THE ANAEROBIC DECOMPOSITION OF AROMATIC COMPOUNDS DURING METHANE FERMENTATION

by

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TABLE OF CONTENTS

INTRODUCTION

The multiplicity of processes performed by all biological systems may be traced, directly or indirectly, to certain chemical reactions. The ability of microorganisms to perform unique biochemical transformations is well known. However, knowledge of the pathways that occur during these transformations, is in many cases incomplete. The processes of nitrogen fixation and photosynthesis are classic examples.

The structural material of the plant world is largely cellulose. In wood the cellulose fibers are embedded in an amorphous material of high molecular weight known as lignin. Lignin comprises 25-30% of the material called wood. The structure of the lignin monomer is still not completely known. Its similarity to the aromatic compound, coniferyl alcohol, noted more than 50 years ago, was confirmed by the fact that it can be oxidized to vanillin and hydrogenated to compounds of the cyclohexylpropyl type. After cellulose, lignin may be the most abundant single organic compound known.

Lignin, detergents, plastics, synthetic fibers, dyes, and industrial wastes are important sources of aromatic compounds in nature. The sulfite waste liquors from paper mills contain up to 6% lignin. Every household contributes detergents daily to our sewage systems. The biological degradation of lignin and related aromatic compounds to simpler substances is an integral part of the complex ecological systems of nature.

Modern technology constantly produces new aromatic compounds which make their way into our water supplies and sewage systems. The most important series of new aromatic compounds has been the synthetic detergents. With the increased usage of synthetic detergents there has been an increase in sewage treatment problems which were blamed on the presence of syndets in sewage. The aromatic compound, alkyl benzene sulfonate (ABS), as of 1959, made up 70% of the syndets produced (McKinney and Symons, 1959). ABS is resistant to both aerobic and

anaerobic attack. The fate of ABS during sewage treatment and its effect on sewage bacteria are unknown. The increase in ABS concentration of ground water is becoming a serious problem in some localities.

Two places where anaerobic decomposition of aromatic compounds are of prime importance are the rumen of herbivorous animals and the digestors of municipal sewage systems. Anaerobic methane bacteria may play a very important part in the decomposition of aromatic compounds; however, their place in the overall picture of aromatic decomposition in nature is rather obscure. It would appear, however, that they are a factor in returning aromatic type materials back to inorganic compounds.

Little attention has been given to the anaerobic utilization of aromatic compounds. One reason might be that there was a general belief that microorganisms were not capable of attacking ring compounds in the absence of unbound oxygen.

The conversion of aromatic compounds to methane and carbon dioxide by the methane bacteria was first reported by Tarvin and Buswell (1934). At the time the research in this thesis was undertaken, only the fact that anaerobic rupture occurs, had been clearly demonstrated.

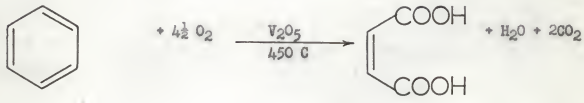
It was the purpose of this study to isolate intermediates in the anaerobic decomposition of benzoic acid. It was of interest to examine the fate of some of the carbons of the benzoic acid ring; that is, had they become methane or carbon dioxide. It was hoped that the results of this study would suggest a possible anaerobic aromatic pathway. The knowledge of this pathway might then suggest mechanisms for the anaerobic decomposition, in nature, of compounds such as lignin, aromatic detergents, and industrial wastes.

LITERATURE REVIEW

Properties of Aromaticity

There is no agreement on the definition of aromatic character (aromaticity). It is associated vaguely in the minds of chemists with the peculiarly unreactive double bonds supposed to be in benzene and certain other cyclic compounds and with their susceptibility to certain types of substitution reactions such as nitration, sulfonation, the Friedel-Crafts reaction, and mercuration.

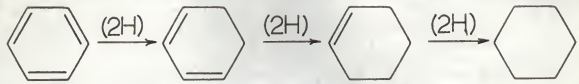
There are some chemical properties which are characteristic of benzene and its derivatives and these are often spoken of as aromatic properties. These properties were listed by Conant and Blatt (1949) and include: (a) resistance to oxidation; (b) the ready substitution of hydrogen by other atoms or groups; (c) acidic properties of the hydroxyl derivative; (d) failure to add reagents which usually add to the unsaturated compounds. The first characteristic is of special interest. Although the ring resists oxidation, if strong oxidizers are used the ring ruptures (Muldoon and Blake, 1947).



benzene maleic acid

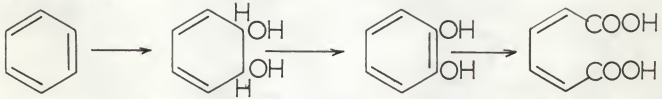
Because it is a resonance hybrid, benzene usually reacts as if it were saturated, but vaporized benzene can be hydrogenated in the presence of nickel to form cyclohexane $(CH_2)_6$. The reduction which is exothermic by 49.8 K cal

per mole, proceeds through the following steps (Muldoon and Blake, 1957).

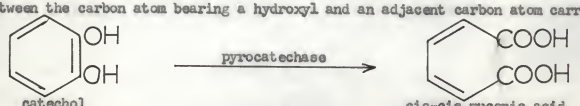


Biological Oxidative Rupture

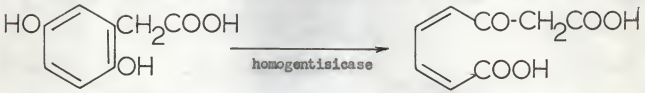
The benzene ring's resistance to oxidation and cleavage can be overcome by oxidative microbial enzymes. The ring can be ruptured under the severe conditions of strong reagents and high temperatures in the chemical laboratory. The microbial enzyme systems can perform this rupture without these requirements. Although the initial attack on the benzene ring by biological systems has yet to be elucidated, the following sequence of events has been postulated by Marr and Stone (1960).



Until the work of Evans et al. (1960), only two methods of cleavage of the aromatic nucleus by microbial enzymes had been demonstrated. These were: (1) oxidative fission of the bond between carbon atoms bearing the hydroxyl groups of an o-dihydroxyphenol by pyrocatechase as shown by Evans et al. (1951) and as shown below by Hayaishi and Hashimoto (1950). (2) Rupture of the bond between the carbon atom bearing a hydroxyl and an adjacent carbon atom carrying



a carbon side-chain or carboxyl as in the case of the enzymatic cleavage of homogentisic acid by homogentisicase. This reaction was demonstrated by Knox and Edwards (1955) and by Chapman and Dagley (1960) as shown below. Both methods

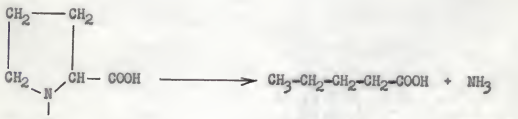


homogentisic acid maleyl acetoacetate

were demonstrated with purified enzyme systems of pyrocatechase and homogentisicase obtained from organisms of the genus Pseudomonas. Evans et al. (1960) were able to show the oxidative microbial attack on the protocatechuic ring resulted in the formation of a four and a three carbon compound. They were able to identify these as malate and pyruvate.

Biological Anaerobic Rupture

The work reviewed in this thesis indicated the oxidative cleavage of aromatic compounds, when used as a sole carbon source, usually led to the formation of Krebs Cycle intermediates. However, the anaerobic attack on the proline ring reported by Dehority et al. (1958) showed proline underwent reductive ring cleavage and deamination to form valeric acid as shown below.



proline valeric ammonia

The difference in types of end products could be significant and perhaps is dependent on the compound used as a final hydrogen acceptor.

The first report of anaerobic utilization of benzoic acid was made by Tarvin and Buswell (1934) while they were studying fatty acid oxidation during methane fermentation. They had attempted to apply Knoop's method of feeding phenyl-substituted fatty acids to enriched cultures of methane bacteria and determining the resulting substances. Complete destruction and gasification of the ring and attendant side chains resulted. Although this made the proposed method of study useless it did indicate, for the first time, that benzoic acid could be attacked anaerobically.

This work was confirmed by Clark and Fina (1952). Fina and Fiskin (1960) showed that carbon-1 of the benzoic acid ring became methane while carbon-7 became carbon dioxide when benzoic acid was used as a substrate for their enriched cultures of methane bacteria.

EXPERIMENTAL METHODS AND RESULTS

Preparation of Experimental Cultures

Cultures of bacteria capable of producing methane were developed from sludge obtained from anaerobic digestors at the Manhattan, Kansas sewage disposal plant. The fluid from the digestors was black and had a thick consistency. This material was incubated anaerobically for several days at 37°C to remove much of the soluble available organic substrate and solid matter. When the evolution of gas had subsided, the material was used as an inoculum.

The cultures were developed by inoculating all glass fermentation flasks, as shown in Plate I, with the above material. The flasks were prepared by adding shredded asbestos as suggested by Breden and Buswell (1933), the modified Barker's mineral salt solution of Fina and Fiskin (1960), and tap water. After

the sludge was added to the system, the flasks were immediately flushed out with oxygen free nitrogen.

When the inoculum from the sewage digestors was placed in the fermentation flasks it contained many types of organisms. Substrate and environmental conditions were chosen in such a way as to favor strongly the development of benzoic acid utilizing organisms. However, other organisms were known to exist in the young cultures. It was found that the fermentation could be carried on at 60°C and that this appeared to eliminate all but the anaerobic, thermophilic, organisms capable of metabolizing benzoic acid as a sole carbon source, at about pH 6.2, under anaerobic conditions. Five million units of penicillin, chloramphenicol, streptomycin, oxytetracycline, and chlorotetracycline were also added to the cultures along with 0.5 grams of sodium azide. The fermentation continued after the addition of each of these usually inhibitory substances. Culture fluid removed aseptically and streaked on nutrient agar plates failed to produce growth of any kind. Plates using benzoic acid as an only carbon source, inoculated in the same manner as above, failed to produce growth under anaerobic or aerobic incubation. It took several months to place these cultures in carbon balance.[1] Enrichment was maintained until the feeding of 0.5 mole of benzoic acid with the recovery of approximately 75 ml of gas in 24 hr could be repeated every 24 hr. At this point, the cultures were considered to be in carbon balance. For carbon balance data see Plate II.

Preparation and Feeding of Substrate

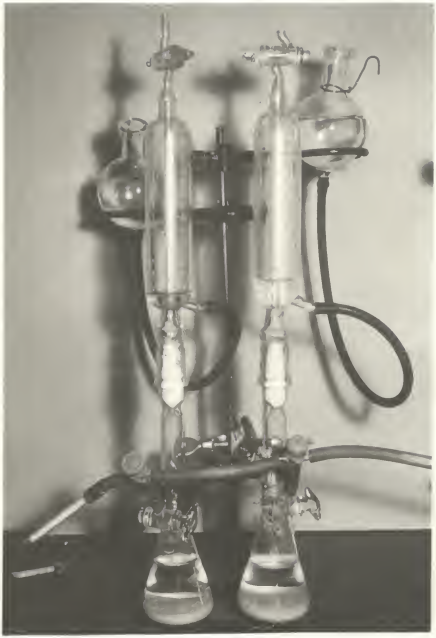
The modification of Barker's mineral salt medium, as described by Fina and Fiskin (1960), with dissolved benzoic acid was used as the substrate for

[1]Carbon balance here refers to a state in which all the substrate fed yielded equivalent amounts of gas. For carbon balance data see Plate II.

EXPLANATION OF PLATE I

Photograph of Benzoic Acid Fermentation Flasks

PLATE I



developing the methane bacteria cultures. The modification contained: $CaCO_3$, 6.33 g; K_2HPO_4, 0.4 g; NH_4Cl, 1.0 g; MgCl, 0.1 g; tap water, 1000 ml; cysteine solution, 30 ml. The cysteine solution contained 1.0 g of cysteine in 100 ml of water. Solid benzoic acid was added in an amount necessary to make an approximately 0.1 N solution. A 1 ml sample of this solution was steam distilled and the distillate was tirated against a standard solution of NaOH. The solution was refrigerated until used as a feeding solution.

The above solution was used as the initial feeding solution until an active fermentation was established. At this point the addition of the cysteine solution to the modified Barker's mineral salts medium was discontinued. For a period of 30 days after the fermentation had been established, the cultures were fed Barker's mineral salts medium without the cysteine solution. At the end of this 30 day period the cultures were fed an aqueous solution of benzoic acid only. A mineral salts balance was maintained by feeding the original Barker's modified benzoic mineral salts medium without cysteine, in place of the aqueous benzoic solution, once every 10 days.

Preliminary Carbon Balance and Steady State Studies

Studies were made to insure that the cultures were in carbon balance during the period of investigation and also in steady state while the actual experiment was being carried out. Plate II shows culture 3# in a state of carbon balance for a period of 14 days prior to determining the fate of carbon four. Table 1 shows culture 3# in steady state just prior to the experiment on the fate of carbon four.

It can be seen from Plate II that this culture was able to return approximately 45 ml of gas during a 24 hour period. It can also be seen that this could be repeated every 24 hours. Table 1 shows that a culture placed in steady

state is able to produce gas at an almost steady rate. The rate in this case was between 5.5 and 6.0 ml of gas per hour.

Table 1. Reaction rates of benzoic culture 3#. Feb. 21, 1961

mmole of feed	total time in hours	time in hours between readings	ml gas produced	ml/hr gas
0.3 mmole	0	0	---	---
0	1	1	6.0	6.00
0.5 mmole	2	1	5.5	5.50
0	3	1	5.5	5.50
0	4	1	6.0	6.00
0.5 mmole	6	2	10.5	5.25
0	8	2	12.5	6.25
0	10	2	11.5	5.75
0	11	1	5.0	5.00
0	12	1	6.0	6.00

Fate of Carbon Four of the Benzoic Acid Ring

It was shown by Clark and Fina (1952) that exogenous carbon dioxide was not further reduced to methane during the benzoic acid-methane fermentation. Fina and Fiskin (1960) showed that carbon-7, of the benzoic acid ring, becomes carbon dioxide while carbon-1 becomes methane. It was thought that if the fate of carbon-4 were known the information might aid in pin-pointing the point of ring rupture.

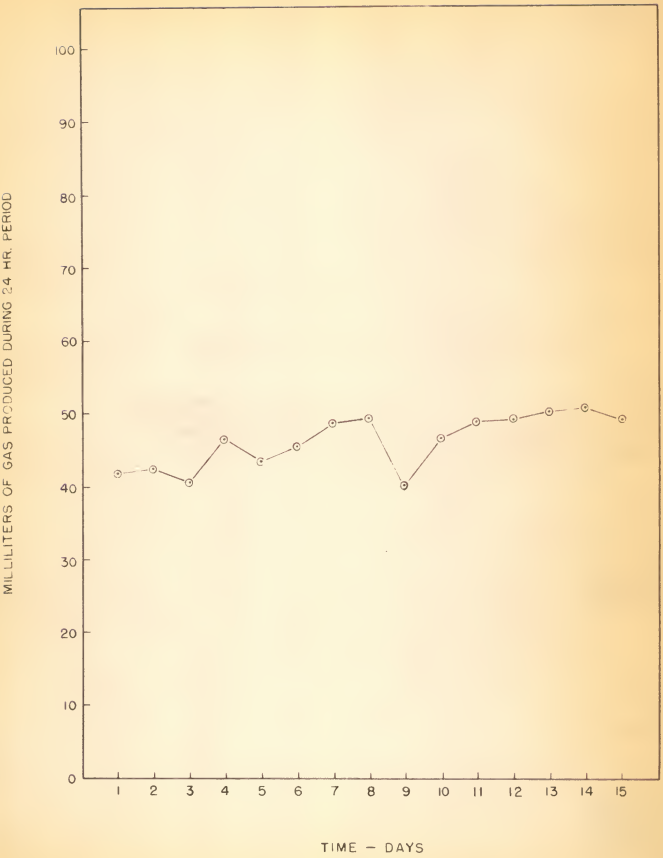
By feeding cultures C^{14} labeled benzoic acid, it is possible to determine the fate of the labeled fourth carbon; that is, had it become carbon dioxide or methane. As complete gasification of the ring takes place, with the production of only methane and carbon dioxide, the gas fraction containing the activity must represent the fate of the fourth carbon.

The techniques used in the determination of the fate of the fourth carbon of the benzoic acid ring were as follows: 0.3 mmole of benzoic-4-C^{14} acid

EXPLANATION OF PLATE II

A carbon balance study. Three mmole of benzoic acid were fed this culture at 0800 every day just after the gas production for the previous 24 hr was recorded.

PLATE II


MILLILITERS OF GAS PRODUCED DURING 24 HR. PERIOD
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
TIME — DAYS

were fed to a culture in carbon balance. The cultures must be in carbon balance so that most of the substrate will be returned as gas rather than be used as cell building material. Benzoic-4-C^{14} acid[1] is commercially unavailable. Upon feeding 1 ml of labeled benzoic-4-C^{14} acid, gas samples were removed as indicated in Table 2. Approximately 25 ml of gas were removed for each sample. The gas was separated into its carbon dioxide and methane fractions by the use of a Burrell Gas Analysis Apparatus. The Burrell gas analysis technique, as described in the Burrell Gas Manual, 7th Edition (1951), is a convenient method for determining exact volumes (± 0.2 ml) and for separating mixtures of gases. The carbon dioxide fraction of the carbon dioxide and methane mixture was absorbed in 1 N NaOH. The methane remaining was mixed with tank oxygen, oxidized by heating to carbon dioxide, and absorbed in additional NaOH. The two fractions were converted to barium carbonate by the method of Calvin et al. (1949), as modified by Fina and Fiskin (1960). The $BaCO_3$ precipitates, representing each of the two fractions, were washed three times in distilled water. The $BaCO_3$ precipitate was suspended in ethyl alcohol and pipetted into separate stainless steel planchets. These planchets, containing the separate $BaCO_3$ precipitates, were placed one at a time into a windowless gas-flow alpha, beta, gamma proportional counter and the C^{14} activities in both the methane and carbon dioxide fractions were determined. The results of this study were recorded in Table 2.

Background counts were determined and a count for each sample was taken three times. The actual counts as seen in Table 2 represent the average of the three determinations for each single sample with corrections for background. The figures under the heading mmole were calculated from the weight of the barium carbonate and checked against the amount of gas produced in a given sample.

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[1]The author was able to use part of 4 ml given to Dr. Louis R Fina by Drs. L. M. Henderson and G. P. Mathur of the Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma.

Specific activity was recorded as activity per millimole. The figures under this heading were calculated by dividing the actual counts by the number of millimoles. The ratio of specific activity of methane to carbon dioxide was the ratio of these two columns. The results in Table 2 indicated the ratio of methane to carbon dioxide activities was 1:4.2 after 21 3/4 hours. These data strongly suggested the oxidation of the fourth carbon of benzoic acid to carbon dioxide. The possibility that the fourth carbon of benzoic acid was reduced to methane during rupture and then oxidized to carbon dioxide does not seem likely as molecular oxygen is not available during the fermentation and hydrogen gas has never been detected. Under the highly reduced conditions that occur in methane cultures it seems probable that an oxidation of the ring carbon would occur in preference to an oxidation of the completely reduced carbon of the methane molecule. Barker (1956) has shown the methyl carbon of acetate gives rise to methane and the carboxyl carbon becomes carbon dioxide. Both of these processes occur by a direct pathway. A direct pathway by the fourth carbon to carbon dioxide is indicated from the results shown in Table 2. Once the fourth carbon is oxidized to carbon dioxide part of it may be reduced to methane; however, reduction to methane appears not to be favored.

Table 2. Fate of the fourth carbon of the benzoic acid ring during anaerobic decomposition.

time hours	CH_4 specific[a] activities x 10^{-3}	CH_4 actual[b] counts x 10^{-3}	CH_4 mmole	CO_2 specific activities x 10^{-3}	CO_2 actual counts x 10^{-3}	CO_2 mmole	ratio spec act CH_4 to CO_2
3.50	1.15	0.57	0.50	4.44	2.18	0.49	1:3.0
7.00	6.15	3.86	0.63	19.07	9.84	0.52	1:3.1
10.75	9.56	5.82	0.61	32.50	12.51	0.39	1:3.4
14.50	26.00	14.80	0.57	93.50	21.60	0.57	1:3.6
21.75	27.00	16.60	0.62	113.70	49.70	0.43	1:4.2

[a]Specific activity as counts/min/mmole
[b]All counts recorded as disintegrations/minute.
Specific activity of benzoic-4-C^{14} was 4.5×10^8 counts min/mmole.

Chromatography of Culture Fluid for Fatty Acid Intermediate Study

The fact that methane fermentations can continue uninterupted for years indicates the lack of accumulation of any compounds in the culture fluid. The substrate is converted almost completely to gas, and the gas is allowed to escape. Other workers have experienced difficulty in isolating intermediates produced by methane bacteria regardless of the substrate used. This study involved a search for any fatty acid intermediate that might be present in the culture fluid. The method of overfeeding the cultures to isolate intermediates was used by the author.

The cultures were placed in carbon balance and steady state. While the cultures were producing gas at a constant rate, 1.0 mmole of benzoic acid was introduced. Normal feeding was 0.3 mmole. The feeding of 1.0 mmole was less than inhibitory but of sufficient concentration to enhance any preferential attack on the substrate.

After a period of 2-3 hours, 50 ml of culture fluid were withdrawn. Subsequent experiments proved this time interval was satisfactory for the isolation of propionic acid. The 50 ml of culture fluid were centrifuged at 2,500 RPM to remove particulate matter. The clear supernatant was drawn off and acidified with 10 ml of 85% phosphoric acid. The phosphoric acid was added to supress salt formation and allow any volatile fatty acid present to be steam distilled easily. The acidified 50 ml of culture fluid were steam distilled and the first 10 ml of the distillate were used to form the hydroxamates of any fatty acid present. Fatty acids are volatile and cannot be chromatogramed in the acid form. The method of forming the hydroxamates of the fatty acid gives a system which is very sensitive (0.01-0.2 uM) to small amounts of fatty acids. It is especially suited for the short chain, one to six carbon, fatty acids.

With the chromatographic method used in this study it was found that distillation was a convenient step in separating any fatty acid intermediate from interfering substances. The percent recovery of the fatty acid intermediate from the culture fluid was of prime importance. Therefore, a study of the yield from the distillation was made. The study was carried out as follows: 50 ml of distilled water containing 0.0901 g-meq wts of propionic acid were steam distilled. Analysis of 10 and 50 ml fractions were repeated three times. The fractions of the distillate were titrated against 0.9225 N NaOH under an atmosphere of nitrogen. Phenolphthalein was used as an internal indicator.

Table 3. Recovery of propionic acid by steam distillation method.

g-meq wt of propionic in original 50 ml	ml of dist	ml of 0.9225 N NaOH needed to titrate dist	g-meq wt recovered in dist	% recovery	g-meq wt recovered per ml
0.0901	10	0.050	0.046	51%	0.0046
0.0901	10	0.052	0.048	53%	0.0048
0.0901	10	0.055	0.051	57%	0.0051
0.0901	50	0.075	0.069	71%	0.0013
0.0901	50	0.071	0.066	73%	0.0014
0.0901	50	0.076	0.076	78%	0.0015

From the data in Table 3 it can be seen that approximately 55% of the fatty acid is recovered in the first 10 ml. Approximately 76% was recovered in the first 50 ml. The fatty acids are separated from interfering substances in either case. The 10 ml fraction not only affects a separation but also concentrates the separated fatty acid. The 10 ml fraction contained approximately 0.0048 g-meq wts/ml of propionic acid as compared to 0.0014 g-meq wts/ml for the 50 ml fraction. The 10 ml fraction is about 3.5 times as concentrated. This was important in hydroxamate formation and spotting the solution on the chromatographic paper.

Hydroxamates were formed from the 10 ml distillate by the method of Block et al. (1955). The 10 ml sample of the distillate was made alkaline with 1 ml of 0.6 N sodium hydroxide and evaporated to dryness overnight in a hot air oven at 110°C. After evaporation, 2 ml of acidified methanol (2.5 ml of concentrated H_2SO_4 sp. gr. 1.84, added to 100 ml of methanol) were added to each tube, and the hard cake of salts was finely powdered with a small nickel spatula. It was necessary to break up the salt cake in order to secure the comple solution of the organic acids. The tubes were tightly stoppered and when esterification reactions had reached equilibrium, the hydroxamates were prepared from the methyl esters by the addition of 3 ml of a freshly mixed alkaline solution of hydroxylamine to each tube. The alkaline solution of hydroxylamine was prepared as follows: nine volumes of 5.0 N sodium methoxide in methanol were mixed with twenty volumes of a 1.7 N hydroxylamine hydrochloride in methanol solution and the mixture cooled to around 4°C. The sodium chloride was removed by centrifugation and washed with sufficient methanol to give the original volume to the hydroxylamine solution.

At least one hour was allowed for the formation of the hydroxamates. After this period of time, the insoluble sodium sulfate had settled in the tubes and the supernatant solution of hydroxamates was easily drawn into a micropipette.

This solution was placed on Whatman No. 1 paper and developed in a descending fashion with amyl alcohol: acetic acid: water in a ratio of 4:1:5 v/v. After the paper was air dried for several hours, the hydroxamates were detected with a ferric chloride solution. The ferric chloride solution consisted of (5% ferric chloride in methanol): acetone in a ratio of 4:3. The solution was filtered immediately prior to use. The hydroxamates appeared as dark blue spots on a pale yellow background.

Spots were located with the first chromatogram. This located the intermediate but did not identify it. Several more chromatograms were run until the

technique became familar and the intermediates could be isolated easily. The RF values of the spots were 0.50. The solvent system used was for the detection of short chain fatty acids. Block et al. (1955) listed the Rf value for the hydroxamate of propionic acid with this system at 0.51. It is not always possible to duplicate Rf values exactly in two different laboratories. It was believed that the Rf value of 0.50 compared closely enough to the value of 0.51 given by Block et al. (1955) to suggest propionic acid as an intermediate.

Hydroxamates were then formed from the following known fatty acids: formic, acetic, propionic, butyric, and valeric. These were run along with the unknown hydroxamate from the culture fluid. The following Rf values were obtained: formis 0.40, acetic 0.47, propionic 0.50, butyric 0.68, valeric 0.77, and the unknown from the culture fluid 0.50. The comparison of Rf values indicated the spot was propionic acid.

This experiment was repeated many times to confirm the first isolation and again many times while looking for other short chain fatty acids. Under these experimental conditions, only propionic acid was isolated and identified. The same techniques were also used in checking for activity in the propionic acid intermediate. A control of propionohydroxamic acid was used in every experiment. See Plate IV for a schematic drawing of a chromatogram. In every case the unknown spot had the same Rf value, 0.50, as the propionohydroxamic acid spot.

Ability of the Culture to Use Propionate

From the chromatographic work it appeared that propionic acid was an intermediate in the decomposition of benzoic acid. If this were true the cultures should be able not only to metabolize propionic acid but they should do it at the same or at an increased rate (Stanier, 1947). Cultures in steady state producing approximately 5.0 ml of gas an hour were fed 0.6 mmole of

propionic acid. The cultures were fed when an apparent decrease in rate of gas production due to substrate decrease was observed. If propionic acid were an intermediate the culture should have responded immediately by the return to the same or increased rate of gas production. From Plate III it can be seen that the culture did respond by returning to a near normal rate. This not only indicated that propionic acid could be metabolized but it suggested it strongly as an intermediate.

Tests for Activity in Intermediate When Culture was Fed Benzoic-1-C^{14}

From chromatographic and propionic acid feeding studies, propionic acid was shown to be an intermediate in the anaerobic decomposition of benzoic acid. Therefore, an attempt was made to find out which carbons of the benzoic ring were incorporated in the propionic acid molecule. By feeding the culture benzoic acid labeled in the first carbon and checking for activity in the isolated propionic acid, it was possible to detect the presence or the absence of the labeled carbon-1.

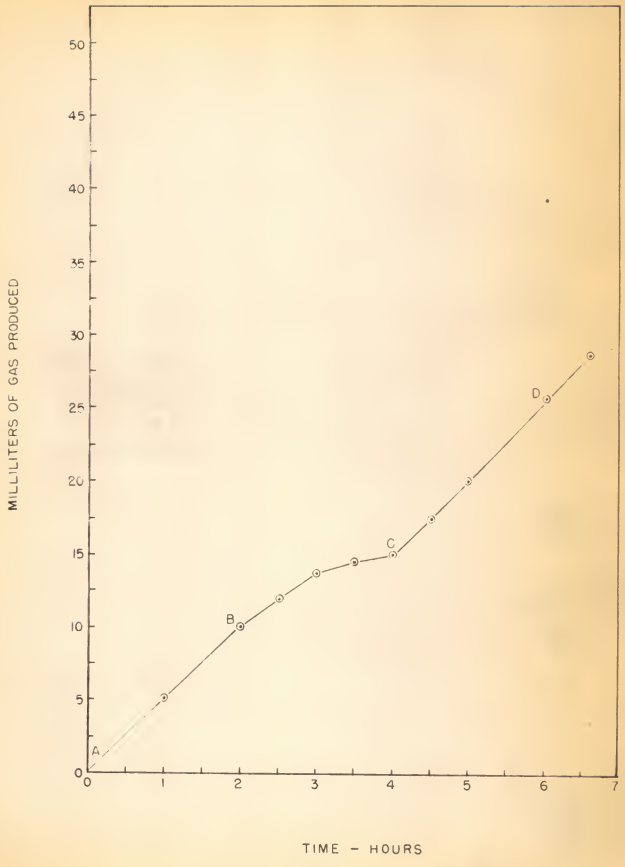
Benzoic-1-C^{14} acid was fed to the culture in the same manner as in the fate of carbon-4 studies and the chromatographic intermediate studies. After feeding 0.3 mmole of benzoic-1-C^{14} acid which had an activity of 9.2×10^8 counts/min/mmole, 50 ml of culture fluid were removed, centrifuged, steam distilled, and the hydroxamates of the propionic acid present were formed. This propionohydroxamic acid solution was spotted on chromatographic paper and the chromatogram was run in the same manner as described previously.

A schematic drawing of the chromatogram from this experiment can be seen in Fig. 1 of Plate IV. The first spot on the left was the propionohydroxamic acid control. The other five spots are from hydroxamates formed from the culture fluid intermediates when the culture was fed benzoic-1-C^{14} acid. Once again the intermediate spots had the same Rf value, 0.50, as the known propionohydroxamic acid.

EXPLANATION OF PLATE III

Results of feeding benzoic acid stabilized culture propionic acid. Line (a) to (b) shows culture in steady state. Line (b) to (c) shows decrease in rate of gas production due to decrease in amount of substrate. The culture was fed 0.3 mmole of propionic acid at point (c). Line (c) to (d) shows an immediate return to normal rate without a lag period.

PLATE III


MILLILITERS OF GAS PRODUCED
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7
TIME - HOURS

After chromatograms were run, and the propionic acid located, the spots were removed. Removal consisted of drying the paper in a current of air and cutting the paper, at each spot, into strips. One end of each strip was cut to form a point. The solvent was allowed to flow down the strips of paper and it was collected at the pointed end. The eluate, containing the propionic acid intermediate, was placed in a stainless steel planchet and dried under an infra red lamp. These planchets, containing the dried hydroxamate derivatives of propionic acid were tested for radioactivity in an alpha, beta, gamma proportional counter of the windowless gas flow type. The results of this study were recorded below in Table 4 as trial 1.

Table 4. Activity of propionate isolated from cultures fed benzoic-1-C^{14} acid.

background spots		1	2	3	4	5	6	trial 1
115		111	108	124	108	105	105	
108		115	106	102	101	109	119	
115		106	101	110	114	115	120	
113[a]	average	110	105	112	106	110	115	
								trial 2
135		120	130	135	115	120	115	
140		123	133	100	130	127	129	
130		114	119	125	129	117	132	
135[a]	average	121	127	120	124	121	125	

[a]All counts are recorded as disintegrations per minute.
Benzoic-1-C^{14} acid had an activity of 9.2×10^8 counts/min/mmole.

This method was employed to detect any activity in the propionic acid above the activity of the background. Detection of activity in the propionic acid molecule should indicate the incorporation of the labeled carbon-1 in the propionic acid intermediate. Absence of activity in the propionic acid should eliminate the possibility of the incorporation of the labeled carbon-1.

It can be seen that carbon-1-C^{14} was not incorporated in the propionic acid intermediate. The propionic acid intermediate did not exhibit activity above the background.

This same study was repeated and the data obtained confirmed the results of the first study. These data are shown in Table 4 as trial 2.

It appears from both of these trials that carbon-1-C^{14} of benzoic acid is not incorporated in the propionic acid intermediate. If carbon-1-C^{14} had been incorporated, the propionic acid intermediate should have exhibited activity above the background.

Tests for Activity in Intermediate When Culture Was Fed Benzoic-4-C^{14}

The analysis for activity in the intermediate when benzoic-1-C^{14} acid was used as a substrate indicated that carbon-1-C^{14} was not incorporated in the propionic acid molecule. This indirectly indicated, because of the relative positions of carbon-1 and carbon-4, that carbon-4 was incorporated in the propionic acid intermediate. See Fig. 1 below.

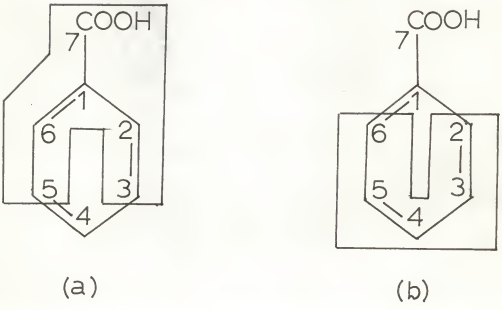


Fig. 1. Possibility of the incorporation of the fourth carbon in the propionic acid intermediate.

It can be seen in Fig. 1 that there are no possible combinations, of three carbons, that can include both carbon one and carbon four. Fig. 1 (a) shows the presence of carbon-1 excludes the presence of carbon-4. Fig. 1 (b) shows how it is possible for propionic acid to contain carbon-4 and not carbon-1. The absence of carbon-1 suggests also three possible combinations, of three carbons each, which could make up the propionic acid molecule. Carbons 6-5-4, 5-4-3, 4-3-2 are possible combinations that do not contain carbon-1 and yet contain carbon-4.

Because the intermediate was shown not to contain carbon-1-C^{14} and because of the inferences drawn from the relative positions of carbons one and four, the propionic intermediate was suspected to contain carbon-4 of benzoic acid.

A test of the hypothesis, that the intermediate contained carbon-4, was made by feeding the culture 0.3 mmole of benzoic-4-C^{14} and testing for activity in the intermediate. The results of this test are recorded in Table 5 and a schematic drawing of the chromatogram can be seen in Fig. 2 of Plate IV.

Table 5. Activity of propionic acid from cultures fed benzoic-4-C^{14}.

background	spots	1[a]	2	3	4	5	6
132[b]		129	534	415	508	510	508
122		135	537	453	490	499	512
119		132	535	434	499	506	510
126	average						

[a]control of known propionohydroxamic acid
[b]All counts are recorded as disintegrations per minute.
Benzoic-4-C^{14} acid had an activity of 4.5×10^8 counts/min/mmole.

From Table 5 it can be seen that the propionic acid intermediate is radioactive and has a count five times greater than the background. It was assumed from this and the data from the carbon-1 experiment that the propionic acid intermediate contained the fourth carbon of benzoic acid.

EXPLANATION OF PLATE IV

Fig. 1 Schematic drawing of one-dimensional chromatogram of hydroxamate preparation from fluid of cultures fed benzoic-1-C^{14}. (a) Indicates position hydroxamate preparation was spotted. (b) Position of hydroxamate spot at time of detection. (c) Position of solvent front at time of detection. Arrow shows direction solvent system moved.

Fig. 2 Schematic drawing of one-dimensional chromatogram of hydroxamate preparation from fluid of cultures fed benzoic-4-C^{14}. (a) Indicates position hydroxamate preparation was spotted. (b) Position of hydroxamate spot at time of detection. (c) Position of solvent front at time of detection. Arrow shows direction solvent system moved.

PLATE IV

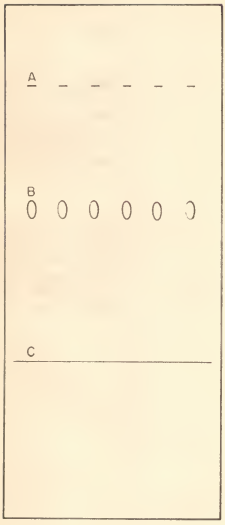


FIG. 1

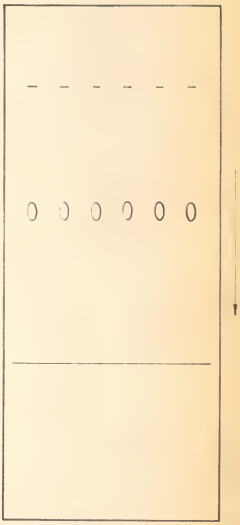


FIG. 2.

Only one experiment was carried out using benzoic-4-C^{14} to measure the activity in the intermediate. It should be stated again that benzoic-4-C^{14} is not available commercially and only enough was available to test for the fate of carbon-4 and detect any activity in the intermediate after feeding benzoic-4-C^{14} as a substrate. It was thought that the results of the carbon-1 experiment demonstrated the absence of carbon-1 in the propionic acid intermediate and strongly suggested the presence of carbon-4. The carbon-1 experiment was repeated twice and the results were the same in both trials. The experiment with carbon-4 served to corroborate the conclusions drawn from the carbon-1 experiment. From the results of these three experiments it was shown that the fourth carbon of benzoic acid is incorporated in the propionic acid intermediate.

Tests for Activity in Intermediate When Culture Was Fed Benzoic-7-C^{14}

At this point propionic acid had been isolated from the benzoic acid fermentation and its use as a substrate, without a lag period, had been demonstrated. The question was did the propionic acid come directly from the benzoic acid molecule or did it arise indirectly by a carboxylation reaction involving compounds such as pyruvate, oxalacetate, succinate, and acetate.

Carbon-7 of the benzoic acid ring was known to become carbon dioxide (Fina and Fiskin, 1960). If propionate arose indirectly its carboxylation could come from both carbon-4 and carbon-7. Therefore a method of testing for the indirect formation of propionate would be to feed 0.3 mmole of benzoic-7-C^{14} acid as a substrate. If the intermediate, propionic acid, were labeled, then the possibility of indirect formation existed. If the propionate were not labeled then the propionate should arise directly by cleavage of the benzoic acid ring. This experiment was carried out in the

same manner as described earlier. Results are listed below.

Table 6. Activity of propionate from cultures fed benzoic-7-C^{14} acid.

background	spots	1[a]	2	3	4	5	6	trial 1
125[b]		120	117	130	119	126	131	
120		128	125	121	125	121	125	
131		132	126	124	127	130	120	
125	average	127	123	125	124	126	125	
background	spots							trial 2
136		135	139	128	131	130	130	
139		131	136	133	134	129	134	
131		134	129	135	137	135	133	
136	average	133	135	132	134	138	132	

[a]Control of known propionohydroxamic acid.
[b]All counts recorded as disintergations per minute.
The benzoic-7-C^{14} had an activity of 5.55×10^6 counts per minute/mmole.

The experiment was repeated twice and the results are reported in Table 6 as trials one and two. The results in both trials show the propionic acid to be unlabeled. This strongly suggests the exclusion of the seventh carbon of the benzoic acid ring from the propionic acid molecule. It would also tend to rule out the indirect method of propionate formation, at least by the carboxylation mechanism. Because of their relative positions, as stated previously for carbon-1, the absence of carbon-7 would favor and suggest the presence of carbon-4 of the benzoic acid ring.

DISCUSSION

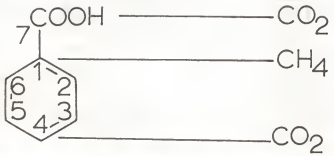
A study was made of the anaerobic utilization of benzoic acid. The methane fermentation was used as the anaerobic system. The methane fermentation was used as a tool in these series of experiments because some methane bacteria were known to use benzoic acid as a substrate under anaerobic conditions. Some

methane bacteria can use benzoic acid as an only carbon source and break it down to methane and carbon dioxide.

It was shown in Plate II and Table 1 that carbon balance and steady state could be achieved with these cultures. It was possible to predict with chemical certainty the ratio of CH_4 to CO_2 and the yield of gas per millimole of feed. Therefore, although the cultures were not pure, their unique qualities made them a valuable tool in this investigation.

The fate of carbons one and seven, of benzoic acid, had been reported prior to the research conducted for this thesis.

Enriched cultures of methane bacteria were fed benzoic-4-C^{14} acid to detect the fate of carbon-4 of the benzoic acid ring. It was hoped this information might suggest a point of ring rupture and perhaps suggest a pathway to methane and carbon dioxide.



It seemed logical at that point to initiate a chromatographic search for intermediates of the fermentation. It was assumed they would be present in u molar amounts and this led to the selection of paper chromatography as a method of isolation and identification. It was known that there was no build up of toxic substances during the fermentation as many of these cultures have been in continuous production for over a year. This also suggested that a large build up of intermediates did not occur. Many chromatographic methods were tried. The hydroxamate method as described in Experimental Methods and Results was successful. The intermediate propionic acid was isolated and

identified. When fed as a substrate propionic acid was used at the same rate as benzoic acid and without a lag period.

Fatty acids are formed in the rumen of some animals and are also found in sewage. This is a prime location for the isolation of organisms capable of carrying on the methane fermentation. Therefore, the fact that the fatty acid, propionic, was formed had some support from nature. The work of Dehority et al. (1958) showed the reductive ring cleavage of proline under reduced anaerobic conditions resulted in the formation of the fatty acid valine. Here the pathways may differ but the type of product formed was the same.

The question of which carbons made up the propionic acid molecule was the next problem to contend with. The oxidation of the fourth carbon of benzoic acid suggested it as a possible point of ring rupture.

Benzoic-1-C^{14} was fed to the enriched cultures to see if it would become incorporated in the propionic acid molecule. Lack of radioactivity in the intermediate after feeding benzoic-1-C^{14} eliminated the possibility of the incorporation of carbon-1 in the propionic acid molecule.

Because of the relative positions of carbons one and four in the benzoic acid molecule, the absence of carbon-1 suggested the incorporation of carbon-4. Both carbons one and four cannot be present at the same time. Proof of this hypothesis came from feeding the methane cultures benzoic-4-C^{14} acid. Activity was detected in the propionic acid intermediate and carbon-4 of the benzoic acid ring was assumed to be incorporated in the propionic acid molecule.

The question of how the propionic acid was formed: that is, was it by direct cleavage or by an indirect mechanism, was studied. If propionate arose directly it should not contain carbon-7 of the benzoic acid ring. If it were formed indirectly by a carboxylation mechanism then the carbon-7 would have an equal chance with the carbon-4 to become incorporated as they both become carbon dioxide. Carbon-1 was shown not to be incorporated. Therefore, benzoic-7-C^{14}

was fed as a substrate to the cultures. The propionic acid isolated exhibited no activity above the background. This excluded the presence of the labeled carbon-7 in the propionate. The carboxylation mechanism would have selected equally carbons-1 and 7. Therefore, the absence of activity in the propionate intermediate eliminated the indirect formation of propionate by a carboxylation mechanism. The exclusion of carbon-7 also strengthened the speculation of the incorporation of the fourth carbon.

Propionic acid has been proposed as an intermediate. The evidence indicates that the fourth carbon of benzoic acid may become the carboxyl carbon of propionic acid. Although this seems to be indicated, the precursor of the propionic acid intermediate is yet to be isolated.

The formation of the three carbon intermediate, propionate, suggests that on ring rupture at least two fragments are formed. The apparent oxidation of the fourth carbon of benzoic acid to carbon dioxide suggests that rupture occurs between carbon-3 and 4 or 4 and 5.

The fate of propionic acid as it is converted to methane and carbon dioxide is unknown. Buswell et al. (1951) did studies with propionic acid labeled in all three positions. They found that all three carbons were capable of giving rise to either carbon dioxide or methane.

The number four carbon, of benzoic acid, may stay with the three carbon fragment as the carboxyl of propionic acid. This is suggested by the presence of activity in the propionic acid isolated when benzoic-4-C^{14} was used as the substrate and the fact that carbon-4 becomes carbon dioxide. Carbons-1,7 of benzoic acid are not incorporated in the isolated intermediate propionic acid. This suggested further that another break occurs between carbons one and two or one and six.

Until the initial attack on the ring is known it would be difficult to compare this pathway with the known aerobic pathways. The term "the aerobic aromatic pathway" is no longer a good one. The attacks on the aromatic nucleus

under aerobic conditions have been shown to be so diverse that no single pathway seems to fit all of the proven mechanisms. Evans et al. (1960) have shown that under aerobic rupture the nucleus undergoes a diversity of attacks. Two intermediates common to most aerobic aromatic pathways are not attacked by benzoic acid enriched cultures under anaerobic conditions. The two intermediates are catechol and protocatechic acid (Fina, 1950). This suggested that the anaerobic and aerobic pathways differ.

This thesis represents research with an isolated system. To suggest the results here as "the anaerobic aromatic pathway" would be premature. It has been shown that benzoic acid is attacked anaerobically with the formation of the intermediate propionic acid. No research can be worthwhile unless it suggests other research. The initial attack on the ring should be elucidated. An investigation to see if carbon-carbon double bond intermediates are formed should be undertaken. Other systems capable of attacking aromatic compounds anaerobically should be investigated. The oxidative attack on aromatic compounds has been shown to occur by a multitude of pathways. To say there is but a single anaerobic aromatic pathway would be without foundation. Only by further research can other possible pathways of aromatic anaerobic metabolism be elucidated.

CONCLUSIONS

Experiments were designed to detect the fate of carbon-4 of the benzoic acid ring, isolate intermediates of an enriched methane fermentation in steady state, and detect any activity in the intermediates which were isolated after feeding labeled benzoic acid. The results of the experiments were recorded and from these data the following conclusions were drawn.

1. The benzoic acid ring is ruptured during this anaerobic fermentation.
2. Carbon-4 of benzoic acid becomes primarily carbon dioxide. Part of it may be reduced to methane; however, reduction to methane appears not to be favored.

3. Propionic acid was isolated from the culture fluid and appears to be an intermediate in the anaerobic rupture of the benzoic acid ring during methane fermentation.

4. The isolation of propionic acid suggests that at least two intermediates are formed.

5. The oxidation of the fourth carbon of benzoic acid to carbon dioxide suggests it as a point of possible ring rupture. The break could occur between carbon three and four or four and five.

6. The fourth carbon may stay with the three carbon fragment as the carboxyl of propionic acid. This is suggested because of the presence of activity in the propionic acid isolated when benzoic-4-C^{14} acid was used as a substrate, and the absence of activity in the propionic acid isolated when benzoic-7-C^{14} acid was used as a substrate.

7. The presence of carbon four in the isolated propionic acid and the absence of carbon one and seven suggests that another break occurs between carbons one and two or one and six. Carbon one and seven have never been found in the propionic acid intermediate.

8. Lack of activity in the propionic acid intermediate when benzoic-7-C^{14} was used as a substrate speaks against formation of propionate by an indirect method such as carboxylation.

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THE ANAEROBIC DECOMPOSITION OF AROMATIC COMPOUNDS DURING METHANE FERMENTATION

by

FOY FARRELL ROBERTS, Jr.

B. S., Kansas State University, 1961

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MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1962

Studies of the anaerobic decomposition of benzoic acid during methane fermentation were undertaken to gain information which might be of value in establishing a general anaerobic aromatic pathway.

Cultures of bacteria, capable of carrying on the methane fermentation, were obtained from anaerobic digestors at the Manhattan, Kansas sewage disposal plant. The cultures were developed in all glass fermentation flasks with benzoic acid in Barker's mineral salt solution as a substrate. Several weeks were required to establish an active fermentation. Anaerobic conditions were always maintained. A carbon balance was established in the flasks so that upon feeding 0.5 mmole of benzoic acid a recovery of 70-80 ml of gas in 24 hours could be repeated every 24 hours. In this condition the cultures would give greater than 90% of the theoretical yield, as gas, during a 24 hour period. In carbon balance little of the substrate was used as cell material. The fermentation experiments were carried out at 37 C although it was found that the fermentation would continue at 50-55 C.

It had been shown that exogenous carbon dioxide was not reduced directly to methane, as the main pathway, during the benzoic acid-methane fermentation. Carbon seven and carbon one were known to become carbon dioxide and methane, respectively, during the methane fermentation. The fate of carbon four was shown by feeding the cultures benzoic-4-C^{14} acid and analyzing the gas produced. Analysis was carried out by first absorbing the carbon dioxide, from the cultures, in 1 N NaOH and next burning the methane to carbon dioxide and absorbing the resulting carbon dioxide in additional NaOH. The two fractions were then converted to barium carbonate. The C^{14} activity from this material was measured in an alpha-beta-gamma proportional counter of the windowless gas flow type. The activity was exhibited in the carbon dioxide fraction by a 4.2 to 1 ratio. From this it can be seen that reduction of carbon dioxide to methane is not favored and the primary pathway of the fourth carbon is to become carbon dioxide.

Chromatographic studies were undertaken in an attempt to isolate possible intermediates between benzoic acid and its gasification to carbon dioxide and methane. The rate of production of intermediate compounds in the cultures used in these experiments was just above the demands for cellular metabolism. Thus, an extensive accumulation of intermediates did not occur. After numerous unsuccessful attempts were made with other methods, a successful isolation was made with a paper chromatographic method employing hydroxamate formation. The spot which was isolated was identified as propionic acid. Propionic acid was fed to the culture and it was used at the same rate and without a lag period.

An attempt was then made to determine which of the carbons of the benzoic acid ring became propionate. First the culture was fed benzoic-1-C^{14} and propionic acid was isolated from the culture fluid. The spot was eluted off of the paper and placed in planchets which were dried. A test for activity was made in the proportional counter. The propionate did not exhibit any activity above the background. This strongly suggested that carbon-1, of the benzoic acid ring, was not incorporated in the propionic acid intermediate.

Because of the relative positions of carbons one and four, the incorporation of one would suggest the exclusion of the other. Therefore, the absence of carbon-1 suggested the presence of carbon-4. A test of this hypothesis was made when the culture was fed benzoic-4-C^{14} acid. The same procedure was followed as above. The propionate contained activity four times greater than the background. It was concluded from this that carbon-4 became part of the propionic acid molecule.

Experiments were run in an attempt to answer the question, did propionate come directly from the benzoic acid molecule or did it arise indirectly by a carboxylation reaction involving compounds such as pyruvate, oxalacetate, succinate, and acetate. If propionate arose indirectly its carboxylation could come from both carbon-4 or carbon-7. A method of testing for indirect formation

of propionate was to feed benzoic-7-C^{14} as a substrate. If the intermediate, propionic acid, were labeled, then the possibility of indirect formation existed. If the propionate were not labeled then the propionate should have arisen by direct cleavage of the benzoic acid ring. The propionate isolated after feeding benzoic-7-C^{14} was not labeled. This suggested strongly that propionate arose directly as a result of the cleavage of the benzoic acid ring.

In summary, carbon-4 of benzoic acid became primarily carbon dioxide. The isolation of propionic acid indicated it as a possible intermediate and suggested that at least two fragments are formed. The oxidation of the fourth carbon suggested it as a point of ring rupture. The break could occur between carbons three and four or carbons four and five. The fourth carbon stays with the three carbon fragment, possibly as the carboxyl carbon of propionate. This was suggested because of the presence of activity in the propionate isolated when benzoic-4-C^{14} was the substrate, and the absence of activity in the propionate isolated when benzoic-1-C^{14} and benzoic -7-C^{14} were used, and the fact that carbon-4 was shown to become carbon dioxide. The presence of carbon-4 in the isolated propionate and the absence of carbons-1 and 7 suggested that another break occurs between carbons one and two or carbons one and six. Carbons-1 and 7 have never been found in the propionate intermediate. Lack of activity in the propionate intermediate when benzoic-7-C^{14} was used as a substrate speaks against formation of propionate by an indirect method such as carboxylation.