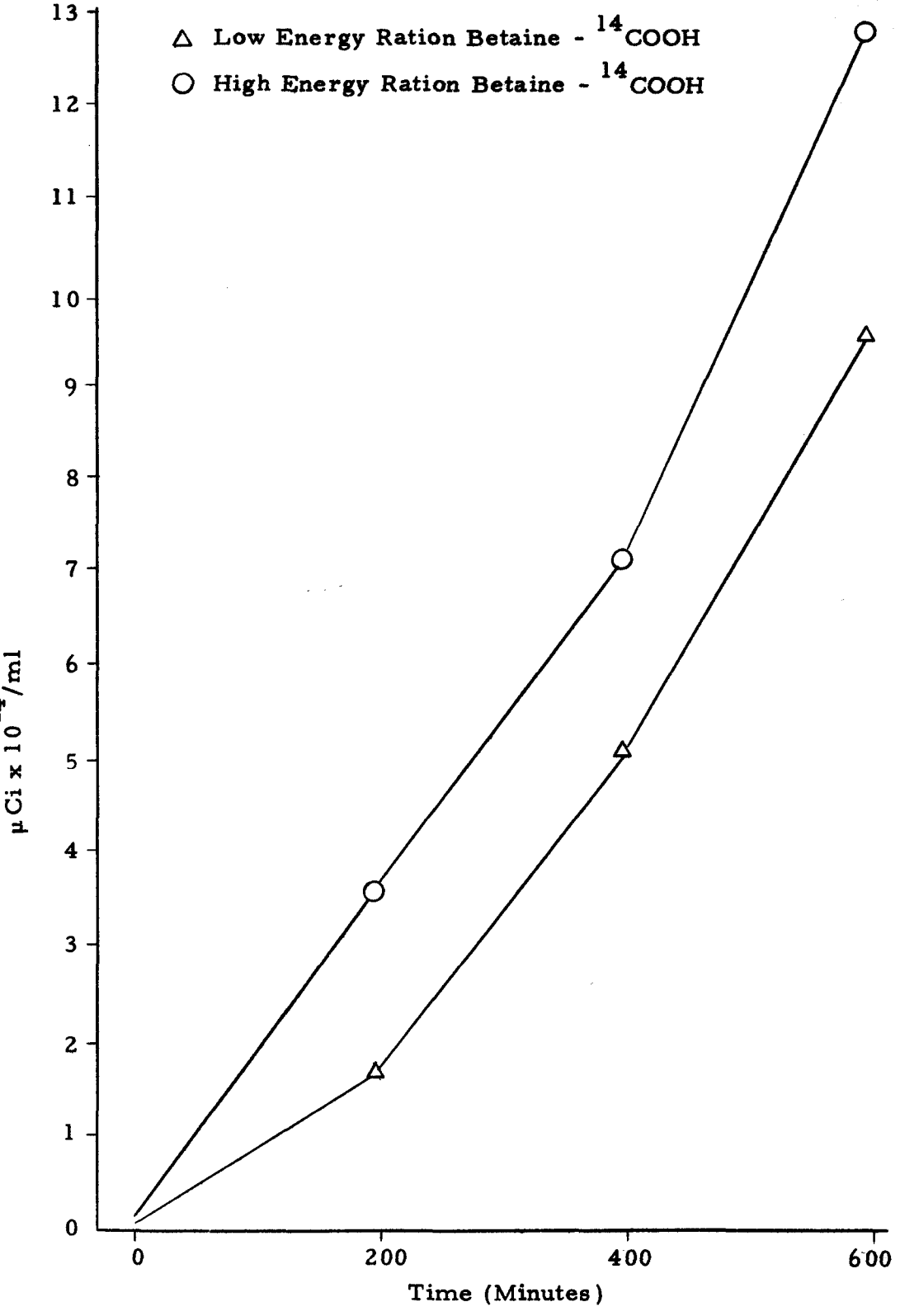

Low Energy Ration Betaine - $^{14}CH_3$
Low Energy Ration Betaine - $^{14}COOH$
High Energy Ration Betaine - $^{14}CH_3$
High Energy Ration Betaine - $^{14}COOH$
500
400
300
200
100
0
meq x 10^{-4}/ml
0
200
400
600
Time (Minutes)

Figure 9. Average Amount of Radioactivity Recovered in Acetic Acid from Carboxyl Labeled Betaine.


△ Low Energy Ration Betaine - ^{14}COOH
○ High Energy Ration Betaine - ^{14}COOH
μCi x 10^{-4}/ml
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0
200
400
600
Time (Minutes)

acetic acid was 24.37 and 68.34 for the low-energy and high-energy ration, respectively, for the 600-minute incubation period (Table 4).

Carbon Dioxide Analysis

The amount of radioactivity recovered in the carbon dioxide is given in Figure 10 (Appendix Table 15). This data showed that the synthesis of carbon dioxide increased with time and that the carbon dioxide was being synthesized from the methyl groups of betaine. The total radioactivity recovered in carbon dioxide accounted for 5.29 and 3.08 per cent of the radioactivity which disappeared from betaine for low-energy and high-energy rations, respectively.

Methane Analysis

Methane was formed from the methyl-labeled betaine. The amount of radioactivity recovered in methane is shown in Figure 11 (Appendix Table 17). The amount of radioactive methane increased during the second 200-minute period of incubation on both rations. The differences were in the last 200-minute incubation period where the low-energy ration showed no change while the high-energy ration continue to increase.

The total amount of radioactivity recovered in methane was 17.65 and 21.69 per cent of the initial radioactivity added for low-energy and high-energy rations, respectively.

Figure 10. Average Amount of ^{14}C Recovered in Carbon Dioxide.

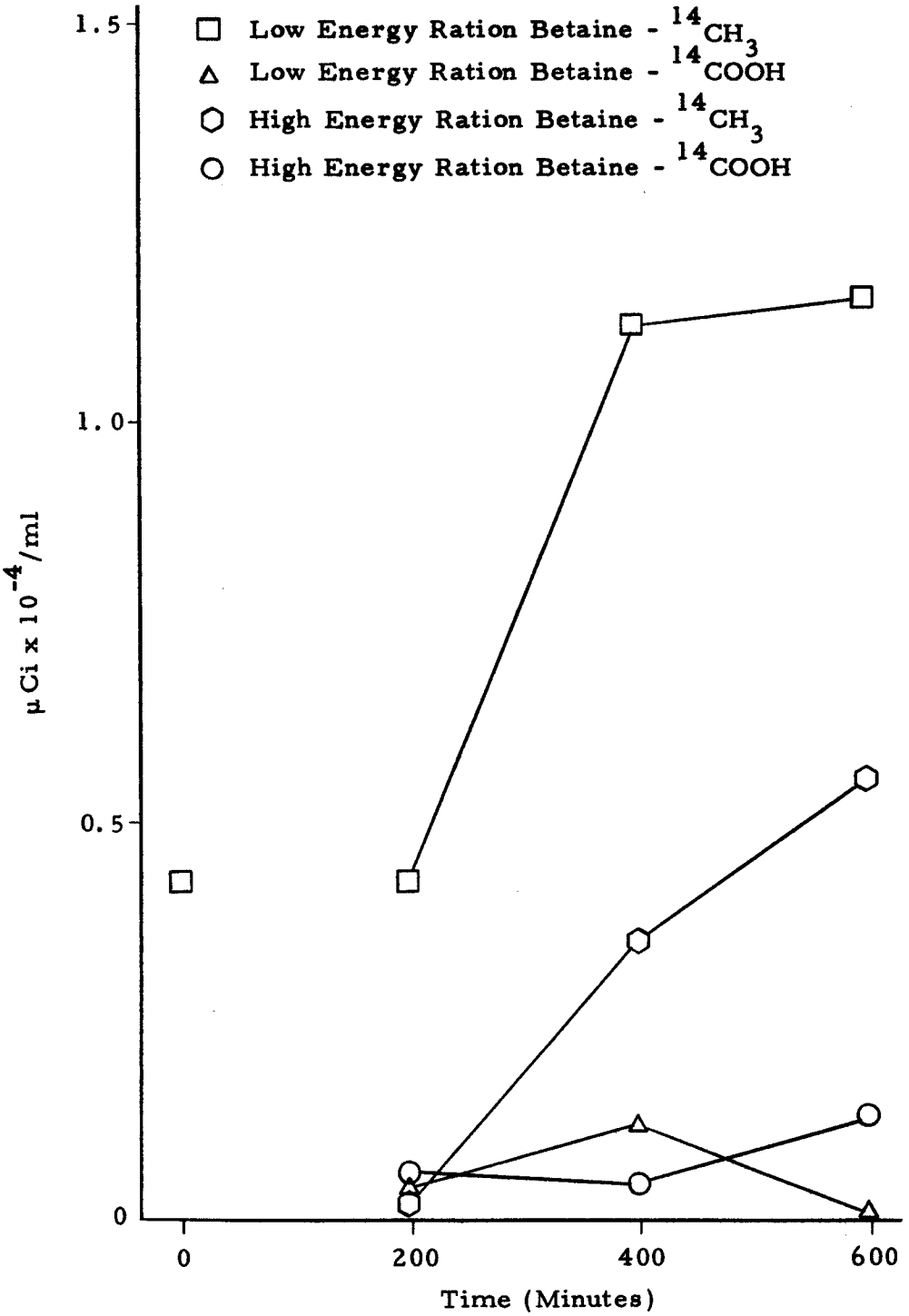
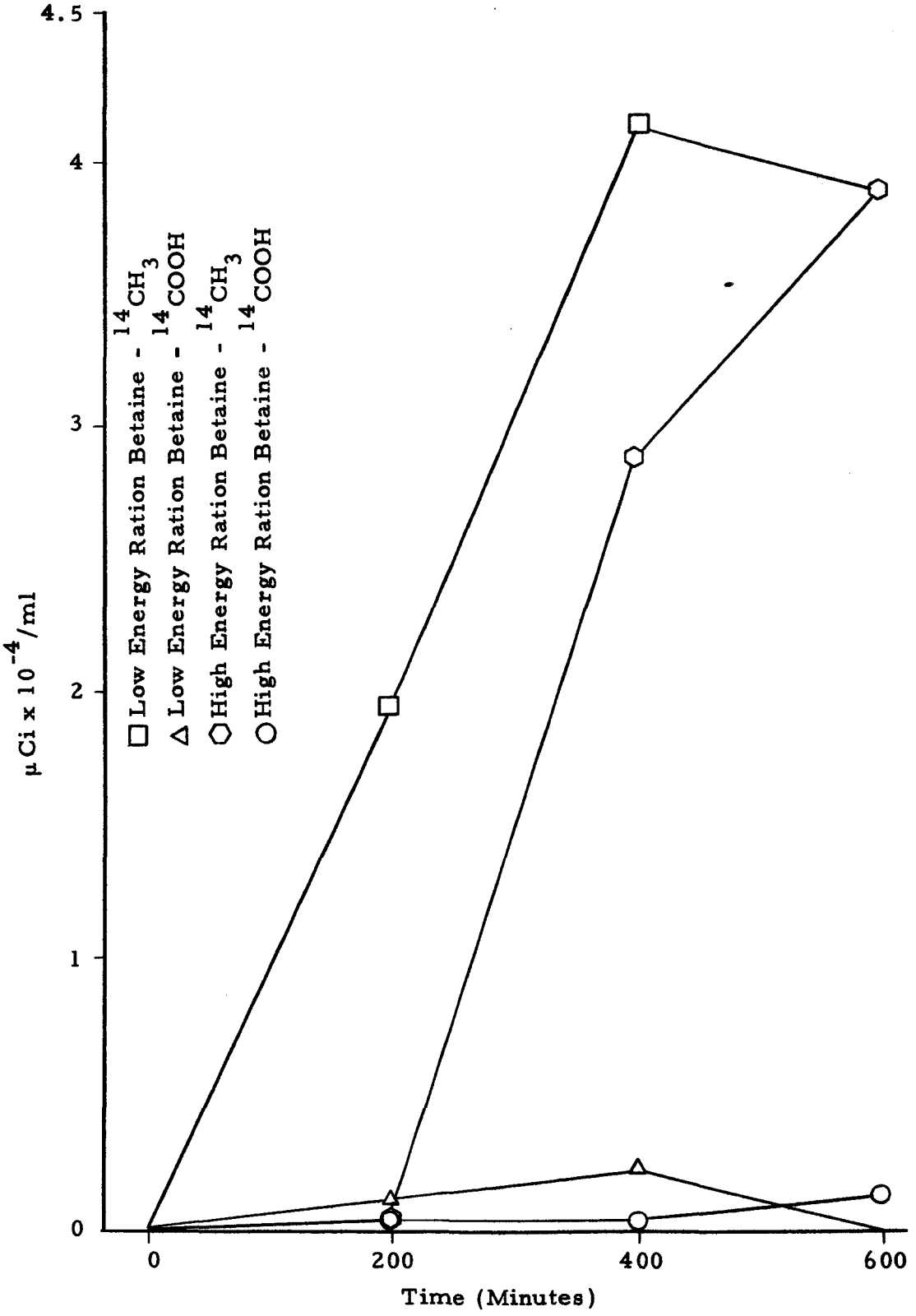

Low Energy Ration Betaine - $^{14}CH_3$
Low Energy Ration Betaine - $^{14}COOH$
High Energy Ration Betaine - $^{14}CH_3$
High Energy Ration Betaine - $^{14}COOH$
1.5
1.0
0.5
0
$\mu Ci \times 10^{-4}/ml$
0
200
400
600
Time (Minutes)

Figure 11. Recovery of ^{14}C in Methane.


4.5
4
3
2
1
0
μCi x 10^-4/ml
□ Low Energy Ration Betaine - 14CH3
△ Low Energy Ration Betaine - 14COOH
⬡ High Energy Ration Betaine - 14CH3
○ High Energy Ration Betaine - 14COOH
0
200
400
600
Time (Minutes)

CHAPTER V

DISCUSSION

Metabolism of Betaine

The data from the betaine analysis (Figure 5 and Appendix Table 2) showed the final concentration of betaine to be significantly lower than the initial concentration. These concentrations indicated that betaine was being metabolized. This observation agreed with previous research indicating that rumen microorganism can metabolize betaine (Velish and Stanek, 1904-5; Voltz, 1907; Kohrausch, 1911, 1912; Brigl and Benedict, 1933; Davies, 1936; Linder, 1967, and Mitchell, 1968).

One of the purposes of this study was to determine if the energy level of the ration had any effect on betaine metabolism. This study showed that the per cent betaine metabolized was greater on the low-energy ration than on the high energy ration. The per cent betaine metabolized during the 10-hour incubation was 41.4 and 23.7 for the low-energy and high-energy rations, respectively. The disappearance rates for betaine were 0.05 mg/ml/hr and 0.03 mg/ml/hr for the low-energy and high-energy rations, respectively. Since the fluid in the fermentation flask was only 60 per cent rumen fluid, a higher disappearance rate should occur *in vivo*, i.e., 0.083 and

0.050 mg/ml/hr. A possible explanation for the differences in betaine utilization may be obtained from the total nitrogen analysis. The data in Appendix Table 5 show that the initial concentration of nitrogen was 1.21 ± 0.12 mg/ml and 2.75 ± 0.20 mg/ml for low-energy and high-energy rations, respectively. The initial concentration of betaine nitrogen (0.14 mg/ml) was approximately the same for both rations. In order to meet the microorganism nitrogen requirement, the microorganism may have had to metabolize more betaine on the low-energy ration. Voltz (1907) and Davies (1936) reported that the amount of betaine nitrogen excreted by ruminants depended on the level of nitrogen intake.

The higher betaine metabolism on the low-energy ration suggested that the microorganism prefer to metabolize high-energy compounds first and to metabolize betaine after the high-energy compounds are utilized. The data dhowed that betaine metabolism continue throughout the 600-minute incubation period (Figure 5), but the synthesis of ammonia (Figure 6) and acetic acid (Figure 8) leveled off after the first 200-minutes of incubation on the low-energy ration. This leveling off indicated that these compounds were being utilized by the microorganism for microbial energy and growth.

Pathways for Betaine Metabolism

Previous results (Mitchell, 1968) showed that betaine was metabolized by rumen bacteria to trimethylamine, methane, carbon

dioxide, and acetate. From his research, Mitchell (1968) concluded that rumen microorganism cleaved the betaine molecule into trimethylamine and acetic acid.

$$(CH_3)_3NCH_2COOH \xrightarrow[H]{} (CH_3)_3N + CH_3COOH$$

This pathway does not account for the synthesis of methane and carbon dioxide (Mitchell, 1968), and the utilization of the betaine nitrogen by ruminants (Linder, 1967, and Davies, 1936). Davies (1936) showed the rapid and almost quantitative excretion of trimethylamine oxide in the unchanged form and also the rapid excretion of trimethylamine as its oxide proved that this was the mode of excretion of tertiary nitrogenous bases in the ruminants, and that with these simple bases, no attack on the methyl groups attached to the nitrogen occurred. Davies (1936) fed trimethylamine hydrochloride and trimethylamine oxide to cows and obtained 93.0 and 95.6 per cent recovery of the tertiary nitrogen respectively, in 16-hour period of urine collection. Davies (1936) stated that the greater utilization of betaine by animals fed regularly on a betaine-containing feed was explained by their greater capacity to metabolize the nitrogen to urea and not to trimethylamine oxide. This process depended on the level of intake of betaine nitrogen. In Mitchell's (1968) study, the amount of radioactivity recovered in trimethylamine was 23.65 and 23.4 per cent for the _in vitro_ and _in vivo_ experiments, respectively.

Therefore, Mitchell (1968) reported that his data supported another pathway whereby betaine was demethylated to glycine which, in turn, was deaminated to ammonia and acetic acid.

$$(CH_3)_3NCH_2COOH \xrightarrow[5H]{} 3CH_4 + H_2NCH_2COOH$$

$$H_2NCH_2COOH \xrightarrow[2H]{} NH_3 + CH_3COOH$$

This pathway can account for the utilization of the betaine nitrogen and the production of methane. The synthesis of carbon dioxide could be an intermediate compound in the demethylation process (this will be explained later in this chapter).

A way to show the existence of the demethylation pathway was to account for the radioactivity which disappeared from the carboxyl-labeled betaine in glycine and acetic acid. The radioactivity which disappeared from the methyl-labeled would be accounted for in methane and its intermediate compound(s), i.e., carbon dioxide. The analysis for glycine failed to detect any ^{14}C-glycine. Wright and Hungate (1967) reported that rumen microorganism rapidly metabolized glycine at rates varying from 0.014 to 0.241 micromole of glycine per ml per min. The main metabolic products were carbon dioxide, acetic acid, and ammonia. They found very little (less than 3.4%) ^{15}N- and ^{14}C-glycine incorporated into bacterial protein. This is a possible explanation for the failure to detect ^{14}C-glycine in this

experiment. Wright and Hungate with the aid of carboxyl-labeled and methylene-labeled glycine showed that the carbon dioxide came mainly from the carboxyl carbon of glycine, whereas, acetic acid was derived partly from the methylene carbon and partly from the carbon dioxide.

Another method of proving the existence of the demethylation pathway was by using ^{15}N-betaine and detecting an increase in ^{15}N-glycine. Since no ^{14}C-glycine was detected, the glycine was not analyzed for ^{15}N.

It was realized that any ^{14}C- and ^{15}N-glycine detected could have been synthesized by the bacteria from intermediate compounds, such as ammonia and acetic acid. Therefore, the glycine would have had to be considered with the other labeled products.

Since Wright and Hungate (1967) showed that glycine was metabolized very rapidly to carbon dioxide, acetic acid, and ammonia, the sample was analyzed for these metabolic products. There was very little, if any, carbon dioxide synthesized from the carboxyl-labeled betaine.

Of the compounds analyzed for ^{14}C activity in this study, acetic acid was the only ^{14}C compound isolated from the carboxyl-labeled betaine. The amount of ^{14}C disappearing from the carboxyl-labeled betaine and recovered in acetic acid was 24.37 and 68.34 per cent for the low-energy and high-energy rations, respectively. The synthesis

of ^{14}C-acetic acid can occur in both pathways. Approximately 100 per cent of the ^{14}C-activity that disappeared from the carboxyl-labeled betaine should have been recovered as acetic acid by way of the trimethylamine-acetic acid pathway. A lesser amount of ^{14}C-activity recovered in acetic acid was expected on the demethylation pathway because there are several intermediate compounds, such as dimethylglycine, methylglycine, and glycine, which would contain some ^{14}C-activity from the carboxyl-labeled betaine. The per cent recovered in acetic acid in this study would support the demethylation pathway, or indicate that demethylation was occurring via other pathways, one being cleavage into trimethylamine and acetic acid.

There are two possible explanations for the failure to detect ^{14}C-carbon dioxide from the carboxyl-labeled betaine. One being that the glycine was deaminated to acetic acid instead of the acetic acid being synthesized as reported by Wright and Hungate (1967). A second explanation being that the carbon dioxide synthesized was not released into the solution, but was retained and used by the bacteria for synthesis of other compounds.

The ammonia recovered in this research showed an increase in ^{15}N. This proved that ^{15}N-ammonia was being synthesized from the ^{15}N-betaine. The study by Davies (1936) ruled out the possibility that this ^{15}N-ammonia came from the metabolism of trimethylamine. This synthesis of ^{15}N-ammonia supported the demethylation

hypothesis, but does not rule out another unknown pathway for betaine metabolism.

The data from the in vitro incubation using methyl-labeled betaine showed that at least three compounds were synthesized from the methyl-end of betaine. The compounds were methane, carbon dioxide, and trimethylamine and they accounted for 19.67, 4.19, and 15.3 per cent of the ^{14}C-activity that disappeared from the ^{14}C-betaine, respectively. Since trimethylamine is an end-product, this small recovery suggests that cleavage to trimethylamine was a minor pathway in betaine metabolism. This observation agreed with the results of Mitchell (1968) who recovered 23.4 per cent of his ^{14}C-activity in trimethylamine and Davies (1936) who recovered between 14 to 43 per cent of the betaine nitrogen in trimethylamine.

The synthesis of methane added support to the demethylation hypothesis. Although approximately one-fifth of the ^{14}C-activity was recovered in methane, this could be the major pathway for betaine metabolism because there are other explanations for the unaccounted ^{14}C-activity, e.g. (1) the methyl-groups removed could be used by the bacteria to synthesize other compounds, and (2) the labeled methyl-group could be in compounds such as dimethylglycine and methylglycine, depending upon the specific methyl groups and the number of methyl groups removed during demethylation. This small recovery of methane could also mean that there are other pathways

for betaine metabolism other than the cleavage to trimethylamine and acetic acid.

An unanswered question of this research and Mitchell's (1968), as well, is the mechanism by which carbon dioxide was formed from the methyl groups of betaine. Shiek (1968) found, in a marine bacteria, that formaldehyde was synthesized from methyl-labeled betaine. Mackenzie (1950) and Mackenzie and Frisell (1958) found that ^{14}C-formate and ^{14}C-carbon dioxide was produced in the oxidation of methyl-labeled dimethylglycine and methyl-labeled methylglycine by the rat liver mitochondria. Most of the formate and carbon dioxide produced in the oxidation of the methyl groups arose by the way of formaldehyde. A possible explanation for the appearance of carbon dioxide is a two-step oxidation reaction:

$$CH_3^+ + O \longrightarrow HCOH + H^+$$

$$HCOH + O_2 \longrightarrow CO_2 + H_2O$$

If this reaction was occurring in the rumen, it could help maintain the anaerobic condition in the rumen.

The data from this research added support to that of Mitchell's (1968) in that betaine was cleaved by the rumen organisms to trimethylamine and acetic acid, but also that this was a minor pathway for betaine metabolism. There are three observations which would suggest that betaine was being demethylated to glycine and the glycine

quickly metabolized to ammonia and acetic acid; (1) the synthesis of labeled methane from the methyl-labeled betaine. The work by Davies (1936) showed that trimethylamine was not metabolized by ruminants, but was absorbed and secreted in the urine unchanged. Therefore, the methane was synthesized from the demethylation of betaine. (2) The synthesis of ^{15}N-ammonia from the ^{15}N-betaine. The work of Wright and Hungate (1967) showed that glycine was metabolized by rumen organisms with ammonia being one of the end-products. (3) The synthesis of ^{14}C-acetic acid from the carboxyl labeled betaine. Acetic acid was an end-product on both pathways, since the per cent acetic acid synthesized exceeded the per cent trimethylamine synthesized. The additional acetic acid could have come from glycine metabolism.

There are two observations in this research that fail to support the hypothesis that betaine was demethylated to glycine and the glycine was converted to acetic acid and ammonia; (1) The failure to detect ^{14}C labeled glycine. In this experiment, betaine was being metabolized at approximately 0.3 to 0.4 micromoles/ml/min (assuming that this was the only pathway for betaine metabolism). Wright and Hungate (1967) reported that glycine was metabolized at a rate of 0.014 to 0.241 micromole/ml/min. Two probable reasons for the failure to detect labeled glycine were that glycine was being metabolized at a rapid rate and could be found only inside the

microbial cells as reported by Wright and Hungate (1967). (2) The failure to recover significant labeled carbon dioxide from the carboxyl-labeled betaine. If the glycine was being metabolized as reported by Wright and Hungate (1967), one of the end-products should have been labeled carbon dioxide. Very little, if any, labeled carbon dioxide was recovered from the carboxyl labeled betaine.

The 68.34 per cent recovery of the ^{14}C-activity which disappeared from the carboxyl labeled betaine in acetic acid indicated that acetic acid was the primary end-product from the carboxyl end of the betaine molecule on the high-energy ration. The 24.37 per cent recovery of the ^{14}C-activity that disappeared from the carboxyl labeled betaine on the low-energy ration indicated that there were other pathways for the carboxyl end of betaine on the low-energy ration. The sum of the per cent ^{14}C-activity recovered for the three compounds $(CH_3)_3N$, CO_2, and CH_4 synthesized from the methyl labeled betaine was 39.16. Therefore, approximately 60 per cent of the ^{14}C-activity that disappeared from the methyl labeled betaine was not accounted for. Some of the unaccounted for methyl labeled ^{14}C-activity could be in dimethylglycine and methylglycine. There are two possible explanations for the unaccounted ^{14}C-activity; one is that there are pathways for betaine metabolism other than the two listed, and that there are other intermediate compounds besides those isolated in this study. The other possibility is that the ^{14}C

activity unaccounted for was incorporated into microbial cells. Any future investigations should determine the incorporation of betaine into microbial cells.

In conclusion, it can be said that this study added support to Mitchell's (1968) hypothesis that betaine was cleaved into trimethylamine and actic acid and suggests the possibility of a pathway whereby betaine is demethylated by rumen microorganism to glycine and the glycine quickly metabolized to ammonia and acetic acid.

CHAPTER VI

SUMMARY

A series of in vitro fermentations were conducted to test the hypothesis that a major pathway of betaine metabolism was the demethylation of the betaine molecule to glycine which was in turn metabolized to ammonia and acetic acid. Two other purposes of this study were to add support to Mitchell's (1968) pathway in which betaine was cleaved into trimethylamine and acetic acid, and to determine the effects of the energy-level of the ration on betaine metabolism.

This research showed that betaine metabolism decreased as the energy level of the ration increased. Approximately equal amounts of betaine hydrochloride were added to all fermentation flasks. On the low-energy ration, 42.1 per cent of the added betaine was metabolized as compared to 21.8 for the high-energy ration. The quantity of betaine that disappeared agreed with the radioactivity that disappeared.

The two-dimensional paper chromatography failed to detect any radioactivity in glycine by scintillation counting and failed to detect any radioactivity amino acids by scanning the whole sheet on a radiochromatogram scanner. A possible explanation for the failure to

detect glycine was that the glycine was being metabolized as fast as it was being synthesized.

The data showed that ammonia was being synthesized from the ^{15}N-betaine. This ^{15}N-ammonia could have been synthesized from glycine as hypothesized.

The labeled carboxyl group of betaine was metabolized to acetic acid. The per cent of ^{14}C-activity that disappeared from the carboxyl labeled betaine that was recovered as acetic acid was 24.37 and 68.34 for low-energy and high-energy rations, respectively. None of the radioactivity from the carboxyl-labeled betaine was recovered in carbon dioxide, methane, or trimethylamine.

The labeled methyl-groups of betaine were found in three labeled betaine compounds: trimethylamine, carbon dioxide, and methane. Methane, carbon dioxide, and trimethylamine accounted for 19.67, 4.19, and 15.3 per cent of the ^{14}C-activity that disappeared from the methyl-labeled betaine. The method by which the carbon dioxide was synthesized is unknown. The synthesis of methane indicated that demethylation of betaine was occurring.

This research failed to prove conclusively that betaine was demethylated to glycine, but added support to the probability of this pathway. This study showed that the cleavage of the betaine molecule into trimethylamine and acetic acid was probably a minor pathway. The large amount of radioactivity unaccounted for indicated that there are other theoretical pathways to be investigated.

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APPENDIX

Appendix Table 1. Activity in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		$\mu Ci \times 10^{-4}$/ml			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	43.9	51.0	47.8	46.1
	Betaine-$^{14}COOH$	58.0	55.1	44.4	54.0
II	Betaine-$^{14}CH_3$	52.3	58.4	50.7	53.7
	Betaine-$^{14}COOH$	54.7	54.1	46.0	54.4
III	Betaine-$^{14}CH_3$	67.5	61.8	-	49.5
	Betaine-$^{14}COOH$	70.8	64.2	60.5	57.9
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	76.4	58.1	61.7	70.7
	Betaine-$^{14}COOH$	71.7	80.6	61.8	68.6
V	Betaine-$^{14}CH_3$	65.3	56.7	50.4	52.6
	Betaine-$^{14}COOH$	74.3	60.5	61.2	57.0
VI	Betaine-$^{14}CH_3$	85.9	91.3	85.8	49.8
	Betaine-$^{14}COOH$	80.0	65.0	66.0	69.6

Appendix Table 2. Concentration of Betaine in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		mg/ml			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	1.131	0.860	0.693	0.462
	Betaine-$^{14}COOH$	1.093	0.814	0.740	0.535
II	Betaine-$^{14}CH_3$	1.098	1.011	0.926	0.779
	Betaine-$^{14}COOH$	1.098	1.119	0.959	0.833
III	Betaine-$^{14}CH_3$	1.144	1.019	0.880	0.650
	Betaine-$^{14}COOH$	1.167	1.010	0.863	0.687
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	1.153	1.102	1.071	0.959
	Betaine-$^{14}COOH$	1.107	1.164	1.031	0.983
V	Betaine-$^{14}CH_3$	1.077	1.028	0.930	0.775
	Betaine-$^{14}COOH$	1.087	1.017	0.991	0.860
VI	Betaine-$^{14}CH_3$	1.472	1.343	1.189	1.022
	Betaine-$^{14}COOH$	1.253	1.092	1.019	0.857

Appendix Table 3. Activity in Betaine in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		$\mu Ci \times 10^{-4}$/ml			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	63.13	55.75	50.63	41.13
	Betaine-$^{14}COOH$	80.25	66.13	48.50	28.25
II	Betaine-$^{14}CH_3$	73.38	69.00	63.25	57.38
	Betaine-$^{14}COOH$	77.00	74.00	62.13	51.88
III	Betaine-$^{14}CH_3$	70.75	64.00	53.50	43.00
	Betaine-$^{14}COOH$	77.44	66.13	49.75	34.13
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	78.38	79.38	72.63	67.00
	Betaine-$^{14}COOH$	87.25	85.88	78.13	71.13
V	Betaine-$^{14}CH_3$	77.25	76.88	65.89	60.50
	Betaine-$^{14}COOH$	85.00	79.88	72.00	62.13
VI	Betaine-$^{14}CH_3$	90.50	84.13	79.63	65.38
	Betaine-$^{14}COOH$	84.38	79.75	80.50	66.88

Appendix Table 4. Specific Activity in Betaine in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		$\mu Ci \times 10^{-4}/mg$			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	55.82	64.82	73.06	89.03
	Betaine-$^{14}COOH$	75.25	81.24	65.54	52.80
II	Betaine-$^{14}CH_3$	66.83	68.25	68.30	73.66
	Betaine-$^{14}COOH$	70.13	66.13	64.77	62.28
III	Betaine-$^{14}CH_3$	61.84	62.81	60.80	66.15
	Betaine-$^{14}COOH$	66.36	65.48	57.65	49.68
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	67.98	72.03	67.82	69.86
	Betaine-$^{14}COOH$	78.82	73.78	75.78	72.36
V	Betaine-$^{14}CH_3$	71.73	74.78	70.84	78.06
	Betaine-$^{14}COOH$	78.20	78.54	72.65	72.24
VI	Betaine-$^{14}CH_3$	61.48	62.64	66.97	63.97
	Betaine-$^{14}COOH$	67.34	73.03	79.00	78.04

Appendix Table 5. Concentration of Nitrogen in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		mg/ml			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	1.179	1.233	1.109	1.145
	Betaine-$^{14}COOH$	0.991	1.202	1.241	1.117
II	Betaine-$^{14}CH_3$	1.155	1.148	1.101	1.164
	Betaine-$^{14}COOH$	1.319	1.284	1.304	1.083
III	Betaine-$^{14}CH_3$	1.249	1.263	1.233	1.256
	Betaine-$^{14}COOH$	1.364	1.265	1.194	1.247
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	2.849	2.693	2.686	2.627
	Betaine-$^{14}COOH$	2.935	2.420	2.600	2.563
V	Betaine-$^{14}CH_3$	2.537	2.467	2.287	3.076
	Betaine-$^{14}COOH$	2.732	2.116	2.233	2.623
VI	Betaine-$^{14}CH_3$	2.459	1.644	1.887	2.227
	Betaine-$^{14}COOH$	3.017	2.522	1.921	2.057

Appendix Table 6. Per Cent ^{15}N in Nitrogen and Ammonia in the Flask at Each Collection. (Natural Abundance of ^{15}N is 0.366%).

Labeled Added	Time in Minutes			
	0	200	400	600
	Ammonia - Low Energy Ration			
Betaine-$^{14}CH_3$	0.377	0.492	0.511	0.442
Betaine-$^{14}COOH$	0.380	0.412	0.397	0.461
	Total Nitrogen - Low Energy Ration			
Betaine-$^{14}CH_3$	0.463	0.571	1.054	1.138
Betaine-$^{14}COOH$	0.535	0.460	0.478	1.069
	Ammonia - High Energy Ration			
Betaine-$^{14}CH_3$	0.396	0.533	0.618	0.667
Betaine-$^{14}COOH$	0.428	0.543	0.604	0.610
	Total Nitrogen - High Energy Ration			
Betaine-$^{14}CH_3$	-	0.856	0.812	0.767
Betaine-$^{14}COOH$	0.732	0.749	0.802	0.807

Appendix Table 7. Concentration of Ammonia in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		mg/ml			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	1.892	0.403	0.459	0.523
	Betaine-$^{14}COOH$	0.504	0.428	0.487	0.536
II	Betaine-$^{14}CH_3$	0.232	0.393	0.437	0.616
	Betaine-$^{14}COOH$	0.749	0.527	0.448	0.523
III	Betaine-$^{14}CH_3$	1.192	0.456	0.533	0.553
	Betaine-$^{14}COOH$	0.311	0.426	0.525	0.555
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	1.678	0.655	0.969	1.377
	Betaine-$^{14}COOH$	1.031	0.520	1.385	1.066
V	Betaine-$^{14}CH_3$	0.824	0.658	0.933	1.340
	Betaine-$^{14}COOH$	1.165	0.586	1.729	1.241
VI	Betaine-$^{14}CH_3$	0.541	0.384	0.646	0.750
	Betaine-$^{14}COOH$	0.431	1.139	0.639	0.905

Appendix Table 8. Concentration of Trimethylamine in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		mg/ml			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	0.000	0.050	0.075	0.123
	Betaine-$^{14}COOH$	0.000	0.041	0.055	0.076
II	Betaine-$^{14}CH_3$	0.000	0.008	0.011	0.023
	Betaine-$^{14}COOH$	0.000	0.007	0.008	0.022
III	Betaine-$^{14}CH_3$	0.000	0.005	0.016	0.023
	Betaine-$^{14}COOH$	0.000	0.007	0.013	0.024
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	0.037	0.038	0.043	0.051
	Betaine-$^{14}COOH$	0.044	0.040	0.052	0.052
V	Betaine-$^{14}CH_3$	0.014	0.027	0.034	0.041
	Betaine-$^{14}COOH$	0.018	0.032	0.027	0.043
VI	Betaine-$^{14}CH_3$	0.013	0.027	0.058	0.087
	Betaine-$^{14}COOH$	0.024	0.038	0.036	0.076

Appendix Table 9. Activity in Trimethylamine in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		$\mu Ci \times 10^{-4}/ml$			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	0.19	3.35	4.63	7.65
	Betaine-$^{14}COOH$	0.00	0.00	0.00	0.08
II	Betaine-$^{14}CH_3$	0.23	0.60	0.78	1.80
	Betaine-$^{14}COOH$	0.00	0.00	0.06	0.03
III	Betaine-$^{14}CH_3$	0.00	0.66	1.30	1.50
	Betaine-$^{14}COOH$	0.00	0.30	0.15	0.00
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	0.36	0.36	0.84	1.31
	Betaine-$^{14}COOH$	0.00	0.08	0.00	0.08
V	Betaine-$^{14}CH_3$	0.04	0.84	1.56	2.21
	Betaine-$^{14}COOH$	0.00	0.00	0.00	0.00
VI	Betaine-$^{14}CH_3$	0.18	1.08	2.69	3.96
	Betaine-$^{14}COOH$	0.05	0.18	0.00	0.00

Appendix Table 10. Specific Activity of Trimethylamine in the Flask at Each Collection.

Trial Number	Labeled Added	Time in Minutes 0	200	400	600
		$\mu Ci \times 10^{-4}/mg$			
		Low Energy Ration			
I	Betaine-$^{14}CH_3$	0.00	67.00	61.73	62.20
	Betaine-$^{14}COOH$	0.00	00.00	00.00	1.05
II	Betaine-$^{14}CH_3$	0.00	75.00	70.91	78.26
	Betaine-$^{14}COOH$	0.00	00.00	7.50	1.36
III	Betaine-$^{14}CH_3$	0.00	132.00	81.25	65.22
	Betaine-$^{14}COOH$	0.00	42.86	11.54	00.00
		High Energy Ration			
IV	Betaine-$^{14}CH_3$	9.73	9.47	19.53	25.69
	Betaine-$^{14}COOH$	0.00	2.00	0.00	1.54
V	Betaine-$^{14}CH_3$	2.86	31.11	45.88	53.90
	Betaine-$^{14}COOH$	0.00	00.00	00.00	00.00
VI	Betaine-$^{14}CH_3$	13.85	40.00	46.38	45.52
	Betaine-$^{14}COOH$	2.08	4.74	00.00	00.00

Appendix Table 11. Concentration of Volatile Fatty Acids in the Flask at Each Collection on Low-Energy Rations.

Trial Number	Labeled Added	Volatile Fatty Acids	Time in Minutes 0 Unlabeled	0 Labeled	200 Unlabeled	200 Labeled	400 Unlabeled	400 Labeled	600 Unlabeled	600 Labeled
			meq X 10^{-4}/ml							
I	Betaine-$^{14}CH_3$	Acetic	11.92	-	127.87	1.39	117.97	-	-	-
		Propionic	18.36	-	8.38	1.61	9.95	-	-	-
		Butyric	-	-	-	-	18.75	-	-	-
	Betaine-$^{14}COOH$	Acetic	41.13	9.94	-	-	-	-	131.14	131.14
		Propionic	25.29	-	-	-	-	-	15.61	3.83
		Butyric	18.02	0.82	-	-	-	-	13.86	-
II	Betaine-$^{14}CH_3$	Acetic	140.79	-	201.35	-	206.65	-	20.60	-
		Propionic	-	-	-	-	16.08	-	32.86	8.42
		Butyric	-	-	285.34	-	7.34	-	17.07	-
	Betaine-$^{14}COOH$	Acetic	-	-	198.30	190.70	190.75	190.75	202.02	194.73
		Propionic	106.80	23.73	10.71	-	1.07	-	20.67	3.06
		Butyric	8.97	1.63	-	-	-	-	-	-
III	Betaine-$^{14}CH_3$	Acetic	221.29	66.23	214.60	-	121.87	-	-	-
		Propionic	9.95	-	10.71	-	-	-	308.00	-
		Butyric	27.71	-	-	-	-	-	16.08	-
	Betaine-$^{14}COOH$	Acetic	208.23	149.03	147.71	147.71	251.03	249.71	286.80	286.80
		Propionic	6.89	-	6.12	3.06	9.95	-	15.30	4.59
		Butyric	-	-	-	-	-	-	-	-

Appendix Table 12. Concentration of Volatile Fatty Acids in the Flask at Each Collection on High-Energy Ration.

Trial Number	Labeled Added	Volatile Fatty Acids	Time in Minutes 0 Unlabeled	0 Labeled	200 Unlabeled	200 Labeled	400 Unlabeled	400 Labeled	600 Unlabeled	600 Labeled
			meq X 10^{-4}/ml							
I	Betaine-$^{14}CH_3$	Acetic	269.58	-	216.59	41.73	338.42	1.32	445.09	-
		Propionic	54.34	-	29.08	-	45.91	15.30	106.37	2.30
		Butyric	-	-	6.52	0.82	81.50	-	22.01	-
	Betaine-$^{14}COOH$	Acetic	188.11	170.88	306.66	306.00	266.93	263.61	370.91	370.91
		Propionic	36.73	23.04	61.99	-	42.09	-	58.15	-
		Butyric	6.52	-	12.23	-	8.15	-	13.04	7.34
II	Betaine-$^{14}CH_3$	Acetic	224.54	-	324.62	84.78	351.70	37.75	475.56	217.91
		Propionic	22.20	-	35.20	8.42	38.26	-	58.92	14.54
		Butyric	-	-	6.52	-	6.52	-	14.67	-
	Betaine-$^{14}COOH$	Acetic	203.35	196.72	344.42	341.11	386.15	386.15	445.09	445.09
		Propionic	19.14	-	36.73	1.53	51.27	12.24	46.68	-
		Butyric	1.63	0.79	7.34	5.71	10.60	5.71	9.78	5.71
III	Betaine-$^{14}CH_3$	Acetic	236.46	-	283.45	-	492.13	-	267.60	-
		Propionic	16.08	-	31.69	-	59.68	-	14.79	-
		Butyric	2.45	-	10.50	-	30.16	-	2.25	-
	Betaine-$^{14}COOH$	Acetic	221.88	154.32	389.46	388.79	488.81	488.81	606.71	606.71
		Propionic	37.49	-	48.21	-	68.11	1.53	106.37	32.91
		Butyric	6.52	-	17.12	-	29.34	24.45	66.51	57.87

Appendix Table 13. Volatile Fatty Acids Produced from Carboxyl Labeled Betaine.

Trial Number	Volatile Fatty Acids	Time in Minutes 0 Specific Activity	0 ^{14}C Recovered /ml	200 Specific Activity	200 ^{14}C Recovered /ml	400 Specific Activity	400 ^{14}C Recovered /ml	600 Specific Activity	600 ^{14}C Recovered /ml
		$\mu Ci \times 10^{-4}$							
		Low Energy Ration							
I	Acetic	0.412	0.000	-	-	-	-	828.217	10.862
	Propionic	-	-	-	-	-	-	1.630	.001
	Butyric	159.006	0.013	-	-	-	-	-	-
II	Acetic	-	-	99.347	1.599	189.729	3.619	331.891	6.463
	Propionic	-	-	-	-	-	-	17.050	0.005
	Butyric	33.610	0.005	-	-	-	-	-	-
III	Acetic	2.230	0.033	115.451	1.705	258.526	6.456	394.187	11.305
	Propionic	-	-	1.018	.000	-	-	2.807	0.001
	Butyric	-	-	-	-	-	-	-	-
		High Energy Ration							
IV	Acetic	6.753	0.115	80.746	2.471	141.761	3.737	236.014	8.754
	Propionic	8.107	0.002	-	-	-	-	-	-
	Butyric	-	-	-	-	-	-	10.942	0.008
V	Acetic	9.076	0.178	121.008	4.128	228.861	8.838	298.317	13.278
	Propionic	-	-	253.837	0.039	32.375	0.040	-	-
	Butyric	2.146	0.000	-	-	15.188	0.009	6.457	0.004
VI	Acetic	7.480	0.115	106.668	4.147	192.694	8.882	273.245	16.578
	Propionic	-	-	-	-	10.011	0.002	2.158	0.007
	Butyric	-	-	-	-	39.617	0.097	2.875	0.017

Appendix Table 14. *Specific Activity of Carbon Dioxide.*

Trial Number	Labeled Added	Time in Minutes		
		200	400	600
		μCi/gram atom carbon		
		Low Energy Ration		
I	Betaine-$^{14}CH_3$	0.878	5.926	6.979
	Betaine-$^{14}COOH$	0.371	0.549	0.049
II	Betaine-$^{14}CH_3$	1.218	6.612	5.026
	Betaine-$^{14}COOH$	0.224	0.480	0.140
III	Betaine-$^{14}CH_3$	3.885	8.110	12.831
	Betaine-$^{14}COOH$	0.315	0.574	0.154
		High Energy Ration		
IV	Betaine-$^{14}CH_3$	0.434	1.463	2.023
	Betaine-$^{14}COOH$	0.403	0.196	0.105
V	Betaine-$^{14}CH_3$	0.340	0.917	1.533
	Betaine-$^{14}COOH$	0.668	0.340	0.238
VI	Betaine-$^{14}CH_3$	0.445	2.121	3.063
	Betaine-$^{14}COOH$	0.655	0.273	0.172

Appendix Table 15. Amount of ^{14}C Recovered in Carbon Dioxide.

Trial Number	Labeled Added	Time in Minutes		
		200	400	600
		$\mu Ci \times 10^{-4}/ml$		
		Low Energy Ration		
I	Betaine-$^{14}CH_3$	0.264	1.925	0.810
	Betaine-$^{14}COOH$	0.087	0.264	0.041
II	Betaine-$^{14}CH_3$	0.433	1.014	1.572
	Betaine-$^{14}COOH$	0.022	0.038	0.010
III	Betaine-$^{14}CH_3$	0.577	0.428	1.090
	Betaine-$^{14}COOH$	0.032	0.074	0.008
		High Energy Ration		
IV	Betaine-$^{14}CH_3$	0.042	0.440	0.557
	Betaine-$^{14}COOH$	0.033	0.069	0.024
V	Betaine-$^{14}CH_3$	0.027	0.186	0.663
	Betaine-$^{14}COOH$	0.061	0.038	0.298
VI	Betaine-$^{14}CH_3$	0.015	0.421	0.443
	Betaine-$^{14}COOH$	0.065	0.039	0.074

Appendix Table 16. Specific Activity of Methane.

Trial Number	Labeled Added	Time in Minutes		
		200	400	600
		μCi/gram atom carbon		
		Low Energy Ration		
I	Betaine-$^{14}CH_3$	1.348	29.904	3.458
	Betaine-$^{14}COOH$	1.243	0.179	0.070
II	Betaine-$^{14}CH_3$	23.888	52.241	33.607
	Betaine-$^{14}COOH$	1.039	2.415	0.490
III	Betaine-$^{14}CH_3$	74.725	80.469	64.677
	Betaine-$^{14}COOH$	2.719	3.882	0.000
		High Energy Ration		
IV	Betaine-$^{14}CH_3$	4.259	12.620	14.749
	Betaine-$^{14}COOH$	1.155	0.151	0.088
V	Betaine-$^{14}CH_3$	0.091	21.172	26.702
	Betaine-$^{14}COOH$	0.291	0.396	1.659
VI	Betaine-$^{14}CH_3$	0.231	28.147	17.822
	Betaine-$^{14}COOH$	0.175	0.161	0.168

Appendix Table 17. Amount of ^{14}C Recovered in Methane.

Trial Number	Labeled Added	Time in Minutes 200	400	600
		$\mu Ci \times 10^{-4}/ml$		
		Low Energy Ration		
I	Betaine-$^{14}CH_3$	0.231	4.529	2.879
	Betaine-$^{14}COOH$	0.141	0.029	0.028
II	Betaine-$^{14}CH_3$	0.988	2.996	3.901
	Betaine-$^{14}COOH$	0.054	0.180	0.041
III	Betaine-$^{14}CH_3$	4.526	4.796	4.831
	Betaine-$^{14}COOH$	0.131	0.491	0.000
		High Energy Ration		
IV	Betaine-$^{14}CH_3$	0.144	2.223	3.272
	Betaine-$^{14}COOH$	0.095	0.008	0.026
V	Betaine-$^{14}CH_3$	0.014	2.393	5.718
	Betaine-$^{14}COOH$	0.025	0.076	0.321
VI	Betaine-$^{14}CH_3$	0.004	3.902	2.621
	Betaine-$^{14}COOH$	0.008	0.008	0.025