THE FORMATION OF METHANE FROM PROPIONATE

by

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To My Wife

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TABLE OF CONTENTS

INTRODUCTION

Methanogenic bacteria are present wherever there is natural microbial degradation of vegetation under anaerobic conditions. The usual places include the ruminants, marsh lands, swamps, ponds and sewage plants. The methane fermentation is widely used for disposal of industrial wastes and domestic sewage. Methane, carbon dioxide and minerals are the main products in the breakdown process. Although this phenomenon has been studied for several centuries, and several possible mechanisms have been suggested, the actual mechanisms are still unknown, and relatively little is known of the microorganisms.

Methane formation by microorganisms occurs best in nature, and in the laboratory, under mixed culture conditions. Purification and isolation of methanogenic bacterial species is rather difficult for they are highly sensitive to oxygen and have a slow rate of growth. The fermentation can be studied in the laboratory using all glass fermentors.

Many have studied this fermentation, but the mechanisms whereby organic acids, namely volatile fatty acids (VFAs), are converted into methane and carbon dioxide remains obscure. Most studies have been done with VFA, having an even number of carbon atoms but very little is known about the utilization of odd carbon VFAs. Odd carbon VFAs are generally difficult to degrade, and show a slower rate of gas production. Propionic acid has been studied by several workers, but there is no uniform concept concerning its metabolic pathway. Further study is necessary to determine the mechanism whereby propionate is converted to methane and carbon dioxide.

This investigation was an attempt to learn more about the possible organic compounds involved in degradation of propionate to methane and carbon dioxide. The fate of the three carbon atoms of propionate was studied. The information gained from this investigation should be useful in determining how propionate is converted to methane and carbon dioxide.

LITERATURE REVIEW

Methane formed in nature originally was not considered a microbial end product. Volta, in 1776, was the first serious investigator (Barker, 1956). He concluded that the combustible gas was derived from plant materials. In 1863, Reiset (Fifield, 1956) suggested that methanogenic bacteria were the causative agents, and that they were abundant in the anaerobic conditions necessary for methane formation. He showed these conditions also occurred in ruminants. In 1904, Omelianskii (Fifield, 1956) showed that methane formation was associated with the microbial process in the anaerobic decomposition of cellulose. This concept was accepted until 1910 when Sohngen demonstrated that formate, acetate, butyrate, ethanol, hydrogen and carbon dioxide (Barker, 1936), the products of cellulose fermentation, were also utilized by methanogenic bacteria. Attempts to isolate and purify the culture was unsuccessful until 1936 (Barker, 1936).

The Methanogenic Bacteria

Methanobacillus omelianskii was the first methanogenic organism successfully isolated (Barker, 1936). This organism has been described

as a Gram-variable, thin, unbranched rod. Later other methane organisms were isolated: Methanobacterium formicicum (Schnellen, 1947; Barker, 1956), Methanosarcina barkerii (Schnellen, 1947), and Methanococcus vannielii (Stadtman and Barker, 1951).

The methanogenic organisms are either rod-shaped or spherical. The rod-shaped cells included the non-sporulating methanobacteria such as Methanobacterium formicicum, Methanobacterium propionicum and Methanobacterium sogngenii and the sporulating methanobacillus such as Methanobacillus omelianskii. The spherical cells included non-sarcina arranged Methanococcus such as Methanococcus mazei and Methanococcus vannielii; the sarcina arranged methanosarcina such as Methanosarcina barkerii and Methanosarcina methanica (Barker, 1956). Only organisms mentioned in the previous paragraph have been isolated in pure culture.

The nutritional requirements of the methanogenic bacteria are relatively simple. Generally an organic acid plus inorganic mineral salts and the ammonium ion are sufficient for growth and methane production. But each different species has relatively specific requirements for carbon and energy source: Methanobactericum propionicum requires propionate (Stadtman, Barker, 1951); Methanobacillus omelianskii is specific for primary and secondary shortchain aliphatic alcohols (Barker, 1941) and hydrogen (Barker, 1943); Methanococcus mazei utilizes acetate and butyrate (Barker, 1936); Methanosarcina barkerii utilizes methanol, acetate and carbon dioxide (Schnellen, 1947). For the decomposition of a more complex substrate such as benzoate, Fina (1950, 1960) has reported that more than one species of bacteria may be present.

Commonly, the methanogenic cultures can be maintained indefinitely in the laboratory. No toxic by-products are formed. The major products, carbon dioxide and methane are easily removed.

The methanogenic organisms are so highly sensitive to oxygen or other oxidizing agents that the isolation and maintenance of pure cultures is difficult. Generally, studies on methanogenic characteristics are carried out with highly enriched culture.

Barker (1936) reported that reducing agents were helpful for methanogenic cultures. These reducing agents include sodium sulfide, sodium hydrosulfide, or cysteine. Upon further study of the influence of reducing agents, it was suggested that a stable low oxidation-reduction potential correlated with the rate of gas production and with the shortening of the lag period of gas production (Sakazawa et al., 1963). Stimulatory factors which increase the rate of gas production, such as purine derivatives and a group of amino acids were reported by Sakazawa et al. (1964, 1965). Solid sediments, such as shredded asbestos (Breden and Buswell, 1933) when added to the liquid media, were beneficial in mechanically shielding the bacteria from dissolved oxygen (Fifield, 1956).

Chemistry of Bacterial Methanogenesis

Decomposition of fatty acid to methane and carbon dioxide can be represented by the following formula, suggested by Buswell and Neave (1930):

$$C_nH_{2n}O_2 + \frac{n-2}{2} H_2O \longrightarrow \frac{n+2}{4} CO_2 + \frac{3n-2}{4} CH_4$$

Van Niel 1933 (Barker, 1936) suggested the general equation:

$$4H_2A + CO_2 \longrightarrow 4A + CH_4 + 2H_2O$$

The H_2A represents any compound to be activated by methanogenic bacteria. Organic substrates donate hydrogen and electrons and are thus oxidized. Carbon dioxide is reduced to methane. An example is the incomplete oxidation of ethyl alcohol to acetic acid by Methanobacillus omelianskii:

$$2CH_3CH_2OH + CO_2 \longrightarrow 2CH_3COOH + CH_4$$

In this type of CO_2 reduction ($CO_2 + 8H \longrightarrow CH_4 + 2H_2O$) CO_2 acts as a hydrogen acceptor and is reduced to CH_4 just as O_2 acts as a hydrogen acceptor and is reduced to H_2O in aerobic conditions.

There are, however, other ways methane is formed. The decomposition of acetic acid by methanogenic bacteria is regarded as a fermentation:

$$CH_3COOH \longrightarrow CH_4 + CO_2$$

The methyl group yields methane, and the carboxyl group yields carbon dioxide. This was demonstrated by Buswell et al. (1948), and Barker et al. (1949, 1951, 1956).

Stadtman and Barker (1951) and Pine and Vishniac (1957) reported that methyl alcohol could also be fermented. Three molecules of methanol were reduced to methane, coupled with the oxidation of one molecule to CO_2:

$$4CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O$$

Pine and Vishniac also reported that methane could be formed directly from methanol, but that methanol was not the usual intermediate in the degradation of natural compounds.

In the complete oxidation of ethanol, Barker (1941), and Stadtman and Barker (1949) proposed the following:

$$2CH_3CH_2OH + CO_2 \longrightarrow 2CH_3COOH + CH_4$$

$$2CH_3COOH \longrightarrow 2CH_4 + 2CO_2$$

$$\overline{2CH_3CH_2OH \longrightarrow 3CH_4 + CO_2}$$

First, ethanol was oxidized to acetic acid by the removal of hydrogen. Second, carbon dioxide acted as a hydrogen acceptor and was reduced to methane gas. Third, acetic acid was converted into methane and carbon dioxide. In total, one mole of methane was formed from carbon dioxide reduction, and two moles were formed directly from acetic acid.

Stadtman and Barker (1951) investigated the decomposition of propionic acid to carbon dioxide and acetic acid by Methanobacterium propionicum. In the complete degradation of propionic acid, three fourths of the CO_2 could be converted to CH_4, and acetic acid could be degraded to CH_4 and CO_2. The equations were shown as follows:

$$4CH_3CH_2COOH + 8H_2O \longrightarrow 4CH_3COOH + 4CO_2 + 24H$$

$$3CO_2 + 24H \longrightarrow 3CH_4 + 6H_2O$$

$$4CH_3COOH \longrightarrow 4CH_4 + 4CO_2$$

$$4CH_3CH_2COOH + 8H_2O \longrightarrow 5CO_2 + 7CH_4$$

But Buswell, Fina et al. (1951) indicated that all carbons from the decomposition of propionate, in three different positions, could yield both methane and carbon dioxide. Their conversion ratios were different for each carbon. The α and β carbons yielded more CH_4 and the carboxyl group more CO_2.

Fina et al. (1960) studied methane production from formic acid, and suggested that formic acid may be reduced to methane without passing through a carbon dioxide step. They indicated an exchange of the carbons of HCOOH and CO_2 did not necessarily occur during this fermentation. The formation of methane in the anaerobic decomposition of benzoic acid did not result from a direct reduction of carbon dioxide (Fina, 1950; Fina et al., 1960).

They found that carbon-1 of benzoic acid went to methane and carbon-7 of benzoic acid was converted to carbon dioxide. Propionic acid was revealed as an intermediate in benzoic acid decomposition by Roberts (1962).

Butyric acid was found in the methanogenic sludge of propionate degradation (McCarty, 1962). It was considered that the build up of butyrate in the propionate culture was caused by either a backup, or a biochemical side reaction. They concluded that in propionic acid degradation the synthesis of all higher acids might be explained as combinations of propionic acid and/or acetic acid. For example, butyric acid could be formed by joining two acetic acid molecules, valeric acid by joining a propionic and an acetic acid molecule, and caproic acid by joining either two propionic or three acetic acid molecules. These "side-acids" were always in lesser concentrations and only the major acids which were added should be considered of importance (McCarty, 1962).

A number of workers have tried to study methanogenesis using cell-free extracts prepared from Methanobacillus omelianskii. Wolin and Wolfe (1963) learned that the precursors of CH_4 could be CO_2, pyruvate or serine, and that the formation of CH_4 was ATP dependent. CoA stimulation and ferredoxin were needed. Blaylock and Stadtman (1963, 1964) demonstrated that methyl cobalamin was a possible intermediate in the formation of CH_4 from methanol. Wolin et al. (1964) found that CH_4 and Vitamin B_{12} were the products of methylcobalamin reduction by crude extract of Methanobacillus omelianskii. Moreover, N^{15}-methyltetrahydrofolate was found to be an important intermediate in the formation of CH_4 from CO_2, pyruvate or serine (Wood et al., 1965).

Knight et al. (1966) studied Methanobacillus omelianskii and suggested that in whole cell studies carbons from both carbon dioxide and ethanol were used for biosynthesis of amino acids, and in most cases ethanol was incorporated as a C_2 unit. Also, carbon atoms from ethanol and carbon dioxide were equally distributed in compounds of the cell. These studies revealed that CO_2 was not only reduced to CH_4 but also incorporated in cell constituents.

EXPERIMENTAL METHODS

Preparation of Cultures

The propionic acid-utilizing methanogenic bacteria (Propionate cultures) were obtained by enrichment from a benzoic acid-utilizing culture of methanogenic bacteria (Benzoate cultures). The benzoate cultures were developed either from rumen contents or from sludge obtained from anaerobic digestors at the Manhattan, Kansas sewage disposal plant (Roberts, 1962). They are maintained in this laboratory.

Stock cultures were kept in all glass fermentors, equipped with glass stopcocks and serum stoppers (Roberts, 1962). An asbestos nidus was used in each fermentor to enhance growth and gas production. The benzoate cultures were maintained in carbon balance, the dynamic steady state wherein the amount of substrate fed, in terms of atoms of carbon, is equal to and reflected by the amount of gas produced as methane and carbon dioxide. That is, one mole of benzoic acid, which has 7 carbons, will produce seven moles of gas, as carbon dioxide or methane. Subsequently, yields approach the theoretical 100% return, plus or minus a few per cent depending on the state of the culture.

Propionate cultures obtained by enrichment from benzoate cultures yield gas at a decreased but constant rate (Fig. 1). Upon continuous feeding of the enriched propionate culture and increasing the amount of propionic acid* fed from 0.5 mole to 1.0 mole, the rate of gas production increased. The propionate culture was eventually placed in a steady state and in carbon balance.

Transfer of stock cultures to experimental fermentors under anaerobic conditions was effected by using a V-shaped glass joint and flowing oxygen-free nitrogen gas (Fig. 2). Usually 50 ml of the enriched stock cultures were transferred to a 125 ml glass fermentor (Fig. 2). A modified Barker's mineral medium (Barker, 1936) was used. It was made up in two solutions, A and B, and mixed in a ratio of 100 ml of solution A to 3 ml of solution B just prior to use.

Solution A		Solution B	
$CaCO_3$	2.7 gm	Na_2S	1.0 gm
KH_2PO_4	0.4 gm	Na_2CO_3	5.0 gm
NH_4Cl	1.0 gm	Tap water	100 ml
$MgCl_2$	0.1 gm		
Tap water	1,000 ml		

Sodium sulfide was used to lower the oxidation-reduction potential and the medium was adjusted to pH 6.8. The cultures were kept in a 40 C incubator. Carbon balance was maintained by feeding propionic acid at the appropriate time intervals determined from the graph on each propionate culture in steady state (Fig. 3). The gases were collected over a saturated solution of lithium chloride (Boell et al., 1939; Yahiro, 1959). The

* Fisher certified reagent, 99.92% purity, Fisher Scientific Company.

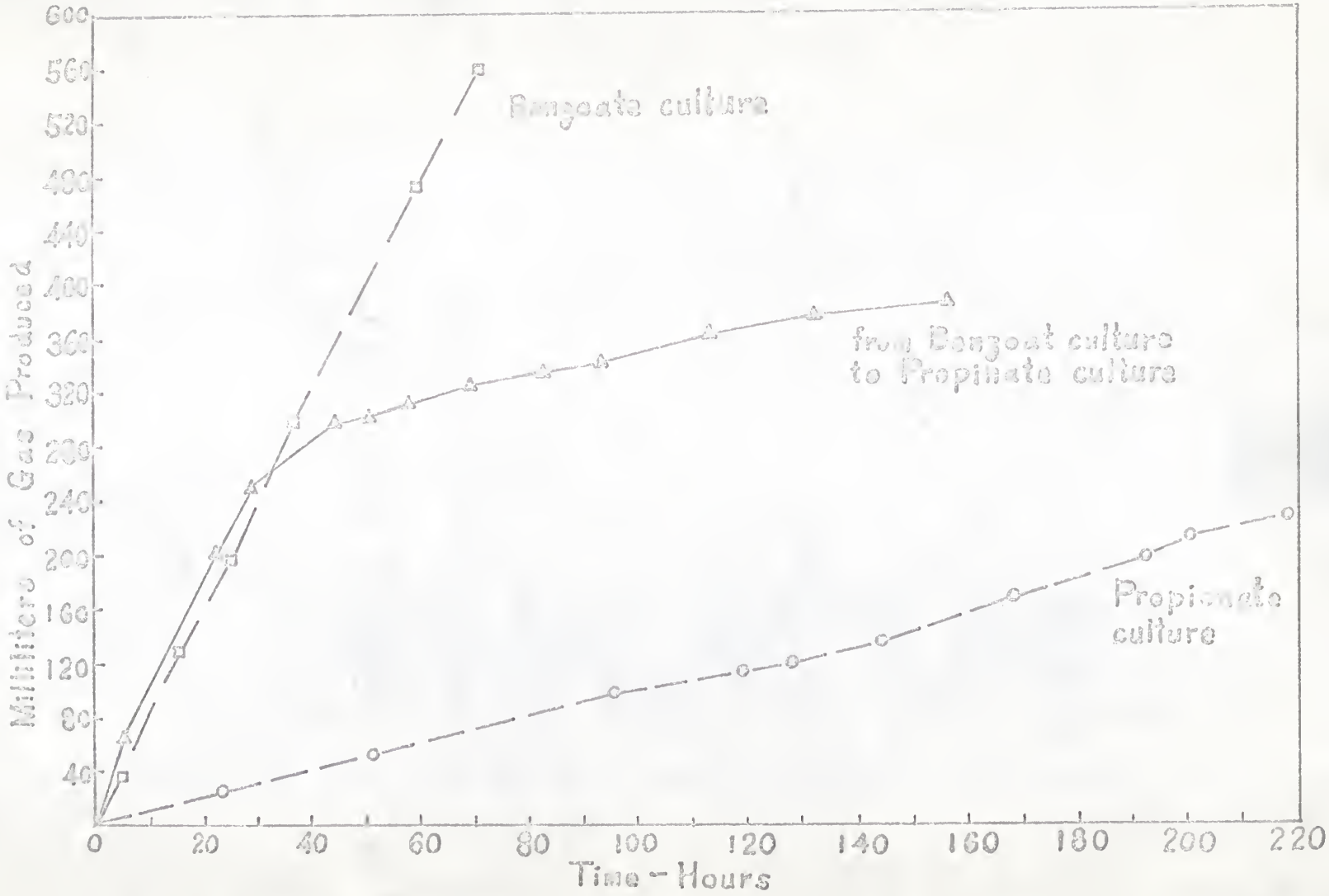

Fig. 1. Conversion of benzoate utilizing culture to a propionate utilizing culture.

Fig. 2. A V-shaped glass joint for insuring anaerobic transfer.

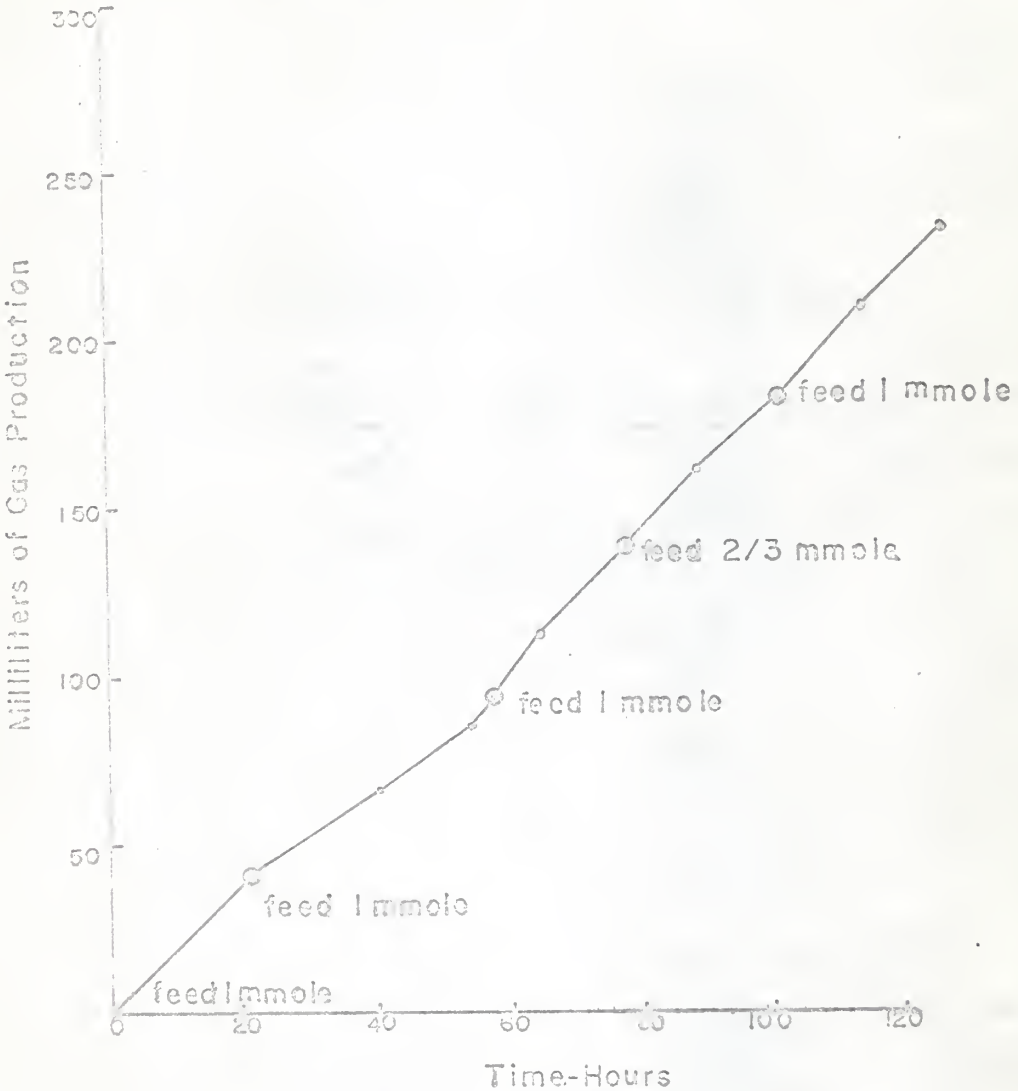

Fig.3. Gas production of propionate culture in a steady state and carbon balance (in a 125ml fermentor).

amount produced was read directly from the calibrated manometer (Fig. 4). Care was always taken to prevent atmospheric O_2 from entering cultures.

Microscopic Observation of the Enriched Propionate Culture

Under microscopic examination the highly enriched propionate cultures appeared to be composed mainly of Gram-negative rods with a few Gram-variable micrococci. Attempts to further purify the culture were unsuccessful.

Volatile Fatty Acid Analysis

The determinations of the volatile fatty acids (VFAs) in the culture medium were performed with a micro steam distillation apparatus. The apparatus and procedure were designed by Fina and Sincher (1959). Orthophosphoric acid (85%) was added to 1.0 ml of culture medium and steam distilled. Forty milliliters of steam distillate were titrated with 1 N NaOH, using a Gilmont ultramicroburet and phenolphthalein (1 gm in 100 ml of 65% ethanol) as an indicator. During titration, CO_2-free air was bubbled through the solution. The CO_2-free air was obtained by using an Oscar air pump to force air through a train consisting of a concentrated liquid KOH solution, solid NaOH and solid $CaSO_4$ (anhydrous Drierite).

Methane and Carbon Dioxide Analysis and Determination of Their Specific Activity

The gasses produced in the propionate cultures were collected and analyzed for methane and carbon dioxide with a modified Burrell gas analysis apparatus (Model JS, equipped with ball and socket joints). In this

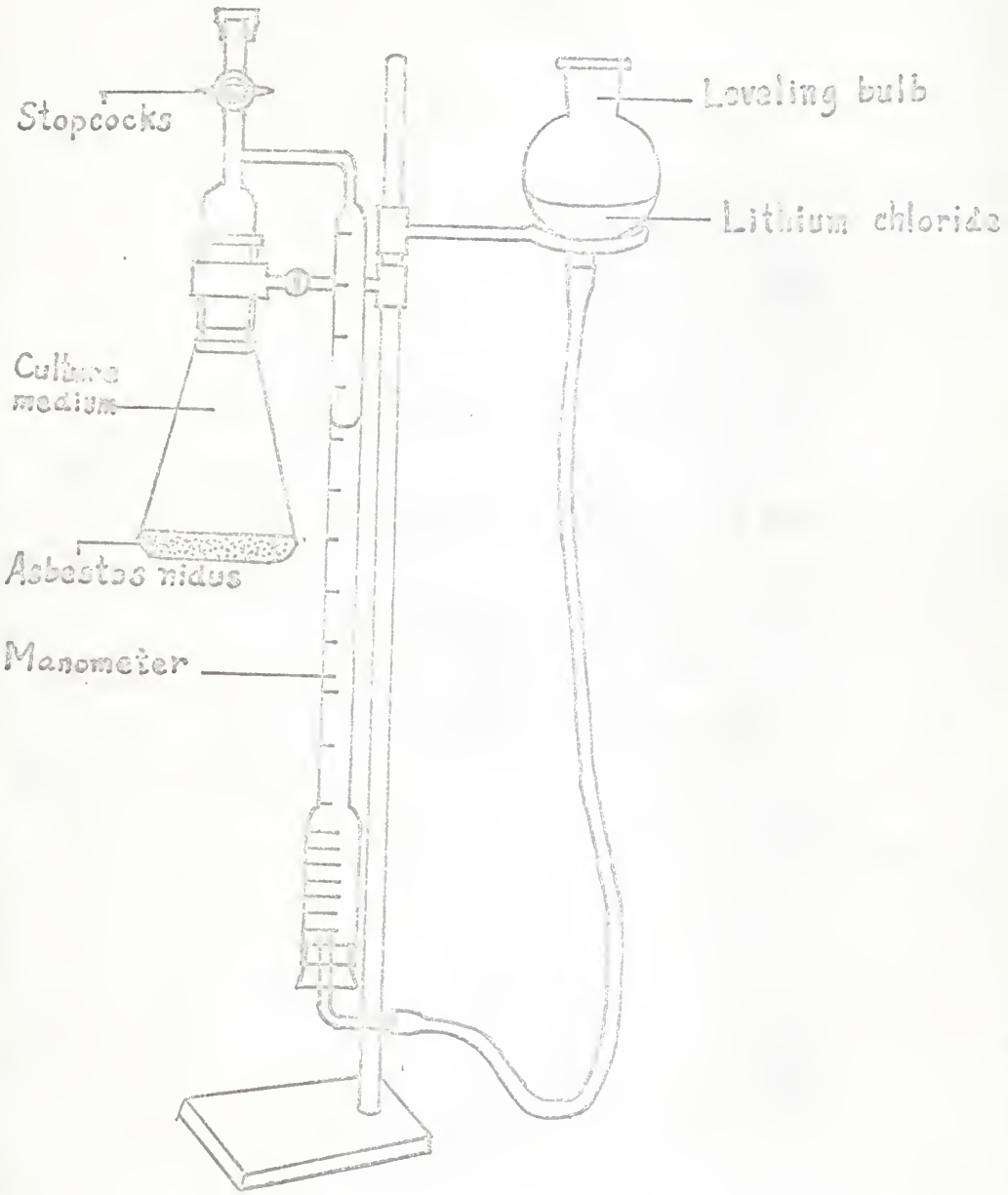

Fig. 4. A 125 ml digester fitted to a calibrated manometer.

procedure the CO_2 was first absorbed in 2 ml 1 N NaOH, then the CH_4 was quantitatively converted to CO_2 and in turn was also absorbed. This method is described in the Burrel Manual for Gas Analysis (1951) and by Fina et al. (1960). Gas was collected and analyzed at various intervals. Gas liquid chromatography was also used and will be described later in this section.

The radioactivity of the gasses, each absorbed in separate solutions of 1 N NaOH, was determined in a Packard liquid scintillation counter. The following phosphor solution was used (Keith, 1963):

p-Dioxane	1000 ml
PPO (2,5-Diphenyloxazole)	6 gm
POPOP (1,4-bis-2-95-Phenyloxazolyl benzene)	0.05 gm
Naphthalene	100 gm

The counting vials were prepared as follows: a 1.0 ml aliquot of the absorbed gas in 1 N NaOH was pipetted to a counting vial. Fourteen ml of the phosphor counting solution were added. The vials were chilled in the refrigerator, dark adapted in the scintillation machine and counted 2-3 times. The quenching effect of the NaOH was determined and taken into account when converting observed counts to disintegrations.

Methane and Carbon Dioxide Analysis Using Gas Liquid Chromatography (GLC)

An aerograph Model A-90 gas chromatograph equipped with a silica gel column* was also used in carbon dioxide and methane analysis. The column temperature was kept at 100 C. The flow rate of the helium carrier gas was adjusted to 60 ml/min. Quantitative estimations were made by cutting

* Varian aerograph, 2700 Mitchell Drive, Walnut Creek, Calif.

and weighing the peaks traced by the recorder. Results were equivalent and in most cases nearly identical to those obtained by the Orsett method.

Paper Chromatographic Analyses of Nonvolatile Organic Acids-^{14}C

The R_f's of cold succinic, malonic and pyruvic acids separated on Whatman No. 1 filter paper using an alkaline-developing solvent of 8:1:1 (v/v) ethanol-water-ammonium hydroxide (Varner, 1957) were determined. A three to six µl portion of an ether or water solution of the standard acid and their mixtures (2 to 20 mg/ml) was spotted on the filter paper sheet. The ascending solvent method was used. Separations were obtained in 24 hours or after the solvent front had advanced 20 to 30 cm. The solvent was removed by steam directed at the face of the paper and the sheet was dried under an infrared lamp. The organic acid indicator consisting of an alkaline alcohol solution of 0.04% bromocresol purple revealed the organic acids as yellow spots.

Spots from unknown ^{14}C tracer experiments with R_f's identical with knowns were cut out and placed into counting vials. They were then eluted with 1.0 ml of water. Scintillation fluids were added and ^{14}C was determined. The quenching effect of the distilled water was determined as previously for the NaOH.

Separation and Collection of Volatile Fatty Acid through GLC

The mixture of volatile fatty acids was separated and collected quantitatively through model A-90 GLC equipped with a Porapak Q column.*

* Waters Associates, Inc., 61 Fountain St., Framingham, Mass.

The column temperature was 200 C, the injector 210 C, the detector 210 C and the collector 170 C. The flow rate of the helium carrier gas was maintained at 100 ml/min.

A ten μl portion of mixed sample was injected into the GLC. The separated organic acids were trapped in individual collector bottles** containing 1 ml of double distilled water. They were kept in an ice water bath during the procedure. Each VFA was automatically recorded as it was collected. The relative position of each VFA was observed and trapped at the proper interval (Fig. 5).

This step was repeated five times so that VFA's from a total of 50 μl were processed. Each separated organic acid collected was decanted into a beaker. The collector bottle was then washed three times successively with double distilled water. The final volume of organic acid in the distilled water was made up to 10 ml and titrated with 1 N NaOH. The quantity of the collected organic acid was calculated from the 1 N NaOH titration. The collection efficiency was determined by using a known quantity (10 μl) of 1 N propionic acid. The collection efficiency was 80%.

When the ^{14}C content of the individual VFAs was to be determined, the titrated collected acids were dried over a low heat electric hot plate, dissolved in 1 ml of distilled water and placed in a counting vial. Scintillation fluid was added and ^{14}C determined. Quenching effect of water was again taken into account.

The ability of the column to purge itself of residual VFA was determined by injection of a ^{14}C-labeled VFA together with a second cold VFA.

** Model 66-023, Varian aerograph, 2700 Mitchell Drive, Walnut Creek, Calif.

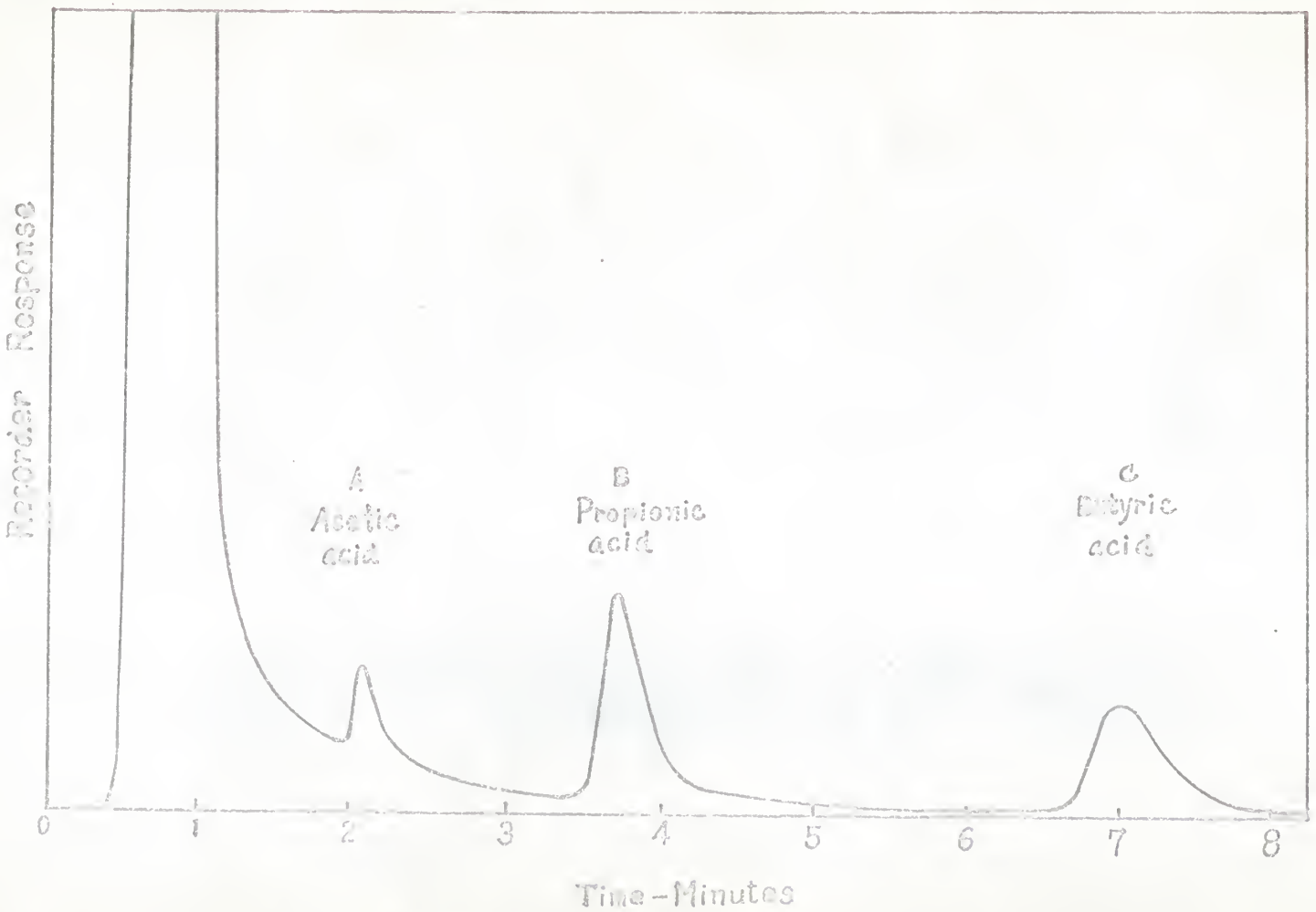

Fig.5. The separation of volatile fatty acids through G.L.C.

Each was then collected and separated as detailed previously. Their activities were determined. A mixture of the same cold VFA was then injected, collected and counted. As seen in Table 1, no residual propionic acid was left on the column to contaminate or interfere in subsequent analyses. Further, it is seen that one labeled VFA does not contaminate or interfere with analysis of another VFA.

Table 1. The ability of the column to purge itself of radioactive VFAs.

Trial No.	Injection mixture* 10 ml	Separated collected samples	CPM minus background**
1	Propionic-^{14}C acid and cold acetic acid	Propionic-^{14}C acid	469
		Acetic acid	0
2	Cold propionic acid and acetic acid	Propionic acid	0
		Acetic acid	0

* Injection mixture: (1) 1 M of propionic-^{14}C acid (0.1 microcurie) equally mixed with 1 M of acetic acid. (2) 1 M of cold propionic acid equally mixed with 1 M acetic acid.

** Background was 35 CPM (14 ml scintillation solution plus 1.0 ml distilled water).

Isolation of Selected Intermediates in Presence of $^{14}CO_2$ by Isotopic Dilution Technique*

The $^{14}CO_2$($NaH^{14}CO_3$) was added to the propionate culture which had been fed 2 m mole of propionic acid 10 hours before. After incubation (20 hours total), 2 ml of a neutralized mixture containing 1 m mole each of butyric,

* See Results and Discussion sections for explanation of why isotopic dilution techniques were employed.

acetic, succinic, malonic and pyruvic acids was added to the culture. These non-radioactive organic acids mixed with the ^{14}C containing acids in the culture. Later (after 4 hours, a total incubation time of 24 hours) an 80 ml sample of culture fluid was withdrawn from the fermentor and filtered through a membrane filter to remove solid materials, mainly bacterial cells.

The VFA was obtained by steam distilling the filtrate, and this then was titrated with 1 N sodium hydroxide. The neutralized distillate was condensed to 1 ml and acidified with 1 drop of orthophosphoric acid. A 50 µl portion was gas chromatographed, and specific activities were determined by procedures already described.

The liquid residues resulting were dried down to 1 ml, extracted with 10 ml of ether and condensed to 1 ml. Non VFA organic acids in these residues were separated , as explained, by paper chromatography. The ^{14}C activities were determined by the method previously described.

Isolation of Selected Intermediates in Presence of Propionic Acid-1-^{14}C, 2-^{14}C and 3-^{14}C Species by Isotopic Dilution Technique*

Propionic-^{14}C acid (labeled singly in 1, 2 or 3 position) was added in the propionate feed to a fermentor in steady state. After incubation (21 hours) 2 ml of a neutralized mixture containing 1 m mole each of cold butyric, acetic, succinic, malonic and pyruvic acids was added. Three hours later 80 ml of culture fluid were removed and filtered through a millipore filter. The filtrate was steam distilled. The VFAs were then

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* See Results and Discussion sections for explanation of why isotopic dilution techniques were employed.

analyzed for ^{14}C as described. The non-VFA organic acids in the liquid residues were determined by the method detailed earlier in this section.

RESULTS

Conversion of the Propionate Carbons to the End Products CO_2 and CH_4

In the first experiment, propionate-1-^{14}C containing 1 microcurie was fed to culture I in 2/3 m mole of propionic acid. At different intervals, gasses were measured and collected. A graph of the gas produced from this experiment is shown in Fig. 6, and as can be seen, the rate was stable within the experiment. In 24 hours 41 ml of gas were produced. The gas production rate was 1.7 ml/hr. The ratios of CO_2 and CH_4 in each interval are recorded in Table 2.

Table 2. Ratio of CO_2 to CH_4, determined for samples taken at indicated interval, from the culture fed with propionate-1-^{14}C.

No.	Time hours	Model A-90 GLC CO_2:CH_4	Burrell analyzer CO_2:CH_4
1	7.5	3.0: 7	3.6: 7
2	14.0	2.9: 7	2.9: 7
3	22.0	3.3: 7	3.4: 7
4	37.5	3.5: 7	3.4: 7

The decreasing amount of VFA (Table 3) showed that propionate* was utilized and converted to end products and intermediates. The specific activity changes of CO_2, CH_4 and total VFA at various times, is graphed

* Identified by GLC. No other VFA was present.

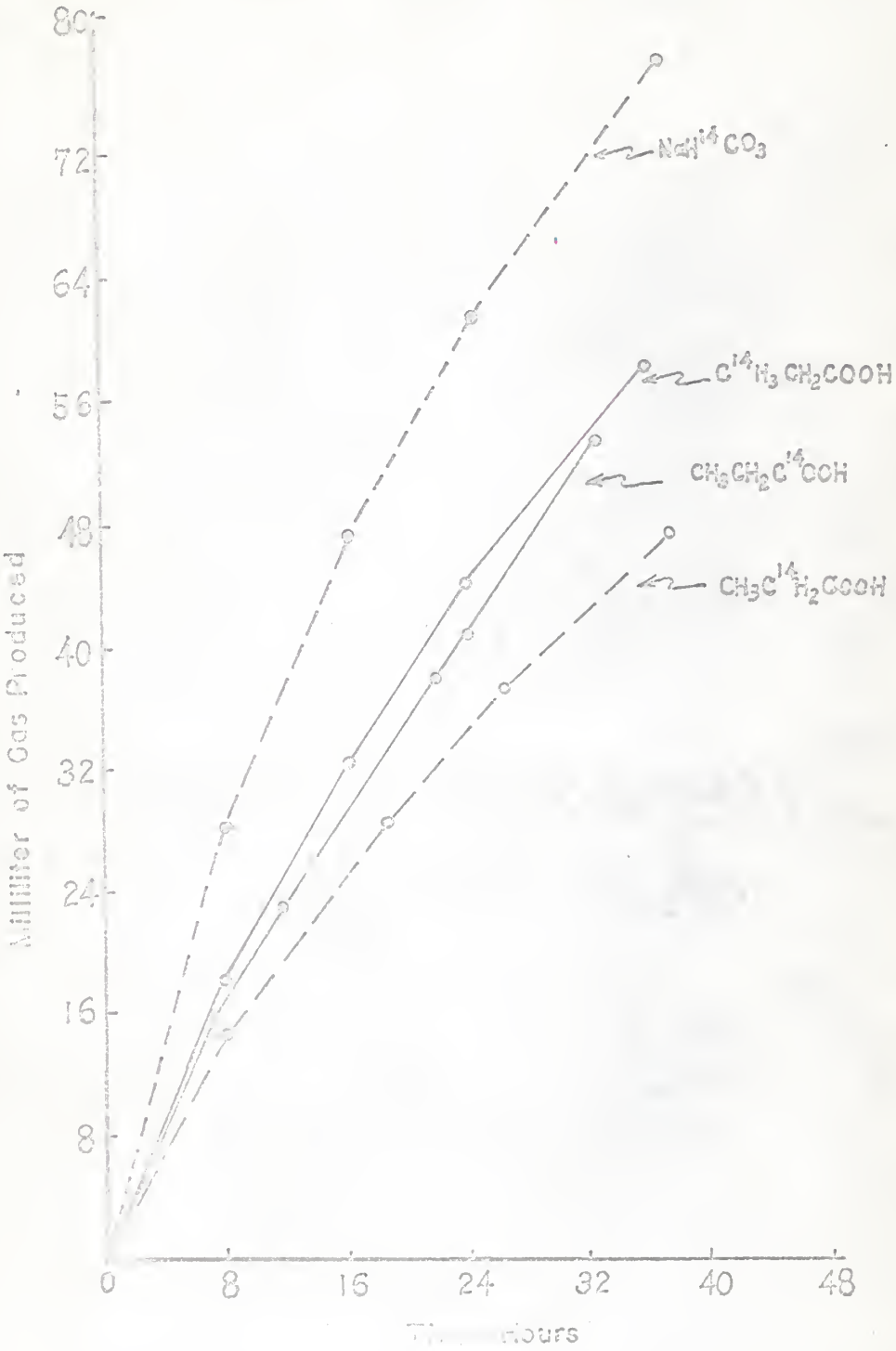

Fig. 6. Gas Production rate of cultures used in each of ^{14}C tracer experiments.

Table 3. Changes in amount of VFA in the propionate-1-^{14}C experiment.

No.	Time hours	The VFA in total culture fluid (m mole)
1	7.5	1.95
2	14.0	1.76
3	22.0	1.42
4	37.5	1.20

in Fig. 7. The results showed that the specific activity of carbon dioxide was always higher than that of methane. The specific activity of the VFA sequentially decreased.

In the next experiment, one microcurie of propionate-2-^{14}C was added to culture II in 2/3 m mole of propionic acid feed. The rate of production curve is presented in Fig. 6. Within 26 hours 37 ml gas were produced. The gas production rate was 1.4 ml/hr. The ratios of CO_2 and CH_4 in each different interval are recorded in Table 4.

Table 4. Ratio of CO_2 to CH_4, determined for samples taken at indicated interval, from the culture fed with propionate-2-^{14}C.

No.	Time hours	Model A-90 GLC CO_2:CH_4	Burrell Analyzer CO_2:CH_4
1	8.5	5.7: 7	5.0: 7
2	18.0	4.7: 7	5.0: 7
3	26.0	2.8: 7	2.4: 7
4	34.5	2.7: 7	2.7: 7

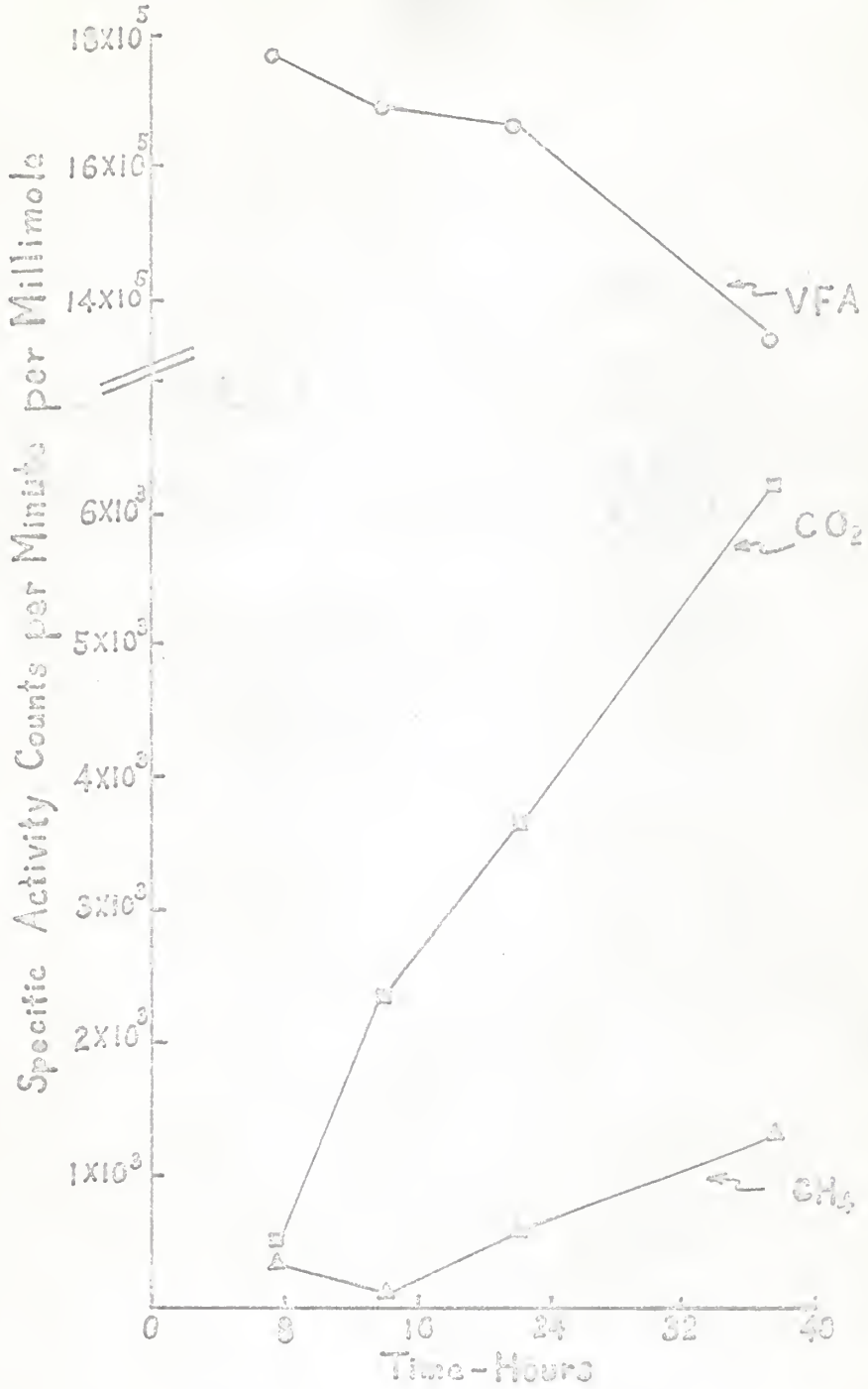

Fig. 7. Time specific activities of CO_2, CH_4 and VFA in the propionate-1-^{14}C experiment.

The VFA in this experiment also indicated a sequential decrease from each time interval (Table 5).

Table 5. Change in amount of VFA in the propionate-2-^{14}C experiment.

No.	Time hours	The VFA in total culture fluid (m mole)
1	8.5	1.95
2	18.0	1.55
3	26.0	1.28
4	34.5	1.14

The results (Fig. 8) showed that the specific activity of methane was always higher than that of CO_2. The specific activity of methane increased faster in the first 8 hours, while the specific activity of CO_2 increased faster after 24 hours. The specific activity of the VFA increased from 8.5 to 26.0 hours, then it decreased, but did not drop to the level of the 8.5 hour sample.

In the last propionate ^{14}C experiment, one microcurie of propionate-3-^{14}C was added to culture III in 1 m mole of propionic acid feed. The rate of gas production is shown in Fig. 6. There were 44 ml of gas produced in 24 hours at the rate of 1.8 ml/hr. The ratios of CO_2 and CH_4 for each different interval are recorded in Table 6. The determination of VFA in this experiment also revealed a sequential decrease per time interval (Table 7). The specific activity of methane was higher than that of CO_2 (Fig. 9). The specific activity in the VFA increased.

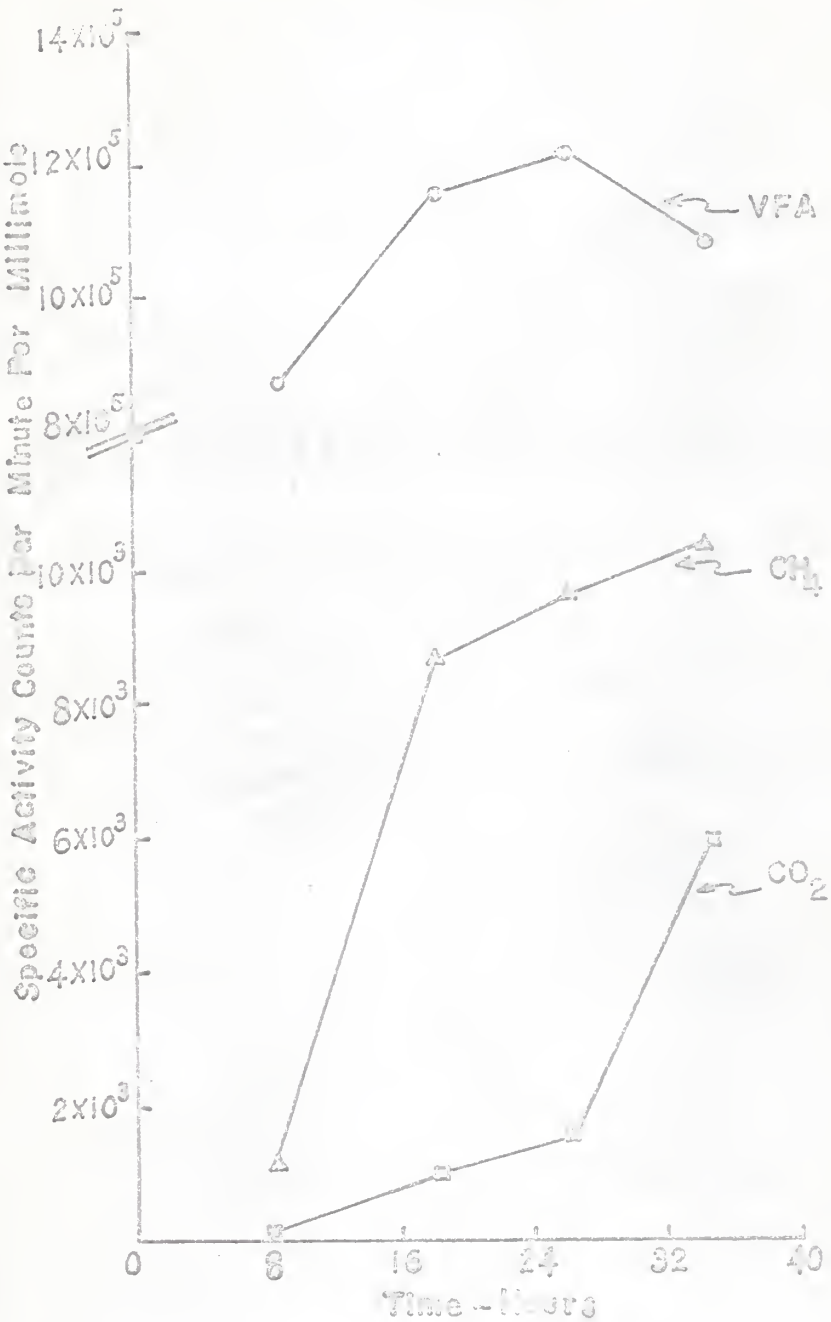

Fig. 8. The specific activities of CO_2, CH_4 and VFA in the propionate-2-^{14}C experiment.

Table 6. Ratio of CO_2 to CH_4, determined for samples taken at indicated interval, from the culture fed with propionate-3-^{14}C.

No.	Time hours	Model A-90 GLC CO_2:CH_4	Burrell analyzer CO_2:CH_4
1	8.0	6.0: 7	6.0: 7
2	16.0	6.5: 7	6.4: 7
3	24.0	6.2: 7	6.3: 7
4	36.0	5.2: 7	5.3: 7

Table 7. Change in amount of VFA in the propionate-3-^{14}C experiment.

No.	Time hours	The VFA in total culture fluid (m mole)
1	8.0	1.71
2	16.0	1.44
3	24.0	1.17
4	36.0	0.77

Fate of the CO_2 During Methanogenesis from Propionate

One microcurie of $NaH^{14}CO_3$ (2.5×10^{-4} m mole in 0.1 ml) and one m mole of propionic acid were added separately to a propionate culture in steady

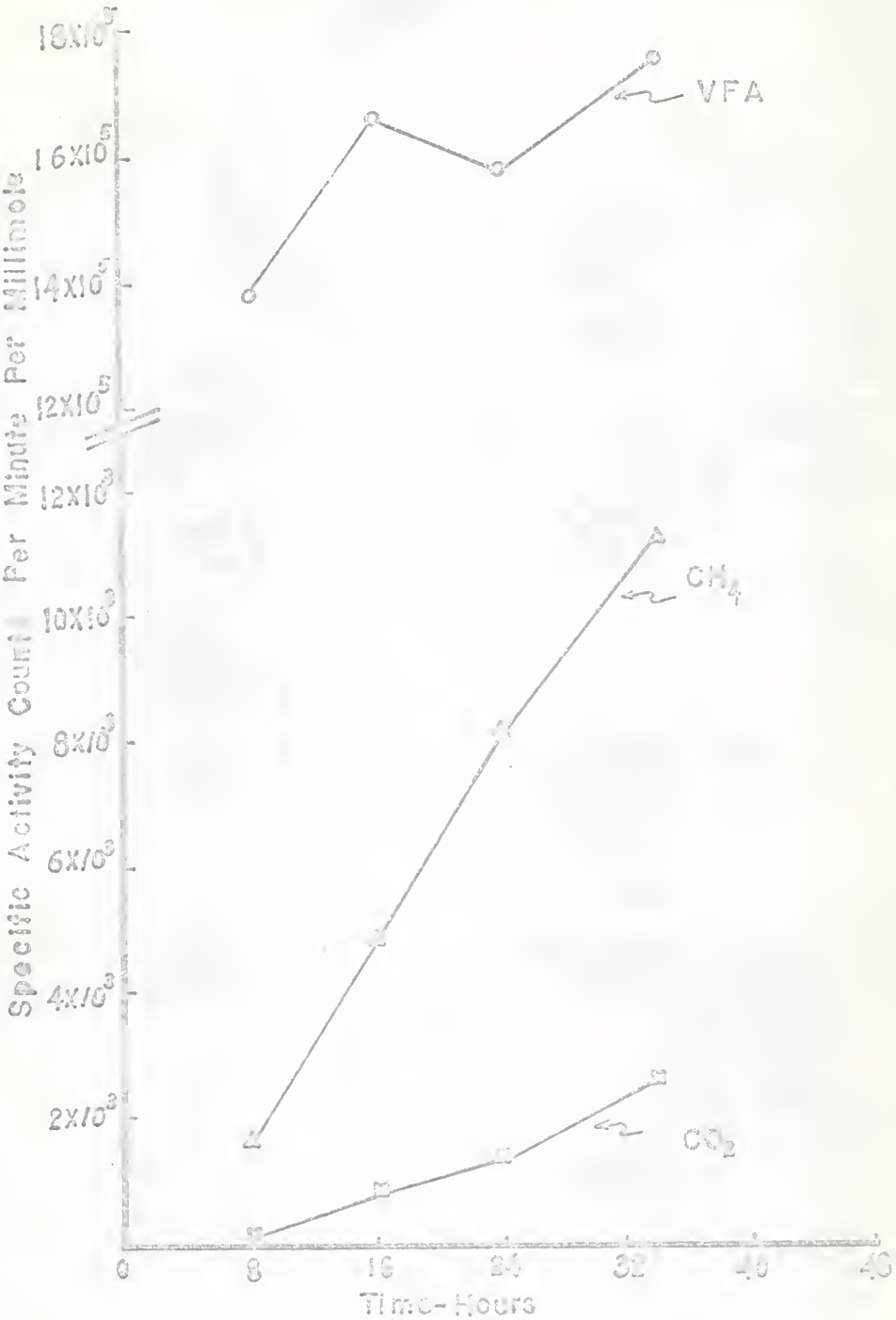

Fig. 9. The specific activities of CO_2, CH_4 and VFA in the propionate-3-^{14}C experiment.

state. The gas producing rate was 2.5 ml/hr. Within 24 hours 61 ml of gas were produced (Fig. 6). The gas ratios between CO_2 and CH_4 are shown in Table 8.

Table 8. Ratio of CO_2 to CH_4, determined for samples taken at indicated interval, from the cold propionate culture to which $NaH^{14}CO_3$ was added.

No.	Time hours	CO_2:CH_4*
1	8.0	7.4: 7
2	16.0	6.4: 7
3	24.0	5.0: 7
4	36.0	4.0: 7

* Using only the Burrell analyzer.

The amount of VFA once again continuously decreased (Table 9).

Table 9. Change in amount of VFA in the $NaH^{14}CO_3$ experiment.

No.	Time hours	The VFA in total culture fluid (m mole)
1	8.0	1.40
2	16.0	1.20
3	24.0	1.06
4	36.0	0.74

In this experiment, the specific activity in CO_2 rapidly decreased while the specific activity in methane slowly increased (Fig. 10). After

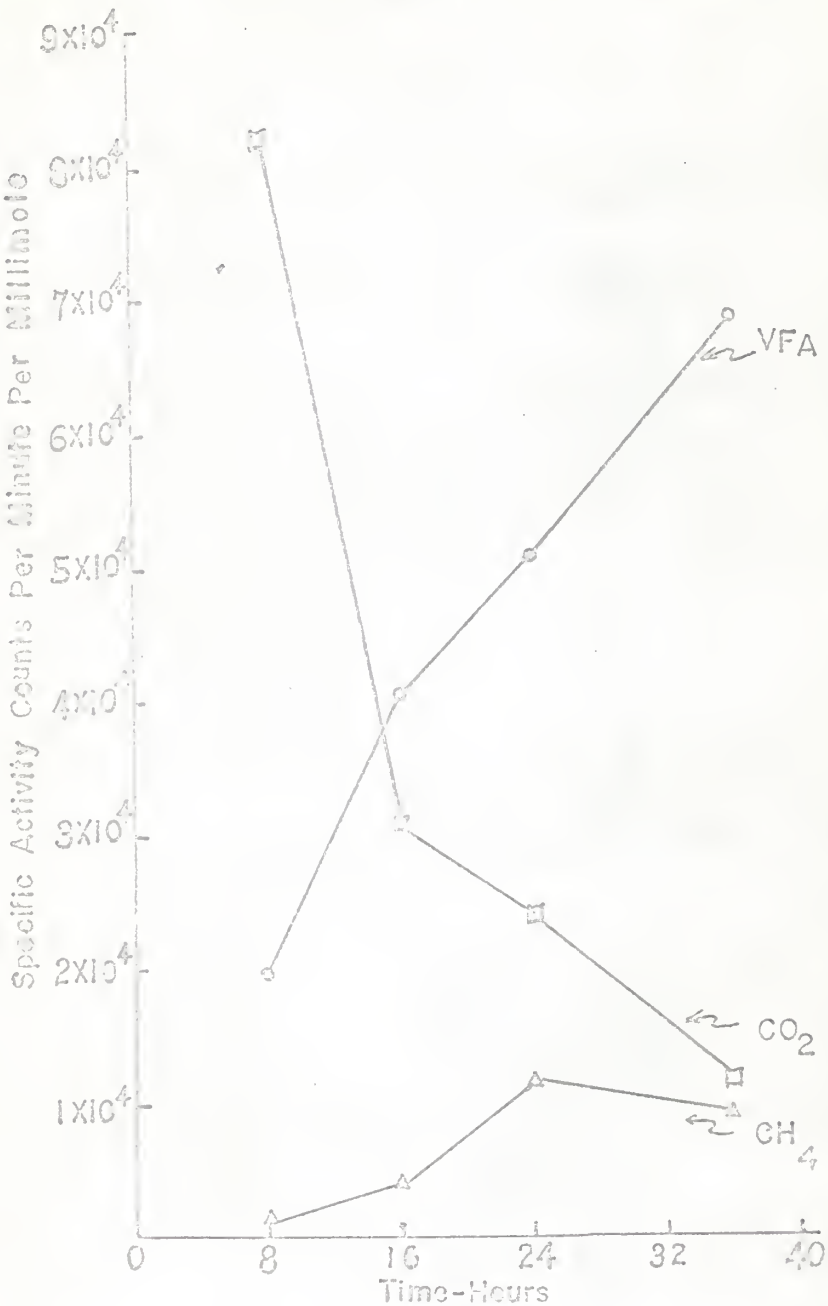

Fig. 10. The specific activies of CO_2, CH_4 and VFA in the sodium bicarbonate-^{14}C experiment.

36 hours, the specific activity in CO_2 and CH_4 was almost the same. The specific activity of VFA also increased continuously.

Distribution of ^{14}C in Selected Organic Acids (VFAs and Non VFAs) in the Presence of $^{14}CO_2$ Using Isotopic Dilution Technique

Attempts to isolate intermediates were unsuccessful even when using GLC methods. Isotopic dilution techniques were then employed. When sodium bicarbonate-^{14}C was added to the propionate culture, as described in the methods section, ^{14}C was found in the VFA portion. From an 80 ml aliquot, 1.114 m mole of VFA were obtained. Initially 1.0 m mole each of the cold VFA and non-VFA organic acid were added to 130 ml of culture. The ratio in this aliquot of acetic, propionic and butyric acids isolated, however, were 1:12:4. All three acids showed ^{14}C activity. The highest specific activity was in propionic acid, the lowest in butyric acid (Table 10).

Table 10. The quantity and specific activities of the GLC separated VFAs in the isotopic dilution $NaH^{14}CO_3$ experiment.

VFAs separated	m mole of VFA in the 50 µl concensed distillate	CPM minus background in 50 µl*	CPM corrected for efficiency in 50 µl **	Specific activity DPM/m M x 10^4
Acetic acid	0.002	45	94	4.7
Propionic acid	0.0236	897	1879	8.0
Butyric acid	0.0075	44	92	1.2

* The background was 31 counts.

** The counting efficiency was $\frac{4853}{8136} \times 100 = 60\%$.
The separation efficiency of GLC was 80%.
The total efficiency was 48%.

Organic acids trapped by isotopic dilution were separated by paper chromatography. They were identified as malonic acid, succinic acid, pyruvic acid and one unknown. These acids chromatographed three times were pooled, that is, three of the same spots were put in a counting vial and eluted to measure individual acid's radioactivity. No counts in any of the separated spots were found (Table 11).

Table 11. Lack of radioactivity in organic acid separated by paper chromatography in the isotopic dilution $NaH^{14}CO_3$ experiment.

Organic acids	Average CPM	CPM minus background*
Malonic acid	23	0
Succinic acid	22	0
Pyruvic acid	20	0
Unknown spot	22	0

* The background was 35 counts per minute.

Distribution of ^{14}C in Selected VFAs and Non VFAs When Species of Propionate-^{14}C were Used as Substrates During Methanogenesis by Isotopic Dilution Technique

In all cases 1 m mole each of the cold VFA and non VFA organic acids was added to each culture. In the culture given carboxyl labeled propionate, 0.316 m mole of VFAs was obtained in 80 ml of filtered culture fluid. The ratios of acetic, propionic and butyric acids were not 1:1:1 but 1:8:4. In the propionate-2-^{14}C experiment, 0.466 m mole of VFAs was obtained in 80 ml of filtrate. Again the ratio of three acids varied and was 1:5:3.

In the propionate-3-^{14}C experiment, 0.478 m mole of VFAs was found. The varied ratio of three acids was 1:4:3. The specific activity of the three VFAs in the three different labeled cultures is shown as Table 12. More butyric acid than acetic acid was isolated in the three cultures. The specific activity was higher in butyric acid than in acetic acid in propionate-1-^{14}C and propionate-2-^{14}C cultures, but it was lower in propionate-3-^{14}C culture.

In organic acids separated by paper chromatography in this same experiment, there were no significant counts in each of three pooled separated spots of malonic acid, succinic acid, pyruvic acid and unknown (Table 13). However, there was some activity in 5, 10 and 50 µl of total condensed ether extract (Table 14).

DISCUSSION

Methanogenic propionate enriched cultures were obtained from a methanogenic benzoate culture maintained in this laboratory. When the methanogenic propionic acid utilizing culture (propionate culture) was selectively enriched from a methanogenic benzoic acid utilizing culture (benzoate culture), an initial decreased but continuous rate of gas production resulted. The benzoate-derived propionate culture was maintained by continuous feeding of propionic acid. The optimal amount fed was determined from the graph of the rate of gas production for each culture. In several weeks the continuously enriched culture increased from its initial limits to an optimal and stable steady state. The propionate culture yields equalled that of the original parent benzoate culture. This state was maintained by feeding measured amounts of propionate. Full

Table 12. The quantity and specific activities of the GLC separated VFAs in the isotopic dilution experiments using various species of propionate ^{14}C.

Labeled propionate	VFAs separated	m mole of VFA in the 1 ml condensed distillate*	CPM minus background in 50 µl **	CPM corrected for efficiency in 50 µl ***	Species activity DPM/m M x 10^6
Propionate-1-^{14}C	Acetic acid	0.01	546	1,163	2.33
	Propionic acid	0.08	3,578	7,625	1.91
	Butyric acid	0.04	13,853	29,522	14.76
Propionate-2-^{14}C	Acetic acid	0.03	306	652	0.43
	Propionic acid	0.16	26,377	56,217	7.03
	Butyric acid	0.10	9,003	19,187	3.84
Propionate-3-^{14}C	Acetic acid	0.05	930	1,982	0.79
	Propionic acid	0.18	39,045	83,199	9.24
	Butyric acid	0.16	1,655	3,527	0.44

* The m moles in 50 µl x 20 = m moles per ml.

** The background was 31 counts.

*** The counting efficiency was 58.7%.
The separation efficiency of GLC was 80%.
The total efficiency was 47%.

Table 13. Organic acid separated by paper chromatography in the experiment using various species of propionate-^{14}C.

Labeled propionate species	Organic acids	Average CPM	CPM minus background *
Propionate-1-^{14}C	Malonic acid	27	3
	Succinic acid	23	0
	Pyruvic acid	31	7
	Unknown spot	40	16
Propionate-2-^{14}C	Malonic acid	19	0
	Succinic acid	27	3
	Pyruvic acid	32	8
	Unknown spot	45	21
Propionate-3-^{14}C	Malonic acid	30	6
	Succinic acid	38	14
	Pyruvic acid	36	12
	Unknown spot	39	15

* The background was 24 counts per minute.

Table 14. Activities in condensed ether extract residues in which VFAs were removed.

Labeled propionate experiment	Amounts µl	Average CPM	CPM minus background *
Propionate-1-^{14}C	5	265	240
	10	904	879
	50	3,510	3,485
Propionate-2-^{14}C	5	243	218
	10	375	350
	50	1,782	1,757
Propionate-3-^{14}C	5	244	219
	10	380	355
	50	2,154	2,129

* The background was 25 counts per minute.

selective adaptation was indicated when refeeding of benzoic acid to the propionate cultures resulted in no utilization of the benzoic acid. Stanier's (1947) simultaneous adaptation theory can still be used to explain this occurrence.

Arguments concerning methane formation from propionic acid have existed since Stadtman and Barker (1951) suggested that the decomposition of propionic acid results in carbon dioxide and acetic acid and that three fourths of the CO_2 would be converted to methane. Further, they stated that the acetic acid would be broken down into methane and carbon dioxide. In the breakdown of acetic acid, methane was formed from the methyl group, and carbon dioxide from the carboxyl group. The investigation of Buswell, Fina et al. (1951) indicated, however, that all carbons of propionate could yield methane and carbon dioxide, but that the ratios were different for each carbon. That is, the carboxyl group of propionate yielded more carbon dioxide, and the alpha and beta carbons yielded more methane. The results of propionate-1-^{14}C degradation (Fig. 7) confirmed that the carboxyl group of propionate favors a yield to carbon dioxide instead of methane. Moreover, there was no evidence that the carboxyl carbon was reduced to methane as reported by Stadtman and Barker. The propionate-2-^{14}C degradation results (Fig. 8) revealed a higher specific activity in methane than in carbon dioxide. Hence the scheme wherein carbon-2 yields CO_2, after the initial carboxyl cleavage of propionate into acetic acid and CO_2, was not necessarily favored. The propionate-3-^{14}C degradation results as expected (Fig. 9) contained a higher specific activity in methane than in carbon dioxide. The Stadtman and Barker scheme did not adequately account for the results noted in this study.

In order to find possible intermediates in the culture tracer experiments using ^{14}C were designed to study organic acids involved during methanogenesis. No intermediates could be isolated during experiments in which propionate alone was fed. By use of isotopic dilution techniques, acetic-^{14}C and butyric-^{14}C acids were isolated from propionate cultures fed three differently labeled propionic acid species in separate experiments (Table 12). The presence of ^{14}C in acetic acid indicated that propionate was degraded to acetate during methanogenesis. The ^{14}C activity in butyric acid showed that acetic acid could be converted to butyric acid possibly by a back-up reaction (McCarty, 1962). The specific activity of butyric acid (Table 12) was much higher than that of acetic acid in the experiments using either propionate-1-^{14}C or 2-^{14}C. The specific activity of butyric acid was somewhat more than half of that of acetic acid in the experiment using propionate-3-^{14}C. If the back-up reaction of butyric acid from acetic acid is considered to be right, a possibility is that decomposition of propionic acid occurred first to form acetic acid and methane by a cleavage of C-3 rather than a decarboxylation. The following scheme illustrates:

A. $2CH_3CH_2\overset{*}{C}OOH \xrightarrow{2H} 2CH_4 + 2CH_3\overset{*}{C}OOH \longrightarrow CH_3\overset{*}{C}H_2CH_2\overset{*}{C}OOH$

B. $2CH_3\overset{*}{C}H_2COOH \xrightarrow{2H} 2CH_4 + 2\overset{*}{C}H_3COOH \longrightarrow \overset{*}{C}H_3CH_2\overset{*}{C}H_2COOH$

C. $2\overset{*}{C}H_3CH_2COOH \xrightarrow{2H} 2\overset{*}{C}H_4 + 2CH_3COOH \longrightarrow CH_3CH_2CH_2COOH$

In 1965, Wood et al. showed methane formation from carbon three of serine using methyl cobalamin and N^5-methyltetrahydrofolate as intermediates. The cleavage of the 3rd carbon of a C-3 amino acid to form methane was possible. However, in the experiment of propionate-3-^{14}C there was considerable activities in acetic acid and butyric acid, instead of low activities. The decarboxylation of propionate to form acetic acid and carbon dioxide (Stadtman and Barker, 1951) possibly occurs simultaneously. Consequently, it appears that at least two mechanisms operate in methane formation from propionate. Although when using the isotopic dilution technique the same quantities of trapping VFAs were added to cultures, a lower amount of acetic acid was obtained compared with butyric acid after separation through GLC. It is possible that acetic acid was utilized faster than butyric acid, and that any butyric acid formed from two molecules of acetic acid accumulated.

The conversion of carbon dioxide to methane was studied using ^{14}C labeled sodium bicarbonate. The results (Fig. 10) indicate that some carbon dioxide could possibly be converted to methane. This is probably not the main pathway. The primary reasoning against this is the slow rate of ^{14}C appearance in the methane. There was a relatively rapid rate of ^{14}C appearance in the VFA portion. This finding led to the next study.

In the isotopic dilution $NaH^{14}CO_3$ experiment, all separated VFAs contained activity (Table 10). Most total activity was found in propionic acid, but there was a high specific activity in acetic and butyric acids. It is possible that reformations of the propionate, acetate, butyrate substrates and intermediates with carbon dioxide occurred. It would not be too unusual for this to happen. The synthesis of acetate from carbon dioxide, with Co-methylcobyric acid and Co-(methyl)-5-methoxybenzimidazolylcobamide as intermediates, with Clostridium thermoaceticum, has been shown by Ljungdahl et al. (1965).

In the methane formation from propionic acid, possible intermediates such as succinic, malonic and pyruvic acids were tested for. Using species of propionate-^{14}C and paper chromatographic isolations, of these acids from the culture fluid, only very, very low activities were found. Although these organic acids were not indicated as direct intermediates, further study is required of these and other organic acids, particularly with regard to reformation and rearrangement of VFAs. Also, in addition to enrichment cultures, studies should be made with isolated enzymes and pure cultures to determine precisely how methane is formed from propionate and/or other odd numbered organic acids.

SUMMARY

A series of studies was made on the fate of the three carbons of propionate in a methanogenic propionic utilizing culture. The data obtained indicate that:

1. Methanogenic cultures utilizing propionate can be easily selected from methanogenic cultures using benzoate.

2. All three carbons of propionic acid yield methane and carbon dioxide. The carboxyl group of propionate preferentially yields carbon dioxide, but carbon-2 and carbon-3 of propionate occur more frequently in methane than carbon dioxide.

3. Acetic acid appears to be an intermediate in methane formation from propionic acid.

4. Carbon dioxide can be reduced to methane, but CO_2 is not the major precursor of CH_4 in this culture.

5. Butyric acid can be recovered from propionate cultures. It is suggested that it is formed from two molecules of acetic acid.

6. Possible mechanisms are discussed for further study.

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THE FORMATION OF METHANE FROM PROPIONATE

by

JU-FUNG CHEN

B. S., National Taiwan University, 1961

AN ABSTRACT OF A MASTER'S THESIS

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The purpose of this investigation was to attempt to determine how propionate is converted to methane and carbon dioxide. The fate of the three carbons atoms of propionate during methanogenesis was studied. Several selected organic acids, possible intermediates, involved in propionate degradation were investigated.

A methanogenic propionate utilizing culture was converted from a methanogenic benzoate utilizing culture. All glass fermentors, equipped with glass stopcocks and serum stoppers, were used to maintain anaerobic conditions. A modified Barker's mineral salts medium plus the propionic acid was used. The medium was adjusted to pH 6.8. The cultures were kept in a 40 C incubator.

Various labeled species of propionate-^{14}C were fed individually to the cultures maintained in a steady state. By determining the specific activity of the gases derived from the catabolism of different propionate-^{14}C species, the fate of each carbon was followed. Each of the three carbons of propionate yielded CO_2 and CH_4, but carbon-1 preferentially yielded CO_2, whereas the 2nd and 3rd carbons appear more frequently in CH_4 and CO_2. Using the same techniques, when sodium bicarbonate-^{14}C was added and cold propionate was fed, the results indicated some CO_2 was reduced to methane.

The quantitative decrease of volatile fatty acid (VFA) in the cultures showed that propionate was utilized; however, when species of propionate-^{14}C were individually fed the specific activities of the VFA did not remain constant. They either decreased as in propionate-^{14}C or increased first, then decreased, as in propionate-2-^{14}C, or showed an increasing trend as in propionate-3-^{14}C. When bicarbonate-^{14}C was used, the VFA specific activities increased markedly. It would appear from these results that carbon dioxide

was fixed to organic acids and that the carbons of propionate are individually removed by a yet to be established mechanism.

Repeating the experiments above, distribution of ^{14}C in selected VFAs and non VFAs was investigated. The isotope dilution technique was used to help determine intermediates present in trace amounts. Attempts to isolate them otherwise failed. Species of propionate-^{14}C and bicarbonate-^{14}C were added individually. Cold acetic, propionic and butyric acids were used to entrap VFA-^{14}C traces. They were isolated by steam distilling filtrates, that had been passed through membrane filters. They were then titrated, acidified and separated by gas chromatography. All three were found to contain ^{14}C and the quantity and specific activity of each was determined. It appears that acetic acid was utilized faster than butyric acid. It may be possible that butyric acid was reformed from two molecules of acetic acid and that butyrate accumulated. The data indicated that propionate may be cleaved in two ways: either decarboxylation to yield CO_2 and acetic acid, or demethylation to yield CH_4 and acetic acid. Non VFAs (malonic, succinic and pyruvic acids) were apparently not involved. No significant counts were found in the separated spots, using isotope dilution paper chromatography and the scintillation counter.

In summary, methanogenic propionate cultures can be selected from methanogenic benzoate cultures. All three carbons of propionate yield methane and carbon dioxide. The carbon-1 of propionate preferentially yields CO_2, but carbon-2 and carbon-3 of propionate appear more frequently in CH_4 than CO_2. Acetic acid appears to be an intermediate in methane formation, either by demethylation or decarboxylation of propionate. Carbon dioxide can be

reduced to methane, but it is not the main pathway in this culture. Butyric acid can be recovered from propionate cultures. It is possible formed from two molecules of acetic acid. Non VFAs do not appear to be involved.