Studies of cyanide assimilation in Klebsiella pneumoniae by using high resolution carbon-13 and nitrogen-15 nuclear magnetic resonance spectroscopy

by Ju Mee Lee

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 cyanide assimilation in Klebsiella pneumoniae and ammonia incorporation in K. pneumoniae M5al strain were studied under anaerobic and aerobic conditions. Cyanide reduction, utilization of glucose and ammonia assimilation were observed by carbon-13 nmr spectroscopy and nitrogen nmr spectroscopy.

In addition to metabolic products from glucose, under anaerobic conditions reduction of cyanide ion can be observed leading to an unknown compound with a methyl resonance at 16.5 ppm. Under the constraints of low concentration imposed by cyanide toxicity, no intermediates were detected nor was methylamine observed. Cyanide was not metabolized under aerobic conditions, consistent with an earlier suggestion and also consistent with the anaerobic metabolism of cyanide being mediated by nitrogenase. The ammonia assimilation in K. pneumoniae with glucose as an energy source was not observed despite extensive variation of experimental conditions. However, the nmr results obtained clearly show that using nitrogen-15 nmr to study ammonia assimilation In vivo is feasible from the concentration point of view.

The conditions for observing cyanide metabolism and related reactions In vivo by nmr are discussed. Chemical shifts of products and reactants are reported along with the relative rates of appearance or disappearance. The natural abundance carbon-13 spectra of K. pneumoniae under anaerobic and aerobic conditions have been observed and are shown to be significantly different.

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STUDIES OF CYANIDE ASSIMILATION IN *KLEBSIELLA PNEUMONIAE* BY USING HIGH RESOLUTION CARBON-13 AND NITROGEN-15 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

by

JU MEE LEE

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Chemistry

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August, 1982

ACKNOWLEDGEMENT

The author wishes to express her gratitude to her major advisor, Dr. Edwin H. Abbott for his teaching, support and encouragement. She would also like to express her thanks to Dr. Jesse Jaynes for his help with preparation of *K. pneumoniae* strains and to Dr. John E. Robbins for his helpful discussion and advice. She wishes to express her deep gratitude to Dr. Bradford P. Mundy, who continuously encouraged her research work.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

ABSTRACT

The cyanide assimilation in Klebsiella pneumoniae and ammonia incorporation in K. pneumoniae M5al strain were studied under anaerobic and aerobic conditions. Cyanide reduction, utilization of glucose and ammonia assimilation were observed by carbon-13 nmr spectroscopy and nitrogen nmr spectroscopy.

In addition to metabolic products from glucose, under anaerobic conditions reduction of cyanide ion can be observed leading to an unknown compound with a methyl resonance at 16.5 ppm. Under the constraints of low concentration imposed by cyanide toxicity, no intermediates were detected nor was methylamine observed. Cyanide was not metabolized under aerobic conditions, consistent with an earlier suggestion and also consistent with the anaerobic metabolism of cyanide being mediated by nitrogenase. The ammonia assimilation in K. pneumoniae with glucose as an energy source was not observed despite extensive variation of experimental conditions. However, the nmr results obtained clearly show that using nitrogen-15 nmr to study ammonia assimilation in vivo is feasible from the concentration point of view.

The conditions for observing cyanide metabolism and related reactions in vivo by nmr are discussed. Chemical shifts of products and reactants are reported along with the relative rates of appearance or disappearance. The natural abundance carbon-13 spectra of K. pneumoniae under anaerobic and aerobic conditions have been observed and are shown to be significantly different.

INTRODUCTION

A. Goals of this research

This work was undertaken to explore the application of nuclear magnetic resonance (nmr) techniques to the in vivo study of chemical reactions and structure determination. Nmr holds great promise for such studies because it is nearly perfectly non-invasive. However, few studies have been reported and generally on rather well understood systems under the most favorable conditions. The reduction of cyanide ion by nitrogen fixing bacteria was selected for the present study. Cyanide ion is isoelectronic with dinitrogen and may react via a similar pathway. At the most, we hoped to discover new intermediates in this important and poorly understood reaction. At the least we wanted to explore the application of the new and potentially very important nmr methods to a more difficult system at the very limits of concentration imposed by current state-of-the-art instrumentation.

B. The current state of application of nuclear magnetic resonance (nmr) to in vivo biochemical studies

Since the discovery of nuclear magnetic resonance (nmr) nearly thirty years ago, there have been a great number of applications by chemists, mostly to problems of structure elucidation of organic compounds by proton and carbon-13 techniques. Only since 1970, have nmr methods been employed as non-invasive techniques to investigate biological and biochemical problems in vivo. This is largely because it has only been in the last 15 years that nmr spectrometers have been built sufficiently sensitive to detect nuclear magnetic resonance signals at biological concentrations. Even with the most sensitive spectrometers available today, only the most concentrated cell systems can be studied. Moreover, only the most straightforward methods of direct observation have been used in vivo despite the fact that there has been a great deal of work developing dynamic methods and applying them to the study of pure compounds. For example, the application of relaxation phenomena, nuclear overhauser enhancement measurement, or two-dimensional fourier techniques have not been made to in vivo system despite the considerable amount of useful information they have yielded in simpler systems.

To apply nmr spectroscopy to intact cells, especially carbon-13 and/or nitrogen-15 nmr, there are some problems which should be dealt with in order to use nmr more effectively in the study of biologically significant molecules occurring in vivo (1). First, there is the sensitivity problem, which has been greatly improved by

Fourier Transform techniques but is clearly not sufficient to study the lowest concentrations yet.

For example, from the concentration point of view, studying glycolysis with carbon-13 nmr in microorganisms is a far different problem than studying cyanide reduction. To study cyanide reduction in K. pneumoniae the maximum concentration which can be used is only 20 micrograms per mL because of high toxicity of cyanide. In contrast, 20 mM of glucose was the concentration used for glycolysis studies in E. coli. As will be shown later, cyanide at this concentration is barely observable whereas the glucose is easily detected.

The second main problem of biological nmr is the assignment of resonances to a molecular structure. Unless the compounds of interest have already been assigned under similar conditions, the standard method (experimental section) is the only way to assign resonances.

As far as nmr studies of metabolism of intact cells are concerned, there has not been much work reported. The few studies that have been done show that application of nuclear magnetic resonance spectroscopy is a potentially powerful method to trace metabolic pathways. For example, in 1972, Matiwiyoff and Needham

(2), reported the carbon-13 nmr study of red blood cell suspensions. They showed that carbon-13 nmr will be a powerful tool for the study of the analysis, structure, and dynamics of intra-cellular constituents and the binding of extra-cellular substrates in cell suspension. In 1975, Schaefer, Slejskal, and Beard (3), reported the study of metabolism in intact, fresh soybean ovules harvested from pods, which showed that comparison of the carbon-13 nmr spectra permited a qualitative estimate of sugar metabolism and rates of lipid synthesis and provided a way of estimating the extent to which glucose is degraded by phosphogluconate pathway. Recent elegant studies of intact cell using phosphous-31, carbon-13 nmr have been performed by Richard (4), and Schulman (5-9). Those studies have shown that this non-invasive technique can be utilized to follow concentration variation and kinetics of metabolites in vivo. They succeeded in tracing glucose metabolism in Escherichia coli in 1978, anaerobic glycolysis in suspension of yeast cell in 1979, and gluconogensis from alanine in hepatocytes from euthyroid and hyperthyroid rats in 1980. In 1981, Robbins et. al. (41-45) reported the studies of metabolic pathways in anaerobic digestors using carbon-13 nmr spectroscopy. These studies clearly demonstrated that nmr can be an extremely powerful way to measure the comparative rates of utilization of a variety of substrates (44) and to study catabolism in living cells in vivo (43).

C. Application of carbon-13 and nitrogen-15 nmr spectroscopy to nitrogen fixation

Through 1981, only two different investigations of nitrogen fixation or related reactions using nmr spectroscopy have been reported. The first was by J. R. Dilworth and R. L. Richard (37), stimulated by M. E. Val'pin (10, cited from 37). This paper concerns metal complexes relevant to nitrogen fixation. It shows that nitrogen-15 nuclear magnetic resonance spectroscopy provides a means of tracing alternate mechanisms of reaction of substrates proposed by several different authors (11, cited from 37) in vitro. The work involved diazenide-, nitrene-, and nitrido complex intermediates (Figure 1) in cell free system. It concluded that nitrogen-15 nmr spectroscopy can be applied to studies of protonation of dinitrogen complex in solution, where hydrazido(2-) complex intermediates have been clearly observed, and to other reactions which lead to the degradation or substitution of nitrogen.

The other investigation was by B. E. Smith, and N. F. Thorneley (12). It is the study of N_2-reduction by nitrogenases from K. pneumoniae using nitrogen-15 nmr spectroscopy. This work was

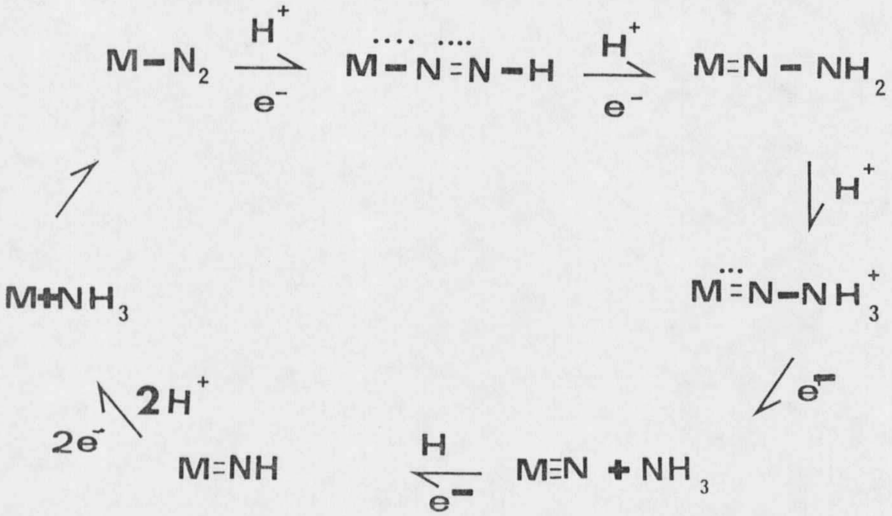


Figure 1. The reactions of dinitrogen complexes leading to the formation of ammonia. A suggested mechanism for reductive degradation of dinitrogen to ammonia (37).

restricted to the study of disappearance of dinitrogen. They reported at least one very strange phenomenum which could not be explained. No resonances could be observed associated with the formation either of intermediates or nitrogen-15 labeled product resonances despite varying the temperature of experiment.

D. Glucose metabolism in *K. pneumoniae* as a facultative bacterium

Anaerobic bacteria have been classified into three major groups (33). Bryant (35) has classified three major groups as the fermentative, acetogenic, and methanogenic bacteria. *K. pneumoniae* which is a facultative bacterium, can metabolize glucose both anaerobically and aerobically. Those metabolic pathways are shown in Figure 2.

The production of methane by polysaccharide metabolism results from the interaction of the three groups of anaerobic bacteria. Although the fermentative group, so called the acid former, does not produce methane, they have an important role in its production. They can convert complex organic material into usable substrates for the methanogens, i.e., those bacteria that have the function of breaking down the polymeric substrates into simple organic molecules such as organic acids, alcohols, H_2O, and CO_2 (35).

A. Under anaerobic conditions.

glucose

fermentation

fermentation product

(acetate, formate)

B. Under aerobic conditions.

glucose

fermentation

(fermentation product)

O_2, respiration

$CO_2 + H_2O$

Figure 2. The pattern of glucose utilization in facultative organisms. The fermentative pathway is common to both the anaerobic (A) and the aerobic (B) pathway of glucose utilization (39).

Most strains of K. pneumoniae can use citrate and glucose as the sole carbon source and ammonia as the nitrogen source. Glucose is fermented with the production of acid and gas (more CO_2 than H_2). Most strains also produce 2,3-butanediol as a major product of the fermentation of glucose. Lactic, acetic and formic acids are formed in smaller amounts. Ethanol is a suggested second major product.

E. Environmental pollution and microbial degradation of cyanide

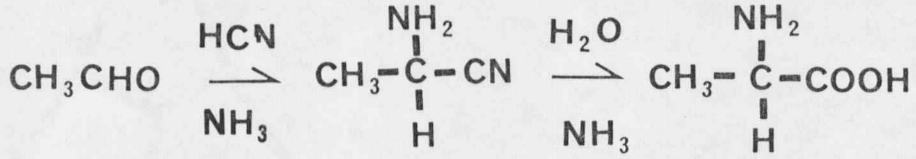
While we were studying cyanide as a model for dinitrogen reduction in K. pneumoniae to trace the pathway of nitrogen fixation, we found that cyanide assimilation and detoxification is a major topic not only to biochemists, but also to the environmental and ecological research workers. Cyanide is one of the major components in waste metal plating, metal hardening, pharmaceuticals, synthetic fiber, and plastic industries. Treatment of cyanide waste has become a great issue because of the expensiveness of chemical methods of treating inorganic, organic, and even complex forms of cyanide. Pretreatments of cyanide by biological processes by using microorganisms which can metabolize cyanide complexes have been utilized (13-16). So far, microorganisms which can be grown with cyanide as a sole source of carbon and nitrogen have not been found. However, two microorganisms Baccllus and Klebsiella appear to be the prominent species which can metabolize cyanide in nature. Experiments which have been performed to determine whether or not cyanide was being metabolized by Klebsiella were done at the School of Biological Science, Oklahoma State University (16).

F. Cyanide assimilation by microorganism other than K. pneumoniae

As a one carbon compound, cyanide utilization has been extensively studied especially because of its scientific (17), and industrial (18) importance. There have been no reports of the metabolic pathways of cyanide assimilation in non-cyanogenic bacteria although they are able to utilize it as a nitrogen source. However, cyanide is a relatively simple ion, and it is possible to predict some of the alternate pathways of its incorporation.

Formamide hydrolyase (cyanide hydratase), purified from the Fungus D. loti, degrades cyanide to formamide (19). Many methylotrophic bacteria can grow on formamide as a carbon and nitrogen source (17), and it is therefore possible that some methylotrophs or closely related organisms are able to utilize cyanide (20). Cyanide assimilation into several other fungi has been tested (21), and as a result, Pholiota adiposa, Pholiota aurivella, and Pholiota praecox have been found to transform cyanide into alanine and, to a lesser extent, into other amino acids. *Fusarium nivale* incorporated cyanide only into asparagine.

According to Strobel (22), labeled alpha-aminopropionitrile and L-alanine were formed when myclia of snow mold *basidiomycete* were incubated with cyanic acid. The following metabolic pathway has been suggested (Figure 3). Also glutamic acid formation, alpha-aminobutyric acid formation, beta-cyanoalanine formation, and gamma-cyano-alpha-amionbutyric acid formation have been suggested in several different studies (23-26). Even though it is known that cyanide is readily converted to the amino acid, alanine, and other biologically important compounds (27, 28) virtually nothing is understood about cyanide assimilation by anaerobic microorganisms (20).



$$CH_3CHO \xrightarrow[NH_3]{HCN} CH_3-\overset{NH_2}{\underset{H}{C}}-CN \xrightarrow[NH_3]{H_2O} CH_3-\overset{NH_2}{\underset{H}{C}}-COOH$$

Figure 3. Suggested metabolic pathway cyanide into alaine in mycelia of the snow mold *basidiomycete* (22).

Hardy et. al. (29, 30) suggested that nitrogenases are able to reduce compounds such as N_2O, azide, acetylene and relate compounds including conversion of hydrogen cyanide to methane and ammonia, where CH_3NH_2 has been proposed as the intermediate (38). The suggested optimal concentration of cyanide for reduction by nitrogenases in _Azobacter vinelandii_ was found to be 40 mg per liter. Dihydrogen, which inhibited nitrogen fixation completely, did not inhibit reduction of azide, acetylene and cyanide (31). When _Klebsiella pneumoniae_ is grown aerobically, no nitrogenase is synthesized. The reason proposed by Brill (32) is that O_2 rapidly inhibits all the proteins related to nitrogenase, i.e., O_2 inactivates the nitrogenases.

G. Current research about cyanide assimilation in _K. pneumoniae_

As mentioned above (Introduction - Section C) most studies of the cyanide assimilation in living cells has been performed by environmental, and ecological research workers. Of relevance to the present work little has been illustrated except that aerobically _K. pneumoniae_ cannot metabolize cyanide (16) (Figure 4).

There is a single interesting paper by Kelly et. al. (34) about _in vitro_ studies of nitrogenase from _Klebsiella pneumoniae_ with

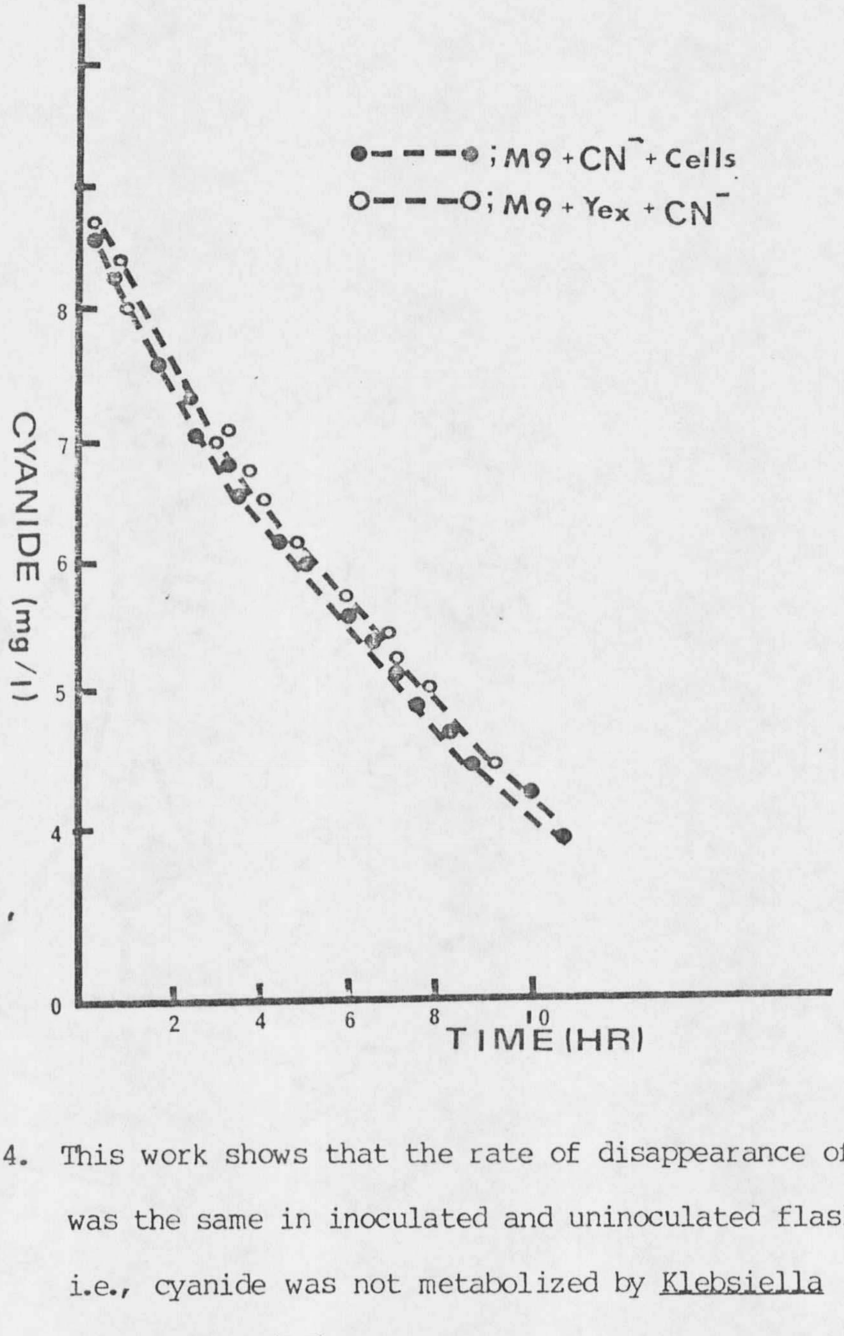


Figure 4. This work shows that the rate of disappearance of cyanide was the same in inoculated and uninoculated flask, i.e., cyanide was not metabolized by Klebsiella aerobically (16).

Y_{ex} - Yeast Extract

cyanide. That work has shown that methane was formed anaerobically in K. pneumoniae, and that a very small amount of acetylene was detected in the absence of nitrogenases or $Na_2S_2O_4$.

H. Relationship between cyanide reduction and nitrogen fixation in microorganisms and in nitrogen-fixing bacterium

There has been an assumption that cyanic acid reduction is catalyzed by nitrogenases in nitrogen fixing bacteria. This assumption is based on the observations of in vitro reactivity of cyanide ion with several nitrogenases. It has also been stated that cyanide can be a misleading model for understanding dinitrogen reduction and that the reaction of cyanide by nitrogenase is a non-specific process probably not connected with dinitrogen fixation (34).

It has been generally agreed that K. pneumoniae and Baccillus ploymyxa are not able to fix dinitrogen aerobically. It has been shown that alternative substrates for reduction of dinitrogen are mostly the low molecular weight compounds with triple bonds between two nitrogen atoms (e.g., azide), between two carbon atoms (e.g., acetylene), or between carbon and nitrogen atoms (e.g., cyanide). It is often stated that the compounds can only be reduced by nitrogenases under anaerobic conditions.

In summary, on the basis of the literature we can tentatively assume that in Klebsiella pneumoniae, cyanide reduction can be brought about by nitrogenases anaerobically, and the end products by cyanide assimilation should be ammonia and methane as major products and methylamine as a minor product (Figure 5).

Major product

HCN $CH_4 + C_2H_6 + C_2H_4 + NH_3$

anaerobically

by nitrogenase $+ CH_3NH_2$

Substrate Minor product

Figure 5. Suggested products of cyanide reduction.

I. Assimilation of ammonia in microorganisms

Although amino acids are of central importance in metabolism of all organisms, the ability to synthesize amino acids varies considerably. For example, the bacterium Leuconostoc mesenteroids cannot grow unless it is supplied with a total of 16 different amino acids. Another bacterium, such as E. coli can manufacture all amino acids starting from ammonia. In this study, we have attempted to trace ammonia assimilation in Klebsiella pneumoniae by using nitrogen-15 nuclear magnetic resonance spectroscopy.

NH_3 + alpha-Ketoglutarate + NADPH + H^+

L-Glutamate + $NADP^+$ + H_2O

NH_3 + GLUTAMATE + ATP

glutamine
synthetase

GLUTAMINE + ADP + P

Figure 6. Ammonia assimilation in microorganism

MATERIALS AND METHODS

A. Carbon-13 nmr

1. Growing Conditions

The Klebsiella pneumoniae strain M5al was grown in two different ways. One way was aerobically at 37°C in M9 minimal medium (Table 1) containing NH_4Cl as a nitrogen source, 20 mL of 20% glucose, 1 mL 1.0 M $MgSO_4$, and 1 mL 0.1 M $CaCl_2$. The other way was in nutrient broth medium without any supplement.

Cells were harvested by centrifugation at 4°C for 10 minutes, washed twice and suspended in distilled water.

2. Special treatment for anaerobic and aerobic conditions

For anaerobic experiments, 2 mL of suspended cell extracts were kept under argon for 24 hours at room temperature. For aerobic conditions, O_2 gas was bubbled through the suspension for approximately 24 hours until it was studied by nmr spectroscopy. Nmr tubes were sealed with parafin film with argon or oxygen gas for the duration of the experiment. Even though the high density of extracts used in our samples will result in the gradual conversion from aerobic to anaerobic conditions during the nmr experiment, it can be accepted that all the nitrogenases reduce cyanide to methane

TABLE 1

Components of M9 Minimal Medium (g/L)

60 g	Na_2HPO_4	
30 g	KH_2PO_4	
5 g	NaCl	
10 g	NH_4Cl/1000 mL H_2O	
20 mL	20% glucose	
1 mL	.1 M $MgSO_4$	added after
1 mL	.1 M $CaCl_2$	autoclaving

will have been destroyed by bubbling O_2 through the suspension for 24 hours at the rate of approximately 20 mL per minute. To maintain anaerobic or aerobic conditions, a syringe was used to inject the substrate and the energy source into the nmr sample. The pH was adjusted to near 7.0 using buffer solution (Table 2). The nmr tube, containing the cyanide, was then sealed with parafin film without further bubbling with argon, nitrogen, or oxygen to avoid the loss of HCN to the gas phase which otherwise occurred at pH near 7.0 and at 24°C - 30°C.

TABLE 2

Buffer components for *K. pneumoniae* M5al strain (pH = 7.0)

8.5 g	NaCl
5.7 g	K_2HPO_4
3.4 g	KH_2PO_4 in 1 L

3. Spectrometer parameters

Carbon-13 nmr spectra at 62.83 MHz were obtained using a Bruker WM-250 MHz Fourier Transform nmr spectrometer. A delay time of 0.1 second, 45° degree pulse, and overall repetition times of 0.2028 seconds were used unless otherwise specified. For kinetic work, spectral accumulation time varied according to the experiment and the FID was stored continuously on a disk using an autoprogram (all details appear in Appendix 1).

To maintain the sample rigorously anaerobic without adding deuterium, the spectra were run without an external reference. The shim coil was pretuned with the model sample which has exactly the same contents as the sample tube except that it also contained D_2O. The sample was then run with the lock circuit switched off. Linewidths of less than 2 Hz were achieved in this way, indicative of good magnet stability and field homogeneity. In order to reference the chemical shift, the spectrum of para-dioxane and buffer (pH = 7.0) was recorded separately and added to each spectrum. All chemical shifts were referred to tetramethylsilane by adding 67.4 ppm to the shift referenced to the para-dioxane resonance. These techniques minimized sample handling and maintained the nmr sample tube anaerobic or aerobic more effectively than if a lock substance were added. Temperature variation is a third control factor besides NH_4^+ and O_2 governing the expression of nif-genes of K. pneumoniae (36). Therefore during long accumulations, temperature was set at 30°C and it was maintained by flowing air into the probe.

B. Nitrogen-15 nmr

1. Sample preparation

The Klebsiella pneumoniae strain M5al was grown aerobically at 37°C in M9 minimal medium containing NH_4^+Cl only as a nitrogen source. Cells were harvested in the same way as in the carbon-13 experiments except that they were suspended in a buffer solution at pH 7.0.

As an energy source glucose was added without any special treatment. Argon gas was used to maintain the 15 mm diameter nmr tube in an anaerobic state. The other parts of sample preparation were exactly the same as in the carbon-nmr experiment.

2. Spectrometer procedure

All the techniques described above - unlocked running, sample spinning, temperature control, and autoprogram were applied to nitrogen-15 nmr experiment except that chemical shifts were referred to NH_4^+ at 25.47 ppm.

A delay time of 0.3 second, 30° degree pulses, and an overall repetition time of 0.759 second were used for acquisition parameters.

C. Assignment of each resonance

1. CIS computer chemical research system

To assign resonances produced by cyanide reduction, the CIS research system in Washington D.C. was used. Deviation of 1 ppm was allowed.

2. Standard method

In order to confirm assignments of the resonances, the method of standard addition was employed. A small amount of the possible compound was added directly to the nmr sample tube containing the compound in question.

Comparison between the spectra prior to any addition and one with the compound added, was the main tool in assigning the resonance.

D. Chemicals used

Carbon-13 labeled cyanide and nitrogen-15 labeled ammonia were purchased from Stohler Isotope Chemicals. Normal dextrose, anhydrouse (D-glucose) was purchased from J. T. Baker Chemical. L-alanine, valine α-amino-n-butyric acid, and L-cysteine were purchased from Sigma. Those chemicals were used without further purification.

RESULTS

A. Natural abundance carbon-13 nmr spectra of the *K. pneumoniae* M5al strain under anaerobic and aerobic conditions

Carbon-13 nmr spectroscopy has become so sensitive that natural abundance carbon-13 signals can readily be observed in living systems. Natural abundance carbon-13 spectra of *K. pneumoniae* are of interest for two reasons in the present study. Primarily, when one is interested in detecting low concentration intermediates, from the reaction of enriched molecules, one must be able to distinguish the intermediates from the natural abundance background. Secondly, the *K. pneumoniae* M5al strain has different metabolic pathways depending on whether it is living anaerobically or aerobically. It is of interest to determine what differences are observed between the anaerobic and aerobic natural abundance spectra. Figure 7 illustrates the spectra for these two conditions. Although it is possible to assign all those resonances in principle, these resonances arising from the many different chemical components are far too complex to interpret. However, general assignments can be made from the natural components of microorganisms. For example, the resonances in the region of 15-30 ppm are largely due to

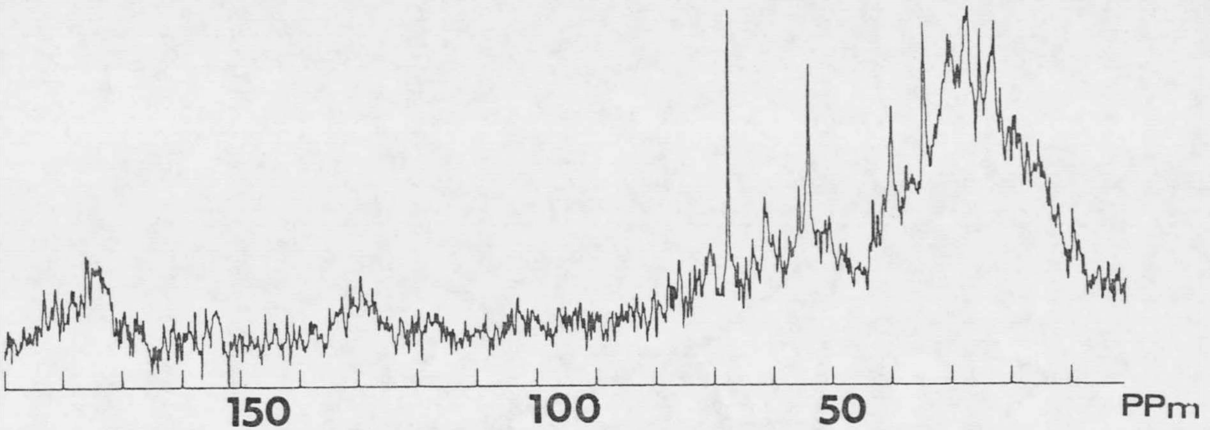


Figure 7a. Natural abundance carbon-13 spectrum of anaerobic <u>K. pneumoniae</u> M5al strain (NS = 100,000)

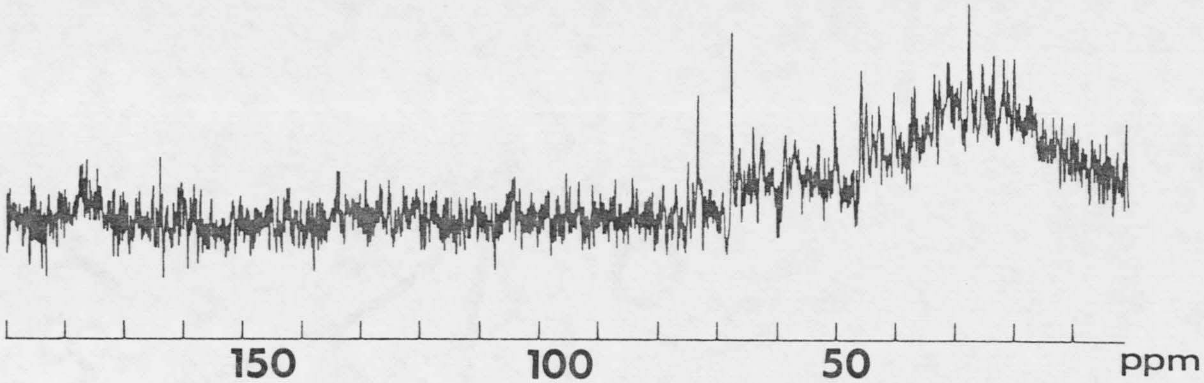


Figure 7b. Natural abundance carbon-13 spectrum of aerobic K. pneumoniae M5al strain (NS = 100,000)

saturated carbon atoms such as those in fatty acids and peptides while 60-80 ppm are largely those of carbohydrates.

As illustrated in Table 3, many resonances which are observed in the anaerobic case are absent under aerobic conditions. Generally, there is a difference in relative intensity when a resonance is common to both sets of conditions. This observation is not unexpected in view of the different metabolic characteristics of the aerobic and anaerobic Klebsiella pneumoniae M5al strains.

B. Conditions which inhibit metabolism of cyanide in K. pneumoniae

Experiments performed without any energy sources show no cyanide reactions at all. Also at concentrations of cyanide above 50 mg per liter, metabolism is virtually completely halted. This is demonstrated by the slow glucose consumption rate and the absence of new resonances except for traces of 2,3-butanediol at 20.5 ppm and 68.9 ppm.

C. Products of cyanide metabolism

1. Nutrient broth (NB) medium

a) Attempted experiment to trace the metabolism of cyanide

The first attempts to follow the cyanide reduction by nmr spectroscopy did not succeed because of the extremely low

TABLE 3

Intensities and Chemical Shifts

Comparison Between Anaerobic and Aerobic Natural Carbon-13 Abundunce

Tables for Figures 7a and 7b

Aerobic		Anaerobic	
Chemical Shifts	Intensity	Chemical Shifts	Intensity
53.7	2.0		
		42.8	2.1
		42.5	1.9
34.5	4.0		
		30.4	2.1
		29.5	2.0
		28.2	2.2
27.2	2.1		
26.6	2.0		
		25.3	2.6
24.5	2.0	24.5	3.0
		24.0	2.39
22.1	1.9		
21.2	1.7		
		20.5	2.17
		20.3	2.37
		18.6	2.3
		18.0	2.0
17.4	4.0		

concentration of intermediate and substrate.

At higher cyanide concentrations reactions are observed (Figures 8a and 8b). Even though it is nearly impossible to interpret all resonances in Figure 8a and 8b, by subtracting those two spectra (Figure 9, Table 4) we can readily observe which resonances are produced by cyanide and/or glucose, and which arise from natural carbon-13 abundance in Klebsiella pneumoniae.

An insurmountable difficulty was observed with the NB medium. Although we used identical experimental conditions after receiving the bacterial cultures we did not get reproducible results with this medium. While it is possible that a variation of cyanide and glucose concentration occurred by experimental error since maximum allowed concentrations of cyanide was only 20 mg per liter, i.e., 40 microgram per 2 mL, it is more likely that the different results stem from different sample preparation step-growing times, washing, and suspending solutions.

An example of this problem is shown in Figure 10a and 10b. In this case (NS = 10,000) spectra showed a different pattern when compared to Figure 8a and Figure 8b which is hard to explain. In Figure 10 there are 20.5 ppm and 69 ppm resonances arising from the products of glucose metabolism (Result Section 5) and a broad 29 ppm

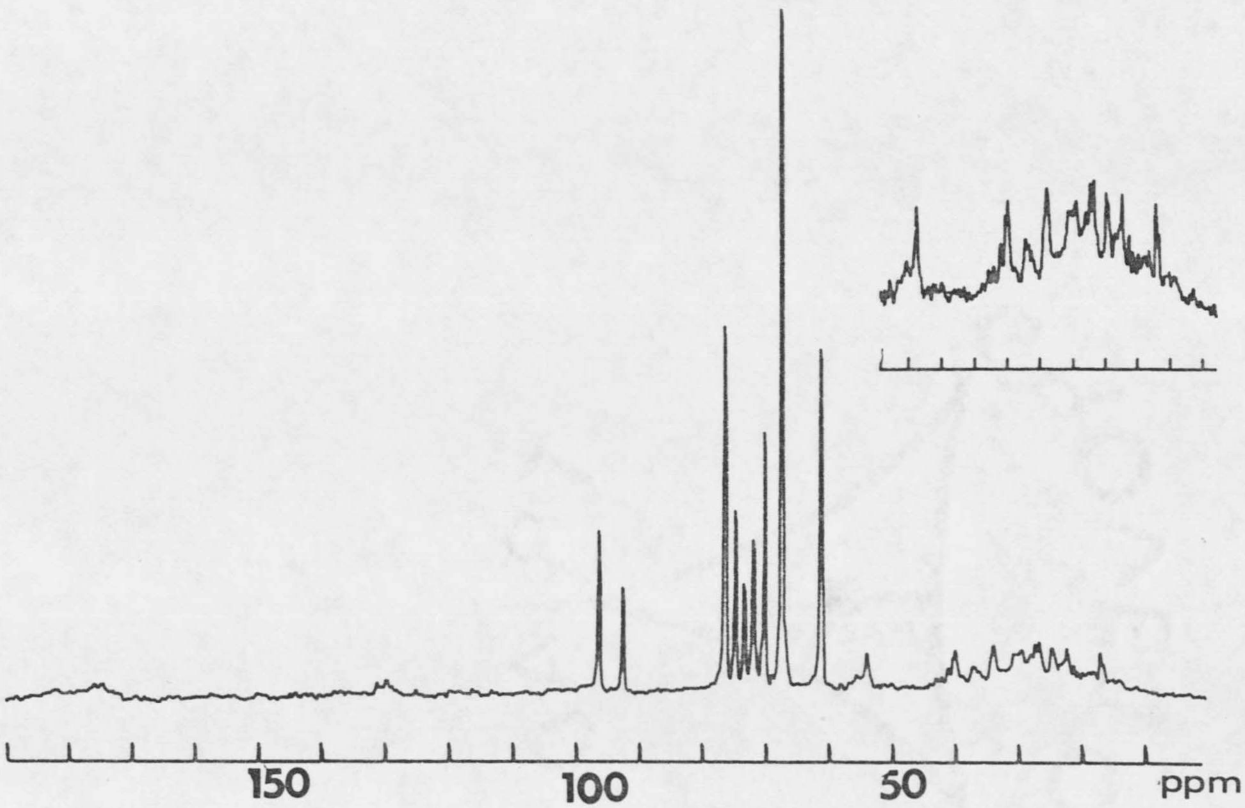


Figure 8a. The spectrum with glucose and K. pneumonia M5a1 strain only. Nutrient broth medium, argon treated sample (NS = 100,000)

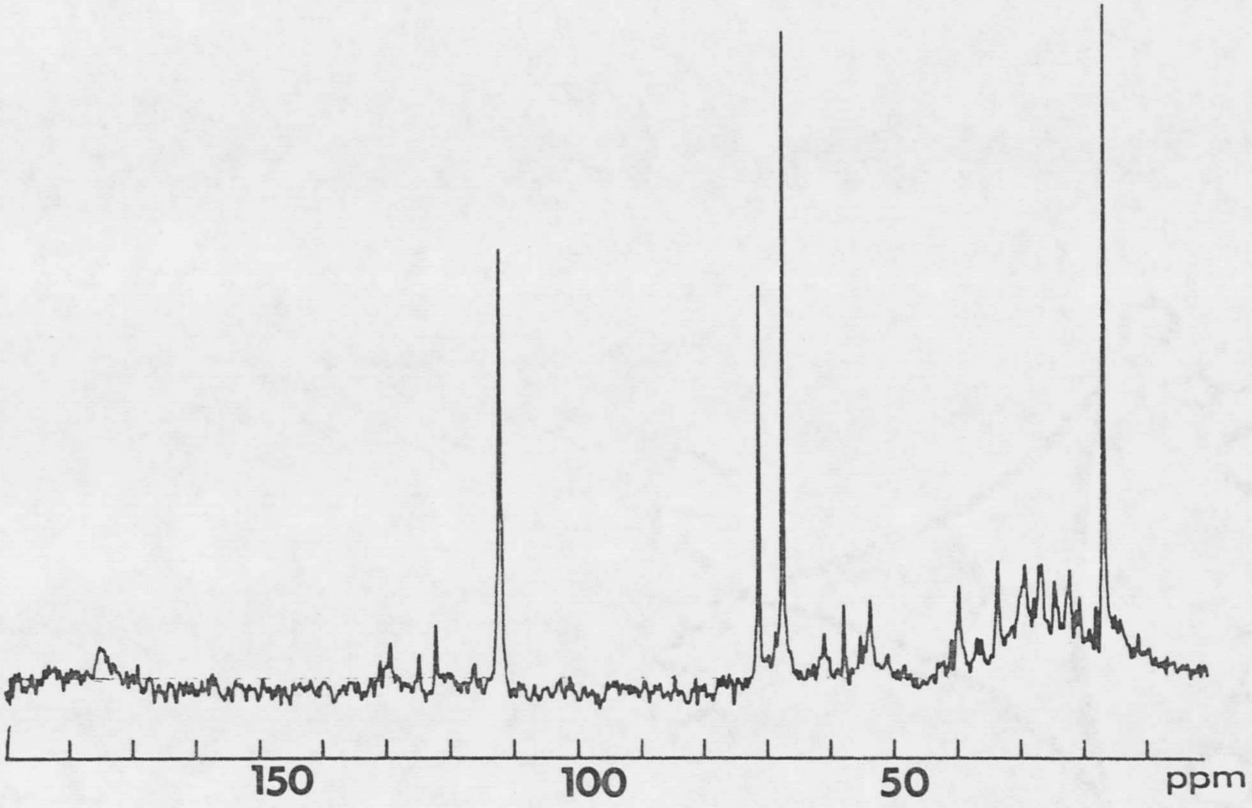


Figure 8b. The spectrum which obtained three days after glucose and cyanide were added to the same sample as that of Figure 8a (NS = 150,000)

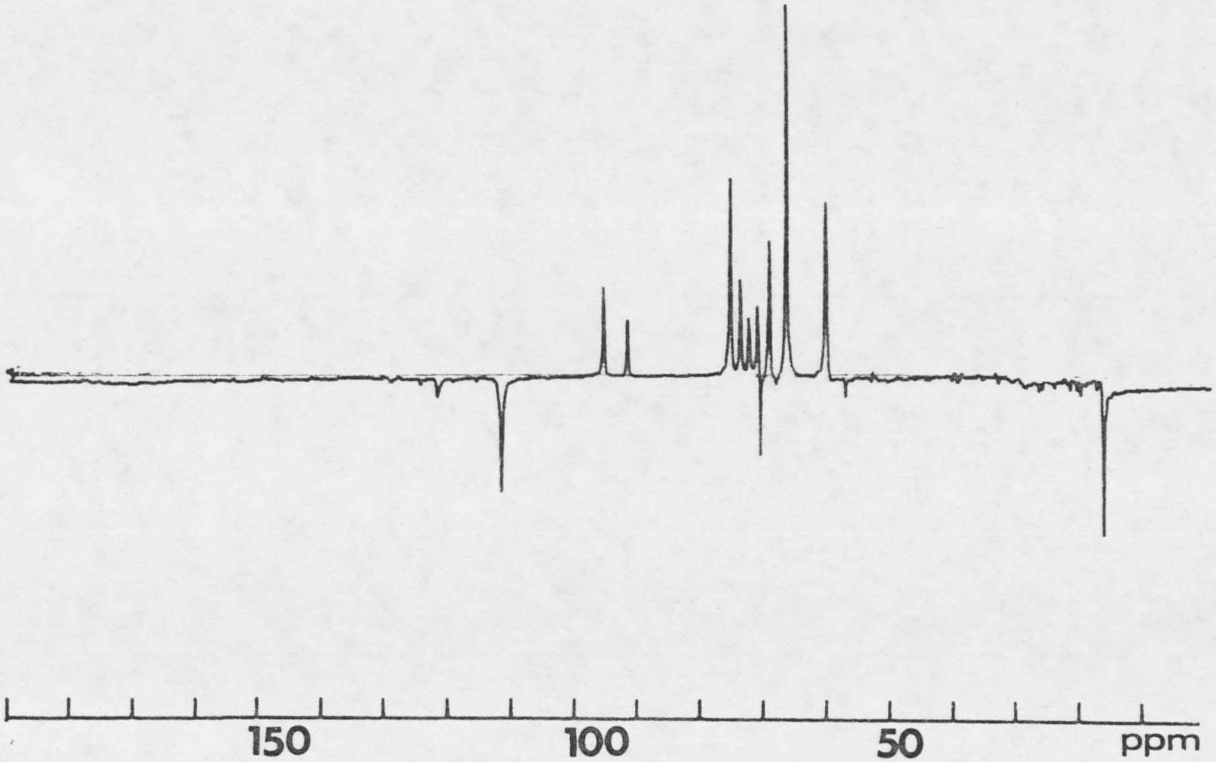


Figure 9. Subtrated spectrum (Figure 8a and 8b)

TABLE 4

Chemical Shifts

Table for Figures 8a and 8b

Chemical Shifts	Glucose only N. B. Anaerobic Figure 8a	Glucose and Cyanide (3 days later) Figure 8b
129.8		X
122.0		X
71.0		X
68.8		X
57.4		X
53.8	X	X
39.9	X	X
33.9	X	X
(30.2)		X
29.7	X	X
29.5		X
28.0	X	X
26.8	X	X
24.6	X	X
22.5	X	X
17.2	X	X
16.9		X

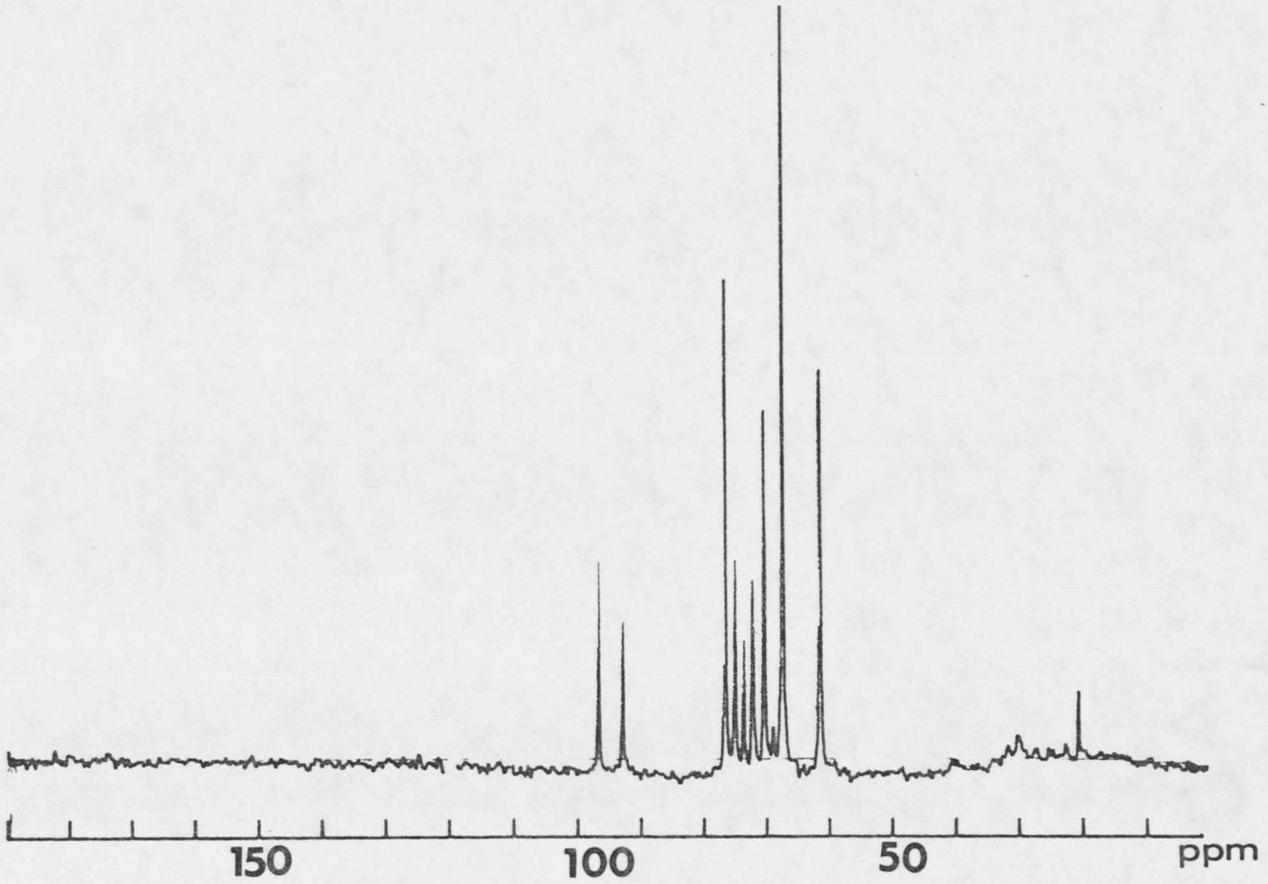


Figure 10a. The spectrum with glucose and K. pneumonia M5al strain only. Nutrient broth medium, argon treated sample, number of scans are 10,000

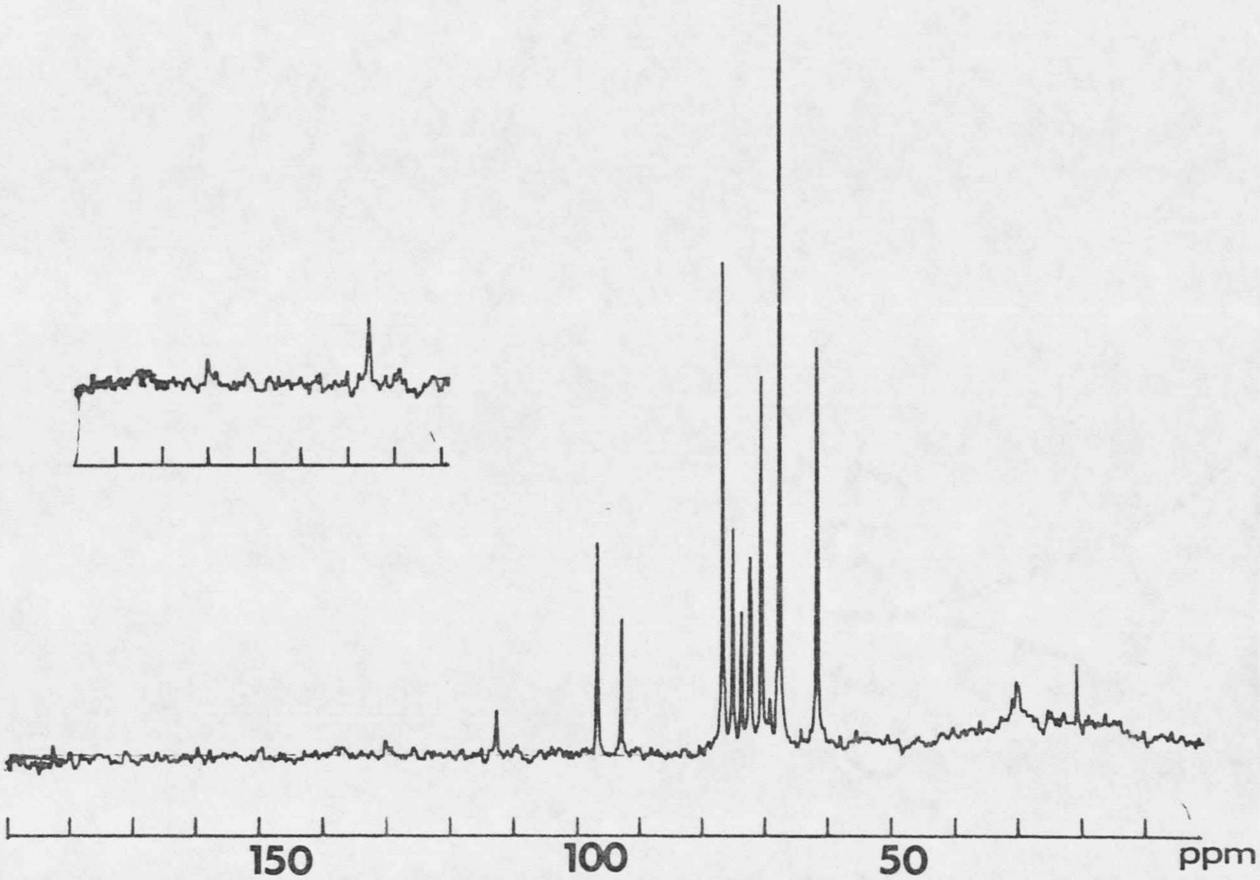


Figure 10b. The spectrum obtained just after glucose and cyanide were added to the same sample as that of Figure 10a (NS = 10,000)

TABLE 5a

Tables for Figure 10a and 10b.

Glucose Only N. B. Medium, Anaerobic		Glucose and Cyanide N. B. Medium, Anaerobic (Immediately)	
Chemical Shifts	Intensity	Chemical Shifts	Intensity
		130.0	0.3
		112.6	0.7
		71.0	4.6
68.9	0.52	68.9	0.9
30.0	0.36	30.0	1.14
		28.0	0.7
		24.5	0.7
20.5	1.0	20.5	1.4
		18.6	0.6
		16.7	0.5
		13.7	0.6

Ref. p-dioxane 67.4 ppm
Intensity 10

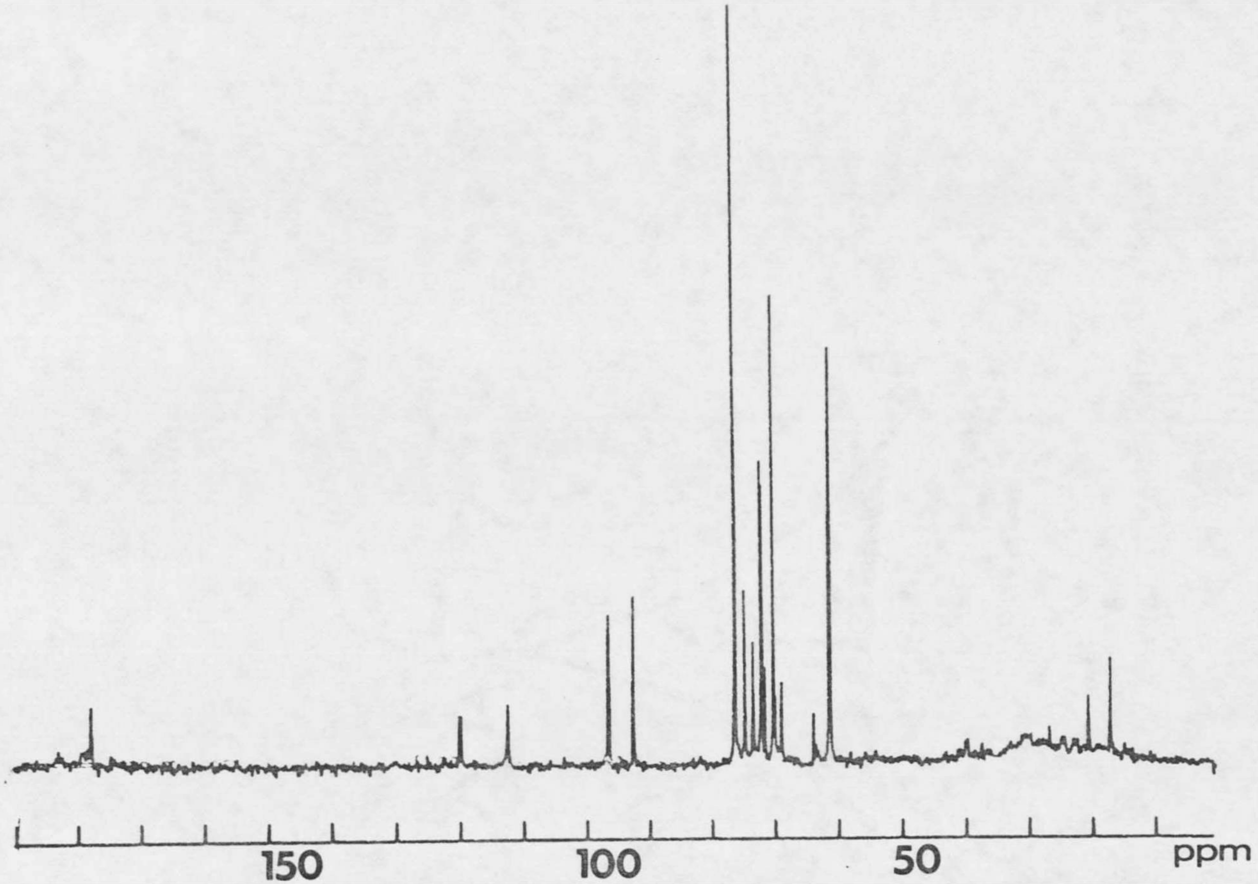


Figure 10c. The spectrum obtained just after glucose and cyanide were simultaneously added

(NS = 50,000)

TABLE 5b

Table for Figure 10c

Chemical Shifts	Intensity
177.66	0.877
119.9	0.734
119.6	0.710
112.3	0.895
68.9	4.173
26.5	0.513
20.4	1.017

Ref. p-dioxane 67.4 ppm

Intensity 8

resonance which was not seen in the spectra of Figure 8. Also there is no 16.5 ppm resonance which was shown to be from cyanide metabolism (Result Section 5). These are only two of the different results obtained when the NB media was used under insignificantly different conditions. Table 6 shows some of the result variations under the same experimental condition.

Generally, irregular decreasing or increasing progression was also observed for the resonances. It is still unclear why these intensities show such patterns for long term experiments (Figure 11), although it should be noted that signals and noise are of comparable intensity.

Despite the difficulties with the NB medium, results of the next section with a different medium lead us to conclude that 16.5 ppm, 20.5 ppm and 69.0 ppm are end products from cyanide or glucose metabolism in Klebsiella pneumoniae M5al strain.

b) Study of end products by glucose and/or cyanide -- a possible explanation for the difficulties with the NB medium

In the course of attempting to identify the products of cyanide reduction with the NB medium we made some observations which may reveal the problems in using NB. We studied spectra with glucose

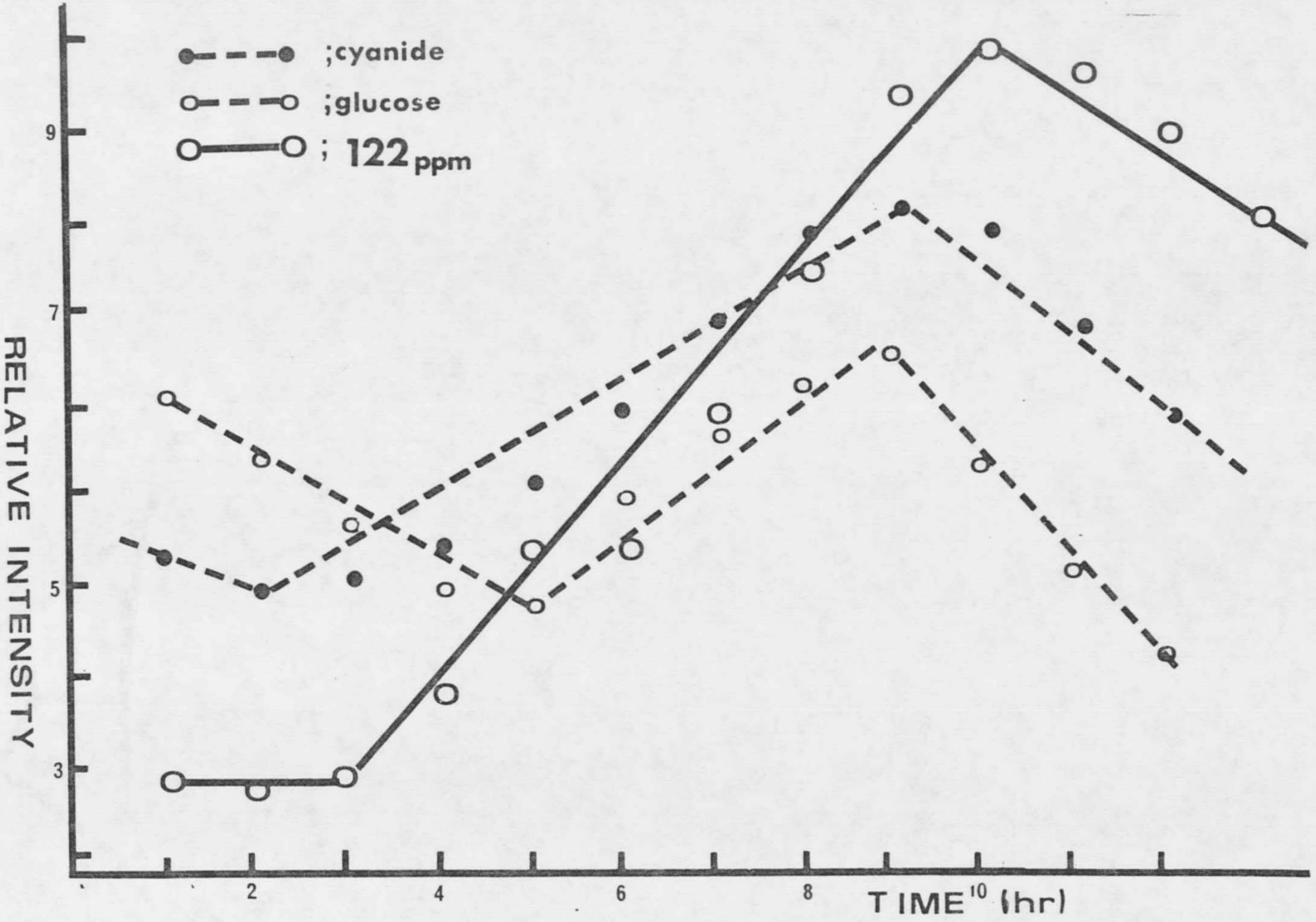


Figure 11. Intensity variations of each resonance, especially for 20 ppm, 112 ppm (cyanide resonance), and 122 ppm

TABLE 6

N. B. Medium, Anaerobic

Experiments Chemical Shift	pattern 1	pattern 2	pattern 3	pattern 4	pattern 5	pattern 6	pattern 7
184.0	X	X		X			
178.6		X					
178.5		X					
129.9		X					
129.8		X					X
122.0	X	X	X		X		X
71.1							X
68.9	X	X	X	X	X	X	
57.4							X
39.4		X	X				
39.6		X	X				
33.9	X						X
26.5			X			X	
24.4							X
20.5	X	X	X	X	X	X	
17.8		X					
16.9		X	X				X
15.8				X			
15.4				X			

and the M5al strain only to check the end products of glucose metabolism in the absence of added cyanide. Figure 8a and Table 4 are from a spectrum obtained in a 10 hour accumulation (number of scans was 100,000). This spectrum does not show any distinctive glucose metabolism product except 53 ppm. In this case we can explain the slow glucose consumption rate in terms of medium effects nutrients left over after centrifugation and are evidently a more favorable energy source than glucose. This explanation is supported by Spectra 8b (number of scans is 100,000) which was obtained three days later after glucose and cyanide had been added (see Figure 8a). Figure 8b shows that all glucose was consumed by K. pneumoniae during the three days and that cells were active compared with cells under high cyanide concentration.

Considering the variation of results with this medium, we have concluded that care should be taken to prepare bacteria whenever one studies the reactions in vivo, especially those of the low concentration metabolic pathways. Left over medium like bacto-beef extract and bacto-peptone apparently can be a more favorable energy source than the glucose supplied.

2. M9 medium

Compared to the reaction in M5al strain grown in nutrient broth medium, those in the M5al strain grown in M9 minimal medium shows relatively regular results. Figures 12a, 12b and Table 7, which show the end products of cyanide reduction using labeled cyanide and normal glucose display distinctive three resonances at 20.5 ppm, 16.5 ppm and 26.6 ppm that were demonstrated below to be products of cyanide and/or glucose metabolism.

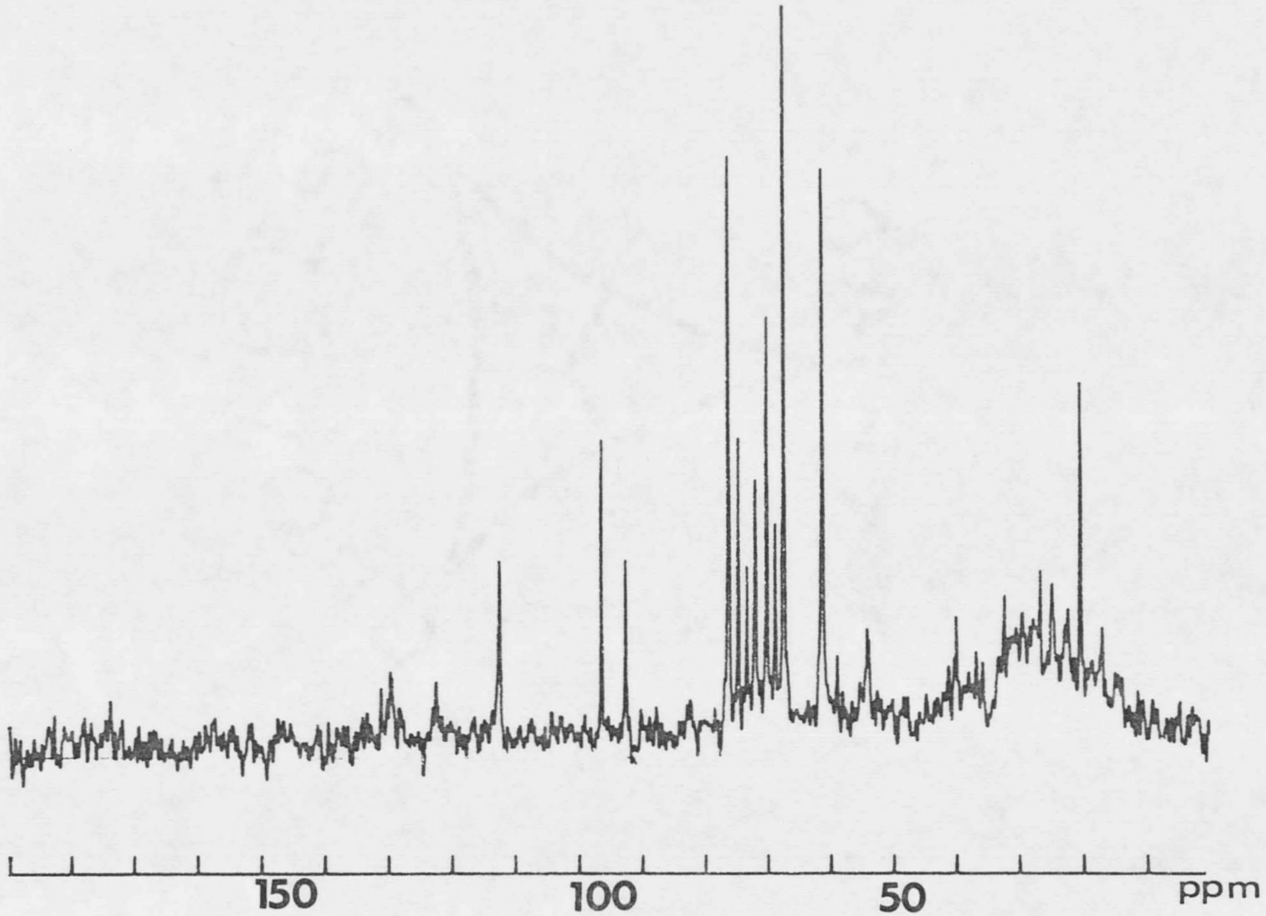


Figure 12a. The spectrum obtained just after glucose and cyanide were added to the argon treated anaerobic sample. (nutrient broth medium), added spectrum of Figure 12a (NS = 15,000)

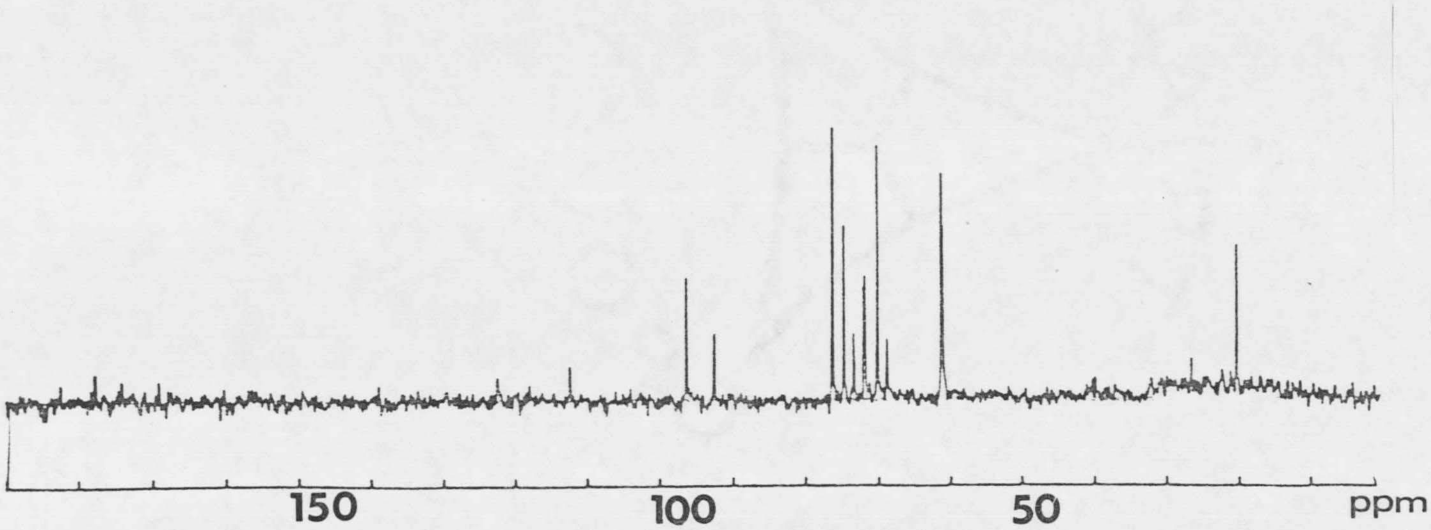


Figure 12b. The spectrum obtained just after glucose and cyanide were added to the oxygen treated aerobic sample. Added spectrum of Figure 12b (NS = 15,000)

TABLE 7

Table for Figures 12a and 12b

Anaerobic N. B. Medium, Argon		Aerobic N. B. Medium, Oxygen	
Chemical Shifts	Intensity	Chemical Shifts	Intensity
130.0	1.3		
71.0	3.7		
68.8	3.2	68.9	2.85
39.8	2.0	39.8	2.24
32.3	2.3		
29.4	2.0		
29.3	2.1		
26.4	2.7		
25.9	4.0		
24.9	2.4		
22.3	2.0		
20.5	5.0	20.5	4.9
17.2	1.5		
16.9	1.9		

COMPARISON BETWEEN AEROBIC AND ANAEROBIC CYANIDE METABOLISM

The results of experiments to determine the differences between anaerobic and aerobic cyanide metabolism are displayed in Figure 12a, 12b, Figure 13 and Figure 14. Comparison of these two kinetic spectra clearly show the differences. First, there are no 16.5 ppm and 40.9 ppm resonances and there is a significant intensity difference of 26.6 ppm and 20.5 ppm in the aerobic case. The resonance at 16.5 ppm, which is considered the product from cyanide reduction, only appears under the anaerobic condition not under the aerobic condition, leading to the immediate conclusion that there is no cyanide reduction under the aerobic conditions which agrees with the previous experimental result by G. Brueggemann, et. al. (16), that there is no cyanide assimilation under the aerobic conditions.

This result is suggestive that cyanide reduction in _K. pneumoniae_ is mediated by nitrogenase which only can be active under the absence of air or oxygen.

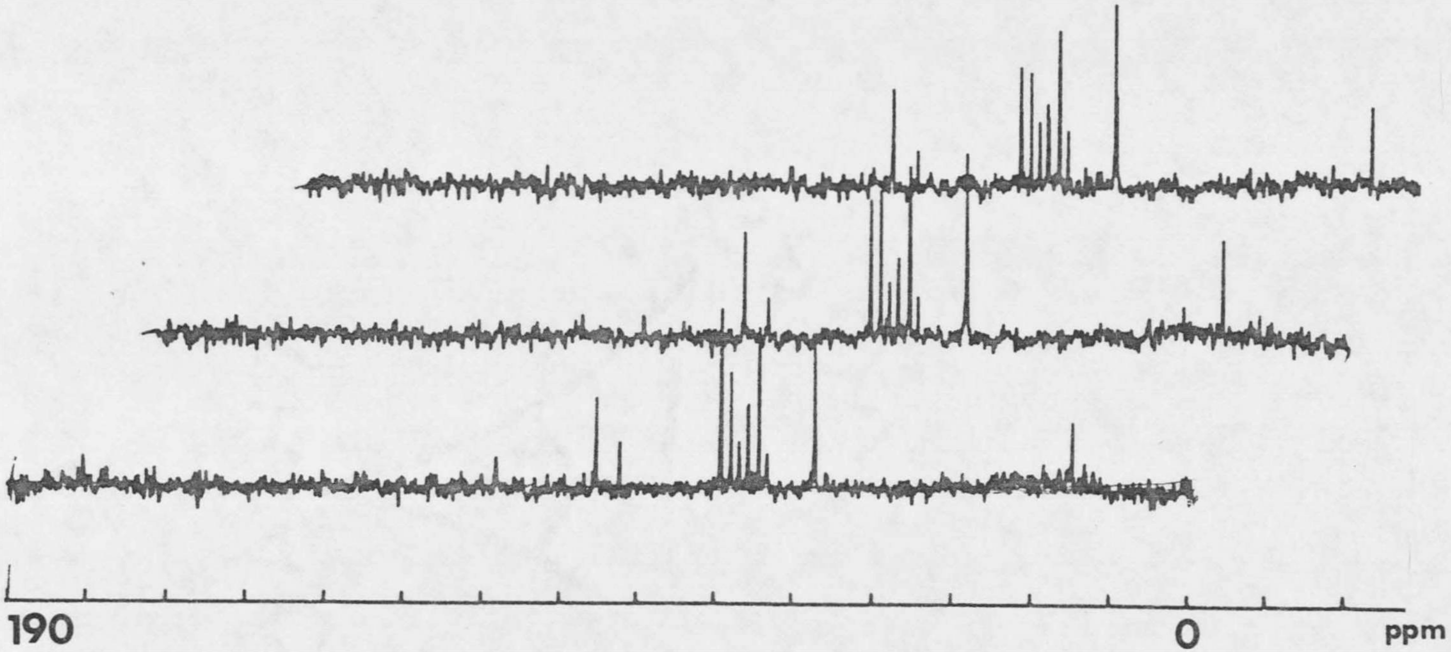


Figure 13. Kinetic study of anaerobic cyanide reduction. Nutrient broth medium, argon treated sample (NS = 5,000)

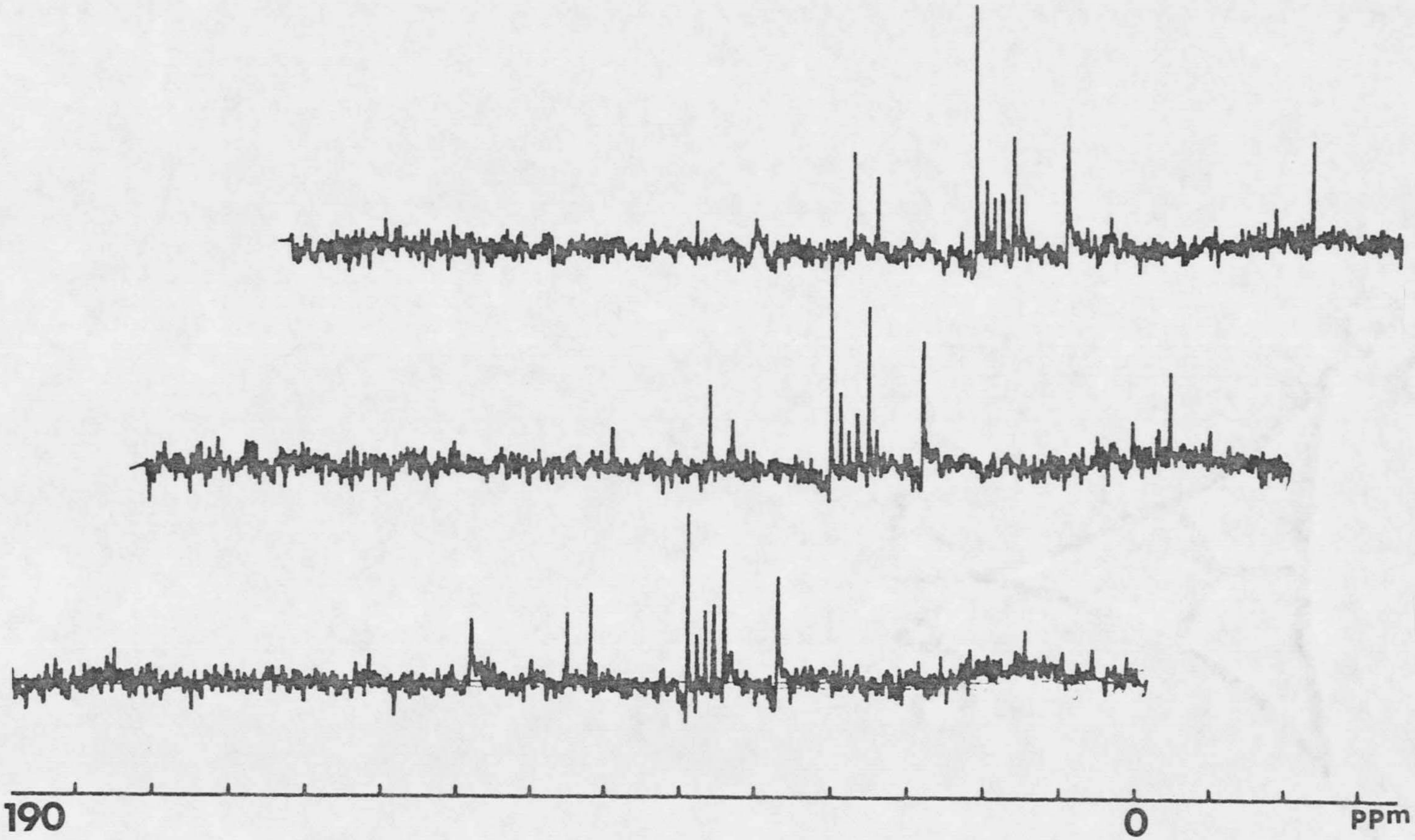


Figure 14. Kinetic study of aerobic cyanide reduction. Nutrient broth medium, oxygen treated sample (NS = 5,000)

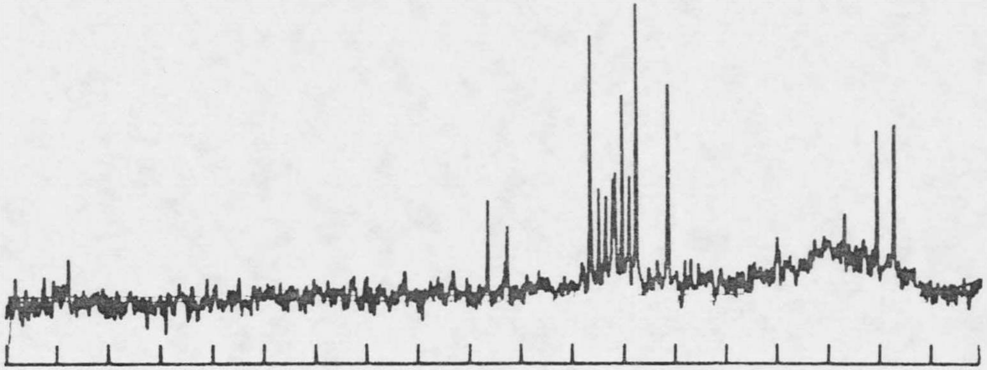
EVIDENCE THAT CYANIDE IS REDUCED

(Experiments that show 16.5 ppm resonance definitely arises from labeled carbon-13 cyanide)

A. Using normal cyanide

Although we could not assign all resonances, we can show which of those resonances came from cyanide reduction by using labeled cyanide (Figure 15a) or normal cyanide (Figure 15b). Comparing these two spectra, the 20.5 ppm resonance is still present with normal cyanide while 16.5 ppm resonance is absent. That proves that the 20.5 ppm resonance comes from glucose and the 16.5 ppm resonance comes from labeled cyanide.

The 16.5 ppm from cyanide reduction can only be a methyl group in a simple compound such as CH_3-, or CH_3-CH_2-X. By using the CIS computer search system, it turns out that there are 330 compounds that can give 16.5 ppm resonance (deviation 0.5 ppm). All of those are inconsistent with any end product yet suggested by cyanide reduction studies. By standard addition (Experiment Section) $CH_3NH_4^+$ has been tested as a suggested end product. That test showed that none of these resonances were methylamine including the one at 16.5 ppm. Valine, alanine and cysteine have been tested,



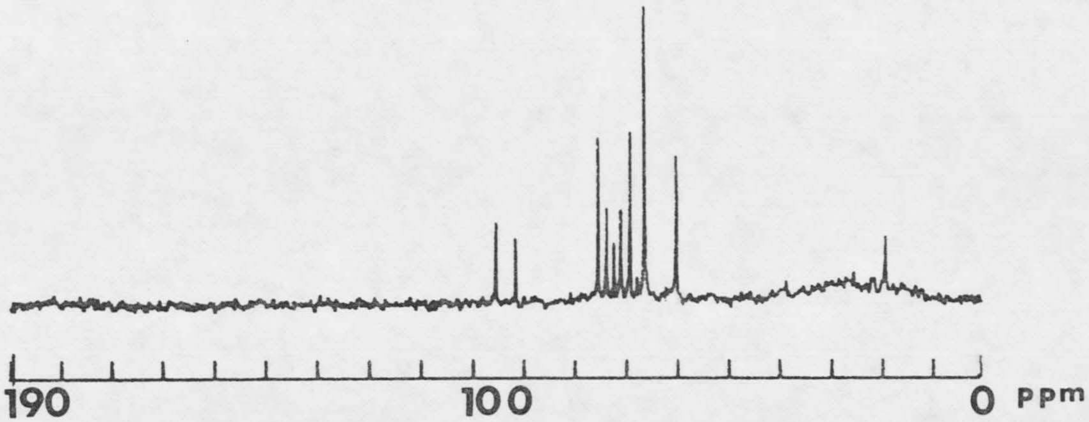


Figure 15a. The spectrum with normal cyanide and normal glucose, argon treated anaerobic sample, M9 medium (NS = 10,000)

Figure 15b. The spectrum with labeled cyanide and normal glucose, argon treated anaerobic sample M9 medium (NS = 10,000)

TABLE 8

Table for Figures 15a and 15b

Normal Glucose Labled Cyanide Argon, M9 Medium		Normal Glucose Normal Cyanide Argon, M9 Medium	
Figure 15a		Figure 15b	
Chemical Shift	Intensity	Chemical Shift	Intensity
71.0	2.8		
68.9	1.7	68.9	2.3
26.6	1.25	26.6	0.5
20.5	2.40	20.5	0.9
16.5	2.5		

p-dioxane = 4.0

p-dioxane = 4.0

and none of them have been produced. Also this test shows that the 20.5 ppm is the methyl group of 2,3-butanediol.

B. Using 1-^{13}C-glucose (Figure 16a and Figure 16b)

Figure 16a, obtained by using labeled cyanide as a substrate and labeled glucose as energy source has been compared to spectra 16b taken just after 2 mg of labeled glucose was added (Figure 17).

That indicates the sharp increase of 20.5 ppm relative to 17.3 ppm or 23 ppm and no 16.5 resonance detected, which further shows that the 20.5 ppm resonance definitely comes from glucose metabolism and 16.5 ppm comes from cyanide.

As a minor result, there are different glucose consumption rates between the two isomer- alpha and beta glucose (Figure 18). That shows the faster consumption rate of alpha-glucose than beta-glucose at the very early state of reaction. But after some time (2 hours), it displays constant ratio between two alpha and beta isomers.

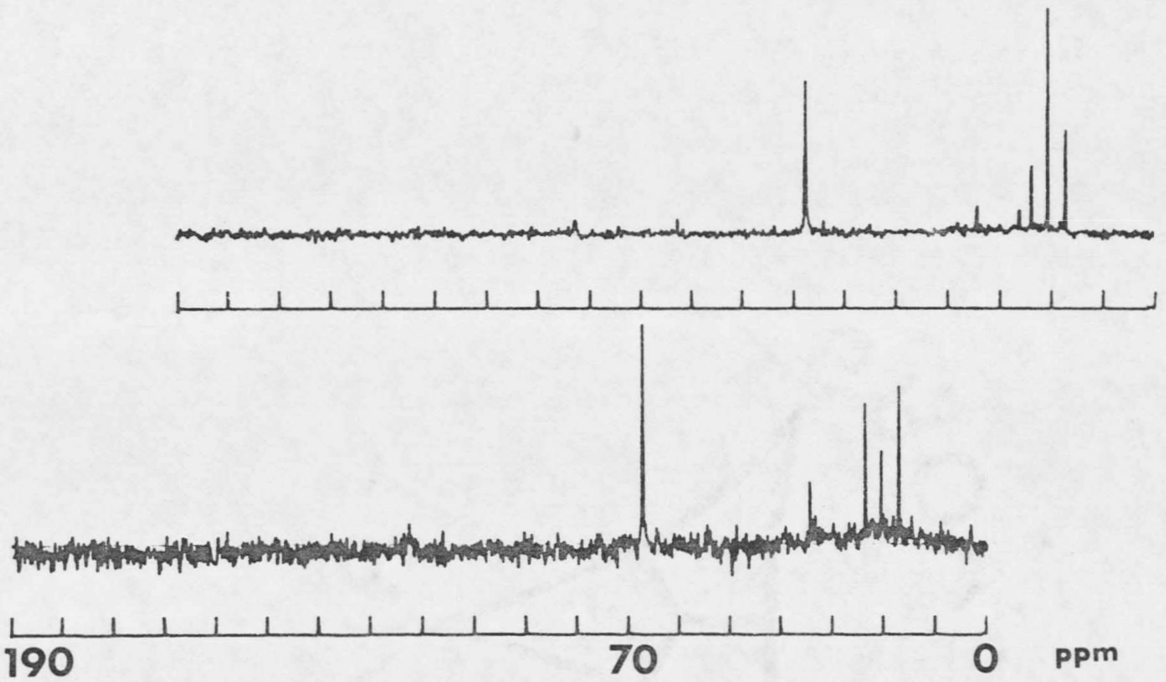


Figure 16a. The spectrum with labeled glucose and labeled cyanide, argon treated anaerobic sample, M9 medium (NS = 12,000)

Figure 16b. The spectrum after adding labeled glucose to Figure 15a sample (NS = 12,000)

TABLE 9

Tables for Figure 16a and 16b

Labled Glucose Labled Cyanide		Labled Glucose Labled Cyanide	
34.5	0.9	34.5	0.6
23.0	2.0	23.0	1.5
20.5	1.4	20.5	4.5
17.3	1.0	17.3	1.3

Ref. p-dioxane = 3.0

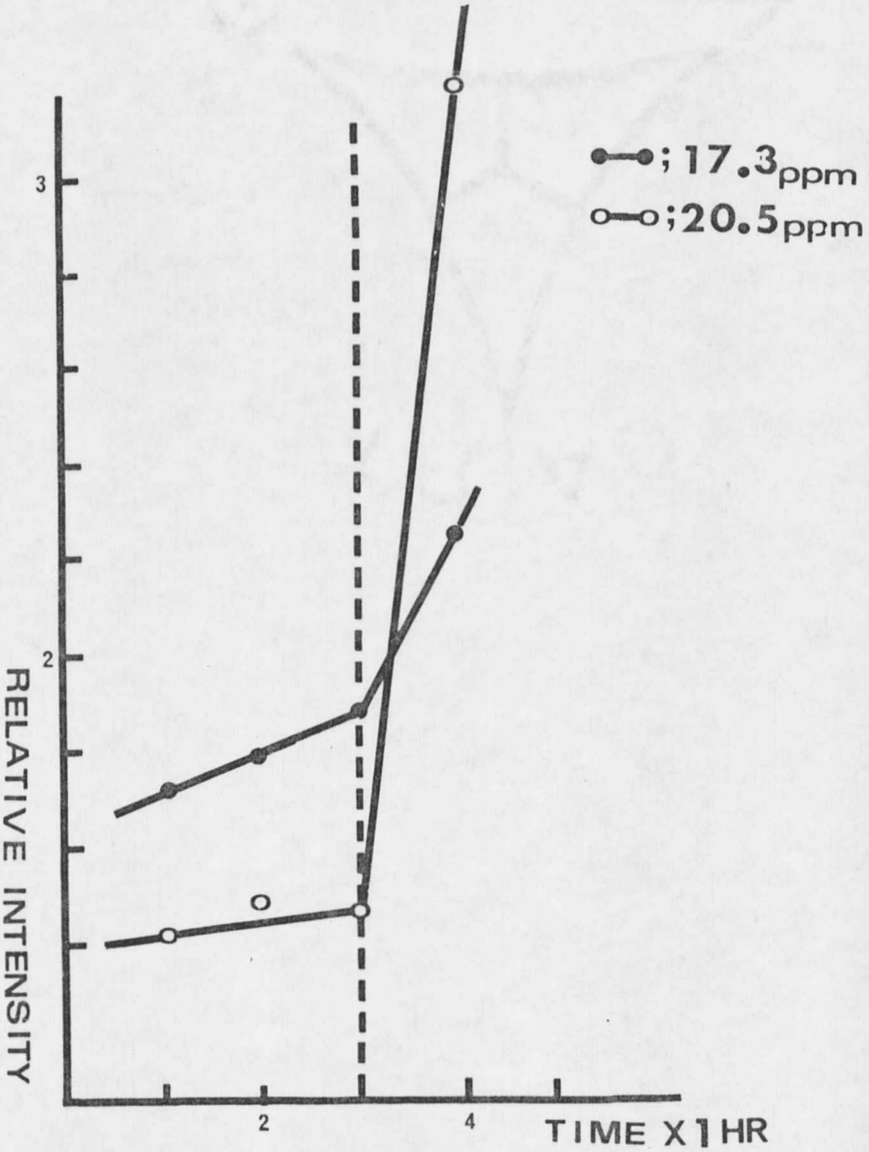


Figure 17. Intensity variation of 17.3 and 20.5 ppm by adding labeled glucose

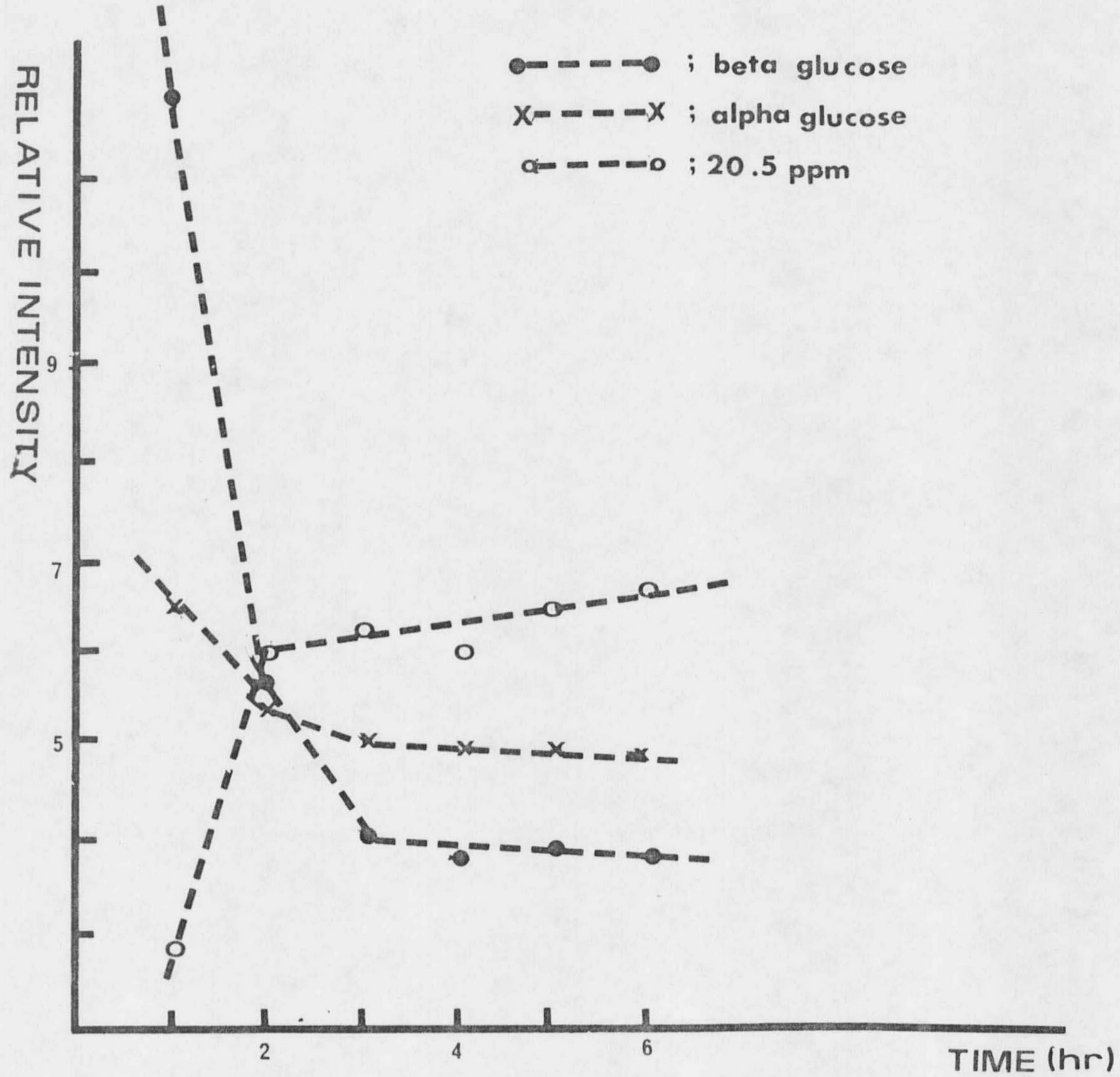


Figure 18. Different glucose consumption rate between two isomers, alpha and beta glucoses

ATTEMPTED NITROGEN-15 NMR EXPERIMENTS

We have tried the assimilation of NH_4^+ in K. pneumoniae using variations of experimental conditions in many ways. No incorporation of nitrogen-15 could be detected. Temperature was varied from 25°C to 40°C, but no difference was observed.

Additions of alpha-keto-glutarate, glucose and ammonia to living cell were tried for the purpose of inducing enzyme for nitrogen-15 incorporation (Figure 19). Various anaerobic and aerobic conditions were tried, nonetheless, no incorporation of nitrogen-15 ammonia could be detected. The reason for this is not yet known. On the more positive side, we have clearly demonstrated that nitrogen-15 spectroscopy is feasible in living cell systems if enriched compounds are used. Figure 19 shows a typical spectrum of enriched ammonia at a concentration of 10 mM. The spectrum has a signal to noise ratio better than 40:1 and was acquired in less than four hours.

Considering the fact that relaxation properties are more favorable for larger molecules, nitrogen-15 nmr spectroscopy is clearly applicable in living system with modern nmr spectrometers.

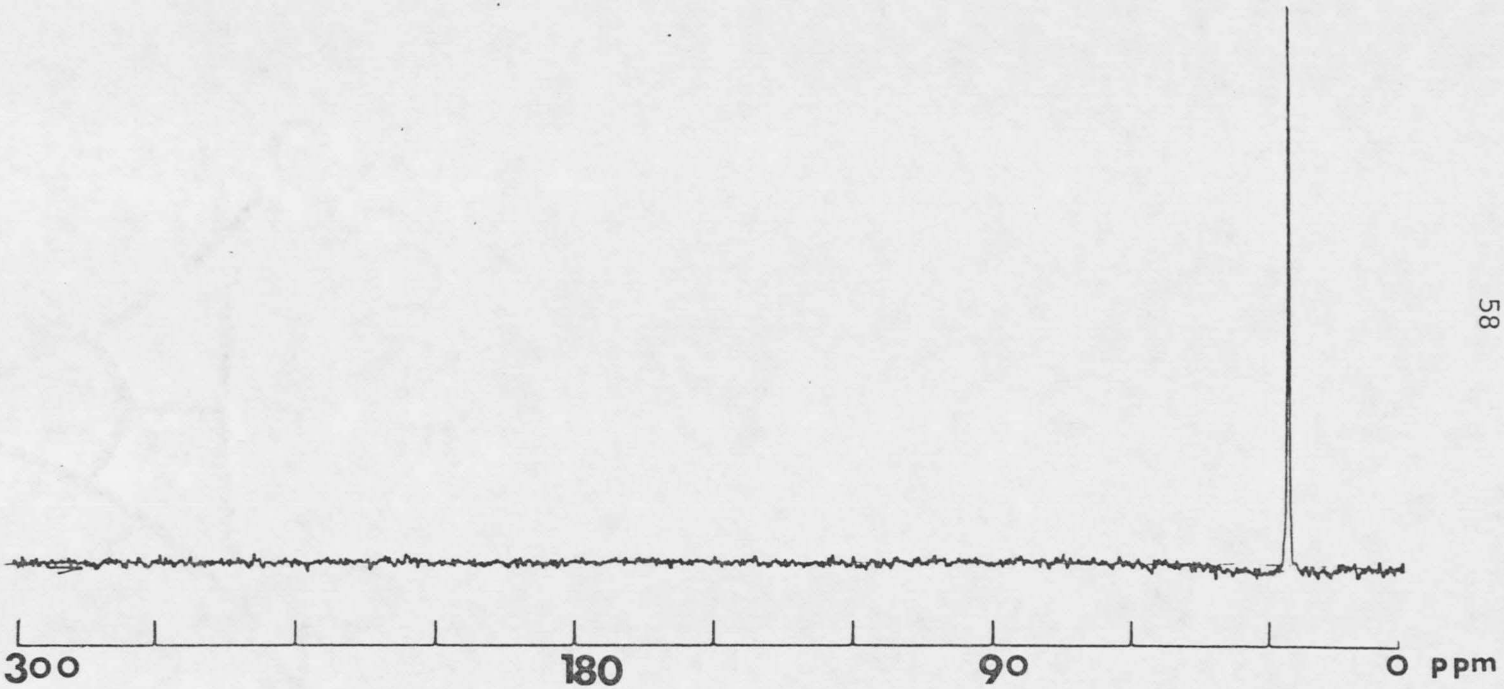


Figure 19. The spectrum of ammonia assimilation in K. pneumonia anaerobic, argon treated sample, M9 medium (NS = 30,000)

DISCUSSION

The nmr study of cyanide reduction by K. pneumoniae in vivo was undertaken in the hope of elucidating information on the pathways of dinitrogen by nitrogenas using carbon-13 labeled cyanide. It was shown that reactions can definitely be monitored in vivo, although with difficulty because of the low concentration.

The production of 2,3 butanediol, which is a major product of glucose metabolism in K. pneumoniae is readily observed as is the production of a resonance at 16.5 ppm which is a product of cyanide reduction. Although the latter resonance has not been identified yet, it clearly arises from a methyl resonance -- proof that reduction has occurred. It is not from the methyl group of methylamine or other suggested amino acid as might be an end product of cyanide assimilation in cyano-bacteria. Cyanide reduction is only observed when the bacteria are in an anaerobic state. This is consistent with reduction by nitrogenase which is only produced in the absence of oxygen.

Among the other results are the following: the natural abundance carbon-13 spectra of K. pneumoniae can be observed and is significantly different when the organism is operating with anaerobic metabolism as opposed to aerobic metabolism. Medium selection appears to be a very important consideration in obtaining consistent results. The nutrient broth media created particular difficulties under the conditions of the experiments, perhaps because it could be stored by *Klebsiella* and metabolized subsequently. Lastly, conditions have been found whereby nitrogen-15 spectroscopy can be applied to living cells. Resonances of useful intensity can be observed for N-15 enriched ammonium ions at biologically useful concentrations.

REFERENCES

1. James, T. L. Nuclear magnetic resonance in biochemistry. Academic Press, 1975.

2. Needham, T. E., and Matiwiyoff, N. A. Carbon-13 nmr spectroscopy of red blood cell suspensions. Biochem. Biophys. Res. Comm., 49 (5).

3. Beard, C. F., Stejskal, E. D., and Schaefer, J. Carbon-13 nuclear magnetic resonance analysis of metabolism in soybeans labeled by $^{13}CO_2$. Plant Physiol., 1975, 55, 1048-1053.

4. Seely, D. J., and Hoult, D. I. Observation of tissue metabolites using phosphorus-31 nuclear magnetic resonance. Nature, 1974, 252, 285-287.

5. Yamane, T., and Schulman, R. G. ^{31}P Nuclear magnetic resonance studies of Ehrilichascites tumor cells. Proc. Natl. Acad. Sci., 1977, 74, 87-91.

6. Schulman, R. G., and Ugurbil, K. R. Phosphorus-31 nuclear magnetic resonance studies of bioenergetics and glycolysis in anaerobic Escherichia coli cells. Proc. Natl. Acad. Sci., 1978 75, 2244-2248.

7. Schulman, R. G., Ugurbil, K., Den Hollander, J. A., Brown, T. R., and Glynn, P. High resolution carbon-13 nmr studies of glucose metabolism in Escherichia coli. Proc. Natl. Acad. Sci. 1978, 75, 3742-3746.

8. Schulman, R. G., Den Hollander, J. A., Brown, T. R., and Ugurbil, K. Carbon-13 nmr studies of anerbic glycolisis in suspension of yeast cells. Proc. Natl. Acad. Sci. 1979, 76, 6096-6100.

9. Schulman, R. G., Glynn, P., and When, S. M. Carbon-13 nmr studies of gluconeogenesis from labeled alanine in hepatocytes from euthyroid and hyperthyroid rats. Proc. Natl. Acad. Sci. 1981, 78, 60-64.

10. Vol'pin, M. E., and Blumenfield, A. L. Dokl. Akad. Nauk. 1980, S.S.R. 251, 611.

11. Thornley, N. F., and Chatt, J. D. Nitrogen Fixation Vol. 1, Ed. W. E. Newton and W. H. Orme-Johnson, University Park Press, 1980. p. 171.

12. Thornley, R. F., Richard, R. L., Postape, J. R., Lowe, J., and Smith, B. E. Studies of dinitrogen reducton by nitrogenase from K. pneumoniae. Curr. Perspect. Nitrogen Fixation, Proc. Int. Symp. 4th, 1981, 67-70.

13. Nesbitt, J. B. et al. Aerobic metabolism of potassium cyanide. Journal San Eng. Div. Proc. Amer. Soc. Civil Engineering, 1960, 86, SA1.

14. Nesbitt, J. B., and Murphy, R. S. Biological treatment of cyanide waste. Eng. Res. Bull. B-88, College of Engineering, Pennsylvania State University, University Park.

15. Howe, R. H. L., Toxic wastes degradation and disposal. Process Biochem (Ct. B.), 1969, 25.

16. Brueggemann, G. Gaudy, A. F. Jr., Gaundy, E. T., and Feng, Y. J. Treatment of cyanide waste by the extended aeration process. Journal WPCF, Volume 54(#2), 1982, 153-164.

17. Quayle, J. R. The metabolism of one-carbon compounds by microorganisms, p. 119-202. In A. H. Rose and D. W. Tempest (ed.), Advances in microbial Physiology, 1972, Vol. 7.

18. Kosaric, N., and Zajic, J. E. Microbial oxidation of methane and methanol. In T. K. Ghose, A. Fiechter, and N. Blakesbrough (ed.), Advances in Biochemical Engineering, 1974, 364-392.

19. Fry, W. E., and Millar, R. L. Cyanide degradation by an enzyme from *Stemphylium loti*. *Arch. Biochem. Biophys.*, 1972, *151*, 468-474.

20. Knowles, Christopher J. Microorganisms and cyanide. *Bacteriol. Rev.*, Vol. 40, 652-674.

21. Strobel, G. A. and Alleng, J. The assimilation of $H^{14}CN$ by a variety of fungi. *Can. J. Microbiol.*, 1966, *12*, 414-416.

22. Strobel, G. A. The fixation of hydrocyanic acid assimilation by a *Psychrophilic basidiomycete*. *J. Biol. Chem.*, 1966, *241*, 2618-2621.

23. Strobel, G. A. Hydrocyanic acid assimilation by a *Psychrophilic basidiomycete*. *Can. J. Biochem.*, 1964, *42*, 1637-1639.

24. Mundy, B. P., Liu, F. H. S., and Strobel, G. A. Alpha-amino butyronitrile as an intermediate in cyanide fixation by *Rhizoctonia solani*. *Can. J. Biochem.*, 1973, *51*, 1440-1442.

25. Fowdem, L., and Bell, E. A. Cyanide metabolism by seedlings. *Nature* (London), 1965, *206*, 110-112.

26. Brysk, M. M., Lauinger, C. and Ressler, C. Gamma-cyano-alpha-L-aminobutyric acid. A new product of cyanide fixation in *Chromobacterium violaceum*. *J. Bio. Chem.*, 1970, *245*, 1156-1160.

27. Oro, J. and Kimball, A. P. Synthesis of purines under possible primitive earth conditions. I. Adenine from hydrogen cyanide. *Arch. Biochem. Biophys.*, 1960, *94*, 217-222.

28. Oro, J., and Kamat, S. S. Amino acid synthesis from hydrogen cyanide under possible primitive earth conditions. *Nature* (London), 1961, *190*, 442-443.

29. Hardy, R. W. F., and Knight, E. Reduction of azide and hydrogen cyanide. *Biochim. Biophys. Acta*, 1967, *139*, 69-90.

30. Kelly, M., Postgate, J. R., and Richards, R. J. Reduction of cyanide and isocyanide by nitrogenase of *Azotobacter chroococeum*, *J. Biochem.*, *102*, 1C.

31. Hwang, J. C., and Burris, R. J. Nitrogenase catalyzed reactions. *Biochim. Biophys. Acta*, 1972, *283*, 339-350.

32. St. John, R. T., Shah, V. K. and Brill, W. J. Regulation of nitrogenase synthesis by oxygen in *Klebsiella pneumoniae*. *J. Bacteriol.*, 1974, *119*, 266-269.

33. Kroeker, E. J., Schulte, D. D., Sparling, A. B., and Lapp, H. M. Anaerobic treatment process stability. *J. Water. Poll. Control Fed.*, 1979, *51*, 718-727.

34. Kelly, M., and Biggins, D. R. Interaction of nitrogenase from *Klebsiella pneumonia* with ATP or cyanide. *Biochim. Biophys. Acta*, 1970, *205*, 288-299.

35. Bryant, M. P. Microbiological methane production; theoretical aspects. *J. Anim. Sci.*, 1979, *48*, 193-201.

36. Shanmugan, K. T., and Hennecke, H. Temperature control of nitrogen fixation in *Klebsiella pneumoniae*. *Arch. Microbiol*, 1979, *123*, 259-265.

37. Richard, R. L., and Dilworth, J. R. Nitrogen-15 nmr spectra of metal complexes relevant to nitrogen fixation. *Inorganica. Chimica. cta.*, 1981, *53*, L162.

38. Hardy, R. W. F., and Parshall, G. W. The biochemistry of nitrogen fixation. *Bioinorganic Chemistry*, 1971, 219-233.

39. Lehninger, A. L. Biochemistry, 2nd edition. Worth Publisher Inc. 1970.

40. Lawrence, A. W. and McCarthy, P. L. Kinetics of methane fermentation in anaerobic treatment. *J. Water Poll. Control Fed.*, 1969, *41*, R1-R17.

41. Runquist, E. A., Abbot, E. H., Armold, M. T. and Robbins, J. E. Application of ^{13}C-nmr to the observation of metabolic interactions in anaerobic digestors. *Appl. Environ. Microbiol.*, 1981, *42*, 556-559.

42. Robbins, J. E. and Runquist, E. A. Application of ^{13}C-nmr spectroscopy to anaerobic digestion. Paper No. 33. 2nd International Symposium on Anaerobic Digestion. Travemunde, Germany, 1981, Sept. 6-11.

43. Runquist, E. A. and Robbins, J. E. Propionate catabolism in anaerobic digestion. Appl. Environ. Microbiol., 1981, submitted.

44. Runquist, E. A., Abbott, E. H. and Robbins, J. E. Comparative Rates of utilization of propionate and acetate in anaerobic digestion in high and low performance digestors. 1981, In preparation.

45. Runquist, E. A. and Robbins, J. E. Glucose metabolism and naerobic digestors receiving high loads of glucose with manure. 1981, In preparation.

46. Gibbons, N. E., and Buchanan, R. E. (co-editors) Bergey's manual of determinative bacteriology, 8th edition. 1974, 322-323.

APPENDICES

APPENDIX 1

Carbon-13 Parameters

The experimental parameters listed below were used in all of the carbon-13 nmr spectra in the results and discussion section.

spectra frequency	62.83 MHz
decoupler power	8H
data points used	8k
sweep width	20000Hz
acquisition time	0.205 second
delay time	0.1 second
pulse width	15.0
pulse angle	45°
synthesizer frequency	93.66 MHz
line broadening factor	3 or 10 Hz
temperature	297 k unless specified
receiver gain	800

broad band proton decoupling - low pass frequency filter

line broadening factor

Nitrogen-15 nmr Spectra Parameters

The experimental parameters listed below were used in all of the nitrogen-15 nuclear magnetic resonance spectra in the results and discussion section.

spectra frequency	25.349 MHz
decoupler power	6H
data points used	8k
sweep width	15000
acquisition time	0.759 second
delay time	0.3 second
pulse width	30
pulse angle	30°
synthesizer frequency	112.4
line broadening factor	3 Hz
temperature	297 k unless specified
receiver gain	20
broad band decoupling	proton

APPENDIX 2

Microprogram Used for All Carbon-13 and Nitrogen-15 Experiments

A. For kinetics

1. ZE
2. GO= 2
3. WR Filename
4. IF File name
5. Lo To 1 times n
6. Exit

B. For addition of spectrum

1. RE file name 1
2. IF file name 1
3. AT file name 1
4. WR file name 2
5. IF file name 1
6. Lo To 3 times (numbers of spectrum)
7. EM
8. FT
9. PK
10. Exit

APPENDIX 3

Chemical Shifts of Possible Endproduct

(Ref. p-dioxane = 67.4 ppm TMS = 0.0 ppm)

Aliphatics

	C1	C2
CH_4	-2.1	
C_2H_6	5.9	5.9
C_2H_4	123	123
C_2H_2	~70	~70

Ether

	-C	-C
CH_3OCH_3	59.4	
$C_2H_5OC_2H_5$	67.4	17.1

Acid, Ketone

	C_1	C_2
CH_3COOH	177.2	20.5
$OH(CH_2)_2OH$	63.4	

Saturated Nitrogen Compound

CH_3NH_2	28.3
$CH_3NH_4Cl^-$	25.8
$CH_3COO^-NH_4^+$	24.5

Alcohol

	C_1	C_2
CH_3OH	89.3	
C_2H_5OH	57.3	17.9
$CH_3(CHOH)_2CH_3$	68.9	20.0

Amino Acid

	C_1	C_2	C_3	C_4
$H_2COO^-NH_3^+$	42.1			
$(CH_3)_2CHNH_3^+COO^-$	61.0	29.7	18.4	17.1
$CH_3CHNH_3^+COO^-$	51.1	16.6		

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