Civil Engineering Technical Report No. 222

Prepared for the
Division of Solar Energy
U.S. Energy Research and Development Administration
Under Grant No. ERDA-EY-76-S-03-0326-DA-44 initiated on June 1, 1976

HEAT TREATMENT OF ORGANICS FOR INCREASING ANAEROBIC BIODEGRADABILITY

by

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June 30, 1977
Annual Progress Report
Covering the period June 1, 1976 to May 31, 1977

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SUMMARY STATEMENT

This report represents the results of the first year of study on the heat treatment of organics to increase its biodegradability by anaerobic bacteria for the microbial production of methane. The purpose of this study is to develop a means for increasing the yield and reducing the cost of methane, a useful energy source. The procedures being evaluated are heat treatment at temperatures up to 250°C, under pH ranges of 1 to 13. Included in this report are results on (1) lignocellulose digestion and acclimation to its products from heat treatment, (2) the fate of waste activated sludge and its cellular nitrogenous compounds, and (3) the biodegradability of model compounds likely to be formed during heat treatment.

The studies on lignocellulose digestion indicated that a 2-stage thermochemical digestion system approximately doubled methane yields from newsprint, a lignocellulose source, when compared to conventional single-stage digestion. In this system, the effluent from the first stage digester is heat treated at 200°C and pH 13, for 1 hr, then fed to a second stage digester. Organisms were found to acclimate to low concentrations of lignocellulose heat treatment products but are inhibited when soluble lignin concentrations approximate 2 g/l. Furthermore, anaerobic digester populations also could acclimate to heat-treated alkali-lignin converting approximately 14% into methane. The practical implications of these results is that this amount represents about 1 to 6% of the total methane potential for a biomass residue of interest and this fraction is generally considered refractory. In addition, organisms must be acclimated to fully evaluate the effect of heat treatment for increasing anaerobic biodegradability.

Initial experiments on the degradability of heat-treated waste activated sludge (WAS) indicate significant increase in degradability, ranging from 25 to 63% over untreated WAS. Heat treatment also appears to increase the solubilization of organics reaching a maximum at 225°C, after which soluble organic concentration decreased. Alkaline or acid conditions did not enhance this solubilization to any great extent. Increasing temperatures appears to hydrolyze the soluble organic nitrogen (SON) fraction causing an increase in amino acids and ammonia. Acid conditions greatly increased this hydrolysis compared to alkaline conditions. Biodegradability studies indicated that decomposition of WAS decreased with temperature increases during heat treatment.

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Formation of inhibitory compounds were demonstrated and is likely to be a factor with regard to the apparent decrease in biodegradability.

Microbiological studies on degradation of model compounds indicated predictable stoichiometric amounts of CO_2 and CH_4 are produced from phenol, vanillic acid, and ferulic acid. Values obtained showed more than half of the organic carbon is converted to CH_4. Cross acclimation experiments indicated that populations acclimated to one compound can be simultaneously acclimated to other compounds which often are otherwise more refractory. Acetate was found to be a key intermediate during ferulic acid decomposition. The organisms responsible for these transformations appear to be a consortium of at least 3 to 4 species. The initial rate-limiting steps appear to be the selection, adaption and increase of the acid-forming and ring-cleaving population rather than that of the methanogens.

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I. INTRODUCTION

A. Objectives of Project

The microbiological conversion of organic material such as sewage sludge, agricultural and industrial residue, and refuse to methane gas has the potential of supplying about 11-20 percent of the current natural gas demand in the United States. Studies on the bioconversion of organic residues to methane have emphasized a significant problem which both adds to the cost of methane production and reduces the actual gas production from 20 to 60 percent of the potential yield; that is, the inability of microorganisms to completely decompose all the organic matter, thus leaving a stable refractory residue requiring further disposal.

The objective of this study is to increase the biodegradability and, hence, methane production from residue organic materials by heat treatment under pressure. This study is to include a range of organic materials including farm residues, forest product residues, and municipal wastewater sludges. The aim is to develop a general model for heat treatment which is applicable to all organic materials.

The major variables proposed for study are temperature of treatment over the range of 25° to 250°C, time of treatment over the range from 0 to 3 hours, and pH over the range from 1 to 13. Based on ongoing studies, heat treatment is expected to solubilize and simplify the structure of wastes which would increase the biodegradability significantly. The heat treatment process is expected to be compatible with the normal process train through which organic materials are digested for conversion to methane gas.

B. Approach

This project is being conducted in several phases, one of which is an evaluation of the effect of heat treatment and pH on anaerobic biodegradability of various organic residues. Information on this phase is reported here and was also reported at the quarterly progress meeting in June 1977. At this time, work in this phase is continuing.

A second phase of the study is to evaluate the effects of treatment on the various typical individual components that comprise residual organics and to determine the degree and the nature of toxic products produced during

heat treatment. Results from these continuing studies were reported at the quarterly progress meetings in March and June, 1977, and are reported here.

A third phase was the determination of the biodegradability of refractory compounds released during heat treatment and the characteristics of their conversion to methane. Aspects of this phase were reported at all the quarterly progress meetings and are also presented in this report.

The fourth phase consists of semi-continuous digester studies on different organic residues at mesophilic and thermophilic temperatures. Preliminary results are reported here and were also reported at the quarterly progress meetings.

The fifth phase involves development of a model which incorporates physical and operating parameters for computing methane production. A preliminary model was developed and reported in previous project reports. Further work in this phase will take place as more experimental data result.

II. EXPERIMENTAL STUDIES

A. Lignocellulose Digestion and Pretreatment Alternatives

W. F. Owen and P. L. McCarty

1. Previous Work

The potential of thermochemical treatment for increasing the anaerobic degradability of lignocellulosic materials was evaluated over a broad range of temperature and pH with a short-term bioassay technique (Gossett and McCarty, 1975a; Gossett et al., 1975b; and Gossett et al., 1976b). Municipal refuse was used as a representative lignocellulosic substrate. In these studies the maximum increase in biodegradability of previously digested material was found with pretreatment conditions of pH 13 for one hour at 200°C. This represented a 100-percent improvement in the degradability of untreated digested material, but a 15-percent improvement when referenced to untreated undigested material. Additional studies with a variety of substrates (Douglas Fir, newsprint, cardboard, bond paper, raw refuse, digested refuse, and cellulose) demonstrated a good correlation between the digestibility of an untreated lignocellulosic sample and its respective lignin content (Gossett, 1976). Lignocellulosics with a high lignin content (e.g. Douglas Fir and newsprint) were considerably less degradable than those with a low lignin content (e.g. cellulose and bond paper). It was further shown that improvement in digestibility, as facilitated by alkaline heat treatment, is related to the solubilization of lignin. This is not a direct relationship since the amount of degradable carbohydrate converted to refractory products increases as the severity of treatment increases (i.e. as the solubilization of lignin increases). Therefore, a maximum increase in degradability was found to occur when the difference between the percentage of original cellulose remaining and the percentage of original lignin remaining was a maximum. These results are tempered by two facts: (1) the short-term bioassay technique employed does not permit organism acclimation to uncommon or toxic heat-treatment products, and (2) heat-treated lignin products in concentrations greater than 1 g/l were found inhibitory to an unacclimated seed.

Semi-continuous, mesophilic digestion studies were conducted to more fully define the potential of alkaline heat treatment for increasing digestibility (Gossett et al., 1976b). The most successful scheme evaluated

employed thermochemical treatment on a recycle stream with the recycle flowrate equal to the influent flowrate. Using "optimum" heat treatment conditions, as determined by the short-term assays, this unit exhibited a 49-percent increase in methane yield from undigested municipal refuse when referenced to a 15-day SRT control digester. This increase is approximately three times greater than predicted by the short-term assay procedure (which predicts a 15-percent increase), and the difference in undoubtedly due to organism acclimation. Despite a significant increase in digestibility effected by thermochemical treatment, the semi-continuous digester appeared to be "stressed" as witnessed by a relatively high volatile acids concentration (1,100 mg/l) and erratic gas production. The stressed conditions are believed the result of soluble, heat-treated lignin products which were estimated at a concentration of 2.5 g/l.

Based on the short-term assays and the semi-continuous digester results, a two-stage digestion scheme was proposed for obtaining maximum methane yields from lignocellulosics (see Figure A-1 after McCarty et al., 1976). In this scheme, readily degradable organics would be fermented to methane in the first-stage digester and the effluent would be subjected to thermochemical treatment to make the remaining organics more degradable. The thermochemically treated effluent would then be fed to a second-stage digester for additional methane generation. This arrangement was postulated to minimize the amount of degradable organics converted to refractory matter by thermochemical treatment, and was estimated to produce 73 percent more methane from municipal refuse than conventional digestion.

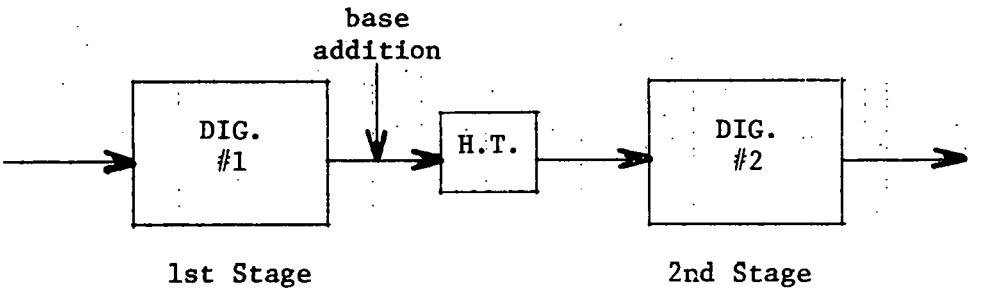

FIGURE A-1. Process configuration for two-stage thermochemical digestion of organics.

Supplemental studies indicated that many of the single-ring aromatic products reported formed during alkaline heat treatment of lignin are fermentable to methane gas (Gossett et al., 1976a; Healy and Young, 1976). These results are important when considering: (1) whether lignin (a complex polymer which is reported as refractory under anaerobic conditions in situ) can contribute a significant quantity of methane when subjected to alkaline heat treatment, and (2) whether the toxic materials formed by alkaline heat treatment of lignin are degradable or otherwise subject to organism acclimation.

2. Objectives of Study

The primary objectives of the research conducted this past year were: (1) to verify the potential of the two-stage thermochemical digestion scheme for increasing methane yield from lignocellulose, (2) to develop an anaerobic culture acclimated to products from the alkaline heat treatment of lignocellulose, and (3) to develop long-term, batch bioassay techniques for future study with acclimated seed. In addition, the digestibility of thermochemically treated lignin was investigated by use of the newly developed long-term assay procedures.

The study was conducted in two phases: The first phase was to satisfy objective (1) above; the second phase made use of the acclimated seed developed during the first phase to satisfy objectives (2) and (3), and to determine the digestibility of thermochemically treated lignin.

Previous semi-continuous digestion studies of thermochemically treated lignocellulose were adversely affected by high concentrations of heat-treated lignin products (Gossett et al., 1976b). In order to minimize these effects during the first phase studies, the heat-treated feed to the second-stage digester was diluted to adjust the soluble lignin concentration to approximately 1 g/l (see Figure A-2). Also, a second digester was fed heat-treated effluent from the first stage using less dilution water in order to establish the threshold for heat-treated lignin toxicity to an acclimated culture. This digester operated at a soluble lignin concentration of approximately 2 g/l. In addition, a control digester was operated to determine the amount of digestible organics remaining after first-stage digestion. This enabled a direct computation for determining the benefits of heat treatment. An operation summary for the four digesters is contained in Table A-1.

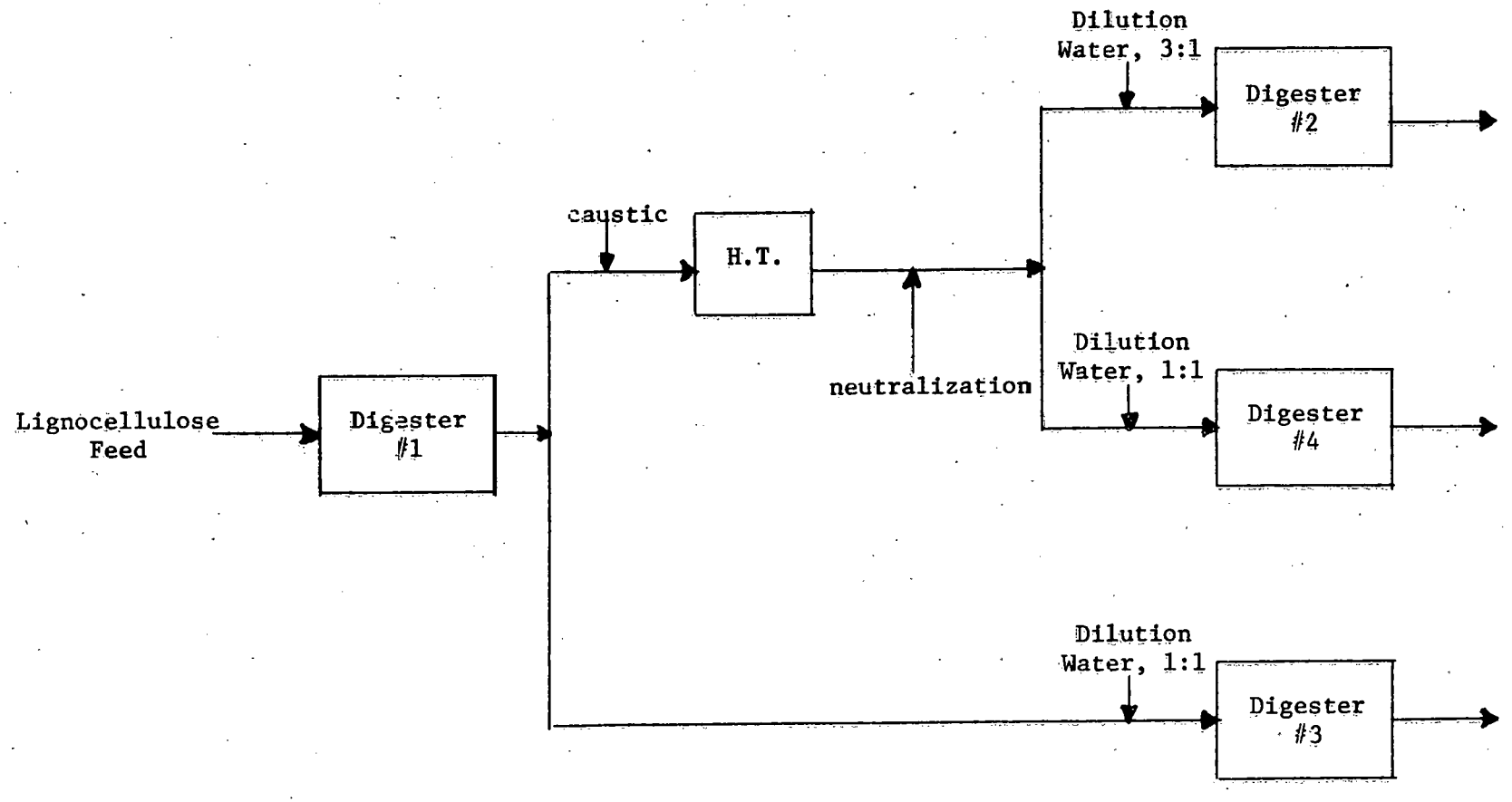

FIGURE A-2. Schematic representation of the two-stage thermochemical digestion study program. All digesters were operated at 35.5°C ± 0.4°C, θ_c = 15 days. Thermochemical treatment conditions: 1 hour @ 200°C, pH 13.

TABLE A-1

DIGESTER OPERATING CHARACTERISTICS FOR TWO-STAGE THERMOCHEMICAL DIGESTION STUDIES

Digester	Volume	Feed	Purpose
1	6 liter	Newsprint/sludge slurry. VS = 20 g/l; COD = 28 g/l.	1st stage digestion, source of lignocellulose-acclimated seed organisms.
2	6 liter	Effluent Digester 1, thermochemically treated, neutralized, diluted 3:1. Soluble lignin ≈ 1 g/l.	2nd stage digestion without lignin toxicity, source of thermochemically treated lignocellulose seed organisms.
3	1.5 liter	Effluent Digester 1, untreated, diluted 1:1.	Control to establish methane production which is not attributable to heat treatment in Digester Nos. 2 and 4.
4	1.5 liter	Same as Digester 2 except diluted 1:1. Soluble lignin ≈ 2 g/l.	Comparison with Digester 2 performance to determine lignin toxicity.

The digestibility of alkaline heat-treated lignin was investigated during the second phase using acclimated seed organisms from the first phase study. The long-term, serum-bottle bioassay technique employed for this study is a modification of the anaerobic enrichment procedure described in Gossett et al. (1976b). In this anaerobic assay, which is analogous to the Biochemical Oxygen Demand (BOD) test, methane production is monitored over a period of time from a chemically defined medium that is inoculated with acclimated seed and appropriate sample aliquot.

3. Experimental Procedures

a. Phase One - Two-Stage Thermochemical Digestion Study. A non-inked newsprint paper was selected as a model lignocellulose material for this study because it has a high lignin content (approximately 23 percent by weight) and its uniformity allows close control of feed composition. A 50:50 mixture (COD basis) of primary and flotation-thickened waste activated sludge, obtained from the San Jose-Santa Clara Regional Wastewater Treatment Plant, was blended with the newsprint feed. Sludge was added to supply growth factors not available in newsprint, and its organic contribution represented

10 percent of the feed COD. Nutrients and alkalinity were added in excess of those in the newsprint/sludge slurry according to the feed concentrations detailed in Table A-2 (after Speece and McCarty, 1962). Two stock nutrient solutions (Table A-3) were used for feed preparation to avoid precipitation during storage. One liter of lignocellulose feed slurry was prepared by combining 20 g newsprint, 80 ml 1M $NaHCO_3$, 10 ml each of stock nutrients, 62.5 ml sludge, and diluting to 1 liter with tap water. This mixture was "pulped" with a Waring blender which incorporated a special "Polytron" cutting head. Feed was stored at 2°C prior to use. An additional quantity of sludge (10 percent of feed COD) was added to the thermochemically treated feed to Digester Nos. 2 and 4 to insure availability of growth factors after heat treatment. No nutrients or alkalinity were added subsequent to the feed addition to Digester No. 1.

Heat treatment was performed in a bomb-type autoclave (Parr Instrument Co., Pressure Reactor No. 4522) which had a 2-liter nominal capacity. A "Pyrex" liner was employed to minimize the introduction of heavy metals during heat treatment. The reactor was continuously stirred and temperature was monitored and controlled by a thermocouple. An opposing heating-cooling system enabled temperature control within ± 5°C.

TABLE A-2

COMPOSITION OF NUTRIENTS ADDED TO THE NEWSPRINT FEED TO DIGESTER NO. 1
(after Speece and McCarty, 1962)

$(NH_4)_2HPO_4$	-	690 mg/l
$MgSO_4$	-	30 mg/l
NH_4Cl	-	840 mg/l
KCl	-	120 mg/l
$MgCl_2$	-	150 mg/l
$FeCl_3$	-	300 mg/l
$CoCl_2 \cdot 6H_2O$	-	15 mg/l
$NaHCO_3$	-	4,000 mg/l as $CaCO_3$

TABLE A-3

COMPOSITION OF STOCK SALT SOLUTIONS ADDED TO LIGNOCELLULOSE FEED SOLUTION. 10 ml OF EACH SOLUTION WAS ADDED TO EACH LITER OF FEED

Salt No. 1		
	$(NH_4)_2 HPO_4$	- 69 g/1
Salt No. 2		
	$MgSO_4$	- 3 g/1
	NH_4Cl	- 84 g/1
	KCl	- 12 g/1
	$MgCl_2$	- 15 g/1
	$FeCl_3$	- 30 g/1
	$CoCl_2 \cdot 6H_2O$	- 1.5 g/1

The treatment procedure was performed as follows: A caustic addition corresponding to an initial pH of 13 (180 meq/1 NaOH) was combined with digested newsprint slurry (effluent from Digester No. 1) prior to heat treatment. The autoclave was assembled, purged with nitrogen for 15 minutes, and heat treatment commenced. The bomb took approximately one hour to reach the desired temperature (200°C), which was held for one hour, and then the system was cooled to room temperature within 45 minutes. The final pH after thermochemical treatment was 10.2. The conditioned mixture was neutralized to pH 7 with concentrated HCl, sludge was added (approximately 10 percent on a COD basis), and the mixture was finally diluted to appropriate feed concentrations with tap water. Feed was stored at 2°C prior to use.

A total of four digesters were employed during this phase (two 6-liter and two 1.5-liter size). All units were continuously stirred and were operated at 35.5°C ± 0.4°C, as summarized in Table A-1 and schematically depicted in Figure A-2. An increase in methane yield, attributable to thermochemical treatment, could be obtained for respective operating conditions by subtracting the "normalized" methane generation of Digester No. 3 (the control unit) from the "normalized" methane generation of both Digester Nos. 2 and 4.

Also, the methane generation from the sludge mixture was monitored in a concurrent study. Thus, methane production from the fermentation of the lignocellulose material itself could be obtained for each digester by subtracting the respective contribution due to sludge addition.

The seed organisms for all units were obtained from a previous semicontinuous digestion study of municipal refuse. Each respective seed population had a 9-month acclimation to a similar feed as that used in this study.

Digester performance was evaluated from daily gas production and routine analyses of chemical oxygen demand (COD), total and volatile solids, volatile acids, pH, alkalinity, and gas composition. Feed mixtures were also analyzed on a regular basis for COD and total and volatile solids to insure uniform composition. All analyses were performed in accordance with Standard Methods for the Examination of Water and Wastewater, 13th ed. (1971).

b. Phase Two - Digestibility of Thermochemically Treated Lignin. The digestibility of thermochemically treated alkali-lignin was monitored with an anaerobic serum-bottle bioassay technique. Indulin AT (Westvaco Chemical Division), a kraft pine lignin, was used as the organic substrate for these studies, and the lignin pretreatment conditions were those previously determined as "optimal" for increasing the digestibility of the lignocellulose complex (one hour @ 200°C, pH 13). Heat treatment was conducted as described in the previous section, except a smaller reactor (600-ml nominal volume, Parr Instrument Co., Pressure Reactor No. 4563) was employed. Three lignin concentrations were evaluated (250, 500, and 750 mg/l), and they were selected to minimize potential toxicity and to maximize gas production.

The long-term, serum-bottle assays were conducted in a constant temperature room at 35°C. In this technique, the sample of interest and seed organisms are anaerobically incubated in a chemically defined media for an extended period of time. The chemically defined media is believed to contain all necessary nutrients and buffers to sustain anaerobic growth; its composition was outlined by Healy and Young (1976). Total gas production is monitored volumetrically by the syringe method and its respective methane fraction is determined by gas chromatography according to Standard Methods (1971). Thus, by keeping close track of methane production from the sample of interest and a blank, the methane contribution due to sample organic decomposition can be computed. In the current study all samples were analyzed in duplicate except the 750 mg/l lignin sample, and methane production is reported as a fraction

of theoretical 100-percent conversion of sample to methane (COD basis, where 395 ml CH_4 at 35°C is equivalent to 1 gram COD destroyed). The seed organisms were obtained from two sources (Table A-1): (1) seed from Digester No. 1, which represent organisms acclimated to lignocellulose, and (2) seed from Digester No. 2, which represents organisms acclimated to thermochemically treated lignocellulose.

4. Results

a. Phase One - Two-Stage Thermochemical Digestion Study. An appropriate system acclimation period was provided to allow stabilization of gas parameters prior to a 36-day data collection period. However, this time was not sufficient to obtain a steady-state system with respect to a solids balance. This was due to the difference in solids loadings from the previous refuse study, and the excessive time required to achieve steady-state in the series system employed. Nevertheless, all units were operating stably as determined by daily gas production and periodic volatile acids analyses, and these data are considered representative of steady-state operation (maintenance of seed digesters [Digester Nos. 1 and 2] in the 5-month time period subsequent to data collection has verified this premise).

During the test period, the influent COD and volatile solids concentrations to the first-stage digester (Digester No. 1) were 27.9 and 19.8 g/l, respectively. Gas production data for these studies are summarized in Table A-4 for the 36-day test period. As previously mentioned, the volatile acids concentrations for all digesters were stable during this time frame and average values for Digester Nos. 1-4 were 80, 140, 40, and 750 mg/l as acetic acid, respectively.

In a concurrent study the methane production of the 50:50 primary/waste activated sludge mixture was determined as 11.0 liters CH_4/liter sludge feed to a 15-day SRT, mesophilic digester. This value was used to determine the methane contribution due to lignocellulose fermentation only, as recorded in Table A-4.

b. Phase Two - Digestibility of Thermochemically Treated Lignin. Two seed inocula were employed for the current study: Seed No. 1 represents organisms that were not acclimated to heat-treated lignin, and Seed No. 2 represents organisms that were acclimated to heat-treated lignin. Briefly, after

TABLE A-4

SUMMARY OF RESULTS FROM TWO-STAGE THERMOCHEMICAL DIGESTION STUDIES

Parameter	Digester			
	No. 1	No. 2	No. 3	No. 4
Description of Digester	Newsprint Feed	HT Effluent Dig. No. 1	Effluent Dig. No. 1	HT Effluent Dig. No. 1
Feed Dilution (volume/volume)	--	3:1	1:1	1:1
Data Base (days)	36	36	36	36
Gas Production @ 35.5°C				
Total (ml/day)	1,770	280	10	100
Standard Deviation (% of mean)	13	14	79	12
CH_4 %	62	88	84	86
CH_4 (ml/day)	1,100	245	8	86
Methane Production Normalized to Digester No. 1 Feed Conditions				
Total CH_4 (ml/day)	1,100	980	67	680
Lignocellulose CH_4 (ml/day)[a]	824	835	67	663
COD Equivalent (g/day)	2.09	2.24	0.17	1.72
Total CH_4 COD as % of Digester No. 1 Total Influent COD	25	22	2	15
Lignocellulose CH_4 COD as % of Digester No. 1 Influent Lignocellulose COD[a,b]	21	23	2	17

[a]Computed as: Total CH_4 produced minus CH_4 from 10% sludge addition.

[b]Computed as: Total influent COD minus COD from 10% sludge addition.

a 48-day incubation period, the unacclimated seed samples showed no significant conversion of lignin to methane, but a measureable amount of conversion did occur in the acclimated seed samples. These results from the acclimated seed are illustrated in Figure A-3 where production is reported as a fraction of theoretical 100-percent conversion of sample to methane (COD basis) versus time. The COD/lignin ratio was 1.45 g/g.

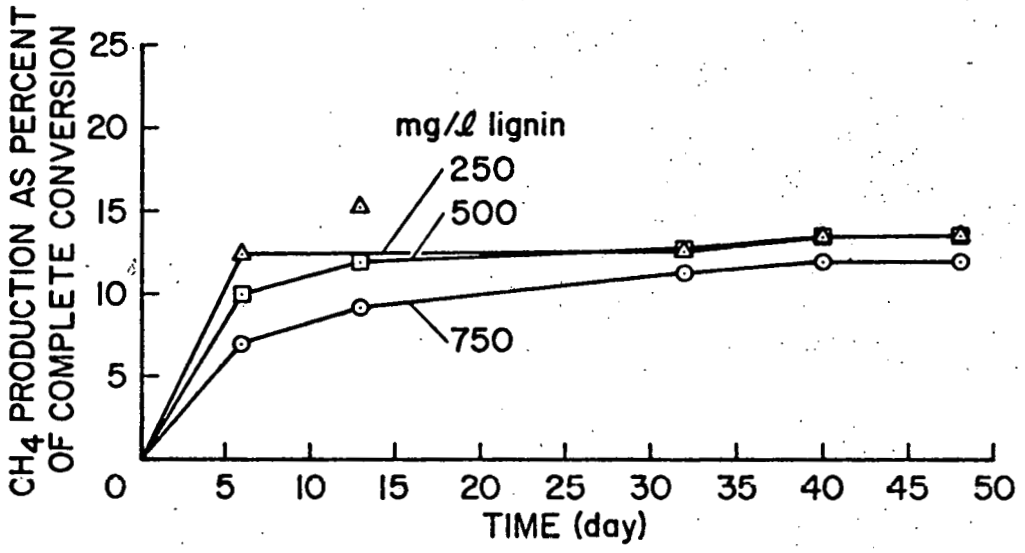

FIGURE A-3. Anaerobic decomposition of thermochemically treated alkali-lignin using acclimated seed. Pretreatment conditions: 1 hour, @ 200°C, pH 13.

5. Discussion

a. Phase One - Two-Stage Thermochemical Digestion Study. As seen in Table A-4, the digestibility of the untreated newsprint feed slurry was, as anticipated, very low. Approximately 25 percent of the influent feed COD was converted to methane by the first-stage digester (Digester No. 1); this corresponded to a 21-percent conversion of lignocellulose to methane and is consistent with previous results. Thermochemical treatment of digested matter from the first stage resulted in an additional 22-percent and 15-percent conversion of COD to methane in Digester Nos. 2 and 4, respectively. The net result was that approximately 50 percent of the lignocellulose slurry was converted to methane by the two-stage process at optimum operating conditions. The effect of heat-treated lignin toxicity can be seen by comparing operating data of the two second-stage digesters. Digester No. 4, with the higher lignin feed concentration of approximately 2 g/l, produced only 69 percent of the methane that Digester No. 2 produced, with a corresponding lignin feed concentration of 1 g/l. Toxicity was also exhibited by a higher steady-state volatile acids concentration in Digester No. 4 (750 mg/l) than in No. 2 (140 mg/l). No toxicity was demonstrated in Digester No. 2. This culture either became acclimated to the lignin feed, or perhaps, 1 g/l of heat-treated lignin was not sufficient to produce adverse effects.

The most significant result of this phase is that a virtual 100-percent increase in methane yield was achieved by thermochemical treatment of digested newsprint. This increase can be attributed to the solubilization of lignin from the lignocellulose complex, thus allowing penetration of extracellular enzymes into the digestible, carbohydrate fraction. Also, the short-term bioassay procedure predicted significantly less improvement in digestibility than observed in the semi-continuous studies. The differences may be attributed to any one, or several of the following factors: (1) organism acclimation to substrates produced by thermochemical treatment, or (2) heat-treated lignin toxicity could not be measured by the short-time bioassay, and (3) conversion of degradable substrates in the original sample into recalcitrant products via thermochemical treatment was avoided by two-stage treatment. (These degradable substrates are fermented in the first-stage of the two-stage scheme employed in the current study.)

b. Phase Two - Digestibility of Thermochemically Treated Lignin. Since the unacclimated seed did not show significant metabolism of heat-treated lignin, this discussion concerns the results from the acclimated seed.

Although the precision of the assay procedure has not yet been quantified, the regularity of data presented in Figure A-3 suggests the information is reasonably reliable. Based on this premise, the following observations can be made: (1) approximately 14 percent of the heat-treated lignin was converted to methane in the samples containing 250 and 500 mg/l of lignin during the 48-day incubation period, and slightly less (12 percent) was converted in the 750-mg/l lignin sample. (This difference may be due to test imprecision.) (2) It appears that the rate of methane production was inhibited at the higher, 750 mg/l, lignin concentration. These results are consistent with earlier studies which indicated methanogen inhibition at lignin concentrations in a range of 1 g/l.

In summary, approximately 14 percent of thermochemically treated lignin was converted to methane by an acclimated anaerobic microflora. This quantity represents anywhere from 1 to 6 percent of the total lignocellulose fraction for organic residues of interest for this study. For the newsprint feed evaluated in Phase One, the lignin content represented approximately 23 percent of the weight and 33 percent of the COD. Therefore, if 14 percent of the lignin fraction were made biodegradable by alkaline heat treatment, this would represent 4.6 percent of the influent lignocellulose COD to Digester No. 1 and 20 percent of the methane production from Digester No. 2.

6. Conclusions

a. The two-stage thermochemical digestion system approximately doubled the methane yield from a relatively refractory lignocellulose source (newsprint with lignin content of about 23 percent by weight), when compared with single-stage conventional digestion.

b. Products, as yet undefined, from the thermochemical solubilization of lignin inhibited methanogenic metabolism at a concentration of approximately 2 g/l with an acclimated culture.

c. Anaerobic organisms can acclimate to products from heat treatment of lignocellulose if the soluble lignin concentration is sufficiently low. Acclimated organisms should thus be used when evaluating the potential of heat treatment for increasing anaerobic biodegradability.

d. An acclimated anaerobic culture can convert approximately 14 percent of thermochemically treated alkali-lignin into methane gas. This would represent 1 to 6 percent of the total methane potential for biomass residues of general interest for energy production.

B. Nitrogenous Organics Digestion and Pretreatment Alternatives

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1. Introduction and Objectives

Previous work in this laboratory has demonstrated that heat treatment of refractory lignocellulosic complexes can result in up to 75 percent increases in anaerobic biodegradability (Gossett et al., 1976b) and that heat treatment can also greatly enhance the degradability of waste activated sludge (WAS) under mesophilic anaerobic conditions (Haug et al., 1977). Hence heat treatment of refractory organics can result in increased methane production and decreased residues for disposal. Since the residue from heat treatment is essentially pathogen-free this reduces some of the problems of disposing digested residues onto land.

Because of the advantages accruing from heat treatment it is desirable to investigate the process more fully, so that the effect of heat treatment on the degradability of any organic material would be predictable.

In considering the refractory nature of organic materials in general it is desirable to divide them into three main groups, carbohydrates, proteins, and lipids. Carbohydrates by themselves, e.g. sugars, starches, and cellulose, are in the main highly degradable. However, when cellulose is combined with lignins as in many natural residues, the result is a highly refractory complex. Studies concerned with lignocellulosic materials are covered in Section A.

Lipids exist in essentially two forms, saponifiables, i.e. hydrolyse with alkali, and the non-saponifiables, e.g. terpenes, steroids. In primary sewage sludge, about eighty percent of the lipids is present in the saponifiable form. A number of workers (Lawrence, 1960; O'Rourke, 1968) have shown that the saponifiable fraction is essentially completely biodegradable, while the non-saponifiables are virtually refractory. However, as a group lipids are considered very degradable (Chynoweth and Mah, 1971).

The final subgroup is proteins. Often this includes any nitrogen-containing compounds as the test used, organic nitrogen, is not specific for proteins. In general there has been very little research concerned with the anaerobic degradation of nitrogenous compounds. Hence it became apparent that to understand the effect of heat treatment on organic residues in general, more research was desirable on its effect on nitrogenous compounds. Since nitrogenous compounds comprise over 60 percent by weight of bacterial cells,

and some success has been achieved in the heat treatment of WAS, it was decided to concentrate specifically on the nitrogenous components of bacterial cells present in WAS. Bacterial cells are also a significant component of residues from ruminating animals and thus of interest in general.

Hence the objectives of this section of the research are:

(1) To understand more fully, through heat treatment, why nitrogenous components of bacterial cells are relatively refractory.

(2) To understand more fully the effect of heat treatment on the degradability of bacterial cells and their nitrogenous constituents, and on the resultant potential production of toxic materials.

2. Background

a. Refractory Compunds. In discussing what refractory compounds are, considerable confusion arises over the definition. Usually the term is defined operationally. For anaerobic digestion of organic sludges, this may mean anything that is not degraded within 30 days. Soil microbiologists define the refractory nature of, say pesticides, in terms of years, and geochemists may think in terms of decades. However, since this study is concerned primarily with the engineering significance of refractory compounds under normally encountered anaerobic digestion conditions, compounds not degraded after approximately 60 days will be considered as refractory. This is a purely operational definition, and will not exclude long-term degradability effects.

There are two opposing points of view with regard to the question of refractory compounds. The first--and older--view relies on the concept of "microbial infallability" (Alexander, 1965). This states that either by mutation and natural selection, or by a non-genetic adaption, one or another species appears always to develop in response to a modification in the habitat, or by the appearance of new types of carbonaceous nutrients. By this adaptation it is felt by supporters of the hypothesis that every biologically synthesized organic molecule will, under some set of circumstances, be destroyed by one or more species. The second view is supported by Alexander (1965) who questions this concept and hypothesizes that recalcitrance could result for at least two reasons: (i) the environment is not conducive to microbial life or to the degradation of a specific compound, or (ii) substances can exist which are resistant, either totally or partially, to biodegradation under all circumstances.

Alexander (1973) has also postulated a large number of environmental conditions which can affect microbial degradation. For the problem under study it would appear that four hypotheses are most plausible:

(i) Lack of an essential nutrient. There is still some controversy over whether oxygen is necessary for complete degradation of organics. Molecular oxygen acts either as a terminal electron acceptor, as in the case of aerobes, or as a direct reactant, as in the case of cleavage of aromatic hydrocarbons (Alexander, 1973).

(ii) Need for different organisms. In some cases it is possible that a compound is refractory simply because the organism, or group of organisms, which degrade it are not present. This becomes more likely when populations become more specialized, as in the case of anaerobic digestion.

(iii) Inaccessibility of site on substrate acted on enzymatically. At times compounds which are normally degradable can either be protected by a refractory "coat," as in the case of spores, or are inaccessible due to steric considerations, e.g. lignocellulosics.

(iv) The compounds in question are inherently refractory.

The question of refractory organics is a relatively new one, and as yet only a small amount of information has been gathered. Some data is available on aerobic degradation; however, very little work has been done on anaerobic degradation.

b. Degradability of Organic Wastes. In the treatment of industrial wastes anaerobic digestion has not been used extensively. However, from the literature (McCarty et al., 1972; McMorrow et al., 1970; Aulenbach and Hallock, 1972; Hiatt et al., 1973; Berg and Lentz, 1972; Chadwick and Schroeder, 1973; Jennett and Dennis, 1975; Arora, 1975), it is apparent that high COD destruction, 75 percent or greater, can be achieved if the substrate is predominantly soluble. This contrasts with insoluble municipal sludges and animal wastes (Hobson and Shaw, 1973), where destructions of only 40 to 60 percent are achieved. Since the wastes treated vary widely in composition this would seem to indicate that accessibility to substrate is one of the factors involved in the refractory nature of some organics.

WAS is about 40 percent degradable anaerobically, while primary sludge is about 60 percent degradable; both of these are under mesophilic conditions (30-35°C). At thermophilic conditions (50-60°C) there is some disagreement as to whether increased overall degradabilities result. Maly and Fadrus

(1971) found with a mixture of primary and WAS that no significant increases in overall degradability resulted at the higher temperatures. Golueke (1958) also found no significant increases in degradability with temperature.

However, Malina (1961) found with WAS that at thermophilic temperature volatile solids destruction was slightly higher than at mesophilic temperatures. Heukelekian (1930) also found that at higher temperatures degradability increased. Using municipal refuse Pfeffer (1973) found that degradabilities increased 25 to 30 percent at thermophilic temperatures over those at mesophilic conditions.

In comparing the degradability of WAS under aerobic versus anaerobic conditions it is apparent that in the main there is no substantial increase, even when the growth of biomass is accounted for. Benedek (1974) found that under aerobic digester conditions only 26 percent of the volatile solids degraded in twelve days. Allowing for a yield coefficient of 40 percent, this corresponds to an overall degradability of 44 percent. Singh and Patterson (1974) found that with a detention time of 4 days, volatile suspended solids destruction was 16 percent, which represents about 27 percent overall. However, the degradability obtainable depends on the conditions of production of the WAS. If short solids retention times are used in the activated sludge unit, then apparent degradation in aerobic digesters can be quite high because of the more easily degradable organics from the aeration tank.

Thermalchemical treatment was attempted in one study to increase overall aerobic degradation. Singh and Patterson (1974) pretreated WAS with concentrated sulphuric acid to pH 1.0 followed by autoclaving at 120°C for 5 hours. They achieved 40 percent volatile suspended solids destruction, compared with 16 percent for the control.

In summary, it would appear that the solubility of a substrate may have an important effect on degradability. The presence of oxygen may affect degradability but studies of aerobic versus anaerobic digestion of WAS do not illustrate that the differences, if any, are very significant. Finally, different microbial populations, as illustrated by thermophilic versus mesophilic anaerobic digestion, may have quite different capabilities in degradation, although even here the differences are controversial.

c. Composition and Structure of Bacterial Cells. In order to help understand why the nitrogenous components of bacterial cells are relatively

refractory under anaerobic conditions, and what effect heat treatment may have on them; it is important to know the structure and composition of bacterial cells. Typical figures for overall composition (dry weight basis) are (Luria, 1960):

Carbon	- 50 ± 5%	Lipids	- 10-15%
Nitrogen	- 8-15%	Protein	- 50%
Hydrogen	- 10%	Carbohydrates	- 10-30%
Oxygen	- 20%	RNA	- 10%
Ash	- 5%	DNA	- 3-4%

These percentages can vary considerably, however, depending on the state of the cell, i.e. whether it is in the log growth or stationary phase (Luria, 1960).

The bacterial cell wall accounts for about 20-30% percent of the dry weight of the cell. The rigid structural framework consists of parallel polysaccharide chains covalently cross-linked by peptide chains. This peptidoglycan structure is resistant to the action of peptide hydrolyzing enzymes, which do not attack peptides containing D-amino acids (Lehninger, 1975).

In addition, gram-positive cell walls are low in lipid (< 2 percent) and high in polysaccharide (40-60 percent), while gram-negative walls have a high lipid content (20 percent) and lower polysaccharides (12-30 percent). With nitrogen compounds gram-positive walls contain 10-30 percent amino sugars, while gram-negative have only 2-7 percent amino sugars (Luria, 1960).

d. Effect of Heat Treatment on Bacterial Cells. Heat treatment has been recognized for many years as a useful aid to dewatering biological sludges, especially in England and Europe. However, until the last decade very little fundamental research had been conducted on the mechanisms of heat treatment, and its effect on specific compounds.

Under heat treatment WAS may generally react in the following ways:

(i) Lipids-(saponifiable fats) hydrolyze easily under acid or alkaline conditions, as follows:

$$\text{fat} + H_2O \rightarrow \text{glycerol} + \text{fatty acids}$$

(ii) Carbohydrates - in general, polysaccharides would be expected to break down to smaller molecular weight polysaccharides, and possibly even to simple sugars. Cellulose, with weak acids, can be hydrolyzed to D-glucose

(Gossett et al., 1975). However, with strong mineral acids dehydration can occur leading to furfural and 5-hydroxymethyl-furfural.

Under strongly alkaline conditions cellulose is more stable, but is still solubilized by an endwise degradation procedure. This "peeling" reaction produces the six-carbon isosaccharinic acid, which can be further degraded as conditions become more severe (Gossett et al., 1976b).

(iii) Proteins-hydrolyzed by acid solutions to amino acid monomers. Some peptide bonds, those of valine, isoleucine and leucine, for example are more stable than others and require longer hydrolysis times or stronger acids. Peptide bond cleavage is noticeably faster in hydrochloric acid than in sulfuric. Continued contact at high temperatures can cause some of the liberated amino groups to undergo degradation and deamination reactions, e.g. tryptophan (Roach and Gehrke, 1969).

Under alkaline conditions proteins are still readily hydrolyzed, however, the conditions are less severe than with acid. Consequently amino acids with unstable functional groups are less likely to undergo degradation under alkaline conditions than under acid conditions.

With heat treatment free amino acids and amino acids formed from protein may form a series of saturated and unsaturated acids, ammonia and some carbon dioxide (Brooks, 1968):

$$RCH_2CHNH_2COOH \xrightarrow{+H_2O} RCH_2CHOHCOOH + NH_3$$

then either

$$RCH_2CHOHCOOH \xrightarrow{-H_2O} RCH{:}CHCOOH \xrightarrow{+2H} RCH_2CH_2COOH \qquad (a)$$

or

$$RCH_2CHOHCOOH \xrightarrow{-2H} RCH_2COCOOH \qquad (b)$$

(e.g. pyruvate from alanine). They can also act as hydrogen donor or acceptors:

$$\underset{\text{(alanine)}}{CH_3CHNH_2COOH} \xrightarrow{+2H_2O} CH_3COOH + CO_2 + NH_3 + 4H$$

$$\underset{\text{(glycine)}}{2CH_2NH_2COOH} + 4H \longrightarrow 2CH_3COOH + 2NH_3$$

(iv) Nucleic acids-would be expected to hydrolyze to produce constituent bases, sugars, and orthophosphate (Brooks, 1968).

Various intermolecular reactions would also be expected, e.g. the "Browning" reaction. This is a complex set of reactions (Hodge, 1953) but in essence involves the polymerization of compounds with a carboxyl group, e.g. aldehydes, ketones, reducing sugars, with amino groups. The end product of these polymerizations are melanoidins, which are described as brown nitrogenous polymers and copolymers. High temperatures and extremes in pH increase the rate of polymerization.

The potential formation of melanoidins is an important consideration in the heat treatment of WAS to increase biodegradability, because due to their structure and complexity they are likely to be difficult to degrade.

Brooks (1968, 1970a,b) carried out extensive experimental work on both oxidative (wet oxidation) and non-oxidative heat treatment of municipal sludge. He used temperatures varying from 100 to 216°C. Parameters monitored included settling velocities, specific resistance to filtration, fate of NH_3 and organic nitrogen, and effects on total and dissolved solids.

Experimentally Brooks (1968) found that at 200°C for 0.5 hours about 2/3 of the organic nitrogen was solubilized. Most of it became soluble organic nitrogen (SON); however, after some time the SON hydrolyzed to NH_3. While the percentage of nitrogen in solution increased with increasing temperature, the pH decreased, indicating the probable release of volatile or other organic acids.

Everett (1972) found the dissolved fraction of digested sludge initially increased with time at constant temperature (> 170°C) but was constant after one hour. With humus sludge at > 200°C he found an actual decrease in the percentage of dissolved organics with time, and suggested that this was due to the precipitation of insoluble polymers formed by the "Browning" reaction.

Everett (1974) also found that with heat treatment over a pH range the specific resistance of sludge dropped by 10^4 with decrease in pH from 10 to 2. At the extremes in pH the percentage solubilities were about the same, hence solubilization did not always explain decreases in resistance to filtration. Erickson and Knapp (1970) studied the biodegradability of the supernatant liquors from heat treatment of mixed primary and activated sludge and found only 7 percent of soluble COD was refractory. Corrie (1972) found that after a 6-fold dilution, 85 percent of the COD in the heat-treated liquor could be removed with extended aeration (θ = 2 days). Everett (1973) cites

other cases where the COD was 90 percent degraded. Thus, the soluble materials released under these conditions of heat treatment appear highly biodegradable.

Anaerobic degradability data, however, is sparse. Kalbskopf (1972) found that for the filtrate from heat conditioned WAS, 68 percent of the potassium permanganate value was removed after 30 days at 36°C, and 78 percent of the BOD_5. Other data (Haug, 1977) reveal 82 percent COD destruction after 15 days at 35°C for the liquor of WAS treated at 175°C.

Previous work in this laboratory (Haug, 1977) has revealed that heat treatment of WAS can produce toxicity to methane bacteria. As the temperature of treatment increased and as extremes in pH were approached, the heat-treated WAS became more toxic. The source and nature of the toxic materials was not determined.

In summary, it would appear that while a considerable amount of data exist regarding the effect of treatment temperature and time on the specific resistance of sludge to filtering, and on solubilization effects, very little data exist on overall anaerobic degradability and on the degradability of specific nitrogen-containing compounds. Also, the source and control of toxicity produced during heat treatment of WAS has not been adequately evaluated.

e. Hypotheses. From the preceding discussion of the relevant literature the following possible hypotheses were formulated to help guide the laboratory investigation:

(i) Nitrogenous components of bacterial cells are relatively refractory because:

(a) of the inability of enzymes excreted by the anaerobes to hydrolyze the nitrogenous material into smaller and more degradable constituents;

(b) oxygen needs to be present to ensure more complete degradation;

(c) due to their structure, they are inherently non-degradable.

(ii) Anaerobic thermophilic cultures have better capability for degradation of nitrogenous materials than comparable mesophilic cultures.

(iii) Heat treatment of nitrogenous components can have several effects:

(a) heat treatment increases the degradability of nitrogenous components of a bacterial cell by solubilizing them, making them more susceptible to bacterial action;

(b) under certain heat treatment conditions condensation products are formed between the nitrogenous components of a bacterial cell, and reducing sugars. These condensation products are refractory and lower the overall degradability of the nitrogenous fraction;

(c) it will be possible to predict the increase in total degradation of the nitrogenous fraction with a knowledge of the behavior after heat treatment of individual nitrogenous components and combinations with reducing sugars.

(iv) Toxic materials formed by the heat treatment of bacterial cells result from the nitrogenous components of the cell.

The experimental study is designed to test these hypotheses.

3. Experimental Procedures

a. Introduction. The aim of these initial studies was to refine experimental technique, and to obtain preliminary data on the effect of heat-treatment variables on anaerobic degradability under mesophilic conditions. Also degradability of nitrogenous components, and the influence of heat-treatment variables on toxicity were evaluated.

The biodegradability as well as toxicity of the samples were determined by a batch-bioassay technique (Gossett et al., 1976b), which enables gas production versus time to be monitored.

b. Source of Material. Air-thickened WAS from the San Jose-Santa Clara Water Pollution Control Plant was used as the source material. By weight the sludge was 4.33 percent total solids and 3.13 percent volatile solids (72.3 percent volatile), with a COD of 46.3 g/l. Sufficient sludge was collected at one time and stored at 3°C to prevent deterioration during the study.

c. Treatment Procedure. The heat-treatment reactor (Parr Instrument Co., No. 4561) had a nominal capacity of 300 ml, and was constructed of Monel, with exposed parts in Hastelloy C to reduce potential heavy-metal toxicity. A teflon liner was also used. The reactor was stirred continuously during the treatment at 400 rpm, and the temperature of the liquid was monitored by a thermocouple which controlled the current to the external heating mantle. A cooling coil inside of the reactor in conjunction with a solenoid valve enables the temperature to be controlled within ± 3°C. The cooling coil, with

mixing, enabled the reactor contents to be cooled to room temperature within fifteen minutes after heating was terminated.

For treatment, 125 ml of WAS was measured into the teflon liner, an appropriate volume of acid or base was added to result in a concentration of 300 meq/l, the slurry was mixed, and the liner was placed into the reaction vessel, which was then sealed. Nitrogen was flushed through the vessel for five minutes, the mantle was fitted around the reactor, and the desired temperature was set. The reactor required about 45 to 60 minutes to reach this temperature (150-250°C), and was held there for an hour, after which it was cooled to room temperature.

d. Batch Bioassay of Biodegradability. Anaerobic biodegradabilities were determined in 250-ml bottles initially gassed with an oxygen-free mixture of 70 percent N_2 and 30 percent CO_2. Prepared nutrient media (Table B-1) was deoxygenated (for a detailed description of this process, refer to the third section of this report). Effluent from a 10-liter seed unit being fed WAS at a detention time of 15 days (at 35°C) was collected anaerobically and centrifuged. The solids were washed twice with a deoxygenated sodium bicarbonate solution (5.38 g/l) to maintain pH at about 7.3. Then 220 ml of the washed seed was added anaerobically to 1.78 liter of the nutrient media, while the media was being stirred to ensure homogeneity.

Then, 7.5 ml of heat-treated WAS was pipetted into the 250-ml bioassay bottles, followed by 140 ml of the inoculated nutrient media. A serum cap was then inserted, and the bottle was allowed to equilibrate for 30 min in an incubator at 35°C ± 0.5°C. Excess gas was released with a syringe and the study commenced.

TABLE B-1

NUTRIENT MEDIA COMPOSITION

Compound	Conc. (g/l)	Compound	Conc. (g/l)
Resazurin	0.001	$CuCl_2 \cdot 2H_2O$	0.0027
KH_2PO_4	0.042	$Na_2MoO_4 \cdot 2H_2O$	0.0025
$MgCl_2 \cdot 6H_2O$	1.8	$ZnCl_2$	0.0021
KCl	1.3	$FeCl_2$	0.23
$MnCl_2 \cdot 4H_2O$	0.02	$NaHCO_3$	5.38
$CoCl_2 \cdot 6H_2O$	0.03	$Na_2S \cdot 9H_2O$	0.55
H_3BO_3	0.0057		

Gas production was monitored with a lubricated 10-ml glass syringe calibrated in 0.2-ml graduations. Gas composition was monitored occasionally using a Fisher-Hamilton Gas Partitioner Model No. 29.

e. Measurement of Inhibition. The degree of inhibition caused by the heat-treated WAS was measured using 125-ml batch systems. Aliquots of the treated WAS were pipetted into gassed 125-ml bottles (70 percent N_2, 30 percent CO_2), deoxygenated bicarbonate buffer solution (5.38 g/l) was then added to make a total volume of 38 ml. Two ml of acetate-propionate (40 mg acetate COD/ml, 20 mg propionate COD/ml) was added, and the bottle was sealed with a serum cap. Ten ml of an anaerobic seed obtained from a 10-liter digester fed WAS was injected into the bottles, and the pressure was reduced to atmospheric with a syringe.

A seed blank and acetate-propionate plus seed blank were also prepared. Gas production was monitored with a lubricated syringe every twelve hours for a period of five days and then less frequently, while gas composition was monitored occasionally.

Inhibition was determined by taking the ratio of the maximum rate of gas production (ml/day) in the samples to that of the fed blank (maximum rate ratio, MRR). A ratio less than 0.9 indicated some degree of inhibition.

f. Analytical Procedures

Chemical Oxygen Demand. COD was used as a measure of the organic content of the sludge, and in most cases reported, represents the average of at least two analyses. The COD procedure (Standard Methods, 1971) was modified slightly by halving all sample and reagent volumes. Soluble COD was determined on centrifuged samples after filtration through a 0.45 micron filter.

Organic and Ammonia Nitrogen. Organic and ammonia nitrogen were determined by the Kjeldahl digestion and distillation methods, respectively (Standard Methods, 1971). Distillates were collected in 0.02N sulfuric acid and ammonia was determined by Nesslerization.

Protein. Total and soluble protein were determined by the Lowry method (Lowry, 1951). Insoluble proteins were released by hydrolyzing a diluted sample (1/10 to 1/100) in 0.1N NaOH for 30 min at 100°C. The color produced with the Folin-Ciocaltau reagent was measured at 750 nm in 1-cm cells. The standard used was powdered bovine albumin (Sigma A4503).

RNA. Both total and soluble RNA were determined by the cupric ion catalyzed orcinol reaction (San Lin and Schjeld, 1969). The method measures only ribose and purine-bound ribose, and depends on the conversion of the pentose in the presence of hot acid to furfural, which then reacts with orcinol to yield a green color (Munro, 1966). The disadvantage of the method is that hydrolysis in strong acid removes mainly purine bases from the polynucleotide chain, the pyrimidine-bound ribose (except some 2 to 6 percent) fails to react (San Lin and Schjeld, 1969). However, if purine and pyrimidine nucleosides are degraded to about the same extent, the analysis should give a good estimate of the fraction degraded.

The method entails heating a 2-ml sample with 2 ml of cupric chloride (0.15 percent w/v) in concentrated hydrochloric acid, and orcinol (1 percent w/v) at 100°C for 35 minutes. The color developed is measured at 666 nm in 1-cm cells. The standard used was sodium ribonucleate extracted from yeast (ICN biological chemicals, Cat. No. 102927).

Total RNA was determined on the hydrolyzed sample prepared for total protein analysis since RNA is also solubilized in dilute hot NaOH.

Protein can interfere with the analysis at high concentrations; however, with the addition of concentrated hydrochloric acid most of the soluble protein precipitates out, and the sample is centrifuged before colorimetric determination.

DNA can also interfere but was found to have a color yield of only 5 percent that of RNA at equal concentrations.

DNA. The method used was that of Hattingh and Siebert (1967), developed for anaerobic sludges. DNA is released from the cells first by ultrasonic lysis for 30 minutes at 0-5°C, and then by centrifugation at 40,000 g for 30 minutes at 2°C with sodium lauryl sulphate. The soluble DNA is precipitated at 2°C with cold 72 percent perchloric acid, centrifuged, and redissolved in 0.5N perchloric acid. The supernatant liquid is mixed with diphenylamine reagent (1.5 percent w/v in concentrated acetic acid) and 2 drops of diluted acetaldehyde, and incubated for 12 to 16 hours at 30°C. The blue color developed is measured at 600 nm in 1 cm cells.

Certain, as yet unknown, compounds can interfere with the color development and produce a green color instead. This interference should be eliminated by the precipitation and removal of DNA, and its subsequent redissolution.

The standard used was sodium desoxyribonucleate isolated from herring sperm (ICN Biological Chemicals, Catalog No. 102907).

Amino Acids. The modified ninhydrin colorimetric analysis was used for free amino acids (Rosen, 1957). Ninhydrin reacts strongly with ammonia (Lehninger, 1975) and the method of Hatting et al. (1967) was used to reduce ammonia to low concentrations. The pH of a 10-ml aliquot of the sample was raised to 12 with a few drops of 15N NaOH and the sample was placed in a dessicator over concentrated sulfuric acid for 48 hours under vacuum and then readjusted to its original volume with deionized water.

Greater than 99 percent removals of NH_3 were achieved in samples containing between 100 and 1100 mg/l NH_3-N. Remaining ammonia was measured by distillation and appropriate corrections were made to measured amino acids concentration.

The ninhydrin method involved the addition of 0.5 ml of cyanide-acetate buffer and 0.5 ml of a 3-percent ninhydrin solution (w/v in Methyl Cellosolve) to a 1-ml sample. The mixture was then heated in a 100°C water bath for 15 minutes, and finally 5 ml of an isopropyl alcohol-water diluent was added to cool the mixture to room temperature. The color developed was measured in 1-cm cells at 570 nm. The standard used was D-L-leucine.

Volatile Acids. A Tracor MT 220 gas chromatograph was used to determine semi-quantitatively the presence of C1 to C10 volatile acids. A 6 ft x 4 mm I.D. glass column was used with a 10°percent SP-1000/1 percent H_3PO_4 on 100/120 Chromosorb WAW packing (Supelco, Inc.); the detector was flame ionization.

The pH of 2-ml samples was lowered to 2 with two drops of concentrated H_2SO_4, 1 ml of ethyl ether was added, and the tube inverted twenty times. The ether layer was separated by centrifugation at 5000 rpm, and was stored up to 3 days at 2°C.

A 1-μl-sample was injected into the chromotograph and temperature programming was used to separate the acetate peak from the solvent tail, Initial column temperature was 115°C, and rose at 15°C/minute to a final temperature of 145°C.

4. Results

a. Effect of Heat Treatment on WAS

Results from the first study are set out in Table B-2. To determine if they are similar to reports by others, certain components were expressed as percentages of the total volatile solids concentration (31.3 g/l). The results are:

	This Study	Others	
N	10.3%	10.7%	(Kincannon and Gaudy, 1965)
Protein	37.4%	47%	"
RNA	7.2%	7.7%	"
DNA	0.14%	3.0%	"
COD/mass of organics	1.48	1.42	(assuming $C_5H_7O_2N$ as WAS composition)

With the exception of DNA, the analyses gave results comparable to those in the literature. DNA gave only 5 percent of the expected value due to problems in centrifuging the insoluble DNA from solution. The centrifuge used was not capable of achieving the 73,000 g required, and hence modifications to the method will be attempted to increase the yield of DNA.

From 5 to 15 percent of the TKN and COD were not recovered. This may be due to some loss due to volatilization or to incomplete recovery of solids thrown to the reactor top by mixing. From Table B-2 the SON concentration increased slightly up to 200°C and dropped sharply at high temperatures. Accompanying this drop was an increase in ammonia due to hydrolysis of organic nitrogen. This behavior was also noted by Brooks (1968) and Gossett et al. (1976b). The use of slaked lime ($Ca(OH)_2$) resulted in lower solubilization of organic nitrogen as well as other components, which reinforces observations made earlier (Gossett, 1976a).

Protein was hydrolyzed with increasing temperatures with only 67 percent remaining at 250°C. The use of HCl also resulted in considerable hydrolysis of protein as expected. The soluble protein concentration increased up to 175°C and then decreased rapidly by hydrolysis of SON to ammonia and amino acids. This decrease was accompanied by increasing concentrations of amino acids up to 225°C.

TABLE B-2

EFFECT OF HEAT TREATMENT ON WAS COMPOSITION

Heat Treatment Conditions			ORGANIC N			NH_3	PROTEIN			Amino Acids	RNA			DNA	COD			% COD Solubilized
Temp. (°C)	Time of Treatment (hrs)	Chemical added (300 meq/l)	TKN mg N/l	SON mg N/l	Insoluble mg N/l	mg N/l	Total mg/l	Soluble mg/l	Insoluble mg/l	mg/l	Total mg/l	Soluble mg/l	Insoluble mg/l	mg/l	Total mg/l	Soluble mg/l	Insoluble mg/l	
Feed WAS			3,220	120	2,420	670	11,705	665	11,040	155	2,255	375	1,880	43	46,305	-	-	-
U.S. lysed WAS			3,195	185	2,245	765	11,560	1,005	10,555	225	2,285	290	1,995	34	46,425	9,220	37,205	20
150	1	None	3,095	1,600	740	755	9,525	7,500	2,025	1,365	1,740	2,115	-	43	39,730	20,930	18,800	45
175	1	None	3,060	1,485	695	870	9,820	8,125	1,695	1,795	1,530	1,420	110	31	43,185	22,130	21,055	48
175	1	$Ca(OH)_2$	3,085	1,290	850	945	8,530	5,400	3,130	1,885	1,295	1,065	230	41	38,720	18,685	20,035	40
175	1	NaOH	2,775	1,605	225	945	9,820	8,570	1,250	3,025	1,530	1,270	260	69	42,440	25,360	17,080	55
175	1	HCl	2,915	1,790	365	760	8,580	5,940	2,640	5,785	140	370	-	63	45,455	24,925	20,530	54
200	1	None	3,300	1,650	710	940	9,225	7,190	2,035	2,140	260	200	60	53	46,835	23,340	23,495	50
200	1	NaOH	3,080	1,510	375	1,195	9,225	7,500	1,725	3,900	1,055	735	320	56	42,300	25,065	17,235	54
200	1	HCl	3,145	1,655	380	1,110	6,745	4,465	2,280	6,260	20	60	-	39	44,170	23,970	20,200	52
225	1	None	3,080	1,555	350	1,175	8,445	5,760	2,685	2,390	90	90	0	48	45,220	23,485	21,735	51
250	1	None	3,105	1,210	545	1,350	7,790	4,730	3,060	1,465	75	50	25	62	40,710	21,545	19,165	47

RNA concentrations decreased rapidly with temperature, especially when HCl was used. This indicates the splitting of the nucleotide into its base and ribose sugar, as the method used to detect RNA was not capable of measuring pyrimidines without the ribose attached.

Soluble COD increased slowly with temperature from 45 percent at 150°C to 51 percent at 225°C. At 250°C there was a relatively sharp drop, perhaps indicating the precipitation of an insoluble polymer. Data for SON indicate that the precipitated material contains nitrogen. The use of NaOH and HCl did not increase the solubilization of organic material markedly and $Ca(OH)_2$ reduced it considerably.

Another way of observing the effect of heat treatment on soluble nitrogen forms is to determine what percentage of SON is accounted for by summing the measured soluble nitrogen. Table B-3 indicates that as temperature rises above 175°C the ratio of soluble nitrogen forms to SON decreased indicating the formation of soluble nitrogen compounds not measured as protein, amino acids or RNA. However, the addition of NaOH, and in one case HCl, appears to have reduced the formation of these soluble nitrogen compounds.

TABLE B-3

EFFECT OF HEAT TREATMENT ON SON

Heat Treatment			$\frac{\text{(Soluble protein + amino acids + soluble RNA)}}{\text{SON}}$
Temp. (°C)	Time (hr)	Chemical added (300 meq/1)	
Feed WAS			10.0
Ultrasonically lysed WAS			8.2
150	1	None	6.9
175	1	None	7.6
175	1	$Ca(OH)_2$	6.5
175	1	NaOH	8.0
175	1	HCl	6.8
200	1	None	5.8
200	1	NaOH	8.0
200	1	HCl	8.5
225	1	None	5.3
250	1	None	5.2

b. Effect of Heat Treatment on Anaerobic Degradability at Mesophilic Temperatures

The biodegradabilities of 10 heat-treated samples, ultrasonically lysed WAS, and normal WAS were determined. Gas production and composition were monitored regularly. From the initial COD and the methane productions, it is possible to calculate the percentage of COD destroyed (1 g COD destroyed is equivalent to 395 ml CH_4 produced). Results are given in Table B-4 for a degradation period of twenty-five days.

Since with time the composition of gas in the bottle approaches the composition of gas produced, methane production was estimated from the final gas composition and the total gas produced. It is unlikely that errors in this estimate would alter recorded degradabilities by more than a few percent.

Heat treatment increased the degradability of WAS considerably. During biological decomposition, about 10 percent of the metabolized organics

TABLE B-4

BIODEGRADABILITIES OF HEAT TREATMENT WAS (25 DAYS)

Heat Treatment			Gas Production		Percent COD destroyed	Increase over feed WAS (percent)
Temp. (°C)	Time (hr)	Chemical added (300 meq/l)	ml (at 35°C)	% CH_4 (est.)		
Feed WAS			113	76.0	48	-
U.S. lysed WAS			126	75.0	53	10
Seed blank			27	77.0	-	-
150	1	None	127	76.5	65	35
175	1	None(15 ml)	264	76.0	70	46
175	1	None	140	76.5	68	42
175	1	$(Ca(OH)_2$	113	79.5	60	25
175	1	NaOH	149	79.5	78	63
175	1	HCl	148	82.0	75	56
200	1	None	137	76.0	60	25
200	1	NaOH	145	79.5	75	56
200	1	HCl	152	79.0	76	58
225	1	None	115	77.5	51	6
250	1	None	92	78.0	43	-10

are converted to bacterial cells. Thus, the maximum short-term COD reduction from complete substrate utilization would be about 90 percent. Thus, Table B-3 results indicate that after heat treatment about 85 percent of the organics can be consumed by anaerobic microorganisms.

Without chemical addition the maximum COD destruction was 68 percent at 175°C and decreased to only 43 percent at 250°C. With either NaOH or HCl the degradability was greater than with no chemical addition, and this difference increased with increasing temperature. On the other hand, heat treatment without chemicals was better than with $Ca(OH)_2$ added.

The degradability of the untreated WAS after twenty-five days correlated well with data obtained previously from semi-continuous digestion of WAS.

The degradability of the ultrasonically lysed sample was only slightly higher than for the untreated sample. This would indicate that either ultrasonic lysis does not increase degradability substantially, or that the sample had not been properly lysed.

Good agreement was obtained between the two bottles containing the same substrate (175-1-no chemical) indicating that the bioassay technique was reproducible within fairly close limits. The bottle containing the larger aliquot (15 ml) gave a slightly higher degradability and possible reasons for this will be discussed in the next section.

Another phase of this study was concerned with the effect of heat treatment on the degradability of specific nitrogen-containing organics. From the composition of the inoculated nutrient media and the heat-treated feed (Table B-2), the initial composition of the substrate in the bioassay bottle was calculated as indicated in Tables B-5 to B-8.

After 25 days of biodegradation, 47.5 ml of the bottle contents were removed anaerobically and analyzed for specific nitrogen compounds as indicated in Tables B-5 to B-8. Because of the large organic content of the nutrient media and other experimental difficulties the conservative parameter TKN, at times, did not balance closely, and overall COD destructions could not be correlated well with destructions predicted by the gas data. However, from the data it is possible to determine some trends.

Soluble COD destruction (Table B-5) was high at low temperatures, but decreased as temperatures rose. This could be due to either the formation of soluble refractory compounds, or to slower rates of degradation due to inhibition. Addition of NaOH had little effect at 175°C, but increased the soluble

TABLE B-5

EFFECT OF HEAT TREATMENT ON BIODECOMPOSITION OF COD

Heat Treatment			Initial COD			COD after 25 days			Percent soluble COD removal
Temp (°C)	Time (hr)	Chemical added (300 meq/l)	Total mg/l	Soluble mg/l	Insoluble mg/l	Total mg/l	Soluble mg/l	Insoluble mg/l	
Nutrient media			3,550	160	3,390	3,190	25	3,165	85
Feed WAS			5,725	-	5,105	3,780	87	3,693	-
U.S. lysed WAS			5,730	619	5,111	3,890	67	3,823	89
150	1	None	5,390	1,215	4,175	2,930	155	2,775	87
175	1	None	5,565	1,275	4,290	3,435	235	3,200	82
175	1	$Ca(OH)_2$	5,340	1,100	4,240	3,350	245	3,105	78
175	1	NaOH	5,530	1,440	4,090	3,815	275	3,540	81
175	1	HCl	5,680	1,420	4,260	3,740	230	3,510	84
200	1	None	5,750	1,335	4,415	3,600	345	3,255	74
200	1	NaOH	5,520	1,425	4,095	3,470	295	3,175	80
200	1	HCl	5,615	1,370	4,245	3,730	220	3,510	84
225	1	None	5,670	1,345	4,325	3,735	435	3,300	68
250	1	None	5,440	1,245	4,195	3,845	505	3,340	60
175	1	None (15 ml)	7,760	2,400	5,360	4,745	379	4,366	84

COD degradability at 200°C. HCl increased degradability at both temperatures, while $Ca(OH)_2$ reduced degradability. In all cases soluble COD destruction was higher than the destruction of insoluble organics. Both bottles containing identical substrate (175°C-1-no chemical) produced similar results for soluble COD destruction.

A substantial portion of the total and soluble organic nitrogen (Table B-6) was degraded at low temperatures. However, as the temperature of treatment rose the portions degraded decreased substantially. Effect of chemical additions are uncertain because of variability in results. Insoluble organic nitrogen either decreased slightly or actually increased due to biomass formation. Due to destruction of both soluble and insoluble organic nitrogen, ammonia increased in proportion to the extent of degradation.

TABLE B-6

EFFECT OF HEAT TREATMENT ON BIODECOMPOSITION OF ORGANIC NITROGEN

Heat Treatment			Initial Nitrogen				Nitrogen after 25 days					
Temp. (°C)	Time (hr)	Chemical added (300 meq/l)	Total organic N(mg/l)	SON (mg/l)	Insoluble organic N(mg/l)	NH_3 (mg N/l)	Total organic N(mg/l)	SON (mg/l)	Insoluble organic N(mg/l)	NH_3 (mg N/l)	Percent SON removal	Percent total org-N removal
Nutrient media			175	14	161	31	157	20	137	49	-	-
Feed WAS			296	19	277	63	200	5	195	114	> 100	64
U.S. lysed WAS			290	23	267	68	200	15	185	124	> 100	63
150	1	None	285	95	190	68	169	50	119	124	63	89
175	1	None	278	89	189	73	186	24	162	142	95	72
175	1	$Ca(OH)_2$	275	79	196	77	185	24	161	123	94	72
175	1	NaOH	260	95	165	77	202	19	183	134	> 100	47
175	1	HCl	276	104	172	68	190	9	181	172	> 100	67
200	1	None	285	97	189	77	202	37	165	138	80	59
200	1	NaOH	262	90	172	90	195	18	177	141	> 100	56
200	1	HCl	269	97	172	86	199	35	164	158	82	55
225	1	None	263	92	171	89	210	48	162	135	64	40
250	1	None	254	75	179	98	187	91	96	124	< 0	62
175	1	None (15 ml)	389	164	225	118	244	45	199	226	83	-

Total and soluble protein, and amino acids (Table B-7) were substantially degraded at lower treatment temperatures, but as the treatment temperature rose the amount degraded decreased, reflecting total nitrogen trends. Again, effect of chemical additions is uncertain. Insoluble protein was only slightly degraded, and under more severe conditions actually increased due, perhaps, to biomass formation.

Soluble RNA (Table B-8) appeared mainly to be highly degraded, although due to the method of measurement this might only indicate the breaking of the pyrimidine (purine) ribose bond.

c. Effect of Heat Treatment on the Toxicity of WAS. The results of toxicity evaluation are presented in Table B-9. As temperature was increased above 175°C, the substrate became more and more toxic. The addition of NaOH seemed to only marginally increase the toxicity; however HCl treatment made the WAS extremely toxic.

Due to the presence of more organic material in the least dilute bottles (25/50), the MRR is often slightly higher than in the (10/50) bottles. This is due to the increased amounts of CO_2 produced, and does not indicate that the more concentrated solutions are less toxic.

Acclimation to the toxicity occurred in some cases after 7 to 24 days indicating either the selection of suitable organisms, the altering of enzyme pathways, or the destruction of the inhibitory compounds.

Gas chromatography data of inhibited samples after 45 days revealed the presence of substantial quantities of C2 to C6 volatile acids. The concentrations of individual acids were as high as 20 mM, and indicated that the inhibitory material affected the methanogenic flora.

5. Discussion

The effect of low temperature heat treatment on WAS appears to be lyses of the cell and release of its contents. However, since some of the internal components of a cell are still insoluble, increasing the temperature of treatment appears to slightly increase the solubilization of organics up to a maximum of 51 percent at 225°C. After this, increasing the temperature reduced the amount of organics in solution. From the data it is not possible to tell whether a limit was reached in the solubilization process, or whether some organics were precipitated simultaneously along with the solubilization of others. Since the cell wall constitutes only 20 to 30

TABLE B-7

EFFECT OF HEAT TREATMENT ON BIODECOMPOSITION OF PROTEIN

Heat Treatment			Initial Composition				Composition after 25 days				Percent soluble protein removal	Percent total protein removal
Temp. (°C)	Time (hr)	Chemical added (300 meq/l)	Total protein (mg/l)	Soluble protein (mg/l)	Insoluble protein (mg/l)	Amino acids (mg/l)	Total protein (mg/l)	Soluble protein (mg/l)	Insoluble protein (mg/l)	Amino acids (mg/l)		
Nutrient media			675	30	645	0	645	0	645	3	100	-
Feed WAS			1,235	60	1,175	8	885	5	880	4	83	57
U.S. lysed WAS			1,230	80	1,150	12	935	3	932	0	94	48
150	1	None	1,125	410	715	70	580	18	582	11	95	114
175	1	None	1,140	440	700	90	700	55	645	0	87	88
175	1	$Ca(OH)_2$	1,075	300	775	95	685	75	610	0	72	90
175	1	NaOH	1,140	460	680	155	805	78	727	14	82	66
175	1	HCl	1,075	330	745	295	670	95	575	0	68	94
200	1	None	1,110	390	720	110	840	145	695	21	60	55
200	1	NaOH	1,110	410	700	200	790	150	640	16	61	67
200	1	HCl	985	255	730	320	830	115	715	5	49	40
225	1	None	1,070	320	750	120	990	180	810	18	38	13
250	1	None	1,035	265	770	75	990	200	790	21	15	4
175	1	None (15 ml)	1,640	855	785	180	1,095	120	975	21	85	-

TABLE B-8

EFFECT OF HEAT TREATMENT ON BIODECOMPOSITION OF RNA

Heat Treatment			Initial RNA			RNA after 25 days			Percent soluble RNA removal	Percent total RNA removal
Temp. (°C)	Time (hr)	Chemical added (300 meq/1)	Total (mg/1)	Soluble (mg/1)	Insoluble (mg/1)	Total (mg/1)	Soluble (mg/1)	Insoluble (mg/1)		
Nutrient media			55	0	55	55	5	50	-	-
Feed WAS			165	20	145	75	4	71	75	82
U.S. lysed WAS			165	15	150	75	5	70	67	82
150	1	None	140	110	30	30	9	21	92	129
175	1	None	130	70	60	45	9	36	87	113
175	1	$Ca(OH)_2$	115	55	60	45	6	39	89	117
175	1	NaOH	130	65	65	60	10	50	85	93
175	1	HCl	60	20	40	70	11	59	45	-200
200	1	None	65	10	55	60	9	51	10	50
200	1	NaOH	105	35	70	55	10	45	71	100
200	1	HCl	50	3	47	60	6	54	-	200
225	1	None	55	4	51	40	8	32	-	-
250	1	None	55	3	52	40	7	33	-	-
175	1	None (15 ml)	205	145	60	45	30	15	79	-

TABLE B-9

MAXIMUM RATE RATIO (MRR) FOR HEAT-TREATED WAS

Heat Treatment			Dilution (sample/total)		
Temp. (°C)	Time (hr)	Chemical added (300 meq/l)	2/50	10/50	25/50
Feed WAS			-	-	1.35
U.S. lysed WAS			-	-	1.26
150	1	None	1.19	1.20	1.24
175	1	NaOH	0.98	0.13 A20*	0.17
175	1	$Ca(OH)_2$	1.06	1.28	0.87 A7
175	1	HCl	0.54 A16	0.14	0.21
175	1	None	1.18	1.02	0.73 A14
200	1	NaOH	1.06	0.13 A20	0.17
200	1	HCl	0.56 A20	0.14	0.17
200	1	None	1.13	0.16 A24	0.19
225	1	None	0.88	0.11	0.16
250	1	None	0.63	0.10	0.13

A20* denotes acclimation after 20 days.

percent by weight of a cell, and hence approximately 20-30 percent of the total COD; it is apparent that some of the internal components of the cell are not soluble, or are precipitated once solubilized. The insoluble material is very low in nitrogen indicating that it is either carbohydrate or non-saponifiable lipids.

The addition of chemicals, except in the case of lime, increased the solubilization marginally. Since soluble COD determinations were carried out on neutralized samples it appears that extremes in pH do not lead to significant increases in hydrolysis of the insoluble organics.

The effect of heat treatment on nitrogenous compounds was similar to that observed by others. Even at low temperatures most of the nitrogen was in a soluble form, predominantly organic (protein, amino acid, RNA, DNA). As the temperature rose the SON hydrolyzed first to amino acids, and finally to ammonia. The use of HCl increased the rate of hydrolysis at the same temperature, as expected. NaOH, however, only increased hydrolysis of SON slightly.

While amino acid concentration increased steadily with temperature, at 250°C there was a sharp drop, with only a small increase in ammonia. Both SON and SCOD dropped also indicating the precipitation of a compound high in nitrogen, possibly in the form of amino acids. Since the Browning reaction involves the polymerization of amino groups and reducing sugars it is possible that this reaction occurred, lowering both SON and SCOD.

Data in Table B-4 indicate that heat treatment of WAS can increase its degradability substantially. This increase comes from both the soluble and insoluble fractions as their respective degradabilities are both higher than the control, except in the case of the 250°C treatment. Hence it would appear that heat treatment can alter the structure of the insoluble fraction to make it more amenable to degradation.

The degradability of the treated WAS sample decreased with increasing temperature above 175°C. This could be due to either the formation of refractory compounds during heat treatment, or to the inhibition of the microorganisms by the treated WAS.

While the data do not allow firm conclusions to be drawn, the inhibition data in Table B-9 reveal that when samples treated at 200°C or above are diluted 25 times only the samples treated at 250°C contain an inhibitory mixture. Hence it would appear that the decreased degradation at 200°C was due primarily to the formation of refractory compounds, both soluble and insoluble, rather than inhibition. Also gas production data after 80 days do not show any increase in degradability.

Chemical addition, except with $Ca(OH)_2$, resulted in substantial increases in degradability, the increases becoming more pronounced at higher temperatures. These increases cannot be explained by solubilization, and hence could be due to alteration of the insoluble organics making them more amenable to attack by extracellular enzymes. The addition of $Ca(OH)_2$ lowers the soluble and overall degradability, perhaps by entrapment of organics or by other means.

Unfortunately, as Table B-9 reveals, heat treatment also produces strongly inhibitory compounds. This is obviously an important consideration in the use of heat treatment as it limits the strength of the heat-treated WAS that can be treated. Hence the most severe conditions that could be used without significant inhibition at half strength is 175°C without chemical addition. Whether digesters could be acclimated to the most degradable WAS

(175-1-NaOH) is questionable, but the fact that acclimation occurred at one-fifth dilution in twenty days holds some promise for complete acclimation to the full strength waste.

Another problem which could become significant is the presence of high levels of ammonia, released by both hydrolysis and degradation, and a high pH. These conditions could lead to ammonia toxicity.

The data obtained partially substantiate some of the hypotheses proposed. By hydrolyzing some nitrogenous components of the cell into smaller constituents, the degradability of WAS was increased. Hence it would appear that WAS is only partially degradable because of the inability of enzymes excreted by the anaerobes to hydrolyze the nitrogenous material into smaller and more degradable constituents. The data would also seem to suggest that some nitrogen compounds, presumably due to their structure, are inherently non-degradable.

Heat treatment appears to increase degradability by solubilizing the nitrogenous components of the cell. However, it also appears to increase the degradability of the insoluble fraction, possibly because it weakens the matrix and makes the insoluble organics more amenable to attack by hydrolyzing enzymes.

While the data are not conclusive it would also appear that heat treatment under severe conditions can lead to the production of nitrogen-containing compounds, both soluble and insoluble, which are refractory and lower the overall degradability of the nitrogenous fraction.

6. Conclusions

1. Non-oxidative heat treatment can lead to substantial increases in the mesophilic anaerobic degradability of WAS. This increase occurs in both the soluble and insoluble portions and is dependent on the temperature of treatment and on the type of chemical added.

2. The effect of heat treatment on WAS is to promote hydrolysis, and to split complex nitrogen polymers into simpler constituent molecules. However, as treatment conditions become more severe, nitrogen-containing organic material is formed which is refractory under the conditions studied.

3. Heat treatment causes the formation of toxic compounds, and this effect becomes more pronounced as treatment conditions become more severe. However, given sufficient time, methanogenic consortiums can acclimate to some extent to these toxins.

C. Biodegradability of Selected Model Compounds

J. B. Healy and L. Y. Young

1. Introduction and Objectives

The aim of heat treatment is to convert natural refractory organic materials such as lignin into less complex substances which are more readily biodegradable by a methanogenic population. The result will be an increase in the methane yield from a given quantity of material and at the same time a reduction in the quantity of residual organics requiring further costly processing and final disposal. These results, therefore, may also improve the economics of bioconversion as an energy source.

Upon heat treatment of lignin, a variety of simple aromatic compounds are expected to be released. Very little is known about the anaerobic biodegradability of simple aromatic compounds under methanogenic conditions. The objective of this part of the study is to determine the manner in which model aromatic compounds are metabolized under methanogenic conditions. The previous studies we carried out (Healy and Young, 1976) provided evidence that model aromatic compounds are converted to methane and carbon dioxide after the aromatic ring is cleaved. This phase of the study reported here, examines in greater detail the microbiology and intermediate metabolites of these anaerobic conversions using four model compounds: phenol, catechol, ferulic acid, and vanillic acid.

2. Background

Lignin is not a constitutionally defined compound but rather is a group or system of high-molecular-weight amorphous materials that are chemically closely related (Pearl, 1967). The lignin molecule is a complex three-dimensional polymer consisting largely of the basic phenylpropane building block. These phenylpropane-type groups include coniferyl or similar guaiacylpropane units, in the case of soft woods, and both coniferyl and syringyl units in the case of hard woods (Cowling and Brown, 1969). These monomer units are joined together by ether linkages.

Lignin is very refractory. Its large molecular size, poor solubility, and complex cross-linking makes it very inaccessible to both microorganisms and enzymes. According to Alexander (1965), there are no known environments in which the rate of lignin degradation through microbial action is rapid.

Some fungi break it down slowly to simple aromatic substances probably using extracellular depolymerases. Hackett et al. (1977), using inoculum from soils, sediments, silage, steer bedding, and rumen contents, observed very slow degradation of synthetic lignin under aerobic conditions. They were not able to detect degradation under anaerobic conditions. Murry (1974) reported that a mixed population from a kraft mill treatment pond was able to anaerobically attack the lignin in kraft black liquor from a batch digester. She concluded that this attack most likely involved depolymerization and aromatic-ring cleavage.

Lignin yields a variety of simple aromatic compounds when its complex structure is broken down by various types of physical, chemical, or biological treatment. Different forms of treatment often yield the same compounds. Pearl (1967) reports that lignin is not appreciably attacked by heat under neutral conditions, but that there is some indication that small amounts of coniferyl aldehyde, as well as vanillin, syringaldehyde, p-hydroxybenzoic acid, and other aromatics are present in hot-water extractives of wood. The effect of acid on lignin is small and essentially the same soluble products are obtained from acid hydrolysis as from hot-water extracts.

Lignin is extensively solubilized in alkaline solution, particularly in hot alkaline solution. Previous work in our lab (Gossett et al., 1976a) has shown the best solubilization results from heat treatment at a temperature of 200°C for 1 hour at a pH of 13. The mechanisms are unknown, but several compounds have been identified in the hydrolyzates of wood lignins by various investigators (Pearl, 1967; Schitzer and Khan, 1972). Among them are guaiacylethane, the 1,3-dimethyl ether of pyrogallylethane, p-hydroxybenzoic acid, vanillin, acetaldehyde, acetovanillone, formaldehyde, syringaldehyde, protocatechuic acid, and catechol.

Aerobically, lignin can be broken down by fungi yielding aromatic compounds such as coniferaldehyde, p-hydroxycinnamaldehyde, ferulic acid, 4-hydroxy-3-methoxyphenylpyruvic acid, p-hydroxycinnamic acid, guaiacylglycerol and its β-caniferyl ether, vanillin, vanillic acid, and dehydrodivanillin. Murry (1974) tentatively identified a number of aromatic compounds from cultures in which lignin from kraft black liquor was anaerobically attacked. These compounds include phenol, hydrocinnamic aldehyde, guaiacol, pyrocatechol-catechol, vanillin, p-hydroxybenzoic acid,

veratric acid, vanillic acid, shikimic acid, syringic acid, and ferulic acid. The choice of model aromatic compounds for lignin degradation studies came from the above lists which show that the same types of aromatic derivatives usually result from different types of lignin treatment.

Several groups have made extensive studies on the aerobic metabolism of aromatic compounds (e.g., Evans, 1963; Ornsten and Stanier, 1964; Ribbons, 1965; and Dagley, 1967). Relatively little attention, however, has been paid to the anaerobic metabolism of aromatic compounds. Early investigators approached the problem using mixed populations of microorganisms. Tarvin and Buswell (1934) obtained mixed cultures from sewage sludge which fermented a range of aromatic compounds to a mixture of CO_2 and CH_4. Clark and Fina (1952) and Fina and Fiskin (1960) used a mixed culture from sewage sludge or rumen fluid and confirmed that benzoate was metabolized to a mixture of CO_2 and CH_4. Barker (1956) described studies in which several aromatic acids were utilized under strictly anaerobic conditions by a heterogeneous population of methanogenic bacteria. Nottingham and Hungate (1969) have also confirmed, using C^{14}-labeled benzoate, that mixed cultures from sewage sludge metabolize benzoate to CO_2 and CH_4. Ferry and Wolfe (1976) reported on the ability of a microbial consortium to anaerobically convert benzoate to CO_2 and CH_4.

Other investigators began to take a closer look at the anaerobic metabolism of aromatic compounds and used isolated species able to carry out these metabolisms. The photosynthetic metabolism of benzoate and other aromatic compounds by the photosynthetic bacterium Rhodopseudemonas palustris has been reported by several groups (Proctor and Scher, 1960; Hegeman, 1967; Guyer and Hegeman, 1969; and Dutton and Evans, 1968, 1969). Anaerobic nitrate respiration has been investigated by Taylor et al. (1970) using a Pseudomonas sp. and Williams and Evans (1975) using a Moraxella sp. isolated from soil. Harary (1956) isolated a Clostridium sp. from river mud that degraded the ring compound nicotinic acid and MacRae et al. (1969) isolated a Clostridium sp. capable of attacking the insecticide lindane, a cycloparaffinic compound. Tsai and Jones (1975) have isolated and identified the rumen bacteria Streptococcus boris and Coprococcus sp. capable of the anaerobic metabolism of phloroglucinol. It is not clear in this last report what electron acceptor was used.

In the course of these studies, some investigators have proposed biochemical pathways for the anaerobic metabolism of aromatic compounds. Oshima (1965) proposed that a mixed population of denitrifying pseudomonads cleaved aromatic rings by the incorporation of oxygen atoms of nitrate into the reaction products. The mechanism was proposed to be similar to those of aerobic systems. Proctor and Scher (1960) proposed a mechanism for the photometabolism of benzoate which involved some of the same intermediates of aerobic mechanisms. Guyer and Hegeman (1969) and Dutton and Evans (1968), on the other hand, proposed reductive pathways for aromatic ring metabolism. Taylor et al. (1970) proposed a different mechanism involving the addition of water molecules to the aromatic ring during the nitrate respiration of benzoate and p-hydroxybenzoate. Evans' group in Wales has taken a very strict biochemical approach to the investigation of mechanisms and has worked at both the photometabolism and nitrate respiration of a large number of aromatic compounds. Their evidence (Dutton and Evans, 1968, 1969; Williams and Evans, 1975) questions the mechanisms proposed by Oshima (1965) and Taylor et al. (1970) and strongly supports a reductive pathway for anaerobic benzoate decomposition. Ferry and Wolfe (1976) identified acetate as an intermediate in the overall conversion of benzoate to methane by a microbial consortium. Their culture also converted acetate cyclohexane carboxylate, 2-hydroxycyclohexane carboxylate, o-hydroxybenzoic acid and pimelic acid to methane without a lag further suggesting that benzoate is degraded by a reductive pathway.

Not all aromatic compounds have been reported to be anaerobically biodegradable. The available information indicates that catechol and protocatechuic acid are very refractory (Clark and Fina, 1952; Taylor et al., 1970; Williams and Evans, 1975). This is of particular interest to us since both catechol and protocatechuic acid are biodegradable in our enrichments.

The refractory nature of some aromatics under anaerobic conditions could be due to toxicity. No methane fermentation occurring with light paraffinous crude oil was ascribed to the presence of aromatics in the gasoline fraction by Muller (1957). Methane fermentation was observed when this fraction was removed. Calder and Lader (1976) described toxic effects of aromatics as decreasing growth rate and maximum cell densities. They found aromatic toxicity to be a function of concentration and to increase as solubility decreases. Chmielowski et al. (1964) reported that shock loadings

of 500 to 1000 ppm of phenol into unadapted cultures strongly inhibited or destroyed the methane fermentation process in a few days. Other hydroxy aromatics are also indicated to be among the toxic extractible substances in wood. Protocatechuic acid is reported to account for the decay resistance of red onion scales (Scheffer and Cowling, 1966). Combinations of 6-, 7-, and 8-carbon straight chain p-hydroxybenzoate esters have been found to have antimicrobial properties towards yeast and malo-lactic fermentations (Chan et al., 1975).

Mass balances have played a big part in most of the investigations of the methanogenic degradation of simple aromatic compounds (Tarvin and Buswell, 1934; Clark and Fina, 1952; Fina and Fiskin, 1960; Chmielowski et al., 1964; Nottingham and Hungate, 1969; and Ferry and Wolfe, 1976). Chmielowski et al. (1964) demonstrated that phenol, p-cresol, resorcinol, and a combination of p-cresol plus resorcinol are anaerobically degradable to methane and carbon dioxide according to the Buswell equation:

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right) H_2O \rightarrow \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right) CO_2 + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right) CH_4$$

They measured the gas production to be 99 to 102% of the theoretical yield calculated from the above equation. This is the only known report on phenol biodegradation under anaerobic conditions. Chmielowski (personal communication) looked at the fate of catechol in the same type of methanogenic systems over an incubation period of three weeks and found that it was quite refractory. That is, catechol was not metabolized in his enrichment cultures. They also adapted a methane fermenting population to the fermentation of phenol as the sole carbon source. This was done by feeding the system 100 to 3000 ppm phenol in increments of 100 ppm doses every two to three days. The methane fermentation of phenol was most efficient at 2000 ppm where it was completely converted to CO_2 and CH_4 in three days.

In a later report investigating the methanogenic metabolism of some phenols, Chmielowski and Wasilewski (1966) found that a temporary accumulation of intermediates appeared to be saturated compounds; although they were not the kinds of volatile acids usually found in the methane fermentation of organic sludges. They found that the early stages of the methanogenic metabolism of phenols were based on the hydrogenation of the aromatic structure with the formation of some alicyclic compounds as intermediate products. The

hydrogenation process is followed by the ring-fission of alicyclic intermediates and then by the methanogenesis from the degradation products. This type of reductive pathway has been proposed and partially demonstrated for other forms of anaerobic metabolism of aromatic ring compounds (Dutton and Evans, 1968; Guyer and Hegeman, 1969; and Williams and Evans, 1975).

A distinct two-stage course of aromatic ring metabolism was observed by Chmielowski and Wasilewski (1966) during the anaerobic degradation of two phenolic substrates. When both resorcinol and p-cresol were provided as a two-component substrate, resorcinol fermentation occurred to completion first, only then followed by the complete fermentation of p-cresol. Facility of the decomposition of resorcinol was explained by less distinct properties of its polyhydric structure than those of p-cresol. This work raises some interesting questions on the role of aromatic structures in the metabolism of simple model compounds. Our group has previously shown (Gossett et al., 1976a) that many of the aromatic compounds expected to be formed from heat treatment of lignin are biodegradable and fermentable to methane. Under conditions of anaerobic nitrate respiration, phenol and benzoate were both decomposed. Benzoate enriched populations readily utilized phenol, whereas adaptation to phenol directly was very slow. Further investigations into the role of structure were carried out in the course of the present biodegradability studies.

Anaerobic test-tube cultures have proved to be a valuable technique for the study of these kinds of methanogenic metabolisms for the following reasons: (1) they are very small and easy to manipulate; (2) they are capable of providing very accurate information as was first demonstrated by Nottingham and Hungate (1969). Using very exact mass balances, these investigations demonstrated that a methanogenic enrichment culture decomposed small concentrations of ^{14}C benzoate to $^{14}CH_4$ and $^{14}CO_2$ under the stringently anaerobic conditions present in the culture tubes. These Hungate tubes were later used by Ferry and Wolfe (1976) to obtain a stabilized consortium of microbes which anaerobically degraded benzoate and produced CH_4. This anaerobic test-tube culturing technique is also a valuable tool in our biodegradability studies. We investigated certain aspects of the manner in which low molecular weight aromatic compounds are biodegradable and fermentable to methane gas.

3. Previous Work

Previous work in our lab (Healy and Young, 1976) has demonstrated that phenol, catechol, benzoic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, vanillin, syringic acid, syringaldehyde, ferulic acid, dimethoxybenzoic acid, trans-cinnamic acid, and trans-cinnamaldehyde are degradable to methane and carbon dioxide in methanogenic enrichments. Simplified mass balance calculations made for most of the above compounds provide evidence that the aromatic ring is cleaved. Acclimation of a microbial population to a compound is evidenced by the fact that additional substrate is utilized immediately. The time required for acclimation in an enrichment varies depending upon the particular aromatic substrate and the source of the inoculum. For example, syringic acid requires less than 1 week for acclimation whereas catechol requires from 3 to 7 weeks. The time required for acclimation is reduced when a microbial population acclimated to heat-treated refuse is used as inoculum instead of a population acclimated to primary settled sludge. Microbial populations acclimated to a particular aromatic substrate can be simultaneously acclimated to other selected aromatic substrates which have similar structural arrangements of side groups on the aromatic ring.

Present work mainly involves the identification of major intermediates formed during the conversion of model aromatic compounds to methane. Ferulic acid and phenol are the main model compounds being looked at. Microscopic observations of the microbial population are being made.

4. Materials and Methods

a. Media. Stock solutions (Table C-1) were used in the preparation of 1800 ml of pre-reduced and defined media in the following manner. To a liter of deionized water is added 1.8 ml of S2, 2.7 ml of S3, 27 ml of S4, and 18 ml of S8. The volumetric flask is then diluted up to the 1800-ml mark. This solution is boiled for 15 minutes while the neck of the volumetric flask is flushed with nitrogen gas which has passed through a heated copper column to remove traces of oxygen. This procedure removes most of the dissolved oxygen. After the solution has been cooled to room temperature in an ice bath, 1.8 ml of S5 and S7 are added to the flask as an additional reductant. The oxygen-free gas, which has been constantly flushing the neck of the flask is now switched to a mixture of 30% CO_2 and 70% nitrogen. This same gas mixture is also bubbled into the solution immediately after 4.752 g of sodium

TABLE C-1

STOCK SOLUTIONS FOR DEFINED MEDIA

Solution No.	Compound	Concentration (g/l)
S1	aromatic substrate	9
S2	resazurin	1
S3	$(NH_4)_2 HPO_4$	26.7
S4	NH_4Cl	13.3
	$MgCl_2 \cdot 6H_2O$	120
	KCl	86.7
	$MnCl_2 \cdot 4H_2O$	1.33
	$CoCl_2 \cdot 6H_2O$	2
	H_3BO_3	0.38
	$CaCl_2 \cdot 2H_2O$	0.18
	$Na_2MoO_4 \cdot 2H_2O$	0.167
	$ZnCl_2$	0.14
S5	$FeCl_2 \cdot 4H_2O$	368
S7	$Na_2S \cdot 9H_2O$	500
S8	biotin	0.002
	folic acid	0.002
	pyridoxine hydrochloride	0.01
	riboflavin	0.005
	thiamin	0.005
	nicotinic acid	0.005
	pantothenic acid	0.005
	B_{12}	0.0001
	p-aminobenzoic acid	0.005
	thioctic acid	0.005

bicarbonate was added in powder form. This eventually lowers the pH to 6.8 where the media is buffered by the carbonate system with a gas atmosphere of 30% CO_2 and 70% nitrogen. Five ml of S1, the aromatic substrate is later added to a 145 ml portion of the defined media in a 250 ml serum bottle, giving a C:N:P molar ratio of 100:15:1. The final composition of the defined media without the aromatic substrate is presented in Table C-2.

Pre-reduced replacement media, used for maintaining stock cultures in serum bottles, was prepared similarly to the above defined media, except that it is boiled under reflux in order to keep the volatile aromatic

TABLE C-2

FINAL COMPOSITION OF THE PRE-REDUCED DEFINED MEDIA WITHOUT AROMATIC SUBSTRATE

DEFINED MEDIA			
Compound	Conc. (g/l)	Compound	Conc. (g/l)
		H_3BO_3	0.0057
resazurin	0.001	$CaCl_2 \cdot 2H_2O$	0.0027
$(NH_4)_2PO_4$	0.04	$Na_2MoO_4 \cdot 2H_2O$	0.0025
NH_4Cl	0.2	$ZnCl_2$	0.0021
$MgCl_2 \cdot 6H_2O$	1.8	$FeCl_2 \cdot 4H_2O$	0.368
KCl	1.3	$NaHCO_3$	2.64
$MnCl_2 \cdot 4H_2O$	0.02	$Na_2S \cdot 9H_2O$	0.5
$CoCl_2 \cdot 6H_2O$	0.03		
VITAMINS			
Compound	Conc. (mg/l)	Compound	Conc. (mg/l)
biotin	0.02	nicotinic acid	0.05
folic acid	0.02	pantothenic acid	0.05
pyridoxine hydrochloride	0.1	B_{12}	0.001
riboflavin	0.05	p-aminobenzoic acid	0.05
thiamin	0.05	thioctic acid	0.05

substrate in solution. Defined media does not contain an aromatic substrate. Because the replacement media contains concentrated substrate at 10 g/l, additional amounts of nitrogen and phosphorus are required in order to sustain the same C:N:P ratio as in the defined media with 300 mg/l substrate. Replacement media was prepared as follows: 3 g of aromatic substrate and the indicated grams of NH_4Cl (Table C-3) were added to 200 ml of deionized water in a 500-ml erlenmeyer flask with a 24/40 ground glass neck for attachment to reflux. In order to neutralize the acidity of the aromatic acids, the indicated ml of NaOH (Table C-3) were added. Then 0.3 ml of S2 (see Table C-1), the indicated ml (Table C-3) of S3, 4.5 ml of S4 and 3 ml of S8 were also added before diluting the solution up to 300 ml. With this procedure, the proper molar ratios of C:N:P are obtained. While constantly flushing the atmosphere with oxygen-free nitrogen gas, the solution was boiled for 15 minutes under reflux and then cooled to room temperature. Then 0.3 ml of S5 and S7 were added as reducing agents, and 0.792 g of $NaHCO_3$ powder was added while flushing with a mixture of 30% CO_2 and 70% N_2 gas. The pH was adjusted to 6.8 by bubbling the same gas mixture in the media.

TABLE C-3

MEDIA ADJUSTMENTS FOR PARTICULAR AROMATIC SUBSTRATES

Substrate	S3 $(NH_4)_2HPO_4$, (ml)	NH_4Cl, (g)	10N NaOH, (ml)
Phenol	9.4	1.273	--
Catechol	8.03	1.08	--
Ferulic acid	7.58	1.013	1.71
Vanillic acid	7.02	0.937	1.92

b. Serum Bottle Enrichments. The defined media was inoculated with a 10-percent-by-volume seed from a laboratory anaerobic digester fed primary settled sludge on a 15-day detention time, and 250-ml serum bottles were flushed with oxygen-free gas for 20 minutes before addition of pre-reduced inoculated media. Solid material and ferrous sulfide precipitate settle to the bottom in these serum bottle cultures. Resazurin, an oxidation reduction indicator, remains clear when the media is reduced and turns pink when oxidized. These cultures are incubated in the dark at 35°C ± 1°C.

Syringes were used to add aromatic substrate, remove portions of the culture for analyses, and monitor the volume of gas produced. Substrate concentration was determined by diluting a centrifuged sample and assaying it in a spectrophotometer at a characteristic uv wavelength for each particular aromatic compound. Gas composition was determined on a Fischer-Hamilton gas partitioner. Gas production and substrate concentration were corrected for background levels by subtracting values measured in a control culture which contained no aromatic substrate.

c. Cross Acclimations. Cross acclimations were set up in the following manner. Hungate tubes were flushed for 10 minutes with oxygen-free gas (Figure C-1). Portions of 7.5 ml of an acclimated stock culture were anaerobically transferred with a syringe to a group of Hungate tubes. These cultures had stopped producing gas several days before the transfer. Then 2.5 ml of various pre-reduced aromatic substrates, stored anaerobically in serum bottles, was added to each of the tubes. These stock solutions of pre-reduced aromatic substrates contained four times the usual concentration of substrate,

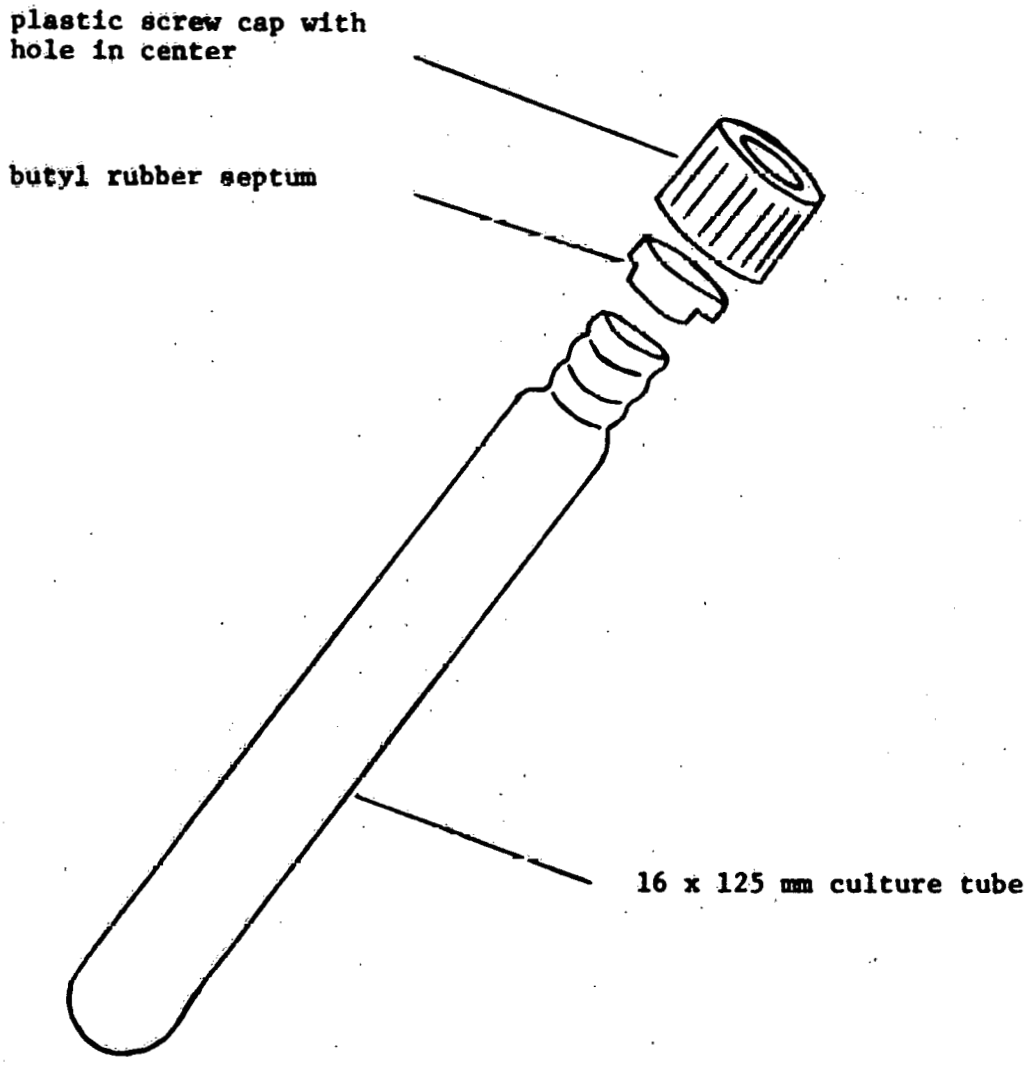

FIGURE C-1. Hungate tube (exploded view).

nitrogen, and phosphorus in order to return the 10-ml culture to original nutrient levels. The control tube contained no aromatic substrate. The onset, rate, and extent of gas production in the tube containing the particular substrate to which the stock culture was acclimated was compared with gas production in the other tubes.

d. Stock Culture Maintenance

Acclimated stock cultures are maintained in serum bottles by regular replacement of 1/5 of the culture volume with fresh pre-reduced media. This replacement is made after the culture finishes converting its previous supply of substrate to gas. After a 150-ml culture is shaken into a homogeneous mixture, 30 ml are withdrawn with a syringe and either wasted or transferred as inoculum to another serum bottle. Then an appropriate volume of concentrated replacement media containing the substrate is added with the balance of 30 ml being defined media. By varying the proportions of concentrated replacement media and defined media, the substrate concentration can be raised up to a maximum of 2000 mg/l.

The above method of stock culture maintenance differs from that of Ferry and Wolfe (1976) in that they only replace the supernatant portion of an unshaken culture in order to maintain high cell numbers. Since the majority of the cells are associated with the FeS precipitate at the bottom of the culture, our replacement method wastes more cells but still maintains a sufficient cell population and simplifies the transfer procedure for our systems.

e. Gas Chromatography

Samples for gas chromatography of volatile fatty acids (C_1-C_7) were prepared by acidifying a 2-ml portion of culture with two drops of concentrated sulfuric acid and extracting this portion with 1 ml of ethyl ether. 1 µl of this ether extract was injected into a 6-foot glass column packed with 10% SP-1000/1% H_3PO_4 on 100/120 Chromosorb W AW (Supelco, Inc.). This column was connected to a hydrogen flame ionization detector in a Tracor MT 220 series gas chromatograph and was temperature programmed to increase 15°C per minute from an initial temperature of 115°C to a final temperature of 145°C. The helium carrier gas flow rate was 54 ml/min. Acetate levels in the culture medium were determined by comparing the peak areas of culture samples and an ether extract from a standard 0.01 M acetate solution. Relative peak areas were determined by weighing the peaks after they were cut from strip chart recorder paper. Under the above conditions, 0.05 mM acetate can be detected in the culture medium.

5. Results

a. Enrichment Cultures

Table C-4 summarizes the results from initial enrichment cultures degrading a range of model compounds. Decomposition in the methanogenic enrichments appears to be slow requiring an acclimation time from 5 days for syringaldehyde to 27 days for catechol. Thirteen days were required for both vanillic acid and ferulic acid.

TABLE C-4

DECOMPOSITION CHARACTERISTICS OF A VARIETY OF AROMATIC COMPOUNDS DURING INITIAL ENRICHMENTS

Substrate	Acclimation Lag (days)	Period of Gas Production (days)	% Conversion of Substrate Carbon to Gas
Vanillin	11	29	86
Vanillic Acid	13	27	86
Ferulic Acid	13	28	83
Cinnamic Acid	14	34	80
Benzoic Acid	11	21	84
Catechol	27	12	59
Protocatechuic Acid	15	17	74
Phenol	16	21	82
p-Hydroxybenzoic Acid	15	17	65
Syringic Acid	5	14	97
Syringealdehyde	5	15	100

Degradation follows this acclimation period, as evidenced by a period of continuous gas production varying from 12 days for catechol to 34 days for cinnamic acid. The conversion of substrate carbon to gas was observed to vary, ranging from 59% in the case of catechol to 98% for syringaldehyde. About 85% of the carbon in both vanillic acid and ferulic acid was converted to gas in 27 and 28 days, respectively.

b. Cross Acclimations

Figure C-2 shows the cumulative volumes of gas produced in four Hungate tubes containing stock culture acclimated to ferulic acid. These results are representative of the type of results obtained in the cross acclimation studies. Very little gas was produced by the control fed no substrate and the culture fed phenol. The cultures fed ferulic acid and cinnamic acid produced gas immediately indicating that the ferulic acid acclimated culture is simultaneously acclimated to cinnamic acid and not to phenol. As shown in Table C-4, an unacclimated culture would have required 14 days to acclimate to cinnamic acid.

Figure C-3 shows similar results for a culture acclimated to syringic acid which is subsequently simultaneously acclimated to syringaldehyde but not to ferulic acid. The onset, rate, and extent of gas production is almost identical for syringic acid and syringaldehyde during the first five days though the final extent of gas produced varies somewhat.

Tables C-5 and C-6 summarize the results of the cross acclimation studies. Structures of the substrates to which individual cultures were originally acclimated are shown in the left-hand column. Substrates to which each of these cultures in the left-hand column are simultaneously acclimated, requiring no additional acclimation lag, are shown in the right-hand column. In all cases shown in Table C-5, there is a high degree of structural similarity among the compounds to which each culture can readily acclimate. For example, the two groups of compounds differ either in the oxidation state of one substituent group, as in the case of the carboxyl group of syringic acid compared to syringaldehyde; or they differ in the presence of an extra substituent group at an additional ring position, as in the case of the methoxy group of syringaldehyde compared to vanillin.

Table C-6 shows the same type of results for vanillic acid acclimated culture. This culture is very interesting because of the number and types of compound structures to which it is simultaneously acclimated. Syringaldehyde, syringic acid, and vanillin are structurally similar to vanillic acid in oxidation state or presence of an additional methoxy group. On the other hand, benzoic acid, catechol, and protocatechuic acid are structurally quite different from vanillic acid in the type of substituent group at identical ring positions. For example, catechol contains a hydroxyl group at the number 3 position compared to the methoxy group of vanillic acid. Also, catechol does not contain a carboxyl group.

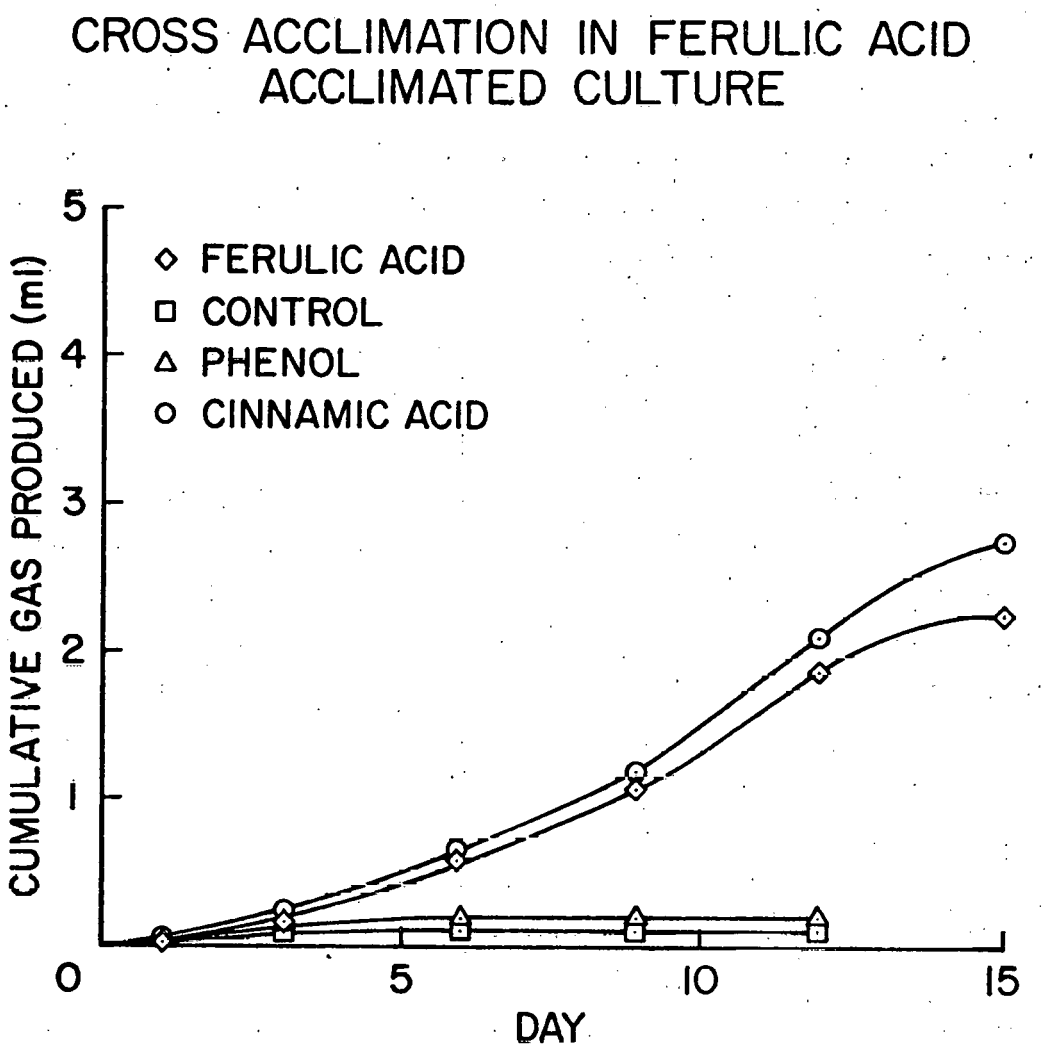

FIGURE C-2. Cross acclimation in ferulic acid acclimated culture.

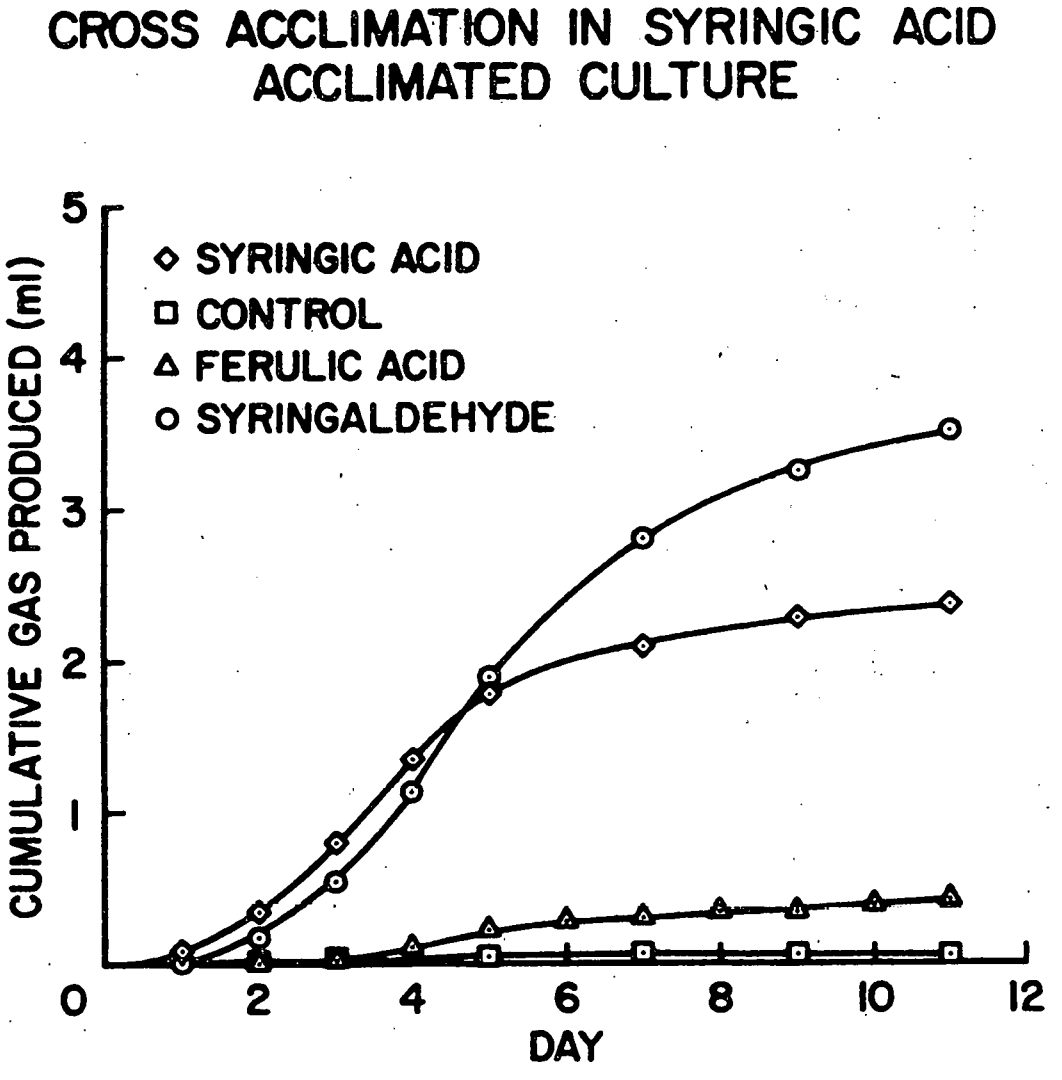

FIGURE C-3. Cross acclimation in syringic acid acclimated culture

TABLE C-5

CROSS ACCLIMATION RESULTS.

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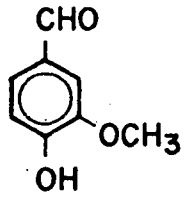
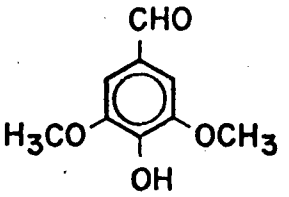
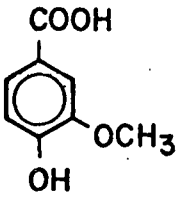
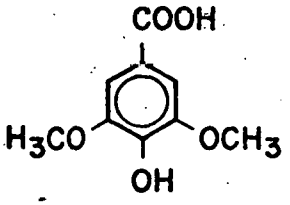
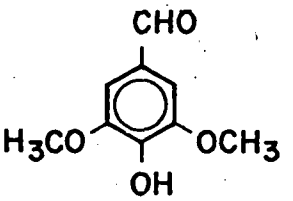
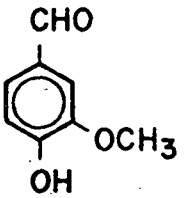
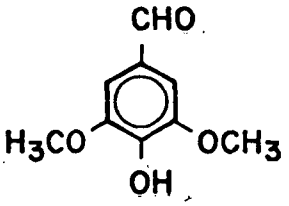
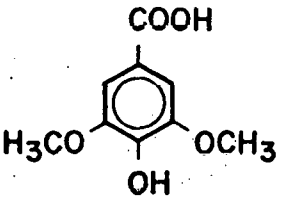
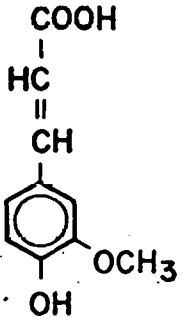
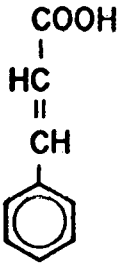
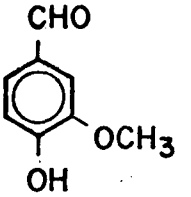
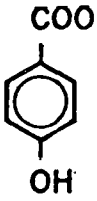
 VANILLIN	 SYRINGALDEHYDE	 VANILLIC ACID
 SYRINGIC ACID	 SYRINGALDEHYDE	 VANILLIN
 SYRINGALDEHYDE	 SYRINGIC ACID	
 FERULIC ACID	 CINNAMIC ACID	 VANILLIN
 P-HYDROXYBENZOIC ACID	BENZOIC ACID	PHENOL

TABLE C-6

CROSS ACCLIMATION RESULTS

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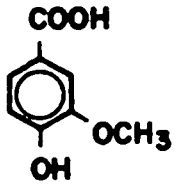
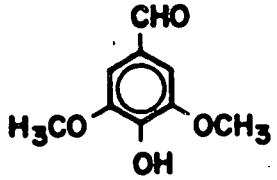
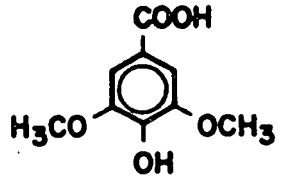
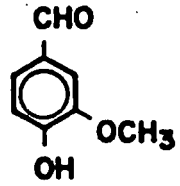
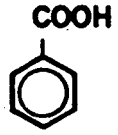
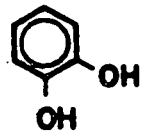
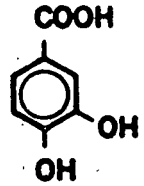
 VANILLIC ACID	 SYRINGALDEHYDE	 SYRINGIC ACID
	 VANILLIN	 BENZOIC ACID
	 CATECHOL	 PROTOCATECHUIC ACID

c. Mass Balances

Figure C-4 illustrates the temporal relationship of substrate degradation and gas production for a phenol acclimated culture fed additional phenol. The conversion of substrate to gas is very rapid as evidenced by the coincidence of substrate disappearance and the production of gas. This additional substrate spike is degraded more rapidly than the initial dose fed to an unacclimated culture. Methane is produced throughout the entire gas producing period.

Figure C-5 presents stoichiometric equations for the anaerobic fermentation of selected substrates to methane and carbon dioxide, where 50 to 60 percent of the organic carbon can potentially be converted to methane gas. Theoretical methane yields for mass balances were based upon the stoichiometry shown in this figure.

Table C-7 summarizes the mass balance studies. Actual values for total gas produced from catechol are expressed as a range because catechol acclimated cultures are much more variable in their response than the other cultures. The actual methane produced agreed extremely well with theoretical values for phenol and vanillic acid and reasonably well for catechol. Experimental error could account for the unusually high methane yield for ferulic acid.

TABLE C-7

MASS BALANCES

Substrate	Total Gas Produced (mmoles)		Methane Produced (mmoles)	
	Actual	Theoretical	Actual	Theoretical
Phenol	2.29	2.44	1.43	1.42
Catechol	1.76 - 2.67	2.87	1.21 - 1.89	1.55
Vanillic Acid	1.73	2.03	1.02	1.02
Ferulic Acid	1.41	1.47	1.07	0.78

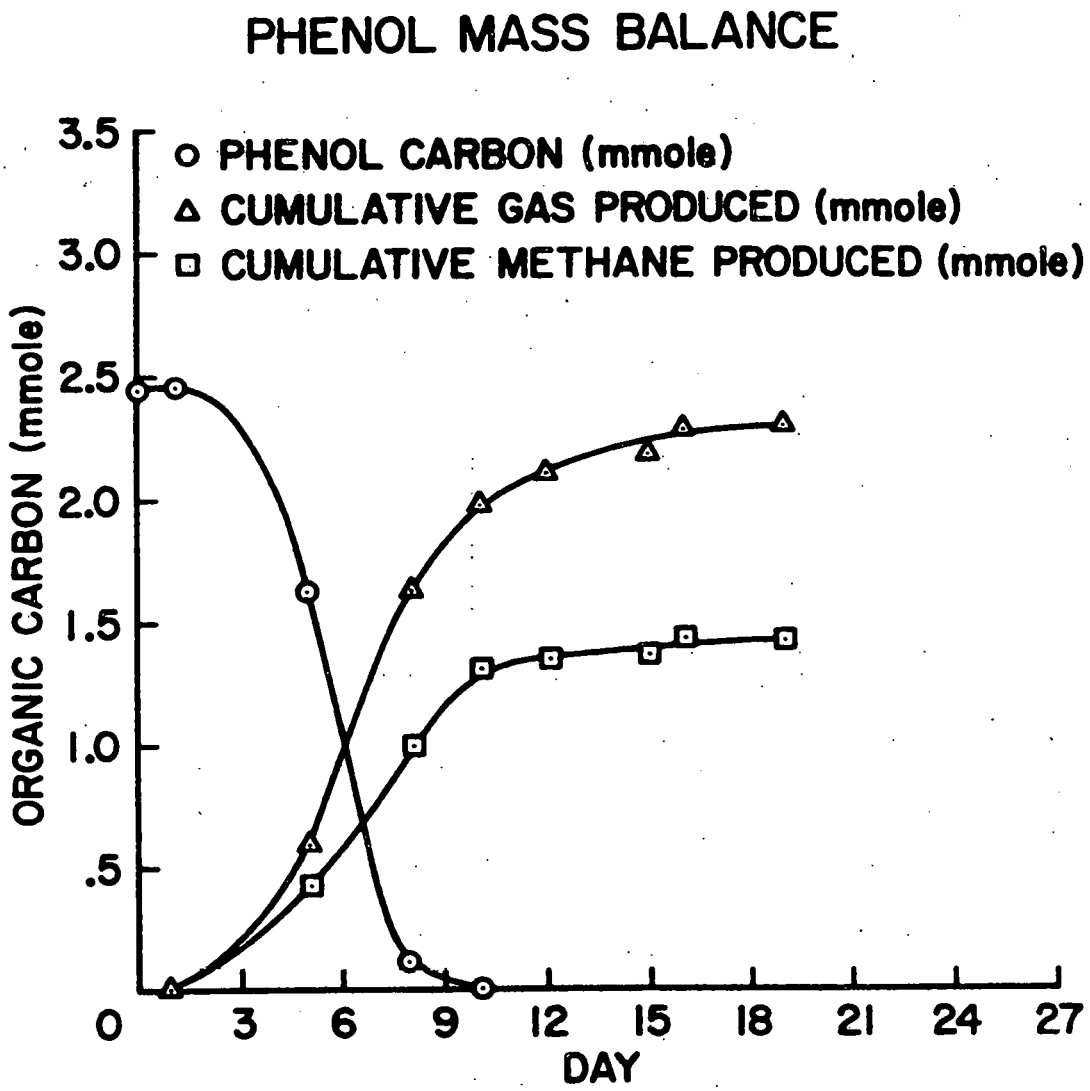


FIGURE C-4. Phenol mass balance

MASS BALANCE STOICHIOMETRY

PHENOL	$C_6H_6O + 4H_2O \rightarrow 2.5CO_2 + 3.5CH_4$
CATECHOL	$C_6H_6O_2 + 3.5H_2O \rightarrow 2.75CO_2 + 3.25CH_4$
VANILLIC ACID	$C_8H_8O_4 + 4H_2O \rightarrow 4CO_2 + 4CH_4$
FERULIC ACID	$C_{10}H_{10}O_4 + 5.5H_2O \rightarrow 4.75CO_2 + 5.25CH_4$

FIGURE C-5. Mass balance stoichiometry

d. Stock Culture Maintenance

Phenol, catechol, ferulic acid, and vanillic acid enrichment cultures, which were started over three months ago, are still being maintained by the media replacement technique at 400 mg/l substrate concentration. All of these cultures have been successfully used as a 20% by volume inoculum in fresh media in order to start new acclimated cultures. Phenol and ferulic acid acclimated inocula begin metabolizing substrate in the new cultures without a lag period. Catechol and vanillic acid acclimated inocula usually require one to two weeks before they begin converting the substrate to gas in the new cultures. By gradually increasing the substrate concentration each time the media is replaced, ferulic acid, vanillic acid, and phenol cultures have been acclimated to substrate concentrations of 1000 mg/l. This facilitates the detection of intermediates which are likely to be present at much lower concentrations than the original aromatic substrate.

Ferulic acid acclimated stock cultures are the most active, converting substrate to gas in less than 2 weeks. The basic parameters monitored for each successive media replacement in all stock cultures are summarized in Table C-8 for a representative ferulic acid acclimated stock culture. This culture does not exhibit a lag regardless of the initial substrate concentration. The gas producing period increases slightly with higher substrate concentrates while the percent of substrate carbon converted to gas remains quite constant.

TABLE C-8

BASIC PARAMETERS OF A REPRESENTATIVE FERULIC ACID ACCLIMATED CULTURE

Substrate concentration (mg/l)	Lag period (days)	Gas producing period (days)	Substrate carbon converted to gas (%)
400	0	11	77
400	0	8	84
400	0	11	90
400	0	10	83
400	0	10	71
800	0	9	81
1000	0	13	78
1000	0	13	79
1600	0	13	84

e. Volatile Acid Intermediates

Acetic acid has been detected in a culture acclimated to 1600 mg/l ferulic acid using gas chromatography. Figure C-6 illustrates the temporal relationship of the conversion of substrate carbon to gas in this culture. Ferulic acid is completely degraded in 3 days while gas production continues for another 8 to 10 days. Significant levels of acetic acid first appear after the culture has begun to produce gas. The amount of substrate carbon tied up in the form of acetic acid remains a little less than 10% of the original 11 mmoles throughout most of the gas producing period. These levels of acetic acid are very similar to those observed by Ferry and Wolfe (1976) during the methanogenic degradation of benzoic acid and seem to indicate that acetate may also be an important intermediate in the conversion of ferulic acid to methane and carbon dioxide.

Acetic acid was also detected in a phenol acclimated culture, but has not yet been detected in catechol and vanillic acid acclimated cultures. If significant levels of acetic acid are being produced, it should be easier to detect these levels in the future when these cultures are acclimated to much higher substrate concentrations. Propionic and butyric acid were also detected in ferulic acid cultures but were not present in significant amounts compared to background levels.

f. Microscopic Observations

A predominance of short rods (2μ x 0.6μ), curved rods (15μ x 0.6μ), and very long rods (25-80μ x 1μ) were observed repeatedly in a ferulic acid acclimated culture using phase contrast microscopy. The very long rods were almost always associated with particulate matter. Occasionally, some of the small rods, which were almost oval in shape, exhibited very rapid motility. A similar mixture of morphological types was also observed in phenol and catechol acclimated cultures. Vanillic acid acclimated cultures contained the same types of organisms but none of the very long rods were observed attached to the particulate matter.

6. Discussion

The initial enrichments conducted at the beginning of this present study support earlier findings (Healy and Young, 1976) on the anaerobic degradability of simple aromatic compounds. Namely, vanillic acid, vanillin, ferulic acid, cinnamic acid, benzoic acid, catechol, protocatechuic acid, phenol, p-hydroxybenzoic acid, syringic acid, and syringaldehyde are

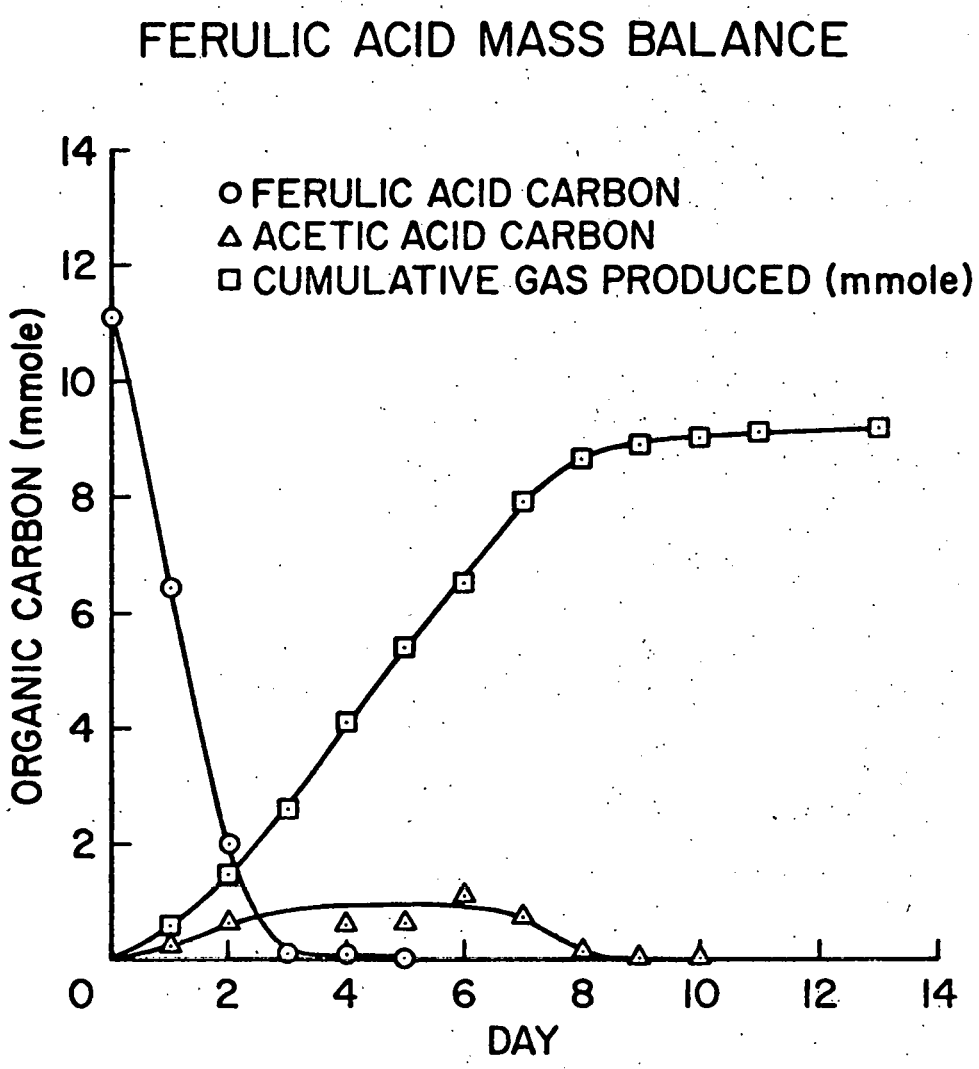


FIGURE C-6. Ferulic acid mass balance

degradable to methane and carbon dioxide under methanogenic conditions. Sixty percent or more of the substrate carbon was converted to gas over a period of 2 to 5 weeks depending on the compound.

After the initial enrichment period these cultures are acclimated to the particular substrate on which they were enriched, such that no lag period is required for the organisms to metabolize additional added substrate. Four of these enrichment cultures were maintained as acclimated stock cultures. Table C-9 compares the behavior of these cultures before and after acclimation. As was expected, the acclimation lag was eliminated after cultures became acclimated to vanillic acid, phenol and ferulic acid. The amount of substrate carbon converted to gas did not change significantly in these cultures indicating that the original enrichment population was just as efficient as the later acclimated population. Unlike the vanillic acid culture, the activity of the cultures acclimated to ferulic acid and phenol increased with acclimation in terms of their ability to convert the substrate to gas in much shorter periods of time. This is likely due to an increase in cell numbers. This was not observed with the vanillic acid acclimated culture, however.

The behavior of catechol acclimated cultures has been found to be much more variable than the other cultures. Catechol always requires the longest acclimation periods in initial enrichments and often requires additional acclimation periods when additional substrate is added. The onset rate and extent of gas production is often irregular in these cultures, varying from one media replacement to the next. The data in Table C-9 illustrate some of this. After the culture has acclimated to catechol in the initial

TABLE C-9

BEHAVIOR OF METHANOGENIC CULTURES BEFORE AND AFTER ACCLIMATION TO PARTICULAR AROMATIC COMPOUNDS

Substrate fed to culture	Acclimation lag (days)		Period of gas production (days)		% Conversion of substrate carbon to gas	
	before	after	before	after	before	after
Vanillic acid	13	0	27	27	86	83
Phenol	16	0	21	11	82	82
Catechol	27	10	12	28	59	70
Ferulic acid	13	0	28	13	86	88

enrichment, it still exhibits an acclimation lag of ten days before metabolizing additional catechol. Increases in both the length of the gas production period and percent conversion of catechol carbon to gas suggest that the initial enrichment may have become inhibited after 12 days of producing gas. At this time the culture had only converted 59 percent of the carbon to gas. Further conversion of carbon to gas can be expected to require additional time. The acclimated culture which is seemingly uninhibited can convert 70 percent or more of the catechol carbon to gas but requires 28 days in which to accomplish the conversion.

The selection of certain types of organisms from an initial mixed population is always involved in an initial enrichment but is not necessarily involved in an acclimation. It is very difficult to determine from our results whether the acclimation process is mainly a population selection or an enzyme induction. Both are likely to be involved.

If the assumption is made that the aromatic substrate is first converted to simple organic acids by acid-forming bacteria and then converted to methane by methane bacteria, it is likely that the initial enrichment primarily involves the selection of sufficient numbers of acid formers. Methane bacteria are probably already present in sufficient numbers in the original inoculum since it came from a methane digester. This hypothesis is supported by comparison of the activity of an initial enrichment culture with the activity of an acclimated stock culture. Typically, a coincidence of gas production and substrate disappearance occurs in initial enrichments. An example of this activity is shown in Fig. C-4, where methane was produced only during the same 9-day period in which phenol was being degraded. This suggests that the rate-limiting step in the conversion of aromatic substrate to methane is the formation of the organic acid intermediates.

This activity changes in acclimated stock cultures which have been fed additional substrate for many months. For example, Fig. C-6 shows that ferulic acid is completely degraded in 3 days while significant gas production continues for another 6 days. In this case it appears that the rate-limiting step is the conversion of simple organic acids, primarily acetate in this culture, to methane. This change in activity can be explained by the fact that acid formers generally grow faster than methane formers. Therefore, the numbers of acid formers have increased faster than the numbers of methane formers. At some point during the maintenance of the stock culture, the activity of the acid formers becomes greater than that of the methane formers,

such that the organic acid intermediates are formed faster than they can be converted to methane. Thus, it is probable that initial enrichments primarily involve the selection and increase in numbers of an acid forming population capable of degrading the aromatic substrate. Enzyme induction is also likely to be involved, but is probably not the rate-limiting process.

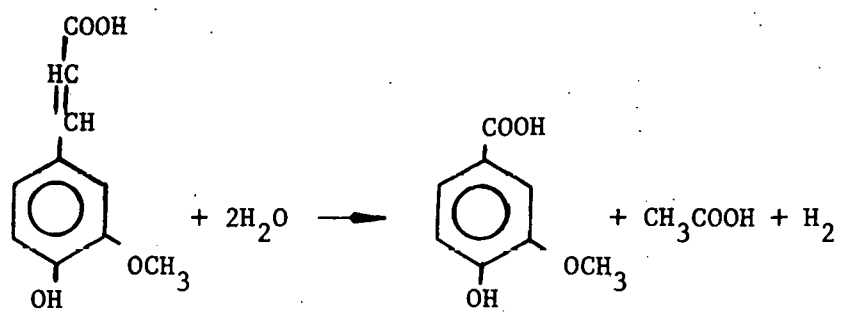
Cross acclimation studies (Figs. C-2 and C-3) strongly suggest that enzyme induction is involved in the ability of the acid forming population to acclimate to other aromatic compounds to which they have not previously been exposed. A culture which is simultaneously acclimated to another aromatic compound produces gas immediately indicating that both the acid formers and the methane formers are acclimated to the new substrate and intermediates. If population selection was involved, an acclimation lag would be expected on the order of days to allow for the growth of a sufficient number of newly selected organisms. Enzyme induction lags are characteristically on the order of hours. It is possible that some cultures, which did not produce gas when fed a new aromatic compound, contain an acid forming population which is simultaneously acclimated to the new compound and a methane forming population which is not acclimated to the new intermediates formed. This type of behavior can be elucidated if substrate concentration is monitored in addition to gas production in the cross acclimation studies.

Results from the cross acclimation studies (Tables C-5 and C-6) indicate that microbial populations acclimated to a particular aromatic compound can be simultaneously acclimated to additional selected aromatic compounds. In most cases these additional compounds are very similar in structure differing only in the oxidation state of one substituent group or in the presence of an extra substituent group at an additional ring position. In a few cases, the compounds to which the same culture is simultaneously acclimated are not similar in structure. They usually differ in the type of substituent group at an identical ring position. A very interesting example of the latter situation is the observation that a vanillic acid acclimated culture is simultaneously acclimated to catechol. This suggests that the 4 weeks required for a digester population to acclimate to catechol in an initial enrichment can be reduced to two weeks if the population is first acclimated to vanillic acid and then fed catechol. Evidence to support this was obtained by feeding 400 mg/l catechol to a 150-ml serum bottle culture acclimated to 400 mg/l vanillic acid. The culture started converting catechol to gas immediately and eventually converted 85% of the catechol carbon to gas in 17 days.

The fact that microbial populations are simultaneously acclimated to more than one aromatic compound suggests that these compounds are degraded to methane via a similar if not the same pathway. It is even possible that some of the compounds are intermediates in the degradation pathway of other compounds to which the same culture is simultaneously acclimated. For example, a ferulic acid acclimated culture is simultaneously acclimated to vanillic acid. It can be hypothesized that the 3-carbon side chain in the number one position on the ferulic acid ring is oxidized to a carboxyl group resulting in vanillic acid as an intermediate (Fig. C-7).

Williams and Evans (1974) proposed a pathway for the anaerobic metabolism of benzoate where the aromatic ring is first reduced to a cyclohexane ring before being cleaved. The final cleavage products proposed are dicarboxylic acids, either adipic or pimelic acid. Ferry and Wolfe (1976) obtained evidence which suggests that pimelic acid is an intermediate in the conversion of benzoate to methane. They also identified acetate, which could easily be obtained from a simple β oxidation of the dicarboxylic acid, as an intermediate in this conversion.

It can be hypothesized that ferulic acid and phenol are converted to methane via a pathway similar to benzoate. Microscopic observations of the morphological types of organisms present in ferulic acid and phenol acclimated cultures are very similar to the observations of a benzoate degrading culture by Ferry and Wolfe (1976). This suggests that a similar consortium of organisms may also be responsible for the methanogenic degradation of ferulic acid and phenol.



Ferulic acid

Vanillic acid + acetate

FIGURE C-7. Possible oxidation of ferulic acid to vanillic acid

Acetate has been detected in both phenol and ferulic acid acclimated cultures using gas chromatography. The levels of acetate observed in the ferulic acid culture were very similar to those observed by Ferry and Wolfe (1976) during the methanogenic degradation of benzoic acid and strongly suggest that acetate is also an important intermediate in the conversion of ferulic acid to methane.

Figures C-8 and C-9 show the proposed steps in the conversion of phenol and ferulic acid to methane with acetate as a key intermediate. The steps also include dicarboxylic acid intermediates. Vanillic acid is incorporated into the ferulic acid scheme based upon the reaction shown in Fig. C-7 and the fact that a ferulic acid culture is simultaneously acclimated to vanillic acid. The net sum of the proposed steps are identical with the mass balance stoichiometry shown in Figure C-5.

The results of mass balances (Table C-7) show that the amount of methane produced from both phenol and vanillic acid closely agrees with the mass balance stoichiometry. Additional mass balances are presently being carried out on ferulic acid acclimated cultures. The actual amount of gas produced indicates that the aromatic ring is cleaved and that phenol, vanillic acid, and ferulic acid are completely degradable to methane and carbon dioxide under reductive conditions.

1. $C_6H_6O + 3H_2O \longrightarrow C_6H_{10}O_4 + H_2$

   phenol — adipic acid

2. $C_6H_{10}O_4 + 2H_2O \longrightarrow 3C_2H_4O_2 + H_2$

   adipic acid — acetate

3. $3C_2H_4O_2 \longrightarrow 3CO_2 + 3CH_4$

   acetate

4. $0.5\ CO_2 + 2H_2 \longrightarrow 0.5\ CH_4 + H_2O$

---

net $C_6H_6O + 4H_2O \longrightarrow 2.5\ CO_2 + 3.5\ CH_4$

phenol

FIGURE C-8. Proposed steps in the conversion of phenol to methane with acetate as a key intermediate

1. $C_{10}H_{10}O_4 + 2H_2O \rightarrow CH_3COOH + C_8H_8O_4 + H_2$

ferulic acid — acetate — vanillic acid

2. $C_8H_8O_4 + 2H_2O \rightarrow C_7H_{12}O_4 + CO_2$

vanillic acid — pimelic acid

3. $C_7H_{12}O_4 + 4H_2O \rightarrow 3CH_3COOH + CO_2 + 4H_2$

pimelic acid — acetate

4. $4CH_3COOH \rightarrow 4CO_2 + 4CH_4$

acetate

5. $2CO_2 + 5H_2 \rightarrow 0.75\ CO_2 + 1.25\ CH_4 + 2.5\ H_2O$

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net $C_{10}H_{10}O_4 + 5.5\ H_2O \rightarrow 4.75\ CO_2 + 5.25\ CH_4$

FIGURE C-9. Proposed steps in the conversion of ferulic acid to methane with acetate as a key intermediate

These results suggest that more than half of the organic carbon of simple aromatic compounds released during the heat treatment of lignin can be potentially converted to methane gas.

7. Conclusions

a. Acetate appears to be an intermediate in the anaerobic conversion of ferulic acid to methane and carbon dioxide.

b. A consortium of organisms similar to that observed in benzoate metabolism also appears to be responsible for the methanogenic degradation of ferulic acid.

c. Mass balances indicate that the aromatic ring is cleaved and that phenol, vanillic acid, and ferulic acid are completely degradable to methane and carbon dioxide under reductive conditions.

d. The amount of methane produced from these substrates closely agrees with stoichiometric values, thus suggesting that more than half of the organic carbon of simple aromatic compounds released during the heat treatment of lignin can be potentially converted to methane gas.

e. Microbial populations acclimated to a particular aromatic substrate can be simultaneously acclimated to other selected aromatic substrates which usually have similar structural arrangements of side groups on the aromatic ring.

f. Stock cultures acclimated to particular aromatic substrates can be maintained for over 6 months in serum bottles with regular replacement of 1/5 of the culture volume. These cultures typically convert 70 to 85% of the substrate carbon to gas.

g. The rate-limiting step during initial enrichments most likely involves the selection and increase in an acid forming population capable of degrading aromatic substrate rather than the methane producers.

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IV. APPENDIX

A. Research Contributors

1. Principal Investigator

Perry L. McCarty, Professor of Environmental Engineering, Department of Civil Engineering, Stanford University.

Education: B.S. in Civil Engineering, Wayne State University, 1949.

S.M. in Sanitary Engineering, Massachusetts Institute of Technology, 1957.

Sc.D. in Sanitary Engineering, Massachusetts Institute of Technology, 1959.

Research Interests: Aerobic and anaerobic biological waste treatment processes; nature and biodegradability of refractory organic materials; microbial transformations in natural waters; nitrogen and phosphorus removal from wastewaters by biological processes; pesticide movement and biodegradation; transformations of mercury by aerobic and anaerobic microorganisms.

2. Co-Principal Investigator

Lily Y. Young, Assistant Professor of Environmental Biology, Department of Civil Engineering, Stanford University.

Education: B.S., Biology, Cornell University, 1965.

M.S., Microbiology, Cornell University, 1967.

Ph.D., Environmental Microbiology, Harvard University, 1972.

Research Interests: Microbiology of natural waters; interrelationships between microbial populations; biological indicators of water quality; characteristics of microbial behavior.

3. Graduate Research Assistants

Joseph B. Healy, Jr.

Education: A.B., Biochemistry, University of California at Berkeley, 1972.

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M.S., Water Resource Engineering, University of Michigan, 1973.

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Education: B.Ch.E. in Chemical Engineering, University of Melbourne, 1969.

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Present: Ph.D. candidate in Environmental Engineering, Stanford University.

B. Project Reports

This is the first annual report for this project.

Technical papers which have resulted to date from this research:

McCarty, P. L., L. Y. Young, J. M. Gossett, D. C. Stuckey, and J. B. Healy, Jr., "Heat Treatment for Increasing Methane Yields from Organic Material," Proceedings of Seminar on Microbial Energy Conversion, Göttingen, Germany (Oct. 5, 1976).

Healy, J. B., Jr., and L. Y. Young, "Methanogenic Decomposition of Simple Aromatic Compounds," Abstracts of the Annual Meeting, American Society for Microbiology, 1977. Q.14.