A STUDY OF THE CHEMOSYNTHETIC GAS EXCHANGER

Period Covered: June 14, 1968 - September 14, 1968

Contract

NASW-1596

National Aeronautics and Space Administration
Washington, D. C. 20546

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

Principal Investigator Dr. Leonard Bongers

INTRODUCTION

In a previous reporting period[1] experiments on the effects of nutrient deprivation upon composition of the cells and their growth characteristics were described. An unusually efficient turnover was observed i.e. the consumption of the three gases involved in the metabolism of hydrogenomonads was not the previously observed[2,3,4] volume ratio of approximately 6/2/1 ($H_2/O_2/CO_2$), but rather a consumption ratio of approximately 4/1/1. The gas consumptions of the efficient and less-efficient turnover can respectively be formulated as:

$$4H_2 + O_2 + CO_2 \rightarrow CH_2O + 3H_2O \qquad (1)$$

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

Results with resting cells[3] and with cells growing under "batch" conditions[4], suggest gas consumption corresponding to equation (2). It was assumed that the more efficient consumption ratio (eq. 1) could be attained only under environmental conditions which permit an exponential rate of cell reproduction.

---

(1) Quarterly Report NASW-1596 June 14, 1968.

(2) W. Ruhland, Jahrbuch f. wiss. Botanik, 63: 321-389 (1924).

(3) L. Packer, W. Vishniac, J. Bact., 70: 216-223 (1955).

(4) L. Bongers, Final Report NASw-971, March 1967.

In order to verify this, attempts were made to accurately determine the simultaneous uptake of CO_2, O_2 and H_2 by hydrogenomonads multiplying "exponentially" in the turbidostat. These results and earlier observations on gas utilization are discussed in this report which covers the period from June 1968 through September 1968.

PROCEDURES

The O_2/CO_2 Ratio. The efficiency of energy conversion by the hydrogenomonads is determined from the coupling of the energy derived from the oxyhydrogen reaction (eq. 3) with the carbon dioxide fixation process (eq. 4).

$$n(H_2 + 1/2\ O_2 \rightarrow H_2O) \qquad (3)$$

$$2H_2 + CO_2 \rightarrow CH_2O + H_2O \qquad (4)$$

Since the majority of oxygen taken up by the cells is consumed in the oxidation of hydrogen, leading to formation of biological energy, the amount of oxygen utilized in carbon dioxide assimilation is an adequate measure of conversion efficiency. Therefore, the conversion efficiency can be expressed as the volume ratio of consumed oxygen to consumed carbon dioxide during cell growth and cell multiplication (O_2/CO_2).

Polarographic Determination O_2/CO_2 by Hydrogen Depletion Method. By this method, described earlier[1], O_2/CO_2 is determined on an aliquot of a growing suspension under conditions of a limited H_2-supply. Briefly, oxygen consumption is measured polarographically on a cell suspension pre-equilibrated with a gas mixture which provides the liquid phase with an excess of O_2 and CO_2, relative to H_2, so that the hydrogen can be completely oxidized.

Oxygen consumption on these pre-equilibrated suspensions is determined both in the presence of an uncoupler* of oxidative phosphorylation so that only reaction (3) occurs, and without the uncoupler so that reactions (4) and (3) occur simultaneously. The difference in the O_2 consumptions with and without an uncoupler then determines the volume of hydrogen used in reaction (4) and thus is a measure of CO_2 fixation (see Fig. 1). From this value and the measured O_2 consumption in the absence of uncoupler (Fig. 1) the conversion efficiency can be calculated. In addition, from the rate of oxygen consumption in the absence of the uncoupler the rate of CO_2 assimilation can be estimated and the concomitant growth rate calculated.

A drawback of this and of the Warburg method used previously[3], is that the environmental conditions under which the efficiency measurements are made are not those usually considered as otpimal for cell growth and multiplication. In addition, the O_2 consumption measurements are made

---

*Carbonylcyanide, m-chlorophenyl hydrazone (cccp).

under conditions of changing oxygen concentration. The relatively high initial O_2 concentration (~ 0.34 mM) may have inhibitory effects, e.g. on hydrogenase, and this may affect the rate of exogenous substrate utilization. Also, the rate of endogenous respiration may be dependent upon O_2 concentration and may affect the correction for respiration as illustrated in Fig. 1. In addition, due to the relatively low solubility coefficient of H_2 and O_2 and the relatively slow response of the polarographic electrode only diluted cell suspension can be employed.

O_2/CO_2 Determination by Gasometer. Due to slow changes in flow controlling parameters such as room temperature and the output pressure of pressure regulators considerable difficulty has been experienced in establishing accurate data on the consumption of hydrogen, oxygen and carbon dioxide by the continuous culture. Gas flow rates had to be frequently recalibrated. The suspension had to be periodically stored off line during these operations, a serious obstacle to true steady state operation. Therefore, a method was designed which permits accurate measurements of the consumption of the three gas components during normal operation of the continuous culture, and reliable calibration of flow rates through gas control valves. The design which is outlined in Fig. 2, depends on the displacement of water by the gas being measured. The unit can be operated manually, or automatically using the control circuit illustrated in Fig. 3.

The gasometer utilizes two magnetic 3-way valves (Fig. 2, S_1 and S_2) which are connected, as indicated, to graduated cylinders A and B. These magnetic valves are energized and de-energized simultaneously. Valve S_1 functions as the gas inlet and S_2 as the gas outlet valve. When S_1 and S_2 are energized the NC ports are open and the NO ports are closed. The gas then enters cylinder A (through S_1 and NC) forcing the liquid through metering valve MV_2 into cylinder B, while the gas in cylinder B exits through the outlet valve (S_2, NC port). When the water level in B reaches probe #2, S_1 and S_2 are de-energized so that the NC ports close and the NO ports open. The process is now reversed; the water is forced into cylinder A from cylinder B and the gas in cylinder A can be fed into the reactor through the NO port of S_2.

The gas flow to the reactor is controlled by a two-way valve (S_3) and a flow rate control valve (MV_1). The opening and closing of S_3 is controlled by the reactor; for details see (4). For accurate volume measurements the outlet gas pressure (PG 2) must be constant. To this end the flow rate through MV_2 is maintained considerably in excess to the flow rate through MV_1.

In automatic operation the number of times the valves are energized and the elapsed time are monitored. From this information the total gas utilization was computed. A volume measurement accuracy of approximately 95% was routinely achieved.

Three of the above described units were used to determine the utilization of H_2, O_2 and CO_2 by the continuous culture. For measurement of the O_2 and CO_2 consumption rate, 100 ml graduated cylinders were used while for H_2 400 ml cylinders were employed.

Dry Weight Measurements. The dry weight content of the suspension was determined on an aliquot which was dried over night in vacuum at approximately 80°C

RESULTS

O_2/CO_2 Under Optimal Conditions. In order to evaluate the efficiency of cell growth and multiplication rather than CO_2 fixation by resting cells, the O_2 and CO_2 consumptions were measured on a steady state culture growing under "optimal" conditions for cell multiplication.

The net volumes of H_2, O_2 and CO_2 consumed by the suspension were determined by the gasometer technique just described (Method B). From the quantitative information on CO_2 consumption the expected dry weight production could be computed, based on the assumption that the carbon content of the cell material is on the order of 45%. The computed dry weight production is compared with the actual dry weight production from the reactor effluent.

Using limited H_2 supply and polarographic technique described earlier (Method A) O_2 consumption values were also measured for small aliquots from the reactor. After a ten-fold dilution of the suspension with fresh medium and equilibration with a gas mixture containing $H_2/O_2/CO_2$ the O_2 consumption was measured in presence and absence of uncoupler. The O_2/CO_2 quotient and the expected cell production were then computed. The results of these experiments are summarized in Table 1.

The results indicate good agreement between the yields calculated from the on stream (Method B) and off stream (Method A) gas uptake values. In general, Method A gives a somewhat lower "production" value than Method B, probably due to the relatively high O_2 concentration utilized for these measurements. A direct inhibition of hydrogenase activity can be expected under these conditions. The results of Method B were usually in good agreement with the actual cell yield (Table 2 and Table 3).

Both methods give good measure of the autotrophic production of organic carbon. The O_2/CO_2 quotient obtained by Method A indicates that, on the average, one mole of O_2 is utilized in hydrogen oxidation for every mole of CO_2 reduced to organic carbon. If this quotient is corrected for endogenous respiration (see also Fig. 1) a somewhat lower value for the quotient is observed (Table 1, Method B). This rate of endogenous respiration was deduced from curves such as those of Fig. 1. Clearly, the rate of O_2 consumption declines sharply upon exhaustion of exogenous substrate (H_2). The endogenous rates are of the order of 20% of the rate in the presence of hydrogen.

The change in endogenous respiratory activity is illustrated in Fig. 4. The endogenous oxygen uptake was measured polarographically on samples withdrawn from the reactor and subsequently equilibrated with air. Results indicate a relatively rapid decline in respiratory activity after utilization of exogenous substrate. Extrapolation of the decay curve to exogenous substrate exhaustion indicates a maximum endogenous rate of 3.4 mmoles O_2/hour and per gram of cells. If a correction for such an

endogenous rate is made, the observed O_2/CO_2 quotients appear to be in good agreement.

From the data on gas consumption in Table 1, Method B, actively growing hydrogenomonads apparently follow an overall conversion corresponding to:

$$4H_2 + O_2 + CO_2 \rightarrow (CH_2O) + 3H_2O \qquad (5)$$

This gas consumption ratio appears to be the best obtainable and indicates an overall conversion efficiency on the order of 50%.

O_2/CO_2 Quotient Under Sub-optimal Conditions. Previously, we observed that O_2/CO_2 quotient was dependent upon environmental conditions in confirmation of observations by Packer et al[3]. They found a decrease in conversion efficiency upon "aging" of the cells, and we observed[1] an increased uptake of H_2 and O_2 for every mole of CO_2 if growth occurred under conditions of Mg limitation. A similar result was observed with growth under conditions of limited iron (see Table 2 and Fig. 5).

Prolonged growth under a limited Fe supply resulted in a decrease in the uptake of O_2 and CO_2 but, while the O_2 uptake declined by a factor of two, the CO_2 assimilation decreased by a factor of three. Consequently, the carbon dioxide fixation process became less efficient as is evident from the O_2/CO_2 quotients in Table 2.

Limited Fe supply also resulted in a rapid decline in dry weight production as illustrated in Fig. 5 (open and closed circles). After 4 to 6 hours of Fe deprivation the observed rate was about one-third the

maximum rate. It is assumed that this final rate is supported by iron carried over as a contaminant in other nutrient salts. Prolonged cultivation under condition of iron limitation is accompanied by suspension foaming, which impedes proper functioning of the turbidostat. Therefore, after 8 hours Fe was added to the suspension.

During Fe starvation, the iron content of the liquid phase declined from approximately 10^{-5}M to less than 10^{-6}M. Half maximum rate of growth occurred at an iron concentration in the order of 0.5×10^{-6} to 10^{-6}M.

O_2/CO_2 Quotient With Recycled Medium. With a continuous cultivation growth-limiting factors are more easily detected than in batch cultures. For example, we can assume that a trace element deficiency would manifest itself only after a relatively long-term continuous cultivation. Since microorganisms in general are good scavengers for inorganic salts, it was assumed that the requirement for a particular trace element could be established even more clearly if growth characteristics were determined in an environment used previously for cultivation of the particular organism. Such an experiment is recorded in Table 3.

During steady state operation of the continuous culture (zero time in this experiment is chosen arbitrarily; the culture used for this experiment was grown continuously for the past four months), reconstituted medium was utilized by the culture instead of fresh medium. The medium was reconstituted by addition of 0.14 gram $MgSO_4 \cdot 7H_2O$ to the 'spent' medium. This addition brought the Mg-concentration up to the level

normally used for the conditions of operation (7×10^{-4} M). No other elements except N were added to the medium. Nitrogen was added directly to the suspension as a concentrated (5 normal) ammonium hydroxide solution in proportion to the rate of N removal by the cells. The nitrogen level was monitored by pH.

As shown in Table 3, neither O_2 and CO_2 uptake or cell yield is affected by this treatment. Reactor effluent thus obtained was, after removal of the cells by centrifugation and addition of $MgSO_4 \cdot 7H_2O$ utilized again. If one takes growth rate as an indicator of environmental sufficiency, addition of trace elements and phosphate to the liquid effluent of the reactor seems unnecessary.

Since the same medium could be utilized three times without affecting the performance, apparently three times the cell density used could be supported by the original medium which then need only be supplemented with Mg and N.

The O_2/CO_2 quotient was essentially the same for the reconstituted and the fresh medium. Since the conversion efficiency and the growth rate are the same for the fresh as well as the reconstituted media, apparently the reconstituted environment accomodates a growth equivalent to the exponential growth, observed in fresh medium.

Conclusions. The observations in the report lead to the following conclusions:

1) Not only resting cells but apparently also actively growing cells can effect an efficiency of energy conversion which is considerably higher than previously assumed possible.

2) An O_2/CO_2 quotient of unity for both CO_2 fixation and generation of new cells indicates that the cell utilizes to the full extent all energy made available by the respiratory chain (assuming a C-fixation pathway via the Calvin cycle and a growth yield (Y_{ATP})[5,6] of 10.

3) Since deficiencies (Mg, Fe) effect the O_2/CO_2 quotient unfavorably and if 2) holds, one can assume that the conditions under which the high conversion efficiency is obtained represent the optimal conditions for cell growth and cell multiplication.

4) Since the efficient energy conversion could be reproduced routinely and maintained in our experimental culture set-up for any desired length of time, we assume that similar conversion efficiencies could also be achieved in a larger, functional unit. If this assumption is borne out the energy which would be consumed for the biosynthetic process involved in bioregeneration of a man's carbon dioxide output would be on the order of 400 watts/man instead of the 600 watts considered previously.

(5) Baushop, T., and S. R. Elsden. J.Gen. Microbiol., 23, 457 (1960).

(6) Hernandex, E., and M. J. Johnson. J. Bacteriol., 94, 991 (1967).

TABLE 1

Conversion Efficiency, Actual and Computed Yields Obtained With a Steady State Culture Under "Optimal" Conditions for Growth

METHOD A

$\frac{a}{b-a}^{*} = \frac{.16}{.33-.16}$ or $O_2/CO_2 = 0.94$

O_2 Uptake: 139 mmoles/hr

CO_2 Uptake: $\frac{O_2 \text{ Uptake}}{O_2/CO_2}$ or

147 mmoles/hr

Computed Yield:

3.9 grams/hr**

METHOD B

Gas Uptake: H_2 = 775 mmoles/hr

O_2 = 196 mmoles/hr

CO_2 = 165 mmoles/hr

$O_2/CO_2 = \frac{196}{165} = 1.19$

$O_2/CO_2(corr)^{\dagger} = \frac{159}{165} = 0.97$

Computed Yield:

4.4 grams/hr**

Reactor Output: 1.28 liter suspension/hr

Dry weight of solids is 3.6 grams/liter

Actual Yield: 4.6 grams/hr

---

*See Fig. 1 for designations.

**Assumed carbon content is 45% of cell mass.

†Corrected for endogenous respiration.

TABLE I

Effect of Fe^{++} Starvation on Growth Characteristics of *H. eutropha*

Time (hrs)	CO_2 l/hr	O_2 l/hr	O_2/CO_2	Yield (gr/hr) Computed*	Actual
			Control Conditions		
0	4.23	4.57	1.09	4.45	4.70
1 1/4	4.23	4.46	1.06	4.45	4.70
1 3/4	4.12	4.30	1.07	4.37	4.48
2 1/4	3.99	4.41	1.11	4.23	4.70
			Fe^{++} Starvation		
3 1/2	4.35	4.50	1.04	4.60	4.70
5	4.15	4.27	1.03	4.40	4.08
5 1/2	3.26	3.65	1.12	3.46	3.26
6 3/4	2.73	3.02	1.12	2.90	2.44
7 1/4	2.33	2.79	1.19	2.47	2.44
8	2.10	2.59	1.23	2.22	2.24
9	1.89	2.46	1.31	2.00	2.04
10	1.50	2.24	1.49	1.59	1.74

*From measured consumption of CO_2.

TABLE 3

Effect of Medium Reconstitution and Reutilization on Yield and Conversion Efficiency in Steady State Cultures

Time (hrs)	Uptake (liters/hr) CO_2	O_2	O_2/CO_2	Yield (grams/hr) Act. Dr. Wt.	Cal. Dr. Wt.
0	3.8	4.6	1.21	4.6	4.5
1	3.8	4.6	1.21	4.4	4.5
1 1/2	3.8	4.5	1.18	4.6	4.5
		Recycled Med. (1X)			
2 1/2	3.8	4.7	1.24	4.3	4.5
3 1/2	3.6	4.5	1.25	4.7	4.3
5 1/2	3.7	4.5	1.21	4.7	4.4
7 1/2	3.7	4.5	1.21	4.6	4.4
9	3.6	4.5	1.25	4.7	4.3
28	4.0	4.9	1.22	4.7	4.7
31	3.9	4.9	1.25	4.6	4.6
53	3.7	4.7	1.27	4.2	4.4
		Recycled Med. (2X)			
55	3.6	4.6	1.28	4.2	4.3
59 1/2	3.9	4.7	1.20	4.2	4.6
77	3.9	4.8	1.23	4.2	4.6

Conditions: Continuous culture; suspension density of 3.3 grams (dry weight) per liter; reactor volume 3 liter of suspension; suspension temperature 33°C. The gas phase controlled at 5% CO_2, and approximately 80% H_2; the dissolved O_2 concentration varied from 0.03 mM to 0.06 mM. The (1X) recycled medium was obtained by addition of .14 gram $MgSO_4 \cdot 7H_2O$ to the liquid of reactor effluent after removing the cells by centrifugation. 0.17 gram $MgSO_4 \cdot 7H_2O$ was added to the medium when it was used for the third time..

Figure 1

Oxygen consumption by H. eutropha measured polarographically in presence (2×10^{-5}M cccp) and absence (-cccp) of uncoupler. The suspension, containing 0.30 mg cells per ml was pre-equilibrated (at 33°C) with a gas mixture containing 60% H_2, 30% O_2 and 10% CO_2. To determine the equilibration dissolved O_2 concentration cells were omitted from the medium. The break in the slopes is due to the cessation of exogenous activity (H_2 exhaustion). Values a and b are corrected for endogenous respiration.

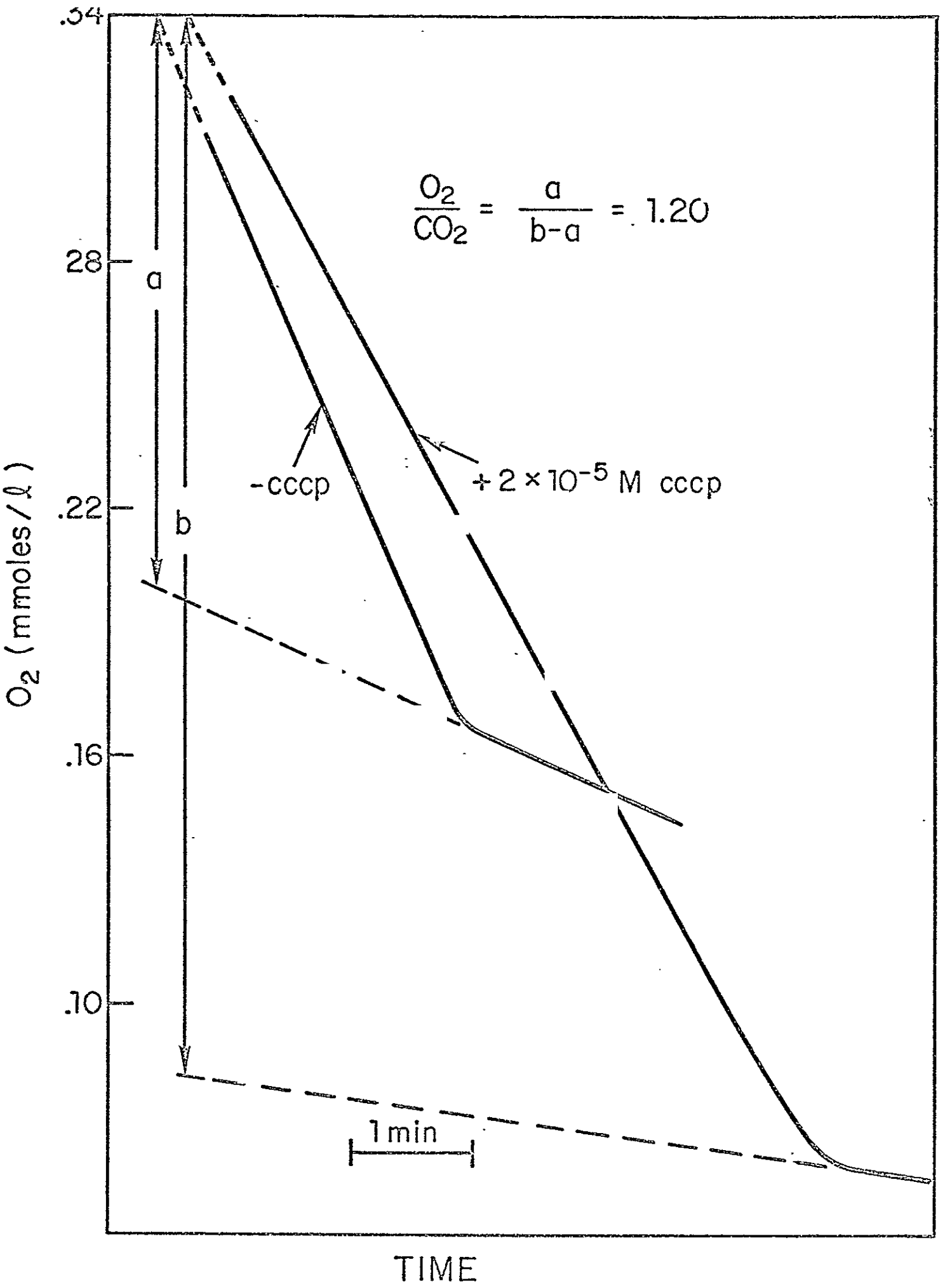

.34
28
.22
.16
.10
O_2 (mmoles/ℓ)
a
b
$\frac{O_2}{CO_2} = \frac{a}{b-a} = 1.20$
-cccp
+ 2×10^{-5} M cccp
1 min
TIME

Figure 2

Schematic of gasometer. For operation, see text. MV = flow-control valve; TV = toggle valve; PG = pressure gauge; S = magnetic valve; NC and NO = ports.

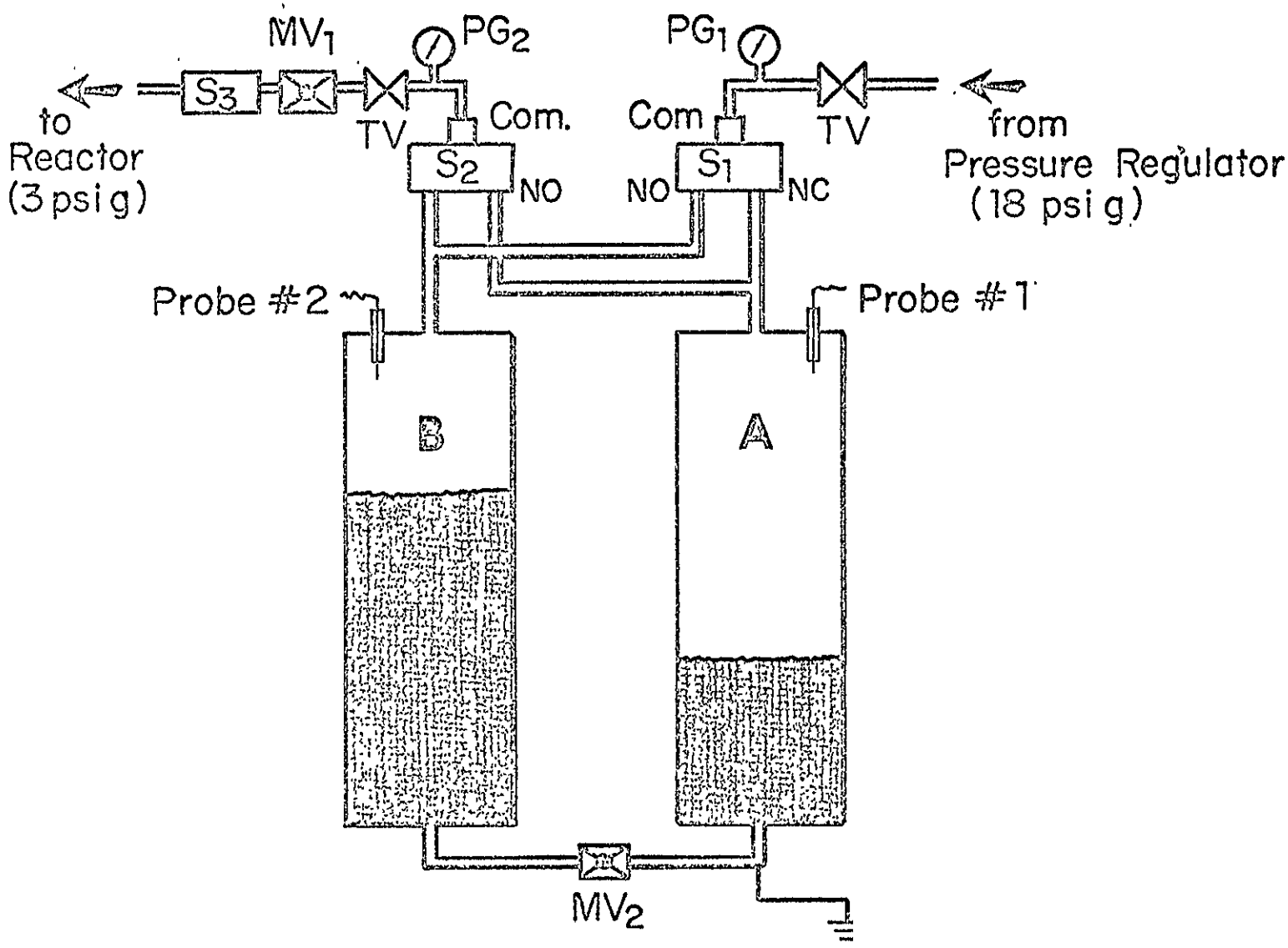

MV1
PG2
PG1
S3
to
Reactor
(3 psig)
TV
Com.
Com
TV
from
Pressure Regulator
(18 psig)
S2
NO
NO
S1
NC
Probe #2
Probe #1
B
A
MV2

Figure 3

Gasometer control circuitry.

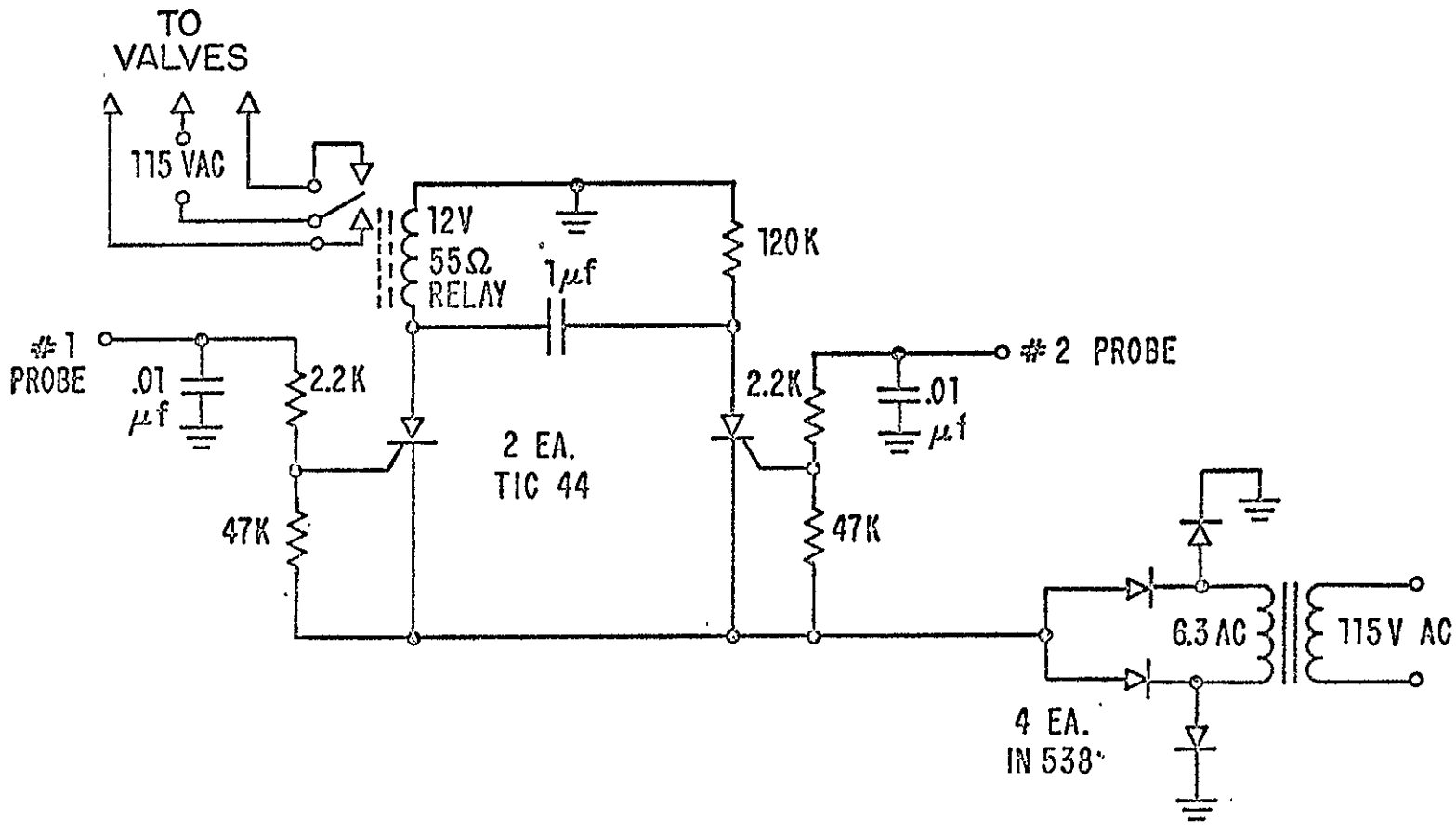

TO
VALVES
115 VAC
12V
55Ω
RELAY
1μf
120K
#1
PROBE
.01
μf
2.2K
2 EA.
TIC 44
47K
2.2K
.01
μf
#2 PROBE
47K
6.3 AC
115 V AC
4 EA.
IN 538

Figure 4

Change in endogenous activity of H. eutropha after utilization of exogenous (H_2) substrate. On an aliquot, removed from the autoculture and diluted four-fold, the oxygen consumption was measured polarographically, after incubation under air for indicated time periods.

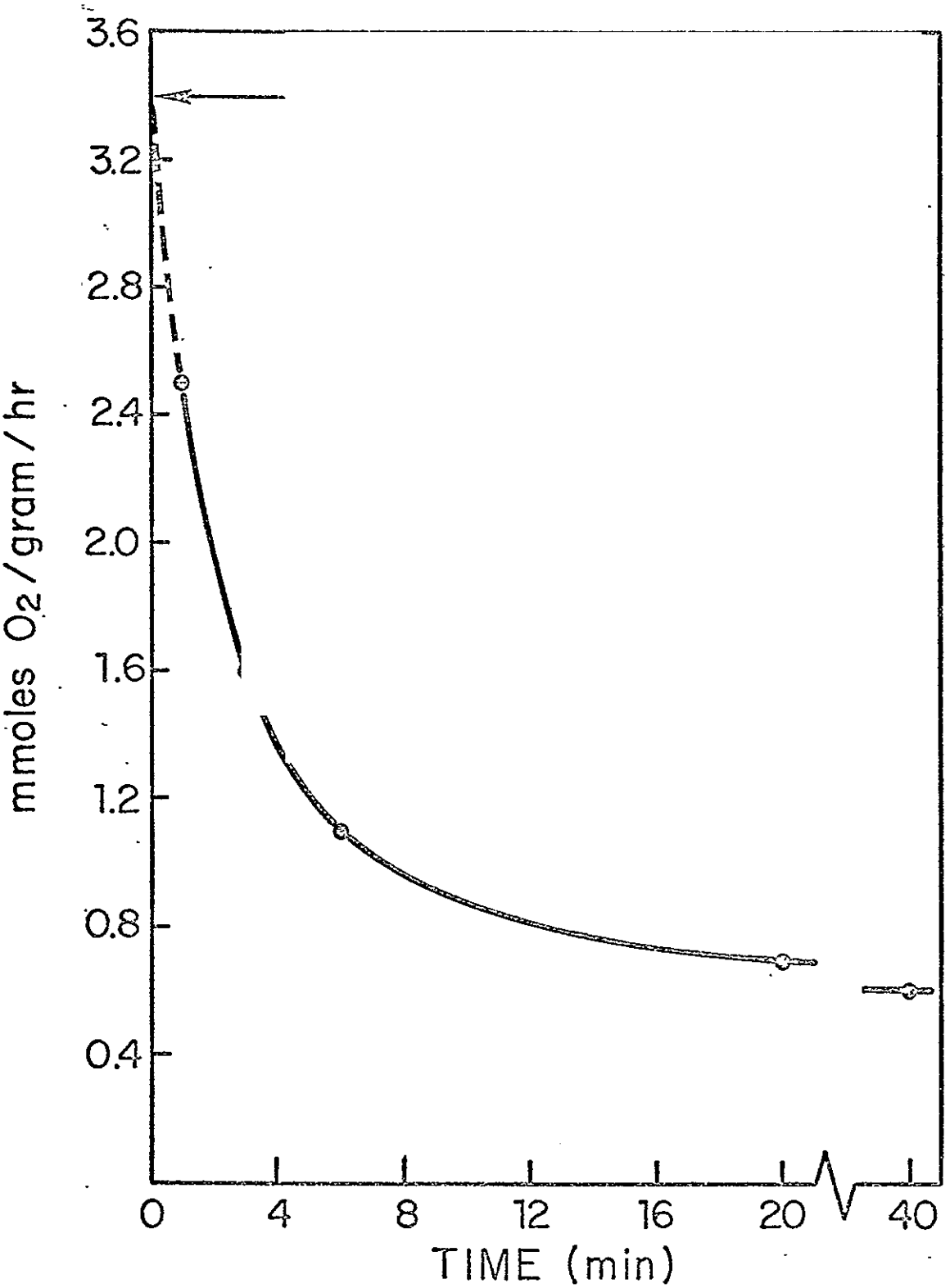
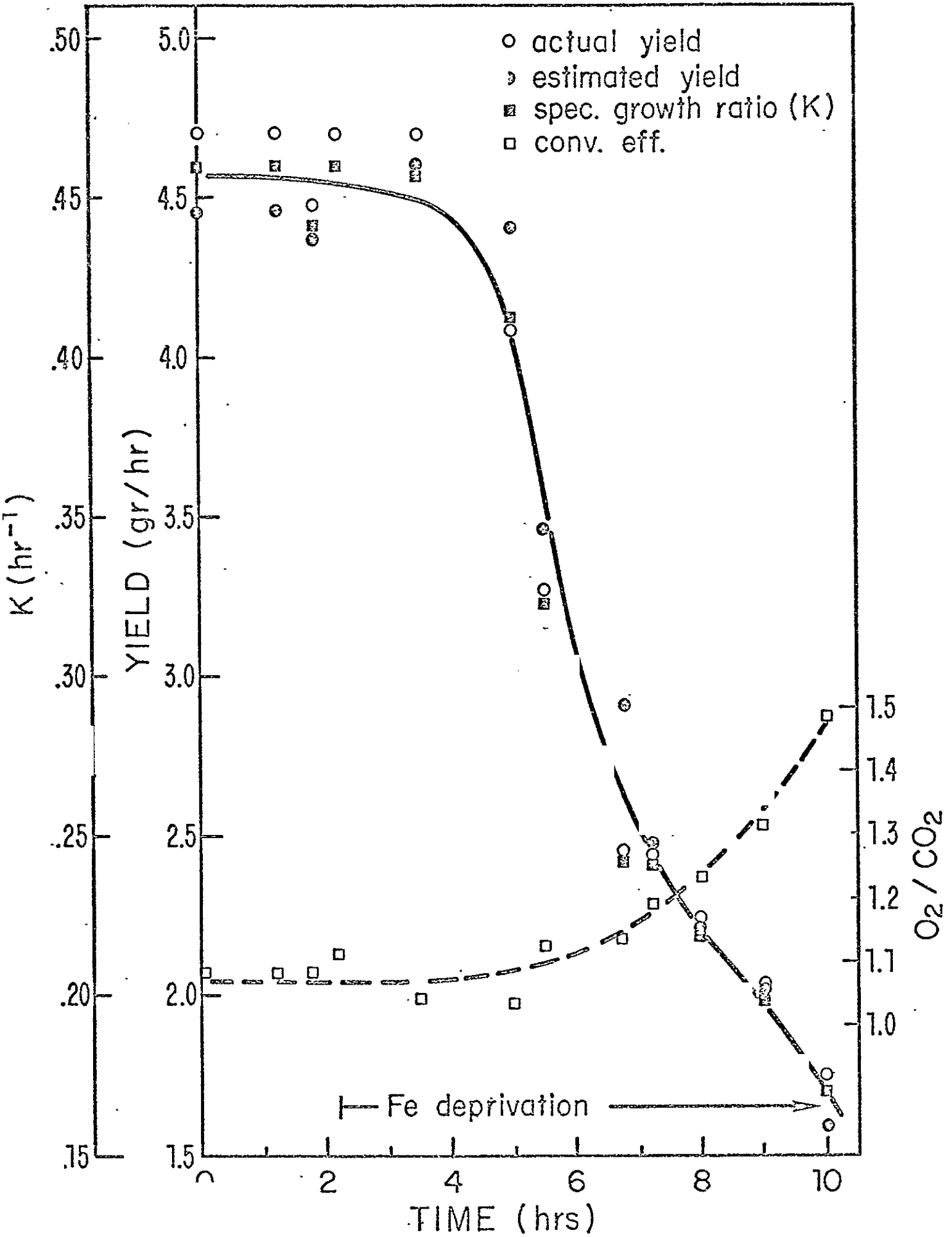

3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
mmoles O2 / gram / hr
0
4
8
12
16
20
40
TIME (min)

Figure 5

Fe-limitation and yield. The iron supply (ferrous ammonium sulphate) was terminated at 2 1/4 hours. The uptake of O_2 and CO_2 was monitored by gasometers. Actual yield was measured as dry weight production on reactor effluent. Estimated yield was computed from the CO_2 consumption by assuming a 45% C content in the cell mass. Conversion efficiency was expressed as the O_2/CO_2 quotient. Auto culture operated at 3.6 grams (dw) per liter.


actual yield
estimated yield
spec. growth ratio (K)
conv. eff.
K (hr⁻¹)
YIELD (gr/hr)
O2 / CO2
TIME (hrs)
Fe deprivation
.50
.45
.40
.35
.30
.25
.20
.15
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.5
1.4
1.3
1.2
1.1
1.0
0
2
4
6
8
10