FINAL REPORT

Period: June 8, 1964 - March 14, 1967

CHEMOSYNTHETIC GAS EXCHANGER

by

Dr. Leonard Bongers, Principal Investigator,
and Dr. John C. Medici

Research Institute for Advanced Studies
(RIAS)
Martin Marietta Corporation
1450 South Rolling Road
Baltimore, Maryland 21227

GPO PRICE $ _____

CFSTI PRICE(S) $ _____

April 1967

Hard copy (HC) _____

Microfiche (MF) _____

ff 653 July 65

Prepared for

N67-36592

(ACCESSION NUMBER)	(THRU)
(PAGES)	(CODE)
(NASA CR OR TMX OR AD NUMBER)	(CATEGORY)

FACILITY FORM 602

FINAL REPORT

Period: June 8, 1964 - March 14, 1967

CHEMOSYNTHETIC GAS EXCHANGER

by

Dr. Leonard Bongers, Principal Investigator,
and Dr. John C. Medici

Research Institute for Advanced Studies
(RIAS)
Martin Marietta Corporation
1450 South Rolling Road
Baltimore, Maryland 21227

April 1967

Prepared for

Office of Space Sciences and Applications
National Aeronautics and Space Administration
Washington, D. C.   20546

Contract No. NASw-971
ER - 13634-11

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I. INTRODUCTION

One solution to participation by man in long-term space exploration is closure of the food-waste loop by biological means. In view of the weight and power limitations a transition from simple storage of commodities to regeneration and reuse of metabolic wastes seems inevitable. The characteristics of a promising biological approach, utilizing the autotrophic metabolism of hydrogen oxidizing bacteria for the regenerative process will be discussed.

The (chemosynthetic) bioregenerative system is a functional coupling of electrolysis of water with biosynthesis by hydrogen bacteria[1,2]. Figure 1 represents a hypothetical flow diagram of chemosynthetically closed ecology; the reactions involved in the overall process are summarized by the chemical equations under the diagram of fig. 1. Electrolysis of water produces oxygen for man and hydrogen for the bacteria. The carbon dioxide produced by man is assimilated by the bacteria, which in turn furnish the proteins, fats, and carbohydrates required for human nutrition. Thus a considerable portion of man's metabolic wastes would be processed and recycled in this ecosystem.

The generation of hydrogen and oxygen by electrolysis is a relatively efficient process; information presently available indicates an efficiency in the order of 80% in the conversion of electrical energy into biological energy (hydrogen and oxygen). Any appraisal of weight and, to a lesser degree, of power requirements for regenerative life support systems must involve a fairly rough approximation because of a rapid technological advance

in the efficiency of power generation and miniaturization of instrumentation. It is therefore not possible, at the present time, to predict with some degree of accuracy the weight penalty of an electrolytic system. However, a weight estimate of 20-40 pounds to support 3 men seems reasonable.

An estimate of the energy which would be required for the regeneration of man's carbon dioxide output are indicated by the chemical equations presented in fig. 1. Electrolytic cleavage of 6 moles of water would suffice to provide the energy for the conversion of one mole of carbon dioxide. Thus, the end result of electrolysis and biosynthesis is the formation of one mole (CH_2O) representing 120 kcal, and one mole (or 22 liters) of oxygen. The products of biosynthesis provide thus, the approximate hourly caloric intake and oxygen requirement of one man.

Because the cleavage of 6 moles of water requires approximately 600 kcals energy input (75% efficiency), the overall efficiency of the energy conversion is of the order of 20%.

Measurements of autotrophic growth rates in batch and in continuous cultures indicate that specific rates of CO_2 conversion of approximately 1 liter can be attained. A suspension volume requirement in the order of 20-30 liters per man seems thus a reasonable estimate.

On the basis of the performance recently obtained with growth in steady-state cultures, it appears that this approach to life support is well on its way to maturity. Therefore, this report will review the present state of the art and discuss in some detail: the techniques employed for cultivation, the demand upon and the response of this organism to its chemical and physical environment, as well as some of the basic aspects* of the conversion process.

---

*Details will be published elsewhere.

II. CULTIVATION PROCEDURES

1. Batch Operation.

Abundant evidence indicates that H_2, O_2 and CO_2 are consumed by hydrogenomonads in a ratio of 6:2:1. This corresponds to a gas composition of 70% hydrogen, 20% oxygen and approximately 10% CO_2. This gas mixture is most convenient for use when growth is obtained by the "passive" gas diffusion, a method described by Repaske[3]. It permits sterile operation and is a satisfactory method for most experiments.

Briefly, the system consists of a gyrotory water-bath-shaker (New Brunswick Model G76) with a capacity for six 500 ml Erlenmeyer culture flasks. The culture flasks are connected to a manifold, which in turn is connected either to a water aspirator or to a reservoir containing the gas mixture. The pressure of the gas reservoir is maintained constant via a water bridge connected to a water reservoir which is open to the atmosphere.

The rate of cell reproduction in this system is limited by the diffusion rate of oxygen into the suspension. The maximum rate of oxygen diffusion can be determined manometrically, utilizing sulfite (0.2N) dissolved in the medium utilized for cultivation. Measurements indicate an oxygen transfer rate of approximately 100 ml per hour with baffled flasks (conditions: air, 1 atm. 30°C, 0.2N sulfite in 120 ml culture medium). The rate of oxygen transfer is 5 to 10 fold lower in an unbaffled flask. The rate of hydrogen diffusion is assumed to be three times the rate of oxygen diffusion, under similar conditions.

The maximum rate of gas consumption and growth can thus, for a given set of conditions, be estimated. If one assumes a gas mixture containing a

hydrogen concentration three times that of oxygen, and a hydrogen to oxygen consumption ratio of three to one, it is evident that with a progressively increasing rate of gas consumption, oxygen diffusion will ultimately become the growth limiting factor.

A disadvantage of this method is that relatively large quantities of the explosive gas mixture must be available to sustain a suspension over night. By using a simple pressure actuated switch (activating pressure range 4-5 cm Hg) and an oxygen electrode-amplifier, we are able to mix the gasses automatically. Oxygen (100%) is fed via solenoid valve, actuated by the oxygen controller, into a gas-mixing chamber (approximately 2 liters) containing the oxygen sensor. A mixture of 86% H_2 + 14% CO_2 is fed to the same mixing chamber the flow being regulated by a solenoid valve actuated by the pressure switch connected to the mixing chamber. A small pump (Dyna Pump Model 4K) circulates the gas mixture to the cultures and back to the mixing chamber.

The same design was used to provide large batches (volumes of 10-12 liters) of cells with gas mixture. The gas mixture was circulated through the suspension by two pumps via four gas dispersion tubes (fritted cylinders) mounted near the bottom of the suspension container. The gas mixture was returned to the mixing chamber via an outlet fitted into the top of the container. Best results were obtained with the oxygen electrode mounted in the suspension instead of in the mixing chamber. This procedure allows for an automatic adjustment of oxygen transfer (by increasing the gas phase oxygen concentration) proportional to the rate of oxygen consumption by the suspension. This system permits cultivation of relatively large volumes of

suspension of medium population density. Routinely, a dry weight production of approximately 20 grams was obtained in 24 hours with heavy inoculum.

2. Continuous Operation.

To achieve continuous culture several steady-state parameters such as pH, concentration of nutrients, oxygen, hydrogen and carbon dioxide must be continuously balanced. These parameters are subject to considerable change in batch cultures. A steady-state culture in which the rate of growth is controlled by a single factor (e.g. the carbon source) is defined as a chemostat; in such cultures, the concentration of nutrients other than the limiting ones, the concentration of products excreted into the medium in the course of cell reproduction and the pH are more or less independent of dilution rate. The background of this type of culture has been well developed by the theoretical work of Monod[4], Novick and Szilard[5] and the experimental approach of Herbert et al[6].

A continuous culture in which the dilution rate is set by the growth rate is called a turbidostat or product-limited culture. In order to allow the most efficient utilization of both power and weight a system of this type is required, in which the growth of the cell is entirely controlled by its internal characteristics, rather than by an environmental factor (as would be in the case of a chemostat).

Little theoretical background on turbidostats has yet been accumulated, but clearly the chemical and physical environments are influenced by the dilution rate. It is therefore necessary to determine the optimal concentration of the essential constituents of the feed medium for each cell density at which the culture is operated. Our progress in establishing and

maintaining these optimal concentrations is discussed in Section III, 3 and Section V.

Our turbidostatically controlled apparatus is an adaptation of a unit fabricated by Battelle Memorial Institute[7]. The configuration (see fig. 2) conforms to a design described by Finn[8]. Cultivation takes place in a closed chamber (3-4 liters of working suspension in total volume of 5 liters) provided with a baffle and impeller arrangement. Rapid gas transfer is accomplished by vigorous agitation and recirculation of the gas phase through the liquid phase. Recent additions include a compact pH (see fig. 3) and a density control system (see fig. 4). The control systems used will be briefly described.

In an earlier arrangement (see fig. 2) the influx of CO_2 to the suspension was controlled by a CO_2 electrode (converted pH electrode, Beckman) in the suspension. When the dissolved CO_2 concentration dropped below a preset level, the sensor actuated solenoid valve (S4), thus admitting a carbon dioxide - hydrogen mixture (86%/14%) to the reaction chamber via a pre-calibrated meter valve. The total H_2/CO_2 flow was monitored by a digital elapsed time meter (C2). A gas mixture of hydrogen and carbon dioxide was used to offset the slow response of the CO_2 sensor. As will be discussed later, this method of carbon dioxide control was inadequate at higher population densities. Presently, a gas phase carbon dioxide - controller is in use and has resulted in improved system performance.

Oxygen supply is controlled by a dissolved-oxygen sensor (gold-silver Clark-type electrode) and measured in a similar fashion as described above. This sensor has a fast response and has performed satisfactorily in the liquid phase.

The pressure is maintained at 3 psig with hydrogen. The hydrogen influx is controlled by pressure switch - solenoid valve arrangement and metered in a similar fashion as the oxygen and carbon dioxide consumption.

The pH of the suspension could be controlled by the pH sensor, mounted in an external sampling loop (see fig. 2). A cut-away diagram of the pH chamber is shown in fig. 3. The combined pH electrode was inserted through the top of the lucite block. A tight seal was provided with a rubber "O" ring and nut arrangement. This arrangement permitted easy removal of the pH electrode for the purpose of cleaning and testing. The pH electrode was connected to a recorder-controller which permits automatic addition of either acid or base. Presently, this equipment is used only as a pH indicator.

The density of the suspension is controlled by the flow-through chamber mounted in the external sampling loop (see fig. 4). The stainless steel sample chamber used interchangeable teflon gaskets for monitoring various cell densities. Not shown is the reference chamber, attached to the sampling chamber. A needle in the light path of the reference chamber could be used to balance the light intensities transmitted to the two photo-cells. In addition, an electric balance was provided in the wheatstone bridge (associated circuitry in fig. 4A). Optical density of the suspension was checked periodically against a calibrated instrument. It was found that the population density remained constant (within a few percent) during periods of constant meter setting. One can therefore assume that essentially no cell build-up occurs on the walls of the control chamber. Figure 5 illustrates that the ratio of optical density over dry weight of cells is

essentially constant throughout the test run, indicating that control of optical density is an adequate means for controlling the cell concentration of suspension.

The cell density of the reactor is maintained through automatic additions of fresh medium. The excess suspension leaves the reactor via a stand pipe, a gas-liquid separator and level controller. The level-controller is connected to a harvest meter via a solenoid valve (S-6 fig. 2) which is actuated by the level controller. The harvest is measured and the output monitored by a digital counter (C4). The digitalized outputs of gas consumption and harvest are registered by a camera, set to take a frame at preselected time intervals.

Production rates of 50 to 90 grams (D.W.) per 24 hours were obtained with a working suspension of 3 liters at a population density of 5 to 6 grams (D.W.) per liter. The best rates observed thus far indicate K-values of 5 (day^{-1}) or 0.2 (hr^{-1}).

III. THE CHEMICAL AND PHYSICAL ENVIRONMENT

1. Temperature

The maximum rate of cell reproduction (growth) occurred[9] at 34° to $35^\circ C$. At $40^\circ C$ an enhanced rate of gas consumption was observed for a short time but growth was negligible. At $20^\circ C$ the rate of growth was approximately one-fourth the rate observed at $35^\circ C$.

Within a temperature range of 25° to $35^\circ C$ conversion efficiency was essentially constant (Table 1); Q_{10} - values for growth were of the order of 1.5 to 2.0.

TABLE 1

Effect of Temperature on Conversion Efficiency[*]

# of Exps.	Temperature ($^{\circ}$C)	O_2/CO_2
13	35	2.4 ± 0.1
10	25	2.8 ± 0.2

---

[*]Conversion efficiency is expressed as the number of oxygens utilized (in the combustion of hydrogen) for the conversion of each carbon dioxide.

2.   pH Effects.

A neutral pH produced the optimal growth rate.

3.   Inorganic Nutrition.

The nutritional requirements for Hydrogenomonas eutropha were described by Repaske[3]. Bartha[10] found a requirement for Ni and Eberhardt[11] observed stimulation of hydrogenase activity by Mn and Co. A simplified medium was described by Bongers[12]. He found rapid growth with H. eutropha and H-20 in media containing either urea or ammonia nitrogen in the presence of Mg, P, and ferrous iron. Contaminants in the major salts made supplementation with trace elements unnecessary. This information on inorganic nutrition for batch cultures is summarized in Table 2.

In the turbidostat the steady-state nutrient concentrations, cell multiplication rate and all other properties of the cell suspension must be kept at a constant level. To maintain the optimal steady-state nutrient

TABLE 2

GROWTH REQUIREMENTS OF HYDROGENOMONAS EUTROPHA AND H-20

Temperature	33C - 35C
Hydrogen	50-560 mm
Oxygen	120-150 mm
Carbon dioxide	70-80 mm
$CO(NH_2)_2$	0.5 g/liter
$MgSO_4 \cdot 7H_2O$	0.1 g/liter
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.008 g/liter
Phosphate (Na, K)	0.03M
pH	6.5-7.5

concentrations, the concentration of the nutrients in the feed medium must be higher than those in the suspending medium, since the inflowing medium is diluted by the suspending medium to an extent which is a function of the consumption of the medium constituents by the cells. The total mass balance for nitrogen is illustrated in the following equation:

$$N_m = N_s + N_p$$

where N_m is the nitrogen concentration of the feed medium, N_s the concentration of the substrate nitrogen and, N_p the concentration of the protein (expressed as nitrogen) in the suspension. Thus, the nitrogen concentration of the feed medium depends upon the protein concentration of the suspension (which determines the nitrogen consumption rate) and upon the level of substrate nitrogen maintained. With suspensions of relatively low population density (low protein concentration) and with a relatively low substrate nitrogen concentration, the nitrogen concentration in the feed medium can be relatively low. With a high cell density (high protein concentration) a relatively high nitrogen feed rate is necessary. A mismatched nitrogen feeding rate, and consequently, a transition to a new steady-state occurs if the actual nitrogen consumption rate deviates from the expected value. Deviations in the nitrogen consumption rate may occur if the population characteristics of suspension change with time; example given, if the ratio of active metabolizing cells (working suspension) over resting cells (inactive population) change in the course of an experiment. The validity of this formulation for N feed rate was tested for different levels of cell concentrations and the results will be discussed later.

A problem associated with nitrogen is the extremely rapid decomposition of urea, the primary nitrogen source in a closed environment. According to König[13] a rapid conversion of urea into ammonia and carbon dioxide is to be expected in a suspension cultivated in the presence of relatively low concentrations of ammonia. Apparently under these conditions the formation of cells with a high urease activity result (see fig. 6). We confirmed these observations.

It would thus appear that the presence of a given concentration of ammonia in a suspending medium may be necessary to prevent the buildup of relatively high urease activity. We were, however, unable to detect any urea in rapidly growing steady-state cultures in which a constant level of ammonia nitrogen was maintained and urea nitrogen constituted the main nitrogen source. Apparently, also in the presence of ammonia a rapid urea decomposition occurs.

A rationale similar to that for nitrogen applies to other elements (such as P, Mg, Fe and trace elements) required for cell reproduction. However, since these elements are tolerated at relatively high concentrations and because their utilization is limited, one would presume that a substrate concentration approaching the concentration of the feed medium would lead to a steady-state concentration conducive to rapid cell reproduction.

While the requirements for N, P, and Mg for steady-state cultures (up to 5-6 grams (D.W.) of cells per liter) are resolved, those for trace elements still present a problem. We have found a significant increase in the rate of cell production by addition of tap water to a medium which contained all trace elements known to be necessary. No such effect was observed with batch cultures. A study concerning the trace element requirement is presently in progress.

4. Gas Supply.

With regards to the gaseous substrate, which provides the suspension with energy and carbon dioxide, it was found that the hydrogen concentration could be varied widely (40 mm - 600 mm) with little effect upon yield. On the other hand, the regulation of carbon dioxide and oxygen proved more critical. Carbon dioxide pressures up to 90 mm were tolerated; with concentrations in excess of this value growth was progressively impeded.

The oxygen partial pressure of the growing suspension had a relatively strong effect on the rate of growth, the efficiency of energy conversion and the metabolic activity with regard to product formation.

At relatively low oxygen concentrations (35 mm - 60 mm), the rate of carbon dioxide conversion was up to 50% higher than was observed with 150 mm oxygen. However, this relatively high conversion rate was not stable. Upon prolonged incubation under these conditions, the rate of carbon dioxide fixation declined and formation of intracellular lipid inclusions occurred. Subsequently, the rate of cell division diminished.

At relatively high oxygen concentrations (110 mm - 160 mm) no lipid inclusions were formed, and the rates of cell division and carbon dioxide fixation remained constant (fig. 7). The efficiencies of energy conversion were somewhat less than the values observed with low oxygen concentrations (see Table 3).

TABLE 3

Oxygen supply and energy conversion. Gas phase: 70% H_2, 10% CO_2 and O_2 as indicated. Urea medium; temperature $35°C$.

% O_2	5	10	15	20
O_2/CO_2	1.9 ± 0.2	2.1 ± 0.1	2.9 ± 0.2	3.0 ± 0.2

5. Material Balance in a Closed Environment.

In the above discussion the assumption is made that carbon dioxide was the only component of the closed environment which had to be dealt with and that the other ingredients necessary for cell synthesis would be available in the proper ratio to carbon dioxide. This assumption is more or less correct, with the exception of urea. A C/N ratio of approximately 4/1 is required to provide optimal conditions for the biosynthesis, while the ratio at which carbon dioxide and urea are supplied approaches 4/.25 if metabolic wastes are the sole contributors. A material balance with regard to nitrogen can thus not be expected, i.e. optimal conditions for the conversion of all carbon dioxide on hand cannot be maintained. Conditions must therefore be applied to the bacterial suspension which lead to the production of a cell mass which is relatively poor in protein-nitrogen. Nitrogen starvation alters the distribution of products of carbon dioxide fixation and changes cell characteristics in a rather similar fashion as those observed if suspensions are incubated at relatively low oxygen concentrations. It is

obvious that this behavior is not compatible with cultivation under steady-state conditions. In order to cope with this problem, cultures staging is proposed. This technique would involve cultivation in two phases, occurring in two separate chambers: one culture chamber containing all available nitrogen, while in a second chamber growth would occur in the absence of substrate nitrogen. No information on the adequacy of this approach is presently available. It is expected, however, that this technique will produce the best nitrogen economy in a closed environment, but preliminary investigations concerning the suitability of nitrogen-starved material as a food source are not encouraging.

IV ENERGETICS

1. Efficiency of ATP Formation

Hydrogen is utilized by hydrogen bacteria for reductive and energy yielding purposes. Hydrogenase, an enzyme which mediates the utilization of hydrogen, catalyzes the reduction of a yet unidentified primary acceptor which subsequently transfers electrons to oxygen via mediators such as pyridine nucleotide, flavins and cytochromes. In this process, the two reactants essential for CO_2 assimilation are generated: reduced pyridine nucleotide ($NADH_2$) and adenosine triphosphate (ATP).

The hydrogenase-mediated generation of $NADH_2$ has been demonstrated in hydrogenomonads[14,15] and other hydrogenase containing bacteria[16]. However, relatively little is known about the electron transport sequence at the reducing site of the respiratory chain. Repaske[15] assumes a requirement for FMN as a cofactor in the reduction of NAD, and has evidence that another coupling factor between hydrogenase and NAD may be operative[17].

This latter coupling factor is presumed to have a similar function as ferridoxin in anaerobic microorganisms and in green plants.

Oxidation of NADH proceeds at too slow a rate to accomodate the relatively high rates of hydrogen oxidation usually observed. The pathways and the cofactors involved in the oxidation of NADH are not well defined.

The formation of ATP by cell-free preparations of hydrogenomonads grown autotrophically was studied in some detail[18]. The results are summarized in Table 4.

Oxidative and phosphorylative activities were always considerably higher with hydrogen than with other electron donors (Table 4). P/O values with hydrogen varied somewhat, but were never in excess of 0.8. Phosphorylation associated with hydrogen oxidation was unaffected by antimycin A and terminal oxidase inhibitors (CN^-, CO, N_3^-).

With succinate as a substrate instead of hydrogen, the rates of oxidative and phosphorylative activities were some 5-fold lower and the P/O values were equal or somewhat less. This phosphorylation was comparatively sensitive to antimycin A.

With both substrates present $(H_2$, succinate), the rate of phosphorylation was equal to the sum of the rates found with each substrate separately. An equivalent increase in O_2 uptake was also observed.

The simplest interpretation of the observed phenomena is that phosphorylation is occurring independently at two different sites in the respiratory chain. The potential range for phosphorylation apparently is between hydrogen and cytochrome b when hydrogen is the substrate, and between cytochrome b and oxygen when succinate is the substrate. Whether phosphorylating activity with succinate alone as substrate involves one or two phosphorylating sites is unknown.

TABLE 4

The Effect of Various Substrates and Inhibitors on Phosphorylation

| Substrate	Inhibitor	$\dfrac{\Delta \text{ μmoles } P_i}{hr}$	$\dfrac{\Delta \text{ μatom } 0}{hr}$	P/0
H_2	-	6.47	12.4	.52
Succinate	-	1.23	6.8	.18
H_2 + Succinate	-	7.51	16.9	.44
β-OH-Butyrate	-	0.38	1.1	.35
$NADH_2$	-	0.11	5.4	.02
		% of Control[a]		
	3×10^{-4} M CN^-	38	37	.48
	10^{-4} M N_3^-	100	98	.45
H_2	20% CO	93	100	.49
	2.5 γ Antimycin A	97	95	.45
	10 γ Antimycin A	97	62	.30
		% of Control[b]		
Succinate	2.5 γ Antimycin A	84	47	.19
	5 γ Antimycin A	69	0	0

a) Values in absence of inhibitors were 9.2 μatoms of oxygen and 4.26 μmoles of P_i.

b) Values in absence of inhibitors were 4.65 μatoms of oxygen and 1.56 μmoles of P_i.

Our results suggest an oxidation of reduced cytochrome b by oxygen. This could be demonstrated spectrophotometrically (see fig. 8); distinct absorption by cytochrome b obtained from a cell-free preparation incubated with hydrogen, rapidly disappeared upon introduction of O_2 in the presence of cyanide concentrations sufficient to prevent oxidation of cytochrome c. Whether this reaction between oxygen and cytochrome b occurs to the same extent in the absence of CN^- is unclear. However, judging from the relatively low P/O ratios observed with succinate and from the inability of antimycin A to significantly inhibit oxidative activity with this substrate one would assume that even in the absence of CN^- some autooxidation occurs at the cytochrome b level. As will be discussed later, spectroscopic observations on cell suspensions substantiate this assumption.

2. Efficiency of CO_2 Conversion.

Hydrogen bacteria grow by a combination of energy-yielding (eq. 2) and energy-consuming (eq. 1) processes, summarized in the following equations:

$$2H_2 + CO_2 \rightarrow (CH_2O) + H_2O \tag{1}$$

$$4H_2 + 2O_2 \rightarrow 4H_2O \; (+ nATP) \tag{2}$$
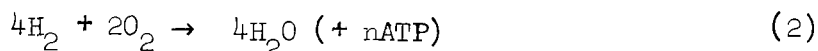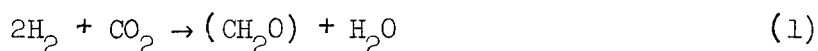
The efficiency of CO_2 conversion can be expressed as an O_2/CO_2 quotient: the molal ratio of molecules of oxygen utilized (eq. 2) for the conversion of one molecule of CO_2. Observed quotients are usually of the order of 2. If ATP generation in intact cells is as efficient as that observed in mitochondrial systems, then approximately 12 moles of ATP should be made available in the combustion reaction (eq. 2), and utilized in the energy-consuming

processes (eq. 1). Such an energy expenditure would be twice the amount required for an efficient system. However, since our experiments on cell-free preparations suggested that cytochrome b has a high affinity for oxygen, one would assume that an "abbreviated" respiratory chain might be operative in the intact cell. The effect of a number of terminal oxidase inhibitors on the efficiency of CO_2-conversion was tested in order to evaluate the significance of the autooxidizability of cytochrome b.

The elucidation of the actual mechanism constitutes not only an area of much interest, but has also its practical implications. If, e.g. 3 ATP instead of 1 ATP could be generated for each oxygen atom utilized, the rate of electrolysis required for waste regeneration could be considerably lower than presently anticipated. This study was undertaken to obtain evidence on this point.

The experiments were conducted at $30^{\circ}C$ in a regular growth medium and an atmosphere of H_2, O_2 and CO_2. The number of inhibitors which can be used with intact cells is limited; only Site III (between cytochrome c and O_2) can effectively be inhibited by cyanide, azide or carbon monoxide. No effective inhibitors are known which block the electron transfer at site II (between cytochrome b and cytochrome c) in intact cells. However, since cytochrome c is not autooxidizable (for spectroscopic evidence see fig. 8 and fig. 9), it is unlikely that in the presence of terminal oxidase inhibitors Site II will contribute substantially in overall ATP generation.

TABLE 5

THE EFFECTS OF INHIBITORS ON CONVERSION EFFICIENCY[*]

Series	Incubation	Condition of Incubation					CO_2 Fixation	O_2 Consumption	Efficiency
	(min.)	Gas phase (%)				Inhibitor	(μmoles/hr)	(μmoles/hr)	O_2/CO_2
		H_2	O_2	CO_2	N_2				
	80	60	15	5	20	Control	32	63	1.95
	80	60	15	5		20% CO	17	43	2.50
I	90	60	15	5	20	Control	41	95	2.30
	90	60	15	5		20% CO	22	45	2.05
	0	70	20	10		Control	3.8	8.0	2.1
	10	70	20	10		10^{-4}M NaN$_3$	4.3	8.7	2.0
II	35	70	20	10		10^{-4}M NaN$_3$	3.7	8.8	2.4
	60	70	20	10		Control	4.3	8.2	1.9

Series	Incubation	Conditions of Incubation			Gas Uptake	CO_2 Fixation	Counts/μl
	(min.)	Gas phase (%)		Inhibitor	(μl/flask)	(CPM)	
		H_2	O_2	N_2			
	10	55	5	40	Control	370	54,000	146
	10	55	5		40% CO	127	23,400	184
	10	80	20		Control	152	16,100	106
	10	80	20		10^{-4}M NaN$_3$	150	15,700	104
	10	80	20		10^{-3}M NaN$_3$	124	9,700	78
III	14	80	20		Control	226	23,000	102
	14	80	20		10^{-4} CN$^-$	86	9,900	115
	10	80	20		Control	165	16,400	100
	10	80	20		5×10^{-5}M CCCP	130	2,500	19

[*]See page 21.

TABLE 5 (contd)

THE EFFECTS OF INHIBITORS ON CONVERSION EFFICIENCY[*]

[*]Experimental conditions were as follows: The starting density of Series I was 2.15 mg/ml (dry weight). The O_2/CO_2 ratio was calculated from the increase in dry weight and the gas uptake. The starting density of Series II was approximately 3.3 mg/ml (calculated from turbidity). The suspension was sampled at specified time intervals, the aliquot diluted with fresh medium containing 10^{-4}M NaN_3 (the control suspension was diluted with medium without azide) to a density of 0.20 mg dry weight/ml (calculated from turbidity readings), and equilibrated with a gas phase containing 68% H_2, 22% O_2 and 10% CO_2. The O_2/CO_2 quotient was calculated from the difference in oxygen uptake with and without iodoacetate (5×10^{-3}M). The experiments, recorded under Series III, were carried out in Warburg flasks; .4 to 6 mg dry weight (in 2 ml) per flask. Carbon dioxide was administered as bicarbonate (specific activity 0.8 µc/µmole). After a pre-incubation of 10 minutes the bicarbonate was added from the side arm, the gas uptake recorded, and the reaction terminated by the addition of acedic acid. The activity was measured in 0.1 ml suspension. The activity of suspensions incubated under air or under O_2 + CO mixtures was 1% to 4% of the activity observed in the presence of H_2 and O_2. Efficiency was expressed as counts fixed per volume of gas consumed.

The three inhibitors cyanide, azide and carbon monoxide not only inhibit electron transfer via cytochrome a to oxygen, but also react with other metalloenzymes (e.g. hydrogenase, catalase, aldolase). Due to these "side" effects a considerable decrease in the rate of growth can be expected; however, of interest is their effect on conversion efficiency.

In Table 5 a number of observations of conversion efficiency in the presence and in the absence of terminal oxidase inhibitors are listed. The conversion efficiencies are expressed as the O_2/CO_2 ratio or, in case of C^{14} as the number of counts over total gas uptake.

Under normal conditions of cultivation the O_2/CO_2 ratio is 2. The data in Table 5 indicate that the ratio is not substantially changed in the presence of cyanide, azide or carbon monoxide. Consequently, one must conclude that Site I also satisfied the necessary ATP requirement in the uninhibited system.

CO_2 assimilation observed in the presence of H_2 and O_2 is dependent upon the formation of ATP, which is generated by oxidative phosphorylation. This is evident from the relatively small amounts of C^{14} assimilated in cells incubated in the presence of an uncoupler (CCCP) of oxidative phosphorylation. The CO_2 assimilation observed is thus a net synthesis which does not depend upon substrate level phosphorylation.

Spectrophotometric observations on the behavior of cytochrome b in intact cells, illustrated in fig. 9 gave results similar to those with cell-free extracts (see fig. 8). In the presence of air and CN^- only absorption due to cytochrome c (fig. 9, curve 2) is observed, while cytochrome b is completely oxidized. The observations support the conclusion that an active cytochrome b-oxidase is operative in the intact cells.

3. Effect of Oxygen on Cytochromes.

In most aerobic cells, electrons released from the substrate are serially transferred via cytochrome $\underline{b}$, cytochrome $\underline{c}$ and cytochrome $\underline{c}$ - oxidase to oxygen. Spectral observations have shown the presence of cytochrome $\underline{b}$ and cytochrome $\underline{c}$ in intact cells and in cell-free preparations, but have failed thus far to establish the presence of cytochrome $\underline{c}$-oxidase in $\underline{H}$. $\underline{eutropha}$ and $\underline{H}$-$\underline{20}$. As described in the previous section, there is good evidence that cytochrome $\underline{b}$ can function as terminal oxidase in the presence of CO, CN^- and N_3^- since it is an autooxidizable component which presumably does not combine with these inhibitors. The main unsolved questions are:

a) The effectiveness of cytochrome $\underline{b}$ as terminal oxidase in normal conditions of growth (i.e. in the absence of inhibitors).

b) The mechanism by which the c-type cytochrome is reduced and oxidized.

A detailed study of these aspects of electron transport in hydrogenomonads is underway; some preliminary results of the effect of oxygen/hydrogen mixtures on the redox state of cytochrome $\underline{b}$ and cytochrome $\underline{c}$ will be briefly discussed.

Spectral observations were made with a dual-beam spectrophotometer. The cell suspension, contained in a (3 ml) thermostated cuvette, could be equilibrated with oxygen/hydrogen mixtures of known composition and simultaneously, the steady-state absorption characteristics of the cytochromes recorded.   A reference wavelength of 650 mμ was used to measure the response of cytochrome $\underline{b}$ (absorption maximum 560 mμ) and of cytochrome $\underline{c}$ (absorption maximum of 552 mμ). Since the total absorption due to cytochrome $\underline{c}$

is approximately twice that of cytochrome $\underline{b}$, changes at the cytochrome $\underline{c}$ level were measured at 548 to 549 mμ. In this fashion equal absorption of both cytochromes is obtained.

In fig. 10 the redox states of cytochrome $\underline{b}$ (closed symbols) and cytochrome $\underline{c}$ (open symbols) as a function of O_2/H_2 mixtures are illustrated. Complete reduction of the cytochromes is assumed if the suspension is flushed with hydrogen. This is indeed so in case of cytochrome $\underline{c}$. The absorption difference between the hydrogen reduced and air oxidized suspension (H_2-O_2 difference) usually equaled the difference obtained with dithionite reduced and ferricyanide oxidized suspensions (D-F difference). However, complete reduction of cytochrome $\underline{b}$ was never observed in the presence of hydrogen. The discrepancy between the (D-F) and (H_2-O_2) absorption increased with decreasing temperatures. At 30°C, the (H_2-O_2) difference was approximately 75% to 90% of the (D-F) difference, while at 1°C hydrogen was capable of reducing only some 60% of cytochrome $\underline{b}$. This temperature response of cytochrome $\underline{b}$ corroborates the oxidation kinetics illustrated in fig. 10. Apparently, the trace amounts of oxygen present in hydrogen are sufficient to partially oxidize cytochrome $\underline{b}$.

Equilibration with increasing amounts of oxygen (up to approximately 5% in H_2) results in further oxidation of cytochrome $\underline{b}$. With mixture containing 5% to 25% oxygen, the redox state of cytochrome $\underline{b}$ is little affected. Apparently, at these O_2/H_2 ratios, reduction and oxidation are in balance.

Little change in the redox state of cytochrome c is observed with O_2/H_2 mixtures containing up to 5% O_2 (see fig. 10, open symbols). If the proportion of O_2 in the mixtures is further increased, a gradual oxidation of cytochrome c is observed. Complete oxidation of cytochrome c usually occurs at lower O_2/H_2 ratios than does cytochrome b.

The observations of the redox state of cytochrome c as a function of O_2/H_2 concentrations is provisionally interpreted on the basis of the assumption that oxidation of cytochrome c is mediated by a relatively "poor" oxidase. This would account for the rapid oxidation of cytochrome c with mixtures containing 15% to 25% O_2.

At low oxygen concentrations (up to 5% in H_2), cytochrome c is virtually in a reduced state, and thus is an inefficient electron acceptor. Presumably, under these conditions cytochrome b-oxidase is predominant, and the electron transport to oxygen occurs (at a reduced rate) via this pathway.

It is not clear whether at the higher oxygen concentrations ferro-cytochrome b is also oxidized by ferri-cytochrome c. However, some insight to the sequence of reduction and oxidation of the two cytochromes was gained, suggesting that under certain conditions such a transfer is possible. The curves of fig. 11, obtained by measuring the transition from oxidized to the reduced state, show that upon the introduction of hydrogen into air-equilibrated suspension, cytochrome b is initially reduced more rapidly than cytochrome c, although a steady-state is subsequently established. A rapid transition then occurs, indicating that cytochrome c

is being reduced further than cytochrome b. Upon further substitution of oxygen with hydrogen, cytochrome b becomes gradually reduced.

Introduction of air (fig. 11, solid line) in the hydrogen-reduced sample results in a relatively fast oxidation of cytochrome b, and a relatively slow oxidation of cytochrome c as indicated by an absorption change in the direction of (reduced) cytochrome c. After a steady-state (b and c oxidize at the same rate) cytochrome c becomes completely oxidized. If the oxidation is carried out at a lower temperature (fig. 11, dashed curve) an "overshoot" in the direction of reduced cytochrome b is observed. This indicates that complete oxidation of cytochrome b occurs after complete oxidation of cytochrome c.

The results illustrated here give only an indication with respect to the sequence of reduction and oxidation, but do not represent quantitatively the redox state of the cytochromes. The oxidation kinetics, however, are in complete agreement with the results illustrated in fig. 10.

Data presented indicate that the oxidative energy-generating system of hydrogenomonads can, under certain conditions, utilize an abbreviated electron transport chain, terminating at cytochrome b. Evidence supporting this view was obtained from ATP formation and efficiency of CO_2 fixation in the presence and in the absence of certain inhibitors, as well as from observations concerning the redox states of cytochrome b and c as a function of oxygen concentration. Possibly the best interpretation of these data is that at relatively low oxygen concentrations, the usual growth conditions of the cells, the abbreviated chain provides the bulk of energy for synthesis.

But this assumption does not provide a solution for the puzzling behavior of cytochrome c. If one assumes that cytochrome c is mainly reduced by NAD - cytochrome c - reductase, a pathway also known to be present in hydrogenomonads[19], then one could conclude that the oxidation of cytochrome c results in expenditure of NADH. Because conversion efficiencies are consistently lower at relatively high oxygen concentrations (see Table 3), this pathway must be defined as uneconomical for the cell.

A tentative electron transport system which in part accounts for the above is illustrated in fig. 12. This scheme includes "X", a primary, yet unidentified acceptor, presumably having a redox potential of the order of ferridoxin, and an unidentified quinone "Q"[18]. NAD is not included in the abbreviated electron transport sequence for reasons discussed earlier[18]. Briefly, it is considered that the main function of NADH is to provide the cell with reducing equivalent for CO_2 reduction, rather than to provide energy via oxidative phosphorylation. The scheme is a tentative one, because determinations of the turn-over rates of cytochrome b and cytochrome c oxidase and characterization of the oxidases will be necessary to define their contribution in electron transport.

V. TURN-OVER CHARACTERISTICS

Long-term performance of a continuous (pilot-size) culture of bacteria (Aerobacter cloacae) was most informatively characterized by Herbert and co-workers[6] in the statement; "The longest individual run lasted 108 days and was still free from contamination when terminated voluntarily". Their results indicate that, (1) reliable continuous operation can be achieved, provided proper aseptic techniques are employed,(2) no detectable mutations developed (genetic stability) and (3) constant metabolic activity can be obtained (physiological stability).

The same level of sophistication has yet to be reached with continuous cultures of hydrogen bacteria. Our longest continuous run to date has been 34 days, the first 25 days of which are summarized in fig. 13. A malfunction in the H_2-pressure regulator forced a shut-down on the 26th day, recovery of the suspension was incomplete and the culture was subsequently discontinued on the 34th day.

The objective of the experiment to be described was to establish the relationship of the combined effects of the cell population density and the composition of the input medium upon the production rate. The culture was started by heavy innoculation at the indicated density (see fig. 13, solid curve) and maintained at this level for 5 days. During this interval cell concentration was relatively constant; variations were less than 4%. Cell production (average specific rates, see fig. 13, dashed curve) varied considerably (10% to 20%).

TABLE 6

Nutritional Composition of Input Medium (Run #21; H-20)

Nutrients		0	4	5	6	11	15	18	20	23	25
							Days				
Urea	(g N/l)	0.47	0.52	0.57	0.57	.72	.72	.86	.89	.72	.72
$(NH_4)_2 SO_4$	(g N/l)	0.23	0.23	0.29	0.29	.36	.36	.36	.36	.48	.48
$MgSO_4 \cdot 7H_2O$	(M)	0.006	0.006	0.006	.008	.008	.008	.008	.008	.008	.008
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	(M)	4×10^{-5}	4×10^{-5}	4×10^{-5}	4×10^{-5}	4×10^{-5}	4×10^{-5}	4×10^{-5}	4×10^{-5}	4×10^{-5}	4×10^{-5}
$\overline{PO_4}$ (K/Na = 3/1)	(M)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Trace Elements*	(ml/l)	1	1	1	1	1	3	3	3	5	3

* Trace Element (Stock Solution)

	$M \times 10^{-3}$
H_3BO_3	1.62
$ZnSO_4 \cdot 7H_2O$	.53
$MnSO_4 \cdot 4H_2O$	.45
$CoCl_2 \cdot 6H_2O$	.42
$Na_2MoO_4 \cdot 2H_2O$	.41
$CuSO_4$	.63
$NiCl_2$	.10

Continuous culture of H-20, turbidostatically controlled. Temperature: 32°C; oxygen concentration maintained at approximately .12 mM; carbon dioxide partial pressure varied from approximately 50 to 80 mm; pressure maintained with H_2 at 2 to 3 psig.

Population density expressed as optical density (an OD of 10 equals approximately 10 grams wet and 3 grams dry weight per liter). The rate of harvest output is expressed as grams of dry weight per liter and per hour.

The population was allowed to increase (e.g. on the 5th day) in small increments (of approximately one OD) by inactivating the density control. After the small increment was attained, the culture was operated automatically for approximately half an hour. During this interval, cell concentration was maintained constant by the addition of the more concentrated medium (for composition see Table 6). The incremental steps were repeated until the desired population density was achieved.

The specific output rate was fairly constant during the first nine days. Then (10th to 15th day) a significant decrease was observed, presumably caused by malfunctioning oxygen sensor. However, after adjustment of the sensor recovery was incomplete, until the trace element concentration was tripled on the 15th day. No direct relationship between cause and cure is evident, however. Supplementation with trace elements was accompanied by a voluntary increase in population density. Good production was subsequently obtained indicating that all nutrient demands were satisfied. On further increasing the density (19th day) the harvest output declined sharply. This decline in production was probably due to lack of CO_2 (see section II, 2), but other nutritional deficiencies may have also contributed. The operation at these relatively high densities is currently being investigated.

Phosphate and nitrogen concentrations in the suspending medium were determined daily. The phosphate concentration (.03M) of the input medium proved adequate. The phosphate concentration of the suspending medium stabilized at a slightly lower level (.029 to .027), depending on population density.

During the first 17 days of operation, the nitrogen concentration in the suspending medium was balanced at ~ .30 grams per liter: the expected level for the combination of cell population and nitrogen input employed. The results satisfy the mass balance relationship (see III, 3). The low specific rate of cell production observed during the last 5 days of operation is accompanied by an increase in the level of suspension nitrogen; one would expect that this increase is caused by decomposition of cells.

Nitrogen was supplied as a urea-ammonia mixture (see Table 6); it was found earlier that the suspension-pH could thus be maintained by relatively low phosphate buffer concentration. While the feed medium contained twice as much urea as ammonia, little or no urea was detected in the suspending medium. Since all urea is rapidly decomposed, urease control by ammonia is obviously not operative under the conditions employed (c.f. fig. 6).

The best specific output rates (fig. 13, 15th to 19th day) were of the order of .80 grams (D.W.) indicating a K-value of .16 to .20 (hr^{-1}). If this rate is expressed as volume of CO_2 converted per gram (D.W.) of working suspension, the hourly specific rate is 150 ml*. A value of the same order is observed at the relatively low cell densities (0 to 10 days, fig. 13). Presumably such rates can be maintained at still higher cell concentrations (8 to 10 grams), but it is not expected that a substantial increase in the specific rate can be realized. The earlier calculations[1,2] indicating

---

*Values of the same order of magnitude obtained with batch cultures were reported earlier[1,2].

that a suspension volume of some 20 liters would provide sufficient regenerative capacity for the carbon dioxide output ($\sim$ 22 liters) of one man seem to be borne out by the results described here.

No attempts were made to resolve simultaneously the efficiency of energy conversion in continuous cultures. However, the measured specific rates warrant the expectation that energy utilization, which is fairly constant under average conditions, will not greatly exceed the values stated in fig. 1.
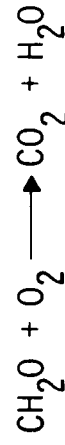
VI. FIGURES

Figure 1 - Flow diagram of a life support system based on the coupling
electrolysis of water and biosynthesis by hydrogen bacteria.

CHEMOSYNTHETIC ECOSYSTEM

HUMAN RESP: $CH_2O + O_2 \longrightarrow CO_2 + H_2O$
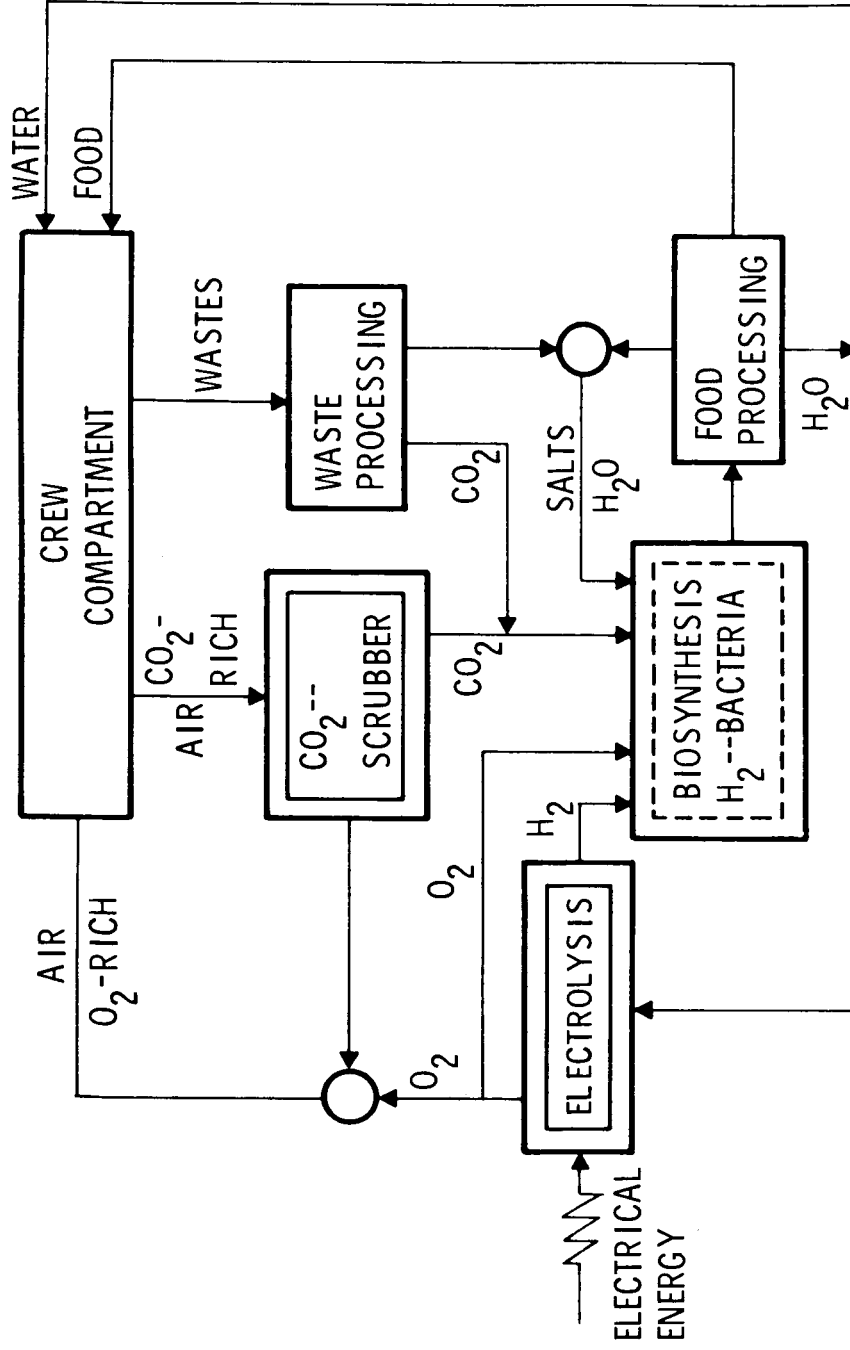
BIOSYNTHESIS: $6 H_2 + 2 O_2 + CO_2 \longrightarrow CH_2O + 5 H_2O$

ELECTROLYSIS: $6 H_2O \longrightarrow 6 H_2 + 3 O_2$

ELECTRICAL $\left(\begin{array}{c} 600 \text{ to } 1000 \\ \text{WATTS CONT} \end{array} \right)$
ENERGY

Figure 2 - Continuous culture apparatus, turbidity controlled.

Camera

O₂   H₂/CO₂   H₂

S₅   S₄   S₃

Mv   Mv   Mv

C₁ C₂ C₃ C₄

Pressure Sensor

B

P₁

P₂

CO₂ Sensor

O₂ Sensor

Acid or Base

Nutrient

pH Controller

S   S

Density Controller

Level Control

Harvest Meter

S₆

HARVEST

P₁ Gas recirculation pump
P₂ Control loop pump
S   Solenoid valve
B   Baffle
C₁ thru C₃ Digital (elapsed-time) clocks
C₄ Digital counter
Mv Metering valve
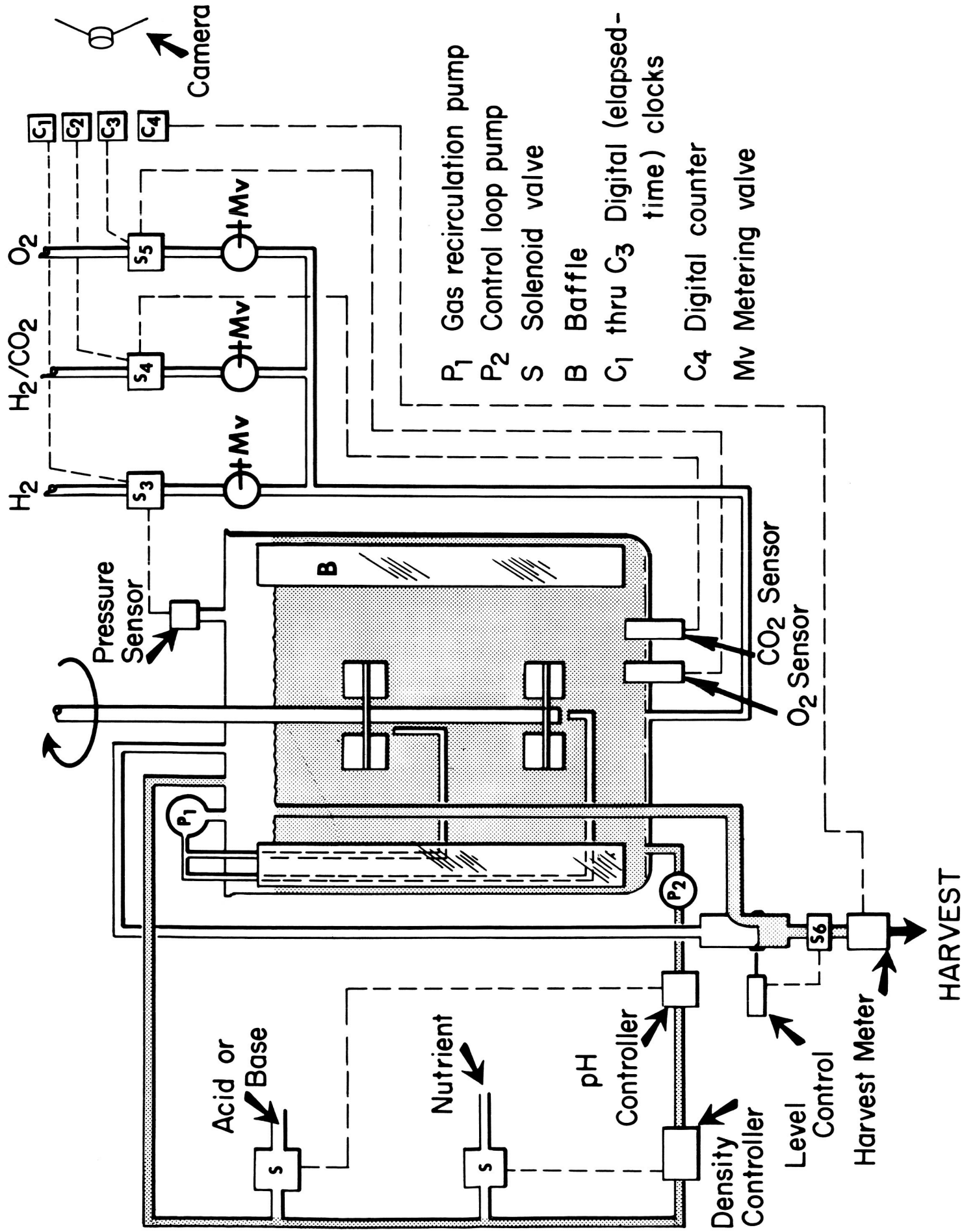
Figure 3 - pH chamber (lucite), actual size.

Combination Electrode

Lucite Block
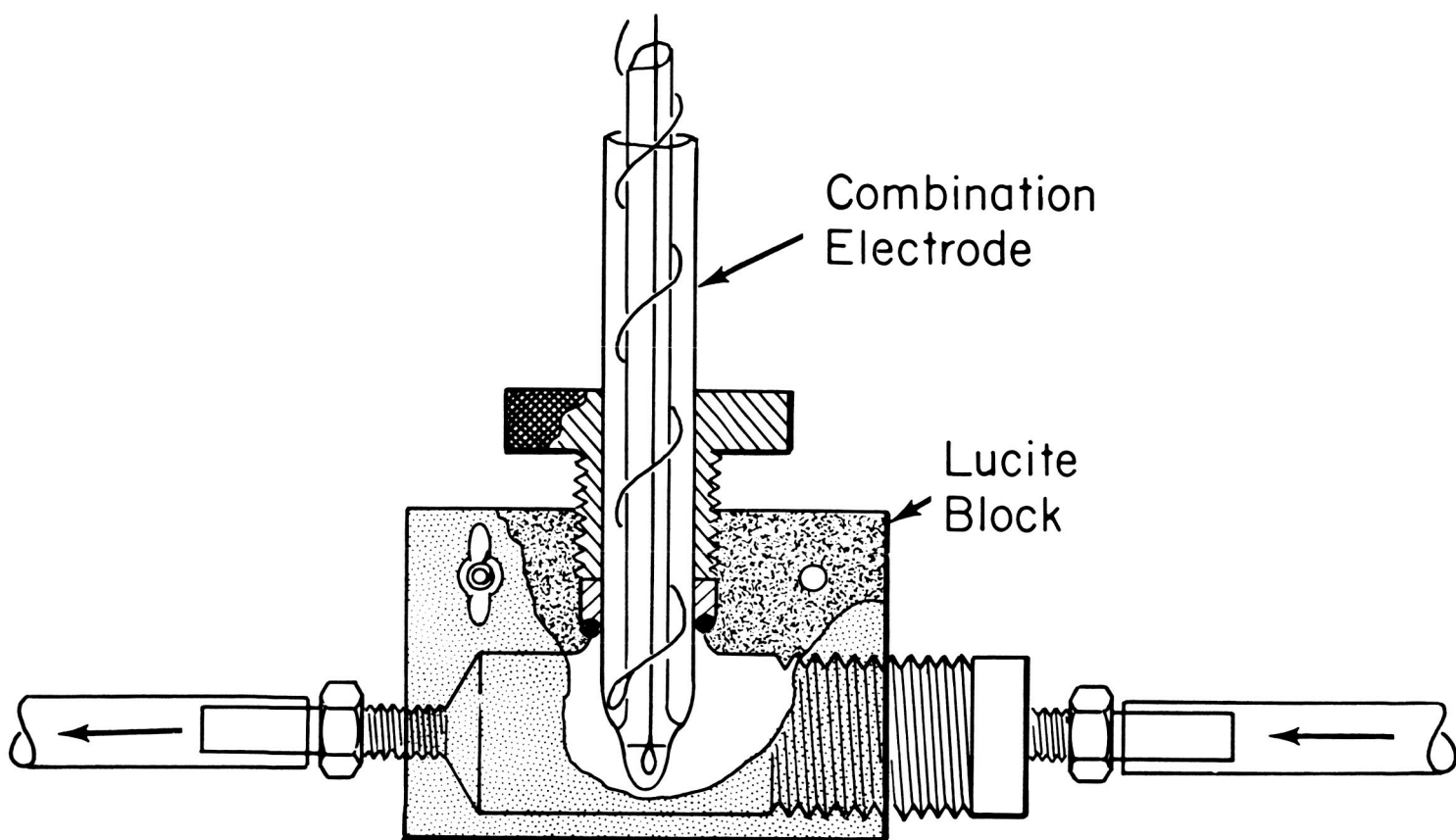
Figure 4 - Density control (sample) chamber, actual size.

GE 253X

Glass Window

.8 mm
1.6 mm
3.2 mm

Teflon
Gasket

Glass Window

CLAIREX
CL 607
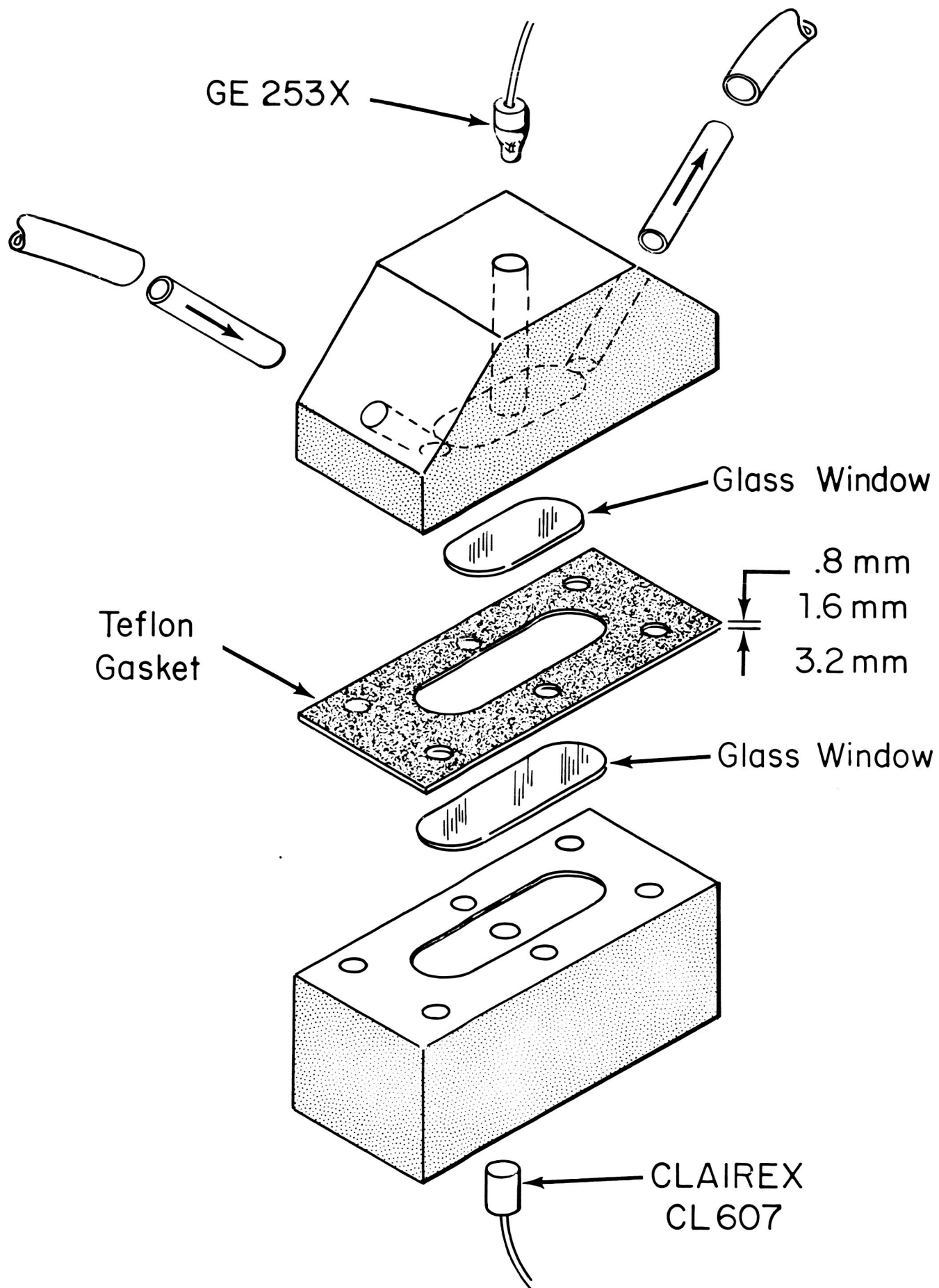
Figure 4A - Density control circuitry
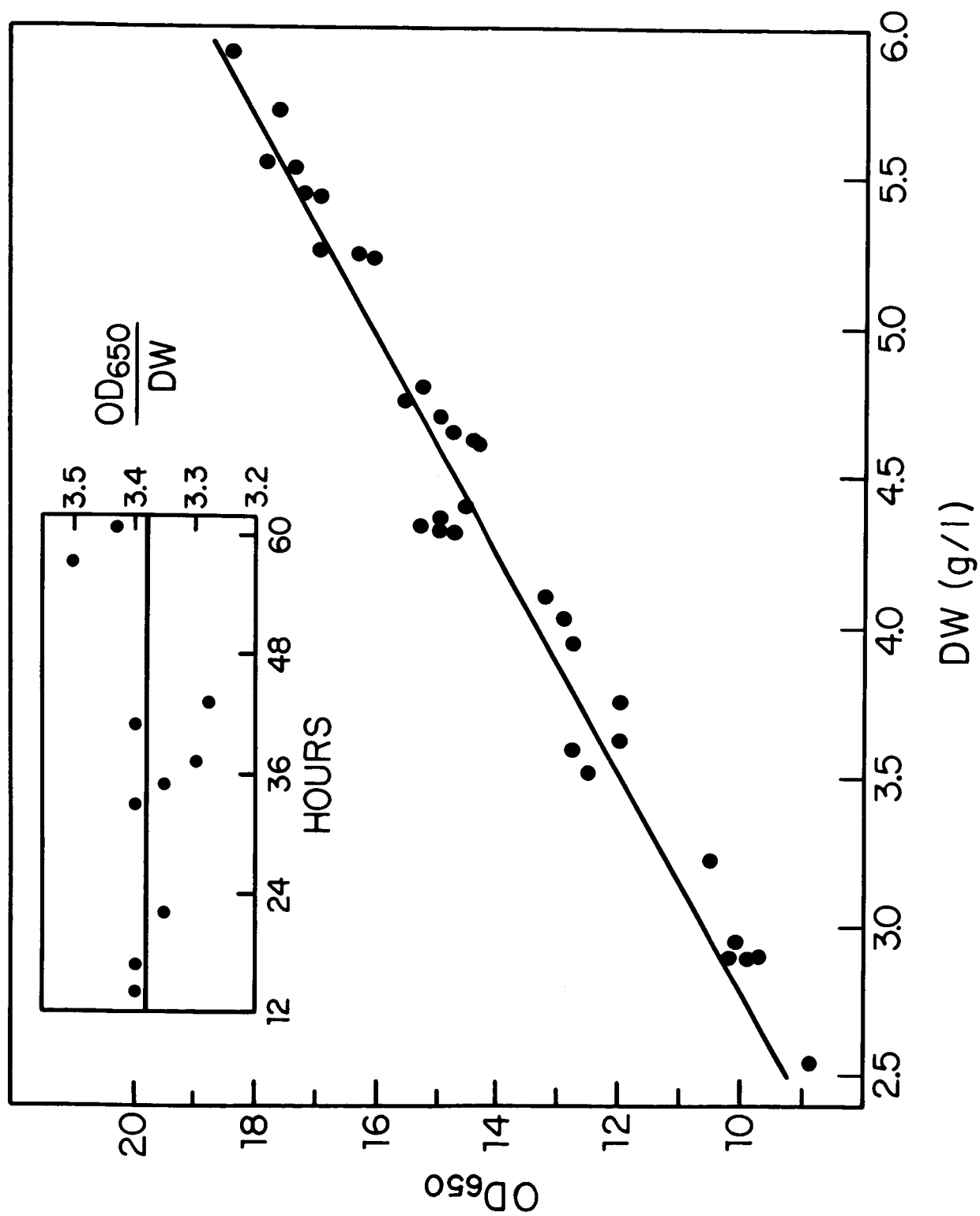
Figure 6 - Effect of ammonia on urease activity of H-16 (König[13]).
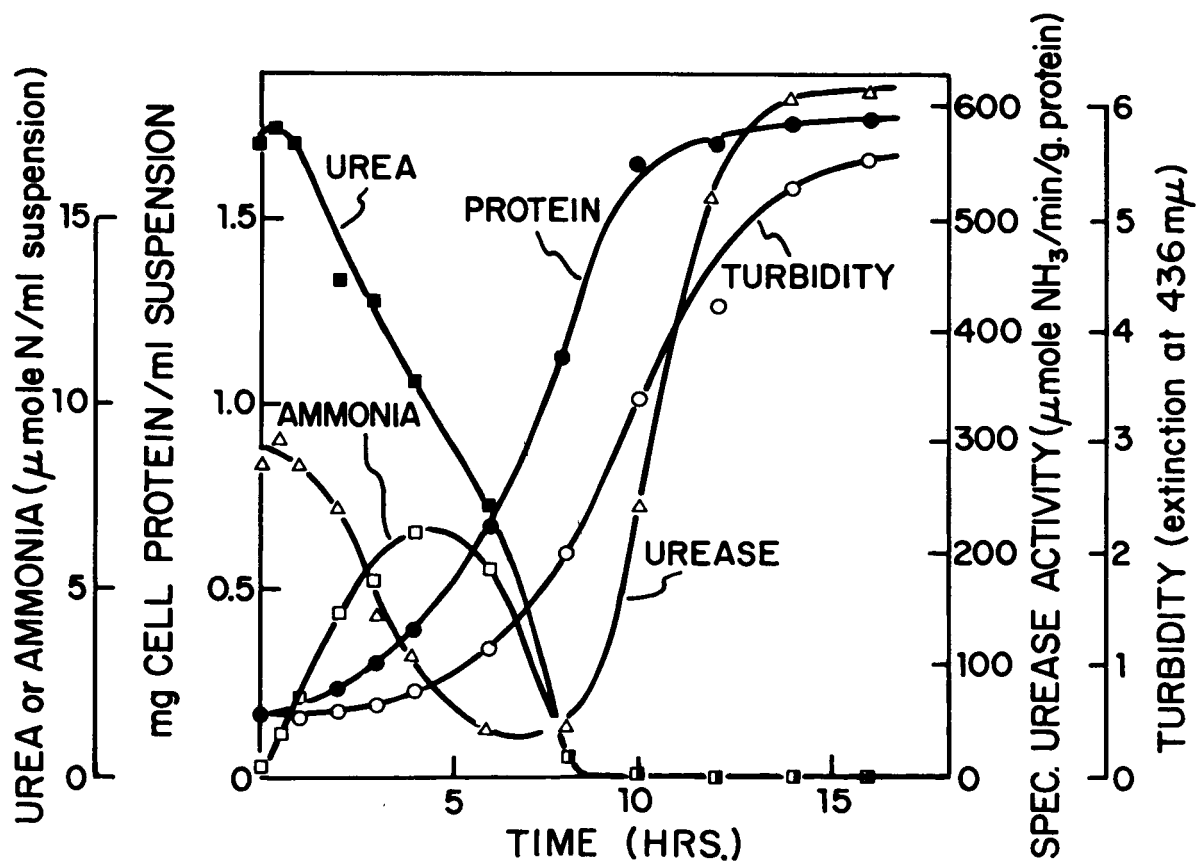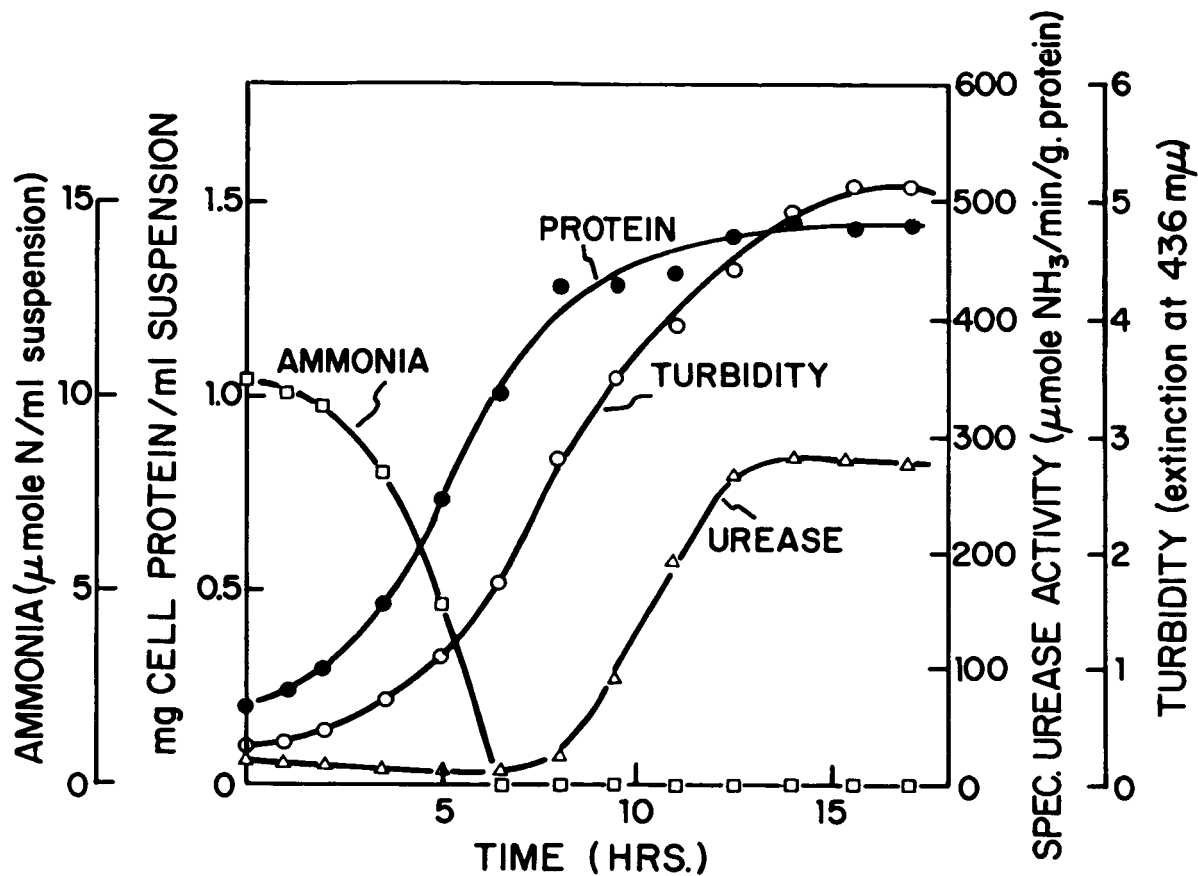
Figure 7 - Rate of CO_2 consumption (top) and formation of intracellular (lipid) inclusion as a function of oxygen concentration at constant H_2 and CO_2 supply.

Lamps GE253X — Reference Chamber, Sample Chamber, CLAIREX CL607, 100K, 25K BALANCE, 100K, 10μA METER RELAY (API), 10Ω 1W, 115V AC ON, 6.3 AC, SARKES TARZIAN F2, 10K SENSITIVITY, 100μf 25V, CONTACTS ON METER RELAY, NC, C, NO, N.C OUTLET, N.O. OUTLET
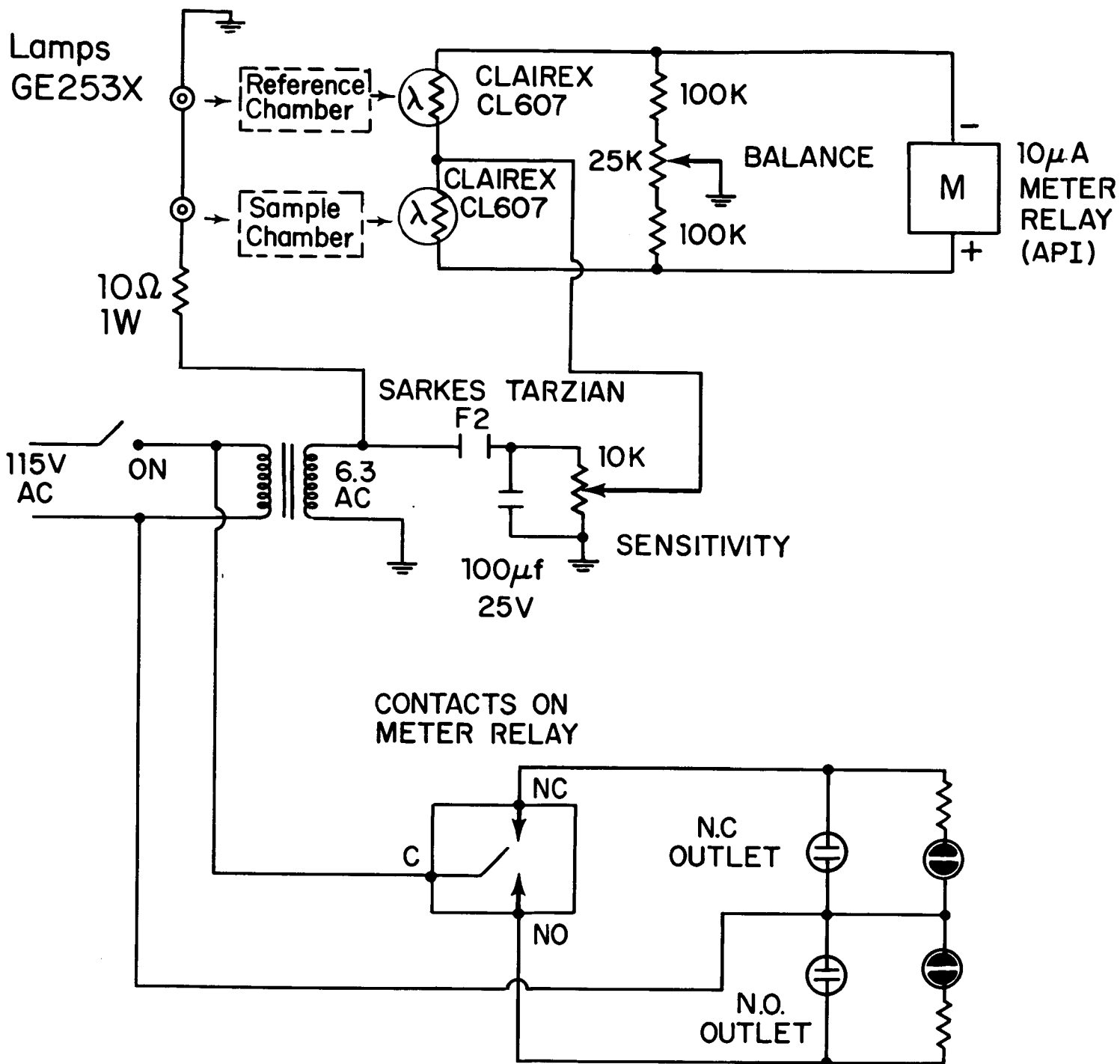
Figure 5 - OD/DW Relationship. Inset: OD/DW ratio at constant density.
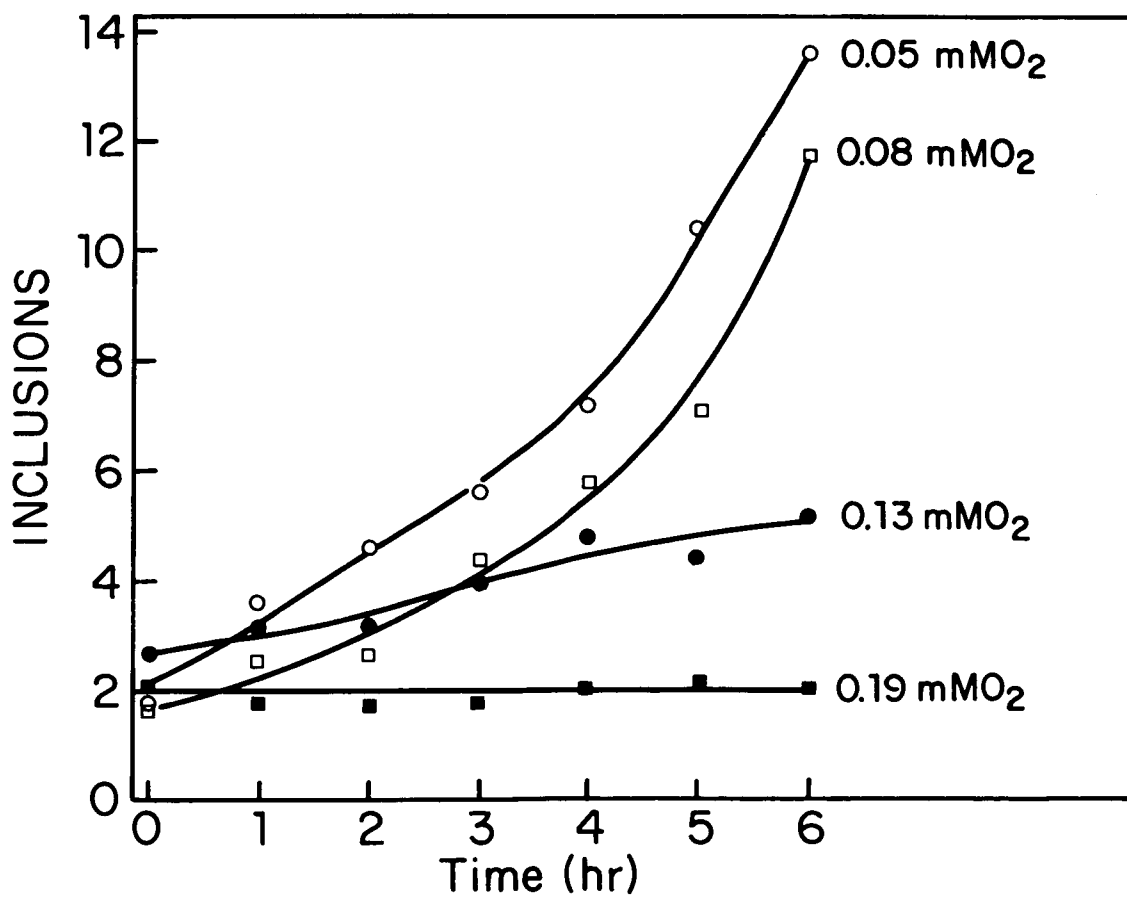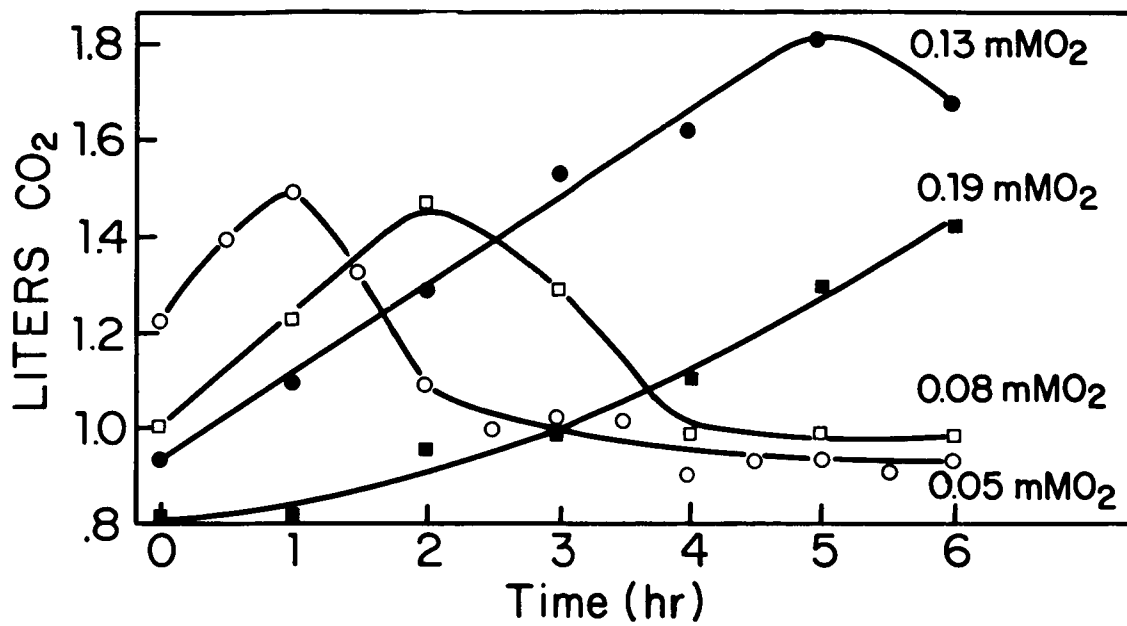
Figure 8 - Oxidation of reduced cytochrome $\underline{b}$ by oxygen. Sample (Thunberg) cuvette containing 2 ml extract (10mg protein per ml) was gassed with hydrogen, reference cuvette with air. After an incubation of 5 minutes at room temperature, spectrum 1 was obtained. Subsequently, cyanide was added from the side-arm (final conc. 10^{-4}M), the hydrogen in the sample cuvette replaced by air and spectrum 2 was obtained. Spectra 3 and 4 were obtained with 10^{-4}M CN$^-$ in both reference and sample cuvette. Spectrum 3 was measured after flushing the sample with hydrogen and the reference with air. Spectrum 4 was obtained subsequently after flushing the sample cuvette with air. Measurements were made at room temperature in Cary model 15.

.01 OD

2

3

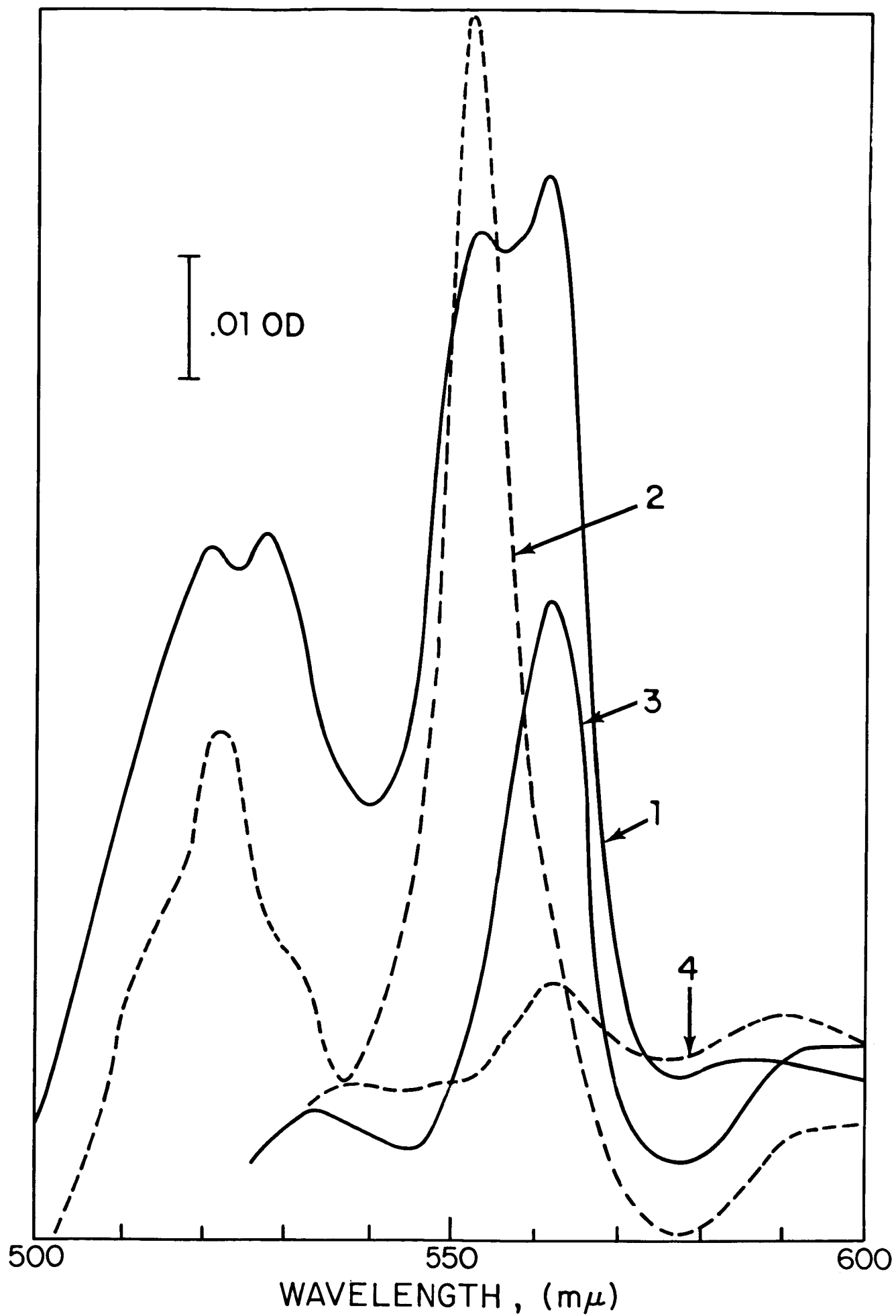1

4

WAVELENGTH , (mμ)

500

550

600

Figure 9 - Absorption spectrum of intact H-20.

Curve 1:   Sample hydrogen reduced and reference air oxidized; absorption

maxima 552 mμ (cytochrome c) and 560 mμ (cytochrome b).

Curve 2:   Sample oxidized in air in the presence of CN⁻, reference in air

in the absence of CN⁻.

H-20

1. H₂- AIR (b/c = .57)
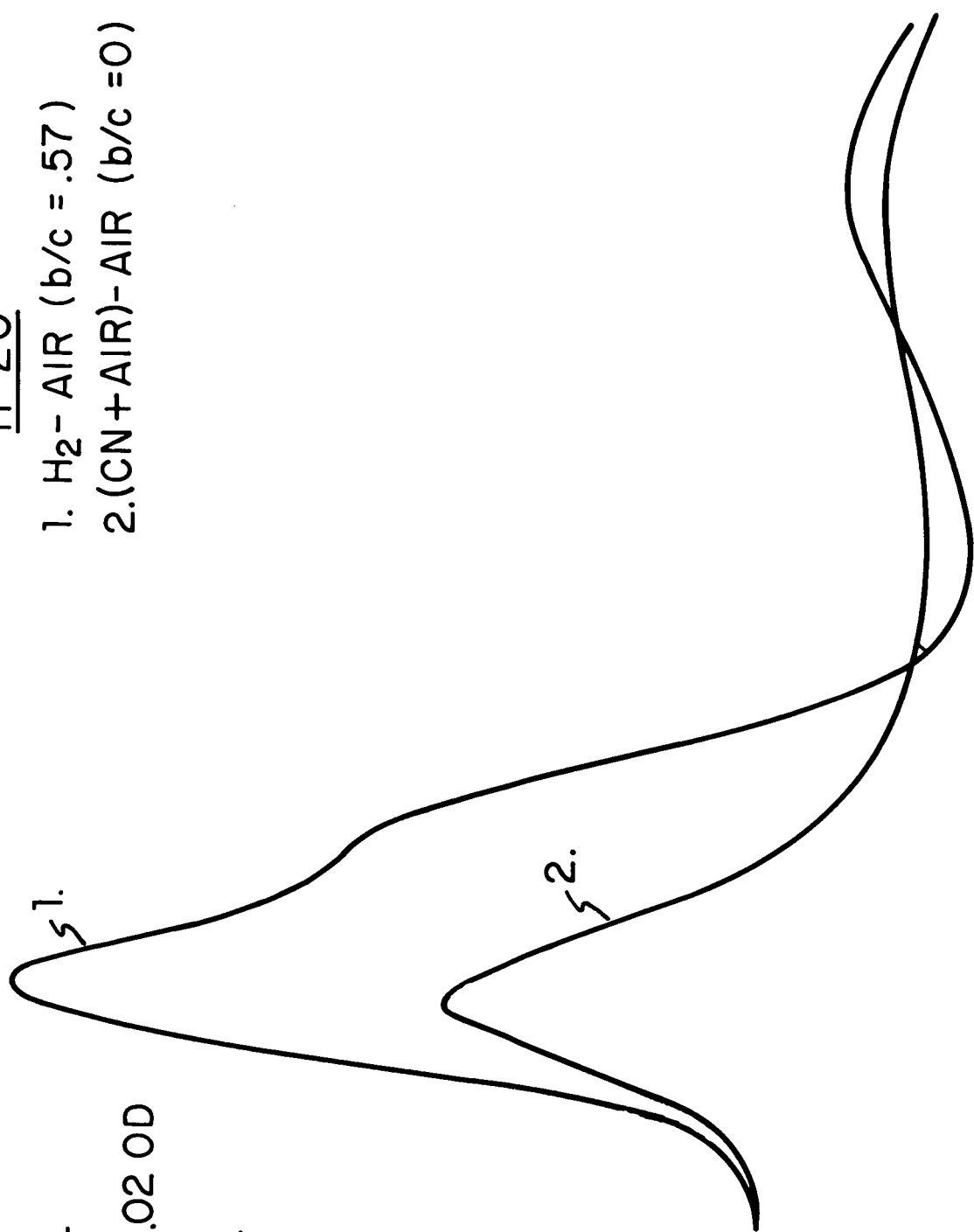2. (CN+AIR)- AIR (b/c = 0)

.02 OD

WAVELENGTH (mμ)

Figure 10 - Redox state of cytochrome b and cytochrome c as a function

of oxygen (hydrogen as balance). The suspension (intact cells)

reduced by hydrogen was subsequently flushed with oxygen/hydrogen

mixtures of known composition, and redox state of cytochrome b

and c (steady-state values) plotted as a function of oxygen.

Measurements were made sequentially in a dual beam spectro-

photometer; reference wavelength 650 mμ.

% REDUCTION

% OXYGEN IN HYDROGEN

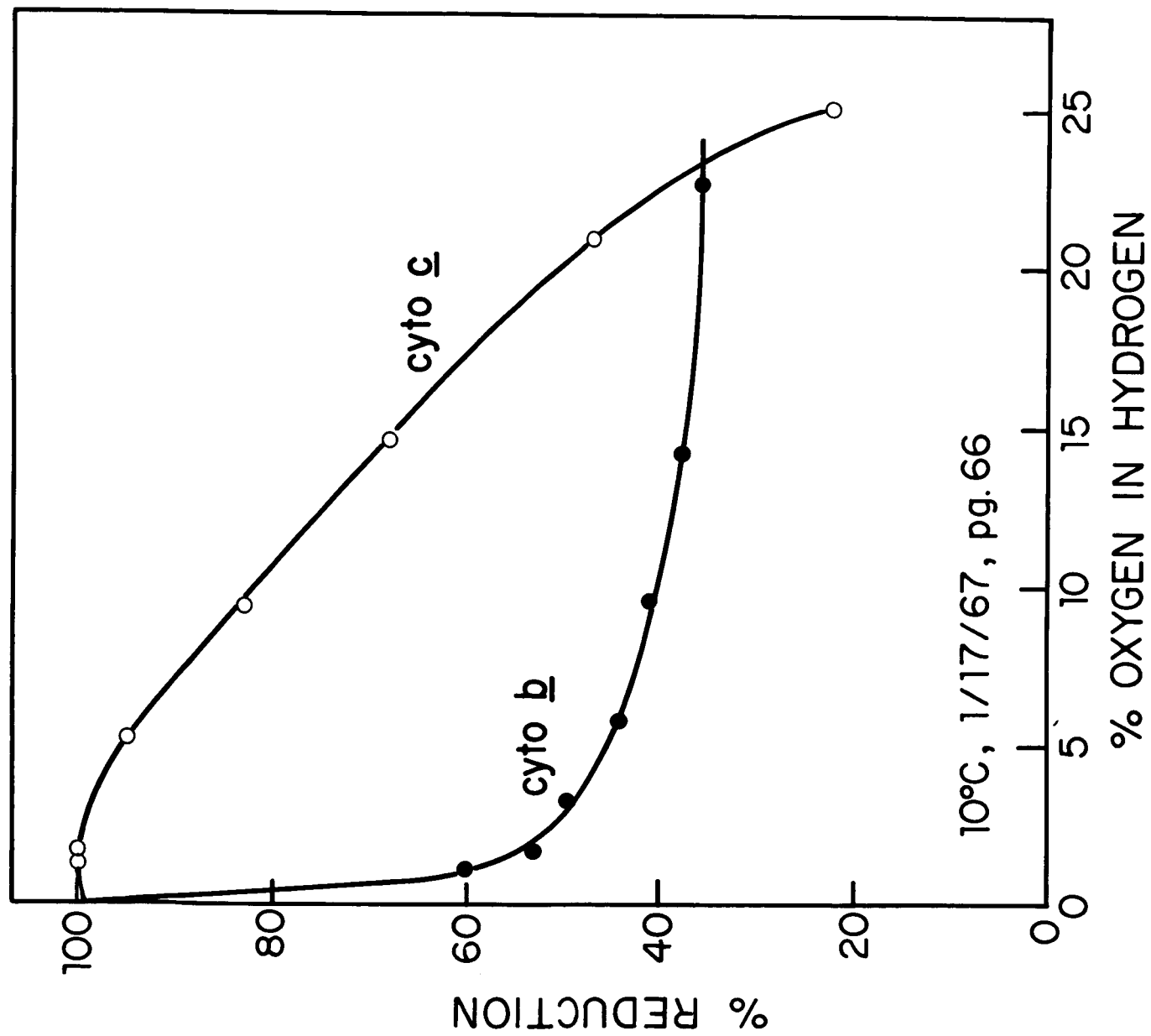cyto c

cyto b

10°C, 1/17/67, pg.66

Figure 11 - Transition changes of cytochrome b and of cytochrome c in

a suspension of Hydrogenomonas sp. Suspension is reduced

by flushing with hydrogen, and oxidized by flushing the sample

with air. A wavelength of approximately 548 mμ (cytochrome c,

positive deflection) was used as sample beam, and a wavelength

of approximately 562 mμ (cytochrome b absorption, negative

deflection) as reference beam. The selection of the two wave-

lengths was such that the absorption due to cytochrome c was

balanced (zero deflection) by the absorption of cytochrome b,
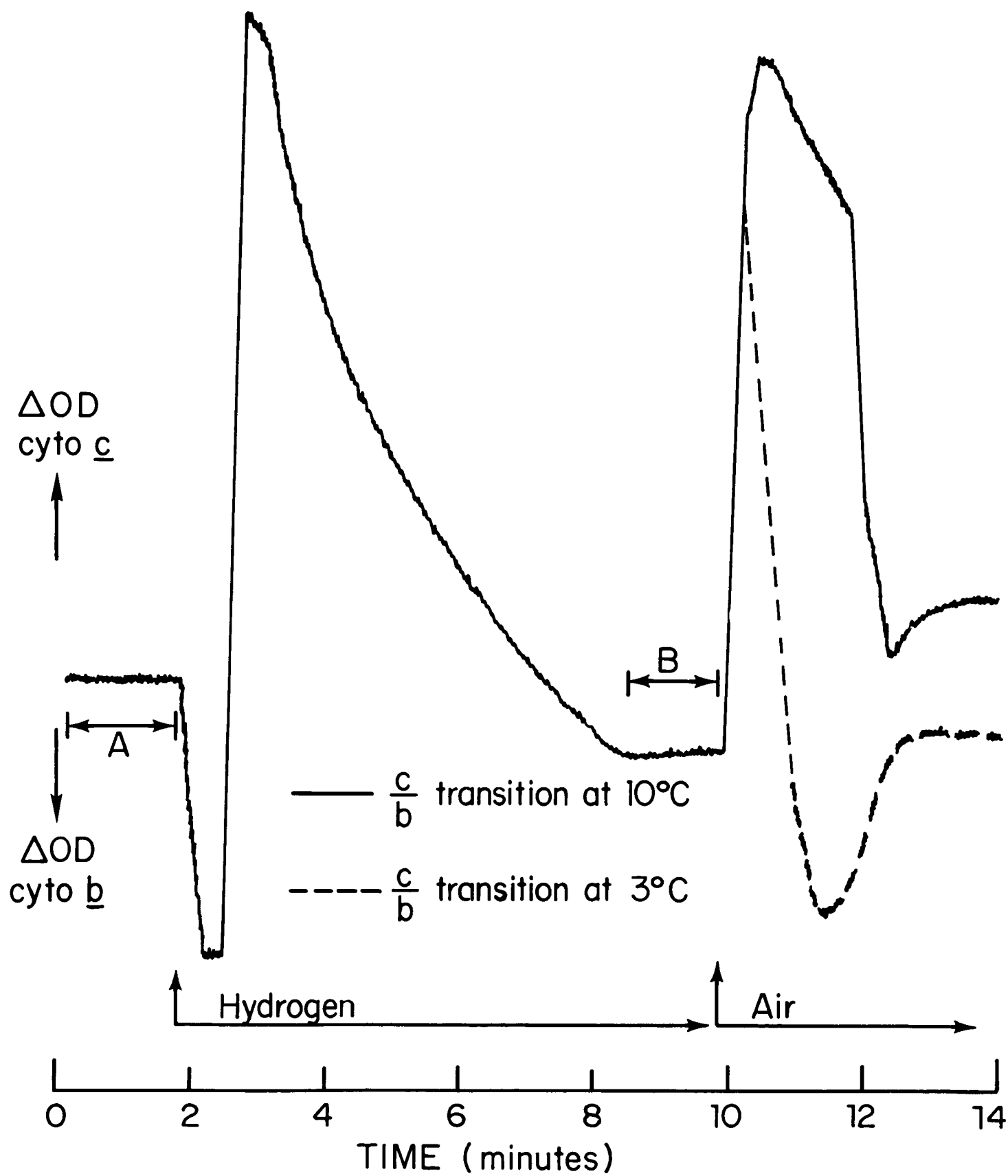
both in oxidized (A) and reduced states (B).

$\triangle OD$ cyto $\underline{c}$

A

$\triangle OD$ cyto $\underline{b}$

B

——— $\dfrac{c}{b}$ transition at 10°C

‑ ‑ ‑ $\dfrac{c}{b}$ transition at 3°C

Hydrogen

Air

TIME (minutes)

Figure 12 - Tentative electron transport scheme of <u>Hydrogenomonas</u> <u>sp</u>.

(see text).

H_2

NAD

X

NAD

cyt. c

Q cyt. b

ADP → ATP

?

?

O_2

O_2

CO_2

(CH_2O)

Figure 13 - Production rate of $\underline{H}$-$\underline{20}$ in a turbidostatically controlled
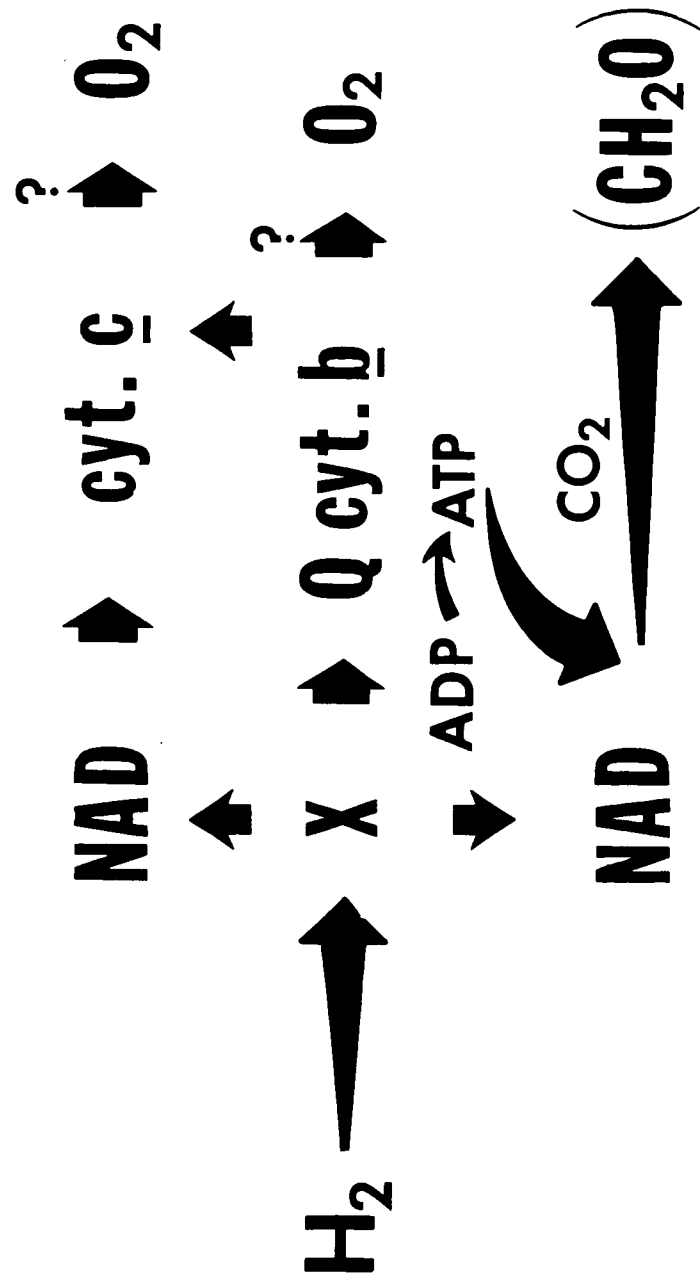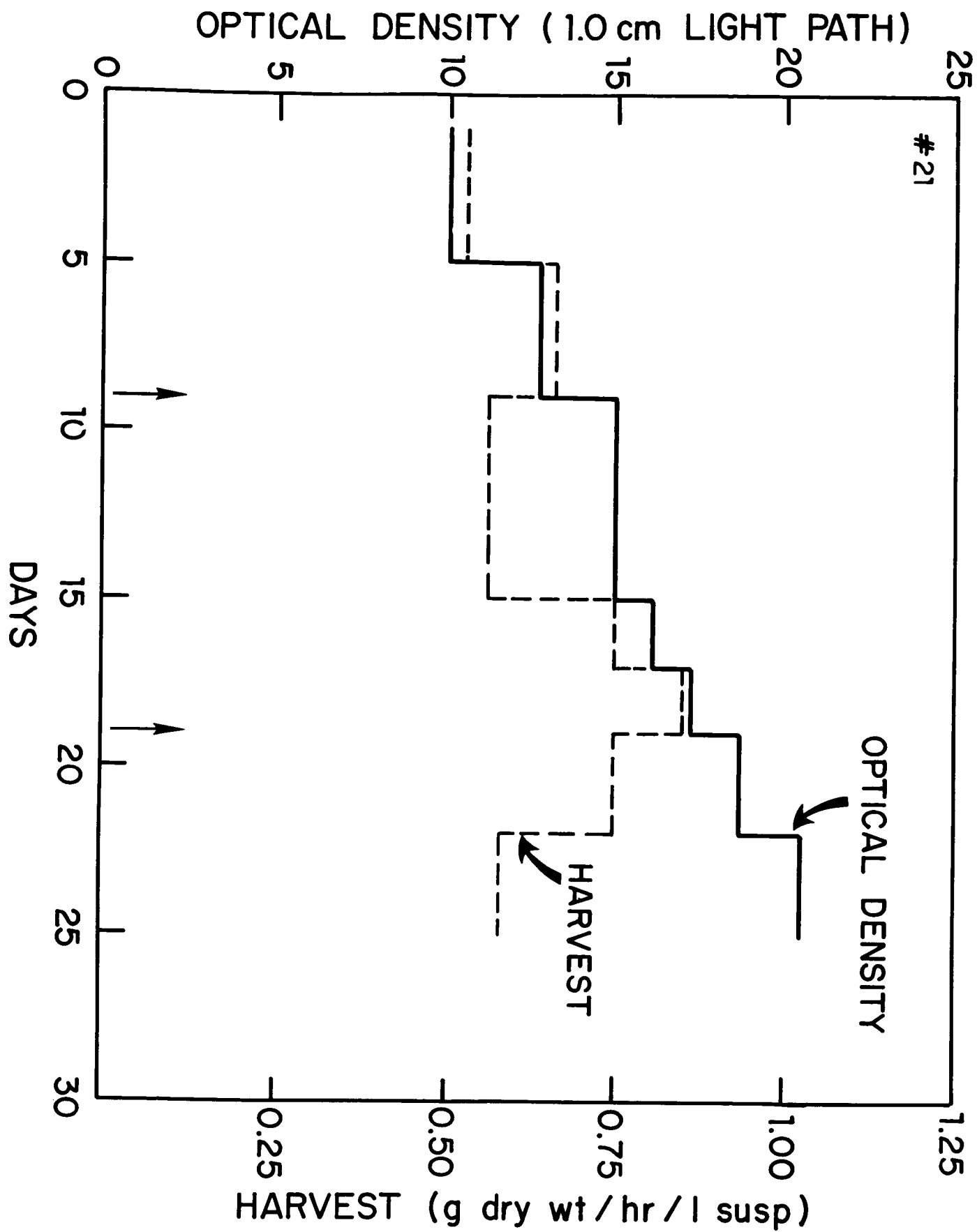
continuous culture.   For conditions see Table 6.

VII

REFERENCES

1. Bongers, L., Aerospace Medicine, 35, 139, 1964.

2. Bongers, L., B. Kok, Dev. in Industr. Microbiol., 5, 183, 1964.

3. Repaske, R., J. Bacteriol., 83, 418, 1962

4. Monod, J., Ann. Inst. Pasteur., 79, 390, 1950.

5. Novick, A. and L. Szillard, Proc. Nat. Acad. Sci., 36, 708, 1950.

6. Herbert, D., R. Elsworth and R. Telling., J. Gen. Microbiol., 14, 601, 1956.

7. Foster, J. and J. Litchfield, Biotech. and Bioeng., 6, 441, 1964.

8. Finn, R., Bacteriol. Rev., 18, 254, 1954.

9. Bongers, L., Final Report ER 13270-4 (NASw-713) July, 1964.

10. Bartha, R. and E. Ordal, J. Bacteriol., 89, 1015, 1965.

11. Eberhardt, U., Arch. Mikrobiol., 53,.288, 1966.

12. Bongers, L. and J. Medici, Paper presented at AIBS/NASA Meeting on Regenerative Systems, Washington, 1966.

13. König, C., H. Kaltwasser and H. Schlegel, Arch. Mikrobiol., 53, 231, 1966.

14. Packer, L. and W. Vishniac, Biochem. Biophys. Acta, 17, 153, 1955.

15. Repaske, R., J. Biol. Chem., 237, 1357, 1962.

16. Peck, H. and H. Gest, Biochem. Biophys. Acta, 15, 587, 1954.

17. Repaske, R., Biochem. Biophys. Res. Comm. (In Prep.)

18. Bongers, L., J. Bact., 93, (May) 1967.

19. Repaske, R., C. Lizotte, J. Biol. Chem., 240, 4774, 1965.