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Biochemical Fuel Cell

Report Nr. 3

Contract Nr. DA 36-039 SC-90878

Task Nr. 3A99-09-001-01

THIRD QUARTERLY PROGRESS REPORT

1 January 1963 to 31 March 1963

U. S. ARMY ELECTRONICS RESEARCH
AND DEVELOPMENT LABORATORY
Fort Monmouth, New Jersey

MELPAR  **INC**

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Biochemical Fuel Cell
Report Nr. 3

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Third Quarterly Progress Report
1 January 1963 - 31 March 1963

Object: The object of this research is to conduct investigations pertinent to biochemical fuel cells. Specifically, the generation of hydrogen by microorganisms, the utilization of this hydrogen, and the usage of readily available hydrogen sources will be studied.

Prepared by: Paul S. May, G. C. Blanchard, and R. T. Foley

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PURPOSE

The purpose of this program is to conduct investigations pertinent to biochemical fuel cells. More specifically, these 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. The latter determinations will be qualitative and quantitative.
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 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

The experimental investigation of microorganisms capable of producing hydrogen for fuel use is described.

The microorganisms, Clostridium butyricum, Clostridium butylicum, Photobacterium phosphoreum and Erwinia nimipressuralis were investigated utilizing various substrates.

A statistical study utilizing a Fractional Factorial design was applied to the study of four microorganism - substrate systems. The design, involved seven factors at two levels. These were pH, temperature, substrate concentration, cell count, buffer concentration, agitation, and oxygen tension during growth. The results showed that different factors governed the H₂ producing capacity of the different systems. The data will be applied to a complete factorial study to optimize conditions for hydrogen production.

Rate studies with E. coli-glucose showed that high-temperature (35°C), anaerobic growth and glucose-supplemented media are required for maximum H₂ production. This study investigated H₂ evolution as a function of time as well as other experimental conditions.

Preliminary experiments demonstrated that the hydrogen-producing capacity of an organism is related to the level of hydrogenase and formic dehydrogenase. The demonstration of this correlation is important for the genetic studies planned for the second and third year of this work.

Work for the next quarter will include further screening studies, rate studies, statistical experimentation, and scale-up studies.

PUBLICATIONS, LECTURES, REPORTS, AND CONFERENCES

There were no publications, lectures, reports, or conferences during this period.

FACTUAL DATA

1. INTRODUCTION

The technical work during the third quarter was devoted to continuation of the screening program, hydrogen evolution rate studies, a fractional factorial design study, determination of hydrogen-producing enzyme systems, and preliminary scale-up studies.

In the screening program, organisms were examined for their hydrogen-producing capabilities. The fractional factorial design study was employed in an attempt to optimize the conditions for hydrogen evolution. The plan involved eight studies for examination of the effects of seven factors at two levels on one organism. A complete factorial design with these factors would require 256 studies.

The combined use of the fractional factorial design of seven factors at two levels, and the complete factorial design with remaining factors should pinpoint the optimum conditions for each system. The required conditions for each system can be determined with considerably fewer experiments with an obvious saving in time.

To understand the mechanism of hydrogen evolution, it is necessary to correlate the specific activity of the hydrogen-evolving enzymes with the hydrogen evolution. Many experiments to increase the hydrogen-producing capacity of an organism are possible if the mechanism is clearly understood. Attempts to correlate enzyme activity and hydrogen evolution were made during this quarter.

By establishing the kinetics of hydrogen evolution by a given organism, much more information can be learned than by a point analysis. For example, it can be determined whether the organism produces gas immediately or requires a lag period, whether the evolution continues at a slow rate or peaks and remains constant, or if the gas is re-utilized. This type of information is valuable from the biochemical viewpoint because one learns when the reaction is completed and whether the organism is grown on a proper substrate for synthesis of the necessary enzyme systems.

The data obtained from the above studies will be used to set up systems capable of larger production levels. In industrial fermentations, it is necessary to gradually increase the volume of the reactions from the flask to small fermenters, and to pilot plant before actual large-scale fermentation tanks are put into operation. Each level of scale-up requires special conditions which must be determined and which are dependent on the criteria for the previous level. The experiments carried out to date have been with small flask systems. Attempts to scale-up are now being made with growing cells.

2. SCREENING PROGRAM

During this quarter, the screening program was devoted to studies of the hydrogen-producing capabilities of Cl. butyricum, Cl. butylicum, Photobacterium phosphoreum, and Erwinia nimipressuralis.

2.1 Clostridium butyricum and Clostridium butylicum

Clostridia of the butyric-butylic fermentative type are capable of producing large quantities of hydrogen when grown in the presence of suitable carbohydrates.^{1 3} The fermentation balances depicted by the equation



fit the data for Cl. butyricum.² This equation was based on the yield of butyric acid, acetic acid, CO₂, and H₂ from glucose.²

The mechanisms by which Clostridia are believed to produce hydrogen include the phosphoroclastic reaction, vinylacetate oxidation, acetaldehyde oxidation and amino acid decomposition. Reference is made to the First Quarterly Report⁷ for a discussion of these reactions. The production of hydrogen by the Clostridia differs from the mechanisms involved in the colon-aerogenes group.³

In the last report⁵ difficulties in procedures for obtaining active resting cell suspensions were noted. These cells were grown in thioglycollate or Difco AC broth and resulted in inactive, or very slowly active, ropy, cell suspensions. The procedure now in use eliminates this problem.

The following medium (Deep Liver Medium⁶) was used for culture maintenance:

Liver extract	100 ml
Yeast extract	5.0 g
Tryptone	10.0 g
K ₂ HPO ₄	2.0 g
Glucose	5.0 g
Distilled H ₂ O	900 ml

pH adjusted to 7.4.

Before dispensing to test tubes, add a small quantity of dried liver particles to each tube.

The liver extract is made by adding 2 liters of distilled water to each pound of finely ground beef liver and heating in flowing steam until all the redness is gone from the liver. This will require about three hours during which the concoction should be stirred frequently in order to break up clumps.

When the supernatant becomes yellowish fluorescent it is filtered through several layers of cheese cloth. The clear supernatant is then bottled and sterilized.

The residue which remains after the above extraction is spread on shallow trays and dried at 50°C. This constitutes the dried liver particles mentioned above.

For the inoculum preparation, the same medium, excluding the solid liver particles, was employed. The resulting cell suspension was uniform and devoid of the sliminess, characteristic of previous inoculum preparations. Cells were grown under nitrogen in 600 ml volumes at 30°C. Cells were harvested by centrifugation, washed 3 times with 0.85% saline and finally re-suspended in saline to final concentrations of between 10^8 and 10^{10} cells/ml. Tests were conducted at pH 7.4, in 0.1M Sorenson buffer with a 1% concentration of substrate. The data for these studies are found in tables 1 and 2. *Cl. butyricum* produced smaller volumes of hydrogen from glucose, maltose and lactose at pH 7.4 than were obtained with parallel studies at pH 6.8. With agitation, greater yields were obtained with sucrose, maltose and lactose than in those tubes lacking agitation. The results of *Cl. butylicum* studies show generally low yields at pH 7.4 with glucose, maltose and lactose. Gas production was delayed past 18 hours and occurred between 18 and 40 hours. Results are from 40-hour samples. There are two possible hypotheses which may help explain the data and the delay in gas evolution. These are enzyme induction in the reaction vessel and the size of the inoculum. Ordal and Halvorsen⁸ report that large inocula may decrease measurable hydrogenase activity.

Further studies with these organisms will be conducted in the next quarter.

2.2 Photobacterium phosphoreum

Studies with this organism were conducted in an attempt to measure its H₂ producing properties. Although the literature² reports H₂ and CO₂ as products of metabolism, no gas was detected in the gas tubes in 36 hours by the detection methods employed. Because this organism requires a high NaCl concentration in the growth medium, the effect of 3% NaCl on its evolution was tested. No gas production occurred, however, in the presence or absence of NaCl.

Further studies with this organism will be confined to relating the procedures for the preparation of cell suspensions with variations in the O₂ tension in the reaction vessels.

2.3 Erwinia nimipressuralis

This organism is a member of a large group of plant pathogens and was selected for study on the basis of its reported ability to produce hydrogen

TABLE 1
TOTAL GAS & HYDROGEN PRODUCTION BY CLOSTRIDIUM BUTYRICUM
(ATCC 859) (INOC. 3.55×10^{10} , pH 6.8, 40 HOURS, 30°C, 1% SUBSTRATES)

SUBSTRATE	GAS-TOTAL ml		% HYDROGEN		VOL. HYDROGEN, ml		pH		
							INITIAL	FINAL	
GLUCOSE	3.0	2.9	79	69	2.27	1.96	7.4	6.8	6.8
	0.5	2.0	84	84	0.42	1.68	6.8	6.6	6.6
GLUCOSE + AGITATION	3.7	2.4	84	80	3.1	1.92	6.8	6.5	6.6
SUCROSE + AGITATION	4.9	5.2	79	79	3.87	4.11	6.8	6.6	6.5
SUCROSE	1.8	1.8	88	88	1.58	1.58	6.8	6.6	6.6
MALTOSE + AGITATION	9.0	7.3	80	86	7.2	6.28	6.8	6.6	6.6
MALTOSE	2.1	1.0	72	75	1.51	0.75	7.4	6.7	7.1 (1)
	3.7	4.0	86	86	3.18	3.44	6.8	6.5	6.5
LACTOSE + AGITATION	2.15	2.2	86	80	1.85	1.76	6.8	6.7	6.7
LACTOSE	—	—	—	—	—	—	7.4	—	—
	1.2	1.2	80	80	0.96	0.96	6.8	6.7	6.7

(1) INOC. 1×10^8 , 40 hours, pH 7.4, 1% Substrates, 30°C

TABLE 2

TOTAL GAS AND HYDROGEN PRODUCTION BY CLOSTRIDIUM BUTYLICUM (NRRL-B593)

(Inoc. 1.2×10^8 , 40 hours, p^H 7.4, 30°C, 1% Substrates)

SUBSTRATE	TOTAL GAS EVOLVED ml		% HYDROGEN		VOL. HYDROGEN, ml		p^H INITIAL FINAL		
GLUCOSE	0.1	0.1	79	76	0.079	0.076	7.4	7.3	7.3
MALTOSE	1.8	2.2	75	75	1.35	1.65	7.4	6.8	6.8
LACTOSE	0.1 (tr)	1.7	-	79	-	1.34	7.4	7.3	6.8

and its ability to degrade complex carbohydrates in plant tissues.¹¹ The latter consideration was based on possible future application to H₂ generation from cheap substrates. Although growth occurred under aerobic conditions, no hydrogen was evolved by the cells. No growth occurred under anaerobic conditions. This organism will not be studied further at this time. This organism should be reexamined when methods are available for adapting aerobically grown cells to produce hydrogen.

3. FRACTIONAL FACTORIAL DESIGN EXPERIMENTS

One of the experimental designs, which has been used successfully to evaluate many factors at different levels, is the factorial design. A complete factorial study with seven factors at two levels would require 256 tubes to elucidate all the possible effects and interactions of the factors contributing to the desired result. A fractional factorial design will enable the size of an experiment to be reduced to a fraction of that of a full factorial experiment while still providing all the important information. In this study we have begun with a 1/32 fractional factorial or an 8-tube study.

Seven factors at two levels were studied with this experimental design. These factors and their levels were:

	FACTOR	LEVELS	
		High	Low
A.	Temperature °C	35	25
B.	pH	7.4	6.2
C.	Oxygen tension	Aerobic	Anaerobic
D.	Substrate Concentration	5%	1%
E.	Cell Count	10 ¹²	10 ¹⁰
F.	Buffer Concentration	0.5M	0.1M
G.	Agitation	Present	Absent

These factors were assigned the letters A,B,C,D,E,F,G. The high and low levels were indicated in column 1 of table 4 by the plus (+) and minus (-) signs, respectively. Alternatively, the high levels are designated by the lower case letter corresponding to the letter assigned the factors.

The organism-substrate systems examined in this study were selected because of their high hydrogen-producing capabilities. These had been studied during the previous quarter. The systems were:

E. coli - glucose, maltose
Pseudomonas Sp. (G4A) - glucose, formate
Aerobacter cloacae - galactose
Aerobacter aerogenes - fructose

The experimental conditions (factors) and the setup of each reaction are found in table 3. The treatment combinations, or combination of factors used in each experiment, and the calculations used in the analyses are found in table 4. The experimental plan and calculations were based on suggestions by Mr. C. Daniel, Consultant to USAERDL and on those found in Davies.¹¹ The responses obtained from the treatment combinations are found in table 5.

The statistical analyses appear in table 6.

TABLE 3
TREATMENT COMBINATIONS USED IN THE FRACTIONAL FACTORIAL DESIGN

P	TUBE No.	TEMPERATURE	pH	OXYGEN TENSION	SUBSTRATE CONCENTRATION	CELL COUNT	BUFFER CONCENTRATION	AGITATION
adef	1	35	6.2	Anaerobic	5%	10 ¹²	0.1M	Yes
bdfg	2	25	7.4	Anaerobic	5%	10 ¹⁰	0.5M	Yes
abef	3	35	7.4	Anaerobic	1%	10 ¹²	0.5M	No
cefg	4	25	6.2	Aerobic	1%	10 ¹²	0.5M	Yes
acdg	5	35	5.2	Aerobic	5%	10 ¹⁰	0.5M	No
bode	6	25	7.4	Aerobic	5%	10 ¹²	0.1M	Yes
abce	7	35	7.4	Aerobic	1%	10 ¹⁰	0.1M	Yes
(1)	8	25	6.2	Anaerobic	1%	10 ¹⁰	0.1M	No
		A	B	C	D	E	F	G
+ = aerob								

TABLE 4

DETAILED FACTORIAL DESIGN EXPERIMENT-7 FACTORS*

TABLE OF SIGNS A B C D E F G							TREATMENT COMBINATIONS	RESPONSE	(1)	(2)	EFFECT (3)	SUM OF SQUARES -MEAN SQUARE $\frac{(4)}{8}$ $\text{COL}(3)\frac{2}{8}$
-	-	-	-	-	-	-	(1)	y_1	$y_1 + y_2$	$(y_1 + y_2) + (y_3 + y_4) = x_1$	$x_1 + x_2$	
+	-	-	+	-	+	-	adeg	y_2	$y_3 + y_4$	$(y_5 + y_6) + (y_7 + y_8) = x_2$	$x_3 + x_4$	
-	+	-	+	-	+	+	bdfg	y_3	$y_5 + y_6$	$(y_2 - y_1) + (y_4 - y_3) = x_3$	$x_5 + x_6$	
+	+	-	-	+	+	-	abef	y_4	$y_7 + y_8$	$(y_6 - y_5) + (y_8 - y_7) = x_4$	$x_7 + x_8$	
-	-	+	-	+	+	+	cefg	y_5	$y_2 - y_1$	$(y_3 + y_4) - (y_1 + y_2) = x_5$	$x_2 - x_1$	
+	-	+	-	-	+	-	acdf	y_6	$y_4 - y_3$	$(y_7 + y_8) - (y_5 + y_6) = x_6$	$x_4 - x_3$	
-	+	+	+	-	-	-	bede	y_7	$y_6 - y_5$	$(y_4 - y_3) - (y_2 - y_1) = x_7$	$x_6 - x_5$	
+	+	+	-	-	-	+	abce	y_8	$y_8 - y_7$	$(y_8 - y_7) - (y_6 - y_5) = x_8$	$x_8 - x_7$	

* from Davies (11).

TABLE 5

TOTAL GAS AND HYDROGEN EVOLUTION - FRACTIONAL FACTORIAL 1

ORGANISM	SUBSTRATE	RESPONSE (ml.)											
		1		2		3		4		5		6	
		GAS TOTAL	H ₂	GAS TOTAL	H ₂	GAS TOTAL	H ₂	GAS TOTAL	H ₂	GAS TOTAL	H ₂	GAS TOTAL	H ₂
E. coli	GLUCOSE	2.3	1.33	8.0	5.28	4.6	3.08	2.3	1.63	0	0	1.8	1
E. coli	MALTOSE	5.0	3.35	4.0	3.16	3.0	2.52	4.4	3.26	0.4	0.29	4.2	3
PSEUDOMONAS Sp	GLUCOSE	1.7	1.07	10.0	4.5	10.0	6.3	1.5	0.84	0	0	3.7	2
PSEUDOMONAS Sp	FORMATE	2.4	1.75	10+	8.3	10+	8.5	10.0	7.0	0	0	4.8	4
AEROBACTER AEROGENES	FRUCTOSE	8.2	4.10	10.0	2.5	7.2	4.03	3.0	0.96	0.1	0.05	0.3	0
AEROBACTER CLOACAE	GALACTOSE	1.8	1.22	1.2	0.79	0.2	0.12	1.4	0.92	0.1	0.52	0.1	0

1

TABLE 5

GAS AND HYDROGEN EVOLUTION - FRACTIONAL FACTORIAL DESIGN STUDIES

RESPONSE (ml.)											
3		4		5		6		7		8	
GAS TOTAL	H ₂	GAS TOTAL	H ₂	GAS TOTAL	H ₂	GAS TOTAL	H ₂	GAS TOTAL	H ₂	GAS TOTAL	H ₂
4.6	3.08	2.3	1.63	0	0	1.8	1.33	7.9	5.06	1.0	0.62
3.0	2.52	4.4	3.26	0.4	0.29	4.2	3.32	10.0	7.00	0.3	0.20
10.0	6.3	1.5	0.84	0	0	3.7	2.33	4.6	2.90	6.2	3.91
10+	8.5	10.0	7.0	0	0	4.8	4.03	10.0	7.80	10+	7.30
7.2	4.03	3.0	0.96	0.1	0.05	0.3	0.19	5.9	3.54	0.7	0.36
0.2	0.12	1.4	0.92	0.1	0.52	0.1	0.66	0.4	0.26	1.5	0.99



TABLE 6

STATISTICAL ANALYSIS FOR FRACTIONAL FACTORIAL DESIGN

FACTOR	E. COLI GLUCOSE			E. COLI MALTOSE			PSEUDOMONAS Sp. (GHA) GLUCOSE			AEROGENES FRUCTOSE			A. CLOACAE GALACTOSE		
	EFFECT	SQUARE	MEAN	EFFECT	SQUARE	MEAN	EFFECT	SQUARE	MEAN	EFFECT	SQUARE	MEAN	EFFECT	SQUARE	MEAN
A	-0.61	0.046*	-3.22	-3.22	1.296*	1.31	0.215*	6.05	4.575	2.05	0.525*	-0.90	0.10		
B	2.45	0.750*	2.86	1.022*	6.05	4.575	2.05	0.525*	-0.90	0.10					
C	-4.31	2.322	-1.48	0.274*	-3.57	1.718*	-7.45	6.938	-0.62	0.048					
D	11.17	15.596	8.90	9.900	10.21	13.030	4.79	2.868	-1.82	0.414					
E	5.61	3.934	4.32	2.333	-5.37	3.604	-1.63	0.322*	-0.50	0.030*					
F	-6.25	4.883	-4.32	2.333	-2.91	1.058*	-5.27	3.471	1.04	0.135					
G	-0.37	0.017*	-10.76	14.472	7.57	7.163	-1.85	0.428*	-0.64	0.051*					
ϕ_1	1	1	1	1	1	1	1	1	1	1					
ϕ_2	3	3	3	3	3	3	3	3	3	3					
Error Mean Sq.	0.271	0.864	0.997	0.997	0.997	0.997	0.997	0.997	0.997	0.997					
F value a 5%	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1	10.1					
Limit of															
Significance	2.737	8.726	10.069	10.069	10.069	10.069	10.069	10.069	10.069	10.069					
Significant															
Factors	D,E,F	D,G	D	D	D	D	D	D	D	D					
Possible Signi-															
ficant Factors	C	-	-	-	-	-	-	-	-	-					

*Interactions Used for Determination of Internal Estimate of Error Variance

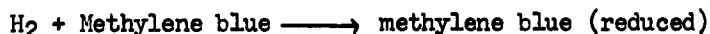
In the E. coli - glucose system, the higher substrate concentration, higher cell count, 0.1M phosphate buffer, and anaerobic conditions appear to be significant factors. In the E. coli - maltose system, the higher substrate concentration and the absence of agitation appear to be significant factors. Only the higher substrate concentration appears significant in the Pseudomonas - glucose system. Low incubation temperatures, anaerobic growth, and 0.1M phosphate buffer show significance in the A. aerogenes - fructose system. Low substrate concentration was the only apparently significant factor in the A. cloacae - galactose system. These results show some of the diversity of factors which contribute to the maximum hydrogen production of the various systems.

Significance testing requires an estimate of experimental error, preferably determined independently of the factors being tested. Because no prior error estimate is available, and because the results are based on an unreplicated study, the estimate of error can only be derived from the interaction mean squares. The mean squares used for the error variance determinations were selected because of their apparent insignificance. This was based on their relative numerical values.

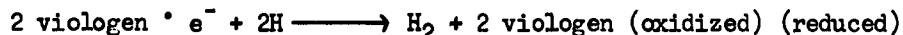
The data obtained from the above study will be used as a basis for further statistical studies. The factors which were found significant for each system will be held constant and the remaining factors examined as variables in a complete factorial design. In addition to the specific factors, anaerobic conditions will be a constant in the complete factorial studies. This condition has been shown to be a requisite for the formation and function of hydrogen-producing systems^{3 9}

4. HYDROGENASE - FORMIC DEHYDROGENASE - HYDROGENLYASE ACTIVITY OF E. COLI

Hydrogenase activity of bacterial enzyme systems can be measured by several reactions. Among these are the reduction of methylene blue and the evolution of H_2 from Methyl viologen when it is reduced. The reduction of methylene blue may be described as occurring according to the reaction.¹

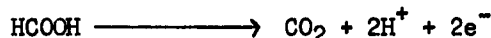


Methylene blue acts as the electron acceptor in the assay procedure reverting, on reduction, to the leuco or colorless form. Another assay procedure is based on the release of molecular hydrogen from the reduced form of methyl viologen, a "one electron" dye.⁴ This dye is colorless in the oxidized state and blue to violet in the reduced state. The stable reduced form at pH 12 is obtained by the addition of one electron. The reaction may be written as follows:

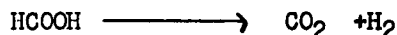


This type of assay is referred to as an evolution assay.

Formic dehydrogenase can be described as active according to the following reaction:



Formic hydrogenlyase acts according to the reaction



The effect of aerobic and anaerobic growth conditions on the hydrogenase-formic and dehydrogenase-hydrogenlyase enzyme complex of E. coli was determined manometrically according to the methods of Gest and Peck.¹²

E. coli was grown aerobically and anaerobically for 18 hours at 35°C for two successive transfers, on trypticase soy agar (TSA). Prior to use in the assay, the cells were harvested, washed three times with 0.85% saline, and resuspended to a volume of 5 ml in saline. Activity was measured in the Warburg Constant Volume Respirometer. (Precision Scientific Co.).

The enzyme assays were conducted as follows: Each reaction vessel contained 0.7 ml of 0.1M phosphate buffer (KH_2PO_4 , K_2HPO_4) pH 6.6., 1 ml methylene blue (12 μM /ml) and 0.3 ml of the enzyme preparation (bacterial cells). One ml of sodium formate (15 μM /ml) was present in the sidearm of each flask. Endogenous respiration was determined for each enzyme by omitting the formate substrate. Flasks for the hydrogenase assays were flushed with hydrogen; those for use with the formic dehydrogenase and hydrogenlyase assays were flushed with nitrogen.

Hydrogenase activity was based on the uptake of hydrogen as indicated by a pressure decrease. Activity measurements for the dehydrogenase and hydrogenlyase were based on gas evolution (H_2 and CO_2) and a corresponding increase in pressure. Potassium hydroxide (20%) and convoluted Whatman No. 1 filter paper were used to absorb the CO_2 evolved in the hydrogenase and hydrogenlyase flasks so that pressure changes were due to hydrogen. Final volume in each reaction flask was 3.2 ml. Manometric calibrations were done according to standard methods.¹³ The results of the studies are found in table 7.

The data show that the enzyme systems primarily responsible for hydrogen evolution are not formed in cells grown under aerobic conditions.

These data can be correlated with the results obtained in the hydrogen evolution studies. For example, on the basis of only the hydrogenase system, 1×10^{11} cells would produce approximately 2.1 ml of hydrogen in 8 hours. The value obtained in the rate study representing the activity of all the hydrogen-evolving systems is approximately 4.0 ml. Thus, a relationship between the two approaches can be shown. This correlation can be applied to future studies, whereby attempts will be made to produce genetically bacteria with higher enzyme levels.

Continued work with the hydrogenase-formic dehydrogenase process utilizing the above systems will be done during the next quarter. Also the hydrogenlyase will be studied.

More preliminary work correlating the enzyme content of a good hydrogen producer with that of a poor producer should be carried out before definitive genetic studies are attempted.

Following these experiments, tests can be conducted utilizing a redox dye in agar as an indicator. This will allow the selection of desirable mutants in genetic experiments.

TABLE 7
MANOMETRIC MEASUREMENT OF THE HYDROGENASE AND DEHYDROGEASE ACTIVITY
of E. COLI

GROWTH	CELL COUNT/ml	HYDROGENASE (ML H ₂ UPTAKE/CELL/45 min)	DEHYDROGENASE (ML CO ₂ EVOLVED/CELL/45 MIN)
AEROBIC	1.2×10^{11}	12×10^{-11}	45×10^{-10}
		7×10^{-11}	43×10^{-10}
	2.2×10^{11}	6.7×10^{-11}	18.6×10^{-11}
		9.3×10^{-11}	24.8×10^{-11}
ANAEROBIC	1.0×10^{11}	198.8×10^{-11}	474×10^{-10}
	5.4×10^{10}	56.5×10^{-10}	104.3×10^{-10}
		54.8×10^{-10}	100×10^{-10}

5. RATE STUDY, E. COLI - GLUCOSE

Previous to this study all analyses were done on gas samples obtained after 18 hours. This study was conducted to determine if maximum hydrogen was being measured in 18 hours; if production occurred rapidly, or after an initial lag; if the reaction was completed rapidly or was continuing at 18 hours; and to determine if any of the produced gases were re-utilized.

The inoculum consisted of twice subcultured E. coli grown on trypticase soy agar (TSA) and trypticase soy agar + 0.25% glucose (TSA + G). Cell suspensions were washed 3 times with 0.85% saline and resuspended to a final concentration of 1×10^{10} cells/ml in 0.85% saline. One ml of this suspension was used for each reaction tube. The substrate was 1% glucose dissolved in 0.1 M, Sorenson Buffer, pH 7.4. The gas tubes and other procedures were as described in the previous reports.^{5 7} The parameters which were considered were temperature (25° and 35°C), oxygen tension (aerobic and anaerobic) and growth substrate variations (TSA and TSA + G). Analyses for gas composition were begun 4 hours after inoculation using 10 μ l gas samples. Samples were taken at 2-hour intervals for 12 hours and again at 15 and 25 hours. The data from this study are found in table 8, and in figures 1 through 5.

The results of these experiments show that maximum hydrogen production is obtained from cells grown anaerobically at 35°C on TSA + G medium. Furthermore, hydrogen evolution is at a maximum in 6-8 hours under these conditions. Cells grown in the absence of glucose showed a peak lower than that on TSA + G at 10 hours with a gradual decrease in 25 hours. Cells grown aerobically on both media showed an initial lag followed by a gradual increase in hydrogen levels. Maximum levels obtained did not approach those of the anaerobic studies.

The results of some of the previously reported data on the effect of aerobic growth on hydrogen evolution were questioned because of the lack of adequate replication. To appraise these data ten replicates were run on aerobically grown cells and seven were run on the anaerobically grown cells. The data are found in table 9. The average value for the volume of H₂ produced in 18 hours by the aerobic group was 2.49 ml (1.07 - 3.57 ml) as against 3.23 ml (1.53 - 4.03 ml) for the anaerobic group. The results in 40 hours were 7.3 ml (6.8 - 7.8 ml) and 6.3 ml (5.3 - 6.93 ml) for the aerobic and anaerobic groups, respectively. The fact that anaerobic values exceed the aerobic values in this replicate study at 18 hours agrees with the data of the rate study, the literature, and with the enzyme data. The increase in the aerobic values over the anaerobic values in 40 hours is small and clearly explained. In the rate study (figure 2) the two curves approximate one another at 25 hours. If the final points for these curves were based on the highest value in the range for the aerobic data and the lowest value for the anaerobic data, then one could estimate that, indeed, the aerobically grown cells were the more active H₂ producers. Therefore,

TABLE 8

RATE OF TOTAL GAS AND HYDROGEN EVOLUTION BY E. coli on GLUCOSE (1.0%) 1×10^{10} cell/ml.

pH 7.4

0.1M Sorenson buffer

Sample Injection 10 ml

TIME-HOURS	4	6	8	10	12	15	25
GAS EVOLVED (ml.)							
	TOTAL	TOTAL H ₂		TOTAL	TOTAL	TOTAL	TOTAL
	H ₂	H ₂		H ₂	H ₂	H ₂	H ₂
35°C	2.1	4.9	3.43	6.8	3.81	7.5	3.98
ANAEROBIC	2.1	4.8	3.36	6.9	4.14	8.3	5.40
TSA	-	0.1	0.07	0.7	0.46	1.5	1.02
35°C	TR	0.1	0.07	0.7	0.46	1.5	1.02
AEROBIC	0.1	0.4	0.31	1.1	0.73	2.1	1.47
TSA	0.1	0.4	0.31	1.1	0.73	2.1	1.47
35°C	3.0	7.9	5.85	10+	+	10+	+
ANAEROBIC	3.0	7.9	5.85	10+	+	10+	+
TSA	3.0	7.9	5.85	10+	+	10+	+
35°C	TR	0.3	0.26	0.8	0.52	1.6	1.09
AEROBIC	TR	0.3	0.26	0.8	0.52	1.6	1.09
TSA	TR	0.3	0.26	0.8	0.52	1.6	1.09
35°C	0.15	0.4	0.29	0.9	0.57	1.7	1.07
ANAEROBIC	0.25	0.7	0.54	1.5	0.92	2.6	1.72
TSA	0.25	0.7	0.54	1.5	0.92	2.6	1.72
25°C	TR	0.1	0.06	0.1	0.06	0.3	0.20
AEROBIC	TR	0.1	0.06	0.2	0.11	0.5	0.36
TSA	TR	0.1	0.06	0.2	0.11	0.5	0.36
25°C	TR	0.1	0.06	0.2	0.11	0.5	0.36
TSA	TR	0.1	0.06	0.2	0.11	0.5	0.36

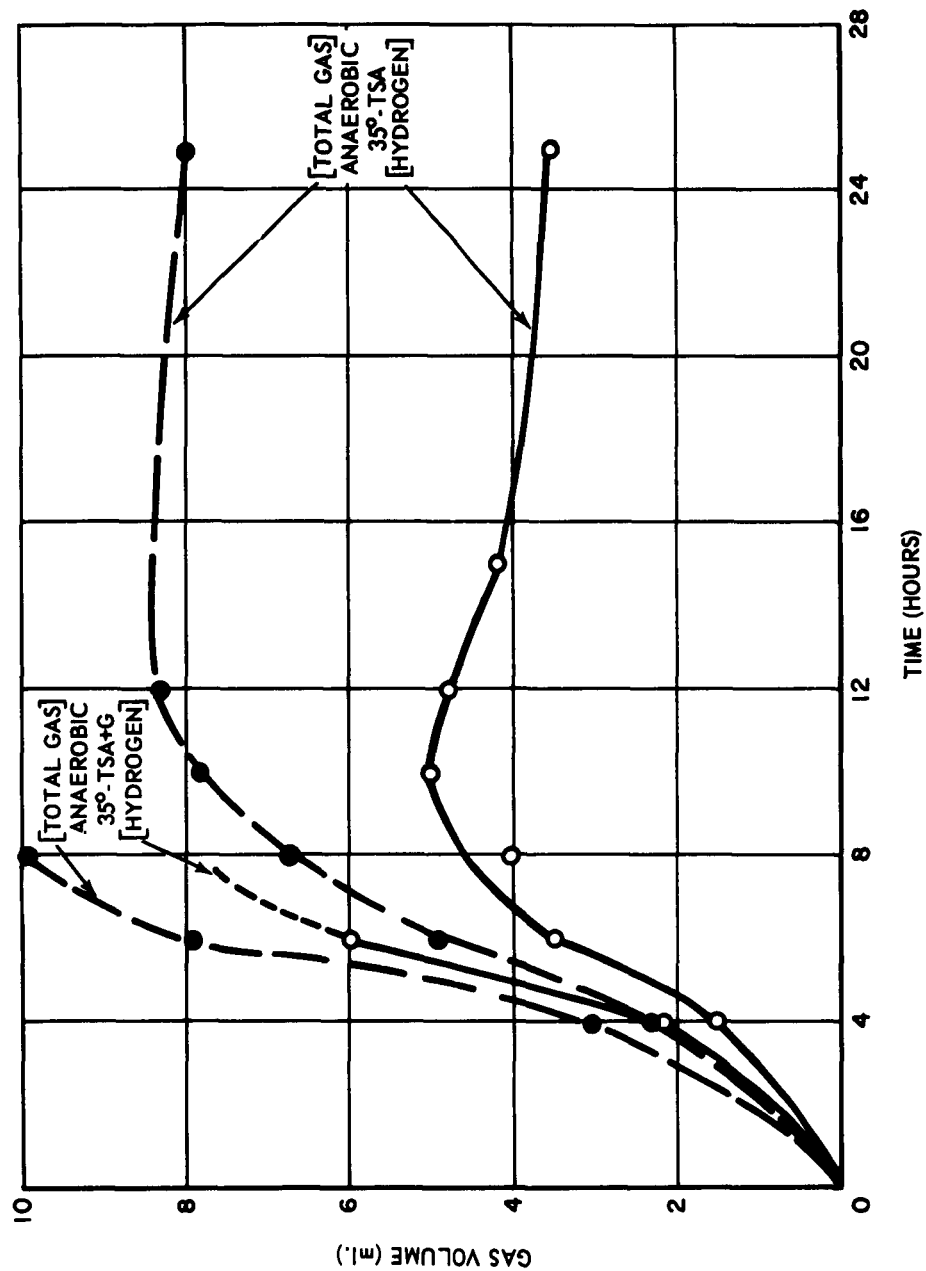


Figure 1. Total Gas and Hydrogen Production - *E. coli* - glucose 1%, Anaerobic, TSA and TSA + glucose, Grown - 35°C.

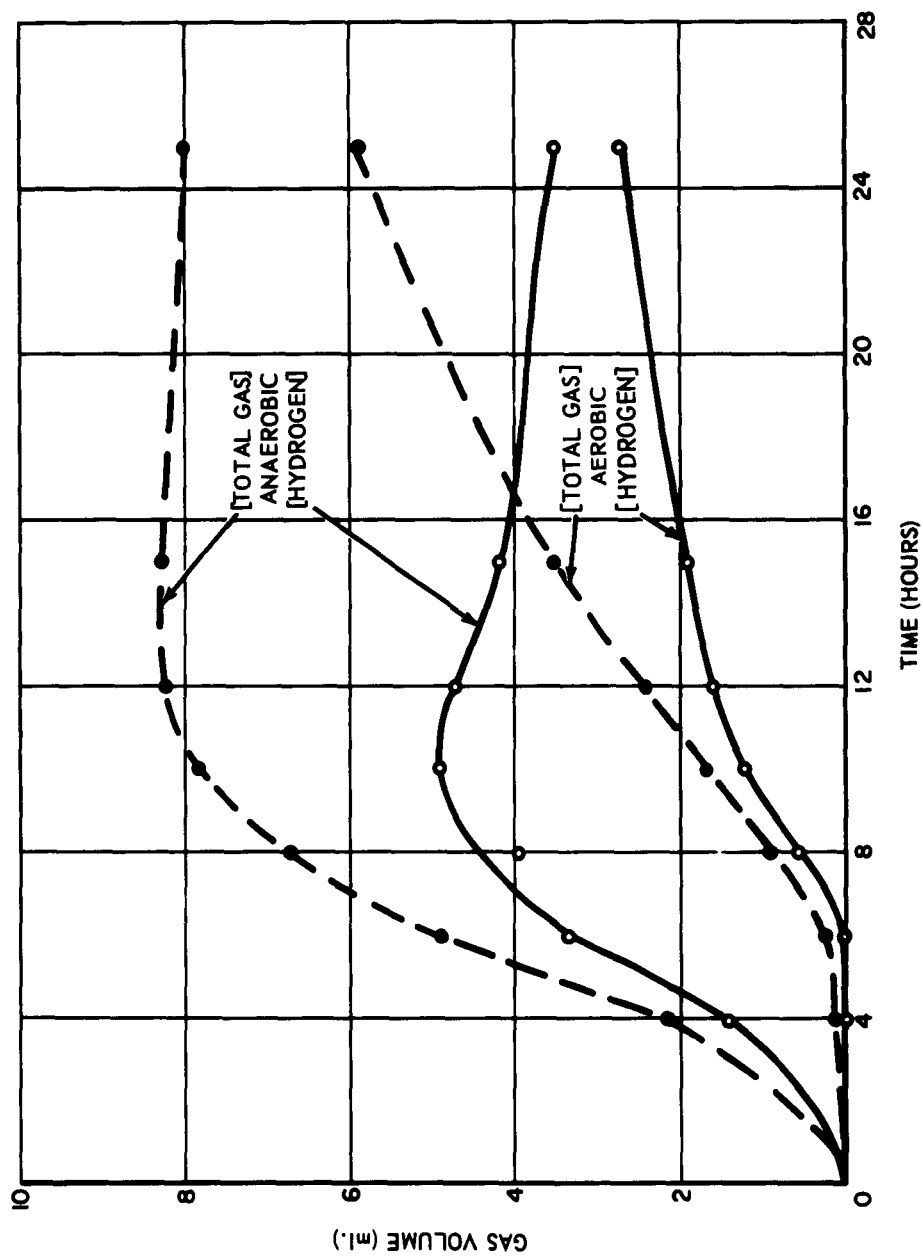


Figure 2. Total Gas and Hydrogen Production - *E. coli* - glucose 1%, Aerobic and Anaerobic - TSA, Grown - 35°C.

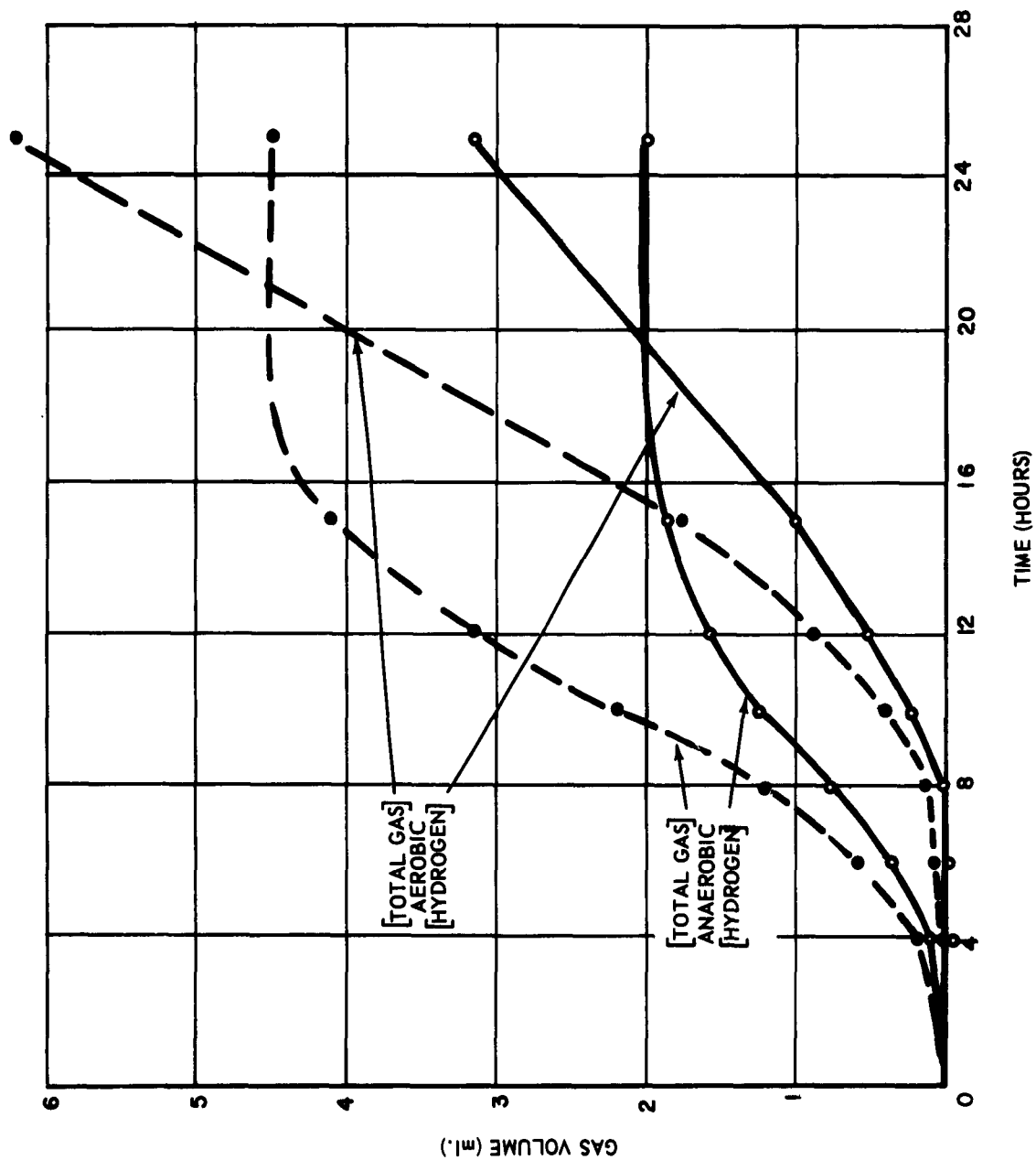


Figure 3. Total Gas and Hydrogen Production - *E. coli* - glucose 1%, Aerobic and Anaerobic - TSA, Grown - 25°C.

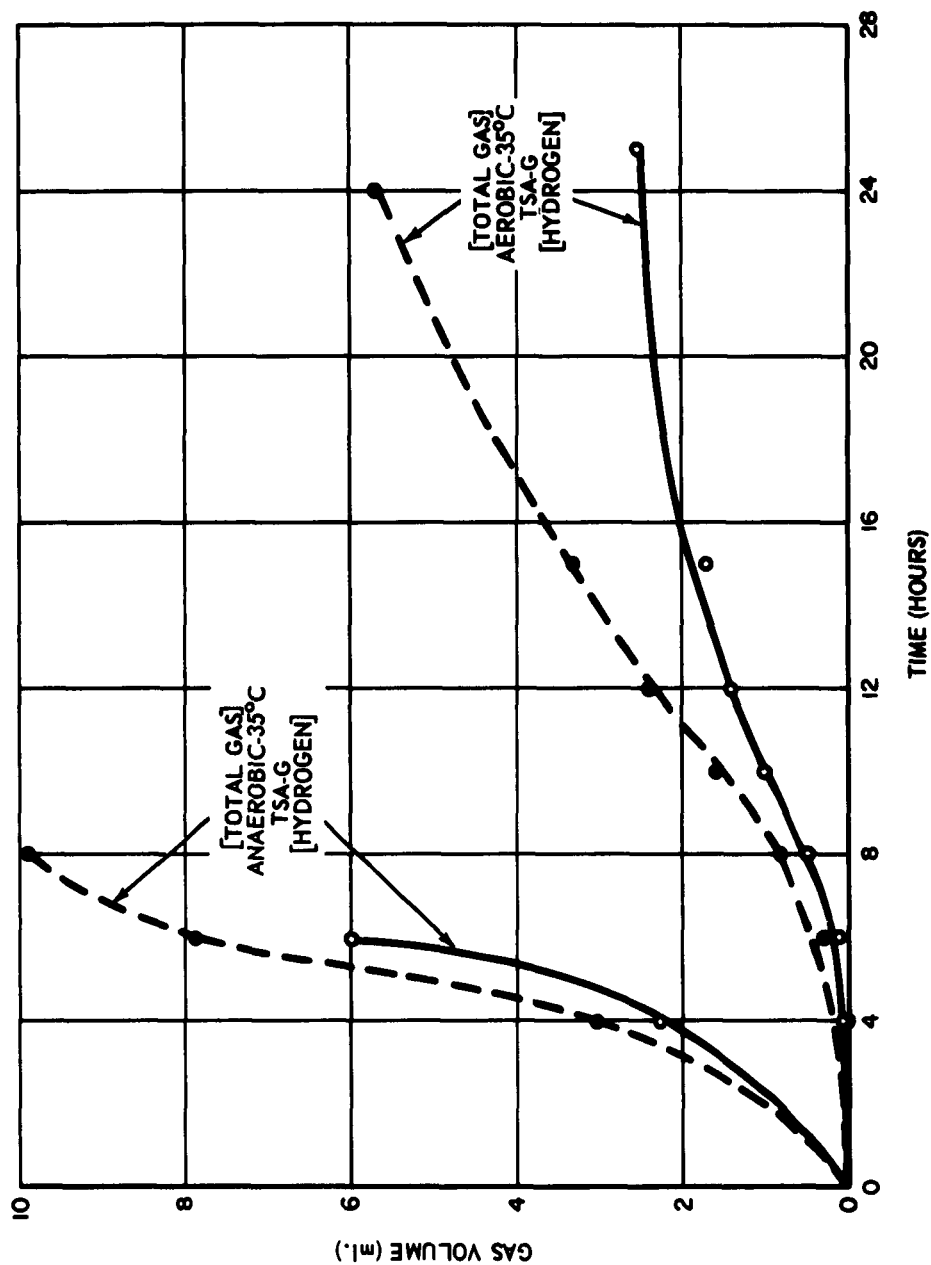


Figure 4. Total Gas and Hydrogen Production - *E. coli* - glucose 1%, Aerobic and Anaerobic - TSA + glucose, Grown - 35°C.

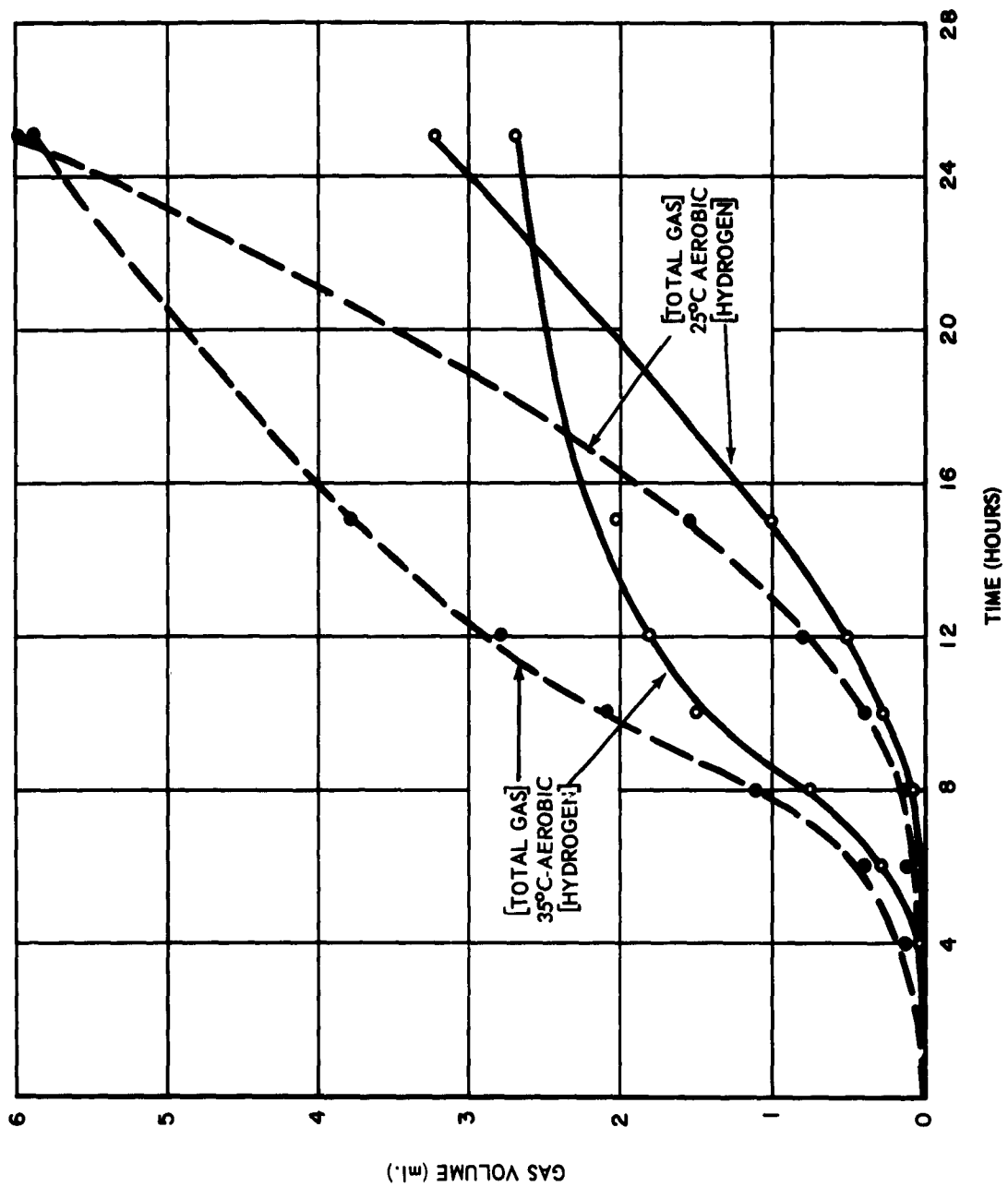


Figure 5. Total Gas and Hydrogen Production - *E. coli* - glucose 1%, Aerobic, TSA, Grown, 25°C. and 35°C.

TABLE 9
TOTAL GAS AND HYDROGEN PRODUCTION - REPLICATE STUDY
E. coli - GLUCOSE (1%)

		TOTAL GAS, ml.		% HYDROGEN		VOL. HYDROGEN	
		18 HOURS	40 HOURS	18 HOURS	40 HOURS	18 HOURS	40 HOURS
AEROBIC	1	4.7	10.0	76	70	3.57	7.00
	2	2.4		82		1.97	
	3	2.6		82		2.13	
	4	1.3		82		1.07	
	5	2.5		84		2.10	
	6	3.9	10.0	78	77	3.04	7.7
	7	2.7	8.4	74	81	2.00	6.8
	8	4.0	10.0	80	78	3.20	7.8
	9	3.4	9.5	77	78	2.61	7.4
	10	4.1	9.2	77	81	3.16	7.4
AVE		3.16	9.5	79.2	77.5	2.49	7.33
ANAEROBIC	1	4.4	9.4	66	65	2.90	6.10
	2	5.7	9.6	66	70	3.76	6.72
	3	5.9	9.9	62	70	3.67	6.93
	4	2.4	7.8	64	68	1.53	5.30
	5	6.5		62		4.03	
	6	4.5	9.7	68	68	2.97	6.80
	7	6.7	9.3	58	68	3.78	6.30
AVE		5.16	9.28	63.8	68.2	3.23	6.36

the particular set of data referred to in the second quarterly report may be at the extremes of range of values. The lag period may be due to time necessary for enzyme synthesis. Pollack¹⁰ in his discussion on enzyme induction, states that enzyme induction can occur in resting cells in an atmosphere lacking an added nitrogen source and in situations where cell proliferation and a net increase in protein cannot occur. This can occur provided specific inhibitors are absent and other conditions are right for the enzyme. The conditions used in the gas tube studies were similar to the above. Thus, enzyme induction is possible. Three hypotheses are offered for the explanation of enzyme induction by resting cells:¹⁰

- a. Activation of a specific enzyme precursor
- b. Selective synthesis of a single enzyme from contaminating traces of N₂.
- c. Protein turnover which could provide the necessary components for new enzyme production from breakdown products of existing proteins.

6. SCALE-UP STUDIES

The data on hydrogen production obtained in the studies to date were derived from systems with 10 ml of substrate. The direct application of these data to actual situations of large-scale production of gas, as would be required in practical fuel cells, cannot be made. The optimum conditions derived from tube studies must be scaled-up as is done in the fermentation industries. It is required that the conditions, determined effective at the tube level of the fermentation, be increased gradually to flasks, to low-scale fermenters and pilot plant scales before application to large-scale fermenters.

The system described in figure 6 was employed in the first attempt in scale-up to a 3- to 5-gallon fermenter

The reaction flask was a 3-gallon pyrex bottle fitted with a 3-hole rubber stopper. The collection bottle was a 4-liter flask also fitted with a 3-hole stopper. The reaction flask was filled with 11.5 liters of sterile medium of the following composition:

Tryptone 1%
Yeast extract 1.0%
Glucose 5%
 K_2HPO_4 0.5%
Salts B 5 ml/L

The stopper had a gas bubbling capillary tube connected to a helium tank; the second port was connected to the collection bottle, and the third was for inoculation purposes. The collection bottle had one port for connection to the reaction flask, one for connection to the manometer, and the third for sampling. The sterile medium was bubbled with helium to displace other gases in the solution. The collection bottle was flushed with helium to displace other gases, and the entire unit sterilized. The system was connected just prior to inoculation. A 1% inoculum (115 ml), prepared from two successive 18 hour-subcultures in the same medium was used to charge the fermenter. Incubation was carried out at 35°C with constant agitation provided by a magnetic stirrer. The volume of gas evolution was assumed to follow the ideal gas laws:

$$PV = nRT$$

where

P = pressure in atmospheres
V = volume of collecting flask
T = temperature - °K
R = constant (= 0.082)
n = calculated = moles of gas

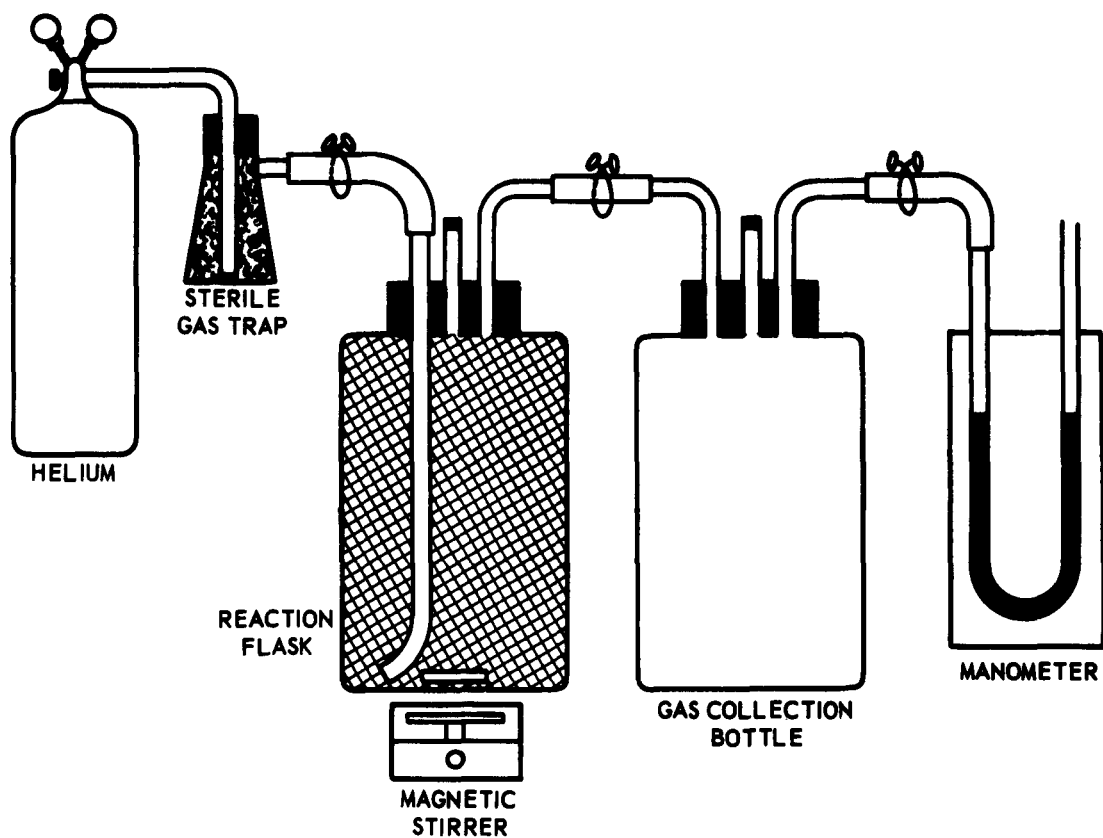


Figure 6. Fermenter for Gas Collection and Production

The actual gas concentration would be determined by converting %H₂ (by gas chromatographic analysis) to moles of gas (n) = moles H₂.

In this preliminary work no significant data were obtained because of foaming and air leaks which developed in the system during the course of the study. It is not intended to discard the system shown in figure 6. This system may turn out to be completely suitable. This type of system should be more readily adapted for practical use. To improve this system an overflow trap will be placed between the fermenter and the collector. An antifoam agent will be introduced to minimize foaming and reduce mechanical carryover of media into the flask trap and the gas collector.

Another system will be studied in the next quarter whereby the evolved gas will be collected over water. The gas volume produced will be determined by measurement of the displaced fluid and gas composition will be determined by gas chromatography.

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CONCLUSIONS

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

a. Further studies regarding methods for preparation of cell suspension and reaction tubes are required to evaluate more thoroughly the hydrogen-producing capability of Photobacterium phosphoreum.

b. The hydrogen-producing capabilities of the Clostridia have been established to some degree. Experimentally, a delay in gas evolution is observed. The preparation of the cell suspensions and the reaction vessels may be the cause of the delay. Further studies should be conducted to elucidate the capabilities of these organisms.

c. The fractional factorial design study showed that the various factors have different effects depending on the organism - substrate systems under investigation. In the E. coli - glucose system, high substrate concentration, high cell count, low buffer concentration and anaerobic conditions were found to be significant. No agitation, and high substrate concentration, were found significant for the E. coli-maltose system. Only the high substrate concentration was found to be significant in the Pseudomonas-glucose system. In the Aerobacter aerogenes-fructose study, low temperature, anaerobic conditions, and low buffer appeared to be significant. Only the use of the low substrate concentration demonstrated significance in the Aerobacter cloacae - galactose system. Further optimization of factors governing H_2 production will be required using additional experimental designs.

d. Rate studies with the E. coli - glucose system have demonstrated that maximum H_2 production was obtained with cultures grown anaerobically on glucose-supplemented media at $35^\circ C$. Maximum H_2 production was obtained at 6-10 hours, indicating the necessity to follow the evolution of gas as a function of time, rather than single point determination.

e. Manometric determination of specific enzyme activities can be correlated with evolution data obtained by the experimental design used in these studies. This relationship can be applied to future studies and can serve as a guide for estimating the potential of genetic variants produced during future investigations.

PROGRAM FOR THE NEXT INTERVAL

The following investigations are planned for the next quarter:

a. The screening program will continue with:

Aeromonas hydrophila

Bacillus polymyxa

Proteus sp. and

Photobacterium phosphoreum

b. Rate Studies, conducted along lines described in section 5 of this report, will be conducted with the systems:

E. coli - maltose

Pseudomonas (G4A) - glucose

Pseudomonas (G4A) - formate

Clostridium butyricum - maltose

Clostridium butyricum - sucrose

c. A further search for optimum conditions employing a 2⁴ factorial design (see table 10, similar to Davies¹¹) will be made with the following systems:

E. coli - glucose

E. coli - maltose

Pseudomonas (G4A) - glucose

Aerobacter aerogenes - fructose

Aerobacter cloacae - galactose

If a trend in the effects of the factors is noted, adjustment in the variables will be made, so that some standardization in conditions is obtained.

d. Further studies in the scale-up of gas production methods using large-volume fermenters will be conducted.

TABLE 10
FACTORS FOR 2⁴ FACTORIAL DESIGN

	CONSTANT	VARIABLE
<u>E. coli</u> - glucose	An aerobic growth 5% substrate conc., temperature 35°C	buffer concentration pH, cell count, agitation
<u>E. coli</u> - maltose	Anaerobic growth, 5% substrate substrate concentration temperature - 35°C	buffer concentration, cell count, agitation
<u>Pseudomonas</u> - glucose	Anaerobic growth, 5% substrate temperature 25°	pH, agitation, cell count, buffer conc.
<u>A. aerogenes</u> - fructose	Anaerobic growth, 0.1M buffer, temp. 25°C	substrate conc. agitation pH, cell count
<u>A. cloacae</u> - galactose	low substrate conc. anaerobic growth, temperature, 25°C.	Agitation, pH, cell count, buffer conc.

IDENTIFICATION OF KEY PERSONNEL

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

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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

Paul S. May 217 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

Ruby L. Brown 129 hours
Biologist with background in antibiotic resistant and antibiotic sensitive microorganisms.

B.A. (Biology, Chemistry) Catawba College
M.S. (Bacteriology) North Carolina State College

Technician, Microbiology 784 hours
Technician, Analytical Chemistry 147 hours

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AD- Meipar, Inc., 3000 Arlington Boulevard, Falls Church, Virginia BIOCHEMICAL FUEL CELL, by Paul S. May, G. C. Blanchard, and R. T. Foley. Third Quarterly Report, 1 January 1963 to 31 March 1963. 39 pp. Incl. illus. tables. (Contract No. DA 36-039-SC-90878) Unclassified Report Statistical study utilizing fractional factorial design was applied to four microorganism-substrate systems. Design involved seven factors - pH, temperature, substrate concentration, cell count, buffer concentration, agitation, and oxygen tension during growth at two levels. Results showed that different factors governed H_2- producing capacity of different systems. Rate studies with <i>E. coli</i>-glucose showed that high-temperature (35°C), anaerobic growth and glucose-supplemented media are required for maximum H_2 production. This study investigated H_2 evolution as a function of time as well as other experimental conditions.	UNCLASSIFIED 1. Microorganism - substrate systems - Statistical study 2. Fractional factorial design - Application 3. H_2 evolution and production - Rate study I. May, Paul S. Blanchard, G. C. Foley, R. T. U.S. Army Electronics Research and Development Laboratory Contract DA 36-039-SC-90878 - II. - III.
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