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Biochemical Fuel Cell

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FIFTH QUARTERLY PROGRESS REPORT

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U. S. ARMY ELECTRONICS RESEARCH
AND DEVELOPMENT LABORATORY
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PURPOSE

The purpose of this program is to conduct investigations pertinent to biochemical fuel cells. More specifically, those investigations are devoted to:

1. The study of hydrogen generation by various microorganisms. This will include a survey of hydrogen-generating microorganisms, the rates at which they generate gases, and the composition of the gases.

2. The consideration of hydrogen utilization. This will include the consideration of the feasibility of utilizing this microorganism-produced hydrogen either at fuel electrodes in a hydrogen-oxygen fuel cell or directly at bioanodes.

3. The study of proper fuels. This evaluation will include the utilization of such materials as carbohydrates, proteins, and organic acids.

The results of this investigation will be evaluated with respect to the total biochemical fuel cell program.

ABSTRACT

This report describes an experimental and theoretical investigation of hydrogen production by microorganisms.

Screening studies were conducted on strains of Clostridium welchii and certain Pseudomonads related to metal corrosion in hydrocarbon fuel systems. These new strains produced hydrogen at rates higher than any organisms so far investigated. The gas generating system was scaled up to 10 liters and in this larger system Clostridium welchii (6785) produced H_2 at the rate of 8.2 liters/hr.

The genetic work, designed to develop mutants with outstanding hydrogen generating capability has been initiated with the development of a simple plate technique for selection of desirable mutants.

A literature survey of potential cheap, available substrates suitable for field use has been made with the starch and sugar content of various materials being tabulated. The possibility of using hydrocarbons is considered.

The rate of hydrogen production observed on this program is expressed in terms of fuel cell consumption to make a rough preliminary appraisal of the biochemical fuel cell.

PUBLICATIONS, LECTURES, REPORTS and CONFERENCES

Publications, Lectures

There were no publications or lectures during this quarter.

Reports

The fourth quarterly report on this project was issued during this quarter.[6]

Conferences

A conference was held at the U.S. Army Electronics Research and Development Laboratory at Ft. Monmouth, New Jersey on July 30, 1963. U. S. Army Electronics Research and Development Laboratory was represented by Dr. H. F. Hunger, Mr. J. Perry, Jr., and Mr. B.Resnic with Dr. C. Daniel in consultation. Melpar, Inc. was represented by Dr. R. T. Foley, Dr. G. C. Blanchard, Dr. P. S. May, and Mr. H. H. Titus. The program was reviewed with emphasis on the statistical design of experiments and statistical treatment of data.

FACTUAL DATA

1. INTRODUCTION

During the first year of this project the hydrogen generating capability of microorganisms was investigated. Initially a survey was conducted on possible biochemical mechanisms by which various genera produced hydrogen, and the literature was searched with respect to microorganisms (catalysts) and substrates (fuels) which could be employed on the hydrogen utilization side of a biochemical fuel cell. Many organisms were screened and rate data accumulated. It was established that rate curves were required to give a valid picture of the capability of the microorganisms.

Technical work done during the fifth quarter was devoted to the continuation of the screening program for new hydrogen evolving organisms, studies on rates of hydrogen evolution, further scale-up studies, investigations of plate techniques for the detection and evaluation of hydrogen producing organisms, and initial surveys of possible natural substrates which would be suitable for field application.

A preliminary calculation was made of the power output of a fuel cell utilizing hydrogen at an output already achieved on this program. The objective was one of preliminary orientation of the biochemical fuel cell in fuel cell technology.

2. SCREENING AND RATE STUDIES

2.1 Pseudomonads No. 108, 109, 110, 112

A group of organisms related to the Pseudomonas sp. (G4A) were found to produce gas in studies relating to metal corrosion[1]. The organisms, identified as Pseudomonads 108, 109, 110 and 112, were examined for their hydrogen producing capability in gas tube experiments. The inocula were prepared as previously described[2] and tested with 1% glucose in 0.1 M phosphate buffer at pH 7.4. Incubation was at 30°C. The data for these studies are found in table 1. Traces of gas were found in all tubes at four hours. Because of foaming in the tubes, no analyses could be begun until 8 hours when approximately 0.3 ml of gas was evolved. In addition to the gas tube studies manometric measurements of the hydrogenase activity for these organisms were made. The procedures for measuring hydrogen utilization were those of Peck and Gest.[3] The data for these studies are found in table 2. It should be noted that although hydrogen evolution was obtained with all the organisms, cultures No. 109 and 110 were not able to utilize hydrogen readily. These organisms, cultures 109 and 110, will be particularly valuable in studies of the mechanism of hydrogen evolution because two types of hydrogenase containing organisms have been described in the literature. The first type are organisms which can either produce hydrogen from rich substrates or utilize hydrogen gas in the presence of appropriate dyes. The second type are organisms which cannot produce hydrogen from rich substrates but can utilize it. No reports however could be found where organisms can produce hydrogen from rich substrates but lack the ability to utilize hydrogen. The study of the ferredoxin, formic

TABLE 1

Hydrogen Production by Corrosion Associated Pseudomonads No. 108, 109, 110, 112

Glucose 1% 30°C   0.1 M PO_4 Buffer, pH 7.4 2×10^{10} cells/ml

TIME (HOURS)	TOTAL GAS (ml)				HYDROGEN (ml)			
	108	109	110	112	108	109	110	112
4	Trace Trace				Trace Trace			
8	0.5 0.6	0.4 0.2	0.3 0.3	0.7 0.5	0.35 0.41	0.26 -	- -	0.48 0.35
12	0.9 1.2	0.5 0.3	0.9 0.5	1.0 1.2	0.65 0.76	0.33 0.19	0.35 0.23	0.84 0.86
24	3.2 3.4	1.8 0.7	2.0 2.2	3.2 2.8	1.82 1.92	0.99 0.38	1.18 1.30	1.82 1.65

TABLE 2

HYDROGENASE DETERMINATION OF CORROSION ASSOCIATED PSEUDOMONADS NO. 108, 109, 110, 112

Organism	ml H_2 uptake x 10^{-9}/30 min/cell
E. coli (control)	30.6 38.7 34.8
108	26 17
109	8.7 4.2
110	0 0
112	46.5 44.7

dehydrogenase and hydrogenase levels in all three types of organisms may provide some clues not only to the basic mechanism of hydrogen production but also to methods of increasing hydrogen yields. These studies will be considered in the future.

Of greater interest is the fact that these organisms were isolated from hydrocarbon fuel-water mixtures and have the demonstrated ability to use the hydrocarbons as carbon sources. If these organisms can produce hydrogen from hydrocarbons, an entirely new area of cheap substrates is made available. Further discussion of this subject will be found in Section 5.2.

2.2 Clostridium welchii, Strains (ATCC 9081) and (NCIB 6785)

In the previous report,[6] strains 9856 and 10543 were screened and found to produce hydrogen at the rate of 0.67 ml/hour and 0.9 ml/hour, respectively. During this period, two new strains, ATCC 9081, and NCIB 6785, were screened for hydrogen production by previously described procedures.[2] Strain 9081, with 1% glucose and 1% starch produced only negligible amounts of gas in 24 hours. On the other hand, strain 6785 produced large volumes of hydrogen in 4.5 hours. The rate of gas, hydrogen and acid production during the five hour incubation is shown in table 3 and figure 1. This experiment was set up with five replicates, each, to be analyzed during a set interval. One of these replicates, taken at random, was used to determine the change in pH during the incubation. Examination of the data in table 3 shows an apparent decrease in total gas production. This is not an actual decrease, but is explained by the fact that the data are representative of a series of tubes. The column

TABLE 3

Hydrogen Production by *Clostridium welchii* (6785) - 1% Glucose

Buffer: 0.1% PO_4, pH 6.8
Temp.: 37°C
Inoculum: 1 ml of 1×10^{10} cells

Tube No.	Time Hrs.	Total Gas Volume (ml)	Total Hydrogen Volume (ml)	pH
	0			6.8
(1)	1.17	1.7	1.63	
(2)	1.33	2.0	2.0	
(3)	1.5	2.3	2.2	
(4)	1.66	3.0	3.0	
(5)	1.83	4.9	4.8	6.69
(1)	2.66	6.0	5.9	
(2)	3.17	6.3	6.2	
(3)	3.33	6.5	6.2	
(4)	3.5	8.0	7.1	
(5)	3.66	8.3	(6.1)	6.58
(1)	3.83	8.2	7.3	
(2)	4.33	8.2	4.4	
(4)	4.5	10.0	4.6	
(5)	4.66	10.0	4.6	6.56

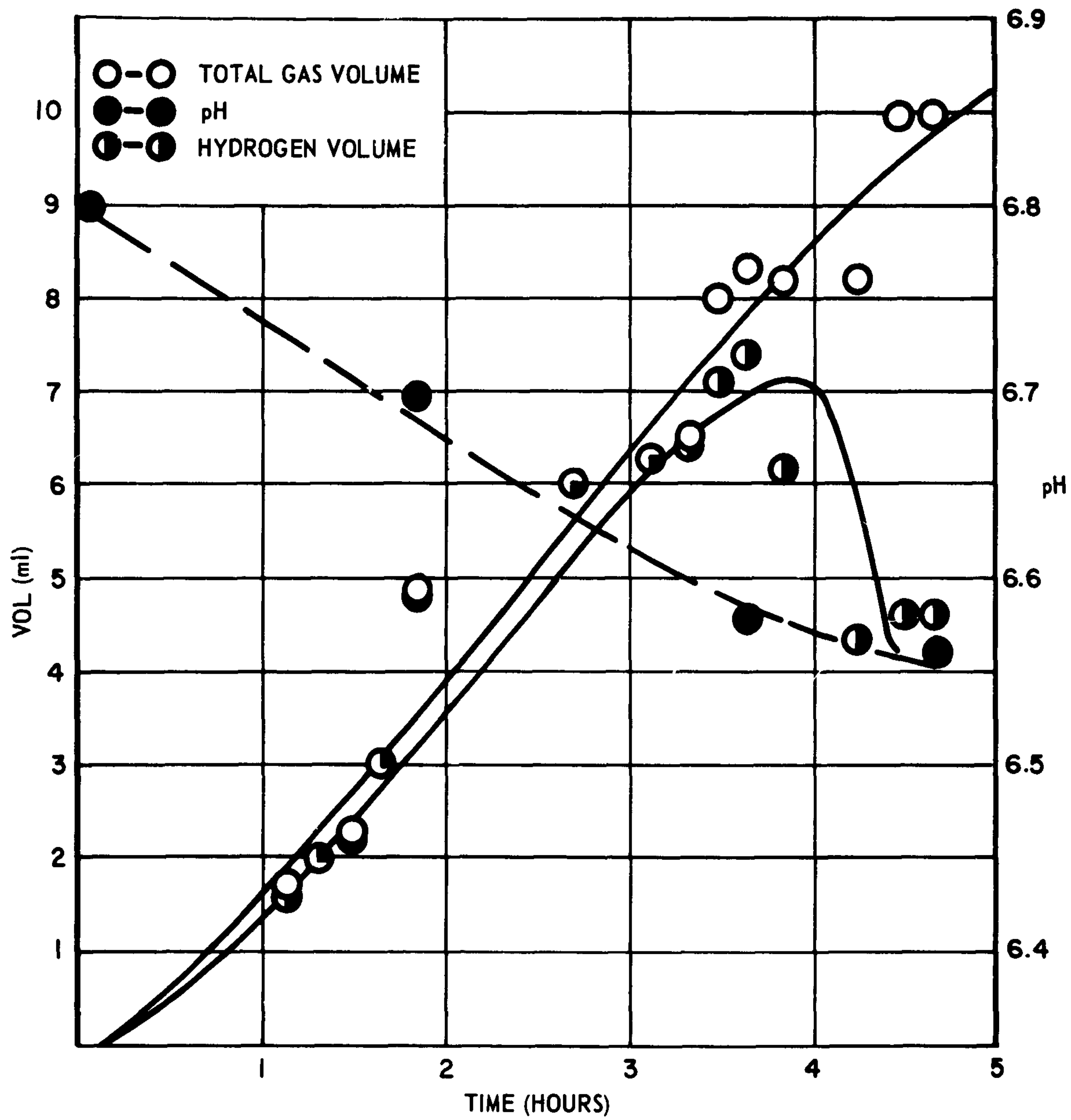

Figure 1. Hydrogen Production by Cl. welchii (6785) Glucose - 1% Gas tube Study

headed tube number indicates the values obtained from a specific tube. It was originally intended that the data from the five tubes be averaged together for the value of a particular interval. But, because of the rapid gas evolution, this was not feasible.

In considering hydrogen evolution the question arises as to how to represent the kinetics accurately and simply. A generalized hydrogen evolution curve is given in figure 2. Most of the experimental runs resemble this curve. In some cases region 1 is absent with gas generation starting immediately. With others there is a drop off in region 3. However, most of the generation curves include the features given in figure 2. Some characteristics of the three regions may be briefly mentioned.

Region 1 is a lag time before gas production begins. This may be attributed to

1. adaptation of microorganism to media

2. alteration of environment, for example, the creation of anaerobicity. _E. coli_ has a long lag period until the system became anaerobic.

3. Enzyme induction

This lag period can be altered by

1. change in media
2. control of environment
3. volume of inoculum

It is pertinent that the extent of the lag is not fixed but can be altered by these parameters. Region 2, appears to be the significant part of the

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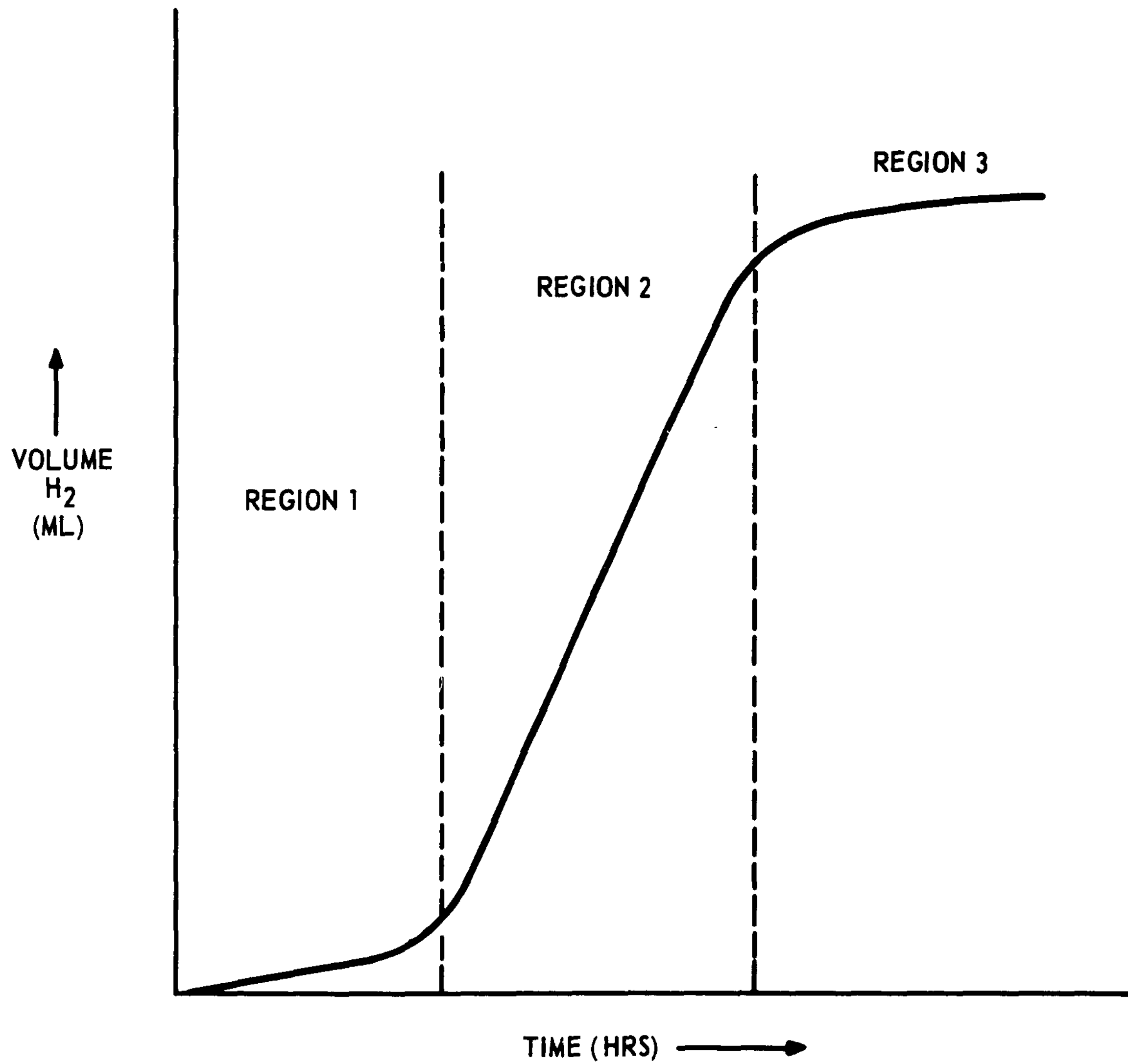

Figure 2. Typical Hydrogen Generation Curve

curve from the standpoint of biochemical fuel cell technology. This part of the curve

1. represents the greatest rate of output of metabolic product, H_2 gas, and presumably the rate which would govern fuel cell application

2. This portion is characteristic of the organism with growing cells. It is proposed here to use this linear part of the curve to characterize the particular microorganism.

Region 3 is the stationary state and one of reduced activity. The cell concentration is relatively constant or, if toxic materials build up, gas production will drop off. Here the significant point is that the "plateau" can be altered or eliminated by

1. increasing substrate concentration

2. continuous culture

Or, in other words, this region 3 is not an accurate measurement of microorganism capability of producing H_2 for fuel cell application.

Calculated on this basis, the hydrogen generation rate, "k", which is equal to $\Delta V/\Delta t$, from figure 1 is approximately

$$\Delta V/\Delta t = 6.6 \text{ ml}/3 \text{ hrs} = 2.2 \text{ ml/hr}.$$

<u>Cl. welchii</u>, strain 6785 was also tested for hydrogen production from starch and maltose. Negligible amounts (less than 0.2 ml) of gas were produced after 24 hours with starch as the substrate. This suggests either insufficient development or absence of the amylases required for starch utilization.

Gas and hydrogen production from maltose did not begin until about 6 hours after inoculation (table 4 and figure 3). The total gas volume rose

TABLE 4

Hydrogen Production by Clostridium welchii (6785)
Maltose 1%

Buffer: 0.1 M PO_4, pH 6.8
Inoculum: 1 ml of 1.2×10^{10} cells
Temp: 37°C

Time Hrs.	Total Gas Volume (ml)	Total Hydrogen Volume (ml)	pH
0			6.8
5.75	1.8	1.2	
9.00	4.7	3.5	6.55
11.00	6.2	5.4	
22.50	10.0	8.0	6.2

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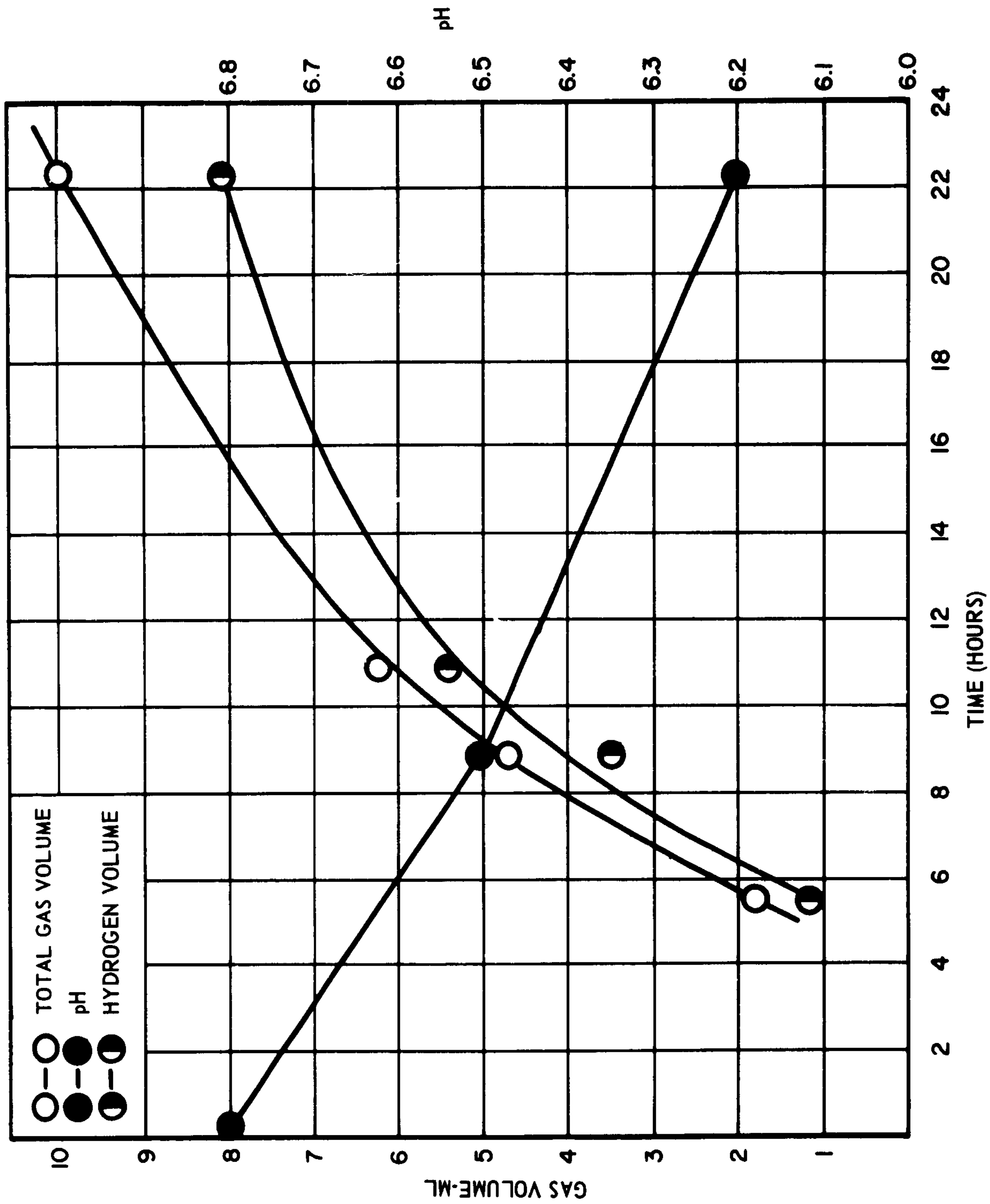

Figure 3. Hydrogen Production by Cl. welchii (6785) Maltose - 1% Gas tube Study

steadily as did the percentage of hydrogen. With glucose, however, peak hydrogen levels were obtained in four hours, and dropped off during the fifth hour. Carbon dioxide levels remained very low throughout both series. The pH dropped from pH 6.8 to pH 6.2 in the 24 hour period.

The rate of production of hydrogen was calculated as:

$$\frac{\Delta V}{\Delta t} = \frac{4.6 \text{ ml}}{6 \text{ hrs}} = 0.77 \text{ ml/hr.}$$

3. SCALE UP STUDIES

3.1 Clostridium welchii 6785 - 10L Fermenter, Growing Cells

The rapid, and high hydrogen rate obtained with this organism, prompted immediate scale up studies. A ten liter fermenter was prepared containing the same media described in the Third Quarterly Report[4] for culture maintenance and inoculum preparation. This medium was prepared without glucose and sterilized for 60 minutes at 121°C. The glucose was added aseptically as a 50% solution to a final concentration of one percent. The pH after autoclaving and before inoculation was 6.75. An antifoam, SAG 470* was aseptically added to a final concentration of 1:100,000. The inoculum for this fermenter was prepared in Deep Liver Medium. Inoculum size was 100 ml of 8×10^9 cells/ml. The uninoculated fermenter, set up as in the Fourth Quarterly Report[6] was deaerated by bubbling helium through a sparger for 8 hours. Following inoculation the fermenter was incubated at 37°C with constant agitation. The data for this experiment are found in table 5 and figure 4.

The rate of hydrogen evolution, using 4 and 6 hour measurements to calculate the curve slope, was 8.2 liters per hour. Total hydrogen produced was 29.14 liters or 54% of the total gas volume of 53.96 liters.

The real efficiency of substrate utilization and product yield cannot be determined unless the substrate remaining at the end of the experiment is known. However, an approximate calculation can be made recognizing that these organisms follow the butyric fermentation and the following equation may be expected to hold[7].

$$4\ \text{Glucose} \longrightarrow 2\ \text{Acetate} + 3\ \text{Butyrate} + 8CO_2 + 8H_2$$

---

* Union Carbide Corp., Silicones Div., New York 17, N.Y.

TABLE 5

Hydrogen Production by _Clostridium welchii_ (6785) - 1% Glucose

10L Fermenter - Growing Cells

Inoculum: 100 ml of 8 x 10^9 cells/ml.
Temp: 37°C

Time Hrs.	Total Gas Volume (Liters)	Hydrogen Volume (Liters)	pH
0			6.75
2.00	0.42		
2.50	1.04		
3.00	3.2		
4.00	23.5	11.75	
4.33	27.73	13.87	5.05
4.58	30.0		
4.83	36.55	17.54	
5.00	43.50		5.00
5.50	45.25		
5.75	48.0	27.84	
6.00	50.10	28.06	4.85
6.50	53.80	26.36	
6.75	53.96	29.14	4.70*
7.00	53.98	28.07	4.85
7.50	53.90	29.11	5.10**
7.90	53.90	26.41	5.2

* 25 ml. - NaOH - 20% added

** 10 ml. - NaOH - 20% added

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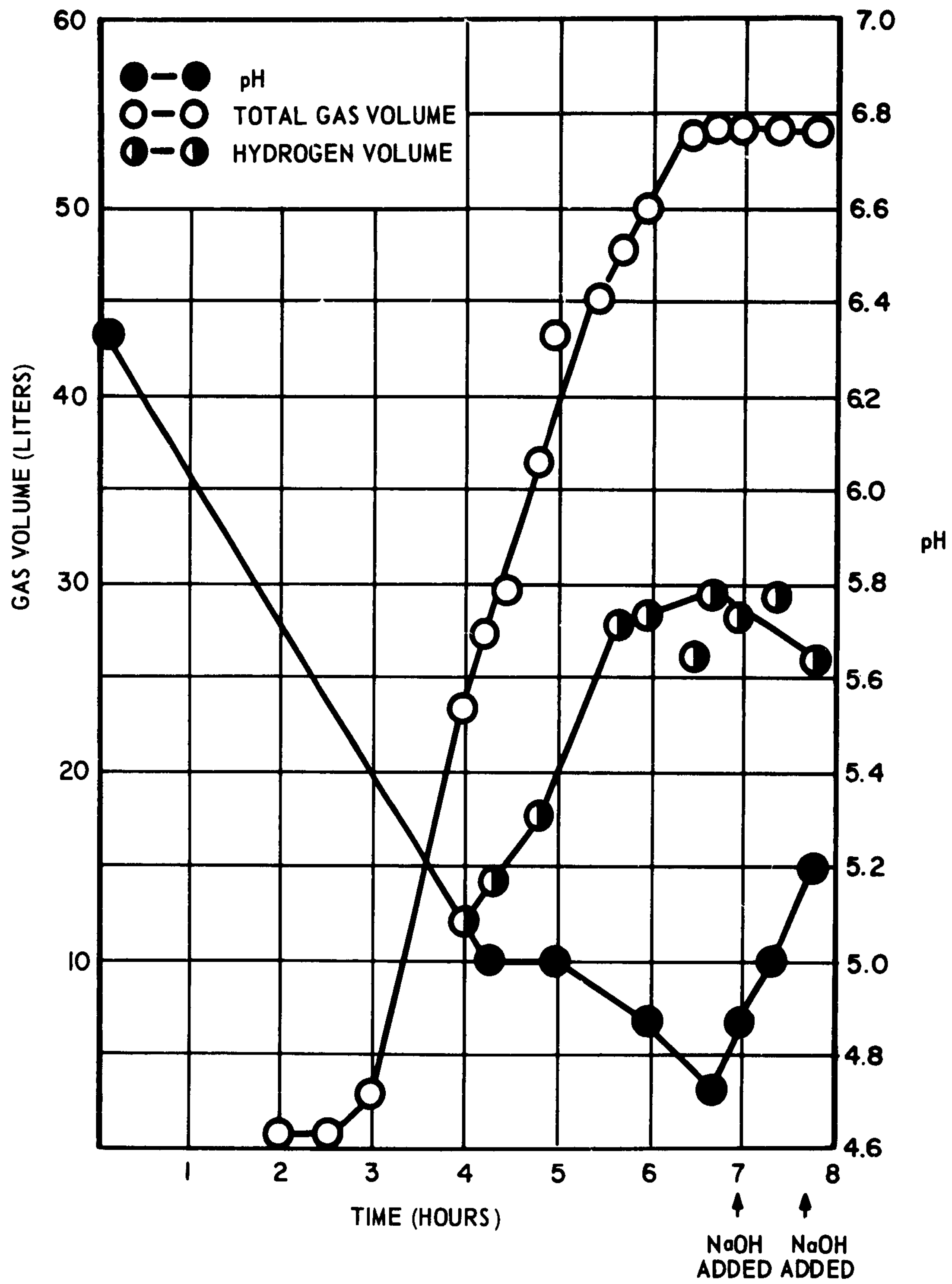

Figure 4. Hydrogen Production by Cl. welchii (6785) Glucose 1% Growing Cells - 10L Fermenter

Assuming that this reaction actually represents the case, the fermentation should return 25.1 liters of hydrogen for 100 gms or 0.56 moles of glucose. The yield obtained in this study was approximately equal to that amount theoretically obtainable. This means that the organisms, under the specified conditions were about 100% efficient in utilizing the available glucose. What is required now are methods for increasing the amount of enzyme per organism and for maintaining conditions for maximum hydrogen production throughout the fermentation.

3.2 Cl. welchii 6785, 2.5L Fermenter - Growing Cells

The results from the above experiment utilizing this particular strain of Cl. welchii were checked in a brief experiment. The total volume of the fermenter in this experiment was 2.5 liters. It was hoped that by adding $CaCO_3$ and increasing the initial pH from 6.8 to 7.1 the effects of acid produced would be reduced. The results did not indicate any improvement. The data for this experiment are found in table 6 and figure 5. As with the previous fermenter gas production did not begin until approximately 4 hours after inoculation. The rate of gas production between 7 and 10 hours was 2.2 liters of hydrogen per hour, which was comparable to the 8.2 liter/hour rate of the previous fermenter when the respective fermenter volumes and inoculums are considered.

3.3 Clostridium welchii (6785)-10L Fermenter-Resting Cells

The employment of resting cells hold an advantage over growing cells. With resting cells, the major requirement is a carbon source. The requirements for a nitrogen source, the procurement of which, under practical field conditions, would constitute a problem, is eliminated. This experiment

TABLE 6

Hydrogen Production by Clostridium welchii (6785) - 1% Glucose

2.5 Liters + $CaCO_3$ (Fermenter)

Inoculum: 25 ml of 1.1 x 10^{10} cells/ml.
Temp: 37°C

Time Hours	Total Gas Volume (Liters)	Total Hydrogen Volume (Liters)	pH
0			7.1
4.00	1	0.03	7.3
7.00	2	0.36	7.1
7.83	3	1.53	*
8.08	3.8		
8.33	4.9		
8.50	5.35	2.25	*
10.17	8.65	3.63	*
13.75	9.00	4.05	5.8

* Sample port obstructed

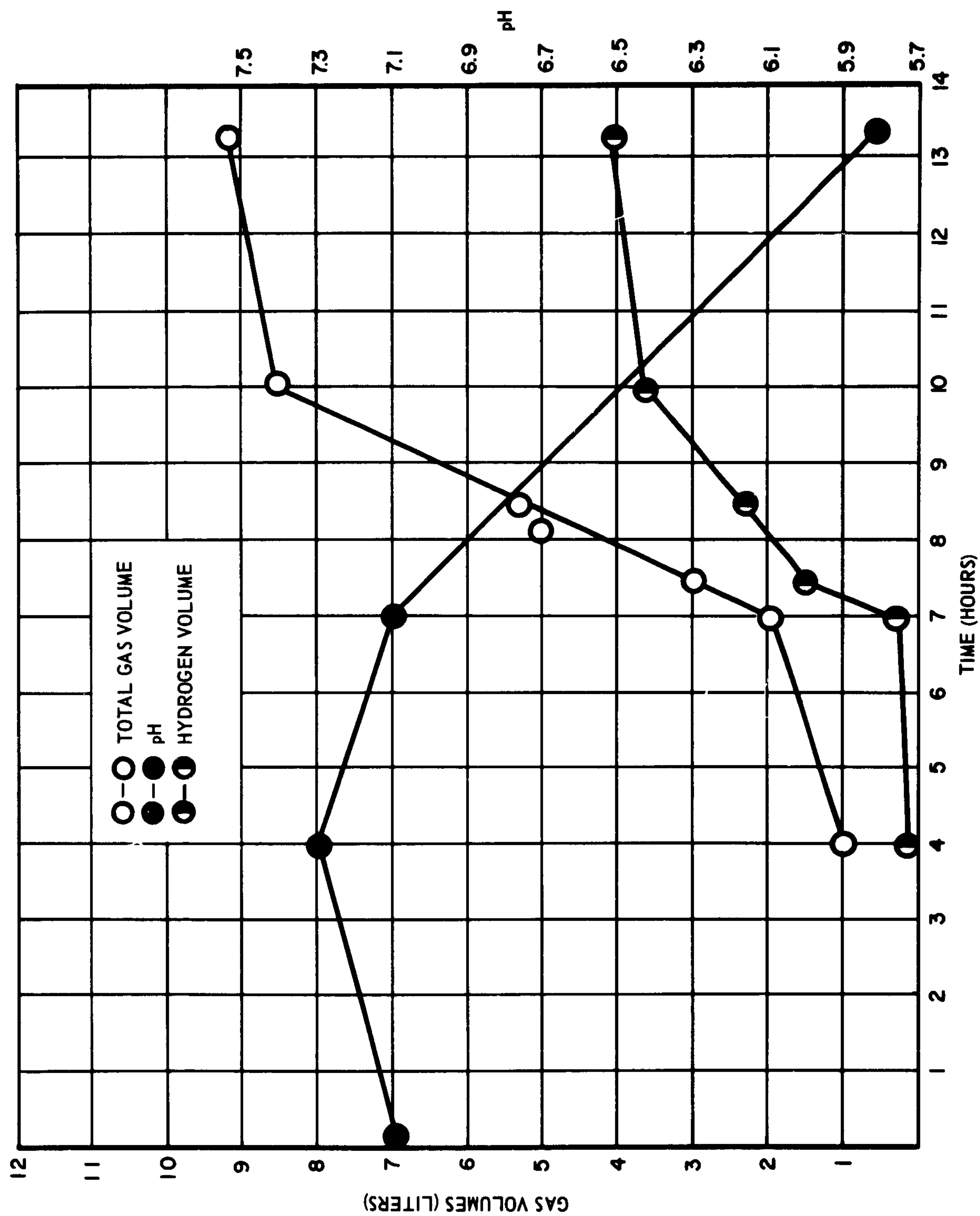

Figure 5. Hydrogen Production by Cl. welchii (6785) Glucose 1% Growing Cells-2.5L Fermenter

is a direct scale up of the resting cell - tube studies and cannot because of inoculum size, be compared with the 10 liter fermenter using growing cells.

The fermenter was set up as previously described,[6] containing 0.01 M, pH 6.8, phosphate buffer. The inoculum, prepared from 8 liters of Deep Liver Medium was 595 ml of $1x10^{10}$ cells/ml. The data for this experiment are found in table 7 and figure 6. An accurate scale-up from the gas tubes would indicate an inoculum size of 667 ml of 1×10^{10} cells 1 ml. The inoculum size used in the 10 liter resting cell fermenter was calculated on the basis of one ml. of 1×10^{10} cells/15 ml substrate. This expands to 667 ml of 1×10^{10} cells for 10 liters. The inoculum used represented 90% of this. The maximum hydrogen production rate was 0.66 liters/hour, measured between 2.5 and 5.5 hours incubation. If the gas tube rate of 2.2 ml H_2/hour were multiplied by the appropriate scale up factor, a rate of 1.46 liters/hour would be expected. Considering the lower inoculation, the volume obtained was only 50% of that anticipated from the direct proportional scale up. The pH range during the tube study was pH 6.8 - 6.56. The range for the fermenter was pH 6.75 - 6.0. Further studies to determine whether maximum substrate conversion can be or was obtained are required. To compare 10 liter fermenters of growing cells and resting cells, the cell concentration at the time of maximum hydrogen production will be used in the resting cell study. The cells from the growing fermenter will be concentrated, washed and used to inoculate the resting cell study.

3.4 Fermentation Analysis

To properly establish the efficiency of an organism-substrate system in fermenters or in other scale-up studies, certain analyses must be carried

TABLE 7

Hydrogen Production by Clostridium welchii (6785) - 1% Glucose

Resting Cells 10L Fermenter

Inoculum: 595 ml of 1 x 10^{10} cells/ml
Temp: 37°C

Time (Hours)	Total Gas Volume (Liters)	Hydrogen Volume (Liters)	pH
0			6.75
0.50	0.25		
1.33	1.50		
1.83	2.05		
2.00	2.40		
2.50	3.01	.60	6.6
3.00	4.45		
3.50	5.75	1.38	
4.00	5.95	1.67	
4.50	6.75	2.30	6.55
5.00	7.35	2.21	
5.50	7.80	2.85	
6.00	8.30	3.57	6.35
8.00	10.00	3.70	6.15
20.50	15.44		6.0

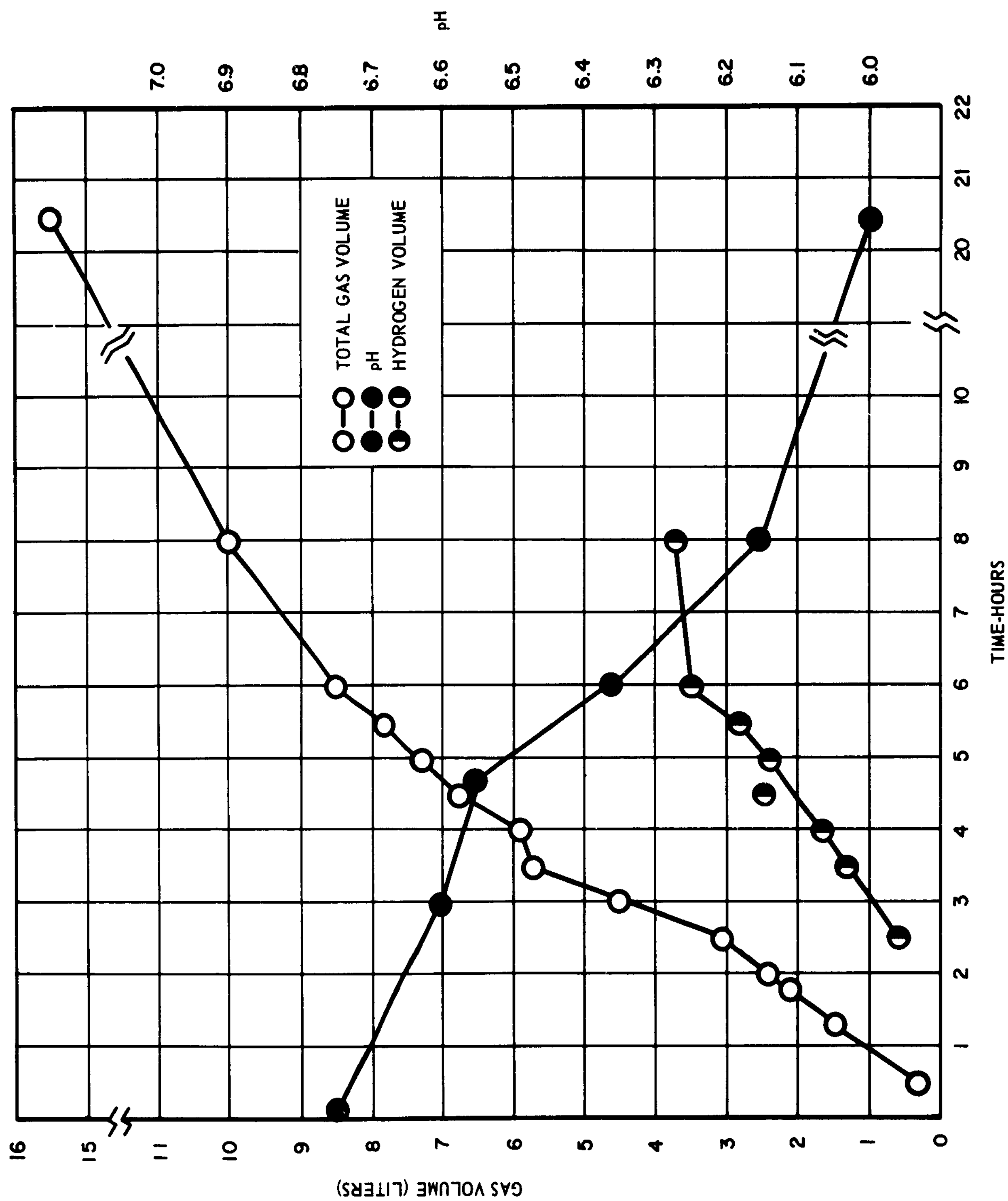

Figure 6. Hydrogen Production by Cl. welchii (6785) Glucose 1% Resting Cells - 10L Fermenter

out. It is required to establish the fermentation balance and accurately determine the relation of products formed to the amount of substrate used. The _Cl. welchii_ fermentation follows the butyric fermentation pathway and to establish the complete balance analyses for H_2 and CO_2, butyric, acetic and lactic acids and ethanol would be required. Initially, it is planned to analyze for glucose utilization and hydrogen production. Other intermediates will be considered if found necessary. Methods for analysis will be those suggested by Neish[8] and other suitable sources.

Errors can be expected from several sources. These include

a. that a large portion of the carbon in the substrate is converted to cell material

b. other components in the medium contribute to product formation

c. CO_2 is fixed by the cells.

3.5 Attempted Studies

Rate and statistical studies on _Cl. butyricum_, _Cl. butylicum_ and _Pseudomonas_ sp. (G4A) were planned, but not completed. Previous data on the Clostridia showed gas production no earlier than 18 to 40 hours. It was expected that methods to improve this output would be developed. These methods were not forthcoming. Continued effort will be directed toward achieving improved gas production.

Similar studies as well as scale up studies were scheduled for _Pseudomonas_ sp. (G4A) and _E. coli_. It was found that a physiological alteration had occurred in these two organisms that affected their hydrogen producing ability. Studies with these organisms will resume when the original strains can be recovered.

4. PLATE STUDIES

The assumption has been made that in spite of diligent screening tests it is unlikely that microorganisms will be found with sufficient hydrogen generating ability. For this reason it is intended to use genetic techniques to increase the amount of hydrogen produced per cell of microorganism. The feasibility of this approach has been demonstrated by the classic work with penicillin. The original strain of the penicillin organism, *Penicillium notatum*, which Fleming isolated in 1929, produced only 2-3 units of penicillin per milliliter in small flasks. By isolation, selection, mutation and kinetic studies it was possible to increase production to 6-10,000 units/ml in large fermentation tanks. This represented a 10^3 - 10^4 improvement factor.

A general plan to develop desirable mutants is as follows:

(a) Treatment of the culture with a mutagenic agent (U.V. light, X-ray or chemical agent) to product a 95-99% kill.

(b) Agar plating of the treated suspension.

(c) Selection of the mutant colony from the agar.

(d) Isolation of the mutant in pure culture.

The procedure can be repeated. Obviously a critical phase of the whole process is to find the proper technique of selecting the mutant. Attention has been directed to this problem during this quarter. *E. coli* was grown, anaerobically, in a liquid medium in one case with 0.25 % glucose as the substrate and the other with 1% formate as the substrate. Following incubation at 37°C for 18 hours, 10 fold serial dilutions in sterile buffer, pH 7.4, were prepared. One ml of each dilution was added to each of four plates. The inoculum was thoroughly mixed with 25-30 ml of trypticase soy agar (BBL) with 1% formate added. Duplicates of each

dilution were incubated aerobically and anaerobically at 37°C for 18 hours. Examination of the plates following incubation showed numerous gas bubbles within the agar. Representatives of the plates may be found in figures 7, 8.

The description of the plates is as follows:

A - formate grown inoculum - formate agar

B - formate grown inoculum - formate agar

C - glucose grown inoculum - formate agar

D - glucose grown inoculum - formate agar

By measuring the size of the bubble, it should be possible to detect mutant colonies with greater hydrogen producing capability than the parent strain.

In the case of the Clostridia, not normally considered able to utilize formate, it may be possible to detect mutants by their ability to produce gas on other substrates, i.e., glucose.

It is encouraging that such a simple technique should be suitable for selection of variants. Further studies with differing agar concentrations, other solidifying media and overlay techniques should better define a more exact procedure.

Other approaches may be used in collaboration with these techniques. These will include correlation of dehydrogenase activity, particularly formic dehydrogenase, with hydrogen production and possible adaptation of the above data to a visual readout using redox dyes such as benzyl viologen.

J1040.01-3

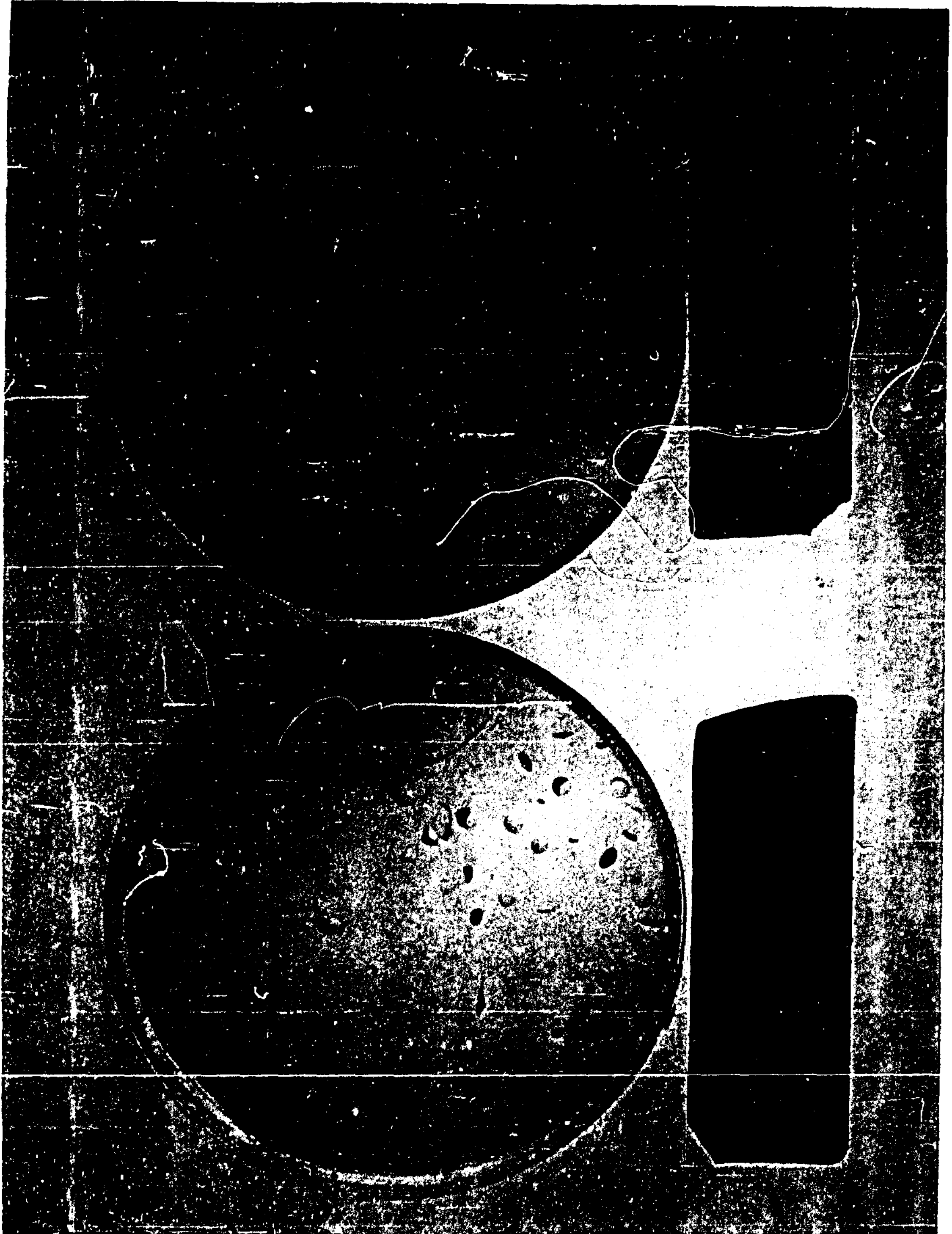

Figure 7 Plate Test for Dehydrogenase Activity - FORMATE

J1040.01-2

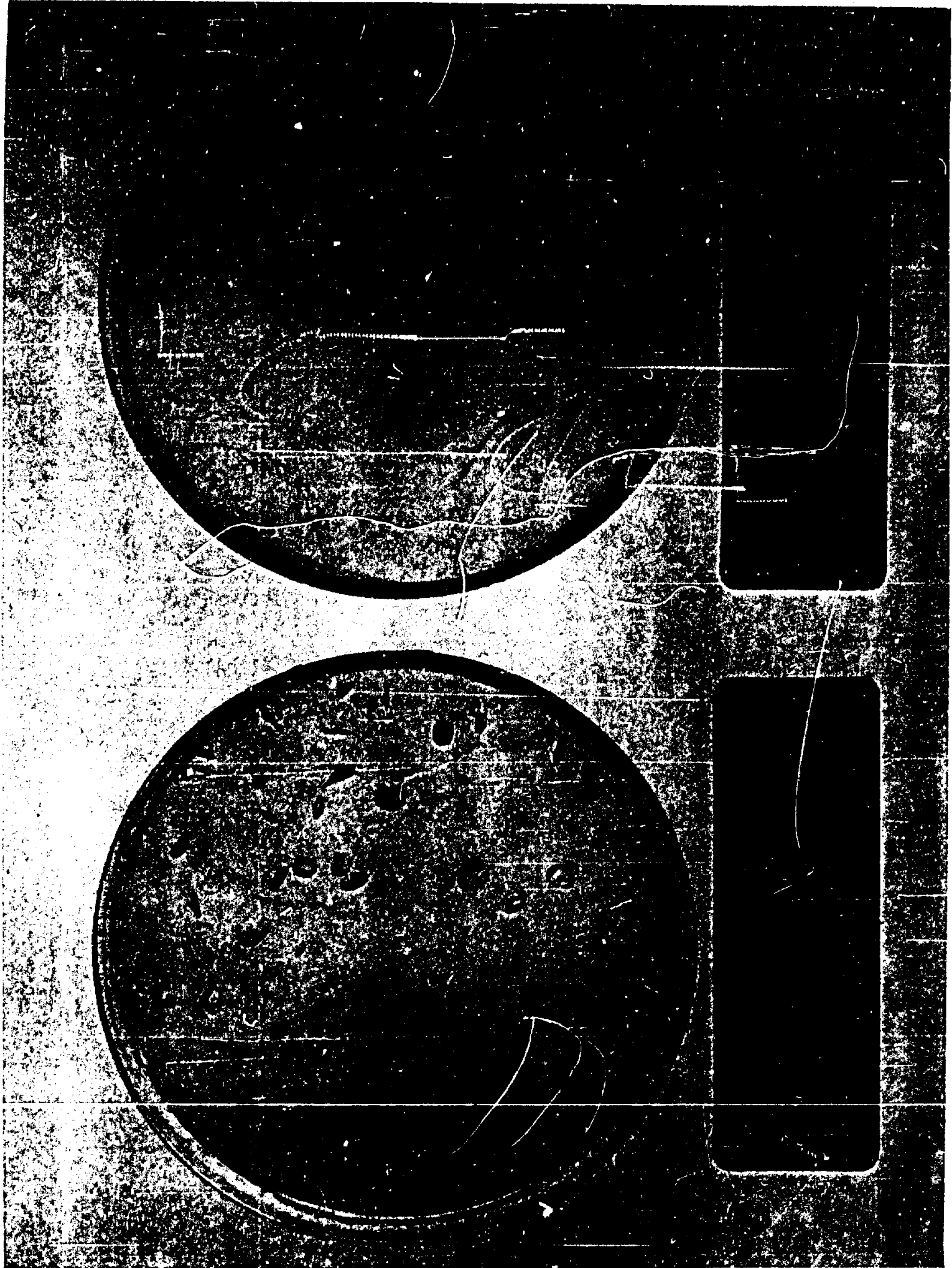

Figure 8. Plate Test for Dehydrogenase Activity - GLUCOSE

5. SUBSTRATES FOR FIELD USE

5.1 Natural Vegetation

The possibility of the ultimate employment of the biochemical fuel cell in the field would be greatly enhanced if cheap available substrates could be utilized. In this connotation a cheap, available substrate implies a source of carbohydrate which can be found at the site in a form that can be metabolized by microorganisms to the desired product (hydrogen gas).

Under most field conditions, some sort of vegetation such as grasses, seeds, grains, roots and leaves can be expected to be present. Chemically, these materials are composed of cellulose, lignin, hemicelluloses, starches, and sugars. From the standpoint of rapid utilization by microbes, only sugars and starches appear to merit extensive consideration.

Before these raw materials can be considered as substrates, the concentration of fermentable components must be established. The available literature on this subject is scarce and the reports that are available do not usually indicate the concentration of carbohydrates or fermentable components. What is listed in the approximate analysis of these products are agricultural and feed terms. The analyses are based on methods which are official with the Association of Official Agricultural Chemists (A.O.A.C.)

Several large symposia have been conducted (10, 11, 12) under the aegis of the National Academy of Sciences. The published proceedings contain the most up-to-date surveys of the approximate composition of the various feed compounds, many of which can be considered for field use in the biochemical fuel cell. In table 8, a list of several grains and grasses and the approximate content of the readily fermentable components is given. The limited

TABLE 8

APPROXIMATE SUGAR COMPOSITION OF SELECTED GRAINS AND GRASSES*

Grains	Starch (% Range)	Total (% Range)	Reducing Sugars				Acid Hydrolyzable	
			Total	Glucose	Fructose	Pentose	Sucrose	Maltose
Barley	60.1-67.1	2.5						
Corn	22.6-73.7	1.5-9.1						
Oats	38.7-62.3	1.2-1.6						
Rice	63.8-66.1	0.3-4.5						
Sorghum	65.9-73.3	0.8-3.1						
Wheat	6.17-69.3	2.7-4.5						
Dry Roughage								
Alfalfa, All Forms	1.6-4.1	3.2-10.8						
Brome Hay, Smooth	-	3.7						
Clover, All Forms	-	2.4-8.6						
Orchard Grass	1.1	5.1-5.7						
Sorghum, All Forms	2.8	0.6-34.8						
Timothy	-	4.4						
Green Roughages								
Alfalfa Forage		4.7-10.5						
Blue Grass Forage		5.0-16.3	3.22-3.32				4.11-5.15	
Blue Stem Forage		1.3-5.4						
Brome Forage		2.8-13.6	2.51-2.58				8.17-8.25	
Canarygrass Forage		5.1-10.3	2.84-3.15				4.43-5.65	
Clover, Forage		3.1						
Fescue, Forage		3.2-14.9	3.74-4.17				4.51-7.36	
Oatgrass, Forage		5.6-16.9	3.00-3.13				7.81-9.65	

TABLE 8

APPROXIMATE SUGAR COMPOSITION OF SELECTED GRAINS AND GRASSES (Continued)*

Grains	Starch (% Range)	Total (% Range)	Reducing Sugars				Acid Hydrolyzable	
			Total	Glucose	Fructose	Pentose	Sucrose	Maltose
Redtop, Forage		4.6-15.6	1.85-2.63				4.44-9.25	
Sorghum, Fodder		3.2-9.1						
Timothy Forage		2.4-9.7	2.44-2.88	1.05	1.02		4.22-12.14	
Orchard Grass Forage			4.11-4.51				4.32-4.54	
Ryegrass				2.00	1.81		6.62-9.69	

* Adapted from References 10, 13, 14.

coverage of the table is not due to selection, but to the small amount of information available in the literature. From the table it can be seen that starch is the predominating fermentable component in the grains. Dry grasses (dry roughages) contain small amounts of starch and varying concentrations of fermentable sugars. Green cut grasses (green roughage) contain virtually no starch but have a variety of sugars. It appears that, of the fermentable sugars present in these materials, sucrose predominates over the reducing sugars.

A partial bibliography of the literature in the area of the composition of field crops and forages is appended to this report. The literature of the U.S. Department of Agriculture and the state departments of agriculture, agricultural colleges and experiment stations is now being collected and will be collated.

The questions regarding the potential of utilizing field vegetation, whether or not plants can actually be considered as substrate material, cannot be answered until some experimentation is completed. But some speculation is possible. The starches in grain materials, such as corn and corn products, have been used, but some pretreatment, such as chopping, shredding or grinding might be required. How the natural flora of these materials would affect the desired microbial activity would have to be established. These questions must be answered before the use of these materials can be fully appraised.

5.2 Hydrocarbons

Up to now consideration of cheap substrates has been directed primarily to vegetation which may be found in the field-substrates which are sugar or

polysaccharide in nature. Comparable consideration should be also given to hydrocarbons as substrates for organisms capable of producing hydrogen. If hydrogen in these compounds can be liberated by these organisms further study is warranted. Preliminary studies with organisms known to be able to metabolize hydrocarbons has been conducted. These organisms, classed as Pseudomonads, produced gas on glucose under the experimental conditions used in Table 1. Whether gas, especially hydrogen can be liberated by microorganisms growing in fuel is not known but experiments are in progress which should answer this question. The results from this study will be reported in the future.

6. UTILIZATION OF MICROORGANISM-PRODUCED HYDROGEN IN FUEL CELL

It is desirable to appraise the rate of hydrogen production achieved on this project in terms of operating a hydrogen-oxygen fuel cell. This is done here in a preliminary way, making what are admittedly some gross assumptions. However, this analysis reveals the order of magnitude of improvements that must be made.

The assumption is made that a one watt unit will operate at 0.6 volt and 1.67 amp, which would require 6000 coulombs, 0.031 moles or about 0.7 liters of H_2 per hour.

Hydrogen generation rates of 8.2 liter/hr have been obtained in the 10 liter fermenter. It is assumed that 0.7 liters would be produced in a fermenter occupying 0.85 liters, or 0.03 cubic feet, and weighing 1.9 lbs.

The unit would thus approximate a rating of about 0.5 watts/lb. or 33 watts/cubic ft.

The hydrogen generation rate should be increased by a factor of 100-1000 to make such systems of interest to the fuel cell field. This order of improvement appears feasible and following are suggested ways in which this may be accomplished.

1. Hydrogen output per organism increases - an improvement of 10-100 may be anticipated here - see section 4.

2. Increased cell content per volume of fermenter - an improvement of 100 fold appears feasible here.

3. Optimize conditions, e.g. temperature, pH, substrate concentration - an improvement of 10 fold appears reasonable. Within the scope of what has been achieved in the laboratory in recent years an increased output of 10^4 over present rates of hydrogen generation appears feasible.

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CONCLUSIONS

The following conclusions may be drawn from the data in this report.

(1) Further screening studies with various Clostridia species and strains should be conducted on new substrates in the form of rate studies with pH determination. The pH control is not only required to maintain hydrogen output but also is required to achieve valid comparisons between test systems.

Species of the Clostridia have produced more hydrogen than other organisms to date. It would be desirable to concentrate on these organisms in future studies. The future studies should be concerned with obtaining a nonpathogenic strain with high hydrogen producing potential and with methods for improving current strains.

(2) Cl. welchii (6785) produced hydrogen at the rate of 8.2 liters/hours in active fermentation. Resting cells of this strain do not produce as much hydrogen as growing cells. Also large scale studies with resting cells show that only 50% of the anticipated level was realized in the scaling up.

Further evaluations with this organism should be conducted with active fermentations with sucrose as a substrate because sucrose constitutes a major portion of the fermentable sugars found in grass cuttings. While it appears that theoretical biological yield of hydrogen has been achieved, adjustment of the conditions of the experiment and substrate concentration may serve to increase yields of hydrogen. But, these studies should be conducted in fermenters so that actual conversion efficiences can be measured.

3. A plate technique has been developed which should make it possible to detect cells with greater hydrogen producing ability than the parent cells. Further studies to establish exact experimental conditions are required.

4. Preliminary examination of the literature pertaining to the composition of substrates available in the field shows that, of those available, grasses and forages appear to possess the greatest potential. However, the percentages of fermentable components is low and this would necessitate the use of large amounts of raw material to achieve a desired substrate concentration. A major problem that enters here, beside the handling of this material, is the contamination from other soil flora. The question of whether this will render this source useless must await the results of further study.

In the same area of consideration, that of inexpensive and available substrates, the hydrocarbons should be considered. There are organisms which can accommodate these materials as energy sources and they should be studied.

5. An improvement of hydrogen generation by a factor of 100-1000 is required before microorganism-generated hydrogen will be of significance in fuel cell technology. However, certain possible ways of accomplishing the required improvement are visualized.

PROGRAM FOR THE NEXT INTERVAL

Work during the next quarter will be conducted primarily in the following areas.

1. Analysis of the glucose utilization efficiency during fermentation with Cl. welchii (6785) and studies on increasing hydrogen production as discussed in Section 3.4.

2. Measurement of hydrogen production by new Cl. welchii isolates obtained from active, human infections.

3. Consideration of nonpathogeni Clostridia species.

4. Improvement of the agar plate technique described in this report by using different media (i.e., silica gel).

5. Investigation of the correlation of formic dehydrogenase activity with hydrogen production.

6. Establishment of ultraviolet dose response curves as preliminary to genetic investigation.

IDENTIFICATION OF KEY PERSONNEL

The following personnel performed the approximate number of man-hours during the fourth quarter of the project.

Robert T. Foley — 68 hours

Project scientist with background in electrochemistry and surface chemistry.

B.S. (Chemistry) University of Massachusetts
M.S. (Physical Chemistry) Lafayette College
(Graduate Work) New York University
Ph. D. (Physical Chemistry) University of Texas

Gordon C. Blanchard — 8 hours

Senior microbiologist with background in heterotropic and autotropic metabolism.

B.A. (Zoology) University of Vermont
M.S. (Biochemistry) University of Vermont
Ph. D. (Microbiology) Syracuse University

Paul S. May — 216 hours

Microbiologist with background in waste conversion and sewage decomposition by fecal microorganisms.

B.S. (Biology) City College of New York
M.S. (Industrial Microbiology) Syracuse University
D.Sc. (Microbiology) Philadelphia College of Pharmacy & Science

Harry H. Titus — 60 hours

Mathematician with background in statistical design of experiments

B. S. (Mathematics) Hampden-Sydney College, Computer
Programming Courses - International Business Machines

Technician, Microbiology — 518 hours

Technician, Analytical Chemistry — 41 hours

APPENDIX A - BIBLIOGRAPHY OF SUBSTRATE COMPOSITION

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SUBSTRATES COMPOSITION

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Unclassified Report

This report describes an experimental and theoretical investigation of hydrogen production by microorganisms Screening studies were conducted on Cl. welchii and certain Pseudomonads related to metal corrosion in hydrocarbon fuel systems. Cl welchii (NC1B6785) produced hydrogen in larger quantities than any organisms tested to date. The development of a simple plate technique has initiated studies to develop mutants with outstanding hydrogen producing capabilities. A survey of economical and available substrates suitable for field use has been made. Hydrocarbons as substrates will be considered. To make a preliminary appraisal of the biochemical fuel cell, the hydrogen production rate is observed and expressed in terms of fuel cell consumption.

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1. Biochemical Fuel Cell
2. Fuel Cell - Biochemical
3. Hydrogen Production - Microorganism source
4. Microorganisms - utilization for fuel cells

I. May, P.S., Blanchard, G.C., and Foley, R.T.
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