FOREWORD

This study was initiated by the Biomedical Laboratory of the 6570th Aerospace Medical Research Laboratories, Aerospace Medical Division, Wright-Patterson Air Force Base, Ohio. The research was conducted by the Magna Corporation, Research and Development Division, Anaheim, California, under Contract No. AF 33(657)-9633. Dr. James H. Canfield, Head of Life Sciences at Magna, was the project manager, and Max D. Lechtman acted as principle investigator. Dr. Sheldon A. London of the Biospecialties Branch, Physiology Division, was the Contract Monitor for the 6570th Aerospace Medical Research Laboratories. The work was performed in support of Project No. 7164, "Biomedical Criteria for Aerospace Flight," and Task No. 716405, "Aerospace Nutrition and Sustenance." The research sponsored by this contract was started in September 1962 and completed in December 1963.

The authors wish to acknowledge the assistance of Dr. Carlton Bovell, Life Sciences Division, University of California, Riverside, California; Dr. Herbert P. Silverman, Head of Physical Sciences, and Mr. Bernard H. Goldner, Research Microbiologist at Magna Corporation.

ABSTRACT

The feasibility of using hydrogen-oxidizing bacteria in conjunction with electrolysis to regenerate oxygen and foodstuff from carbon dioxide and water was investigated. Results showed that 75 liters of Hydrogenomonas eutropha culture can fix the CO_2 and supply oxygen for one man. The cell yield is rich in proteins and deficient in carbohydrates and lipids for direct consumption by man. The feasibility of integrating the culture with the electrolysis was also investigated, with incompatibility of the bacteria with high salt content being the limiting factor. The ability of a hydrogen cathode to supply hydrogen to the culture was shown.

PUBLICATION REVIEW

This technical documentary report is approved.

Wayne H. Mc Candless
WAYNE H. Mc CANDLESS
Technical Director
Biomedical Laboratory

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

As the duration planned for manned space flights increases, more complete closure of environmental systems employed for maintenance of habitable conditions in the spacecraft must be sought. Closure of both the food and oxygen loops can be most efficiently accomplished through biological treatment of respired carbon dioxide, water, and other wastes.

The most prominently studied biological systems for life support in closed systems are those which depend on photosynthesis, the photosynthetic gas exchangers. The greatest utility of photosynthetic devices lies in their ability to use light radiation directly. When artificial illumination must be considered, however, photosynthetic processes suffer from the poor efficiency of converting electrical to light energy. A loss of 85% of the energy will be encountered in this step alone, the efficiency of the photosynthetic conversion of light to chemical energy resulting in additional loss.

Combination of electrolysis with a microbial culture that can consume by-product hydrogen and respiratory carbon dioxide provides the same function as the photosynthetic gas exchangers - regeneration of oxygen and potential foodstuffs from waste water, carbon dioxide, and other wastes. Such a combination also promises greater efficiency in utilization of electrical energy than does a photosynthetic process because of the relatively high efficiency (up to 60%) with which electrolysis can convert electrical to chemical energy. Further, greater economy of volume is seen for the electrolysis-bacteria combination because constraints on effective illumination of the microbial culture are not limiting. There appear to be no reasons that microbial cell matter, from such an approach, would not serve as well as algae in human nutrition.

While a wide variety of microorganisms can fix both hydrogen and carbon dioxide, those of greatest interest in this application are the varieties that consume these gases stoichiometrically. Accordingly, a literature search was conducted for the purpose of finding what organisms had been reported to consume hydrogen and carbon dioxide in this fashion. Based on information available in the literature, several organisms were considered attractive for laboratory screening. The literature search is recounted in its entirety in Appendix I. Only the pertinent conclusions drawn from the literature search are discussed below.

The laboratory screening effort was undertaken to confirm literature information and to explore certain related strains of hydrogen-consuming bacteria for their applicability to bioregenerative life support systems. Based on the laboratory screening, final selection of two organisms, Hydrogenomonas eutropha and Hydrogenomonas ruhlandii, was made for intensive study in an experimental program.

The experimental program followed two pathways. One was an examination of the nutritional and physiological parameters leading to and culminating in continuous

culture of H. eutropha. The other pathway was to explore the feasibility of integrating the hydrogen-producing function of the electrolysis cell with the bacterial culture, thus eliminating the need for transport of hydrogen from the electrolysis cell to the culture, and leading ultimately to decreased power and weight requirements. The study of the latter pathway was predicated on the basis of a previously successful integration of biological and electrochemical processes. This was the use of another hydrogen-consuming bacteria, Desulfovibrio desulfuricans, at a cathodically polarized electrode for the enhancement of power production from cells using that electrode.

FACTUAL DATA

Literature Search and Preliminary Selection

Search of the literature for microorganisms reported to consume hydrogen and carbon dioxide stoichiometrically revealed a broad spectrum of potential candidates. The genus Hydrogenomonas, the photosynthetic bacteria, and the family Methanobacteriaceae (methane bacteria) all possess members with the required characteristics. In addition, a Clostridium sp. Cl.aceticum, and several algal species were found.

Preliminary selection of candidates for laboratory screening was made not only on the hydrogen and carbon dioxide metabolism of candidate organisms, but also on reported growth and metabolism rates, on the products of metabolism, and on the ease of handling the organisms. Candidates selected for laboratory screening were the following:

Hydrogenomonas facilis
Hydrogenomonas ruhlandii
Hydrogenomonas eutropha
Chlorobium limicola
Rhodospirillum rubrum
Chromatium sp.

The first three have been reported to metabolize hydrogen and carbon dioxide autotrophically, energy for the synthesis of cell material being obtained from the reaction of hydrogen with oxygen. The others are photosynthetic bacteria, each a representative of the three families of photosynthetic bacteria, Chlorobacteriaceae (Chlorobium limicola), Thiorhodaceae (Chromatium sp.), and Athiorhodaceae (Rhodospirillum rubrum). All have been reported to metabolize hydrogen and carbon dioxide autotrophically using light as their energy source.

Selection of the hydrogenomonads was based on reports that these organisms show the highest rates of carbon dioxide and hydrogen metabolism of any of the hydrogen-consuming bacteria surveyed. Their characteristics appear entirely compatible with their application to life support systems. The photosynthetic bacteria were selected for additional examination because it was felt that insufficient data with respect to metabolic rates were available in the literature to make a conclusive judgment. Experimental screening of the photosynthetic bacteria was decided upon with the realization that complexity in their handling and the possible incompatibility of their pigments in human nutrition might adversely affect their ultimate choice.

No candidates were selected from the methane bacteria, because of their metabolic production of methane. Their use in a life support system would appear to provide no advantage over the production of hydrogen. The metabolism of hydrogen by algae species is an adaptive capability and is readily lost. Thus algae were rejected as candidates. Clostridium aceticum, while reported in the literature, has evidently been lost and no sources of this organism could be found.

Laboratory Screening and Selection of Candidates

The screening of preliminary candidates for intensive evaluation was performed to establish insofar as possible for the preliminary candidates, the rates and proportions of utilization of hydrogen and carbon dioxide and the rates of growth and reproducibility of growth rates. In addition, the utilization of organic substrates was considered, with an eye to the effectiveness with which the bacteria might be capable of aiding in waste (urine and feces) degradation.

In addition to the candidate species discussed above, two other hydrogenomonads (see Appendix II) were subjected to scrutiny for their potential.

In sum, Hydrogenomonas eutropha and Hydrogenomonas ruhlandii were the only organisms which exhibited adequate metabolic rates and consistency and they were selected as the candidates for intensive study in the experimental program. Results of the screening as applied to specific organisms were as discussed below. See Appendix II for experimental details.

Microbiological Screening

1. Rhodospirillum rubrum (Esmarch) Molisch Strain S1, (ATCC 11170). A procedure reported by Ormerod, Ormerod, and Gest[1] to result in photoautotrophic growth of this bacterium was used: The inoculum (3%) consisted of washed cells derived from heterotrophic medium. The inoculated medium was placed in a flask, fitted with a stopcock and a gas sampling port, under an atmosphere of 10% CO_2 and 90% H_2. Air was excluded. Heterotrophic

medium inoculated with the same quantity of washed cells served as a control. The cultures were illuminated with fluorescent lamps and incubated at 28°C. No autotrophic growth was observed during 10 days of incubation, while normal growth occurred heterotrophically.

The experiment was repeated with the following modification: The inoculum consisted of washed cells obtained from a culture growing heterotrophically under an atmosphere of 10% CO_2 and 90% H_2. Autotrophic growth as judged visually was evident after 6 days of incubation; heterotrophic growth after 3 days. On subsequent subculture (1% inoculum) of the autotrophic culture to the same medium, growth was evident only after 5 days incubation.

2. Chlorobium limicola, Larsen Strain YG2A, (NCIB 8325).
Growth of this organism in the light with H_2 and CO_2 could not be obtained.

3. Chromatium sp. (NCIB 9425)
A Chromatium sp. of unspecified history was acquired from the National Collection of Industrial Bacteria, Scotland, but was obtained too late in the screening program to be examined culturally. No source was found for a Chromatium sp. known to metabolize H_2. This organism was not considered further.

4. Hydrogenomonas facilis (ATCC 11228)
Growth of this organism was slow. Approximately ten days were required before visual evidence of turbidity resulted. In an attempt to obtain more rapid growth, reported optimum conditions were examined, i.e., maintenance of atmospheric pressure, 30°C, and a gas mixture of 70/20/10, $H_2/O_2/CO_2$ (6.5/1.8/1 by analysis). Also included in the study was the effect of the buffering capacity of the medium.

To accomplish these conditions, variations in the medium were made as follows: The autotrophic medium for the hydrogenomonads, buffered with tris (hydroxymethyl) aminomethane (tris buffer) 0.054 M, pH 7.0; and the medium prepared without buffer, also at pH 7.0. Each medium received a 5% inoculum, ten ml were dispensed into Warburg flasks, gassed, and after five days of observation, there was no apparent gas consumption or turbidity. A second attempt was performed using the same conditions, with a new lyophilized culture as the inoculum. Ten ml amounts were dispensed into Warburg flasks and gassed. No gas uptake was observed during five days of observation.

5. Hydrogenomonas eutropha.
Three quantitative experiments were performed with this organism to determine the stoichiometry and rate of gas utilization. For each experiment 100 ml

of autotrophic medium was inoculated with one ml of 20-24 hr. culture of the organism. Five ml quantitites were dispensed into each sterile microrespirometer unit. The microrespirometers were placed in the Warburg water bath at 30°C and gassed with a mixture of 70/20/10, $H_2/O_2/CO_2$. The linear period of gas uptake began approximately 18 hours after inoculation and was usually observed for 5-6 hours. Gas uptake during this period was an average of 0.3 ml of gas per ml of culture per hour. The stoichiometry observed was too variable to be reliable. Good growth of the organism was obtained, as evidenced by turbidity (optical density) (OD_{660}) values of 0.91-1.50. Figure 1 presents some gas uptake data observed during these studies.

6. Hydrogenomonas ruhlandii.

The initial subculturing of organisms from lyophile demonstrated poor growth (seven days to initial turbidity), and, therefore, it was decided to open a new lyophile and grow the organism under optimum conditions in uncalibrated Warburg flasks using a gas mixture of 82/8/10, $H_2/O_2/CO_2$. This culture demonstrated slight gas uptake during a four-day period and on the fifth day consumed gas rapidly. During this study with the new lyophile, a previous subculture began to grow exceptionally well (visual observation). This increased growth rate allowed the consideration of H. ruhlandii for further experimentation.

One quantitative experiment was performed with 5 ml cultures of this organism. The culture for inoculum was 34-36 hours old; a 5% inoculum was used to insure reasonable growth. The gas mixture used was 82/8/10, $H_2/O_2/CO_2$. Gas uptake during the linear period of uptake (20-26 hours after inoculation) was 0.27 ml of gas per ml of culture per hour. The culture grew well as indicated by the final OD_{660} of 0.7-1.5.

7. Other Hydrogenomonas sp.

Hydrogenomonas sp. H-10. Two experiments were performed and out of a total of four microrespirometers, only one demonstrated apparent gas utilization. Experimental conditions were the same as used for H. eutropha. Gas uptake observed for the one culture was in the order of 0.16 ml of gas per ml of culture per hour, during the linear uptake phase. (18-24 hours after inoculation.) Growth was erratic in subsequent subcultures.

Hydrogenomonas sp. H-20. One quantitative experiment was performed with the organism. The cultural conditions and procedure are the same as for H. ruhlandii with the exception that only 1 ml of culture was used. The gas mixture supplied was made up to 82/8/10, $H_2/O_2/CO_2$, but was not analyzed. Gas uptake was 0.45 ml of gas per ml of culture per hour during the linear uptake phase (22-36 hours after inoculation). Growth in subsequent subcultures was erratic.

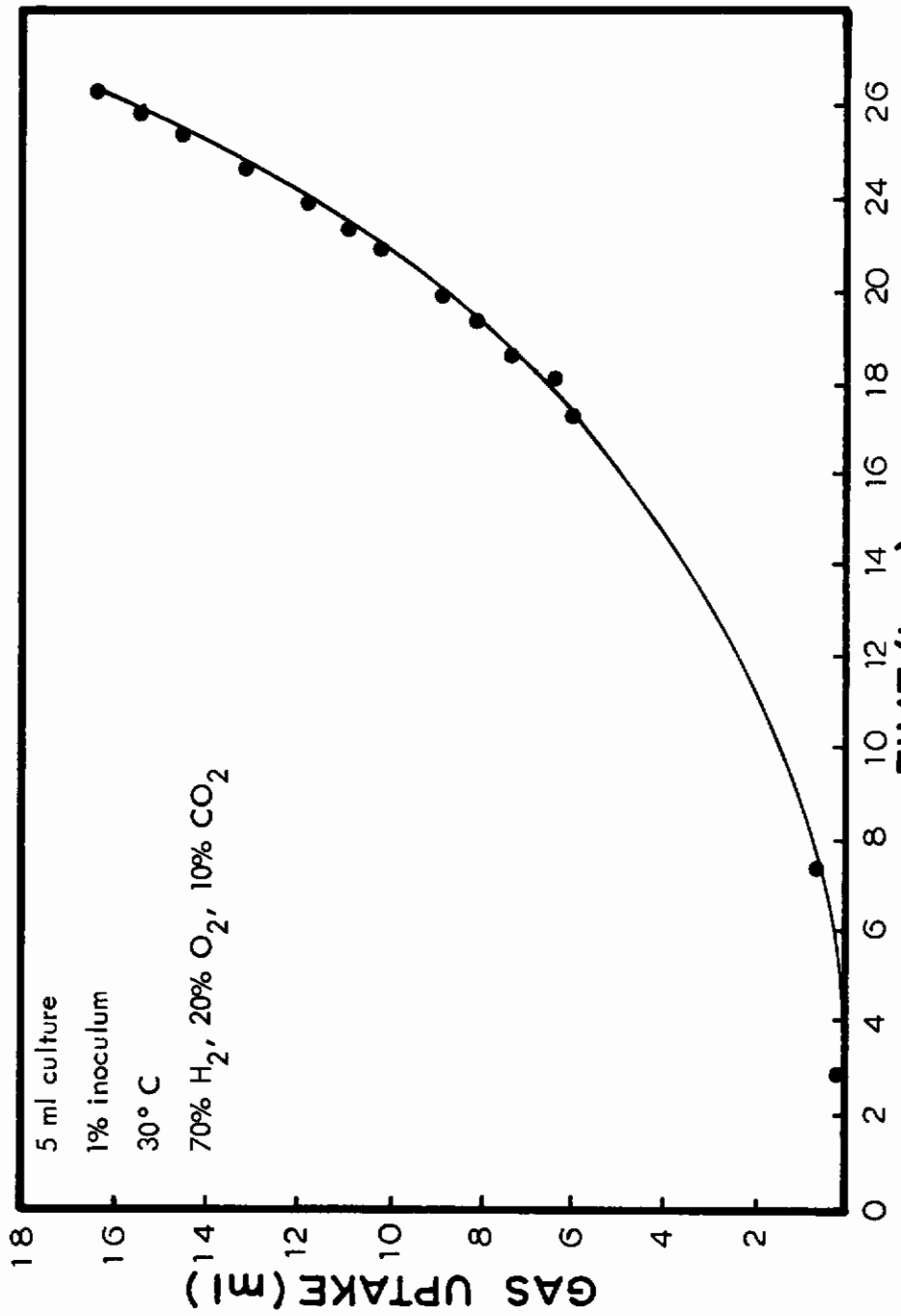


FIGURE 1

Gas Comsumption by H. eutropha, Experiment No. 1

Experimental Program

The experimental program was undertaken to obtain detailed information on the parameters of continuous cultivation of the candidate organisms, H. eutropha and H. ruhlandii. To do so, batch culture experiments were performed to establish the broad range of the parameters involved, prior to continuous culture experiments. Certain of the parameters under investigation, i.e., nitrogen sources, pH and temperature optima for H. eutropha, have been examined in the literature. Our purpose for examining these parameters has not been to question the validity of the data but has been based upon past experience with microbial variability. Bacteria are subject to genetic changes which can alter important characteristics of their physiology, and it was thus considered necessary to corroborate literature reports for our cultures. The experimental program also included bioelectrochemical experimentation on the integration of water electrolysis and culture of H. eutropha. The details of the experimental work performed are given in Appendix III.

Nutritional Studies

1. Growth Response to NH_4Cl and Urea

It has been reported by Repaske[2] that H. eutropha would grow well with ammonium chloride or urea as sources of nitrogen. The main purpose for the examination of the growth response to various concentrations of these compounds was to determine a growth-limiting concentration for application to chemostatic control of continuous culture.

James (ref. 3) reported that a simple and reliable method controlling bacterial growth in continuous culture was to develop an equilibrium between population growth and loss by overflow through the use of a fixed input feed rate of a growth-limiting medium, and that the medium should be limiting with respect to only one essential growth factor, all of the other nutrients being in excess.

a. Hydrogenomonas eutropha

The growth response of H. eutropha to NH_4Cl and to urea (as nitrogen sources) is presented in Figure 2. Good reproducibility was found between two experiments and we can state with reasonable assurance that growth of H. eutropha was limited at concentrations of 0.0095 M NH_4Cl and 0.0084 M urea. These data are of importance due to their application to continuous culture.

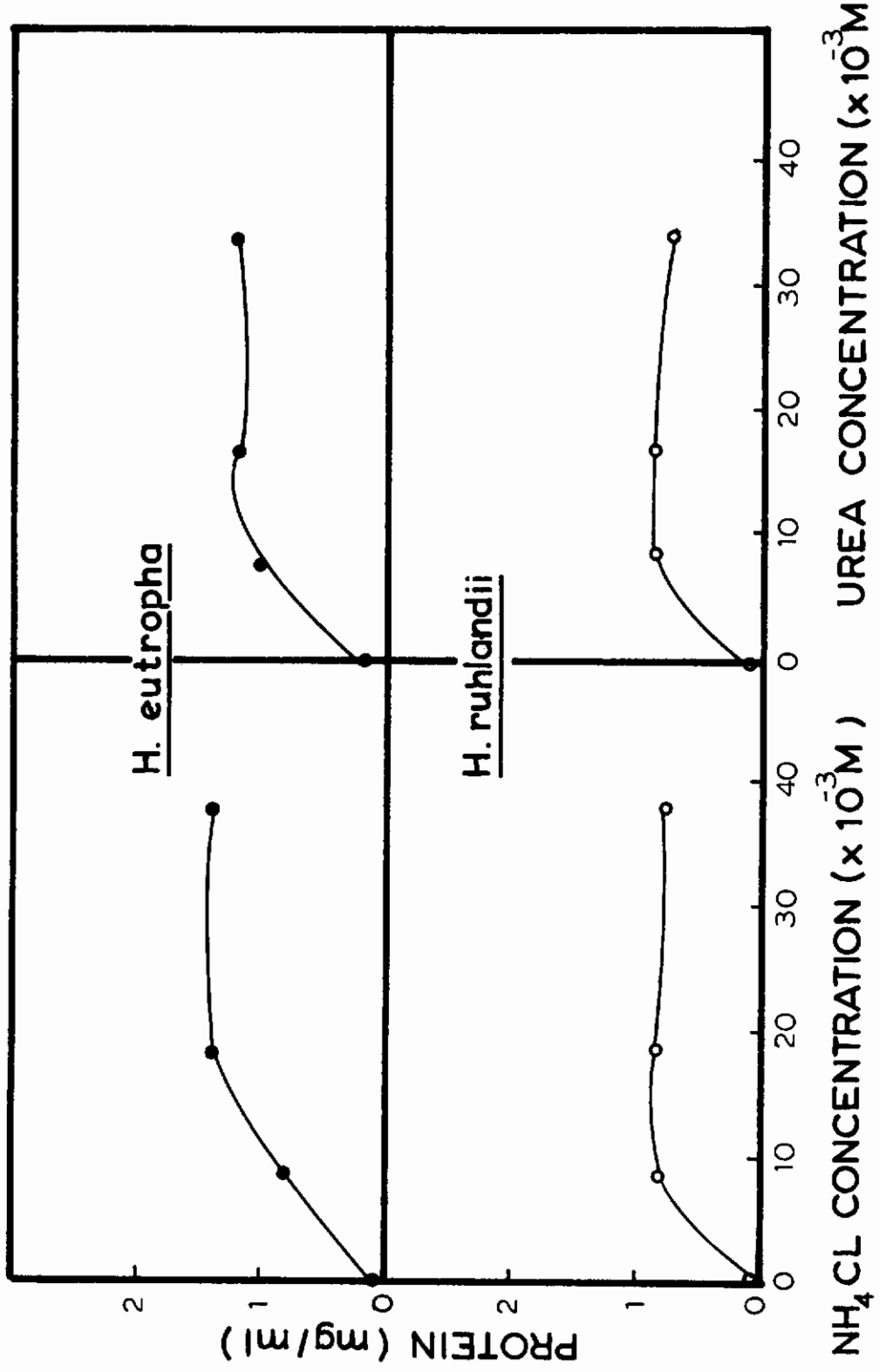


FIGURE 2

Autotrophic Growth of Hydrogenomonas eutropha and Hydrogenomonas ruhlandii in various concentrations of NH_4Cl and Urea

b. Hydrogenomonas ruhlandii

The growth response of H. ruhlandii to NH_4Cl and to urea (as nitrogen sources) is presented in Figure 2.

The data indicate that concentrations below 0.0095 M NH_4Cl or 0.0084 M urea are probably growth limiting for this organism.

2. Medium Composition

Few media formulations are derived experimentally and, consequently, usually contain components which are not nutritionally required or may be present in excess. A quantitative study was performed to examine the requirement for the major mineral components composing the basal medium. It was found that the autotrophic growth of H. eutropha was severely limited when $MgSO_4$, $Fe(NH_4)_2(SO_4)_2$ or NH_4Cl were omitted from the medium. It was apparent that sufficient trace elements were evidently available through the water supply and the other chemicals.

3. Spent Medium

An important consideration for a closed loop biological system is the extent to which the culture medium may be re-used. This consideration becomes critical for long-term operation due to the weight of medium components required for operation of the system, and because of the possible accumulation of deleterious metabolites in the medium. Table I presents the data obtained during two studies concerning the growth of the organism in spent medium, including the effect of adjusting pH.

An additional reinoculation study of spent medium was performed with H. eutropha. Table II shows the results of this study. On the basis of the data it is clear that ammonium ion is the limiting factor with regard to reutilization of media.

4. Carbon Monoxide

The potential for the production of carbon monoxide by electronic instrumentation leads one to expect, as pointed out by Chappelle,(ref. 4) that carbon monoxide will be present in the space craft atmosphere. Chappelle also states that a Hydrogenomonas sp. has been shown to have the ability to fix carbon monoxide, although it can only do so in the absence of some other oxidizable substrate. We therefore studied the ability of H. eutropha to fix carbon monoxide in presence of the normal atmosphere for growth of

TABLE I

The Effect of Spent Medium on the Autotrophic Growth Response of H. eutropha

Experiment	Medium	OD_{660} (1/5)	pH (final)	Protein (final) mg/ml
#1 (1)	Basal	0.67	-	0.97
	Basal, in which organism has been grown once	0.64	5.1	0.36
	Basal, in which organism has been grown twice	0.06	5.4	0.08
#11 (2)	Basal	0.78	6.1	1.29
	Basal, in which organism has been grown once	0.11	6.6	0.20
	Basal, in which organism has been grown three times	0.03	7.0	0.11

(1) Medium pH not adjusted

(2) Medium pH adjusted to 7.0

TABLE II

The Growth Response of H. eutropha in Spent Media Adjusted with Various Medium Components

Medium	Initial pH	Final pH	Final OD_{660}(1/5)	Final protein mg/ml
Spent Medium	7.0	6.4	0.17	0.09
Spent Medium + NH_4Cl	7.0	6.1	0.75	0.43
Spent Medium + $MgSO_4$	7.0	6.4	0.075	0.06
Spent Medium + $Fe(NH_4)_2(SO_4)_2$	7.0	6.5	0.37	0.15
Spent Medium + NH_4Cl + $MgSO_4$ + $Fe(NH_4)_2(SO_4)_2$	7.0	5.8	0.52	0.48

this organism, 70, H_2/20, O_2/10, CO_2. Table III presents the growth response of H. eutropha to two concentrations of carbon monoxide in the atmosphere. It is evident that concentrations of carbon monoxide of 1-5% are inhibitory. Because of this inhibition, it was not considered worthwhile to establish the extent of carbon monoxide fixation.

5. Human Waste Media

The growth response of H. eutropha in urine and a feces extract was performed to examine the feasibility of considering human waste material as a source of nutrients for this organism.

It was found that good growth occurred in 24 hours in urine, while no growth was observed at 24 hours in the fecal extract. Urea was added to a final concentration of 0.0084 M to the fecal extract, and the culture was reinoculated. The culture grew well during an additional 24 hours of incubation. A subsequent experiment was performed in the modified macro-respirometers with urine and urine supplemented with fecal extract. Duplicate flasks of urine plus distilled H_2O (1:1) gave a gas uptake of 1.02 ml of gas per ml of culture per hour, while duplicate flasks of urine plus fecal extract (1:1) gave a gas uptake of 1.30. Stoichiometry observed for this gas utilization was 18/7/1 and 15/6/1, $H_2/O_2/CO_2$, for urine plus distilled water and 18/7/1 and 37/16/1 $H_2/O_2/CO_2$ for urine plus fecal extract. Repeated subculture of H. eutropha in urine was carried out for 24 days and, although considerable discrepancies were observed with optical density and protein analyses, microscopic evaluations indicated that the cultures were thriving under these conditions.

Physical Requirements

1. Growth Response at Various pH levels

Hydrogenomonas eutropha

In the first set of pH studies using phosphate buffers, the data indicate a pH optimum for growth near 7.6. These findings did not agree with those reported by Repaske, which indicate a pH optimum near 6.5. In experiments using tris (hydroxy methyl) amino methane buffer at higher pH values, the pH optimum was between 6.4 and 6.8, as shown in Figure 3. It may be appropriate to report these as optimum initial pH ranges. The striking break in the pH curve for H. eutropha might well be an artifact of the two-buffer (phosphate and "tris") test system, although a comparable discontinuity was not observed with H. ruhlandii.

TABLE III

The Effect of CO on the Autotrophic Growth of H. eutropha *

Concentration of CO (%)	Experiment No.	Flask No.	Final pH	Final OD_{660} (1/5)	Final Protein (mg/ml)
0	I	1	6.7**	0.21*	0.33*
		2	6.2	0.69	0.99
		3	6.2	0.68	1.11
	II	1	6.2	0.83	1.07
		2	6.2	0.83	1.13
<1	II	1	6.7	0.31	0.51
		2	6.7	0.29	0.20
3-5	I	1	6.8	0.05	0.06
		2	6.8	0.08	0.09
		3	6.8	0.05	0.07

* 20-hour growth.

** Poor growth not readily explainable.

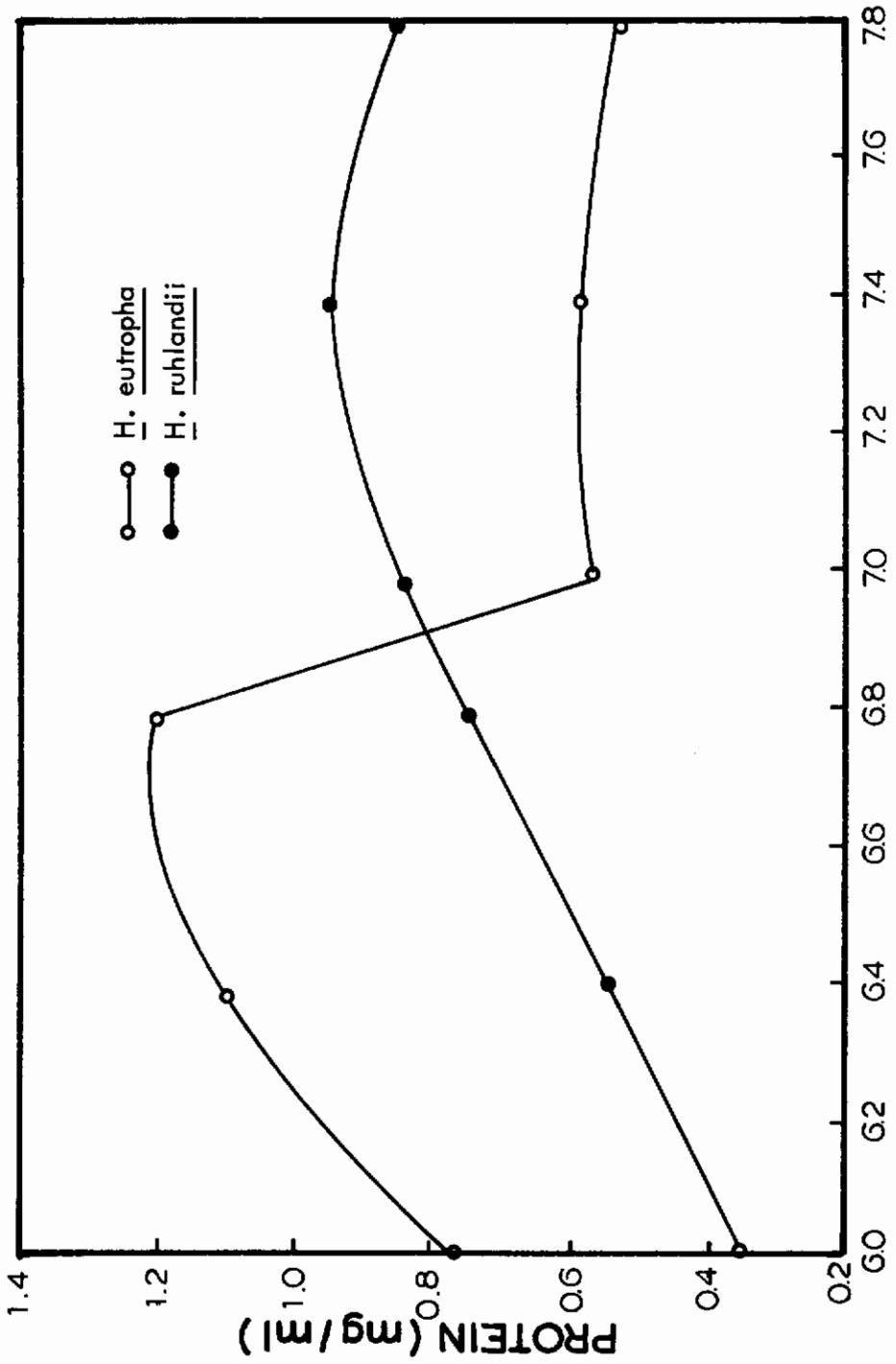


FIGURE 3

Effect of Varying pH on the Autotrophic Growth of Hydrogenomonas eutropha and Hydrogenomonas ruhlandii

Hydrogenomonas ruhlandii

These studies were initiated subsequent to the pH studies with H. eutropha and for that reason were performed only with the system using tris buffer for the high pH values. Figure 3 presents the growth response of H. ruhlandii at the various pH levels. The data indicate an optimum growth pH near 7.4.

2. Temperature

The data obtained during controlled temperature studies with H. eutropha and H. ruhlandii are represented in Table IV. Table IV also contains average control data for the growth of H. eutropha and H. ruhlandii at 30°C. It appears that 25-30°C is optimum, while 35°C is inhibitory.

3. Ionic Strength

The high resistivity of the H. eutropha culture medium interfered with interpretation of results from the bioelectrochemical studies. Various ionic strength media were tested for their effect on the autotrophic growth of H. eutropha as an approach to decreasing resistivity of the medium.

Although spurious results were obtained when the phosphate buffer was altered, the data with $NaHCO_3$, Na_2SO_4 and NaCl show a definite inhibition at ionic strengths above 0.1, the ionic strength of the basal medium, and are represented by data obtained with Na_2SO_4, Table V.

Growth Curves

The growth response of an organism as indicated by its generation time, i.e., the period required for a doubling of protein, gives a good picture of the suitability of the growth environment.

H. eutropha

Duplicate cultures of H. eutropha were grown in macro-respirometers for determination of growth rates. The average values are plotted in Figure 4. Considering a doubling of protein to be comparable with generation time, we obtained values of 3.5 - 4 hours, which agree with values published by Repaske.

TABLE IV

The Effect of Temperature on the Autotrophic Growth of *Hydrogenomonas eutropha* and *Hydrogenomonas ruhlandii*

Temperature (C)	Average Final Protein Concentration (mg/ml)	
	H. eutropha	*H. ruhlandii*
25	1.10	0.89
30	1.24	0.82
35	1.00	0.74

TABLE V

The Effect of Ion Concentration on the Autotrophic Growth of *Hydrogenomonas eutropha*

Na_2SO_4 Concentration (M)	Average Final Protein Concentration (mg/ml)
0.0	1.24
0.1	1.05
0.2	0.27
0.3	0.05

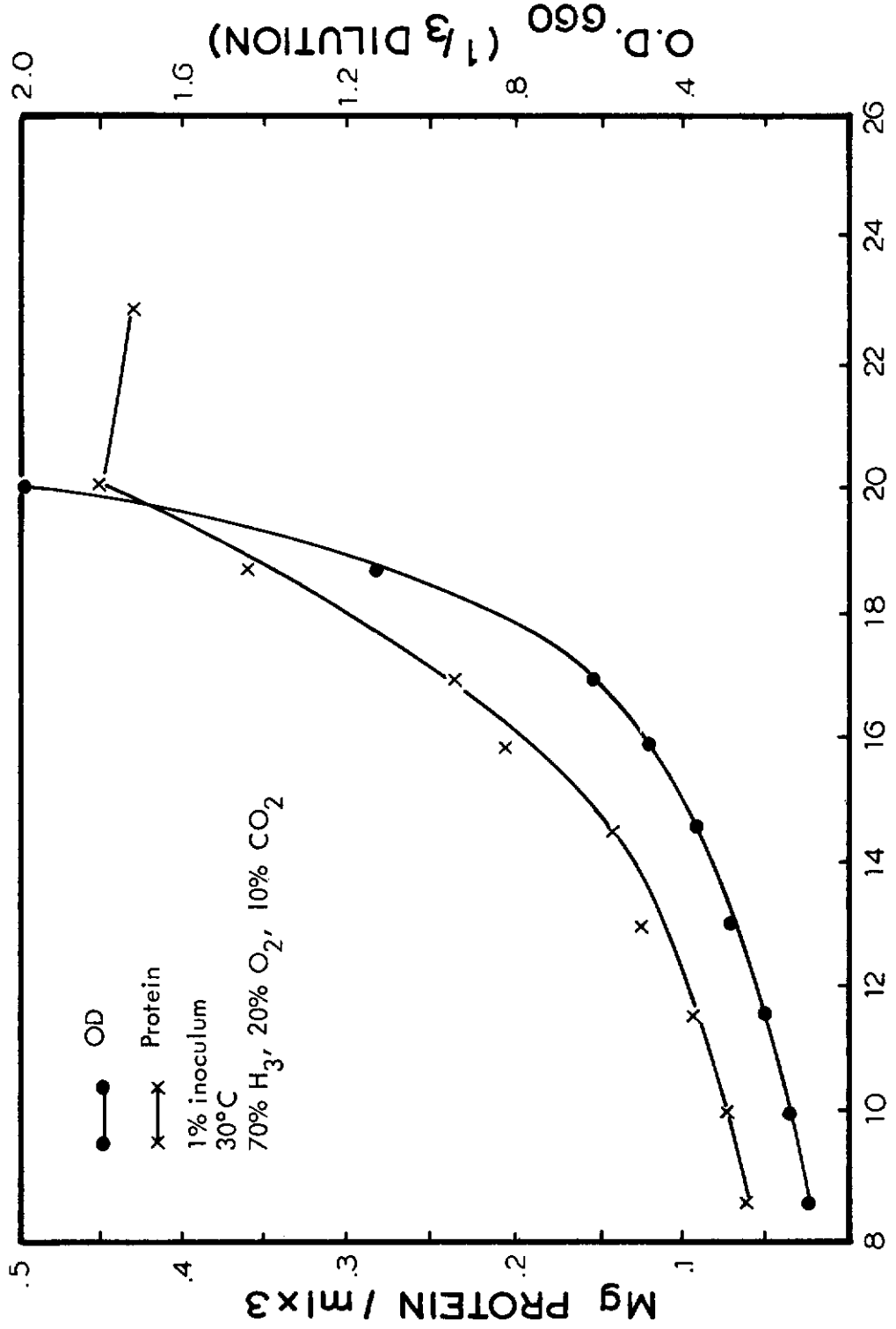


FIGURE 4

Growth Curves for H. eutropha

Hydrogenonomas ruhlandii

Data on growth rates of H. ruhlandii were obtained in the macro-respirometer devices. Average values for protein are plotted in Figure 5. Although the protein data for H. ruhlandii are not as clear as those for H. eutropha, a tentative generation time of 6.5 - 7 hours may be estimated from these data.

Batch Culture Gas Utilization Studies

Hydrogenomonas eutropha

Use of 5 ml of culture in screening experiments appeared to limit the range of observations that could be made, due to the magnitude of gas volume change during metabolism. Gas uptake was rechecked with 1 ml of culture. The data from three replicates using 1% inoculum showed gas uptake of 1.2 ml of gas per ml of culture per hour during the linear uptake phase with growth response better than observed in screening experiments (final OD_{660}(1/3 dilution)= 1.75)

Further examination of gas uptake with H. eutropha was performed by growing cultures in the macro-respirometers, and then, during the log phase of growth, transferring 2 ml samples of the culture without dilution to Warburg growth vessels. Gas uptake during seven hours of study averaged 1.50 ml of gas per ml of culture per hour. There was also a 27.5% increase in the culture protein.

During these studies, stoichiometric analyses of gas utilization were as difficult to obtain as those reported in the screening program. It was considered likely that utilization of gases in the Warburg flasks decreased pressure so far below atmospheric as to be responsible for poor analyses. Two techniques were considered and tested to solve this problem: addition of H_2 to maintain pressure near atmospheric and use of constant pressure manometry using a 20 ml piston burette.

Six subsequent experiments concerning gas utilization were performed. The first two studies utilized log phase cells grown in stoppered flasks; all subsequent studies used log phase cells grown in a macro-respirometer. Log phase cells were then transferred to the micro-respirometer. Stoichiometric analyses were not sufficiently consistent to determine whether constant pressure manometry or the addition of pure H_2 was the better technique for analysis. It was

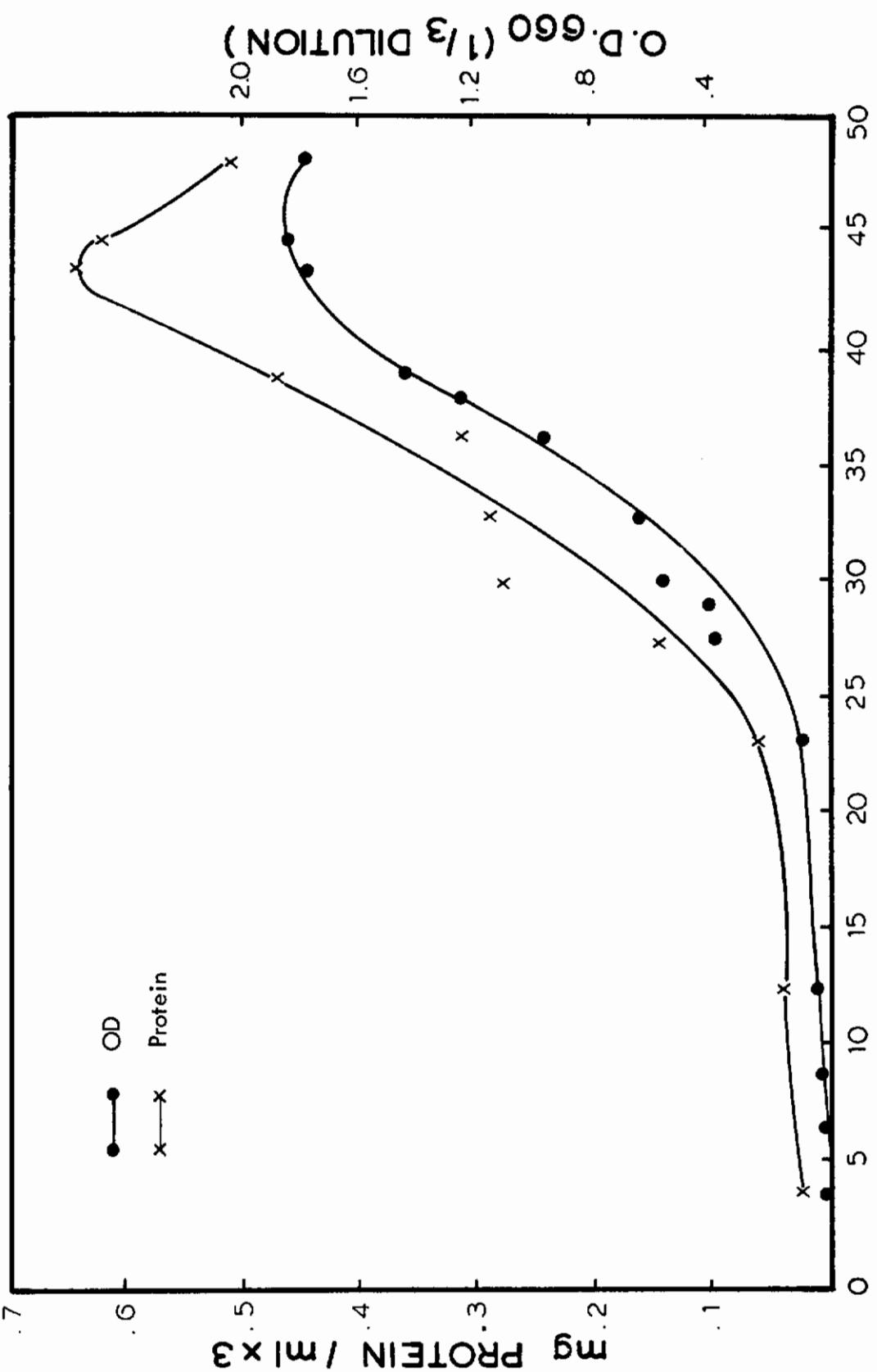


FIGURE 5

Growth Curve for H. ruhlandii

learned, however, that maintenance of pressure in the system near atmospheric was accompanied by improved gas uptake rates. Five experiments gave rates of 1.26 to 2.07 ml of gas per ml of culture per hour with most values between 1.56 - 1.87. During these studies the gas analyses showed approximately 70, H_2/20, O_2/10, CO_2, with CO_2 usually being above 10%. In the sixth experiment the CO_2 content was at 8.6% and an average gas uptake with constant pressure manometry was 2.95 ml of gas per ml of culture per hours, while H_2 addition gave a value of 2.17. As a result of these data, it was decided to use macro-respirometers for gas uptake and stoichiometry at lowered CO_2 content. Further, more reproducible stoichiometric data were expected due to the central position of the gas sampling port, which allows access to the gas atmosphere directly above the culture surface.

It can be seen from Table VI that our expectations were justified. Gas uptake during the linear uptake phase was 2.67 - 2.89 ml of gas per ml of culture per hour, with reasonably consistent stoichiometries near 6 H_2/2 O_2/1 CO_2 indicative of young vigorous cultures.

H. ruhlandii

As with H. eutropha, gas uptake by 1 ml cultures of H. ruhlandii was rechecked in Warburg growth vessels. The gas mixture was made up to have nominal $H_2/O_2/CO_2$ proportions of 82/8/10, but was not checked by analysis.

Each of two Warburg flasks received one ml of culture seeded with a 5% inoculum and other conditions were consistent with those previously noted. Gas uptake during this experiment averaged 0.67 ml of gas per ml of culture per hour. A subsequent study was designed to test gas uptake and stoichiometry in macro-respirometers.

Very low gas uptake rates were observed in three experiments, and stoichiometry varied to a large degree. The gas uptake rates ranged from 0.01 - 0.24 ml of gas/ml culture/hour for H. ruhlandii as compared to 2.7 - 2.9 ml of gas/ml culture/hour for H. eutropha. Therefore, H. ruhlandii at best consumes gas less than one third as fast as H. eutropha and for this reason we consider the organism unattractive for further work.

TABLE VI

Gas Utilization by Cultures of H. eutropha Growing in Macro-Respirometers

Exper. #	Flask #	Initial Gas Mixture $H_2/O_2/CO_2$	Gas Uptake ml gas/ml culture/hr			Stoichiometry		
			0-20 hrs	20-23 hrs***	0-23 hrs	0-20 hrs $H_2/O_2/CO_2$	20-23 hrs*** $H_2/O_2/CO_2$	0-23 hrs $H_2/O_2/CO_2$
	1	72.2/18.2/9.6	-	-	1.1	-	-	5.0/2.2/1
I*	2	72.0/18.1/9.9	-	-	1.2	-	-	5.2/2.0/1
	1	70/21/9.0	1.29	2.89	1.50	4.7/2.2/1	5.6/2.1/1	4.9/2.4/1
II**	2	71/21/8	1.20	2.67	1.39	6.3/3.0/1	4.6/1.6/1	5.7/2.5/1
	3	72/21/7	1.28	2.84	1.50	6.1/2.6/1	4.8/2.0/1	5.7/2.4/1

* 10 ml of culture/flask
** 7.5 ml of culture/flask
*** Log phase gas uptake

Continuous Culture

1. Gas Mixing for Continuous Culture

The gas mixing and continuous culture apparatus (Figure 9, Appendix III) was operated at preselected settings to flush the system. Samples for gas analyses were taken through rubber tubing which connected the gas sterilizing unit and the culture vessel. It was found that approximately 30 minutes of operation were required to flush all the air from the system. In preliminary checkout, a gas mixture of 70 -H_2/20-O_2/10-CO_2, as required for H. eutropha, was established and maintained (actual analysis was 71.0/18.7/10.3).

2. Series I

Four continuous culture studies were performed with the apparatus described in Figure 10, (Appendix III). The first study was essentially a test run to examine culture feed rates and to confirm procedures for gas sampling and measurement of flow rates. In the second study, steady state variables were examined, i.e., gas uptake, protein content, OD_{660}, pH, using ammonium chloride for chemostatic control of growth.

When it was observed that gas uptake was poorer than in batch cultures, a third study was performed to determine if the limiting NH_4Cl concentration was responsible for the poor gas uptake rates. The fourth study examined the use of column packing (porcelain berl saddles) as a means of improving mass transport across the gas-liquid interface.

Steady state operation could not be achieved in the first continuous culture. The gas input control was good according to the two analyses performed, i.e., 71.4, H_2:18.9, O_2: 9.7, CO_2 at zero time and 71.5, H_2: 18.7, O_2: 9.8, CO_2 at 144.5 hours. The single stoichiometric analysis was near the expected 6/2/1, $H_2/O_2/CO_2$, i.e., 5.85/2.12/1. Gas uptake ranged from 0.91 - 1.54 ml of gas per ml of culture per hour.

Data were obtained during ten days operation of continuous culture with H. eutropha in Experiment 2. Selected data from this experiment are presented in Table VII. It can be seen that reasonable steady state conditions were obtained, in that gas uptake, OD_{660}, and protein content did not fluctuate to any great degree. The expected shift in stoichiometry occurred subsequent to the increase in feed rate

TABLE VII

Selected Data Obtained During the Second Continuous Culture Study with Hydrogenomonas eutropha[1], Series I

Time (Hours after start of continuous culture)	Inlet Gas Analysis (%)[2] H_2	O_2	CO_2	OD_{660} (1/5 dilution)	Protein (mg/ml)	Gas Utilization: Uptake - (ml of gas per ml of culture per hour)	Gas Utilization: Mole Ratio $H_2/O_2/CO_2$
74[3]	73.4	17.1	9.5	0.62	0.74	1.25	8.2/1.6/1.0
78[3]	———			0.59	0.74	1.22	———
90[3]	76.3	16.7	9.7	0.56	0.71	1.26	7.7/2.1/1.0
96[3]	72.4	17.6	10.0	0.53	0.65	1.26	6.2/2.0/1.0
140[4]	73.6	17.0	9.4	0.60	0.64	1.09	8.1/2.1/1.0
144[4]	73.8	16.5	9.7	0.60	0.72	1.09	8.6/1.4/1.0
165[4]	———			0.77	0.74	———	———

1 Culture volume - 530 ml.

2 Gas flow rate - 50-60 ml/min.

3 Nutrient feed rate, 0.388 ml/min; retention time, 22.76 hours.

4 Nutrient feed rate, 0.194 ml/min; retention time, 45.52 hours.

to 0.388 ml per minute between 72 - 115 hours; i.e., 8.2/1.6/1.0 - 6.2/2.0/1.0. $H_2/O_2/CO_2$. This shift indicated that the culture became younger during this period due to the input of nutrients at a faster rate. In both Experiments 1 and 2, however, gas uptake was considerably poorer than the 2.7 - 2.9 ml of gas per ml of culture per hour observed in batch cultures. Samples of the culture were obtained at termination and the protein and dry weight of the culture were determined. Protein was found to be 0.753 mg/ml and the dry weight was determined to be 1.454 mg/ml. Calculation indicates that protein is 51.8% of the dry weight.

The third continuous culture was performed with the basal medium (0.019 M NH_4Cl) and four days results indicate that the NH_4Cl concentration (0.0095 M) of Experiments 1 and 2 was not limiting gas uptake in those experiments.

An estimate of the gas-liquid interfacial area for macro-respirometers indicated it to be approximately ten times that estimated for the continuous culture growth chamber; that is, ratios of interfacial area to liquid volumes were estimated at 53.6 and 6, respectively. Assumptions leading to these numbers were extremes and it is probable that the ratio for continuous culture is considerably smaller than 6 and that the ratio for static culture is much more than ten times the ratio for continuous culture. The implication of these calculations is that mass transport across the gas-liquid interface is probably limiting gas uptake rates in continuous culture. If the supply of one or more gases to the bacteria is indeed limiting, this would explain the need for slow culture flow rates with the resultant increase retention times, compared with the growth rate of 35 hours seen in batch culture.

In the fourth continuous culture experiment, the growth vessel was packed with porcelain berl saddles to increase gas retention volume and thus to improve the mass transfer of gas from the gas phase into the liquid phase. Table VIII presents selected data observed during 14 days of operation. A single flow rate, and thus, a single retention time of 29 hours was used. The gas uptake did improve somewhat, in that most values ranged from 1.3 - 1.7 ml of gas per ml of culture per hour, compared with maxima of about 1.3 ml/ml/hour in Experiments 1-3. During this experiment we also attempted to observe the response of growth to varying concentrations of oxygen. Oxygen concentration was progressively decreased to as little as 7% without greatly affecting the gas uptake rate. Limiting success in improving gas uptake in the fourth continuous culture prompted redesign of the continuous culture growth chamber. (Figure II, Appendix III.)

TABLE VIII

Evaluation of Column Packing as a Means of Improving Gas Uptake by Hydrogenomonas eutropha during the Continuous Culture Study[1], Series I

Time (Hours after start of Continuous culture)	Inlet Gas Analysis (%)[2]			OD_{660}	Protein	Gas Utilization	
	H_2	O_2	CO_2	(1/5 dilution)	(mg/ml)	Uptake- (ml of gas per ml of culture per hour)	Mole Ratio $H_2/O_2/CO_2$
48	74.0	17.7	8.3	0.63	0.61	1.3	10.0/3.0/1.0
56	- -	- -	- -	0.76	0.56	1.7	- - - -
68	77.2	14.1	8.7	0.88	- - -	1.3	9.2/2.4/1.0
96	80.3	10.3	9.4	0.77	0.57	1.3	10.9/1.8/1.0
119	83.0	7.0	10.0	0.68	0.69	1.3	15.7/1.7/1.0
192	74.2	17.0	8.8	0.56	0.55	1.5	7.9/2.1/1.0
216	74.4	17.0	8.6	0.63	0.71	1.0	13.4/2.9/1.0

1 Culture volume - 335 ml; nutrient feed rate, 0.194 ml/min; retention time, 28.78 hours.

2 Gas flow rate - 50-60 ml/min.

3. Continuous Culture, Series II

The first successful continuous culture, Series II, was set up with a flexible impeller pump for culture recirculation. This pump had a carbon bearing, and a considerable quantity of carbon particles were liberated into the culture. The gas mixture could not be checked due to difficulties with the chromatograph, and therefore rotameter flow settings were based upon previous data. The calculated gas uptake rate after 8 hours of growth was 2.01 ml of gas per ml of culture per hour. During the next 9.5 hours, temperature control was lost and at 17.5 hours the uptake rate was 0.63 ml/ml/hour. Temperature control was then re-established and during the following days at 63.5, 66.5 and 80 hours the gas uptake rates were 0.96, 0.84 and 0.97 ml/ml/hour. After 80 hours the carbon from the recirculation pump had coated the culture chamber to such a degree as to necessitate cleaning of the chamber.

The second continuous culture was set up in the same manner as culture No. 1, except that a pump with teflon bearings was used. The pump still threw out black material (possibly teflon-carbon mixture) but to much less a degree. A gas analysis near the 16-hour point showed that the culture was receiving a gas mixture very low in oxygen, and after the readjustment the gas uptake improved. The medium feed rate was 0.194 ml per min. and the culture volume was 540 ml. Table IX presents the data obtained during this experiment. The experiment was stopped after 76.5 hours in order to clean the chamber of deposited material.

The third continuous culture was set up in like manner to the second study. Table X presents the data obtained during two days of growth. The growth appeared to fall off considerably after 24-48 hours and did not re-establish itself even when the medium feed rate was stopped after 82 hours.

The fourth continuous culture established itself well, as shown in Table XI. It can be seen that culture flow rate and oxygen concentration are very important variables. Considerable foaming occurred transiently but the degree of foaming could not be correlated with gas uptake. It can be seen that the highest feed rate, corresponding to a generation time of 21.5 hours, combined with an oxygen level of approximately 14%, resulted in gas uptake comparable with the best values obtained in batch cultures.

TABLE IX

Gas Utilization Data Obtained During the Second Continuous Culture Study with H. eutropha, Series II

Time (hrs)	Medium Feed Rate (ml/min)	Inlet Gas Mixture (% $H_2/O_2/CO_2$)	Inlet Gas Flow Rate (ml/min)	Gas Utilization	
				Gas Uptake (ml/ml/hr)	Stoichiometry ($H_2/O_2/CO_2$)
0	0.194	-	-	-	-
16.25	"	90/1/9 approx.	50.41	0.67	-
21.75	"	-	60.46	1.35	-
23.50	"	75.8/16.5/7.7	59.20	1.41	-
42.50	"	-	60.90	0.91	-
42.50	0.388	-	-	-	-
53.00	"	-	61.24	1.24	-
68.25	"	-	63.74	1.45	-
76.50	"	74.9/16.6/8.5	62.35	0.96	12.2/2.4/1

Temperature - 30 °C

Culture volume - 540 ml

Retention time - 46.4 hours at 0.194 ml/min

23.2 hours at 0.388 ml/min

TABLE X

Gas Utilization Data Obtained During the Third Continuous Culture Study with H. eutropha, Series II

Time (hrs)	Medium Feed Rate (ml/min)	Inlet Gas Mixture (%H_2/O_2/CO_2)	Inlet Gas Flow Rate (ml/min)	OD_{660} (1/5)	Gas Utilization	
					Gas Uptake (ml/ml/hr)	Stoichiometry (H_2/O_2/CO_2)
0	0.194	-	-	-	-	-
2	"	75.1/16.2/8.7	-	-	-	-
22	"	73.7/16.4/9.9	60.83	-	1.97	8.6/2.5/1
29.75	"	-	62.39	-	1.51	-
32.50	"	-	60.22	-	1.13	-
46.50	"	-	58.92	-	0.67	-
48.50	"	-	-	0.80	-	-
50.50	"	-	61.73	-	0.82	-
51.25	0.30	-	-	-	-	-
60.50	"	-	57.32	-	0.13	-
69.25	"	73.4/16.8/9.8	61.62	0.24	0.56	5.7/2.9/1
74.25	"	-	60.89	-	0.63	-
74.25	0.194	-	-	-	-	-
82.0	"	-	58.03	0.07	0.35	-

Temperature - 30°C
Culture volume - 540 ml
Retention time - 46.4 hours at 0.194 ml/min
30.0 hours at 0.30 ml/min

TABLE XI

Gas Utilization Data Obtained During the Fourth Continuous Culture Study with H. eutropha, Series II[1]

Time (hrs)	Medium Feed Rate (ml/min)	Inlet Gas Mixture (% $H_2/O_2/CO_2$)	Inlet Gas Flow (ml/min)	OD_{660} (1/5)	Protein (mg/ml)	Gas Utilization	
						Gas Uptake	Stoichiometry
0*	0.194	77.6/13.7/8.7	-	0.052	0.022	-	-
20	"	75.2/14.5/10.3	-	0.44	0.15	-	-
22	"	-	65.95	-	-	0.92	-
44	"	-	63.40	0.38	0.16	0.66	-
70.5	"	-	64.86	0.44	0.19	0.96	-
95.5	"	-	-	0.34	0.18	-	-
114.5	"	-	67.1	-	-	1.40	-
118	"	-	63.9	0.34	0.25	0.87	-
119	0	-	-	-	-	-	-
140	"	-	-	0.40	0.21	-	-
145	0.194	-	-	-	-	-	-
168**	-	-	-	0.34	0.22	-	-
173	0.388	-	63.1	0.42	0.24	0.76	-
177.5	0.388	-	63.80	0.52	0.28	0.91	-
195	"	70.8/19/10.2	69.3	-	-	1.62	-
197.5	"	-	66.50	0.95	0.50	1.71	-
220.5	"	lowered O_2 level	64.3	0.98	0.57	1.53	-
232	"	-	69.2	-	-	2.64	-

(continued)

TABLE XI (continued)

Gas Utilization Data Obtained During the Fourth Continuous Culture Study with H. eutropha, Series II

Time (hrs)	Medium Feed Rate (ml/min)	Inlet Gas Mixture (% $H_2/O_2/CO_2$)	Inlet Gas Flow (ml/min)	OD_{660} (1/5)	Protein (mg/ml)	Gas Utilization	
						Gas Uptake	Stoichiometry
242	0.388	-	64.7	-	-	2.31	-
246	"	75.4/14/10.6	63.8	0.98	0.60	2.24	10.2/3.4/1
256	"	-	66.2	-	-	2.94	-
281	"	-	61.9	-	-	2.70	-
283	"	76.4/13.8/9.8	-	1.78	1.04	-	14.2/5.9/1

---

1 30°C, culture volume - 500 ml, retention time - 0.194 ml/min = 42.96 hrs.

* Culture medium with limiting nitrogen conc. (0.0095 M NH_4Cl)

** Culture medium with optimum nitrogen conc. (0.019 M NH_4Cl)

Bioelectrochemical Studies

1. Resting Cell Experiments

Examination of suspensions of H. eutropha demonstrated that metabolism of the bacteria proceeded normally when a cathodically polarized electrode served as the sole source of hydrogen. Depolarization of the cathodically polarized electrode, however, was not observed under the conditions of the experiment.

Data in Table XII illustrated the occurrence of metabolism of H. eutropha as described. Experiment No. 1 was performed to find the rate of hydrogen evolution from electrolysis of the medium in the absence of the bacteria. Experiment No. 2 was run to show that evolution of hydrogen occurred even when oxygen was present. The difference in gas evolution rates between Experiments No. 1 and 2 is accounted for by the electrochemical consumption of oxygen. Experiments No. 3 and 4 illustrate that under conditions such that hydrogen is otherwise evolved by this system (Experiment No. 2), the presence of H. eutropha prevents evolution of hydrogen. At the same time, gas consumption occurred over that accountable by endogenous oxygen uptake by the cells. Experiment No. 5 was run to determine the endogenous hydrogen consumption rate by cells in the presence of the polarized electrode. Comparison with Experiment No. 1 indicates this to be negligible.

These data lead to the conclusion that H. eutropha metabolizes hydrogen at a cathodically polarized electrode when also supplied with oxygen. The alternative, that H. eutropha catalyzes electrochemical reduction of oxygen, is indistinguishable, but not likely.

The influence of H. eutropha cells on the potential of a cathodically polarized electrode is shown in Table XIII. These experiments are the same as described in Table XII. The electrodes were passing identical current (1 ma). The polarized potentials are the same within experimental error, considered to be $\pm$ 0.01 volt.

Comparison of data of Experiments No. 3 and 4 with that of Experiment No. 2 show that no particular influence on the electrode potential was exerted by the H. eutropha cells. Differences that exist in these data could be accounted for on the basis of differences in cell resistance.

TABLE XII

Influence of H. eutropha on Hydrogen Evolution at a Cathodically Polarized Electrode

Experiment No.	1	2	3	4	5
Gas Mixture Used					
% He	100	90	90	90	100
% O_2	-	10	10	10	-
H. eutropha cells present	no	no	yes	yes	yes
Duration of experiment, min.	45	120	45	145	60
Gas change rate*, µl/min.	+6.7	+2.6	-1.7	-1.9	+7.2
Test for H_2 at end of experiment	pos.	pos.	neg.	neg.	pos.
Endogenous O_2 uptake rate, µl/min.	-	-	3.6	2.5	-

Cell current was 1 ma; cell electrolyte was 0.1 M phosphate buffer, pH 7; gas change measurements were made only with the cathode compartment.

* Corrected for endogenous O_2 uptake rate.

TABLE XIII

Influence of H. eutropha on the Potential of a Cathodically Polarized Electrode

	Cathode Potential vs. Standard Calomel Electrode (SCE)				
	Experiment Number				
	1	2	3	4	5
Time from start of experiment, min.					
5	-1.72	-	-1.53	-1.80	-1.53
15	-	-1.65	-	-	-1.53
30	-1.82	-1.70	-	-	-
45	-1.82*	-1.72	-1.63*	-	-1.56
60	-	-	-	-1.88	-1.58*
120	-	-1.74*	-	-1.92	-
145	-	-	-	-1.95*	-
Cell Resistance, ohms	1080	940	-	900	780

* End of experiment.

2. Growth Experiments

Two gas uptake control studies were performed in the Warburg electrolysis cell, prior to a growth experiment, in which H2 was supplied electrolytically. The two studies, as biological controls, showed that H. eutropha could metabolize the gasses well, under the conditions used in the cell. Table XIV shows gas utilization data for two dilutions of washed cultures. In the latter study, it can be seen that the organisms, although diluted into 0.2 ionic strength medium, took up gas at the steady rate of 0.24 ml of gas per ml of culture per hour with an over-all stoichiometry of 4.82, H_2/ 1.61, O_2/ 1, CO_2. This stoichiometry is indicative of a young, vigorous culture.

A growth study with H. eutropha was performed in the Warburg electrolysis cell during a period of 142 hours. Protein increased from 0.09 to 0.23 mg per ml. C.s uptake averaged 0.07 ml of gas per ml of culture per hour. The initial gas mixture was 71.35, He/ 19.70, O_2/ 8.95, CO_2. The culture medium was 0.2 ionic strength basal medium, increased ionic strength was adjusted with increased buffer concentration.

3. Immobilization of H. eutropha at Electrodes*

Porous gold disks were inoculated with H. eutropha in two ways: A culture was filtered onto the disk or a disk seeded with H. eutropha was laid on agar nutrient and exposed to the appropriate gas mixture. Initial attempts to inoculate disks by the growth technique were unsuccessful; a zone of inhibition could be seen at the periphery of the disk. This difficulty was obviated by pre-reducing the disk, that is, by using it as the cathode of an electrochemical cell.

In Table XV, the biological evaluation of two successfully inoculated disks is compared with a nonbiological control, in terms of consumption in the Warburg of a $H_2/O_2/CO_2$ mixture. Because the filtration-inoculated electrode had not been pre-reduced, it is not possible to reject the possibility that inhibition of the bacteria was important in that case. The results are indicative of the presence of biological activity on the disks that was approximately the equivalent of organisms in 0.1 to 0.2 ml of active culture per cm^2 of electrode.

Bioelectrochemical data observed with the H. eutropha on porous gold disks are presented in Tables XVI and XVII. Neither electrode described above gave evidence of depolarization when operated as a cathode. This is not surprising since it was necessary to operate the control electrode at a

* Immobilization studies reported here, as well as work on ionic strength of H. eutropha media and isolation of a marine hydrogenomonad, were performed by Magna Corp. under Contract NObs 84243 to the Dept. of the Navy, Bureau of Ships.

TABLE XIV

H. eutropha: Gas Utilitization in Warburg Electrolysis Cell

Experiment No.	Time (min)	Gas Uptake (ml of gas/ml cult/hr)
1*	5	0.84
	15	0.66
	30	0.66
	45	0.72
	60	0.84
2**	10	0.36
	45	0.24
	60	0.24
	90	0.24
	120	0.24
	150	0.24
	185	0.24
	210	0.24

* 20 hour culture, washed 1 x in 0.2 ionic strength basal medium, pH7.2, gas mixture - 78, H2/14, O_2/ 8, CO_2, OD_{660}(1/5) = 0.80, 15 ml culture, initial protein = 0.62 mg/ml.

** 20 hour culture, washed 1 x in 0.2 ionic strength basal medium, 15 ml culture, pH 7.0, OD_{660} = 0.90, protein = 0.13 mg/ml, gas mixture = 76.4, H_2/14.8, O_2/8.8, CO_2.

Final conditions = pH 6.75, protein = 0.14 mg/ml, gas mixture 80.9, H_2/1.73, O_2/7.0, CO_2.

Stoichiometry = 4.82, H_2/1.6, O_2/1, CO_2.

TABLE XV

Gas Uptake by Electrodes* Inoculated with
H. eutropha (μl/hr/cm^2)

Experiment	Conditions	Gas Uptake 0-2 hrs	Gas Uptake 0-3 hrs	Gas Uptake 2-8 hrs
1	Nonbiological control	0.04		0.05
2	Filtration inoculated electrode	0.26		0.09
3	Organisms grown on electrode		0.55	

* 2.4 cm^2 gold disk

TABLE XVI

Bioelectrochemical Studies with H. eutropha[1]: Electrochemical and Chromatographic Data

Experiment	Cell Resistance (Ω) Initial	Final	Coulombs Passed	Hydrogen Present Initial	Final (approx.%)	pH Initial	Final	Calculated Faradaic Efficiency for H_2 Evolution (ave. %)
#1 Nonbiological Control Electrode[2]	490	430	39.3	-	7	7.0	7.5	67.3
#2 Filtration Inoculated Electrode	510	460	18.8	-	3	7.0	7.2	86.7
#3 Organisms Grown on Electrode	500	465	6.6	-	1.5	7.0	7.0	72.3

1. Warburg electrolysis cell, catholyte - 0.2 ionic strength basal medium, initial gas mixture - 70%He/20% O_2/10% CO_2.

2. Electrodes were 2.4 cm^2 gold disks.

TABLE XVII

Bioelectrochemical Studies with H. eutropha[1]: Current-Potential and Manometric Data

Experiment		Current Density (mA/cm^2)					
		0.1	0.2	0.4	0.8	1.2	1.4
#1							
Nonbiological	Cathode Potential vs SCE (V)	-0.44	-0.56	-0.87	-1.00	-1.12	-
Control Electrode[2]	Gas Volume Change ($\mu L/min$)	-1.4	-2.5	-2.8	+1.8	+6.0	-
#2							
Filtration Inoculated	Cathode Potential vs SCE (V)	-	-	-0.88	-	-	-1.11
Electrode	Gas Volume Change ($\mu L/min$)	-	-	-2.8	-	-	+9.2
#3							
Organisms Grown	Cathode Potential vs SCE (V)	-	-	-	-1.06	-1.11	-
on Electrode	Gas Volume Change ($\mu L/min$)	-	-	-	+2.1	+7.8	-

1,2 Please refer to Table XVI

minimum density of 0.8 ma/cm^2 to evolved hydrogen (gas analysis at end of experiment). Hydrogen is then evolved from the nonbiological control electrode at a rate of 4 to 5 μl/min/cm^2 (one atmosphere pressure, 30°C and a Faradaic efficiency for hydrogen evolution of 80%). However, the biological gas consumption of the unpolarized disk was less than 1 μl/min/cm^2. It appears, however, that hydrogen was consumed in that approximately 7% hydrogen was found in the final gas sample when an uninoculated disk (the nonbiological control) was polarized while only 1.5 to 3% hydrogen was observed in the final gas mixtures when bioelectrodes were polarized. On the other hand, manometric data on the polarized electrodes show essentially little or no difference in gas uptake between the nonbiological control and the bioelectrodes.

Isolation of Marine Hydrogenomonas

As it became apparent that H. eutropha was particularly sensitive to ionic strength above 0.1, we then set out to isolate a marine hydrogenomonad. The marine Hydrogenomonas enrichments were allowed to incubate for several days and then regassed. It was found that a fair amount of gas uptake, indicated by a partial vacuum, developed in the 20% oxygen flasks. Less vacuum was observed in the 8% oxygen flasks and none in the cultures with no oxygen.

Microscopic analyses of gram strains from the aerobic cultures showed that the predominating organism was a small, slender, gram-negative rod. Other forms observed were a few cocci, vibrios, and small, plump, gram-negative rods.

After regassing and incubation, a partial vacuum was observed in all flasks, including those without oxygen. These enrichments were subcultured in the sea water basal medium with tris-HCl buffer, to dilute the carry-over nutrients from the mud. Periodically, samples of the enrichments were streaked onto the solidfied basal medium and colonies were observed for purity. A pure colony has been obtained from the anaerobic flasks.

The pure colony obtained from anaerobic enrichment was found to grow best when supplied with 0.1% yeast extract. This organism was tested for hydrogenase activity by the Thunberg hydrogenase test, uptake of H_2 in Warburg micro-respirometers, and uptake of 90, H_2/10,CO_2 in macro-respirometers. With the Thunberg hydrogenase test, a weak positive was observed after overnight incubation. This experiment was repeated a second time and the same results were observed. The uptake of hydrogen by the

organism in Warburg flasks was observed in only one of duplicate flasks. During the first 90 minutes approximately 53 µl of gas was produced in each flask; subsequent to this time one flask maintained this positive trend while the duplicate flask proceeded to an uptake of 51 µl at 565 minutes, the termination of the experiment.

The uptake of 90, H_2/10, CO_2 was attempted in macro-respirometers and by 66 hours of growth the average gas uptake was 27.3 µl per ml of culture per hour.

Nutritional Requirements

When it was observed that significantly increased growth of the organism would occur if 0.1% yeast extract were added to the sea water basal medium, an attempt was made to determine whether the stimulation were due to some amino acids, vitamins, or Krebs cycle intermediates. It was particularly interesting to note that the compounds allowing improved growth were all Krebs cycle intermediates, i.e.,

1. ketoglutarate or succinate
2. citrate
3. fumarate
4. oxalacetate
5. malate

DISCUSSION

Selection and Study of Hydrogen-Oxidizing Bacteria

Literature Search and Selection of Candidates

Examination of the literature for microorganisms which are known to metabolize hydrogen and carbon dioxide stoichiometrically revealed several photosynthetic bacteria, members of the genus Hydrogenomonas and the family Methanobacteriaceae, a Clostridium, and certain algae. Three Hydrogenomonas spp. and three photosynthetic bacteria were selected for experimental screening. These were H. eutropha, H. facilis, H. ruhlandii, Rhodospirillum rubrum, Chlorobium Timicola, and Chromatium sp. The screening study consisted of laboratory verification of reported growth and gas utilization characteristics of the selected bacteria. Only H. eutropha and H. ruhlandii demonstrated reproducible growth and consumption of hydrogen and carbon dioxide. These two organisms were selected for more intensive study. An additional factor in their selection was that these two species are representative of the two physiological classes of Hydrogenomonas with respect to oxygen tolerance. H. eutropha is reported to grow best under oxygen tensions of 20% and tolerates 30% while H. ruhlandii is reported to grow best at 8% oxygen tension and is inhibited at higher oxygen tensions.

The reaction classically attributed to members of the genus Hydrogenomonas (hydrogenomonads) is that shown in equation (1).

$$6\,H_2 + 2\,O_2 + CO_2 \longrightarrow (CH_2O) + 5\,H_2O \qquad (1)$$

The expression in parentheses represents carbohydrate. When combined with the reaction for electrolysis of six moles of water, equation (2),

$$6\,H_2O \longrightarrow 6\,H_2 + 3\,O_2 \qquad (2)$$

a net production of oxygen and carbohydrate results, equation (3).

$$H_2O + CO_2 \longrightarrow (CH_2O) + O_2 \qquad (3)$$

The identity of equation (3) with that classically given for the photosynthesis reaction serves to illustrate the utility of electrolysis with hydrogen bacteria for oxygen regeneration. The primary difference between the processes is the form in which energy is introduced.

Batch Culture Experiments

The candidate organisms H. eutropha and H. ruhlandii were subjected to considerable examination in batch culture preliminary to continuous culture experiments. Batch culture was used to establish broadly the parameters involved in culture of these organisms. Further, certain aspects of culturing these organisms were examined only in batch culture.

Nutritional requirements aside from hydrogen, oxygen, and carbon dioxide of the hydrogenomonads are readily met by a relatively simple medium containing a number of inorganic salts. As a medium component attractive for use in control of continuous culture, nitrogen sources were examined for their effect on microbial growth. Both ammonium chloride and urea were studied. Ammonium chloride at 9.5×10^{-3} M and urea at 8.4×10^{-3} M limited growth of both organisms. At twice these concentrations and above, no effect on growth could be seen. The utility of urea as a nitrogen source is of particular importance. Urea from human urine in a closed system can be considered a useful nitrogen source for the bacteria.

The complement of trace materials and other inorganics used in culture of H. eutropha was also examined. Only magnesium sulfate, ferrous ammonium sulfate and ammonium chloride were critical for growth. Omission of sodium bicarbonate, sodium chloride and trace elements from culture medium had no effect. It is probable that trace requirements exist but that these were satisfied by trace contamination of the distilled water or of the other salts used to prepare media. The requirement for a source of nitrogen and of ferrous ion is consistent with literature reports. The requirement for magnesium is not surprising in the light of the dependence of many enzyme systems on this ion. For closed system operation, it appears likely that trace requirements can readily be met either by expendable supplies or by recycling materials from the wastes of occupants of the system. Consideration was given to the use of chelating agents for the maintenance of ferrous ion availability to the bacteria. However, the pH range found suitable for growth of H. eutropha in particular obviated the need for such aids in solubilizing ferrous ion. Indeed, the use of chelating agents under conditions such that ferrous salt solubility does not limit the availability of ferrous ion would tend to make the ferrous ion less available to the bacteria.

The pH requirements for growth of H. eutropha and H. ruhlandii were checked against the literature and established as 6.4 - 6.8 and 7.2 - 7.4 respectively. Temperature requirements were confirmed to be in the range of 25 - 30°C for both organisms, with inhibition of growth occurring at 35°C.

Poor tolerance for ionic strength greater than in basal medium was shown for H. eutropha. The basal medium has an ionic strength of approximately 0.1. Increasing this by increasing the concentrations of sodium chloride,

sodium bicarbonate, or phosphate buffer, or by adding sodium sulfate resulted in severe inhibition. Thus, electrolytic conductivity of the culture medium for H. eutropha can be increased only at the expense of decreased activity of the bacteria under otherwise normal conditions. It is conceivable that one effect of increasing ionic strength was to decrease solubility of the gaseous substrates, thus impeding transport of the gases to the bacterial cells. This is not to say that other effects of increasing salt concentration are not exerted on the bacterial processes.

Growth rates of H. eutropha and H. ruhlandii were examined in batch culture as a guide to what might be expected in continuous culture. H. eutropha exhibited a generation time of 3.5 - 4.0 hours and H. ruhlandii 6.5 - 7.0 hours. This difference in generation time is reflected further in the differences in gas uptake rates discussed below.

Stoichiometry and gas uptake rates were evaluated in batch culture. Both H. eutropha and H. ruhlandii exhibited stoichiometry approximating the reported optimum of 6/2/1, $H_2/O_2/CO_2$.

The stoichiometry of gas consumption is indicative of the efficiency with which the bacteria use the energy available to them. The reactions attributed to the hydrogenomonads are the "Knallgas" reaction, equation (4), and reduction of carbon dioxide, equation (5).

$$2\,H_2 + O_2 \longrightarrow 2\,H_2O \qquad (4)$$

$$2\,H_2 + CO_2 \longrightarrow (CH_2O) + H_2O \qquad (5)$$

The sum of two times the reaction of equation (4) and the reaction of equation (5) gives the over-all reaction, equation (1), and the stoichiometry of $H_2/O_2/CO_2$ consumption of 6/2/1. If the hydrox reaction occurs more than twice

$$6\,H_2 + 2\,O_2 + CO_2 \longrightarrow (CH_2O) + 5\,H_2O \qquad (1)$$

for each carbon dioxide reduction, the energy utilization is less efficient. This appears to be the case with older cultures of the hydrogenomonads. While H. eutropha cultures were capable of consuming approximately three times their volume of gas with the foregoing stoichiometry in one hour, H. ruhlandii cultures only consumed about one-fourth their volume per hour. Occasional gas consumption rates by H. ruhlandii cultures were as high as two-thirds their volume per hour, but this could not be reproduced. Because of the wide divergence between gas uptake rates by H. eutropha and H. ruhlandii cultures, work with the latter was not carried to continuous culture; the considerably poorer gas uptake rate by H. ruhlandii rendered that organism unattractive for further consideration in life support systems.

Batch culture studies were also made with H. eutropha for evaluation of certain other properties that were not subsequently studied in continuous culture. The tolerance of biological systems contemplated for use in space vehicles to human waste products is an important consideration from a number of points of view. The possible utilization of human wastes as primary or secondary nutrients would minimize requirements for expendable supplies and for reprocessing equipment. The possibility that a dual function could be performed, such as atmosphere control and waste degradation, would minimize weight requirements for waste management. Finally, the possibility of contamination in such a confined environment clearly exists. Accordingly, H. eutropha was cultured in urine, in urine supplemented by a fecal extract, and in a fecal extract. Growth occured in media containing urine but not in the fecal extract alone. Gas uptake in the urine media was approximately one-third that in conventional medium. Growth in urine media can be attributed to the presence of most nutritional requirements including urea in the urine. It is probable that the major limitation on growth was ferrous ion. The inability of the fecal extract to support growth parallels results indicating that feces is a poor nutrient for algae because it lacks an assimilable nitrogen source (ref 5). That severe inhibition did not occur in the presence of the fecal extract is indicative of the tolerance of H. eutropha to hot-water soluble fecal components.

Tolerance to its own excretion products is another capacity sought for microorganisms in closed systems. The re-use of culture medium is desirable to minimize processing requirements and to maximize efficiency of utilizing medium components. Batch culture experiments made with spent H. eutropha medium from which cells had been removed by centrifugation showed that reconstitution of nitrogen and ferrous ion content was required. Without reconstitution, the spent medium would not support growth. However, successful re-use of the medium twice after adding ammonium chloride and ferrous ammonium sulfate indicated that soluble metabolites are not severely inhibitory.

Because of the potential for formation of carbon monoxide that exists in closed systems containing electrical equipment, and because a hydrogenomonad has been reported to utilize carbon monoxide as an oxidizable substrate, the effect of carbon monoxide on H. eutropha cultures was examined. Both 1% carbon monoxide and 5% carbon monoxide inhibited growth severely, however. Because utilization of carbon monoxide by the hydrogenomonad occurs only in the absence of other oxidizable substrates, and because of the inhibition observed, this line of study was not pursued.

Continuous Culture Experiments

Gas uptake and stoichiometry of gases consumed were the major concern of continuous culture studies. These parameters are most critical to the sizing and power requirements of a device for use in space vehicles.

Initial continuous culture studies were made in apparatus designed after devices in the literature and modified for feeding of the oxygen-hydrogen-carbon dioxide gas mixture. Although steady-state conditions could be estimated in this culture vessel, gas consumption was less than half that observed in batch culture. Even the use of nonlimiting ammonium chloride concentrations did not show improvement. Further, the stoichiometry of $H_2/O_2/CO_2$ consumed was approximately 8/3/1, indicative of an old culture. Both the feed rate, giving a retention time in the culture (and therefore generation time) of approximately 45 hours and microscopic examination of the cells characterized the culture as an old one. Note that generation time in batch cultures is 3.5 - 4.0 hours.

Estimation of the ratio of gas-liquid interfacial area to culture volume in the continuous culture apparatus and the ratio for batch culture devices suggested that the limitation on gas uptake rates was mass transfer of substrate from the gas phase to the liquid phase.

Confirmation of this limitation was gained by packing the continuous culture vessel to increase gas retention volume. A significant improvement in gas uptake resulted although stoichiometry of $H_2/O_2/CO_2$ consumption remained at approximately 8/3/1.

A different continuous culture vessel was designed to provide improved mass transfer of gaseous substrate. Initial studies still gave poorer gas uptake rates than obtained in batch culture, although feed rates corresponding to a generation time of 21 hours were possible. As in earlier continuous culture experiments, adjustment of ammonium chloride concentration in the nutrient feed had no effect on the culture. A recent report (ref 6) that the optimum oxygen tension for H. eutropha growth was not 20% but nearer to 10 - 12% (ref 6) prompted use of lower oxygen tension in the continuous culture experiments. Indeed, the use of 15% oxygen tension resulted in gas uptake rates comparable with those obtained in batch culture: approximately three volumes of gas per hour by one volume of culture. Stoichiometry of $H_2/O_2/CO_2$ consumed, however, was approximately 10/3/1, indicative of relatively inefficient metabolism. Attempts to improve on this stoichiometry by decreasing generation time (increasing feed rate) were unsuccessful, the culture washing out at a retention time of 8.5 hours.

The manifold implications of these data center on the limitation which mass transfer of gaseous substrates exerts on growth of the hydrogenomonads. Particularly in the light of results reported by Bongers, (ref 8) that gas consumption rates of 30 volumes per hour by one volume of culture could be attained with violent agitation of H. eutropha cultures, it appears that all reported work with the hydrogenomonads has the prospect of substantial improvement in gas uptake rates by this organism. Further, growth of the organism under conditions such

that supply of gaseous substrates is not limiting, may require redefinition of many parameters previously reported.

Integration of H. eutropha Culture and Water Electrolysis

Successful combination of biological functions and electrochemical functions in the generation of power encouraged evaluation of a similar combination of functions in the present study. Many potential advantages can be seen for such a cambination. Significant potential for weight economy lies in eliminating equipment required for transport (and possibly intermediate storage) of hydrogen from electrolysis cell to culture vessel. At the same time, hazards involved in handling hydrogen would be minimized. A further potential weight economy may result from integration because of smaller equipment requirements for water purification: Possible direct utilization of urine, for example, in the culture medium, which serves as catholyte, would eliminate the part of the requirement for water purification equipment for supply of water to the cathode compartment.

Another significant potential advantage for integration exists because the hydrogen resulting from electrolysis is produced in the solution where it is to be used. Thus, the design problems associated with obtaining mass transport of hydrogen from the gas phase to the liquid phase would be minimized. Obviously, mass transport of oxygen and carbon dioxide would still be necessary; a smaller quantity of gas for a given increment of growth would have to be transported, however.

The ability of bacteria in the vicinity of an electrode to remove products of the electrode reaction such as hydrogen should decrease the polarization of the electrode (polarization is the deviation from the theoretical electrode potential at which the electrode operates in practice while passing current). Because polarization is a loss in efficiency, any effect of decreasing polarization should result in improved efficiency of the electrode.

Finally, the possibility that reduction of some intermediate other that water involved in the bacterial reaction process may serve to change the potential at which the electrode operates to a value that results in more efficient utilization of power. The direct transfer of electrons from an electrode to the enzyme system of a bacterium would be such a phenomenon.

Results of bioelectrochemical experiments show the feasibility of supplying hydrogen for H. eutropha culture by maintaining a cathodically polarized electrode in the culture medium. The culture was not able to consume all the hydrogen produced by the electrode when the bacterial population was small. This can be contrasted with the complete consumption of cathodically produced hydrogen by higher concentrations of resting cell

suspensions. The clear implication is that the hydrogen need not be discretely evolved for its consumption by the bacteria.

Results of experiments to immobilize H. euthopha cells on an electrode were clearly not definitive. There is considerable question whether immobilization of the bacteria is even desirable in the light of new reports on oxygen limitations in H. eutropha growth.(ref 6) Immobilization of H. eutropha was originally predicated on reports that hydrogen availability limited growth and that immobilization of the bacteria at the hydrogen-producing electrode would place the organisms in the most favorable physical situation for maximum exposure to hydrogen.

The low conductivity of medium for growth of H. eutropha, and the sensitivity of H. eutropha to increased ionic strength in the medium indicates that some expedient is required to avoid loss in gas uptake rate by the culture while minimizing losses in efficiency due to the resistance of the medium. One approach taken was the isolation of a marine hydrogenomonad. Tolerance of such an organism to a medium as conductive as seawater would significantly improve on such efficiency losses. Only marginal success was obtained in this isolation, however. Further, marine species generally exhibit significantly poorer rates of growth and metabolism than their fresh water counterparts.

The means seen for minimizing the limitations exerted by culture medium conductivity and ionic content, is to avoid use of the culture as the conductive medium for the electrolysis cell. Techniques are available for providing a highly conductive electrolytic path in an electrochemical cell in the presence of fluids with low conductivity. Thus an ion exchange membrane can be used for the cell electrolyte. Maintenance of cell electrodes in close proximity to the membrane electrolyte provides a cell with low internal resistance. An ion exchange membrane is attractive for a number of reasons: High electrolytic conductivity combined with use of thin section allows construction of cells with low internal resistance. Highly mobile ions such as H^+ or OH^- can be used in the membrane and thus as the carriers of electrolytic current in the membrane. Identity of an ionic electrode reaction product and the ion carrying the electrolytic current minimizes build-up of an electrode reaction product in the vicinity of the electrode and thus minimizes concentration polarization due to that component. In addition, changes in conditions in the bulk of the solution would be minimized. Exchange of ions from a solution poor in hydroxyl ions with an anion exchange resin in the hydroxyl form in such a cell would be minimized so long as polarization of the cell maintained a supply of hydroxyl ions by way of the electrode reaction.

Applicability of Hydrogen-Oxidizing Bacteria to Bioregenerative Life Support Systems

Size and Power Requirements

Based on data derived in this study, the size and power requirements can be estimated for a bioregenerative life support system which employs water electrolysis for regeneration of oxygen from water, and which uses Hydrogenomonas eutropha for regeneration of foodstuffs from respired carbon dioxide and hydrogen produced in electrolysis of water. The consumption by one volume of culture of the three volumes of gas per hour with the composition of 6/2/1, $H_2/O_2/CO_2$, related to the approximate production by one man of 22 liters of carbon dioxide per hour leads to a culture volume requirement of 66 liters for support of one man. Production of sufficient hydrogen by electrolysis for the maintenance of bacterial growth would be accompanied by the production of 66 liters of oxygen, 44 of which would be consumed by the culture and 22 of which would be available for man's respiration. Because this is somewhat less than average requirements for respiration (the respiratory quotient is smaller than 1.0), and because other sources of carbon dioxide will be available, such as from waste management, a somewhat larger culture volume, on the order of 75 liters (2.6 cu. ft.) would actually be required for support of one man. This culture volume may be compared with estimates of 28-100 liters (1-3.5 cu. ft.) of algae culture for a photosynthetic gas exchanger.

Based on an estimated power requirement of 0.25 kw for the supply by electrolysis of one man's oxygen requirement, the power requirement for electrolysis to supply both man and enough H. eutropha culture for regeneration of his respired carbon dioxide would be 0.75 kw for the 6/2/1 stoichiometry of $H_2/O_2/CO_2$ metabolism. Power for illumination of an efficient photosynthetic gas exchanger has been estimated at up to ten times this value. Power for operation of auxiliaries - pumps, control equipment, etc., would probably be small compared to the power required for electrolysis. The difference between the power required for oxygen supply alone and the total power requirement as estimated above - 0.5 kw per man based on available data - can be considered the power cost of closing the food loop for a closed system.

Prospects for bioregenerative systems using H. eutropha for regeneration of foodstuffs from respired carbon dioxide are that considerable improvement over the estimated culture volume of 75 liters for maintenance of one man can be realized. Based only on gas uptake rates reported by Bongers (ref 6) a culture volume of 7.5 liters may be adequate for the support of one man. Such a volume would be considerably less than can be contemplated for photosynthetic systems. Similarly, improvement in mass transfer for H. eutropha cultures may also provide significant power improvements over those estimated from present data. Thus operation of the culture with $H_2/O_2/CO_2$ metabolism in the proportions 3/0.5/1 would place the power requirement (for electrolysis) at 0.37 kw for the support of one man.

Data obtained to date do not provide information to estimate specifically what the power and volume requirements would be for integrating electrolysis and bacterial culture. It is reasonably certain that these would be no greater than for the isolated subsystems. The potential of the bioelectrochemical approach is that interactions of the bacteria with the hydrogen-producing electrode will actually lower requirements compared to those for the isolated subsystems and that this interaction may also allow the utilization of smaller volumes (and therefore weights) than are required by the sum of isolated subsystems.

Suitability of H. eutropha for Human Nutrition

Estimates of the suitability of H. eutropha cell material in human nutrition must be made in the consideration of H. eutropha for a bioregenerative life support system. Based on the 75 liters of culture for regeneration of one man's carbon dioxide, on the 20 hour rentention time found in the best continuous culture data, and on the protein content of such a culture of 1.04 g/liter, one would expect approximately 91 g bacterial protein production per day. One analysis of cell material indicated that these were 52% protein, so that total cell yield would be 175 g per day. While H. eutropha cell composition has not been established, extensive analysis of Hydrogenomonas facilis cells, a closely related organism, has been reported (ref 7) These contain all of the essential amino acids except for methionine. Less than 1% lipid was reported, although 5 - 10% would normally be expected for this type of organism. The low lipid analysis may be an artifact of the method used, which did not include an acid hydrolysis to release protein-bound lipids. Using 5% as a reasonable value, the lipid yield of 75 liters of culture may be assumed to be 8.8 g/day. Assuming further that the remainder of cell material, excepting 2% as ash, is carbohydrate, the daily yield of carbohydrate would be 71.7 g. According to one source, (ref 8) daily requirements for protein, lipids and carbohydrate are respectively 65 g, 81 g, and 550 g. Thus, the daily yield of protein from H. eutropha culture would be more than adequate by 36 g. Cell yields would be severly inadequate to provide lipid and carbohydrate requirements, however, only 11% of the lipids and 13% of the carbohydrate being available.

Comparison of the quantity of carbon dioxide reacting per day, 956 g, and the 175 g of cell material produced indicates a significant discrepancy. Theoretically, 956 g of CO_2 should result in 720 g of carbohydrate. However, the reaction used to represent metabolism, equation (1), is at best

$$6\ H_2 + 2\ O_2 + CO_2 \longrightarrow (CH_2O) + 5\ H_2O \qquad (1)$$

an approximation. The whole of the carbon dioxide does not indeed appear as carbohydrate, some 52% being combined as protein in H. eutropha. Further products of

metabolism of the hydrogenomonads are fatty acids and low molecular weight aliphatic acids. The latter do not appear as cell yield but are dissolved in the medium.

CONCLUSIONS

The feasibility of using a culture of the bacterium Hydrogenomonas eutropha in a bioregenerative life support cycle has been demonstrated. These bacteria are able to convert hydrogen, oxygen, and carbon dioxide to potential foodstuffs. The ratio of H_2 to CO_2 to O_2 consumed varied from 10/3/1 to 6/1.5/1 under different conditions.

Present data indicate that a volume of 75 liters of culture would be required to support one man. Considerably smaller volumes can be expected from additional effort to increase efficiency of mass transport between the gas and liquid phases.

The power required by the bioregenerative process is estimated to be 0.75 kw per man, which is 0.5 kw per man greater than that required to supply oxygen by electrolysis of water in an open system. The power requirement is significantly smaller than that for a photosynthetic gas exchange process.

The cell material generated in the culture is rich in proteins and poor in carbohydrates and lipids for direct human consumption. A number of approaches to providing a better balanced material can be suggested.

The culture of H. eutropha is tolerant to urine and to a hot-water extract of human feces. Thus these waste products are promising sources of nutrient for the bacteria.

Hydrogen was successfully supplied to a culture of H. eutropha from the cathode of a cell operating to electrolyze water directly in the culture medium. Attempts to grow the bacteria on the electrode surface were unsuccessful. No attempt was made to optimize the weight, volume, or power of this combination as compared to a separate electrolytic generator for hydrogen and oxygen.

TECHNICAL RECOMMENDATIONS

Based on the results of the work described herein, the following additional work is recommended to develop this approach:

a. Design studies should be carried out to maximize the rate of mass transfer between the gas mixture and the liquid culture. These studies would lead to the minimum volume of culture required.

b. Methods for producing microbial cell material with a higher ratio of carbohydrate-to-protein content should be investigated.

c. The extent to which human urine and feces can be used to supplement the input to cultural growth of H. eutropha should be quantitatively determined.

d. The quantitative comparison of cell growth from hydrogen generated in situ with that from an external source should be made. Also, the effect of carrying out electrolysis in culture on the growth pattern should be investigated.

APPENDIX I

LITERATURE SEARCH

Hydrogenomonas spp.

These organisms are facultative autotrophs whose metabolism is characterized by the aerobic oxidation of molecular hydrogen with CO_2 serving as the sole source of carbon. Members of the genus Hydrogenomonas can also utilize organic compounds such as lactate, glucose, and succinate in their aerobic heterotrophic metabolism.

It was discovered by Kluyver and Manten (ref 9) that the oxidation of molecular hydrogen by molecular oxygen proceeds in the presence of organic matter, demonstrating that two separate enzymatic pathways are operative.

Resting cell suspensions of Hydrogenomonas flava were used to demonstrate the simultaneous oxidation of lactate and hydrogen. This study was later corroborated by Wilson, et al. (ref 10) who showed that lactate disappears from the medium concurrently with oxidation of molecular hydrogen. Washed cell suspensions of H. facilis were employed.

Kluyver and Manten (ref 9) reported that heterotrophically grown cells of H. flava did not possess the ability to respire autotrophically, while autotrophically grown cells could respire autotrophically, but at a reduced rate compared to the autotrophants. They concluded that hydrogenase is, therefore, a constitutive enzyme. This conclusion, however, was disputed by several later studies with several species of hydrogen bacteria. (refs 11,12) These workers showed that heterotrophically grown cells demonstrated little or no hydrogenase activity. Similarly, Wilson et al (ref 10) found that hydrogenase activity developed in H. facilis when the heterotrophically grown cells were cultured under reduced oxygen partial pressure (5% O_2 + 5% CO_2 + 90% N_2). Linday and Syrett (ref 13) reported similar results and concluded that hydrogenase was an adaptive enzyme.

Two distinct groups of Hydrogenomonas spp. are recognized: those which are capable of autotrophic growth under high oxygen tensions (over 15%), and those species which require oxygen tensions between 2-8%. H. pantotropha, H. facilis, H. eutropha, and H. carboxydovorans belong to the former group while H. vitrea, H. flava, and H. ruhlandii occupy the latter class. Schatz and Bovell (ref 14) reported that H. facilis can grow "autotrophically in traces of oxygen up to 30% but not in 40% oxygen," although they routinely grew the organism with 8% oxygen on solid media. Subsequent investigators who have cultivated this organism on solid media have used less than 10% oxygen. Cohen and Burris (ref 15) used from 22 to 30% oxygen and Schlegel (ref 16) reported that optimal growth of H. facilis occurred with20 to 30% oxygen. A summary of gas mixtures employed by various investigators is presented in Table XVIII.

Schatz (ref 17), using a special Warburg technique, was one of the first workers to examine the simultaneous uptake of hydrogen, oxygen, and carbon dioxide by resting cells of H. facilis. He found that this organism consumed these three gases in the ratio of 6 H_2:2 O_2:CO_2 and assumed the following reactions:

TABLE XVIII

Summary of Hydrogenomonas Research in the Literature

Investigator(s)	Species	Gas Mixture	Mole Ratio H_2	Mole Ratio O_2	Mole Ratio CO_2	Rate of Gas Utilization
Schatz and Bovell (ref. 14)	H. facilis resting	25% air, 75% H_2				Q_{H_2} = 400-1100 per mg dry wt of cells/hr
	H. facilis growing	60% H_2, 30% air, 10% CO_2				
Wilson et al. (ref. 10)	H. facilis growing	50% H_2, 40% air, 10% CO_2				
Schatz (ref. 17)	H. facilis resting	89% H_2, 10% air, 1% CO_2	6.0	2.0	1.0	Q_{H_2} = 1784; Q_{O_2} = 592; Q_{CO_2} = 288 per mg dry wt of cells/hr
Marino and Clifton (ref.19)	H. facilis resting	96% H_2, 2% O_2, 2% CO_2	6.4	1.9	1	
	H. facilis growing	82% H_2, 6% O_2, 12% CO_2	5.8	1.8	1	17.2 ml H_2, 5.4 ml O_2 and 3.0 ml CO_2 per 50 ml culture in 24 hours
Cohen and Burris (ref. 15)	H. facilis growing	60% H_2, 30% O_2, 10% CO_2				

TABLE XVIII (cont'd)

Summary of Hydrogenomonas Research in the Literature

Investigator(s)	Species	Gas Mixture	Mole Ratio H_2	Mole Ratio O_2	Mole Ratio CO_2	Rate of Gas Utilization
Packer and Vishniac (ref. 12)	H. ruhlandii	91% H_2, 8% O_2, 1% CO_2	5.5	1.7	1	355 μl total gases per mg weight of cells/hr
Repaske[2]	H. eutropha growing	70% H_2, 20% O_2, 10% CO_2				1 liter total gases 100 ml culture/24 hours
Chapman and Meyer (ref 20)	H. facilis growing	70% H_2, 20% O_2, 10% CO_2	6.0	2.0	1.0	

$$4\,H_2 + 2\,O_2 \longrightarrow 4\,H_2O$$
$$2\,H_2 + CO_2 \longrightarrow H_2O + (CH_2O)$$
$$6\,H_2 + 2\,O_2 + CO_2 \longrightarrow (CH_2O) + 5\,H_2O \text{ (over-all)}$$

Marino, [ref 18] working with the same organism, corroborated the work of Shatz and found that autotrophically-grown H. facilis suspensions utilized hydrogen, oxygen and carbon dioxide in the ratios of 6:2:1 for two-day cells and 8:3:1 for three-day or older cells. Studies with actively proliferating cells gave results which were almost identical to the resting cell values. This demonstrated the similarity of efficiency between resting and growing cells with respect to reduction of carbon dioxide. Packer and Vishniac [ref 12] studied the consumption of hydrogen, oxygen, and carbon dioxide by resting cells of H. ruhlandii and obtained a remarkably efficient reaction for the chemosynthetic assimilation of carbon dioxide under an atmosphere of 5 to 9% oxygen and 91 to 95% hydrogen according to the following equations:

$$2\,H_2 + O_2 \longrightarrow 2\,H_2O$$
$$CO_2 + 2\,H_2 \longrightarrow (CH_2O) + H_2O$$
$$CO_2 + O_2 + 4\,H_2 \longrightarrow (CH_2O) + 3\,H_2O \text{ (over-all)}$$

The results obtained by various workers on the simultaneous consumption of hydrogen, oxygen and carbon dioxide are summarized in Table XVIII.

Rate studies performed with growing cultures of the hydrogenomonads are practically nonexistent in the literature. Marino, [ref 16] using a specially designed "macrorespirometer," obtained gas uptake rates of 25.9 ml H_2, 6.9 ml O_2 and 5.3 ml CO_2 per 50 ml of culture (H. facilis) between the 26th and 50th hours of growth. Repaske [ref 2] reported that, under optimal autotrophic conditions, a 100 ml culture of H. eutropha consumed more than 1 liter of a gas mixture of 70% hydrogen, 20% oxygen and 10% carbon dioxide in 24 hours. Stoichiometric data were not presented.

Rapid autotrophic growth of several species of Hydrogenomonas was achieved with the discovery of Cohen and Burris [ref 15] that H. facilis grew better when the gas pressure in a closed system was maintained at atmospheric pressure. By employing a water displacement system to maintain constant gas pressure, approximately 2 grams/liter wet weight of packed cells was obtained in a 16-18 hour growth period. By supplementing the medium with trace elements, growth of H. facilis was greatly accelerated. Repaske [ref 2] confirmed the observation that a partial vacuum limited growth with another hydrogenomonad, H. eutropha. An extensive cultural and

nutritional study revealed that the maximal growth rate occurred between 30° and 35°C at a pH of 6.4 to 6.7. Ammonium ion, urea, or nitrate ion served as nitrogen sources; nitrate was not utilized. Growth was found to be dependent upon iron, particularly ferrous ion, which has been shown to be a cofactor for hydrogenase.

Photosynthetic Bacteria

The photosynthetic bacteria are divided into three families, Thiorhodaceae (purple sulfur bacteria), Athiorhodaceae (purple nonsulfur bacteria), and Chlorobacteriaceae (green sulfur bacteria). These bacteria are characterized by their ability to utilize light energy without the evolution of oxygen. A summary of the outstanding characteristics of these bacteria is presented in Table XIX.

Roelofsen[ref 21] was the first to report that the Thiorhodaceae could grow photoautotrophically with molecular hydrogen. One year later, Gaffron[ref 22] reported a similar occurrence with the Athiorhodaceae and finally, Larsen[ref 23] found that two species of Chlorobacteriaceae utilized hydrogen photoautotrophically. Van Niel[ref 24] found it was necessary to add small amounts of yeast extract to the medium in order to grow certain nonsulfur purple bacteria. This requirement for growth factors casts some doubt on the autotrophic nature of these bacteria. In general, the photoautotrophic reaction is carried out according to the following equation:

$$2\,H_2 + CO_2 \xrightarrow{\text{light}} (CH_2O) + H_2O$$

Studies on photoautotrophic metabolism with molecular hydrogen have been very limited and primarily restricted to resting cell suspensions. Wassink,[ref 25] working with resting cells of a Chromatium sp., found that the average ratio of the amount of hydrogen and carbon dioxide consumed was $H_2:CO_2 = (2.13 \pm 0.10):1$. Larsen[ref 25] studied the behavior of resting cells of Chlorobium thiosulfatophilum and Chlorobium limicola with respect to their photoautotrophic metabolism of molecular hydrogen and carbon dioxide and found approximately two moles of hydrogen were assimilated for each mole of carbon dioxide. A further study of Larsen et al.[ref 26] with resting cell suspensions of these same two organisms showed rates which corresponded to the consumption of approximately 5-10 ml of hydrogen and 2-5 ml of carbon dioxide per liter of suspension at the highest light intensity investigated.

Ormerod and Gest[ref 27] reported the "unambiguous growth of Rhodospirllum rubrum (SI) on hydrogen and carbon dioxide in a completely synthetic medium, "but admitted growth was erratic and not optimal. The rate of growth in a heterotrophic medium was approximately ten times that of the autotrophic culture. Studies regarding the cultural and physiological characteristics of the photosynthetic bacteria have

TABLE XIX

General Characteristics of Photosynthetic Bacteria

	Green Sulfur Bacteria[1]	Purple Sulfur Bacteria[2]	Purple Nonsulfur Bacteria[3]
Source of reducing power	Sulfide; reduced inorganic sulfur compounds; hydrogen	Inorganic sulfur compounds; organic acids; hydrogen	Organic compounds; inorganic sulfur compounds; hydrogen
Principal source of carbon	Carbon dioxide	Organic compounds; carbon dioxide	Organic compounds; carbon dioxide
Relationship to oxygen	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic or facultatively anaerobic
Growth in dark	None	None	Aerobic on substrates used in light
Requirement for trace organic growth factors	None	None	One or more vitamins

1. e.g., Chlorobium spp.
2. e.g., Chromatium spp.
3. e.g., Rhodospirillum spp.

not been particularly concerned with the photoautotrophic oxidation of molecular hydrogen. Van Niel[ref 28] and Larsen[ref 23] have contributed the majority of available information.

In general, the nutrient requirements of the photosynthetic bacteria are similar to other autotrophic bacteria with some notable exceptions. Rhodospirillum rubrum requires biotin for growth. Ormerod et al[ref 1] reported that the SI strain of this organism grew better in deionized water rather than distilled water, which they attributed to a sensitivity of the bacteria to copper. Chlorobium spp. have been shown by Larsen[ref 23] to grow well only in the presence of iron (salts) which he believes is a cofactor in the synthesis of the bacteriochlorophyll of the green sulfur bacteria. He also observed that the addition of trace elements was necessary for optimal growth. Photosynthetic bacteria absorb light at somewhat longer wave lengths than green plants, therefore, incandescent lamps are usually employed as a light source. The composition of the gas employed for growth of the photoautotrophic bacteria has varied according to the particular investigator but generally consists of 2 to 10% carbon dioxide in hydrogen.

Methane Bacteria

The methane bacteria are a specialized physiological group of anaerobes which, according to the classification of Barker[ref 29] compose the family Methanobacteriaceae. Four species have been demonstrated to produce methane autotrophically from molecular hydrogen and carbon dioxide: Methanobacterium omelianskii (Barker)[ref 30] Methanobacterium barkerii (Schnellen,)[ref 31] Methanobacterium formicicum (Kluyver and Schnellen)[ref 32] and Methanoccoccus vannielii (Stadtman and Barker).[ref 33] This reaction proceeds according to the following equation:

$$4\,H_2 + CO_2 \longrightarrow CH_4 + 2\,H_2O$$

All species are facultative. However, heterotrophic metabolism is restricted to a narrow range of organic compounds: straight chain fatty acids and primary and secondary alcohols having up to six carbons. Because great difficulty in maintaining these organisms in pure culture has been observed, only one species Methanobacterium omelianskii is reported to have been kept in pure culture for prolonged periods.

The rates of metabolism of carbon dioxide and hydrogen by the autotrophic species of the methane bacteria have been studied to a limited degree (Barker,[ref 34] Schnellen,[ref 31] Stadtman and Barker[ref 33]), however, attention has been devoted largely to resting cell suspensions. Kluyver and Schnellen[ref 32] observed that resting cells of this same organism utilized from 5 to 7 ml hydrogen/hour/mg cell nitrogen at high carbon dioxide tensions.

Nutrition of those autotrophic species studied in pure culture is not complex. Most species have an optimum temperature range between 30-40°C. Some thermophiles exist, in addition.

Clostridium aceticum

Clostridium aceticum assimilates carbon dioxide for growth, utilizing molecular hydrogen as the sole energy source according to the following equation:

$$4\ H_2 + 2CO_2 \longrightarrow CH_3COOH + 2\ H_2O$$

Although carbon dioxide apparently serves as the major source of cell carbon, this organism requires complex organic factors for growth.[ref 35] Therefore, this species cannot be considered an autotroph in the usual sense of the term. Karlsson et al [ref 36] observed that Cl. aceticum readily converts hydrogen and carbon dioxide to acetate in a complex medium containing yeast extract and malt extract. Several, but not all, the growth factors have been identified; these include biotin, pyridoxamine, and pantothenic acid.

No report of this organism has appeared in the literature since 1948 and it appears that Cl. aceticum has been lost.

Algae

Several algal species have been shown capable of utilizing molecular hydrogen. [refs 37,38,39] Their normal metabolism is the photosynthetic production of carbohydrate and oxygen from carbon dioxide and water. Special adaptive techniques have been used. Gaffron[ref 40] reported a species of Scenedesmus and Rhaphodium polymorphium have simultaneously metabolized carbon dioxide, hydrogen and oxygen in the dark in the approximate proportions 1:10:3. Other algal species have been adapted to the anaerobic photosynthetic or chemosynthetic metabolism of carbon dioxide and hydrogen, thus producing carbohydrates and water. Special procedures must be used to prevent the loss of this adapted ability. Gaffron[ref 42] found that the aerobic consumption of hydrogen and carbon dioxide by growing cultures of several algal species, resulted in the assimilation of these gases at the respective rates of 0.5 and 0.05 liter per day by one liter of culture.

APPENDIX II

MATERIALS AND METHODS, SCREENING PROGRAM

Organisms and Growth Media

Hydrogenomonas facilis (ATCC 11228)

Growth of H. facilis in an autotrophic medium has been obtained. The liquid phase is made up with 94 ml of buffer solution, 2 ml of solution B and 1 ml each of solutions A, C, D, and E, to make 100 ml of medium. Each of these solutions is made up with the following components diluted to 100 ml with distilled water and sterilized, solutions B and D by filtration.

Buffer, pH 6.3
- 7.75 ml 0.25 M KH_2PO_4
- 2.25 ml 0.25 M K_2HPO_4

Solution A

$CaCl_2 \cdot 2H_2O$	1.0 g
NH_4Cl	10.0
NaCl	1.0

Solution B

$NaHCO_3$	5.0 g

Solution C

$MgSO_4 \cdot 7H_2O$	1.0 g

Solution D

$Fe_2(NH_4)_2SO_4$	0.08 g

Solution E (trace minerals solution)

$CoCl_2 \cdot 6H_2O$	0.2 mg
$MnCl_2 \cdot 4H_2O$	400
$CuSO_4 \cdot 5H_2O$	2.0
$NaMoO_4 \cdot 2H_2O$	10.0
$ZnSO_4 \cdot 7H_2O$	10.0

The gas phase used was 50-40-10, H_2-Air-CO_2

Hydrogenomonas eutropha

A culture of H. eutropha was received from Dr. R. Repaske, U. S. Public Health Service, Bethesda, Md. Ready autotrophic growth of the organism has been obtained in the medium described under H. facilis. The gas phase used

was 50-40-10, H_2-Air-CO_2. Growth was significantly better than observed with H. facilis. Good growth has also been obtained with a gas phase of 70-20-10, H_2-O_2-CO_2.

Hydrogenomonas ruhlandii

This organism was obtained from Dr. L. Packer, Univ. of California, Berkeley, Calif. H. ruhlandii has been grown autotrophically on the medium described under H. facilis, with a gas mixture of 82-8-10, H_2-O_2-CO_2.

Other Hydrogenomonas sp.

Hydrogenomonas sp. H-10

Agar slants of this organism were obtained from Dr. C. R. Bovell, University of California, Riverside, California. Preliminary growth was approximately the same as observed with H. eutropha - one to two days for good turbidity (visual observation) with the previously described conditions for H. eutropha.

Hydrogenomonas sp. H-20

Agar slants of this organism, obtained from Dr. N. Goodman, University of Southern California, grew out essentially as well as H. eutropha in preliminary evaluation, and were cultured in the same manner as H. ruhlandii.

Chlorobium limicola (Larsen Strain YG2A)

A lyophilized culture of Chlorobium limicola was grown readily in the light in autotrophic medium containing sulfide as the reductant. Attempts to grow this organism in autotrophic medium with hydrogen as the reductant have not been successful, despite literature citations to the contrary. Attempts to activate the hydrogenase in these organisms by growing them in a medium containing sulfide under a gas phase of hydrogen and carbon dioxide were unsuccessful. Moreover, only insignificant growth was observed when the hydrogen-carbon dioxide atmosphere was present. Both incandescent and fluorescent light sources were used, as well as several modifications of the media. Good growth was obtained in the following medium[ref 41] under fluorescent lights (liquid-full tubes).

NH_4Cl	1.0 g
KH_2PO_4	1.0
$MgCl_2 \cdot 6H_2O$	1.06
Tap Water	700 ml

Sterilize 15 min at 121°C, then prepare the following, filter-sterilize, and combine with the solution above:

$NaHCO_3$	2.0 g; 100 ml tap water
$Na_2S \cdot 9H_2O$	1.0 g; 100 ml tap water
$FeCl_3 \cdot 6H_2O$	5.0 mg; 100 ml tap water

Adjust final pH to 7.3 with phosphoric acid

Improved growth could be obtained by storing the medium overnight in a refrigerator and adding 1 drop of 10% sodium sulfide solution per 10 ml of medium just before inoculation.[ref 42]

Insignificant or no growth was obtained using the foregoing medium and a gas phase of 98% hydrogen and 2% carbon dioxide, or in the following medium[ref 19] with the same gas mixture:

NH_4Cl	1.0 g
KH_2PO_4	1.0
$Na_2S \cdot 9H_2O$	0.1
$MgCl_2$	0.5
$NaHCO_3$	2.0
Tap Water	to make 1 liter
Final pH	7.3

The sodium sulfide and sodium bicarbonate were filter-sterilized and added to the cooled medium.

Variations of the Larsen medium were studied by adding (a) 5 mg $FeCl_3 \cdot 6H_2O$, (b) 0.2 g Sequestrene H2FE, (c) 0.2 g Sequestrene H2FE and 1 ml of the trace element solution (Solution E, Page 61), or (d) 1 ml 10% sodium sulfide per 10 ml of medium after refrigeration overnight. All these variations failed to provide growth under a hydrogen-carbon dioxide atmosphere.

Rhodospirillum rubrum (ATCC 11170)

Good growth of this organism was obtained with the following heterotrophic medium:

Malate	6 g
K_2HPO_4	900 mg
KH_2PO_4	600
$MgSO_4 \cdot 7H_2O$	200
$CaCl_2 \cdot 2H_2O$	75
$FeSO_4 \cdot 7H_2O$	11.8 (filter sterilized)
EDTA	20
Biotin	15 µg
Trace Elements*	1 ml
$(NH_4)_2SO_4$	500 mg
Deionized H_2O	1 liter
pH	6.8

Only limited growth of R. rubrum occurred on an autotrophic medium supplemented with trace organic growth factors under a hydrogen-carbon dioxide atmosphere. On subsequent subculture to the same medium, no growth occurred. Incandescent illumination was used. The medium used was as follows:

K_2HPO_4	0.1 g
NH_4Cl	0.1
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2$	0.01
Biotin	0.005 mg
Yeast extract	0.005 mg
Trace element solution	(solution E, under H. facilis)
Distilled water to make 1 liter	

To each 100 ml of this medium was added 0.4 ml of 5% sodium bicarbonate and 0.2 ml of 0.1% sodium sulfide, the latter solutions being sterililized by filtration.

Modifications were made by adding an additional 0.2 g of ammonium chloride or 0.5 g sodium sulfide per liter of the medium above. These modifications gave no better growth.

*Trace Element Solution

H_3BO_3	280 mg
$MnSO_4 \cdot 4H_2O$	210
$Na_2MoO_4 \cdot 2H_2O$	75
$ZnSO_4 \cdot 7H_2O$	24
$Cu(NO_3)_2 \cdot 3H_2O$	4
Deionized H_2O	100 ml

Chromatium sp. (NCIB-9425)

A Chromatium sp. of unspecified history was acquired from the National Collection of Industrial Bacteria, Scotland, but was obtained too late in the screening program to be examined culturally. No source was found for a Chromatium sp. known to metabolize H_2. This organism was not considered further.

Screening Criteria, Techniques

The major objective of the Screening Study was the final selection of organisms for further evaluation in the Experimental Program. The most important criteria for this selection are the rates and proportions with which the various organisms can consume hydrogen and carbon dioxide, and in the case of the hydrogenomonads, oxygen. Other criteria are the controllability of growth rates and the ability of the organisms to depolarize a cathodically polarized (hydrogen-producing) electrode. A minor consideration was the ability of these organisms to utilize organic substrates.

The lack of absolute standards and the advanced nature of the work at hand did not permit the establishment of minimum absolute performance levels to be sought in the screening program. Thus, selection of organisms was necessarily based on relative behavior.

Gas Uptake Rates and Proportions

Screening for gas uptake rates and proportions was accomplished by analyzing the gas phase maintained over growing cultures of organisms to be screened. Warburg techniques were employed; however, to be sure of having sufficient numbers of cells, flasks and associated apparatus of 80-150 ml volume were used rather than the 10-15 ml volume assemblies ordinarily used. The desirability of using even larger volumes was negated by the requirements for illumination, in the case of photosynthetic bacteria, and by the hazards involved in handling mixtures of hydrogen and oxygen, in the case of the hydrogenomonads.

Satisfactory separation of hydrogen, carbon dioxide, and oxygen mixtures has been obtained on a silica gel gas chromatography column. The smaller difference between the thermal conductivities of helium and hydrogen than the differences between that of helium and oxygen or carbon dioxide results in a much lower sensitivity of the analysis toward hydrogen. Further, the thermal conductivity behavior of hydrogen-helium mixtures imposes the

requirement of using relatively large gas samples, on the order of 3 ml. Table XX summarizes the conditions employed.

TABLE XX

Conditions for Analysis of Hydrogen-Carbon Dioxide-Oxygen Mixtures by Gas Chromatography: Screening Program

Instrument	Beckman GC-2
Column	30-60 mesh silica gel. 8 ft. x 1/4 in.
Inlet pressure	30 psig
Temperature	100°C
Detector filament current	250 ma
Sample size	3.0 ml
Recorder	Brown 1 mv, equipped with ball-and-disc integrator

Standard curves were prepared, plotting the chromatogram peak areas for hydrogen, carbon dioxide and oxygen as a function of their respective concentrations. Peak areas for both carbon dioxide and oxygen vary in a linear fashion with concentration in the 0-30% range. The peak area for hydrogen, again due to the abnormal thermal conductivity behavior of certain hydrogen-helium mixtures, is not linear in the 70-100% range studied but a satisfactory standard curve has been prepared. Sensitivity of the analytical method to hydrogen available from 3.0 ml samples containing less than 70% hydrogen is too poor to consider. Should quantitative analysis for lower concentrations of hydrogen be required, larger sample sizes would be necessary.

Precision and accuracy of this method should be in the order of $\pm$ 1.5% of the concentration determined.

Preparation of Gas Mixtures and Gas Atmosphere Alteration

The system for gas mixture production and alteration of atmospheres in Warburg flasks was essentially that described in Manometric Techniques.[ref 43] The entire apparatus is pictured in Figure 6. The gas bottle (E) was filled with 1% H_2SO_4 so that CO_2 would not be absorbed from the atmosphere. The reservoir bottle (D) was graduated in 50 ml increments, which allowed a careful measurement of each gas added to the gas bottle. As a rule, 3 liters of gas mixture were made, which meant that to produce a 70:20:10, H_2:O_2:CO_2 mixture, 300 ml

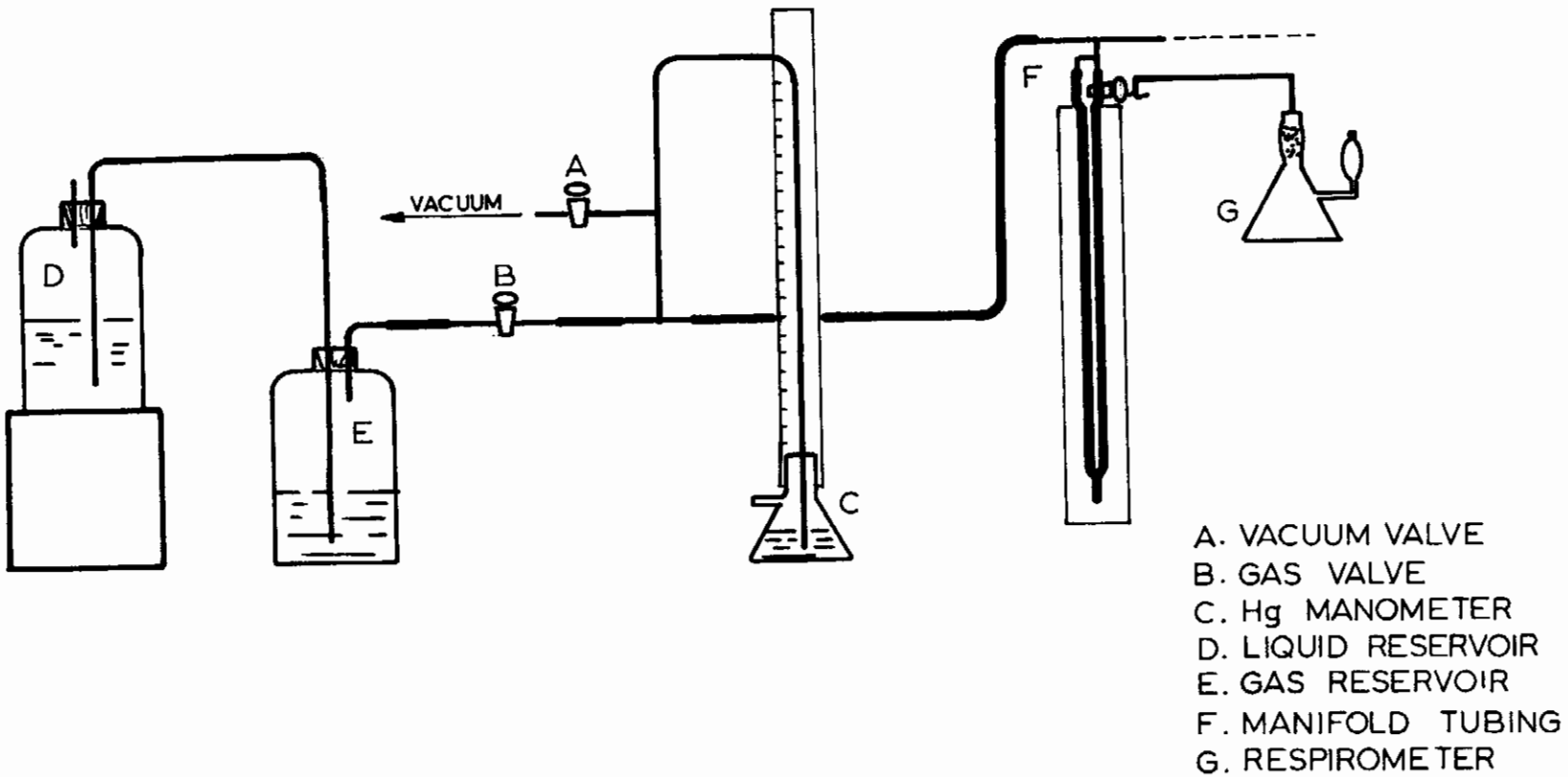


FIGURE 6

APPARATUS FOR PRODUCTION OF GAS MIXTURES AND ALTERATION OF ATMOSPHERES IN THE RESPIROMETER.

of acidified H_2O was displaced for CO_2, 600 ml displaced for O_2, and 2100 ml displaced for H_2, in this order to minimize the possibility of an explosion. While the gas mixture was equilibrating and being analyzed, the respirometers attached, as shown in Figure 6, were being flushed with pure H_2. This procedure involved the attachment of an H_2 line to valve B in place of the gas bottle. The evacuation and filling of the flasks with H_2 was determined by observation of the manometer C. Generally, flasks were evacuated to approximately 60 mm Hg between flushes. Subsequent to the H_2 flush, the gas bottle (E) was attached and the flushing procedure was followed as for H_2. The lack of gas pressure in E necessitated the elevated reservoir D to supply the pressure to flush the system. When the flasks had been flushed the gas atmosphere in each one was analyzed by gas chromatography.

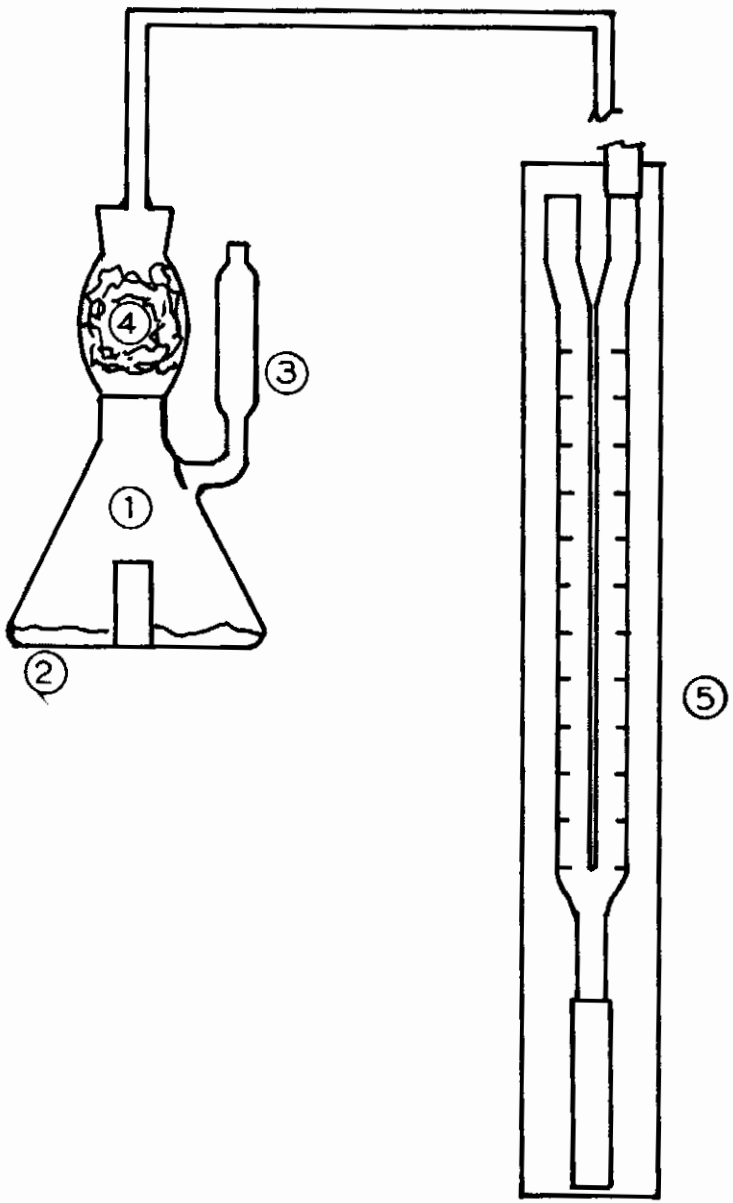
Microrespirometers

Microrespirometers of the Warburg type (Aminco), Figure 7, were made up with a 125 ml culture flask (1), a gas sampling sidearm (3), and a gas sterilizing adapter (4) according to Manometric Techniques.[ref 43] The units were calibrated by the mercury method of Schales, Manometric Techniques. The flask constants were calculated for fluid volumes of 10, 5, 2 and 1 ml.

Optical Density

At the end of experiments described below, samples of cell suspensions were checked for optical density at 660 mμ as a check on growth between replicate experiments.

MICRO - RESPIROMETER



1 WARBURG MICRO-RESPIROMETER FLASK
2 BACTERIAL CULTURE
3 GAS SAMPLING PORT
4 GAS STERILIZING ADAPTOR AND COTTON
5 MERCURY MANOMETER

FIGURE 7

APPENDIX III

MATERIALS AND METHODS, EXPERIMENTAL PROGRAM FOR MICROBIOLOGICAL TECHNIQUES

Growth Media

The media used for growth of H. eutropha and H. ruhlandii was the same as used in the Screening Study and is described under H. facilis.

Apparatus and Techniques

Gas Mixing and Flushing

Apparatus, as described in the Screening Program, was used for the preparation of gas mixtures and introduction of these mixtures into growth vessels.

Growth Vessels

Uncalibrated macro-respirometers with culture flasks of 500 ml volume were assembled. Figure 8 gives a schematic of one of these. The leveling bottle (8) allowed maintenance of atmospheric pressure. The sampling sidearm (4) allowed periodic removal of culture samples for protein analysis and optical density measurements.

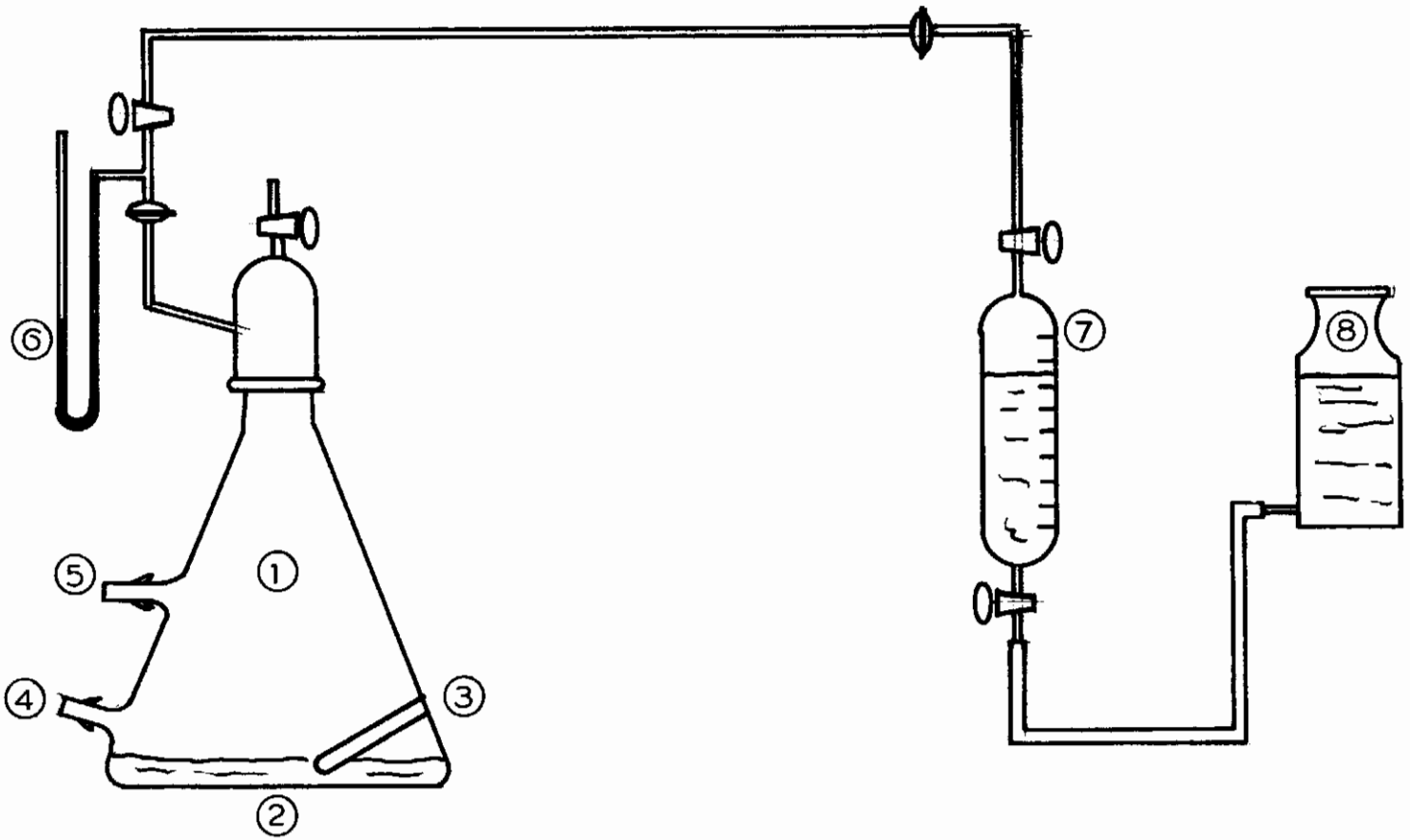
Optical Density

Optical density measurements were made on culture samples withdrawn from the growth vessel by diluting 1/3 with sterile medium and reading at 660 mμ in a spectrophometer.

Protein Analysis

The materials necessary for the determination are as follows:

a. Lowry B Solution:
   Na_2CO_3 20 gm/l
   NaK tartrate 0.2 gm/l
b. 1 N NaOH
c. 5% $CuSO_4$
d. Lowry C Solution:
   50 ml of Lowry B + 1 ml of 5% $CuSO_4$
e. Phenol reagent:
   5 ml of stock Folin phenol reagent added to
   7 ml of distilled water



MACRO-RESPIROMETER

FIGURE 8

1 CULTURE FLASK
2 BACTERIAL CULTURE
3 THERMOMETER WELL
4 GAS SAMPLING PORT
5 CULTURE SAMPLING PORT
6 MERCURY MANOMETER
7 500 ML MANOMETER
8 LEVELING BOTTLE AND ACIDIFIED H_2O

Procedure:

Materials added per tube:

a. 0.5 ml of diluted sample
b. 0.5 ml of 1 N NaOH and place in boiling water bath for 20 min.
c. Cool and then add 5.0 ml of Lowry C, wait 10 min.
d. 0.5 ml of phenol reagent
e. Incubate 30 min at ambient
f. Read at 660 mμ

Calculations:

a. Read μg protein from standard curves
b. $\frac{\mu g \times \text{dilution} \times 2}{1000} = \text{mg protein/ml}$

A standard curve for protein was prepared from data on analysis of a solution of 100 μg/ml bovine serum albumin at various dilutions.

Gas Analysis

The difficulties encountered with the gas chromatographic analysis for hydrogen, oxygen, and carbon dioxide during the Screening Study led to the selection of a new method. Beckman application engineers were consulted for advice and many helpful comments on technique were received. The method has been checked qualitatively and calibration curves with pure gas samples and carefully prepared standard gas mixtures were prepared. Table XXI gives pertinent data on this method. The columns used are arranged in series such that the sample to be analyzed is swept first through the silica gel column, where air and hydrogen are partially resolved from each other, and both gases resolved from carbon dioxide. The sample is then swept through one side of the detector cell and then through the molecular sieve column where hydrogen, oxygen and nitrogen are completely resolved. The output of the molecular sieve column feeds into the reference side of the detector. Separate peaks are obtained for hydrogen, oxygen, nitrogen, and carbon dioxide.

Increased detector filament current over that used in the earlier method makes this method more sensitive. Use of smaller sample size, 0.2 ml rather than 3.0 ml, obviates to a large extent the difficulty associated with decreasing pressure in growth vessels. From a 150 ml volume in growth vessels, only 0.13% is removed for analysis rather than 2%. Further, hydrogen sensitivity, while low compared with that toward the other gases, has not been severely affected by use of smaller samples. This is due to the anomalous thermal conductivity of hydrogen-helium mixtures. The concentration of hydrogen in helium resulting in this analysis remains below that at which inversion of thermal conductivity occurs.

TABLE XXI

Conditions for Analysis of Hydrogen, Oxygen, Nitrogen, and Carbon Dioxide Mixtures by Gas Chromatography

Instrument	Beckman GC-2, equipped with thermal conductivity detector
Columns	30-60 mesh Silica gel, 2 ft x 1/4 in. and 30-60 mesh Molecular Sieve, 5A, 6 ft x 1/4 in.
Swept gas	Helium
Inlet pressure	30 psig
Temperature	40°C
Detector filament current	350 ma
Sample size	0.2 ml
Recorder	Brown 1 mv. equipped with ball-and-disc integrator

Experimental Media

Nitrogen Source

The following concentrations of NH_4Cl and urea were added as a source of nitrogen to NH_4Cl-free basal medium:

NH_4Cl	0.0095, 0.019, and 0.038 M.
Urea	0.0084, 0.017, and 0.034 M.

The inoculated medium was dispensed into flasks, gassed with $H_2/O_2/CO_2$ mixtures of 70/20/10 for H. eutropha and 82/8/10 for H. ruhlandii, stoppered, and incubated on a shaker. H. eutropha was incubated for 20 to 24 hrs. and H. ruhlandii was incubated for 43 hrs. An inoculated control flask containing no NH_4Cl or urea was included with each experiment. A subsequent study with H. ruhlandii was performed with the following concentrations of NH_4Cl and urea:

NH_4Cl	0.0048, 0.0095 and 0.0143 M
Urea	0.0042, 0.0084 and 0.0126 M

Basal Medium Composition

The effect of altering the composition of the basal medium was studied by removing the major components of the medium, one at a time, and observing the results. Eight cultures were prepared according to the following.

1. Basal medium control
2. Basal medium minus $NaHCO_3$
3. Basal medium minus $MgSO_4$
4. Basal medium minus $Fe(NH_4)_2(SO_4)_2$
5. Basal medium minus trace element solution
6. Basal medium minus $CaCl_2 \cdot 2H_2O$
7. Basal medium minus NH_4Cl
8. Basal medium minus NaCl

H. eutropha was cultured, gassed, and incubated as previously described.

Spent Medium Studies

The culture conditions for the studies concerning the spent medium were the same as previously reported for stoppered flask cultures. The medium was centrifuged at 12,000 G and Millipore filter-sterilized

prior to reinoculation with a fresh inoculum. In Study No. 1, the medium was inoculated without adjustment of any kind. The second study involved pH adjustment of pH 7.0. In the third study, the spent medium was adjusted with NH_4Cl, $MgSO_4$, and $Fe(NH_4)_2(SO_4)_2$ individually, and with all three components combined.

Carbon Monoxide Studies

The culture conditions for CO studies were the same as reported for stoppered flask cultures. The CO was tested at two concentrations, 3 - 5% and 1%.

Human Waste Materials

a. Urine

The urine was collected from five donors, and a composite was prepared from equal amounts. The urine was filter-sterilized. Urea was determined by the colorimetric biacetyl monoxime method. (ref. 44) Urea concentration was 23 mg/ml. H. eutropha was studied in stoppered flasks and macro-respiratometers.

b. Fecal Extract

The fecal extract was prepared from a composite of five donors, in equal amounts. The composite feces, 1% in water, was refluxed for one hour and subsequently filter-sterilized. H. eutropha was studied in stoppered flasks and macro-respirometers.

pH

The pH experiments were run with two types of buffers. The first experiments used buffers composed of KH_2PO_4 and Na_2HPO_4 at pH 6.0, 6.4, 6.8, 7.2 and 7.6, according to formulations in the Biochemists' Handbook (ref 45) (ionic strength 0.05 @ 25°C). The second group of pH experiments used buffer systems as above at pH 6.0, 6.4 and 6.8 and tris (hydroxymethyl) amino methane - HCl buffers at pH 7.0, 7.4 and 7.8. The tris buffer was prepared according to the Biochemists' Handbook (ref. 45) (ionic strength 0.05 @ 25°C). Culturing and incubation conditions were the same as described under nitrogen source studies.

Temperature

H. eutropha and H. ruhlandii were cultured in stoppered flasks and incubated in the constant temperature room at 25 and 35°C. The 30°C data was obtained from previous control data.

Ionic Strength

The following concentrations of Na_2SO_4 and NaCl were added to the basal medium:

0.1, 0.2 and 0.3 M

The basal medium which contains phosphate buffer at an ionic strength of 0.05 was adjusted to ionic strength of 0.1 and 0.2 according to formulation in the Biochemists Handbook,[ref 45] page 32. The basal medium also contains $NaHCO_3$ at a concentration of 0.1%; this compound was tested at the following concentrations:

0.1, 0.3 and 0.5%.

Culturing and incubation were the same as described above for H. eutropha.

Batch Culture Gas Utilization

Preliminary studies on gas analysis described in the screening program indicated that the partial vacuum produced during gas utilization interfered with obtaining reproducible analyses (except for the initial samples, which were taken when the flask was nearest to atmospheric pressure). Zero time gas analyses have been reliable.

Two metnods have been used to counter the effect of vacuum. The first consisted of adding pure H_2 to the Warburg flask, just prior to sampling for analysis, in order to produce positive pressure. The change in manometer readings allowed calculation of the volume of gas added and the slight positive pressure allowed more accurate sampling. Subsequent values obtained from the gas chromatograph were then corrected to account for added H_2. An experiment was performed to test the feasibility of this method. Although good results were obtained, subsequent studies with H. eutropha indicate that inadequate mixing of the H_2 with the other gases was creating a false picture of the gas composition.

The second approach involves modification to use the Warburg constant volume system as a constant pressure system. This has been accomplished in two ways: (1) a gas burette and attached leveling bulb filled with acidified water was connected to the Warburg manometer; and (2) a gas-tight piston burette was attached to the Warburg manometer. Gas uptake, in either case, determined by adjusting the burettes until the reading on the Warburg manometer matches the reading on the thermobarometer. With these procedures, gas uptake can be read directly from the burette and slight positive pressure can be applied for sampling.

Continuous Culture

Gas Mixtures for Continuous Culture

The apparatus for producing continuous gas mixtures, an integral part of the continuous culture device for the hydrogenomonads, consists of three rotameters, one each for H_2, O_2, and CO_2, with all three feeding into a manifold for mixing of the gases before passing through a cotton-filled sterilizing tube into the culture medium. The complete apparatus for gas mixing and continuous culture is shown schematically in Figure 9. Figure 10 shows the design of the continuous culture growth chamber used in the first seris of studies with H. eutropha. In this series of studies, the gas mixture was bubbled through a column of medium with or without column packing.

For the second series of continuous culture studies with H. eutropha, a new growth chamber was designed (Figure 11) to provide increased surface area for gas uptake. To accomplish better mass transfer of gas, the culture is pumped from the outlet tube on the chamber to the inlet tube on the cap by a peristaltic pump or a flexible impeller pump. The flow is directed into an overflow ring which allows a continuous wetting of the walls.

The medium was the basal medium in which NH_4Cl was used in normal concentration or at the growth limiting level, 0.0095 M. 20% inoculum was used to hasten establishment of the H. eutropha culture. Growth was followed by optical density (absorbance at 660 mμ, 1/5 dilution), protein (determined by the Lowry method), pH, and gas utilization. Gas uptake was taken as the difference between the gas inlet flow, measured with a Precision Scientific wet test meter connected between the manifold and gas sterilizing tube (cotton-packed), and the gas effluent flow, measured with a 50 ml soap bubble flow meter. All values were corrected for water vapor pressure and reduced to standard temperature and pressure.

Electrochemical Studies

The electrochemical studies were concerned with (1) demonstrating the metabolism of candidate bacteria in the presence of a cathodically polarized electrode and (2) the effects of metabolizing bacteria on the potential of a cathodically polarized electrode.

An electrolysis cell was devised which would allow the use of a small volume of cell suspension and which could be used with Warburg manometers to obtain quantitative gas change data. The cell was designed to allow immersion in the Warburg water bath for constant temperature operation.

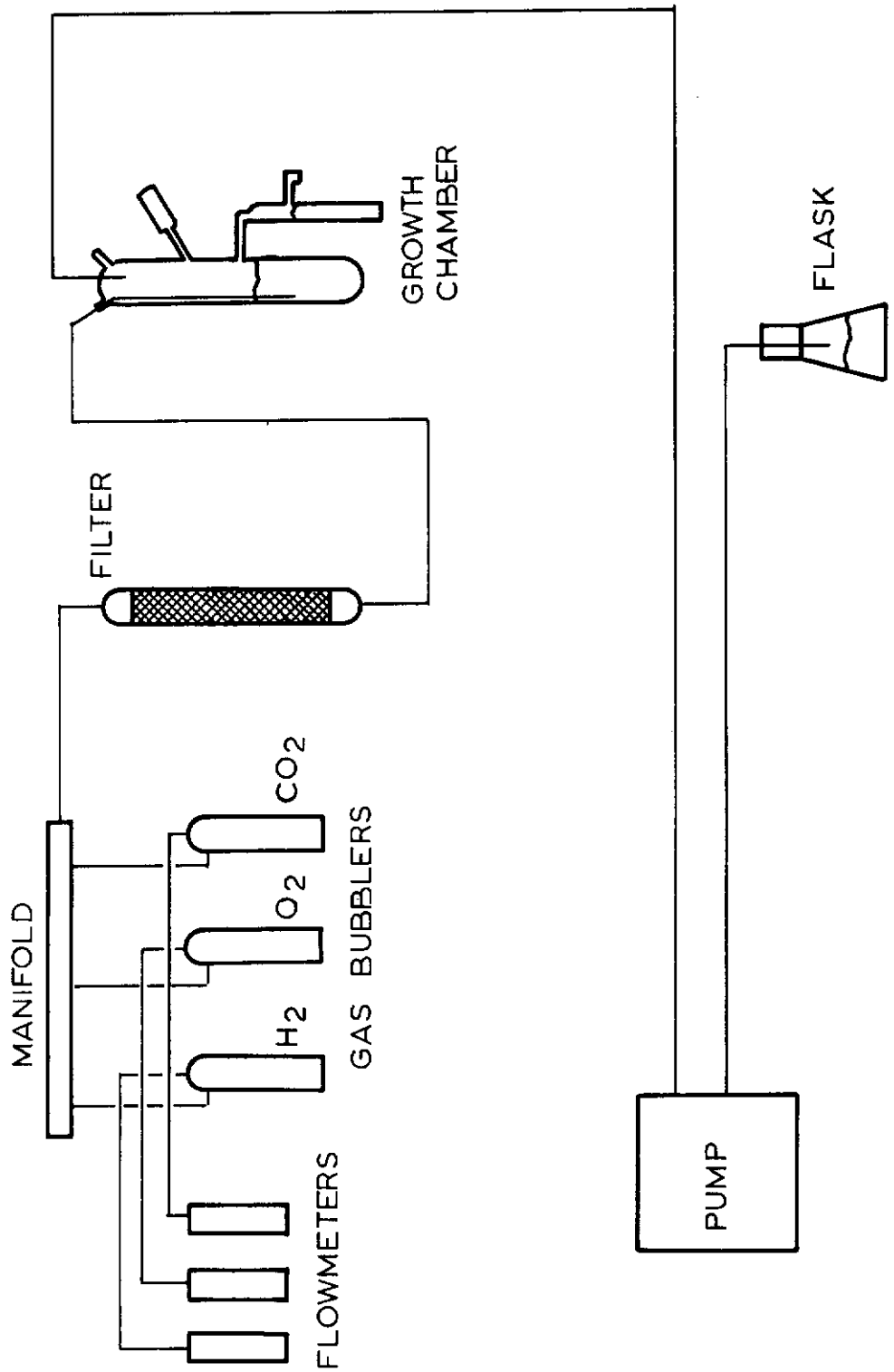
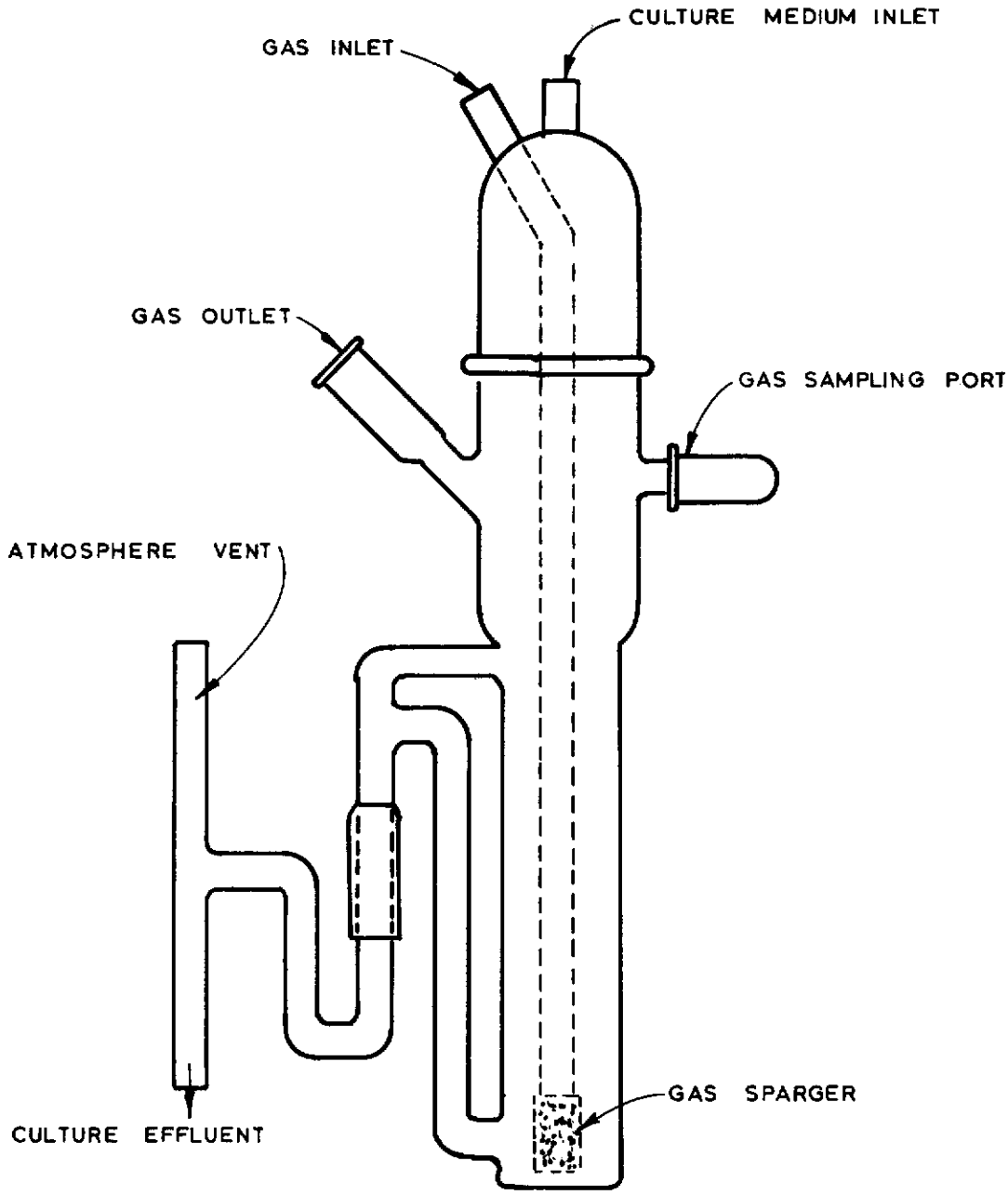


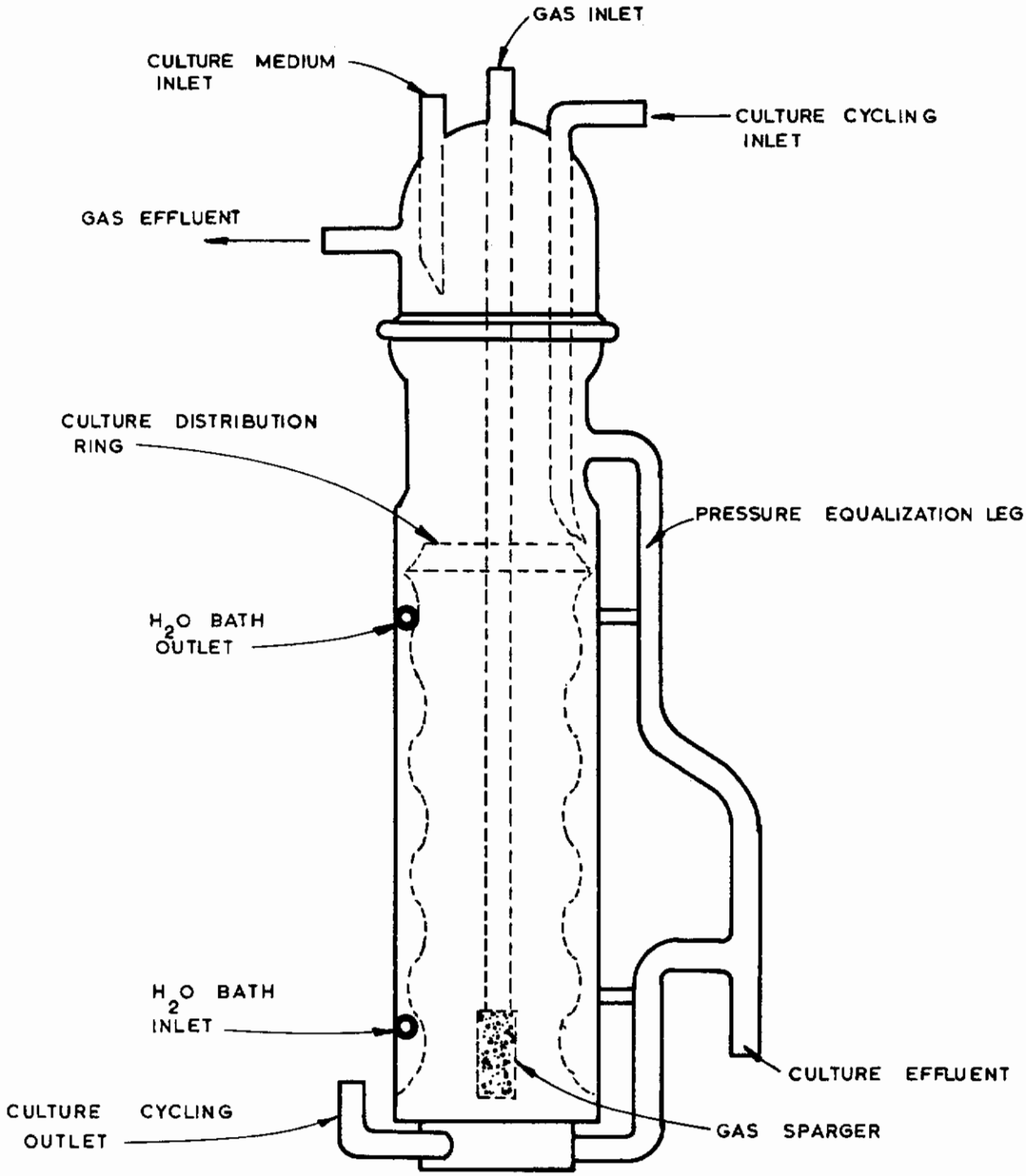
FIGURE 9

Schematic of Gas Mixing and Continuous Culture Apparatus



CONTINUOUS CULTURE GROWTH CHAMBER, SERIES I

FIGURE 10



CONTINUOUS CULTURE GROWTH CHAMBER, SERIES II

FIGURE 11

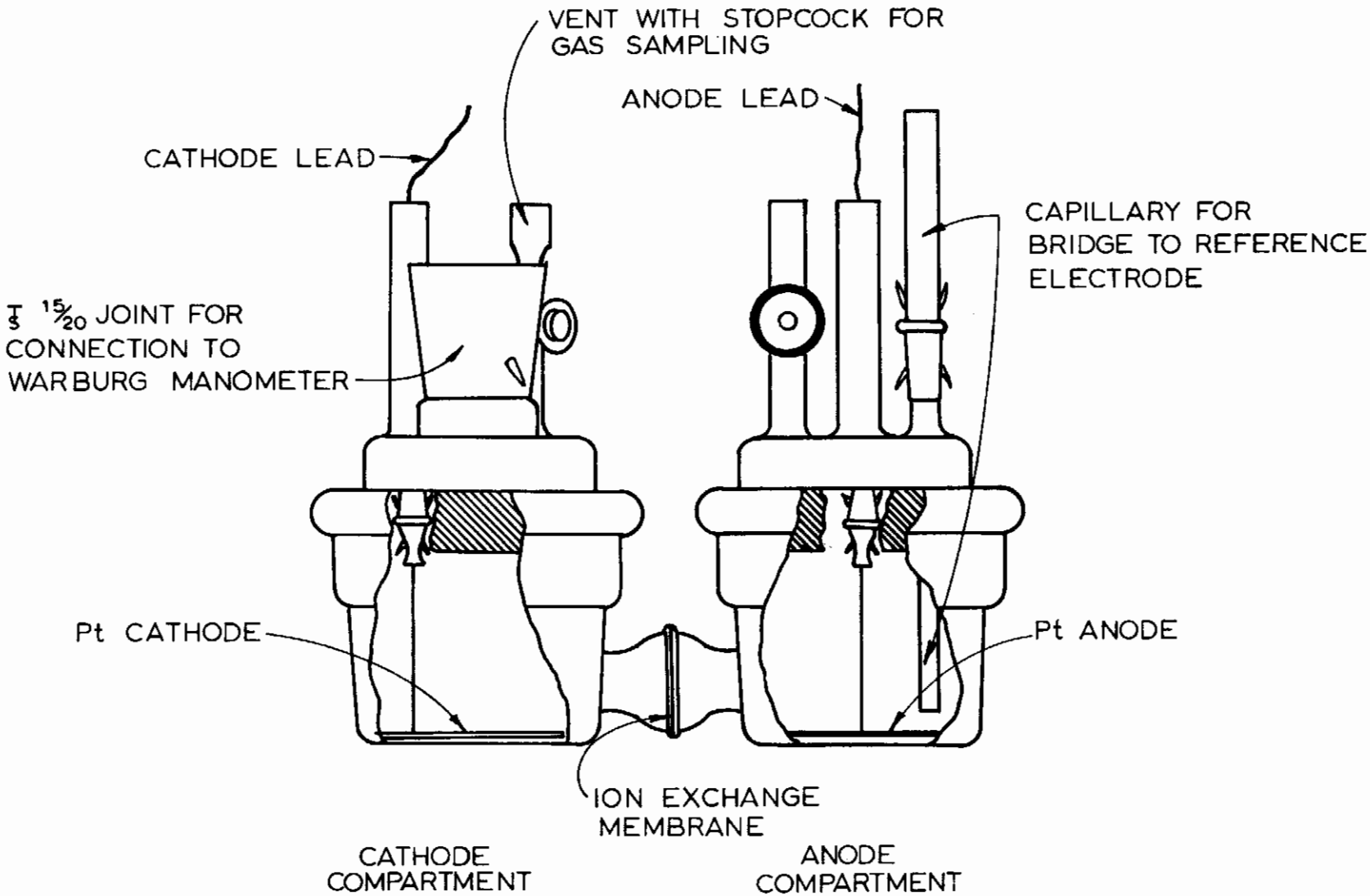
Figure 12 is a drawing of the Warburg electrolysis cell. The cell is constructed of two compartments, an anode compartment and a cathode compartment. Each compartment is a two-piece chamber, the flask, bearing an O-ring joint, and a cover, bearing electrodes and ports. The anode compartment cover has a port with a stopcock for venting and a port for connection of the cell to a reference electrode via an agar-KCl bridge. The cathode compartment cover has a venting port which can be used for gas sampling and a joint for connection of the cell to a Warburg manometer. The electrodes (anode and cathode) are constructed of 0.003" platinum foil. Each electrode has an area of 14.14 cm^2 (both sides) and is spot-welded to platinum wires which serve both as supports and electrical leads. The horizontal placement of electrodes was employed to minimize the electrolyte volume required. Ten ml of electrolyte in each chamber is adequate to maintain coverage of the electrodes and continuity of the electrolytic circuit while the cell is being shaken in the Warburg.

To aid in maintaining the cathode chamber gas-tight and because of space limitations, the reference electrode entry to the cells was placed in the anode chamber. Cathode potentials were measured versus the saturated calomel reference electrode (SCE) and corrected for the potential drop due to current flow through the cell resistance. A check on this was performed by measuring cell voltage and the anode potential vs. the reference electrode.

Isolation of the cathode and anode chambers of the cell was effected by a cation exchange membrane clamped between O-rings at the flask openings. This membrane allows the flow of electrolytic current while minimizing diffusion between compartments.

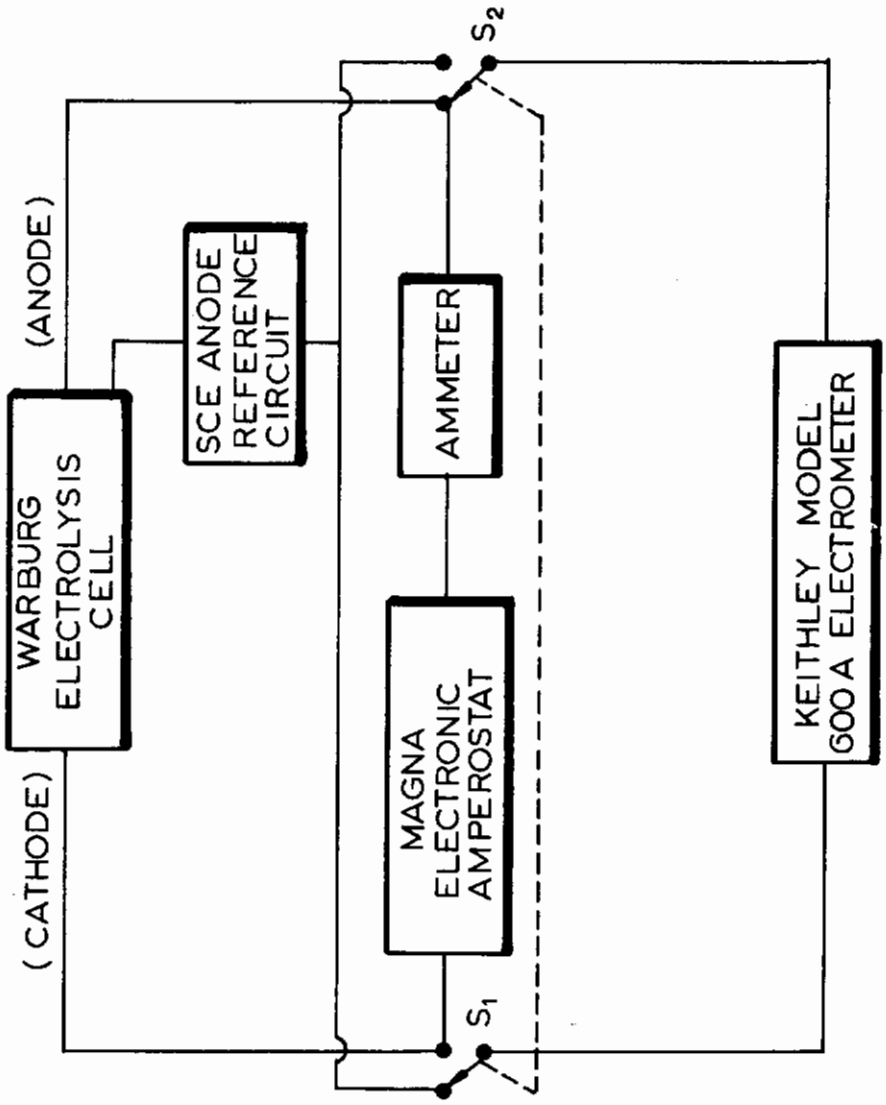
Figure 13 is a schematic of the external electrical circuit used with the electrolysis cell of Figure 12. Cell power was provided by an "amperostat", which consisted of an electronic potentiostat connected to provide D. C. power at constant current. Operation in such a fashion eliminated the need for a coulemeter to measure the quantity of electricity used. Potential measurements were made by connecting an Electrometer between the reference electrode and the anode or cathode and between anode and cathode by appropriate manipulation of the switches marked S1 and S2, Figure 12. Cathode potentials were corrected for the potential drop in the cell.

Cell experiments were conducted at 30°C. During operation, cell suspensions were placed in the cathode compartment and buffer in the anode compartment. The cathode compartment was then flushed with the gas mixture appropriate to the experiment. Cell suspensions were prepared by centrifuging cultures in the log phase of growth, washing and resuspending the cells in 0.1 M phosphate buffer, pH 7. Ten ml portions of cell suspensions were used. The same quantity of 0.1 M phosphate buffer, pH 7, was used in the anode



WARBURG ELECTROLYSIS CELL

FIGURE 12



SCHEMATIC OF ELECTRICAL CIRCUIT UTILIZED IN WARBURG ELECTROLYSIS CELL EXPERIMENTS

FIGURE 13

compartment. Control experiments were performed in the electrolysis cell with the buffer free of cells in the cathode compartment. Endogenous controls were made with portions of the suspension in conventional Warburg appartus. Upon supply of current to the electrolysis cell, electrode potential and manometric measurements were made throughout the run.

Biological Electrode Studies

Several attempts were made to attach H. eutropha to a porous gold electrode. The first procedure consisted of filtering the suspension of organisms onto the electrode, between rubber sheet masking, in a Seitz filter apparatus. The second procedure consisted of inoculating the electrode by dipping into the suspension and then placing the electrode onto an agar plate made from the basal medium solidified with Noble agar (Difco). Inoculated electrodes were tested for the presence of bacteria by placing the electrode into a Warburg respirometer with 3.0 ml of basal medium. The flasks were gassed by flushing with 70-H_2; 20-O_2; 10-CO_2.

During several initial studies, we learned that sterilizing and handling of the gold electrode created sufficient oxidation to be toxic to the bacteria. Subsequently, the electrodes were reduced as a cathode prior to seeding and incubation.

Growth Study with H. eutropha in Warburg Electrolysis Cell

Fifteen ml of 0.2 ionic strength basal medium were inoculated (1%) witn H. eutropha. The medium was placed into the Warburg electrolysis cell, gassed with 71.35, He:19.70, O_2, 8.95 CO_2 and attached to a gas reservoir with a Sigma pump to circulate the gas mixture. Initial current level was 4.0 ma for H_2 production.

Hydrogenase Activity

The hydrogenase activity of several isolates was tested according to the following procedures:

1. Thunberg Hydrogenase Test

In this procedure, 1 ml of 1/10,000 methylene blue and 4 ml of Repaske's Sea Water Medium are placed into the Thunberg test tube and 1 ml of the test suspension is placed into the side arm. The tube

is evacuated and flushed with hydrogen several times to insure a pure hydrogen atmosphere. The tubes are placed into a 30°C water bath and after approximately 10 minutes of temperature equilibration, the test suspension in the side arm is mixed with the reagents. The tubes are then examined for blanching of the methylene blue, which is indicative of hydrogenase.

2. Warburg Hydrogenase Test

In this procedure, 3.2 ml of the test suspension in Repaske's Sea Water Medium is placed into a 16-ml Warburg flask and connected to the manometer. The manometer fluid was Brodie's Solution. The flask system was flushed with hydrogen for approximately 30 minutes and then the manometers were observed for evidence of gas uptake.

Examination for Nutritional Requirements of Marine Organisms

The examination of nutritional requirements was performed with a modified antibiotic test system. The organism is seeded into the agar basal sea water medium in which it will grow, but slowly. The organism is also seeded into the agar medium with 0.1% yeast extract as an "optimum" growth control. The surface of the former plate is then spotted with impregnated sterile filter paper disks or crystals of the test reagent. The backs of the plates are marked to identify the particular reagent. The plates are incubated and a positive test is observed when a zone of improved growth is evident around a particular reagent.

I. Impregnated filter paper disks of the following vitamins were used:

a. Riboflavin - 50 µg/ml
b. Biotin - 0.1 µg/ml
c. Thiamin - 50 µg/ml
d. Nicotinic acid - 50 µg/ml
e. Ascorbic Acid - 50 µg/ml

II. The following materials were spotted in powder form:

a. L-cysteine
b. L-ornithine-HCl
c. Ketoglutarate
d. Succinate
e. Glycine
f. Citrate
g. Fumarate
h. Oxalacetate
i. DL-alanine
j. Urea
k. Cystine
l. Leucine
m. DL-malic acid
n. L-glutamic acid

The plates were flushed with the standard gas mixture and incubated at 30°C.

REFERENCES

1. Ormerod, J. G., K. S. Ormerod, and H. Gest, "Light-Dependent Utilization of Organic Compounds and Photoproduction of Molecular Hydrogen by Photosynthetic Bacteria; Relationships with Nitrogen Metabolism." Arch. Biochem. Biophys., Vol 94, pp 449-463, 1961

2. Repaske, R., "Nutritional Requirements for Hydrogenomonas eutropha." J. Bacteriol. Vol 83, pp 418-422, 1962

3. James. T. W., "Continuous Culture of Microorganisms." Ann. Rev. Microbiol. Vol 15, p 27, 1961

4. Chappelle, E. W., Carbon Monoxide Metabolism, Developments in Industrial Microbiology, Vol 3, p 99, Plenum Press, N. Y., 1962

5. Lancaster, J. H., R. G. Tischer, and R. J. Long, "Human Feces as a Nitrogen Source for some Green Algae." Developments in Industrial Microbiology, Vol 3, pp 25-33, Plenum Press, N.Y., 1962

6. Bongers, L., "Chemosynthetic Gas Exchanger," Martin-Marietta Space Systems Division, Baltimore, Md., Report RM 153-1963.

7. Stern, J. A. and R. Meyer,"Utilization of Hydrogen by Hydrogenomonas," The Boeing Company, Final Report, Contract No. 3614-(00), December, 1962.

8. Spector, W. S., Handbook of Biological Data, Technical Report 56-273, Wright-Patterson Air Development Center, Wright-Patterson Air Force Base, Ohio, ASTIA Document No.AD 110 501, 1956.

9. Kluyver, A. J. and A. Manten, "Some Observations on the Metabolism of Bacteria Oxidizing Molecular Hydrogen," Antoine van Leeuwenhoek, J. Microbiol. Serol., Vol 8, pp 71-85, 1942

10. Wilson, E., H. A. Strout, D. Powelson and H. Koffler, "Comparative Biochemistry of the Hydrogen Bacteria. I. The Simultaneous Oxidation of Hydrogen and Lactate," J. Bacteriol., Vol 65, pp 283-287, 1953

11. Kister, A., "On a Bacterium Oxidizing Carbon Monoxide," Koninkl, Ned. Akad. Wetensch. Proc., Vol 56, pp 443-450, 1953

12. Packer, L. and W. Vishniac, "Chemosynthetic Fixation of Carbon Dioxide and Characteristics of Hydrogenase in Resting Cell Suspensions of Hydrogenomonas ruhlandii, Nov. Spec. J. Bacteriol., Vol 70, pp 216-223, 1955

13. Linday, E. M. and P. J. Syrett, "The Induced Synthesis of Hydrogenase by Hydrogenomonas facilis," J. Gen. Microbiol., Vol 19, pp 223-227, 1958

14. Schatz, A. and C. R. Bovell, "Growth and Hydrogenase Activity of a New Bacterium, Hydrogenomonas facilis," J. Bacteriol., Vol 63, pp 87-98, 1952

15. Cohen, J. S. and R. H. Burris, "A Method for the Culture of Hydrogen Bacteria," J. Bacteriol., Vol 69, pp 316-319, 1955

16. Schlegel, H. M., "Physiologishe Untersuchunger on Wasserstoffoxydierender Bacterium," Arch. Mikrobiol., 18, pp 362-390, 1953

17. Schatz, A., "Uptake of Carbon Dioxide, Hydrogen and Oxygen by Hydrogenomonas facilis," J. Gen. Microbiol., Vol 6, pp 329-335, 1952

18. Marino, R. J., "Assimilation by Hydrogenomonas facilis," Ph. D. thesis, Stanford University, Palo Alto, California

19. Marino, R. J. and C. E. Clifton, "Oxidative Assimilation in Suspensions and Cultures of Hydrogenomonas facilis," J. Bacteriol., Vol 69, pp 188-192, 1955

20. Chapman, D. D. and R. Meyer, "Application of Hydrogen-Utilizing Microorganisms to Respiratory Support in Closed Systems," presented at the 19th Meeting of the Society for Industrial Microbiology, Corvallis, Oregon, 1962

21. Roelofsen, P. A., "On the Metabolism of the Purple Sulphur Bacteria," Koninkl. Ned. Akad. Wetensch. Proc., Vol 37, pp 660-669, 1934

22. Gaffron, H., "Uber den Stoffwechsel der Purpurbakterien. II." Biochem. Vol 275, pp 301-319, 1935

23. Larsen, H., "On the Culture and General Physiology of the Green Sulphur Bacteria," J. Bacteriol. Vol 64, pp 187-196, 1952

24. Van Niel, C. B., "The Bacterial Photosyntheses and Their Importance for the General Problem of Photosynthesis," Advances in Enzymol., Vol I, pp 263-328, 1941

25. Wassink, E. C., "On the Ratio Between the Uptake of Carbon Dioxide and of the Hydrogen Donor in Purple Sulphur Bacteria," Enzymologia, Vol 10, pp 257-268, 1942

26. Larsen, H., C. S. Yocum and C. B. van Niel, "On the Energetics of the Photosyntheses in Green Sulphur Bacteria," J. Gen. Physiol., Vol 36, pp 161-171, 1952

27. Ormerod, J. G. and H. Gest, "Hydrogen Photosynthesis and Alternative Metabolic Pathways in Photosynthetic Bacteria," Bacteriol. Rev., Vol 26, pp 51-66, Symposium on metabolism of inorganic compounds, IV., 1962

28. Van Niel, C. B., "The Culture, General Physiology, Morphology, and Classification of the Nonsulfur Purple and Brown Bacteria," Bacteriol. Rev., Vol 8, pp 1-118, 1944

29. Barker, H. A., "Bacterial Fermentations," John Wiley and Sons, New York, 1956

30. Barker, H. A., "Studies on the Methane Fermentation, VI. The Influence of Carbon Dioxide Concentration on the Rate of Carbon Dioxide Reduction by Molecular Hydrogen," Proc. Natl. Acad. Sci. U.S., Vol 29, pp 184-190, 1943

31. Schnellen, C. G. T. P., "Onderzoekingen over de Methaangisting, Dissertation, Delft.

32. Kluyver, A. J. and C. G. T. P. Schnellen, "On the Fermentation of Carbon Monoxide by Pure Cultures of Methane Bacteria," Arch. Biochem., Vol 14, pp 57-70, 1947

33. Stadtman, T. C. and H. A. Barker, "A New Formate-Decomposing Bacterium, Methanococcus vannielii," J. Bacteriol., Vol 62, pp 269-280.

34. Barker, H. A., "Studies on the Methane Fermentation, V. Biochemical Activities of Methanobacterium omelianskii," J. Biol. Chem., Vol 137, pp 153-167, 1941

35. Wieringa, K. T., "The Formation of Acetic Acid from Carbon Dioxide and Hydrogen by Anaerobic Spore-Forming Bacteria," Antonie von Leeuwenhoek, J. Microbiol. Serol., Vol 6, pp 251-262, 1940

36. Karlsson, J. L., B. E. Volcani, and H. A. Barker, "The Nutritional Requirements of Clostridium aceticum," J. Bacteriol. Vol 56, pp 781-782, 1948

37. Gaffron, H., "Reduction of Carbon Dioxide with Molecular Hydrogen in Green Algae," Nature, Vol 143, pp 204-205, 1939

38. Gaffron, H., "Reduction of Carbon Dioxide Coupled with the Oxyhydrogen Reaction in Algae," J. Gen. Physiol., Vol 26, pp 241-267, 1942

39. Frenkel, A. W. and C. Rieger, "Photoreduction in Algae," Nature, Vol 167, p 1030

40. Gaffron, H., "Photosynthesis, Photoreduction and Dark Reduction of Carbon Dioxide in Certain Algae," Biol. Revs. Cambridge Phil. Soc., Vol 19, pp 1-20, 1944

41. Skerman, V. G. D., Genera of Bacteria, Williams & Wilkins Co., Baltimore, p 127, 1959

42. Postgate, J. R., "Differential Media for Sulphur Bacteria," J. Sci. Food Agric., Vol 10, pp 669-674, 1959

43. Umbreit, W. W., B. H. Burris and J. J. Stauffer, Manometric Techniques, Burgess Publ. Co., 1957.

44. Ormsby, A. A., "A Direct Colormetric Method for the Determination of the Urea in Blood and Urine," J. Biol. Chem., Vol 146, pp 555-604, 1942

45. Biochemists' Handbook, D. Van Norstrand Co., Inc., Princeton, N. J., 1961