Metabolism of glucose and acids in stressed anaerobic digestors : an investigation by 13C-NMR spectroscopy
by Elizabeth Ann Runquist

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Chemistry
Montana State University
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Abstract:
The metabolism of glucose and the acids produced from glucose were studied in non-stressed and stressed anaerobic digesters. The utilization of glucose and the acids, acetate and propionate, were observed by 13C-nmr spectroscopy. Rate constants were obtained for both stressed and non-stressed fermentations. The rate constants obtained for propionate utilization suggested that propionate oxidation was a limiting factor in the anaerobic fermentation of glucose.

The observed carbon flow from 13C-l-glucose to the acids indicated that a major portion of the glucose was initially converted to lactate which was subsequently metabolized to either propionate or acetate. These studies confirmed an earlier suggestion that under high energy loads a major portion of the glucose was converted to reduced acids e.g, propionate. Under these conditions, these investigations indicated that approximately 70% of the glucose fed was converted to propionate. Thus, the methanogenic bacteria obtained a limited suppply of acetate rather than a saturating level. Possible metabolic intermediates of propionate catabolism indicated that both the acrylate and randomizing pathways had been utilized.

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Date 4 August 1981.

METABOLISM OF GLUCOSE AND ACIDS IN STRESSED ANAEROBIC DIGESTORS: AN INVESTIGATION BY ^{13}C-NMR SPECTROSCOPY

by

ELIZABETH ANN RUNQUIST

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ABSTRACT

The metabolism of glucose and the acids produced from glucose were studied in non-stressed and stressed anaerobic digestors. The utilization of glucose and the acids, acetate and propionate, were observed by ^{13}C-nmr spectroscopy. Rate constants were obtained for both stressed and non-stressed fermentations. The rate constants obtained for propionate utilization suggested that propionate oxidation was a limiting factor in the anaerobic fermentation of glucose.

The observed carbon flow from ^{13}C-1-glucose to the acids indicated that a major portion of the glucose was initially converted to lactate which was subsequently metabolized to either propionate or acetate. These studies confirmed an eariler suggestion that under high energy loads a major portion of the glucose was converted to reduced acids e.g, propionate. Under these conditions, these investigations indicated that approximately 70% of the glucose fed was converted to propionate. Thus, the methanogenic bacteria obtained a limited suppply of acetate rather than a saturating level. Possible metabolic intermediates of propionate catabolism indicated that both the acrylate and randomizing pathways had been utilized.

INTRODUCTION

Anaerobic Digestion - the Ecosystem

Today, with much emphasis being placed on the diminution of our energy resources, greater attention is being directed towards the exploration of alternative sources for energy. Anaerobic treatment of waste for the production of methane gas is one such alternative. McCarthy (45) has estimated that twenty percent of the natural gas consumed in the United States could be provided from the anaerobic treatment of municipal sludges and refuse, animal wastes and crop residues.

Since the nineteeth century the process of anaerobic digestion has been employed for the production of energy (40). To date, however, its widespread application has been limited by a lack of resolution of the optimum conditions for the production of methane. Kroeker (40) has suggested that the key to the feasibility of this alternative energy source lies in the understanding of the biochemical aspects of the process and their relationship to the stability of the system.

The production of methane via anaerobic digestion is accomplished through an ecosystem comprised of about twenty-five species of non-spore forming bacteria (67). These bacteria have been classified into three major groups (some authors have divided the microorganisms into only two groups (20,30,40) whereas some authors have referred to four groups present in the process (17)). Bryant (13) has classified the

three major groups as the fermentative, acetogenic and methanogenic bacteria. The production of methane results from the interactions of these three groups of anaerobic bacteria.

The Fermentative Group

The first step in the anaerobic process involves a group of faculative anaerobic bacteria known as the acid formers or the fermentative group. During this stage of the process no methane is produced, however, the importance of this step lies in the process by which complex organic material is converted into usable substrates for the methanogens.

The functional role of the fermentative group in the overall anaerobic digestion process is the conversion of the polymeric substances into simple organic acids, alcohols, H_2O and CO_2 which can be utilized by the methanogens. Substrates which are hydrolyzed by this group include polysaccharides, lipids and proteins. The polymers can be converted into such compounds as butyrate, acetate, propionate, ethanol, H_2 and CO_2. A representative metabolic pathway is summarized in Figure 1. The polysaccharide is hydrolyzed to glucose which is then metabolized to pyruvate by the glycolytic pathway. The fate of pyruvate is determined by metabolic interactions of the entire anaerobic ecosystem. This will be elaborated upon in the section entitled Function of H_2: Ecosystem Interactions. Pyruvate may be converted, by the fermentative group, to acetate, CO_2, H_2, ethanol,

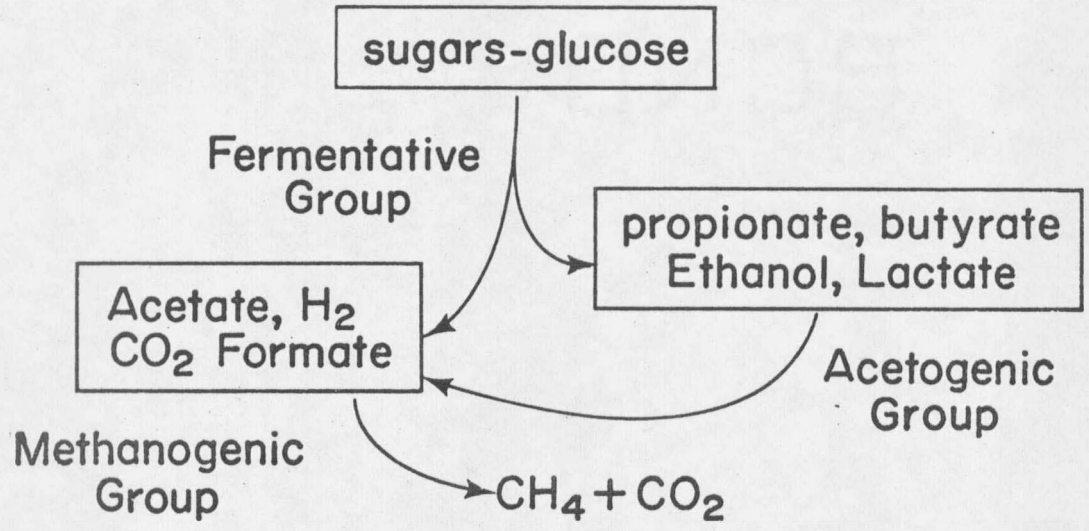


Figure 1. The three stages involved in the production of methane (13).

butyrate or propionate. The general metabolic scheme of the fermentative bacteria is shown in Figure 2.

The H_2-Producing-Acetogenic Bacteria

Some products of the fermentative process are not substates of the methanogenic group. There is a secondary process which must occur that utilizes the products, other than acetate, CO_2 or H_2. This process involves the oxidation of propionate and butyrate to acetate which can be utilized by the methanogens. The group of microorganisms responsible for this step are the acetogenic bacteria.

The thermodynamics of these acetogenic processes are unfavorable. In Table 1 the reactions that have been proposed (13) for the acetogens are listed. The ability for oxidation of propionate and butyrate has been rationalized by a syntrophic association between the acetogenic and methanogenic populations. Bryant (13), has explained this association by the maintance of a low H_2 concentration, thus permitting the oxidation of the substrates, e.g. propionate to produce acetate and H_2 (see Table 1). Studies by Bryant have demonstrated that syntrophic associations between ethanol catabolizing (10,12) and butyrate catabolizing (13) bacteria with methanogens exist. It has also been shown (46) that similar associations exist between lactate catabolizing bacteria and the methanogens. There has been no direct evidence (13) supporting any associations between the propionate catabolizing acetogens and methanogens. It has been shown by Smith (59) that

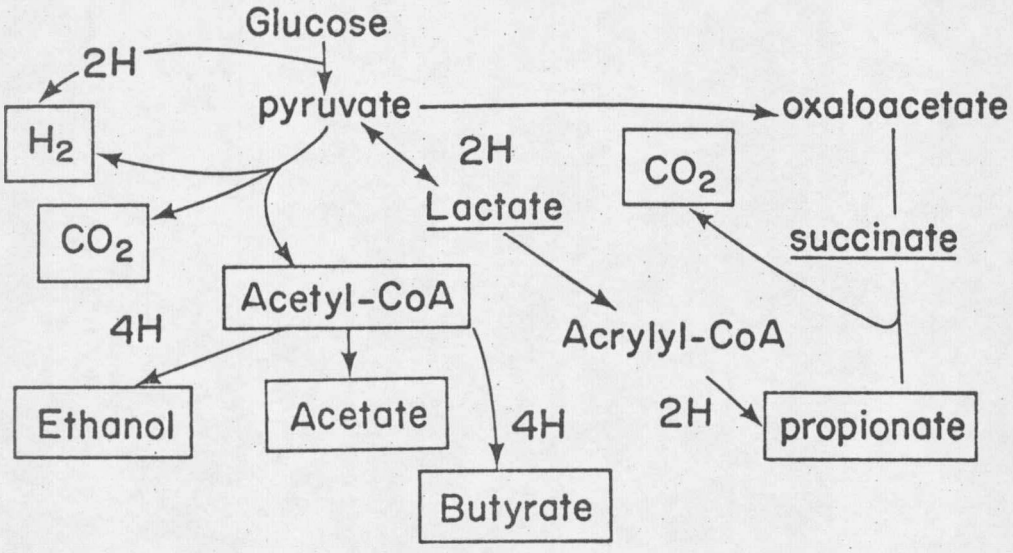


Figure 2. Pathways utilized by the fermentative bacteria (13).

Table 1. Proposed Reactions for the Acetogenic Bacteria

1) Propionate-catabolizing acetogenic bacterium (13).

$$CH_3CH_2COO^- + 3\ H_2O \rightleftharpoons CH_3COO^- + HCO_3^- + H^+ + 3\ H_2$$

ΔG° = +76.15 KJ/reaction

2) Butyrate-catabolizing acetogenic bacterium (13).

$$CH_3CH_2CH_2COO^- + 2\ H_2O \rightleftharpoons 2\ CH_3COO^- + H^+ + 2\ H_2$$

ΔG° = +48.12 KJ/reaction

3) Lactate-catabolizing acetogenic bacterium (61).

$$CH_3CH_2OCOO^- + 2\ H_2O \rightleftharpoons CH_3COO^- + HCO_3^- + H^+ + 2\ H_2$$

ΔG° = -4.2 KJ/reaction

propionate accumulates when H_2 is added. Research by DeMeyer and Van Nevel (25) demonstrated that when methanogenic activity is inhibited, propionate accumulates. These studies indirectly support an association between the propionate catabolizing acetogens and methanogens (13). However, addition of H_2 to digestors or inhibition of H_2 utilization in digestors may stimulate the production of propionate. It is difficult to assess whether one or the other process was primiarily responsible for propionate accumulation.

The Methanogenic Group

The third and final step in anaerobic digestion is the production of methane. The methanogenic bacteria are responsible for this stage of the process. The methanogens are obligate anaerobes which use H_2 as the electron donor to reduce CO_2 to methane (60). Acetate, formate, methanol, methylamines or methylmercaptan can also be reduced to methane (5). The methanogens are one of the few genera which can grow on H_2 as the only electron donor. The absence of cytochromes, flavins and quinones has presented problems in the understanding of the mechanims by which ATP synthesis occurs. The Methanobacterium thermoautrophicum has been shown to grow in the absence of any organic compounds, indicating that ATP synthesis could not occur by substrate level phosphorylation (61). It has been suggested that the formation of ATP occurs by electron-transport phosphorylation (7,61).

The coenzymes, F_{420}, M and Factor B, also make the methanogenic group unique. These coenzymes are involved in the reduction of CO_2 to CH_4. Coenzyme F_{420} is involved in electron transfer. It is a low molecular weight (630), fluorescent electron carrier which is protein bound in the reduced form, but is protein-free and non-fluorescent in the oxidized form. Coenzyme M (2-mercaptoethane-sulfonic acid-$HS-CH_2-CH_2-SO_3^-$) is a growth factor in all methanogens (61). Coenzyme M is a methyl carrier group involved in the reduction of methyl groups to CH_4. The third coenzyme, Factor B, is involved in the production of methane from coenzyme M (13).

Methanogens differ from other bacteria in that they contain 16 S ribosomal RNA and lack the peptidoglycan that is usually found in the cell walls of procaryotes (5). This does not allow the methanogens to be strictly classified as either eucaryotic or procaryotic organisms (5,7).

In Table 2, the energy sources utilized by the methanogens are summarized. In the anaerobic treatment process sixty-five to seventy percent of the methane is derived from acetate (35). However, even with large amounts of acetate present in the system the methanogens multiply slowly on this substrate because of the small amount of free energy available from the reaction (see Table 2) (13). The remaining fraction of methane produced is generated from the oxidation of hydrogen.

Table 2. Energy Sources for the Methanogenic Bacteria (13)

1) $4\ H_2 + HCO_3^- + H^+ \rightleftharpoons CH_4 + 3\ H_2O$

$\Delta C^\circ = -135.56$ KJ/rection

2) $2\ HCOO^- + H_2O + H^+ \rightleftharpoons CH_4 + HCO_3^-$

$\Delta G^\circ = -30.96$ KJ/reaction

3) $CH_3COO^- + H_2O \rightleftharpoons CH_4 + HCO_3^-$

$\Delta G^\circ = -30.96$ KJ/reacation

4) $4\ CH_3OH \rightleftharpoons 3\ CH_4 + HCO_3^- + H^+ + H_2O$

$\Delta G^\circ = -314.64$ KJ/reaction

Function of H_2-Ecosystem Interactions

The interactions of the three groups of microorganisms described above result in the final products, CO_2 and CH_4. As shown in Table 2, the energy sources required by the methanogens are primarily acetate, H_2 and CO_2. The metabolic processes carried out by fermentative and acetogenic groups must result in the needed substrates of the methanogens in order to obtain the desired product, methane.

The key to the control of the quantity and the particular types of products resulting from the metabolic processes of the fermentative and acetogenic groups has been suggested by many investigators (11,61,67) to be dependent on the concentration of H_2 present in the system. The concentration of H_2 is of importance because the utilization of H_2 directly affects the activity of the first two stages of anaerobic fermentation (67). The reaction which associates the H_2 concentration with the formation of the various products is shown below.

Reaction (1) - NADH-linked formation of H_2

$$NADH + H^+ \rightleftharpoons H_2 + NAD^+ \qquad \Delta G^\circ = +17.99 \text{ KJ/mol}$$

The mechanism describing the transfer of electrons is shown in Figure 3. The mechanism shown has been proposed (54) for the S organism (which ferments ethanol to acetate and H_2) and involves NADH-linked formate dehydrogenase, NADH-ferredoxin oxidoreductase and ferredoxin-dependent hydrogenase.

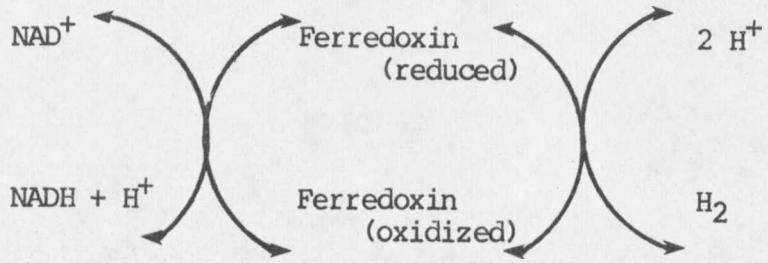


Figure 3. Scheme for electron transfer from NADH to H_2.

In the anaerobic process the removal of H_2 is associated with the formation of methane by the methanogenic group (see Table 2). As the concentration of H_2 decreases the H_2 forming reaction becomes thermodynamically more favorable. Many studies have demonstrated (16,19,34,57,58,67) that in co-cultures of fermentative bacteria with methanogenic bacteria, the products resulting from the fermentative process proceeded towards the production of acetate, CO_2 and H_2; as opposed to the production of the more reduced acids, viz. lactate, propionate, succinate and butyrate. An increase in the H_2 concentration within the system results in the accumulation of NADH, which results in the production of the reduced acids (13). Kaspar and Wuhrmann (36) have demonstrated that the presence of high concentrations of hydrogen inhibits the oxidation of ethanol and propionate to acetate. Thus, the addition of hydrogen into the system results in the accumulation of the more reduced acids. These results further support the importance of Reaction 1; and suggest that the presence or absence of H_2 in the system acts as a regulator by product

inhibition. When H_2 is present, in quantities greater than 0.005 atm, the acetogens are unable to oxidize the propionate to acetate, and when the concentration of H_2 is less than 0.005 atm the propionate is oxidized to acetate and methane production occurs.

The interactions of the fermentative, acetogenic and methanogenic groups based on the hydrogen production are summarized in Figure 4. The fermentative bacteria produce H_2 via Reaction 1, from NADH derived from Stage II of glycolysis, i.e., glyceraldehyde 3-phosphate proceeding to 1,3 bisphosphoglycerate. Hydrogen is also produced from the NADH independent pyruvate: ferredoxin oxidoreductase involved in pyruvate conversion to acetyl-CoA. The methanogens utilize this H_2 and deplete the H_2 concentration in the system. The low concentration of H_2 allows Reaction 1 to favor the product NAD^+. The availability of NAD^+ results in the formation of oxidized products e.g., acetate. If the H_2 concentration builds up in the system, the availability of NAD^+ decreases, and the NADH which results may cause the formation of the reduced products, e.g. butyrate, ethanol or propionate, of the fermentative process. Thus, high concentrations of H_2 can both stimulate reduced acid production and inhibit reduced acid utilization.

Production and Utilization of Acetate

The generation of acetate by the fermentatiave bacteria is of major importance in anaerobic digestion for the production of methane.

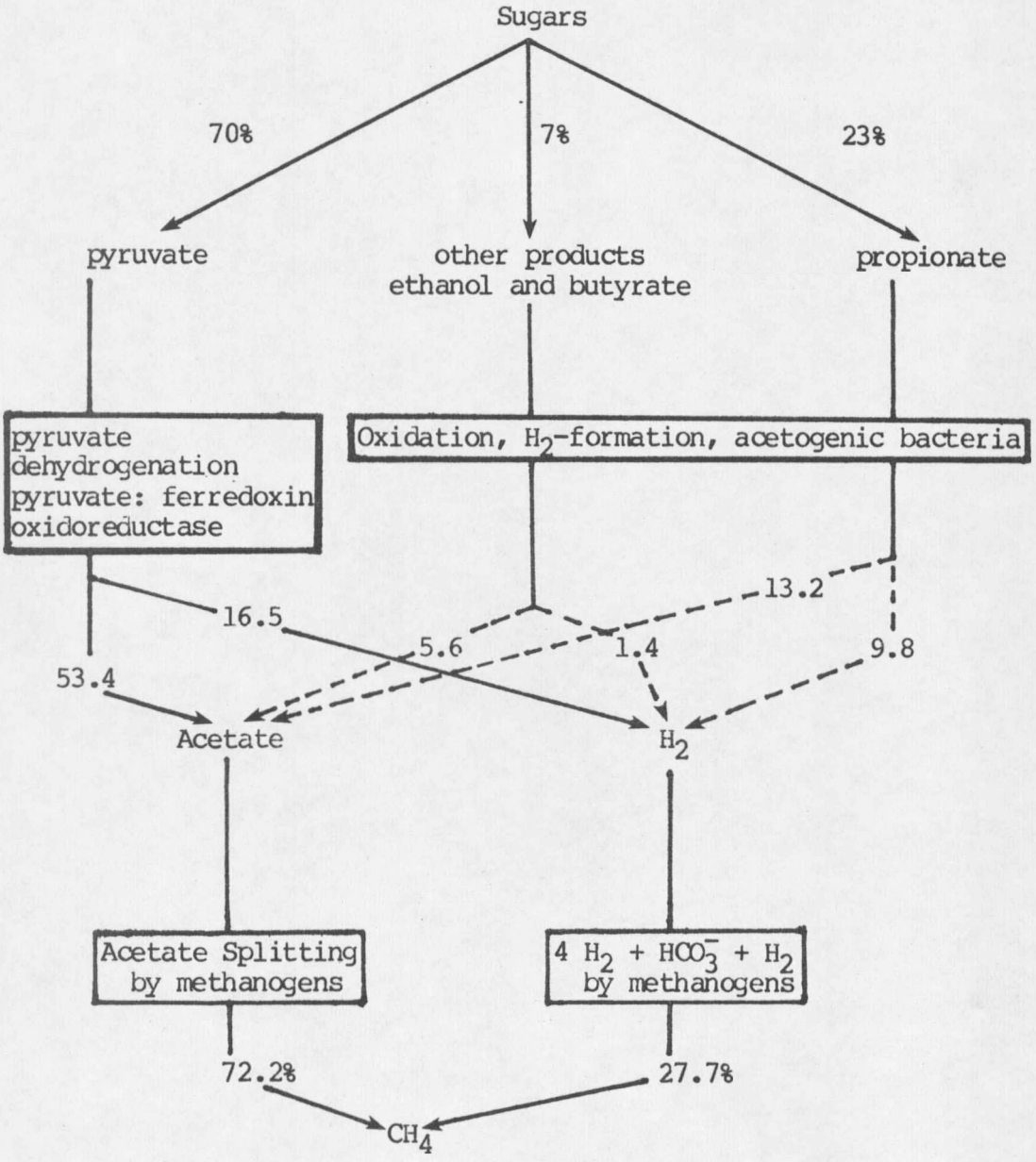


Figure 4. Substrate flow in a steady state anaerobic digestion with H_2 production included (obtained from reference 36).

Acetate provides approximately 70% of the methane produced by the anaerobic digestion process (35). The metabolic pathways which are known to result in acetate, the microorganisms responsible for the generation of acetate, and the pathways by which the acetate is utilized will be discussed in this section.

There are six reactions which could possibly result in the product acetate. These reactions are summarized in Table 3. Evidence (13,46) has shown that the reactants, pyruvate (acetyl CoA), lactate, propionate, ethanol and butyrate could or do exist in the anaerobic digestion process. The two other reactions have been included because the starting materials, succinate and CO_2, are end products for groups of microorganisms which have been found in the anaerobic digestors.

Acetate is considered to be principally derived from pyruvate (13,37). Most all of the fermentative bacteria are capable of producing acetate via this route. The acetyl CoA produced from pyruvate has been shown to involve the enzymes pyruvate: ferredoxin oxidoreductase or pyruvate formate lyase, which are found in anaerobic organisms (61). The pyruvate: ferredoxin oxidoreductase involves the electron acceptor, ferredoxin, which can be utilized for the production of H_2. The acetate and the synthesis of ATP are derived from the reactions shown below (61).

Reaction (2): $\text{acetyl-CoA} + P_i \rightleftharpoons \text{acetyl-P} + \text{CoA}$

$\Delta G^\circ = +9.0$ KJ/mol

enzyme-phosphotransacetylase

Table 3. Possible Reactions Which Can Produce Acetate.

1) $CH_3CHOHCOO^- + 2H_2O \rightleftharpoons CH_3COO^- + HCO_3^- + H^+ + 2\ H_2$ (61).

$\Delta G° = -4.2$ KJ/reaction

2) $CH_3CH_2COO^- + 3\ H_2O \rightleftharpoons CH_3COO^- + HCO_3^- + H^+ + 3\ H_2$ (13).

$\Delta G° = +76.15$ KJ/reaction

3) $CH_3CH_2CH_2COO^- + 2\ H_2O \rightleftharpoons 2\ CH_3COO^- + H^+ + 2\ H_2$ (13).

$\Delta G° = +48.12$ KJ/reaction

4) $CH_3COCOO^- + 2\ H_2O \longrightarrow CH_3COO^- + HCO_3^- + H^+ + H_2$ (61).

$\Delta G° = -47.3$ KJ/reaction

5) $^-OOCCH_2CH_2COO^- + 4\ H_2O \rightleftharpoons CH_3COO^- + 2\ HCO_3^- + H^+ + 3\ H_2$

$\Delta G° = -55.96$ KJ/reaction

(calculated from free energy values obtained from reference 61).

6) $2\ CO_2 + 4\ H_2 \longrightarrow CH_3COO^- + H^+ + 2\ H_2O$ (61).

$\Delta G° = -95$ KJ/mole

Reaction (3): acetyl-P + ADP $\rightleftharpoons$ acetate + ATP

$\Delta G^{\circ} = -13.0$ KJ/mol

enzyme-acetate kinase

The production of acetate from lactate has recently been demonstrated by McInerney and Bryant (46). They have shown that lactate is completely degraded to methane and CO_2, anaerobically, by syntrophic associations of Desulfovibrio vulgaris and Methanosarcina barkeri. The mechansim for the production of acetate from lactate was not demonstrated.

The acetogenic bacteria are responsible for the conversion of propionate, butyrate and ethanol to acetate. However, the species which are involved are largely unknown (13). A syntropic association between an acetogenic species which is believed to degrade butyrate to acetate, and Methanospirillum hungatii has been shown (12). Ethanol was shown to be degraded by syntropic associations of Desulfovibrio species and methanogens (12). The S organism has been shown by Bryant (10) to oxidize ethanol to acetate and H_2. There has been no direct evidence indicating that propionate is fermented to acetate which could be, degraded to methane, by syntrophic associations with methanogens (13).

Succinate, has been included in this section as a possible source of acetate, although no evidence has been found showing any syntrophic associations between succinate utilizing organsims and methanogens.

The succinate, which is an intermediate in the production of propionate, has been shown to be the end-product of the fermentative species - Succinvibrio dextrisolvens, Bacteroides amylophilus, Bacteroides ruminicola, Ruminococcus flavefaciens, Succinimonas amylolytica (67). The succinate produced by these species is an extracellular product which is utilized by the propionate-producing species (see section Propionate: Production and Utilization).

Some species of Clostridia are known to reduce CO_2 to acetate (61) by the reaction shown in Table 3. Mackie and Bryant (47) have recently reported that 1.4 to 1.7% of the acetate produced in mesophilic fermentors appeared to be resulting from the reduction of CO_2.

The acetate produced by the reactions listed in Table 3, provide the source for 70% of the methane produced. The reaction shown in Reaction (4) is for the degradation of acetate into CH_4. This reaction has been referred to as "acetate splitting".

Reaction (4) - "Acetate Splitting"

$$CH_3COO^- + H_2O \rightleftharpoons CH_4 + HCO_3^- \qquad \Delta G^\circ = -30.96 \text{ KJ/reaction}$$

The presence of H_2 plays a role in the acetate production and utilization. It has been demonstrated (10) that the S organism, which oxidizes ethanol to acetate and H_2 can be inhibited by H_2. Baresi et al. (6) have suggested that the effects of H_2 may have a more complex role than just the inhibition of acetate formation. They have

suggested that in systems where acetate and H_2 utilizing methanogens are present there exists "complex regulatory reactions involving as yet unknown soluble factors..." The same year, Kaspar and Wuhrmann (37) investigated the kinetics of propionate oxidation, acetate splitting and the formation of methane from H_2 and CO_2. They suggested that the conversion of acetate to methane was the rate limiting step. However, the estimated maximal rates were 0.23 $mmole \cdot l^{-1} \cdot h^{-1}$, 0.63 $mmole \cdot l^{-1} \cdot h^{-1}$ and 103 $mmole \cdot l^{-1} \cdot h^{-1}$ for propionate, acetate and H_2 utilization, respectively. Kaspar and Wuhrmann (36) earlier reported that product inhibition from high concentrations of H_2 or acetate halts either ethanol or propionate oxidation. Hansson (33) and Khan et al. (38) have recently demonstrated that HCO_3^- or CO_2 have inhibitory effects on the conversion of acetate to methane.

Propionate: Production and Utilization

The metabolite, propionate, which results from the fermentative process, is of major concern to the production of methane. Propionate appears to have an adverse effect on the formation of methane. Andrews and Pearson (3) have demonstrated that propionate accumulated in stressed fermentors. Varel et al. (64) reported that the accumulation of other volatile acids occurred after propionate had accumulated in stressed fermentors. It has been shown by Macy et al. (48) and Wallnofer and Baldwin (65) that propionate formation provides

an alternative to the formation of H_2 as an electron sink. The production of propionate regenerates NAD^+ from NADH.

There are two pathways involved in the production of propionate. These pathways, known as the randomizing pathway and the acrylate pathway, are shown in Figures 5 and 6. Scheifinger and Wolin (57) have shown that when cellulose is fermented the primary pathway of propionate formation is via the randomizing pathway. The acrylate pathway is used to form propionate when starch is fermented, however, the randomizing pathway is also employed during starch fermentation. It has been suggested by Bryant (13) that lactate and succinate, intermediates in the acrylate and randomizing pathways, respectively, may be of importance, as these intermediates may serve as substrates in the formation of propionate. Lactate, has been shown to be fermented by Peptostreptococcus elsdenii (8,9), and by Selenomonas ruminantium (52), to propionate. The end-product of succinate has been shown to be produced by the Succinimonas and Bacteroides species (see page 17).

The randomizing pathway, which utilizes the symmetrical intermediates, succinate and fumarate, has been shown to result in C-3 and C-2 labelled propionate arising from the fermentation of C-1 and C-3 glucose and lactate, respectively (48). The microorganisms which have been demonstrated to utilize this route of propionate production are Bacteroides fragilis (48), Selenomonas ruminatium in a syntrophic association with Bacteroides succinogenes (57).

1) phosphoenolpyruvate + ADP + CO_2 $\xrightarrow{\text{PEP carboxykinase}}$ oxaloacetate + ATP

2) oxaloacetate + NADH + H^+ $\xrightarrow{\text{malate dehydrogenase}}$ malate + NAD^+

3) malate $\xrightarrow{\text{fumarate hydratase}}$ fumarate + H_2O

4) fumarate + NADH + H^+ $\xrightarrow{\text{fumarate reductase}}$ succinate + NAD^+

5) succinate + propionyl-CoA $\xrightarrow[\text{transferase}]{\text{acyl-CoA}}$ succinyl-CoA + propionate

6) succinyl-CoA $\xrightarrow{\text{methylmalonyl-CoA mutase}}$ R-methylmalonyl-CoA

7) R-methylmalonyl-CoA $\xrightarrow[\text{racemase}]{\text{methylmalonyl-CoA}}$ S-methylmalonyl-CoA

8) S-methylmalonyl-CoA $\xrightarrow[\text{decarboxylase}]{\text{methylmalonyl-CoA}}$ propionyl-CoA + CO_2

Figure 5. Propionate production via the randomizing pathway (from reference 48).

1) pyruvate + NADH + H^+ $\xrightarrow{\text{lactate dehydrogenase}}$ lactate + NAD^+

2) lactate + acetyl-CoA $\xrightarrow[\text{transferase}]{\text{Coenzyme-A}}$ lactyl-CoA + acetate

3) lactyl-CoA $\xrightarrow[\text{dehydrase}]{\text{lactyl-CoA}}$ acrylyl-CoA + H_2O

4) acrylyl-CoA + NADH + H^+ $\xrightarrow[\text{dehydrogenase}]{\text{Butyryl-CoA}}$ propionyl-CoA + NAD^+

5) propionyl-CoA + acetate $\xrightarrow[\text{transferase}]{\text{Coenzyme A}}$ propionate + acetyl-CoA

Figure 6. Propionate Production via the acrylate pathway (from references 9,65).

The acrylate pathway, which contains no symmetrical intermediates, utilizes the electron acceptor acrylyl coenzyme A. The microorganisms which have been demonstrated to utilize this pathway to produce propionate are Peptostreptococcus elsdenii (9), Bacteroides ruminicola (65), and Clostridium propionicum (see reference in (65)). The propionate, which results from the fermentative processes of anaerobic digestion, is thought to be oxidized to acetate via the reaction shown below, by propionate catabolizing acetogenic bacterium (13).

Reaction (5) - the oxidation of propionate to acetate:

$$CH_3CH_2COO^- + 3\ H_2O \rightleftharpoons CH_3COO^- + HCO_3^- + H^+ + 3\ H_2$$

$$\Delta G^\circ = +76.15\ KJ/reaction.$$

The thermodynamics for this reaction are unfavorable, however, Lawrence and McCarthy (41) have demonstrated that when proper environmental conditions are maintained in the digestor, propionate is oxidized at a greater rate than acetate. Kaspar and Wuhrmann (36) concluded, "... that the limiting factors for complete anaerobic mineralization of biodegradable organic matter in digesting sludge are to be sought in the boundary conditions for the exergonic oxidation of propionate."

Operational Factors Influencing the Ecosystem

There are other aspects of the anaerobic digestion process which are not directly involved with the biochemistry in the process. These factors are associated with the mechanics of process operation. The

importance of understanding these operational factors is to prevent any operational problems to interfere with the biochemical aspects which are under investigation.

The stability in the anaerobic process is a function of the balance which exists between the three groups of microorganisms. McCarthy (44), described this balance in terms of the methanogenic population in its ability to remove the acid products produced by the fermentative and acetogenic groups as rapidly as they are formed. Instability in the system is evidenced by the accumulation of volatile acids, e.g. butyrate, propionate (40). This volatile acid accumulation indicates that the methanogenic or the acetogenic groups are not in equilibrium with the other groups in this system.

The operational factors which are associated with the disruption of this ecosystem are 1) loading rates, 2) temperature, 3) pH, 4) acclimatization of new substrates, and 5) the presence of toxic materials. These factors are not separate entities from one another, since they interact with each other. However, the optimization of each factor must be addressed in order to stabilize the process.

The loading rate is a function of the retention time and the concentration of waste. Retention time (RT) is the average turnover rate of the material in the system. Converse (24) reported that a minimum of a fifteen day RT with a percent of solids which did not

exceed 6-8% provided optimum gas production. Varel et al. (64) demonstrated that a decrease in methane production is associated with shorter retention times (such as 3 d). The retention times which are utilized must be chosen based on the temperature and the percentage of total solids of raw waste used in the system (24,55). Thus, the optimum RT must be chosen with regard to these factors. Converse (24) reported that in mesophilic conditions the percent methane was greater but the amount of gas produced was less, than in thermophilic conditions, with higher than normal loads. Varel et al. (64) found that the highest methane production occurred with a RT of 3 d at 8.2% solids. Robbins et al. (55) reported that optimum methane yields were obtained from fermentors receiving 5% solids and 3% added carbohydrate on a 16 d RT at 37°C. It has been shown (41) that changes in the RT can affect the acetogenic and methanogenic populations. A very short RT (3 d or less) presents a disadvantage to the population growth of these two groups. The percentage of solids added, however, does not appear to affect these two groups specifically but has an effect on the kinetics of methane production reflected by the entire system.

Rapid temperature fluctuations in the anaerobic system have been associated with instability and failure in the process (40) since the microbial population growth is dependent on temperature (64). It has been shown (49) that there are three temperature zones possible in the anaerobic digestion process: 1) the thermophilic (above 42°C), 2) the

mesophilic region (28-42°C), and 3) the temperate region (below 28°C). The common temperature which has been utilized has been in the mesophilic region. Operations occurring at lower temperatures have been shown to require very long (greater than 20 d) RT's. Chen et al. (18) have demonstrated that temperature changes occurring without proper adjustments in the RT, causes instability to occur. These temperature changes tend to be more critical in systems operating with short RT's. Buhr and Andrews (14) have presented results indicating that small deviations in temperatures of digestors operating in the thermophilic range results in instability.

The effect of pH on the stability of the system is related to the increase in volatile acid production (4). The pH is a function of the bicarbonate and CO_2 concentrations as well as the volatile acids concentrations and $[NH_4^+]$ present in the system. The methanogenic population is affected by slight fluctuations in the pH (34), and an acidic pH results in an unfavorable environment for the methanogens. The optimum pH for methane production is at neutrality (49), however, production will occur in a pH range of 6.0-8.0. Deviations from this range, causes a decrease in methanogenic activity and process instabiltiy could result. The introduction of different substrates into the anaerobic digestion system results in the growth of different microbial populations (43). Such a result could cause an upset in the balance of the ecosystem. Kotze (39) indicated that adaption of

fermentors to new substrates requires an acclimation period. The period which was estimated for the adjustment of enzymatic activity was five weeks.

Toxic materials present in the system have been known to result in the instability of the anaerobic digestion process (40). These materials include high concentrations of volatile acids, e.g. butyrate and propionate, ammonia, alkaline earth metals, heavy metals and sulfides (40). The biochemical impact of these materials, however, is not well understood. The theories which have developed in regards to the effects caused by these toxic materials have been reviewed by Kroeker (40). The effects of the volatile acid propionate have been previously described.

The following operational factors which appear to provide optimum conditions for methane production are: a loading rate in the range of 16-25 d with 5-8% total solids, a temperature in the range of 28-42°C and a pH in the 6.0-8.0 region.

STATEMENT OF THE OBJECTIVES

Heuristic Data

The work of Ghosh and Klass (31) indicated that cellulose hydrolysis was the rate limiting step in the overall process of nonmethanogenic digestion. A logical extention of this conclusion might be to pretreat waste materials with the hydrolysis of cellulose to soluble sugars. Preliminary work in this laboratory and the studies of Cohen et al. (20) indicated that problems could result from such pretreatment. In our laboratory, digestors which were presented with feed stock containing high concentrations of soluble sugar, i.e. glucose, converted a large portion of the sugar to reduced acids, principally propionate. In the studies of Cohen et al., a large amount of the glucose was converted to lactate, butyrate, ethanol and propionate. These compounds, however, are not substrates for the methanogenic group. The studies indicated that a large portion of the microbial work load under these conditions was shifted to the acetogenic group. Thus, the production of methane percursors was due principally to the acetogenic rather than the fermentative bacteria.

The consequences of increased production of reduced acids under these conditions emphasizes the importance of the relative rates of acetate production from the reduced acids versus acetate utilization. If the production of the acetate exceeded its utilization, then the methanogenic metabolic activity would be the rate limiting factor.

Conversely, if acetate utilization were greater than its production, then the acetogenic metabolic activity would be the rate limiting factor. The importance of resolving this question, is that it would explicitly define the problem which confronts digestion efficiency and thus appropriate measures could be taken to improve digestion efficiency. The evidence which is known is that the principle reduced acid, propionate, accumulates in stressed fermentors. However, the research which has been conducted on the rates of propionate and acetate utilization are not in agreement (36,37,41). Thus, the primary objective in this investigation was to evaluate rates of propionate and acetate utilization in stressed or high performance digestors.

The reasons stressed fermentors were chosen for study were: 1) The highest methane yield could be determined under these conditions and 2) the metabolic stress points would be most evident under this mode of operation. Previous experiments were concerned with 1) the energy levels(glucose) which caused instability to occur in fermentation, and 2) whether the failure of the stressed digestors (high glucose concentrations) was due to either an excess or a lack of acetate. In the first series of experiments, digestors were operated with manure feeds containing 1-6% additional solids as glucose. Digestors receiving 1-3% additional glucose were stable and those with the 4-6% glucose were unstable and digestor failure occurred within 8-16 d. In the second series, digestors receiving 6% additional glucose

were compared with those receiving 6% glucose plus 1% acetate as additional solids. Those digestors receiving the added 1% acetate were stable indicating that an excess of acetate was not a source of failure. The digestors receiving the 6% additional glucose, again, failed within 8 d. The results obtained from the 6% glucose and 6% glucose plus 1% acetate digestors indicated that an investigation comparing utilization rates and metabolic activities in the first 5 to 8 d of fermentation would be warranted since this was shown to be the period of destabilization. An investigation of this type could provide insight to the destabilization of the process and thus more clearly define the problems confronting high performance digestors.

The Avenues of Investigation

To investigate the production and utilization of acetate and propionate and glucose utilization in anaerobic digestion, ^{13}C nuclear magnetic resonance (nmr) was employed. ^{13}C-nmr was the tool chosen for this study because of the quality and accuracy of the data which is obtained through a rapid and non-invasive technique.

Proton nuclear magnetic resonance has been applied to metabolic studies (50,51), however, ^{13}C-nmr provides information on the fate of ^{13}C labeled carbon substrates, as only metabolites containing the ^{13}C-label are observed. The method most commonly used in studies of metabolic pathways has been through the use of radioactive tracers. A

comparison of ^{14}C tracer techniques and ^{13}C-nmr, was recently investigated by Cohen et al. (23). This study demonstrated that ^{13}C-nmr has a greater accuracy over the ^{14}C tracer technique. ^{13}C spectroscopy has been shown (15,21,22,23,26,27,28,63) to be a very informative technique for observing metabolic pathways. These studies have demonstrated that this technique provides a rapid and non-invasive method to study metabolism. ^{13}C-nmr has been used in metabolic studies of intact cells (15,21,22,23,26,63), amoeba (27) and cockroaches (28). This technique has also been applied to kinetic studies of glucose utilization in yeast cells (26). In a recent study, it has been demonstrated by Runquist et al. (56) that ^{13}C-nmr has useful applications to the anaerobic digestion process.

The potential application of ^{13}C-nmr to the anaerobic digestion process and the overall intent of this study to investigate the rates of acid utilization in this system has induced the following objectives:

1) to demonstrate that ^{13}C-nmr spectroscopy can be utilized as a method of investigation of the metabolism of glucose in the anaerobic digestion process in the production of methane.

2) To investigate the relative rates of glucose utilization in fermentors receiving high concentrations of glucose and in fermentors receiving acetate plus high concentrations of glucose by ^{13}C-nmr.

3) To investigate the major metabolic pathway or pathways which are utilized in the production of propionate and acetate from glucose in the anaerobic digestion process and the possible effect H_2 may have on the production of propionate and acetate.

4) To investigate the relative rates of acetate utilization in fermentors receiving high concentrations of glucose and in fermentors receiving high concentrations of glucose and acetate.

5) To investigate the relative rates of propionate utilization in fermentors receiving high glucose concentrations and comparing them to fermentors receiving high glucose concentrations plus acetate.

MATERIALS AND METHODS

Manure

The cattle manure utilized in all of the experiments was obtained from the Montana State University dairy. The manure collection and analysis have been described previously (55). The manure was collected from a holding area within two hours after deposition. The cattle from which the manure had been collected had been fed on a 40% grain mix and 60% hay diet. The grain mixture was 50% barely, 25% wheat, 10% soybean meal, 10% sugar beet pulp and 5% mustard meal. The manure composition is shown in Table 4. Total solids and volatile solids were determined by standard methods (2). Fiber content was determined by the method described by Goering and Van Soest (32) and the Kjeldahl method was used to determine the nitrogen content. The manure was diluted with tap water to 5% total solids content. It was then blended for 30 s in a 3.8 l Waring blender, and packaged in 1000 ml plastic bags which were frozen. The feed was thawed and stored at 4°C as needed.

Inoculum

The digest which was used to inoculate the fermentors was obtained from a 3.2 l working volume anaerobic fermentor which had been maintained for over three years on a 16 day retention time (RT) on manure feed with 5% total solids, at a temperature of 37°C.

Table 4. Composition of Dry Matter of Dairy Cattle Waste (55).

	% Dry Matter
Volatile Solids	83
Ether extract	2.5-2.8
Cellulose	31
Hemicellulose	12
Lignin	12.2
Total Nitrogen (crude protein)	2.0
Ammonia	.5
Volatile Acids	
Acetic	.01
Propionic	Trace
Other	-

Fermentors

Fermentors which were used for the ^{13}C-nmr experiments were constructed from 100 ml wide mouth flasks which were sealed with a #9, three hole rubber stopper. The fermentors used for the enzymatic assay experiments were constructed from 250 ml Erylemeyer flasks which were sealed with a #6½, three hole rubber stopper. For each type of fermentor, one of the holes in the rubber stopper was fitted with a y-tube to be used for gas collection and gas sampling. Gas was collected in calibrated tubes that were submerged in 0.1 M HCl solution. The second hole was fitted with a glass tube to which plastic tygon tubing was attached. A polypropylene, female joint (9 mm in diameter) was inserted into the tygon tubing. This provided a point of attachment for 10 ml syringes. Disposable plastic syringes, which had the hub drilled out to provide a 8 mm diameter hole, were used. This provided adequate means by which the digest could be removed and feed added, quantatively, on a daily basis. This also provided the point of entry for additional feeds, e. g. glucose. A screw clamp was used to seal the tygon tubing. The third hole was plugged. The working volume of the fermentors used in the ^{13}C-nmr experiments was 64 ml, and in experiments for chemical analysis of glucose, the digestor working volume was 192 ml. For both types of fermentors, the same regime was used (discussed in the next section). A diagram illustrating the 100

ml fermentor is shown in Figure 7.

Feeds Used

"6% only" Experiments - The fermentors in these experiments received 0.333 M concentration of glucose in the feed on a daily basis. The amount of glucose (720 mg in the 192 ml fermentors and 240 mg in the 64 ml fermentors) resulted in an additional 6% solids in the feed. The concentration of glucose in the fermentor which resulted upon addition was 1/16 of the .333 M value; ca. 20 mM.

"4% only" Experiment - The fermentors in these experiments received 0.22 M concentration of glucose in the feed on a daily basis. The amount of glucose (158 mg in a 64 ml fermentor) resulted in an additional 4% solids in the feed.

"2% only" Experiments - The fermentors in these experiments received 0.11 M concentration of glucose in the feed on a daily basis. The amount of glucose (79 mg in a 64 ml fermentor) resulted in an additional 2% solids in the feed.

"6% and 1%" Experiments - The fermentors in these experiments received 0.33 M concentration of glucose plus 0.17 M concentration of acetate (magnesium salt) in the feed. The amount of acetate (437 mg in the 192 ml fermentors and 77 mg in the 64 ml fermentors) resulted in an additional 1% solids in the feed, in addition to the 6% supplementary solids from the glucose.

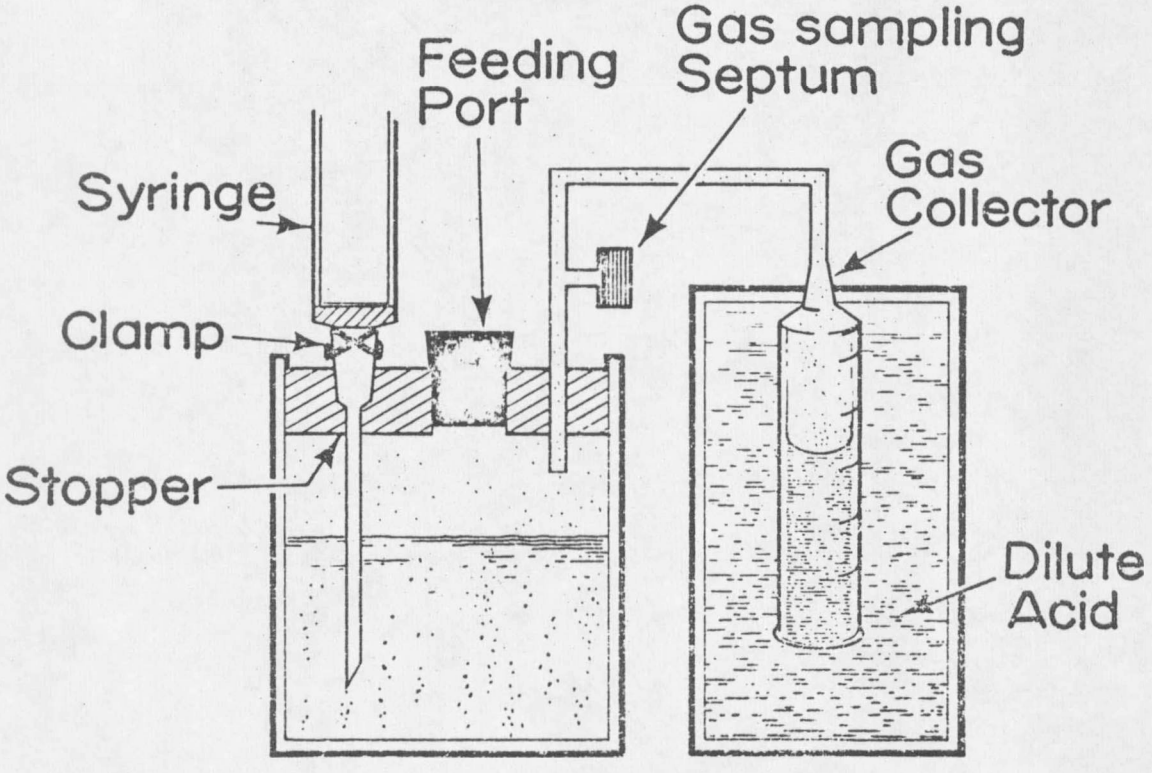


Figure 7. A 100 ml fermentor.

Conditioning and Daily Regime

The fermentors were maintained on a 16 d RT with the 5% total solids, manure feed at 37°C in a water bath. The fermentors were maintained on the manure feed only until the pH and gas compositions obtained were similar to the control, indicating that the fermentors had stabilized. Gas compositions were determined by gas chromatography on a Carle-Model 800 gas chromatograph and a Dohrmann SC 852 recorder. The experiments, which will be subsequently described, were begun after the fermentors had stablized. Each day of the experiment (Day 1 indicated the first time the fermentor received the specified feed) the fermentors were swirled to suspend the particulates, and 4 ml or 12 ml in the 64 ml fermentors and 192 ml fermentors respectively, of the digest were removed. The specified feed was added with 4 ml (12 ml) of the feed manure. The fermentors were then sealed and again, swirled to mix the feed and digest. The digest pH was determined on a Corning pH meter-model 125. No adjustments of pH were made during the experiments.

Glucose Assay Procedure

The enzymic assay experiments were performed on Days land 3 in fermentors receiving the "6% only" feed. Two ml samples were collected for each of the sampling periods designated for each day of the experiment. The sampling periods are listed in Table 5. When the

Table 5. Sampling Periods Used for Glucose Utilization via the Enzymic Method.

Day 1 - Samples were taken at the times shown below, after glucose had been fed to the fermentor.

Time (h)
0.17
1.5
3.0
4.5
5.5
6.0
6.5
7.0
7.5
9.0
10.5

Day 3 - Samples were taken at the times shown below, after glucose had been fed to the fermentor.

Time (min)
10
20
30
40
50
60
70
80
90
100
110
120

samples were removed, they were placed in a boiling water bath for 10 min to stop further metabolic activity. The samples were then frozen and stored until analyzed.

The analysis of glucose involved the following reactions which resulted in a specific assay for glucose.

Reaction (6) glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose 6-phosphate + ADP

Reaction (7) glucose-6-phosphate + $NADP^+$ $\xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}}$ δ lactone + NADPH

The procedure used for the assay has been described by Peterson and Young (53).

The assay required three components; 1) the digest sample, 2) a cocktail solution and 3) an enzyme solution. The cocktail solution, which contained ATP and $NADP^+$, was mixed prior to the assay and frozen in 50 ml containers until needed. This solution contained 0.001 M of both ATP and $NADP^+$, dissolved in a buffer. The buffer contained 0.01 M tris-hydroxymethyl, amino methane, dissolved in distilled water, and glacial acetic acid was added until a pH of 7.5 was obtained. The enzyme solution was prepared each time the assay was performed. This solution contained 100 units of glucose-6-phosphate dehydrogenase and 200 units of hexokinase. The ammonium sulfate suspensions of glucose-6-phosphate dehydrogenase and hexokinase were centrifuged on an

Eppendorf micro-centrifuge - Model 3200 for 3-4 min. The precipitate was dissolved in 1 ml of the tris-hydroxymethyl, amino methane buffer. The 2 ml digest samples, described above, were centrifuged in the micro-centrifuge for 5- 6 min. The clear supernant was removed and 0.2 ml were diluted in 3.8 ml of distilled water and mixed well, resulting in a twenty-fold dilution.

The reaction was monitered at 339 nm on a Varian Techtron Ultra-Violet and Visible Spectrophotometer - Model 635, with a slit of 1 nm. Disposable, standard 12 x 10 mm curvettes were used. In each curvette 0.1 ml of enzyme solution, 0.2 ml of the sample and 2.7 ml of cocktail solution were added. The latter was pipetted forcefully to provide adequate mixing. Immediately upon the addition of the cocktail solution the absorbance was recorded. Twenty minutes after the initial reading the absorbance was again recorded. The change in absorbance was used in the calculation below to determine the glucose concentration.

Equation (1): Determination of glucose concentration:

$$c = \frac{\Delta A_{339}}{6.22} \times 15 \times 20$$

where c = concentration of glucose in mmoles

ΔA_{339} = change in absorbance at 339 nm

15 = dilution factor in the curvette

20 = dilution factor of the digest sample

6.22 = mM extinction coefficient

^{13}C-nmr Procedures

The ^{13}C-nmr experiments performed utilized the following procedures to transfer the digest to the instrument, obtain spectra and identify resonances.

Two ml of digest from a fermentor was transferred to a 10 mm diameter nmr tube containing the ^{13}C-labelled compound used in the prescribed experiment. The tube was flushed with CO_2 or a CO_2, CH_4 gas mixture (50:50) to exclude O_2 and thoroughly mixed to dissolve the added compound. A sealed coaxial tube, 7.5 mm, containing D_2O and dioxane was then inserted into the 10 mm tube. The coaxial tube served a three-fold purpose: to seal the 10 mm tube containing the digest; the D_2O provided a deuterium lock signal; and the dioxane served as a reference to tetramethylsilane in the carbon spectra. The spectrometer was then programmed to monitor the system.

The ^{13}C-nmr spectra at 62.83 MHz were obtained in a fourier transfer mode on a Bruker WM-250 MHz NMR spectrometer. The temperature was maintained at 37°C. The spectra were fourier transforms (with a line broadening factor of 10 Hz) of the accumulations of a number of radio frequency pulses. The parameters and number of scans used for the experiments described are listed in Appendix 1. Four or eight K data points were used for each free induction decay depending on the

sweep width used. The chemical shifts were assigned relative to tetramethylsilane as was determined from the external reference, dioxane.

To identify the metabolites of glucose, the chemical shifts of the possible intermediates and products of glucose metabolism were identified by spiking the digest with the compound and observing the chemical shifts from the natural abundance ^{13}C nuclei. The compounds for which spectra were obtained were acetate, lactate, pyruvate, propionate, butyrate, acrylate, malate, succinate, bicarbonate and ethanol. Care was taken to maintain the pH near 7.5, the value normally encountered in the digest, since the chemical shifts are pH dependent. The chemical shifts for the compounds listed above are reported in Appendix 2.

Glucose Utilization Monitored by ^{13}C nmr Spectroscopy

The information on glucose utilization determined by ^{13}C-nmr were obtained from fermentors that had received ^{13}C-1-glucose. The ^{13}C-nmr experiments were performed on Days 1, 3 and 5 in fermentors that received the "6% only" and "6% and 1%"feeds. Separate fermentors were assembled for each day. The fermentors were maintained on the daily procedures which have been previously described. On the designated days of ^{13}C-nmr spectroscopic analysis of the "6% only" and "6% and 1%" fermentors, 2 ml of the digest was transferred to the 10 mm nmr tube

and 7.2 mg of the ^{13}C-1-glucose was added to provide a 20 mM concentration. The nmr procedures were performed as previously described. The micro-programs used for each of the three days on the spectrometer are outlined in Appendix 3.

Supernatant-products from ^{13}C-glucose

The fermentor used in this experiment, was on the feeding regime of the "6% only" fermentors previously described. The fermentor had received the "6% only" glucose two days prior to the ^{13}C-nmr experiment. On the third day, 4 ml of the digest were removed and 14.4 mg of ^{13}C-1-glucose was added to provide a 20 mM concentration. Two hours after the addition of the ^{13}C-glucose the 4 ml of digest (containing the ^{13}C-glucose) was centrifuged in a Sovall RC2-B Refrigerated Centrifuge at 3020 x g for 5 min, to remove the cells and particulate matter. Two ml of the supernant was removed and used to observe the ^{13}C-nmr resonances. The resonances obtained from this spectra would be those of excreted products from the metabolism of glucose. The procedures used to obtain the spectra have been previously discussed.

Investigation of Spin-Lattice Relaxation* Effects on Acetate, Propionate and Lactate

Twenty ml of digest was boiled for 5 min to destroy any living

*See Appendix 4

microorganisms. Equal concentrations of propionate (potassium salt), acetate (potassium salt) and lactate (lithium salt) were added to the digest. The concentration used was 0.1 M. The pH was adjusted to 7.33. Two ml of the solution was transferred to a 10 mm nmr tube. The spectrum obtained was from 2125 radio frequency pulses. The parameters used are listed in Appendix 1.

Comparisons of "2%, 4% and 6% only" Fermentors by ^{13}C Spectroscopy

A comparison of glucose utilization and the metabolite profiles in "2%, 4% and 6% only" fermentors were studied by ^{13}C-nmr. These fermentors were maintained on the appropriate additional feed and the daily procedures which have been previously described. The ^{13}C-nmr experiments using ^{13}C-1-glucose were performed on Day 3 for each fermentor (2%, 4% and 6%).

Acetate Utilization Monitored by ^{13}C-nmr Spectroscopy

The information on acetate utilization determined by ^{13}C-nmr were obtained from fermentors that had received ^{13}C-2-acetic acid. The ^{13}C-nmr experiments were performed on Days 0, 1, 3 and 5 in fermentors that had received the "6% only" and "6% and 1%" feeds. On day 0, the fermentor received the feed manure only. Separate fermentors were used for each day.

On day 0, the 2 ml of digest was removed and placed in the 10 mm nmr tube to which 1 µl of ^{13}C-2-acetic acid was added to provide a 10

mM concentration. The subsequent nmr procedures have been described.

On day 1, the following protocol was used in both the "6% only" and "6% and 1%" fermentors. Ten hours prior to the nmr experiment the fermentors received the feed manure and "6%" glucose. After the ten hour period, 2 ml of digest was removed, placed in the nmr tube to which the ^{13}C-acetic acid (1 l) was added.

On days 3 and 5 the protocol used differed from that on Day 1 in that the time period from the administering of the "6%" glucose and manure feed to the addition of the ^{13}C-acetic acid was only 2 hrs as opposed to 10 hrs.

Propionate Utilization Monitored by ^{13}C-nmr Spectroscopy

Propionate utilization was studied by addition of ^{13}C-3-sodium propionate. The ^{13}C-nmr experiments were performed on Days 0, 1, 3, 5 and 12* in fermentors that had received the "6% only" and "6% and 1%" feeds. On day 0, the fermentor received the feed manure only. Separate fermentors were assembled for each day.

On day 0, 2 ml of digest was removed and placed in 10 mm nmr tube after the daily feeding (manure only) had been completed. ^{13}C-3-sodium propionate (1.9 mg) was added to provide a 10 mM concentration. On day 1, the following procedure was used in both the "6% only" and

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*^{13}C-nmr experiments were performed in Day 12 in only the "6% and 1%" fermentors

"6% and 1%" fermentors. Ten hours prior to the nmr experiment the fermentors received the feed manure and the additional feeds ("6%" glucose or "6%" glucose plus 1%" acetate). After the ten hour period, 2 ml of the digest was removed and placed in the nmr with the ^{13}C-propionate. On days 3, 5, and 12 the protocol used differed from that on Day 1 in that the time period from the administering of the "6%" glucose (or "6%" glucose and "1%" acetate) and manure feed to the addition of the ^{13}C-propionate was only 2 hrs as opposed to 10 hrs. The nmr procedures were the same as described previously.

Investigation of Metabolites Resulting from ^{13}C-propionate

This series of experiments involved two fermentors, one which was stressed with glucose, propionate and acetate as additional feeds* and the second being a fermentor which had been maintained on the manure feed only. The stressed fermentor had been stabilized on the manure feed (as previously described). The first nine days this fermentor received 123 mg of propionate (sodium salt) resulting in a 0.320 M propionate concentration in the feed, 66 mg of acetate (potassium salt) resulting in a .169 M concentration of acetate in the feed and 4 ml of manure feed. On the tenth day the fermentor received 240 mg of glucose (a .333 M glucose concentration in the feed corresponding to 6%

*the stressed fermentor was part of a series of experiments performed in our laboratory (unpublished results).

additional solids), in addition to the propionate and acetate. The fermentor was maintained on the feedings described above for eighty-five days. On the eighty-sixth day a ^{13}C-propionate nmr experiment was performed. Two ml of the digest was removed after the fermentor had received the 4 ml of the manure feed, and transferred to the 10 mm nmr tube to which 1.9 mg of ^{13}C-3-sodium propionate was added. The nmr procedures used have been described previously. The spectrum obtained in this experiment was a free induction decay containing 6,394 radio frequency pulses.

The fermentor which had been maintained solely on the manure feed was subjected to the same protocol used on the stressed fermentor on the eighty-sixth day. The spectrum which was obtained was the result of 10,394 radio frequency pulses in the free induction decay.

Chemicals Used

The Assay - The hexokinase, glucose-6-phosphate dehydrogense, adenosine triphosphate, nicotinamide adenine dinucleotide phosphate and the tris hydroxymethyl, amino methane (Trizma) were obtained from Sigma Chemical Company and used without further purification.

Additional Feeds - The glucose was obtained from Eastman Chemical Company. The magnesium acetate and potassium acetate were obtained from J. T. Baker Chemical Company. The glucose and acetate were used without further purification. The sodium propionate was made in our

laboratory (the procedure is unpublished research). It was shown to be chromatographically pure.

^{13}C-labelled Compounds - The ^{13}C-1-glucose was a gift from Dr. Jack Nordin, Department of Biochemistry, University of Massachusettes. Spectra obtained indicated that the sample was pure and was used without further purification. The ^{13}C-2-acetic acid and ^{13}C-3-sodium propionate were obtained from Merck, Sharp and Dohme Chemical Company. The spectra obtained on each sample indicated that they were pure and were used without further purification.

RESULTS

The results which have been obtained will be reported in two sections. The first section, titled Major Results, will include those results which are pertinent to the objectives which have been stated in this thesis. The second section, titled Minor Results, will be those results obtained from the data which are not directly associated to the ojectives of this thesis but have implications to the application of ^{13}C-nmr spectroscopy to the study of the anaerobic digestion process.

Section 1 - Major Results

Glucose Utilization From the Enzymic Assay Procedure

The "6% only" fermentations for this experiment were executed as previously described (pg. 37) to ascertain glucose utilization as a function of time on days 1 and 3. The results are shown in Figure 8. There was a significant change in the time period in which glucose was utilized on the first day compared to the third day. Glucose was utilized within 11 h the first day, and on the third day glucose was depleted within 2 h. On the first day there were two distinct rates of utilization. In the first 6.5 h after feeding the glucose utilization rate was slower, a rate constant (obtained from first order rate laws) of 0.02 h^{-1} was obtained. In the 6.5 to 11 h period, a faster rate of utilization was observed, the rate constant calculated was 0.25 h^{-1}. The rate constant for glucose utilization on Day 3 was 1.20 h^{-1} (see Table 6).

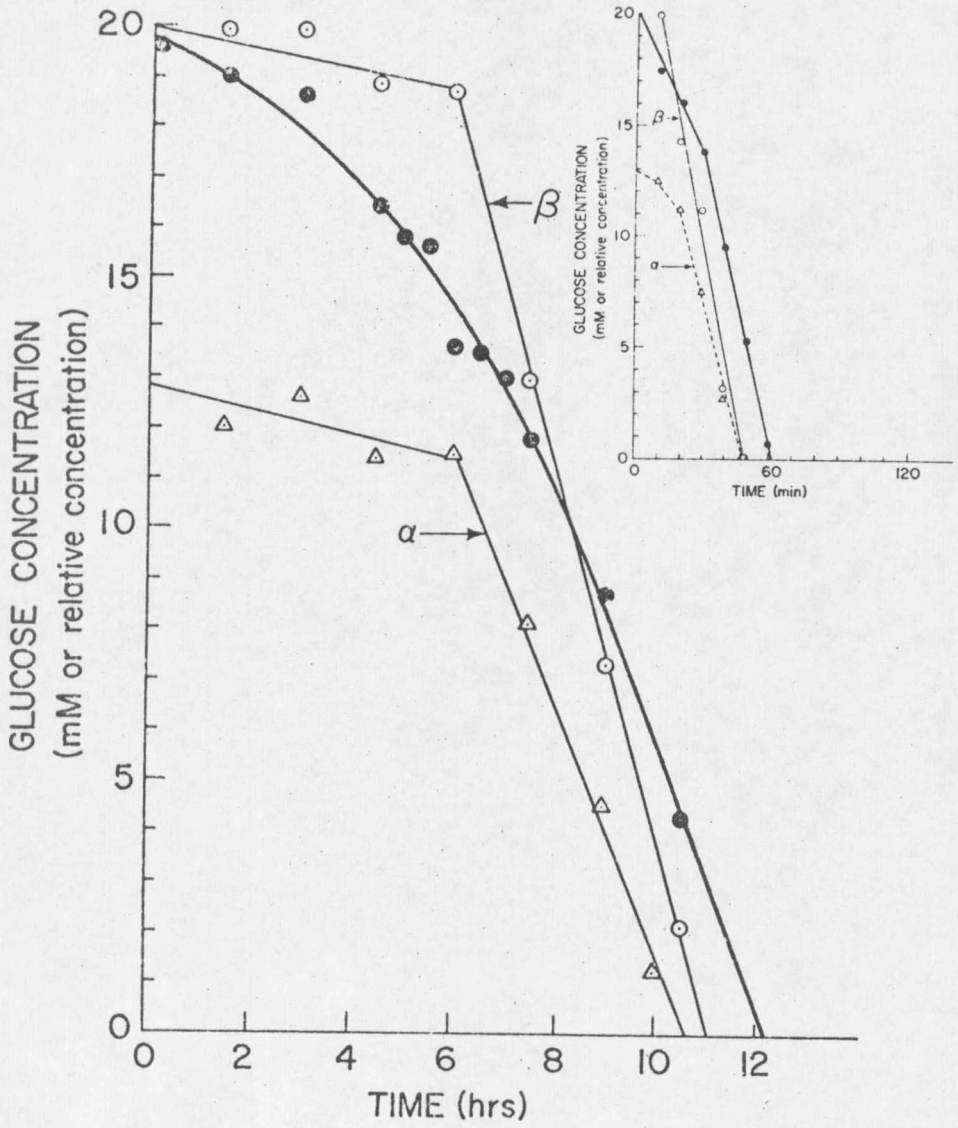


Figure 8. Glucose utilization: enzymic and ^{13}C-nmr methods compared. Glucose concentration as determined by chemical analyses (●), β-glucose (○) and α-glucose (△) as determined by NMR: in fermentors receiving glucose the first time. The latter are relative concentrations (peak intensities). Inset: Glucose utilization in fermentors receiving the third feeding of glucose. The graphic symbols are the same.

Table 6. Rate Constants for Glucose Utilization

6% Glucose Fermentations

^{13}C Method			Enzymic Assay		
	k (h^{-1})	r		k	r
Day 1			Day 1		
t=0-6	0.02	.76	t=0-6	0.020	.96
t=6-11 h	0.31	.98	t=6-11	0.25	.96
Day 3	1.09	.98	Day 3	1.20	.95
Day 5					
t=0-.833 h	0.96	.98			
t=.833-2 h	0.18	.80			

6% Glucose plus 1% Acetate Fermentations

	k	r
Day 1		
t=0-4.5 h	.01	.80
t=4.5-9 h	0.58	.92
Day 3	0.96	.99
Day 5	4.64	-*

*Only 2 points used to calculate

Glucose Utilization From the ^{13}C-nmr Procedure

The "6% only " and "6% and 1%" fermentations were designed and conducted as previously described (pg 42). Metabolic activity was observed on days 1, 3 and 5. A representative stacked spectra obtained by this technique is shown in Figure 9. The spectra shown in this figure represents a "6% only" fermentation which had received the ^{13}C-1-glucose on day 3. The changes in the intensities of the β-1-glucose and α-1-glucose at 96.8 ppm and 93.1 ppm respectively, are shown as a function of time. The results of the glucose utilization on days 1, 3 and 5 obtained by the ^{13}C-nmr method are summarized in Figure 10. The rate constants obtained for glucose utilization by this method have been summarized in Table 6. These rate constants were calculated in the same manner as used in the enzymic experiments.

The rate constants which were obtained for the "6% only" experiments show that on Day 3 and 5 (from t = 0-50m) were nearly the same. However, after t=50m on Day 5 the rate at which glucose was utilized was slower by a factor of 5. In the "6% and 1%" experiments, the glucose utilization rates were similar in pattern to the "6% only" experiments, except for Day 5 where the utilization increasd by a factor of 5 (from Day 3 to Day 5).

Metabolites Resulting From ^{13}C-Glucose

The resonances of metabolites produced from ^{13}C-1-glucose are

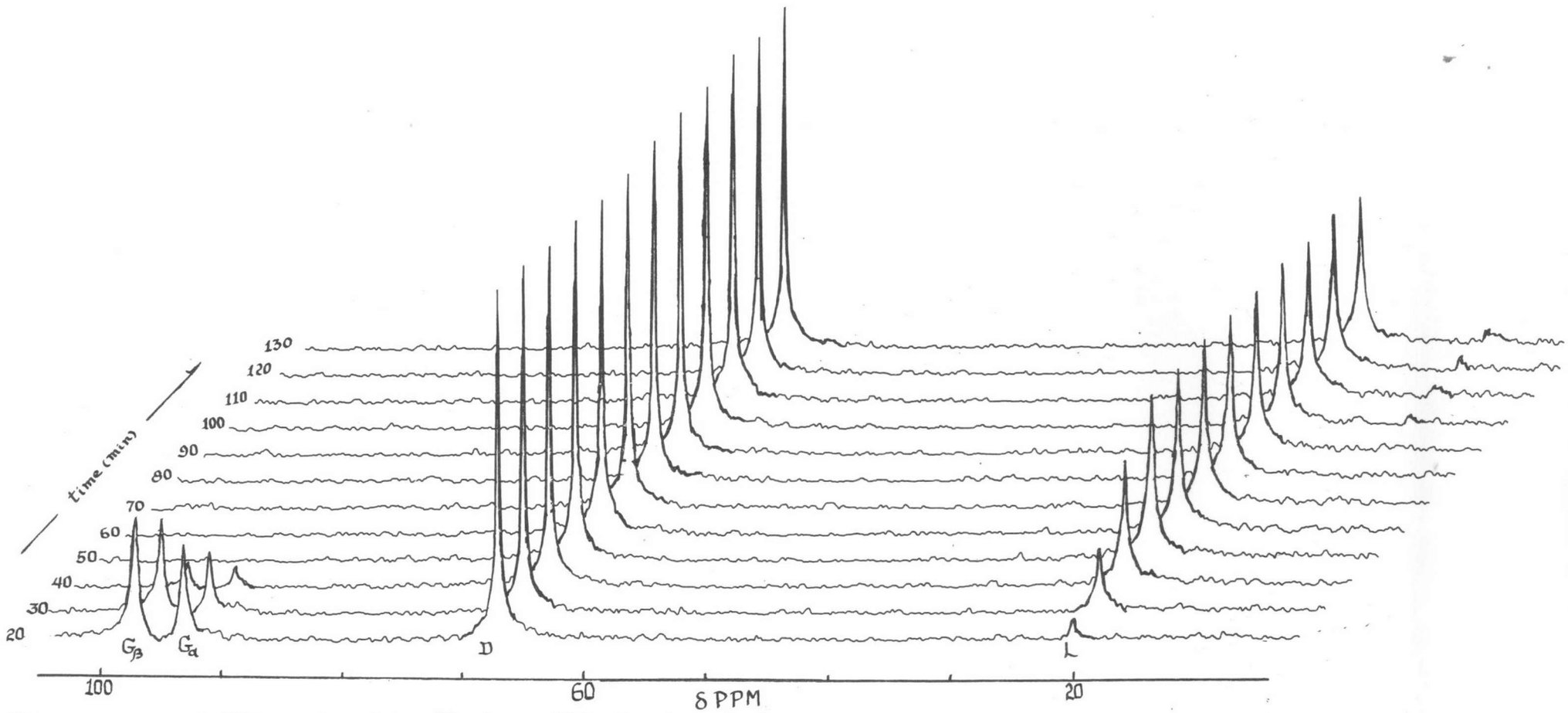


Figure 9. Time sequence of NMR spectra from 20 min to 130 min after feeding. This stacked spectra represents a 6% glucose fermentor which had received glucose for the third time. G_β and G_α = resonance positions for β and α of l-glucose 96.8 and 93.0 ppm, respectively; D = resonance of dioxane at 67.4 ppm and L = resonance of the methyl group of lactate.

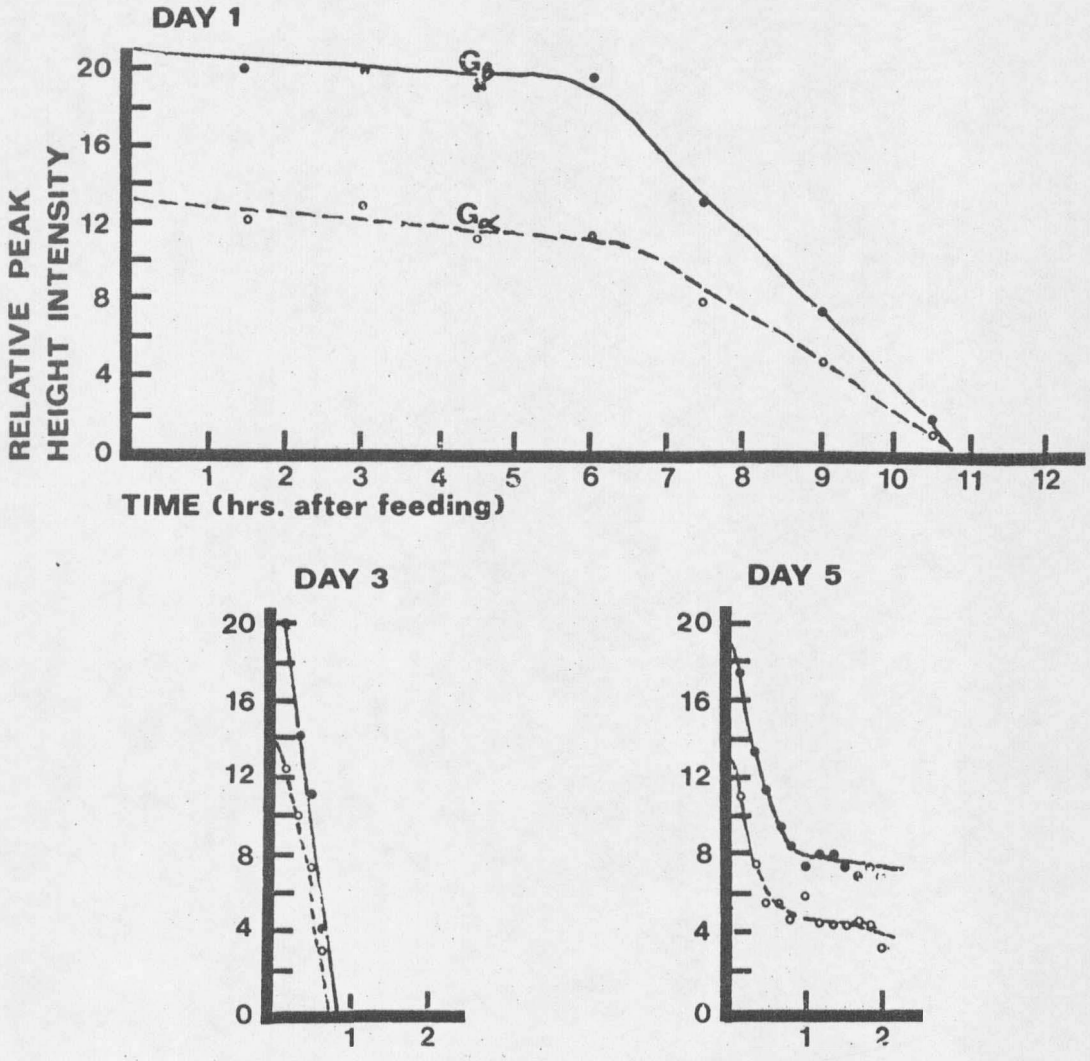


Figure 10. Glucose utilization in 6% glucose fermentations on Days 1, 3 and 5, as determined by the nmr technique. Peak intensities represent relative concentrations. β-l-glucose (•) and α-l-glucose (o).

shown in Figure 11. This spectrum was obtained 12 h after addition of labelled glucose. The products resulting from glucose fermentation were acetate and propionate. The label from the glucose was found to be in the methyl carbons of acetate and propionate at 24.1 ppm and 10.8 ppm, respectively. Labelled methylene of propionate was also detected at 31.2 ppm. In this spectrum it was evident that the resonance from the C-2 of propionate was less intense than the resonance of the C-3-propionate. In a spectrum of natural abundance ^{13}C of propionate the CH_3:CH_2 intensities ratio was 1.16:1. The ratio of intensities in the spectrum shown was 5.25:1.

In a similar experiment but approximately 1.5 h after the addition of ^{13}C-1-glucose, an additional metabolite, lactate, was detected. The spectrum obtained is shown in Figure 12. The label of glucose was detected in the methyl of the lactate at 20.8 ppm. Lactate was an immediate and major product of glucose metabolism (see Figure 14). However, in the 12 h spectrum (Figure 11) the lactate resonance had disappeared indicating that the lactate was an intermediate metabolite in the fermentation of the glucose to the product propionate.

The results from the spin-lattice (T_1) relaxation experiment demonstrated that the ratio of peak height intensities of the methyl groups of lactate, propionate and acetate were found to be 4:3:2, respectively, with equal concentrations of each.

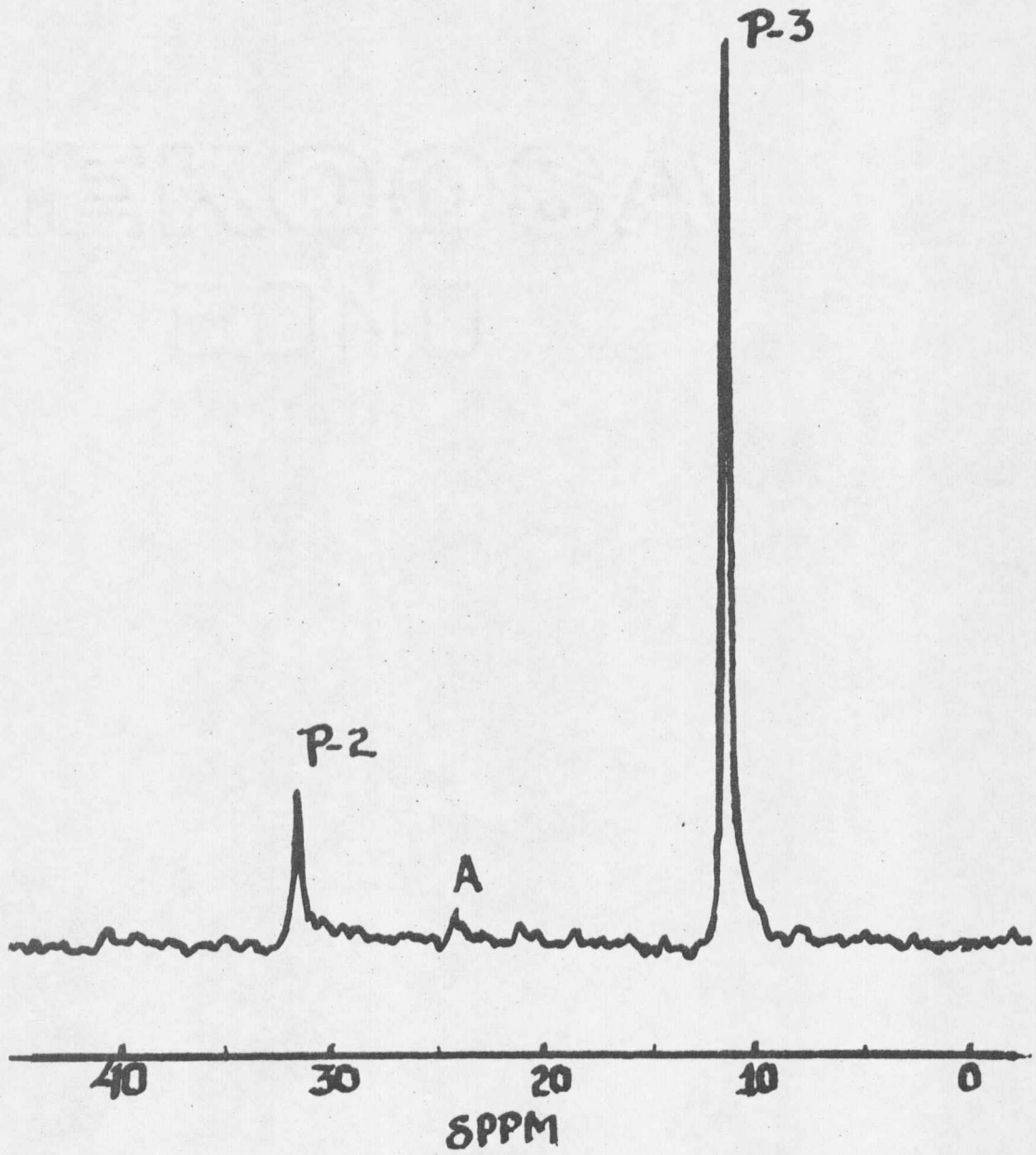


Figure 11. ^{13}C-nmr spectrum obtained 12 h after the addition of ^{13}C-1-glucose. A = methyl group of acetate, 24.1 ppm; P-2 = methylene group of propionate, 31.2 ppm and P-3 = methyl group of propionate, 10.8 ppm.

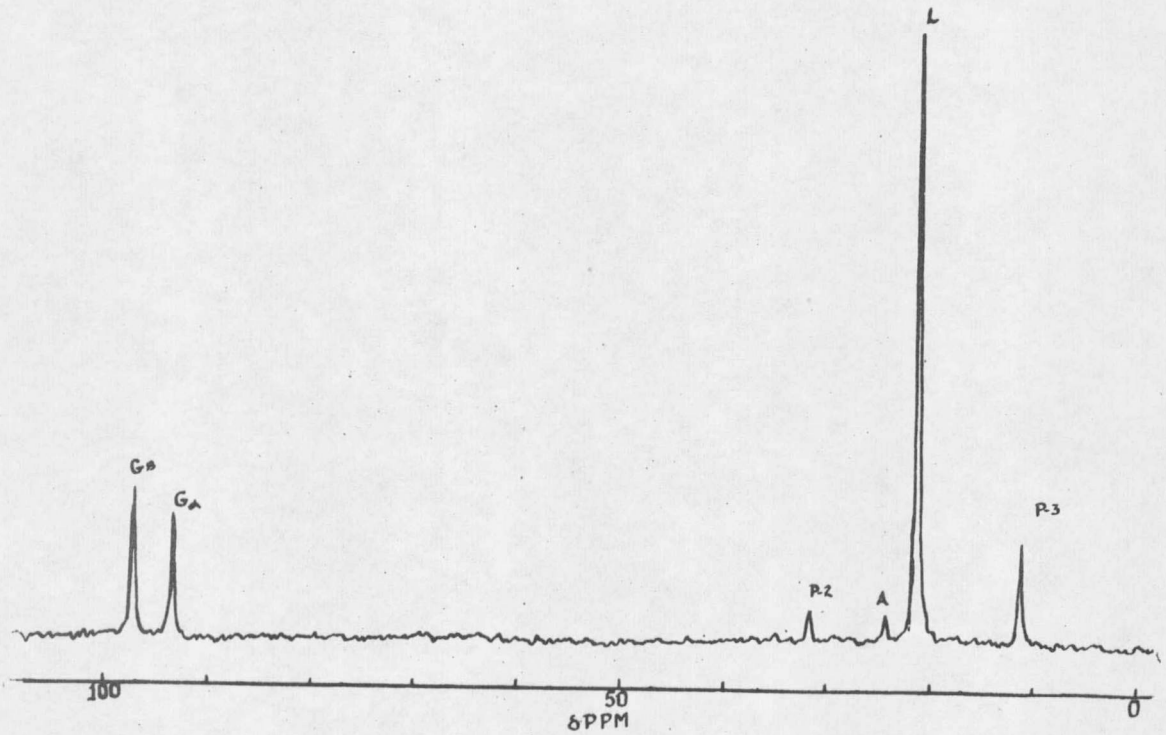


Figure 12. ^{13}C-nmr spectrum obtained 1.5 h after the addition of ^{13}C-1-glucose. A, P-2 and P-3 designated the same as stated in Figure 11; and G_β, G_α and L designated the same as stated in Figure 9.

acetate.

The supernant of the digestor which had received the ^{13}C-1-glucose (described on pg 43), provided information regarding the end products in the fermentation of glucose, which had been excreted into the medium. A spectrum obtained from the supernant is shown in Figure 13. The resonances detected in this spectrum were the methyl groups, of acetate, lactate and propionate at 24.1 ppm, 20.8 ppm and 10.8 ppm, respectively.

The concentration of glucose and metabolites as a function of time are depicted in Figure 14. These results demonstrated that lactate was produced almost immediately after the ^{13}C-1-glucose was administered. As lactate was utilized, propionate and acetate began to accumulate. It can be observed from this figure that the maximum relative concentrations of lactate to propionate to acetate were 10.25:7.8:3.6, respectively. In another experiment performed in our laboratory the concentration of H_2 in solution was monitored by gas chromatography as a function of time after glucose had been fed to "6 % only" fermentors (unpublished results). The results of this H_2 experiment are shown in Figure 15. It was evident from Figures 14 and 15 that both the lactate and H_2 concentrations increase virtually simultaneously and both begin to increase immediately after the addition of glucose.

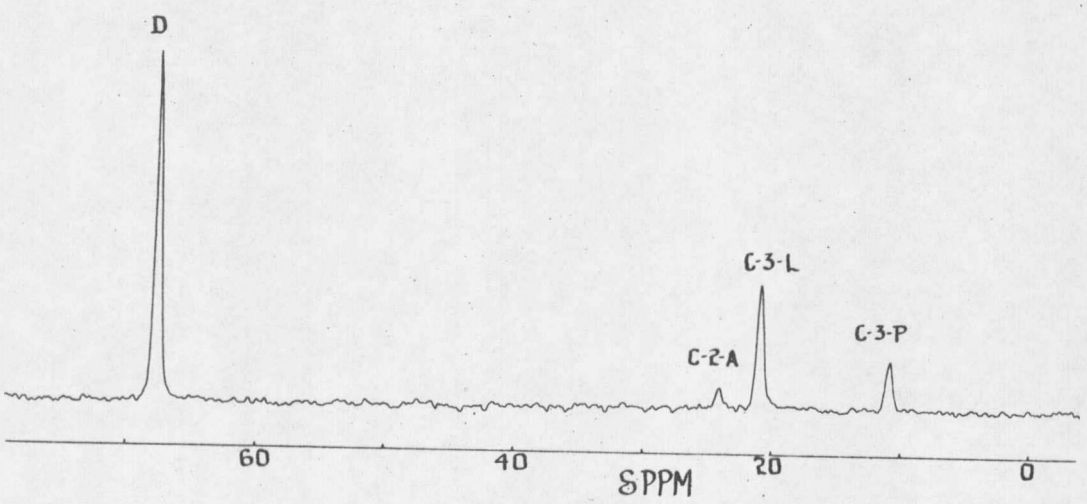


Figure 13. Spectrum of the supernant obtained from a 6% glucose fermentor on Day 3, 2 h after the addition of glucose. A= methyl group of acetate 24.1 ppm; L = methyl group of lactate 20.8 ppm; P = methyl group of propionate 10.8 ppm and D = dioxane at 67.4 ppm.

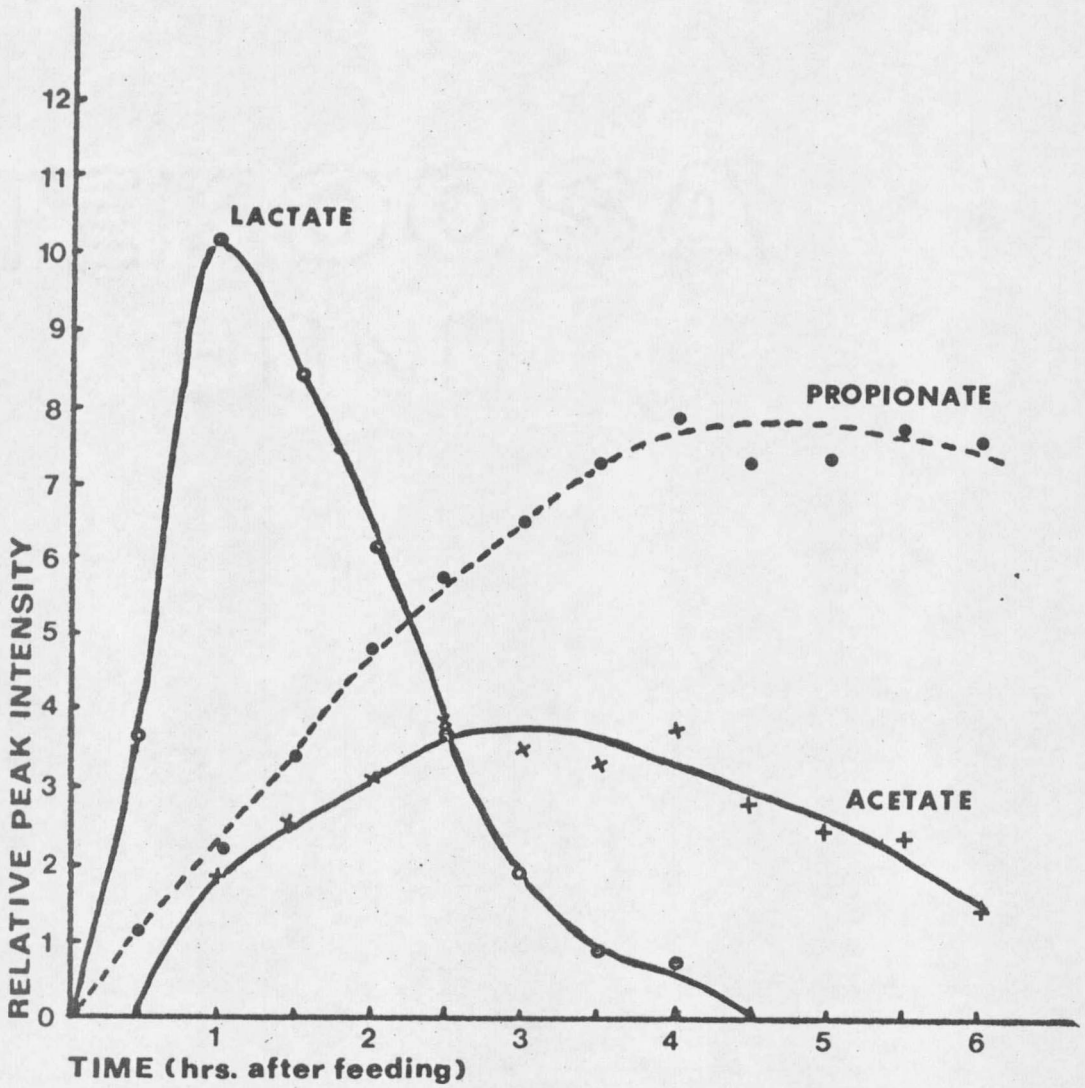


Figure 14. The relative concentrations of glucose and metabolites as a function of time obtained by the nmr technique. The graph represents a 6% glucose digestor which had received glucose for the third time. The relative concentrations of the propionate, lactate and acetate were determined from the peak intensities which had been corrected for T_1 relaxation effects (see text).

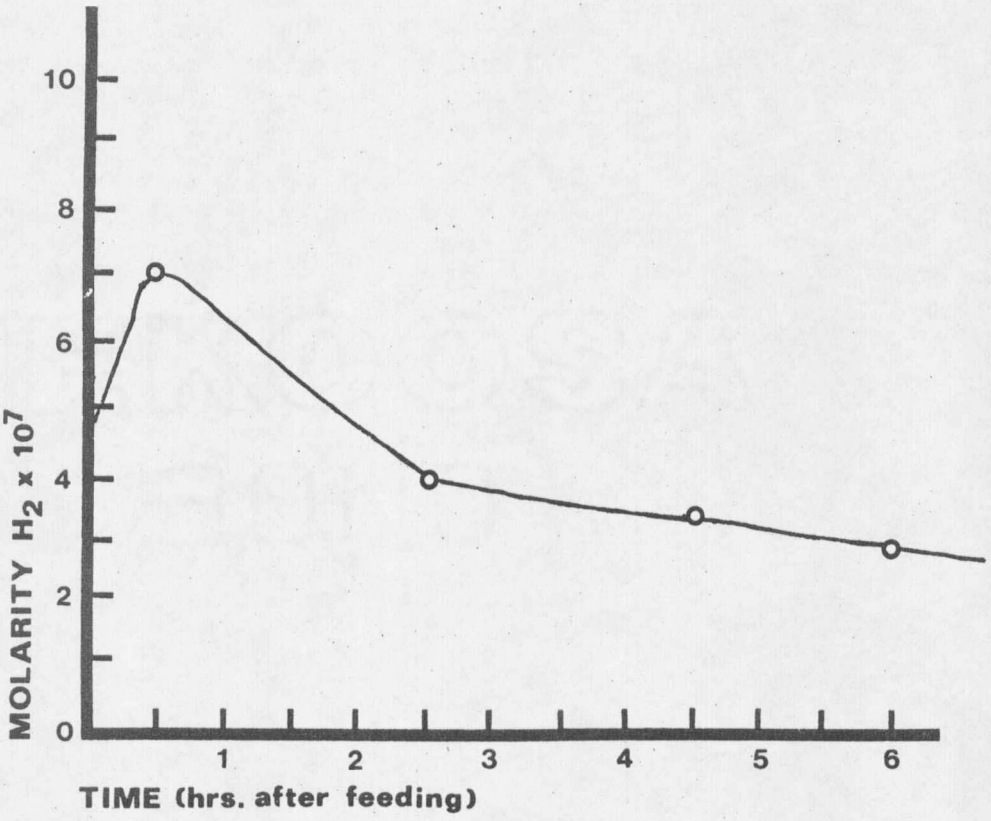


Figure 15. A plot of the H_2 concentration as a function of time after the addition of glucose. This represents a 6% glucose digestor which had received glucose for the third time.

Acetate Utilization as Observed by ^{13}C-nmr

The "6% only" and "6% and 1%" fermentations were conducted as previously described (pg 44). Days 0, 1, 3 and 5 were used to obtain acetate utilization rates. The resonance intensities were used as relative concentrations of the ^{13}C-2-acetic acid to describe the acetate utilization as a function of time. The rate constants and the half-life of acetate utilization for each day in the "6% only" and the "6% and 1%" are shown in Table 7. These rate constants were obtained from application of the first order rate law to acetate utilization.

Propionate Utilization as Observed by ^{13}C-nmr.

The "6% only" and "6% and 1%" fermentations were conducted as described (pg 45). Days 0, 1, 3, 5, and 12* were used to obtain propionate utilization rates. Again, the resonance intensities were used as relative concentrations of the ^{13}C-3-propionate. The rate constants and half-life of propionate utilization for Days 0, 1, and 12** in the "6% only" and "6% and 1%" fermentors are summarized in Table 8. The rate constants were calculated as described for acetate

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*Day 12 the ^{13}C-nmr experiment was performed only on the "6% and 1%" fermentor, as the "6% only" had undergone failure by this time.

**The rate constants and half-life for Days 3 and 5 have been omitted in both the "6% only" and "6% and 1%" fermentations. This point will be taken up in the discussion section.

Table 7. Rate Constants and the Half-life of Acetate Utilization

6% Glucose Fermentations

	Day 1	Day 3	Day 5
$k(h^{-1})$	0.19	0.07	0.05
$t_{\frac{1}{2}}(h)$	3.63	9.24	13.59
r	.97	.86	.92

6% Glucose plus 1% Acetate Fermentations

	Day 1	Day 3	Day 5
$k(h^{-1})$	0.19	0.14	0.44
$t_{\frac{1}{2}}(h)$	3.66	4.87	1.57
r	.85	.70	.84

Day 0 - manure feed only

$k(h^{-1})$	0.19
$t_{\frac{1}{2}}(h)$	3.67
r	.98

Table 8. Rate Constants and Half-life - Propionate Utilization

Day 0 - manure feed only

$k = 0.10\ h^{-1}$

$t_{\frac{1}{2}} = 6.60$ h

$r = .96$

Day 1

	6% Glucose	6% Glucose plus 1% Acetate
$k(h^{-1})$	0.02	0.06
$t_{\frac{1}{2}}(h)$	32.56	11.18
r	.97	.89

Day 12 - 6% Glucose plus 1% Acetate

$k = 0.02\ h^{-1}$

$t_{\frac{1}{2}} = 34.62$ h

$r = .80$

utilization.

Section 2 - Minor Results

Metabolite Profiles in "2%, 4% and 6% only" Fermentors from ^{13}C-1-glucose.

The "2%, 4% and 6% only" fermentors used in these experiments were constructed and maintained as described on pg. 44. The results of these experiments, from Day 3 only, are summarized in Figure 16. The intensities of glucose, acetate, lactate and propionate have been plotted as a function of time, after the ^{13}C-1-glucose had been administered to the digestor. In general, the profiles of glucose and lactate were virtually the same for each concentration of glucose. However, in the "2% only" fermentation there was no appearance of propionate which was evident in both the "4% and 6% only" fermentors.

Metabolites in a Non-stressed Fermentor from ^{13}C-3-propionate

The fermentation was conducted as described previously (pg 46). The spectrum obtained after the ^{13}C-3-propionate had been added is shown in Figure 17. The resonances which have been identified are bicarbonate at 161.0 ppm, C-2 propionate at 31.2 ppm, propionate at 10.8 ppm. The methyl and methylene region of the previous spectrum was expanded. As a result the C-3 of lactate at 20.8 ppm was also detected in this region.

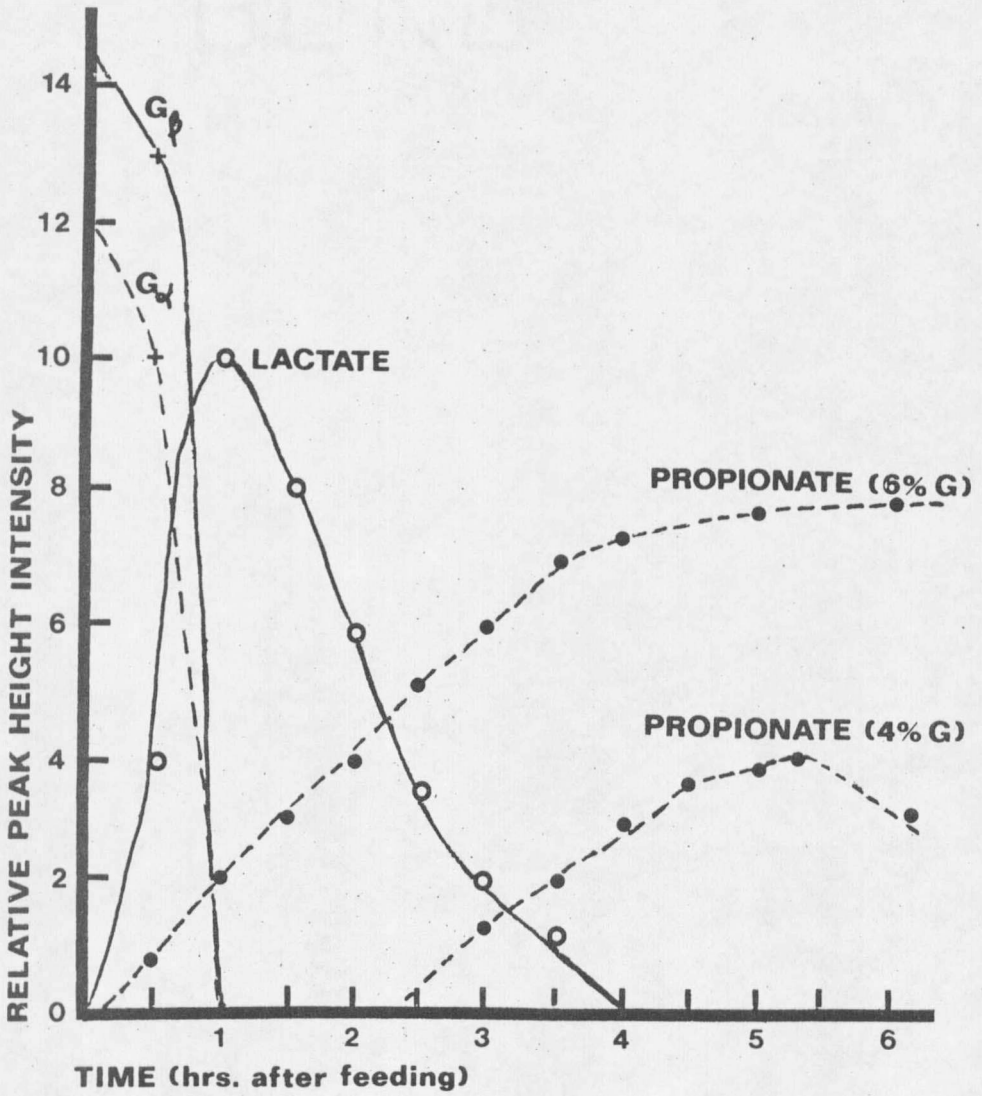


Figure 16. A profile of 2%, 4% and 6% glucose and the resulting metabolites as a function of time as observed by ^{13}C-nmr. The β α-glucose (+) and lactate (O) profiles were virtually the same for all three concentrations. The propionate (•) for the 4% and 6% glucose concentrations are as shown.

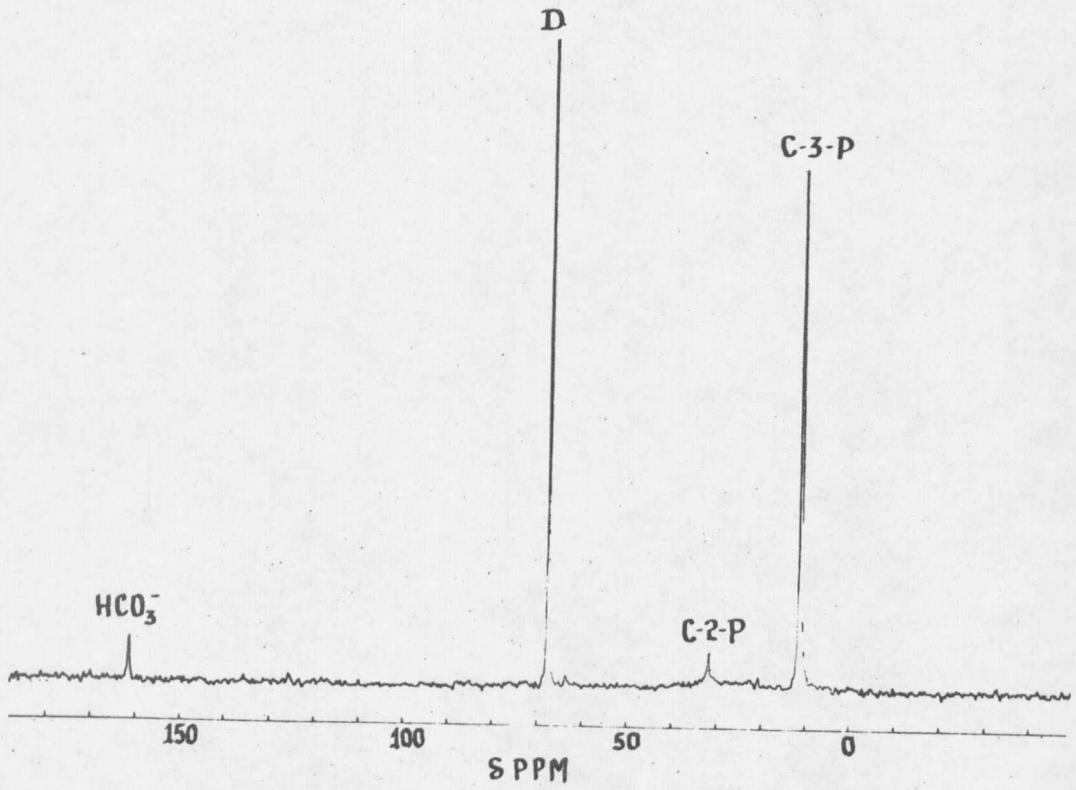


Figure 17. A spectrum of 10,394 radio frequency pulses obtained after the addition of ^{13}C-3-propionate to a non-stressed fermentor. HCO_3^- = bicarbonate, 161.0 ppm; D = dioxane, 67.4 ppm; C-2-P = methylene group of propionate, 31.2 ppm and C-3-P = methyl group of propionate, 10.8 ppm.

Propionate Metabolites in a Stressed Fermentor from ^{13}C-3-Propionate

The fermentation was conducted as described previously (pg. 47). The spectrum obtained after the ^{13}C-3-propionate had been added is shown in Figure 18, the methyl and methylene region have been expanded and is shown in Figure 19. The resonances which have been tentatively* identified are C-1 propionate at 185.0 ppm, C-1 acetate at 182.0 ppm, C-2 malate at 40.4 ppm, C-2 butyrate at 38.3 ppm, C-2 succinate at 29.9 ppm, C-3 pyruvate at 27.8 ppm and C-3 butyrate at 14.0 ppm. The resonances which have been identified with certainty are C-3 propionate at 10.8 ppm, C-3 lactate at 20.8 ppm, C-2 acetate at 24.0 ppm and C-2 propionate at 31.5 ppm.

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*ppm ±1.0 ppm

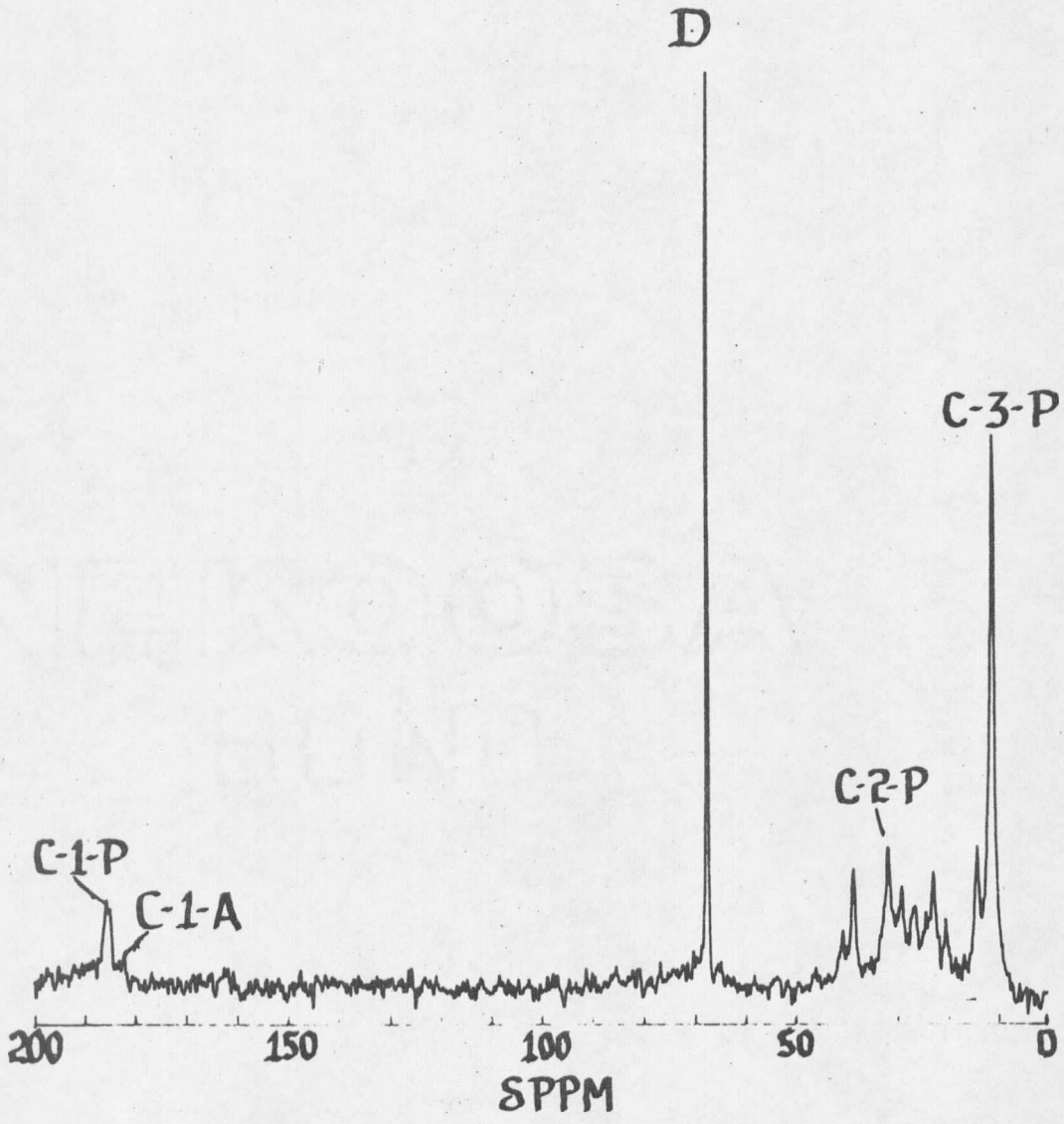


Figure 18. A spectrum of 6,394 radio frequency pulses obtained after the addition of ^{13}C-3-propionate to a stressed fermentor. C-1-P = carboxyl group of propionate, 185.0 ppm and C-1-A = carboxyl group of acetate, 182.0 ppm. The graphic symbols C-2-P, C-3-P and D same as in Figure 17.

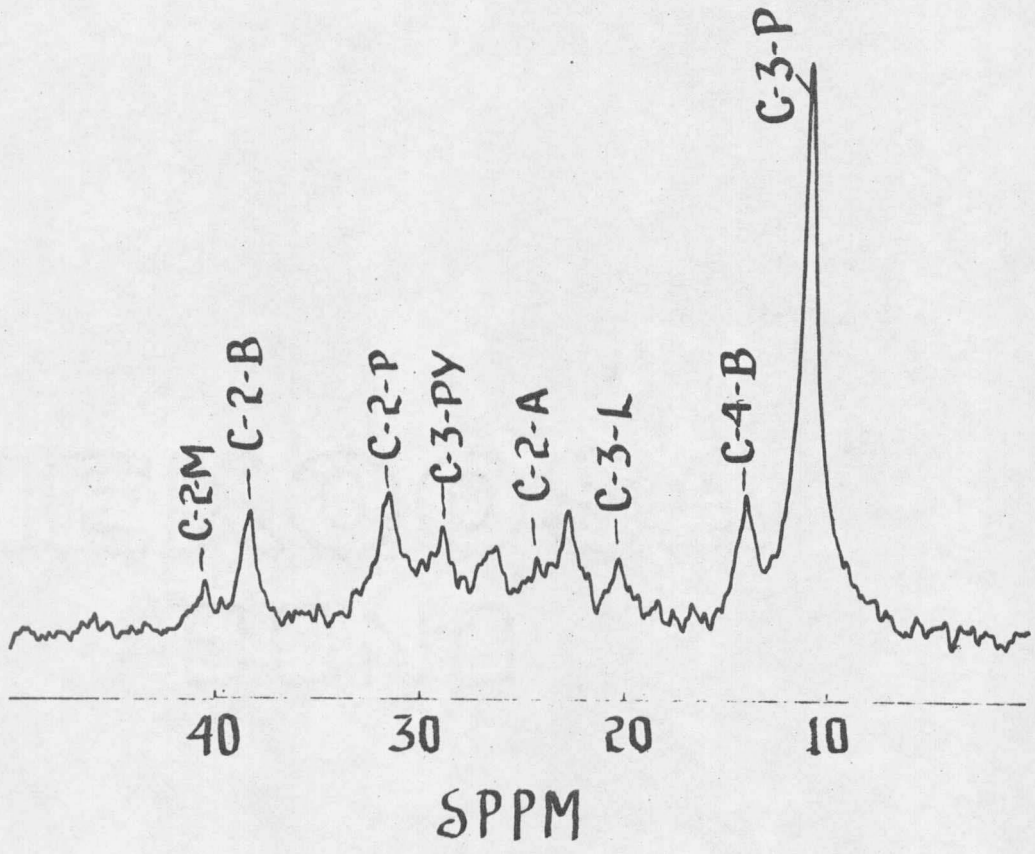


Figure 19. The 10-40 ppm region of previous spectrum (Figure 18) expanded. C-2-M = C-2 malate, 40 ppm; C-2-B = C-2 butyrate, 38 ppm; C-3-Py = methyl group pyruvate, 27 ppm; C-2-A = methyl group acetate, 24.0 ppm; C-3-L = methyl group lactate, 20.8 ppm and C-4-B = C-4 butyrate, 14 ppm. The graphic symbols for C-3-P and C-2-P are the same as in Figure 17. The other resonances are unidentified at this time.

DISCUSSION

Glucose Utilization Rates

Glucose utilization rates were obtained for 6% glucose and 6% glucose plus 1% acetate fermentations. The rate constants which are shown in Table 6, indicated that the pattern of glucose consumption in both of these fermentations were similar. In these fermentations the rate constants obtained for Day 1, indicated that there were two separate rates of utilization. The first rate constant was for the first six hours of fermentation after the ^{13}C-glucose had been administered. After the first six hour period the rate constant obtained for the utilization of glucose increased by a factor of 15 and 60 for the 6% glucose and 6% glucose plus 1% acetate, respectively. The rate constants obtained for Day 3 increased by a factor of 3.5 and 1.6 over the rate constants obtained in the 6-11 h period on Day 1, for the 6% glucose and 6% glucose plus 1% acetate fermentations, respectively. On Day 5 the rate constant obtained for the 6% glucose plus 1% acetate increased by a factor of 5 over Day 3. However, the rate constants obtained for the 6% glucose fermentation on Day 5 remained similar to Day 3 for the first 50 min of the fermentation period. After this period the rate constant decreased by a factor of 5.

The fact that the rate of glucose utilization increased from Day 1

to Day 3, and the concentration of glucose was the same for each suggested that there was an enrichment of the fermentative bacterial population. Since glucose utilization was found to be a first order rate equation this suggested that population changes did not occur during the time of observation on a given day.

Since both the 6% glucose and 6% glucose plus 1% acetate fermentations on Day 1 and 3 were similar, with regard to glucose utilization, it suggested that the fermentative bacteria metabolic activities were similar. This implied that the instability and failure, as seen in previous 6% glucose experiments was not due to the utilization of glucose by the fermentative group.

The results obtained on Day 5 from these two fermentations were not similar. The utilization of glucose in the 6% glucose fermentor did not increase over Day 3 as observed in the 6% glucose plus 1% acetate fermentor. The rate of glucose utilization increased up to Days 3 and 5, in the 6% glucose fermentations, thereafter decreasing whereas, in the 6% glucose plus 1% acetate fermentation the glucose utilization rate continued to increase. These observations along with the fact that glucose is the major energy substrate for the fermentative bacteria suggested that the differences observed in these two fermentations on Day 5 in the rates of glucose utilization may be due to either changes in the acetogenic or methanogenic groups or possibly both.

Comparison of Glucose Utilization by Both the ^{13}C-nmr and Enzymic Methods

Both, ^{13}C spectroscopy and the enzymic assay techniques were employed to monitor the glucose utilization in a 6% glucose fermentor on Day 1 and 3. The purpose was to determine whether there was a correlation between these two techniques. An example of the time sequence of glucose utilization compared by these two methods is shown in Figure 8. In the ^{13}C-nmr technique, the relative glucose concentration was reflected by the peak intensities. Peak intensities do not represent the absolute concentration (21,26,63), however, a plot of intensity vs time (^{13}C-nmr) should correlate with concentration vs time. Figure 8 shows the glucose utilization on Days 1 and 3 were also calculated from both techniques. As seen in Table 6 the rate constants obtained by the enzymic assay of Peterson and Young (53) correlated well with those obtained from nmr measurements. These observations demonstrated that ^{13}C-nmr spectroscopy can be employed as a useful method in the study of anaerobic digestion in general, but also as a method by which rate data can be obtained.

Metabolic Pathways of Propionate and Acetate Production

There are two major pathways in the anaerobic digestion process by which propionate can be produced. The first, being the randomizing pathway, where a label from 1-glucose would result in randomization of

the label in the methyl and methylene of propionate. The second pathway, the acrylate pathway would produce methyl labelled propionate from ^{13}C-1-glucose. In Figure 11, the peak intensities of the methylene and methyl of propionate resulting from ^{13}C-1-glucose are shown. The intensity of ^{13}C-3 propionate in this spectrum was approximately 5 times greater then the ^{13}C-2 propionate. This result and the result obtained from natural abundance ^{13}C propionate, where the CH_3:CH_2 ratio was 1.16:1, indicated that the acrylate pathway was the predominate pathway for propionate production. This conclusion is further supported by the presence of lactate, an intermediate in the acrylate pathway (see Figure 6). Lactate which was observed early in the fermentation of glucose (see Figure 12) but was absent later in fermentation (see Figure 11) suggested that lactate served as a precusor of propionate.

There are six possible reactions in the anaerobic digestion process which could result in acetate (see Table 3). The metabolites which resulted from the fermentation of ^{13}C-1-glucose are shown in Figure 14. The metabolites shown are acetate, lactate and propionate. It can be seen that as the peak intensity of lactate starts to decrease, both the acetate and propionate peak intensities begin to increase. This suggested that the product acetate as well as the propionate (as has been suggested previously) was derived from the intermediate lactate. This suggestion is further supported by the

results (see Figure 14) which illustrated that the maximum relative concentration of lactate, propionate and acetate were 10.25:7.8:3.6, respectively. These results implied that the amount of ^{13}C label in the methyls of both the acetate and propionate accounted for most of the ^{13}C label present in the methyl of lactate and thus, the ^{13}C-1-glucose which is converted to acetate is resulting from the intermediate, lactate.

The spectrum of the supernant (shown in Figure 13) showed that, lactate, acetate and propionate arising from the ^{13}C-1-glucose, were products which had been excreted. These results indicated that lactate, was a product for one group (or groups) of microorganisms which served as a substrate for other microorganisms. Lactate could then be utilized to produce either the acetate or propionate.

H_2 Effects on Acetate and Propionate Production

The results shown in Figure 14 and 15 demonstrated that lactate and H_2 begin to increase immediately and simultaneously upon the addition of glucose. It has been demonstrated by Kaspar and Wuhrmann (36) that high concentrations of H_2 present in the system inhibited the oxidation of propionate to acetate. They have suggested, that the presence of H_2 results in the accumulation of reduced acids. These results suggested that the increase in lactate (a reduced acid) is not a direct result of the increase in H_2. If H_2 were the cause of

increased lactate production one would expect that the lactate concentration would increase after the increase of H_2. However, as both the H_2 and lactate production profiles were virtually identical, it strongly argues that the lactate was produced due to the limited supply of NAD^+.

Application of ^{13}C-NMR to Anaerobic Digestion

The utility of ^{13}C-nmr spectroscopy to the study of anaerobic digestion has been supported in the previous sections. It can be seen from Figures 8,9,10,11 and 12 that both glucose utilization rates and the metabolites of glucose can be monitored. The supernant results (Figure 13) and the results in Figure 14 demonstrated that with this method the interactions of microorganisms which comprise the ecosystem can be conveniently studied. One advantage which this technique has over others is that it allows one to study a relatively complex system non-invasively and obtain information on the interactions of the entire system in a short period of time. This is of particular importance to the anaerobic digestion process, where the interactions which occur among the three groups of microorganisms are greatly dependent on each other (13).

Utilization of Acetate

The observations of the utilization of acetate (see Table 7) indicated that the reaction was a first order rate equation. The data

obtained from Kaspar and Wuhrmann (36) for acetate utilization also fitted a first order rate equation with a correlation of 0.99. The rate constants obtained from the 6% glucose fermentation have shown (see Table 7) that the rate of acetate utilization decreased from Day 1 to Day 5. In the 6% glucose plus 1% acetate fermentation (Table 7) there was an increase in the rate of acetate utilization from Day 1 to Day 5. These results suggested that in the high energy load fermentors (6% glucose) the methanogenic activity of acetate utilization had diminished as the experiment progressed whereas, the utilization of acetate in the 6% glucose plus 1% acetate fermentations increased as the experiment progressed. These results and those of preliminary experiments, where it was demonstrated that the presence of acetate had a sparing effect (prevented digestor failure) suggested that the addition of acetate allowed enrichment of the methanogenic population. The fact that acetate utilization was observed to fit a first order rate equation implied that the change in rates was dependent on the population of the acetate utilizing organisms. Thus, one tentitive conclusion which could be drawn is that the decrease in the methanogenic activity involving the utilization of acetate in the 6% glucose fermentation and the increase in the 6% glucose plus 1% acetate fermentation was a result of a change in the methanogenic population. These results suggested that acetate splitting was not a rate limiting factor. This contradicts the conclusions of Kaspar and Wuhrmann (36),

where acetate splitting was suggested to be rate limiting.

Propionate Utilization

The utilization of propionate was shown to be a first order rate equation (see Table 8). The data on propionate utilization obtained from Kaspar and Wuhrmann (36) also fit a first order rate equation with a correlation of 0.98. The rate constants which were obtained for both the 6% glucose and 6% glucose plus 1% acetate fermentations suggested that propionate utilization was greater in the fermentation which contained the additional 1% acetate. From ^{13}C-1-glucose experiments in Day 1, it was observed that little propionate was produced. This suggested that the 10mM ^{13}C-3-propionate which had been added was a large percentage of the propionate in the digestors during the ^{13}C-nmr experiments on Day 1. One might assume that the rate constants obtained for propionate utilization on Day 1 for both fermentations would be similar to that of the non-stressed fermentor (see Table 8 - Day 0-manure feed only), as was observed for the rate constants of acetate utilization. However, this was not observed.

The rate constants for propionate utilization rates for Days 3 and 5 were virtually undetectable or very small. A logical explanation for these observations might be that since propionate oxidation was observed to be slow (rate constant was -0.10 h^{-1} with a half-life of 6.6 h) in the non-stressed fermentors, and since propionate had

accumulated in the stressed fermentors (80-120mM), the added ^{13}C-propionate (10mM) would have reflected approximately 10% the actual rate. Further, the large positive $\Delta G°$ for propionate oxidation (see Table 1) would indicate that a large $\Delta G°$ of activation would be required. Thus, the observations for Days 3 and 5 for propionate oxidation were unpredicted.

Due to the difficulties encountered with reproducibility in propionate utilization in the non-stressed fermentors, and the ^{13}C-propionate experiments from Day 1 for both fermentations, no firm conclusions can be drawn on propionate utilization rates. However, it is of interest to note that the rate constants for propionate utilization in the 6% glucose plus 1% acetate fermentation was found to be approximately 2 times slower on Day 1 than the non-stressed fermentor.

The rate constants for propionate utilization on Day 12 were obtained for 6% glucose plus 1% acetate fermentation. The rate constant was shown (see Table 8) to be approximately 5 times slower than the rate constant obtained for the non-stressed (Day 0) fermentor. This result did not fit the results from the previous experiments where the propionate which had accumulated in this fermentor (6% glucose plus 1% acetate) had stated to decrease by Day 12. These previous results suggested that the rate of propionate oxidation had increased greatly in order to have utilized the large amount of the propionate which had

accumulated. Thus, one would have expected the rate of propionate utilization to have been similar or faster than the rate observed in the non-stressed fermentor. A possible explanation for the deviation in the results obtained and those which had been hypothesized, could have been that the ^{13}C-propionate (10mM) added may have been 20% of the total amount of propionate which could have been present. From previous experiments it has been shown that on Day 12 the concentration of propionate could have been between 0-50mM. There is need for further study of propionate utilization to clarify the utilization of propionate in stressed fermentors.

Comparisons of Glucose, Acetate and Propionate Utilization

The results of propionate and acetate utilization on Day 0 (see Tables 7 and 8) and the glucose utilization on Day 1 (see Table 6) showed that the glucose and propionate utilization are relatively similar in the first six hours. However, after the first 6 h, glucose utilization was greatly enhanced while the propionate utilization remained the same. A comparison of the rate constants for propionate and acetate utilization showed that propionate utilization was slower by a factor of 2. Acetate and glucose rate constants, after the first 6 h of fermentation were similar. These results suggested that propionate oxidation after the first 6 h period on Day 0, was a slower process than either the rates of glucose or acetate utilization. This

suggested that propionate oxidation was the rate limiting process. These results are inconsistant with the conclusions of Kaspar and Wuhrmann (36) where acetate splitting was suggested to be rate limiting. However, more research is warranted to further clarify the utilization of propionate in stressed fermentors.

Glucose Fermentations of 2%, 4% and 6% Glucose

The results from fermentation of three different glucose concentrations are shown in Figure 16. The general profiles of glucose and lactate in the 2%, 4% and 6% glucose fermentations did not vary significantly. The propionate profiles, however, did show variations within the three concentrations of glucose. No appreciable amount of propionate was produced in the 2% glucose fermentation. In the 4% glucose fermentation propionate was produced, but the propionate concentration which did build up, decreased 5 h after the ^{13}C-1-glucose had been fed. The accumulated propionate in the 6% glucose fermentation did not decrease.

These different profiles seen for the propionate in the 2%, 4% and 6% glucose fermentations are consistent with the previous results from this laboratory. These results showed that the 4% and 6% glucose digestors, propionate accumulated and failure occurred within 16 d whereas, in the 2% glucose fermentation, little propionate accumulated and the fermentor remained productive. It appears from Figure 16 that

the 2% digestor was able to utilize the propionate as it was being produced (or that little propionate was produced from the glucose). The 4% and 6% glucose fermentor showed that either more propionate was produced from the glucose, or it was utilized slowly (or it could be the result of both). This provided some insight into the failure which occurred in these digestors, and supports the tentative conclusion drawn in the previous section that the utilization of propionate appears to be a very critical step in the stability of the anaerobic digestion process.

Intermediates of Propionate Catabolism

The results (not shown) from the propionate utilization experiments demonstrated that the ^{13}C-3-propionate which was administered to a digestor resulted in a small amount of the label was present in the methylene carbon of the propionate. These observations precipated the study of the catabolism of propionate.

The results of the addition of propionate in both non-stressed and stressed fermentors (see Figures 17, 18 and 19) showed that the ^{13}C label of the methyl carbon in propionate has been randomized. This randomizing resulted in the ^{13}C label in the methylene of the propionate.

The presence of the ^{13}C label in the methylene of the propionate indicated that the randomizing pathway has been reversed. The spectrum

shown in Figure 19 with the tentative assignments of succinate (not shown) and malate, intermediates in the randomizing pathway, indicated that the randomizing pathway was employed. The presence of lactate, an intermediate in the acrylate pathway was also present in both the non-stressed and stressed fermentor indicated that the acrylate pathway had also been reversed.

These results suggested that both the acrylate and randomizing pathways which can be used in the anabolism of propionate were used to catabolize the propionate, to the product acetate. The absence of a resonance for the C-2 of lactate suggested that there are at least two different species involved in the propionate catabolism. The C-2 of lactate would have become labeled if there had been one species utilizing both the acrylate and randomizing pathways. The absence of the C-2 labeled lactate also suggested that the randomized propionate and succinate had not been excerted as products into the media. This would have provided randomized propionate available to the acrylate utilizing species resulting in ^{13}C-2-lactate. These results are in need of further investigation.

SUMMARY

The application of ^{13}C-nmr spectrosocpy has been shown to provide a useful method to study the metabolism of glucose in the anaerobic digestion process. Acetate, lactate and propionate, metabolites of glucose were observed to be produced from the ^{13}C-1-glucose. Kinetic data on glucose utilization which was obtained corresponded well to the kinetic data from the enzymic procedures also used. The rate data obtained on glucose utilization was shown to be a first order rate equation. The changes observed in the rate constants in both the 6% glucose and 6% glucose plus 1% acetate fermentations indicated that enrichment had occurred in the fermentative populations. There were no significant changes in the glucose utilization rates between the two types of fermentations used until Day 5. It was observed on Day 5 that there was little increase in the fermentative populations in the 6% glucose fermentors. This suggested that in these fermentations that glucose utilization had been affected by either changes in the acetogenic or methanogenic activity, or both.

The metabolic pathways used for propionate and acetate production were investigated. It was found that the acrylate pathway (utilizing the intermediate lactate) was predominant in the production of propionate, and acetate was produced from lactate. The production of lactate from glucose did not appear to have been a result of the increased H_2 concentration in the system but was assumed to be due to the lack of available NAD^+ present.

The relative rates of acetate utilization were investigated in 6% glucose and 6% glucose plus 1% acetate. It was shown that acetate utilization was a first order rate equation. The decreasing rates obtained in the 6% glucose fermentations over the five day experimental period suggested that the methanogenic activity in these fermentor had greatly diminished. However, the increasing rates observed for the 6% glucose plus 1% acetate fermentation indicated that the addition of acetate provided enrichment of the methanogenic population and that acetate splitting was not rate limiting as suggested in previous studies (36).

The rate of propionate utilization was investigated and was found also to be a first order rate equation. However, the rates which were obtained for Day 1 in the 6% glucose and 6% glucose plus 1% acetate, and Day 0 allowed no firm conclusions to be drawn at the present time concerning propionate utilization in stressed and non-stressed fermentations.

Acetate and propionate utilization were compared in a non-stressed fermentor, only. It was shown that the oxidation of propionate was slower by a factor of 2 than the acetate splitting. These conclusions and the results of the rates of glucose utilization suggested that propionate oxidation is a rate limiting factor in the anaerobic digestion process. This would suggest that this is part of the problem which confronts digestion efficiency in the production of methane.

The spectra which were obtained after the addition of ^{13}C-3-propionate, showed the C-2-propionate, C-3-lactate, succinate and malate resonances. The presence of these metabolites indicated that both the acrylate and randomizing pathways had been utilized in propionate catabolism. Absence of the C-2-lactate resonance suggested that there are at least two different species involved in the utilization of propionate.

APPENDIX 1

^{13}C-NMR Parameters

The experimental parametes listed below were used in all of the ^{13}C-glucose nmr experiments described in the Materials and Methods Chapter.

sweep width - 20,000 Hz

pulse delay - 1.8 s

pulse width - 10.0

pulse angle - 41°

receiver gain - 800

broad band proton decoupling, low pass frequency filter (rejects 250 MHz).

deuterium external lock

data points used - 8K

The number of scans used, varied for each day of the experiment. Day 1 experiments scan number was 2520. Days 3 and 5 experiments scan number for the first two hours after the ^{13}C-glucose was administered were 280, the following 10 h the scan number were 1680.

The experimental parameters listed below were used in all of the ^{13}C-acetate nmr experiments

sweep width - 20,000

pulse delay - 0.6 s

pulse width - 15.0

pulse angle - 61°

receiver gain - 800

broad band proton decoupling, low pass frequency filter (rejects 250 MHz).

deuterium external lock

data points used - 8K

scan number - 1000

The experimental parameters listed below were used in all of the ^{13}C-propionate nmr experiments.

sweep width - 5,000

pulse delay - 0.6 s

pulse width - 15.0

pulse angle - 61°

receiver gain - 800

borad band proton decoupling; low pass frequency filter (rejects 250 MHz)

deuterium external lock

data points used - 4K

scan number 1333

The experimental parameters used in supernant, NOE, and propionate

metabolite experiments were:

sweep width - 20,000

pulse delay - 1.8 s

pulse width - 10

pulse angle - 41°

receiver gain - 800

broad band proton decoupling; low pass frequency filter (rejects 250 MHz).

dueterium external lock

data points used - 16K

APPENDIX 2

Chemical Shifts in ppm

Acrylate - $CH_2 = CH - COO^-$

56.7 62.6 135.0

Ethanol - $CH_3 - CH_2OH$

17.7 58.4

Butyrate - $CH_3 - CH_2 - CH_2 - COO^-$

13.8 19.3 38.0 nd

Propionate - $CH_3 - CH_2 - COO^-$

10.8 31.2 185.0

Acetate - $CH_3^- - COO^-$

24.1 182.0

Lactate - $CH_3 - CHOH - COO^-$

20.8 69.4 183.3

Pyruvate - $CH_3 - CO - COO^-$

27.1 nd nd

Succinate - $^-OOC - CH_2 - CH_2 - COO^-$

nd 30.5 nd

Malate - $^-OOC - CHOH - CH_2 - COO^-$

nd 67.5 39.6 nd

Bicarbonate - HCO_3^-

161.0

APPENDIX 3

Micro-programs used on the spectrometer.

The following micro-program was used for the glucose experiments, with the parameters listed in Appendix 1.

Day 1 - program ZE - zeros the number of scans

GO - starts to accumulate the FID*

WR FILE - write the FID onto disk

IF FILE - incrediments the file

LO 1 TIMES X - collects FID for X times

Day 3 and 5 programs

1 RJ PARA - Reads parameters for first 2 h period

2 IF PARA - Increments parameter table to the parameters for last 10 h period

3 ZE - zeros the FID-starts 1st 2 h period.

4 GO - starts the accumulation

5 WR FILE - writes the FID in a FILE

6 IF FILE - increments the FILE

7 LO 3 TIMES X - collects FID for X times in the 2 h period

8 RJ PARA - reads the parameter listing for 10 h period

9 ZE - zeros the FID

10 GO - starts accumulation

11 WR FILE - writes the FID

12 IF FILE - increments the FILE

13 LO 9 TIMES X - loop to step 9; X times = collect FID for X times in 10 h period.

14 EXIT - stops the program

The following micro-program was used in both the ^{13}C-propionate and ^{13}C-acetate experiments.

1 ZE - zeros the FID

2 D1 - a delay period

3 GO - starts the accumulation

4 WR FILE - WRITES the FID onto the disK

5 IF FILE - incrediments the FILE

6 LO 1 TIMES X - loop to step 1 to start the acquistions of the accumulations

*FID - free induction decay - a sum of radio frequency pulses.

APPENDIX 4

The Nuclear Overhauser Effect

The nuclear Overhauser effect (NOE) is the intensity change of the nmr signal which occurs when carbon nuclei are in close proximity to proton nuclei which have been perturbed by irradiation (66). The maximum increased enhancement of a signal which can result from the NOE is 2.987 in a ^{13}C proton decoupled spectra. However, the enhancement due to the NOE on different carbon resonances can vary depending on the relaxation mechanisms present and the internuclear distance. For the maximum NOE enhancement, the internuclear distance must be short and the relaxation mechanism present must be only that of dipole-dipole interactions (29).

Carbon nuclei possesses two relaxations processes: 1) spin-lattice (T_1) relaxation and 2) spin-spin (T_2) relaxation. These relaxation processes allow the spin populations in the ^{13}C nuclei, which have been perturbed by an electronic pulse in a magnetic field, to return to their equilibrium state. The T_1 process affects the z-component of the magnetization process and T_2 affects the x and y components of the magnetization (29). The T_1 relaxation processes are of primary concern to the NOE, therefore this discussion will include only T_1 mechanisms which are of most concern to the NOE.

The two principle mechanisms which contribute to the T_1 relaxation process and affect the NOE are: 1) dipole-dipole and 2) spin-rotation. The principle relaxation mechanism in carbon atoms containing directly

bonded protons is the dipole-dipole mechanism (1). The NOE is dependent on this T_1 relaxation mechanism (42). The spin-rotation relaxation mechanism is important in small symmetrical molecules. When the spin-rotation mechanism is the principle T_1 relaxation process, the overall relaxation time increases and the enhancement from the NOE does not occur. Signals arising from ^{13}C nuclei affected by this relaxation mechanism are usually lowered in intensity (1).

The enhancement of the NOE is a result of an increase in the spin populations in the lower energy levels in the ^{13}C nuclei (E2 and E4 see Figure 20). When ^{1}H nuclei are fully irradiated, the population in the higher energy levels, E1 and E2, are increased. The transitions W_0 and W_2 from E2$\longleftrightarrow$E3 and E1$\longleftrightarrow$E4, respectively, are normally forbidden in absorption irradiation. However, when dipolar-dipolar relaxation mechanism is present (which is a radiation independent mechanism) the W_0 and W_2 transition are possible. These transitions (W_0 and W_2) allow the populations of the E3 and E4 energy states to increase. Thus, enhancement of the carbon signal due to the NOE will result (66).

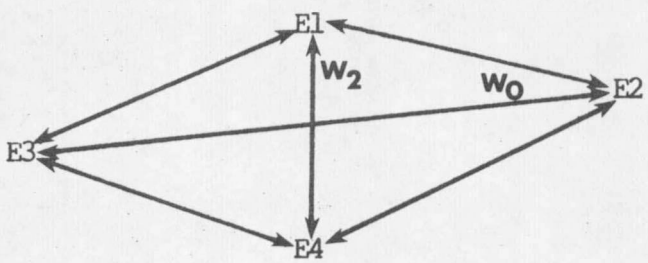


Figure 20. Representative energy levels in ^{13}C and ^{1}H nuclei.

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