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Energy Generation and Utilization in Hydrogen Bacteria

FOREWORD

This progress report on Contract NASW-1596 covers the reporting period from 23 September 1968 through 22 January 1969. In this report the efficiency of growth by hydrogen bacteria and the definition of growth-rate limiting factors on efficiency of energy conversion are considered.

Some previously reported observations and data from the open literature are included to provide a balanced and interpretive discussion.

ENERGY GENERATION AND UTILIZATION IN HYDROGEN BACTERIA

INTRODUCTION

Hydrogen bacteria utilize molecular hydrogen for reductive and energy-yielding purposes. The ability to generate reduced nicotinamide adenine dinucleotide ($NADH_2$) with molecular hydrogen as electron donor was demonstrated in a number of hydrogenase containing organisms (1, 2, 3,). It was also shown that utilizable energy, in the form of adinosine triphosphate (ATP), is released in the oxidation of hydrogen by oxidative phosphorylation (4). While there is a good deal of information on energy transformation in cell free extracts, little is known about the interaction of the growth environment and the efficiency of energy transformation in the intact cell.

The classic studies by Ruhland (5) suggest that autotrophically growing hydrogen bacteria utilize H_2, O_2 and CO_2 in a ratio of approximately 6:2:1, respectively. An amount of energy equivalent to that released in the combustion of 4 moles of H_2 would thus appear to be required in the assimilation of a mole of CO_2. Later studies by Packer and Vishniac (6) on resting Hydrogenomonas ruhlandii confirmed this finding, but they also obtained data suggesting conversion processes of greater and lesser efficiency. Their findings indicate that the observed value for energy transformation is strongly dependent on the history of the sample as well as the conditions

under which the measurements are made. Finally, Marino and Clifton (7) determined gas consumption ratios on Hydrogenomonas facilis and observed similar phenomena. The best values (4.9:1.3:1) were obtained with young, actively growing cultures; 4 day old cultures exhibited a relatively poor energy utilization (8:3.1:1).

The contrast between variation in efficiency of energy conversion and the more constant values recently obtained (8, 9, 10) for the assimilation of organic substrates in other microorganisms and hydrogen bacteria prompted a reinvestigation of the problem. Also, previous observations on energy conservation by subcellular preparations with hydrogen as a substrate (4) would seem to warrant an investigation of energy transformation in the intact cell. The present report deals with the effect of the growth environment on gas consumption characteristics of autotrophically growing H. eutropha. The data presented here suggest that the variation in efficiency of energy conversion is due to a lack of an obligatory coupling between energy-donating and energy-utilizing processes in hydrogen bacteria.

MATERIALS AND METHODS

Culture Procedure. Hydrogenomonads eutropha (ATCC 17697) was grown autotrophically under steady-state and under batch culture conditions.

Steady-state cultures were maintained by utilizing a photoelectric technique for automatic control of cell concentration (turbidistat). The cell concentration was maintained at approximately 3.5 grams (dry weight) per liter, by dilution with fresh medium containing: 0.03 M potassium phosphate (pH 7.0), 1.45×10^{-2} M NH_4Cl, 8×10^{-4} M $MgSO_4 \cdot 7H_2O$, 2.5×10^{-5} M $Fe(NH_4)_2(SO_4)_2$, and the following trace elements: 10^{-6} M Mn, 1.6×10^{-6} M Zn, 1.1×10^{-6} M Ni and a mixture of Li, V, W, Mo, B, Ca, Cu, Co, Al, Cs, each to about 10^{-8} M. The pH of the suspension was automatically maintained at 7.0 ($\pm$ .05) by addition of small aliquots of 5N ammonium hydroxide. Hydrogen, oxygen and carbon dioxide were supplied at a rate which equaled the rate of consumption by the cells ("dead-end" system). The H_2, O_2 and CO_2 content of the gas phase was 80%, 15% and 5% respectively. Under our operating conditions the oxygen concentration of the suspension varied from .07 to .09 mM. This is well above the rate-limiting threshold. Similarly, the rate of H_2 and CO_2 dissolution was sufficiently fast to satisfy demand. Some of the techniques utilized in the operation of this turbidistat, containing 3 liters of working

suspension, were described previously.[11]

Batch cultures were grown in a mineral medium by a method described previously.[4] In addition, by deinactivating the density control device, the turbidistat could be utilized for batch cultivation. This method of cultivation allowed an accurate determination of the consumption of the individual gases. Unless otherwise stated, all cultures were incubated at 33°C.

Gas Consumption Measurements Two methods were used to determine the gas consumption. The uptake of H_2, O_2 and CO_2 in the turbidistat was measured by water displacement with an accuracy of $\pm$ 3%.

The second procedure which can be used with any method of cell cultivation makes use of a Teflon membrane-covered gold/silver Clark-electrode and an uncoupler of oxidative phosphorylation. The method is based on polarographic measurements of oxygen consumption by a cell suspension preequilibrated with a gas mixture containing approximately 60% H_2, 30% O_2 and 10% CO_2. This composition was selected to provide the suspension with an amount of O_2 slightly in excess of that required for complete oxidation of the available (dissolved) H_2. If hydrogen oxidation is the only hydrogen utilizing activity of the sample, the total amount of hydrogen dissolved in the preequilibrated suspension can be determined (equation 1 page 7). This condition can be obtained by measuring oxygen consumption in the presence of an inhibitor of the

reductive reaction (equation 2 page 7)i.e. in the presence of an uncoupler of oxidative phosphorylation [4]. The consumption of hydrogen by both reactions can now be estimated from the difference between oxygen uptake (corrected for endogenous activity) by a sample with uncoupler (3×10^{-5} carbonyl, m-chlorophenyl hydrazone was used in these experiments) and a duplicate sample without an uncoupler. This difference in oxygen consumption is a measure of the amount of hydrogen utilized for CO_2 fixation (see equation 2). From this value and the O_2 consumption determined in the absence of uncoupler* an O/C consumption ratio can be computed.

The above procedure gives best results for O/C = 2. Replicate measurements yielded an average O/C value of $2.4 \pm .1$. Since the denominator of the quotient represents the difference of two oxygen readings, the accuracy of the ratio is dependent upon the magnitude of this difference relative to the amount of O_2 utilized by the oxyhydrogen reaction and total dissolved H_2. Thus for O/C $\approx$ 4 or more, the uncertainty becomes large.

A drawback of this hydrogen "depletion"method, as well as the Warburg method used previously (6) is that the environmental conditions under which the efficiency measurements are made are not those usually considered as optimal for cell multiplication. In addition, due to the low solubility coefficients of H_2 and O_2 and the relatively slow response of the polarographic electrode only diluted cell suspensions (0.3 mg/ml to 1.5 mg/ml, depending on the activity of the material) can be employed.

* The rate of O_2 consumption in presence of uncoupler varied with time, the initial rate being somewhat faster than that in the absence of uncoupler. However, afte an incubation of 5 to 15 minutes this rate usually became less than the rate obtained in the absence of uncoupler.

Cell Concentration - Growth was measured turbidimetrically and by dry weight determination. The dry weight content of the suspension was determined on an aliquot dried overnight in vacuum at approximately 80°C. Turbidimetric readings were made on appropriately diluted suspensions so that observations fell within the range of proportionality. Under constant growth conditions dry weight was indirectly determined from turbidity values utilizing calibration curves relating dry weight to suspension turbidity.

Viable Count - Viable count was determined on diluted samples spread on petri plates of solidified trypticase soy agar (BBL) and on plates of solidified agar containing the same inorganic constituents used in the liquid media. The inorganic plates were incubated at 32°C under an atmosphere of 50% air, 45% H_2 and 5% CO_2. The trypticase soy agar plates were incubated under similar conditions except that He was substituted for H_2. Plates were examined after 4 days of incubation.

CO_2 Retention - A correction for CO_2 retention in the effluent of the turbidistat was made. Bound CO_2, released from the suspension by acidification, was measured manometrically, according to a method described by Umbreit, et al[12].

Incorporation of $C^{14}O_2$ Into Whole Cells - Whole cells were exposed to labeled carbon in conventional Warburg flasks provided with a gas phase containing H_2, O_2 and CO_2. After a preequilibration for 10 minutes, labeled carbon was added

to the reaction mixture and pressure changes were recorded. After given incubation times, the reaction was terminated by addition of acid. Subsequently an aliquot was dried on stainless steel planchets and the fixed radioactivity counted.

Results

Energy Coefficient (O/C) - The stoichiometric relationship of the three gases involved in the autotrophic metabolism of hydrogen bacteria can be expressed by the following two equations:

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

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

Since endogenous activity of autotrophically grown cells is relatively small, the large majority of oxygen consumed by the cells during autotrophic growth is utilized for hydrogen oxidation (equation 1) i.e. generation of energy for biosynthesis. Since biosynthesis leads primarily to the formation of new cell material, the increase in cell weight and the amount of CO_2 consumed can be employed as indices of biosynthetic activity or energy conversion. The conversion efficiency can now be expressed in terms of the ratio of consumed oxygen to consumed carbon dioxide or the ratio of consumed oxygen to carbon fixed as cell material. An O/C = 2 thus indicates that for the conversion of

a gram-atom carbon as CO_2 into cell carbon (equation 2), the energy-yielding reaction (equation 1) must occur twice (n = 2); a higher value denotes a relatively less efficient use of energy.

The energy coefficient is thus an expression of the amount of energy utilized for the conversion of a unit of carbon dioxide into cellular matter. It thus includes energy for the conversion of CO_2 into a hexose monomer and energy for polymerization of these monomers.

If the ratio of ATP synthesized to oxygen consumed, P/O, is fixed and known, the amount of ATP required for the overall conversion process can simply be deduced from the O/C ratio. The energy coefficient as defined here is inversely related to the yield coefficient originally defined by Bauchop and Elsden[13] and subsequently used in relating dry weight production to energy supply[9, 14].

O/C During Growth Cycle - Campbell, Hellebust and Watson[15] reported that with Nitrocystis oceanus the ratio of the rate of CO_2 fixation to the rate of nitrite production decreased with increasing culture density. In addition, a decline occurred in the specific rate of CO_2 fixation with an increase in culture density. Similar observations were obtained with H. eutropha (See Table 1)

TABLE 1

Change in energy coefficient (O/C) and the rate of CO_2 fixation during the growth cycle.

Expt. No.	O/C	O_2 Uptake μmoles/mg/hr	CO_2 Fixation μmole/mg/hr	Physiological "age" of culture
1	2.90	15.3	10.6	a
2	2.78	16.1	11.6	b
3	2.62	19.0	14.5	b
4	2.84	18.7	13.2	b
5	3.30	13.5	8.2	c
6	3.20	13.7	8.5	c
7	2.96	13.4	9.1	c
8	4.20	16.8	8.0	d
9	5.60	18.7	6.7	d
10	4.22	5.9	2.8	d

Cultures were inoculated at approximately 0.15 mg/ml. Final densities were 2.0 to 2.5 mg/ml. Incubation temperature was 33°C. The approximate physiological age of the culture at time of sampling was: a) "early" exponential phase cells, b) "mid" exponential phase cells, c) cells in transition from exponential to stationary phase, d) "late" stationary phase cells. The gas phase of expts. 1 through 9 contained 8% O_2, 5% CO_2 and 87% H_2. Expt. 10 was incubated under 70% O_2, 10% CO_2 and 70% H_2. The suspension pH at time of sampling was in the range of 6.5 to 7.0 for expts. 1 through 9, and 4.2 for expt. 10. Gas exchange was determined by hydrogen depletion method (see Materials and Methods).

The results show that the energy coefficient (O/C) varies according to the physiological age of the cultures. During the exponential growth phase the lowest O/C values were obtained (expts. 1 through 4). A slight increase is observed in the transition from exponential to stationary growth (expts. 5 through 7). Stationary phase cells (expts. 8 and 9) have O/C values in the order of 4 to 6, i.e. exhibit a relatively low conversion efficiency. Also, a decline in specific rate of CO_2 fixation (i.e. growth rate) is obtained during stationary growth. The decline during stationary growth became more pronounced under extremely unfavorable environmental conditions (expt. no. 10).

O/C in Exponentially Growing Cultures - The environmental conditions used for the experiments described above do not necessarily permit all the cells of the culture to multiply exponentially during the exponential growth phase. One can assume that some fraction of the cultures reproduces exponentially while a part of the culture is already in stationary phase. Such a heterogeneous culture would not exhibit the maximum efficiency of energy utilization if indeed exponential growth is a prerequisite for maximum conversion efficiency. In order to determine whether a more efficient conversion is attainable, gas exchange characteristics were determined for a steady-state culture. In Table 2 some of the results observed under such conditions are recorded.

In this continuous process at steady state the cells are assumed to reproduce in an exponential fashion for several reasons. The average rate of CO_2 assimilation is 4.73 liters per hour, a rate equal to above 20 mmoles of CO_2

per hour per gram (dry weight) of cells or some 30% higher than that observed during the exponential phase in static culture (See Table 1). The rate of reproduction indicates a mean generation time of 1.5 to 2 hours, somewhat higher than the highest rates observed[10, 16] in static cultures (Table 1). Viability measurements indicate that the culture contains 2.4×10^9 to 2.9×10^9 viable cells per mg of dry weight. Viability estimates obtained under heterotrophic and autotrophic conditions (see Materials and Methods) fell within this range. Taking into account a total cell number of the order of 3×10^9 cells per mg of dry weight (16) it appears that the large majority of cells are reproductive. These observations are evidence that a gratuitious environment is provided and that the maximum rate of growth is observed.

The rate at which the three gases are consumed under these conditions is virtually independent of time (see Table 2). From the ratio of the rate of oxygen to carbon dioxide consumption, an average O/C value of 2.48 can be computed.

An alternate method of deriving on an O/C quotient is to use the actual cell yield (see Table 2) as a measure of CO_2 fixation. A production of 5.03 grams per hour, containing 45%C (9, 11) indicates a CO_2 fixation rate of 4.25 liters per hour and O/C value of 2.76. This is in close agreement with the value computed from CO_2 consumption.

Oxygen uptake values in Table 2 not only represent oxygen utilized for hydrogen oxidation (i.e. energy supply for anabolic activity) but also oxygen consumed in endogenous respiration. A computation of the actual gas utilization in the anabolic processes requires a correction for endogenous activity. Endogenous activity was estimated from aliquots withdrawn from the turbidistat. After equilibration with air, the endogenous activity was measured polarographically. The results, (See Figure 1) indicate a relatively rapid decline in endogenous respiration; the maximum observable rate was of the order of 2.4 mmoles of oxygen/hour/gram. Total oxygen consumption (in the presence of the complete gas mixture), calculated on the same basis from data in Table 2, indicates an average rate of 25.1 mmoles. Thus, the endogenous oxygen consumption is approximately 10% of the total oxygen consumption.

Taking into account a 10/1 ratio of anabolic (equation 3) to catabolic (equation 4) activity and the approximate values of H_2, O_2 and CO_2 consumption (see Table 2) the following set of equations result.

$$20\ H_2 + 5\ O_2 + 5\ CO_2 \longrightarrow 5\ (CH_2O) + 15\ H_2O \qquad (3)$$

$$0.5\ (CH_2O) + 0.5\ O_2 \longrightarrow 0.5\ CO_2 + 0.5\ H_2O \qquad (4)$$

$$20\ H_2 + 5.5\ O_2 + 4.5\ CO_2 \longrightarrow 4.5\ (CH_2O) + 15.5\ H_2O$$

TABLE 2

Consumption of H_2, O_2 and CO_2 and cell production by a steady state culture of H. eutropha.

Time (Hrs)	CO_2-uptake l/hr	O_2 uptake l/hr	H_2 uptake l/hr	O/C	H_2/O_2	Yield (g/hr)
0	4.80	5.60	22.4	2.34	4.01	
3/4	4.70	5.75	21.3	2.44	3.71	5.0
1 1/2	4.80	6.00	22.5	2.50	3.75	
2	4.85	5.90	23.2	2.44	3.94	
2 1/2	4.85	6.05	23.7	2.50	3.92	5.2
3 1/2	4.60	5.95	22.3	2.58	3.74	
4	4.75	5.90	22.0	2.48	3.73	
4 1/2	4.65	5.85	21.8	2.52	3.73	
5	4.60	5.75	21.9	2.50	3.82	4.9
Average	4.73	5.88	22.3	2.48	3.78	5.03

The 3 liters of suspension were incubated at 33°C under an atmosphere of 80% H_2, 15% O_2 and 5% CO_2. The cell concentration was turbidimetrically controlled at 3.6 grams (dry weight) of cells per liter by dilution with fresh medium. Approximately 15 generations after the culture was permitted to reach steady state

gas consumption characteristics were measured for approximately three cell generations. The CO_2 uptake data are corrected for retention in reactor effluent (see Materials and Methods).

The actually observed O/C value of 2.48 (Table 2) closely compares to the value of 2.44 predicted according to equations 3 and 4. The actual H_2/O_2 ratio of 3.78 (see Table 2) is somewhat higher than equations 3 and 4 predict ($H_2/O_2 = 3.64$). If one assumes that this discrepancy is within the range of experimental uncertainty, the results in Table 2 are consistent with the following assimilatory process:

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

This gas consumption ratio reflects the best conversion obtained thus far for growing hydrogen oxidizing bacteria.

O/C in Stationary Growing Cultures - Experiments recorded in Table I suggest that the coefficient of energy conversion is dependent on culture "age". A similar conclusion is suggested by the change in gas consumption characteristics observed after shifting a culture from steady state to batch growth. Since under these conditions only gas and ammonia utilization are matched by resupply, the suspension

continues to grow at the same rate as under steady-state conditions until one of the other inorganic nutrients constituents becomes rate limiting. By monitoring gas consumption and concomitant cell production the effects of the transition from steady-state to batch growth could be investigated. The results are illustrated in Figure 2.

Discontinuation of supply of fresh medium to the steady-state culture results in an initial rapid increase in cell concentration followed by a somewhat slower increase. This change in cell concentration is paralleled by a decrease in the ratio of the rate of hydrogen to oxygen consumption and in an increase of the ratio of the rate of oxygen to carbon dioxide consumption. The change in these values are indicative of decreased conversion efficiency. The consumption of $H_2/O_2/CO_2$ changed from 5.1/1/2/1 at the onset of batch growth to 7.5/2.5/1 for "late" stationary phase cells.

Not only did the gas consumption ratios change, the specific activities of CO_2 and O_2 uptake were also affected. The decline in these activities follows first order kinetics (see Figure 3). The rate constant found for carbon dioxide ($k = .66\ hr^{-1}$) was about twice the value obtained for oxygen ($k = .38\ hr^{-1}$). The CO_2 assimilatory activity declined to half the steady-state (or maximum) value ($t_{1/2} = 1.0$ hour) in approximately half the mean generation time. This is thought to indicate that in addition to "dilution" of activity some

deactivation occurred. An equivalent decline in oxygen consumption requires approximately 2 hours ($t_{1/2}$ = 1.8 hours). This is approximately equal to the mean generation time observed during steady-state growth. The rate of decline in oxidiative activity can thus be accounted for by dilution through formation of new cells.

Effect of CO_2 Limitation on O/C - A comparison of cell performance in steady state culture (see Table 2) with "static" culture conditions (see Figure 2) suggest that the energy yield coeffient is adversely affected by an environmental constraint. To test this assumption the gas consumption characteristics were determined on a steady state culture of which the rate of growth was limited by CO_2 supply. CO_2 was selected as a limiting factor since it was expected not to interfere with the oxidative activity or produce other, less defined effects such as would result from, for example, a nitrogen limitation. The results of a series of experiments in which the rate of growth was varied by CO_2 supply is presented in Table 3.

Under conditions of limited CO_2 supply the decline in the rate of hydrogen oxidation was less than to be expected from the decline in the rate of CO_2 assimilation. For example, the results show a 25% decline in the rate of hydrogen oxidation while a 50% decline was imposed on the rate of CO_2 consumption. Comparable changes were observed at other CO_2 supply levels

(see Table 3). Since the extent of the reduction in oxidative activity is significantly less than to be expected from the decrease in growth rate, the efficiency of energy utilization decreased and the O/C values increased (see Table 3). The decline in the H_2/O_2 ratio points to a similar conclusion.

Results presented here suggest that under conditions of CO_2 limitations more ATP is available to the cell then strictly required for normal cell synthesis. These observations and the fact that in complete absence of CO_2 the rate of hydrogen oxidation is still some 40% of the rate attained with an ample supply of CO_2 leads one to assume that there is a limited feed-back control between both activities.

Effect of Oxygen Concentration on Conversion Efficiency - The effect of O_2 concentration on the balance between energy-yielding and energy-consuming reactions was measured on cells precultured in the presence of either a relatively high (20% O_2, 5% CO_2, 75% H_2) or a relatively low (5% O_2, 5% CO_2, 75% H_2, 15% N_2) oxygen concentration. Mid-log phase cells, grown under such conditions, were diluted with fresh medium to approximately 0.30 mg cells per ml, transferred to conventional Warburg flasks, and equilibrated with gas mixtures of various oxygen concentrations. The coupling between both activities was assessed in terms of the ratio of the rate of CO_2 uptake (determined from C^{14} fixation) to total gas consumption. The results, plotted as a function of O_2 concentration (see Figure 4) show that the ratio $C^*/(H_2 + O_2)$ declines with increasing O_2 concentrations. This decrease is observed regardless of conditions of precultivation, but the decline is most distinct with cells precultivated at 5% O_2 (see Figure 4, open circles). These results are taken to imply that excess O_2

TABLE 3

Effect of CO_2 Limitation on the Yield Coefficient

% CO_2	H_2 l/hr	Uptake %	CO_2 l/hr	Uptake %	l/hr	%	O/C	H_2O_2
5	19.3	100	4.25	100	5.22	100	2.45	3.70
2	16.3	85	3.29	77	4.63	88	2.82	3.50
1.5	12.7	66	2.20	52	3.96	76	3.55	3.22
0.9	10.9	56	1.72	40	3.63	69	4.22	3.00
0	-	-	0	0	2.00	38		

For normal growth conditions see legend to Table 2. At gas phase CO_2 contents of 2% and less the rate of growth was limited by the rate of CO_2 transfer. The average values recorded here were calculated from gas determinations made at 10 minute intervals for at least 80 minutes. Values recorded for 1.5% CO_2 were determined from observations made for a total period of three hours. Over this period of time consumption rates were virtually constant: O/C value was $3.55 \pm .11$ and the H_2/O_2 value was $3.22 \pm .09$.

(8% or more) results in a less efficient coupling of energy-yielding and energy-consuming processes.

At relatively low O_2 concentrations (see Figure 4, 3% O_2 or less) cells precultivated in a low oxygen environment exhibit significantly higher $C^* / (H_2 + O_2)$ ratios than cells with a high-oxygen history. This response to cultivation conditions may indicate that synthesis of enzymes connected with CO_2 assimilation are more severely affected by excess O_2 than enzymes responsible for oxidative activity. This would tend to support observations by Kuehn and McFadden[10] who found that synthesis of ribulose- 1, 5-diphosphate carboxylase by H. eutropha was adversely affected by relatively high concentrations of oxygen.

DISCUSSION

ATP Requirement and Growth - The reductive pentose phosphate cycle has been implicated as the main pathway for CO_2 fixation by H. eutropha [10] and a number of other Hydrogenomonas specs [17, 18, 19, 20]. Carbon dioxide fixation through this cycle is thought to require 2 moles of reduced nicotinamide adenine dinucleotide ($NADH_2$) and 3 moles of ATP per mole of CO_2. Since H. eutropha can generate $NADH_2$ directly with molecular hydrogen (1) it seems doubtful that ATP is utilized for the generation of reducing equivalents through ATP-dependent reversed electron flow, e.g. as observed in Thiobacillus [21].

In addition to the 3 ATP, assumed to be required for the conversion of a mole of CO_2 into carbohydrate, another 2.5 to 3 moles of ATP (based on molar growth yield determinations of Bauchop and Elsden[13]) must be expended for the conversion of a mole of carbon from carbohydrate to cell material. For autotrophically growing hydrogen bacteria, utilizing exclusively the Calvin-Benson cycle, the total amount of ATP required to convert a mole of CO_2 to cell material can thus be set at 5.5 to 6 moles.

Energy mobilization derives in this organism from the oxidation of hydrogen; the electron transport associated with this oxidation has been shown to be coupled to the generation of ATP (or its equivalent)[4]. The amount of oxygen used during formation of new cell material is thus a measure of ATP formation.

Depending on the mode of growth energy coefficients (O/C) in the range of 2 to 5 were observed (Tables 1 and 2). A high rate of growth (exponential) apparently is correlated with a high efficiency of energy utilization, while a relatively low growth rate corresponds to a relatively low conversion efficiency. For the most efficient conversion process (see equation 5) an average of 2 atoms of oxygen are utilized per mole of carbon dioxide. If electron transport is coupled at three sites, 6 moles of ATP must be available to the cell for biosynthetic activity. This amount would

actually equal the predicted requirement of 6 ATP. If one assumes the same number of phosphorylative sites but a less efficient conversion process (O/C > 2) more energy than the set requirement would be at the disposal of the cell. How the cell would utilize this excess is not clear. It is not likely that this extra amount of energy is utilized for other cellular activities[22] or for energy of cell maintenance[23]. In observations reported here a correction for the endogenous activity is applied and it is assumed that this correction appropriately reflects the energy utilized in cell maintenance.

A number of other possibilities can be considered however. (i) Three coupling sites contribute ATP to the cell, but the enzyme systems concerned with oxidative phosphorylation function at reduced efficiency in aging cells. This would mean that measurements of O_2 overestimate ATP-formation. (ii) At relatively high O/C values, less than three sites contribute ATP to the cells. Such a relationship between the number of coupling sites and O/C values is illustrated in Figure 5. The previously observed[4] relatively low P/O ratios obtained with cell-free preparations would seem to suggest that with H_2 as a substrate two phosphorylating steps have been eliminated and that the electrons are funneled through cytochrome b to oxygen in a non-phosphorylating bypass. The achievement of energy coefficients of the order of 2.4 (see Table 2) would seem to require participation of these phosphorylating steps. However, spectral evidence suggest the absence of cytochrome a[4, 16]. Therefore, a

significant contribution in ATP formation from a coupling site between cytochrome c and oxygen seems unlikely. Although experimental evidence points to the presence of cytochrome o in hydrogen bacteria[24], its role, or that of a cytochrome c peroxidase system in the oxidation of cytochrome c remains unknown at present. However, the easy oxidation and reduction of cytochrome c in intact cells (Bongers, unpublished) suggests a coupling site between the b and c cytochromes. A minimum of two coupling sites would be required to accommodate cell synthesis at an energy coefficient of approximately 3 (see Figure 5: Predicted requirement of 6 ATP/C).

With coefficients of less than 3, the amount of ATP contributed by two coupling sites would appear to be insufficient, at least, on the basis of the predicted requirement of 6 ATP. However, this requirement is based on the assumption that all carbon dioxide is assimilated via the Calvin-Benson cycle. Although experimental evidence indicates that indeed a majority of C flows via this cycle, fixation via minor, but more economical pathways may also occur. A number of routes are suggested for CO_2 fixation in the tricarboxylic acid cycle [25]. An anaplerotic CO_2-fixation, catalyzed by phosphoenol pyruvate carboxylase was described in E coli by Kornberg[26]. A similar role of this carboxylase was found in autotrophic-grown Ferrobacillus ferrooxidans[27]. Since phosphoenol pyruvate carboxylase activity is also observed in H. facilis, it is tempting to assume that

replenishment of tricarboxylic acid cycle intermediates via this fixation route is a more general phenomenon. A contribution from such a pathway could accommodate an energy coefficient of less than 3.0.

If one assumes a requirement of 5 ATP/C and 2 coupling sites then an O/C = 2.5 can be accommodated (See Figure 5). Such a relatively low overall ATP requirement could not only result from a more economical monomer formation, but also from a more economical monomer polymerization. For the computations presented here it is assumed that monomer polymerization occurs at the expense of 2.5 ATP/C, a value based on a Y_{ATP} = 10.5. This value includes energy of cell maintenance and most probably some energy for the transport of the monomer across the cell membrane. Since such expenditures are not involved in calculations presented here, the assumed polymerization expenditure of 2.5 ATP/C might be an overestimation.

Coupling of Formation and Utilization of Energy - A relatively tight coupling between the rate of hydrogen oxidation and the rate of biosynthesis was reported by Schlegel and Bartha for strain H-1.[28, 29, 30] In H. eutropha oxidative activity is not obligatorily linked to the CO_2 assimilatory activity. Consequently, any condition which specifically limits the occurrence of the energy-consuming reaction (equation 2) will automatically diminish the efficiency of energy utilization. Thus, a limited supply of carbon dioxide (see Table 3) or an environmental factor which specifically inhibits an enzyme

involved in CO_2 fixation will interfere with an equilibrium between both activities.

This "idling" phenomenon, first described by Ruhland [5], could be due to inactivation of ribulose-1, 5-diphosphate carboxylase. Kuehn and McFadden[10] found this carboxylase in H. eutropha to be very sensitive to substrate (fructose) limitation, and aeration. These conditions resulted in loss of carboxylase activity. Some of the CO_2 assimilation phenomena described here could be explained by assuming that under autotrophic conditions carboxylase is similarly affected. This could explain the finding that upon medium "exhaustion" the CO_2-assimilatory activity decays rapidly relative to the oxidative activity (see Figure 3). A similar correlation is suggested from the behavior of the cells with respect to high oxygen tension (see Figure 4) .

Also, a close resemblance appears between the correlation of CO_2-assimilatory activity and growth rate (see Figure 3) and the correlation between carboxylase activity and growth rate in heterotrophic-grown cells[10]. From this behavior it would seem that the observed decline in energy utilization by the autotrophic process is at least in part due to a relatively rapid degradation of ribulose-1, 5-diphosphate carboxylase.

Observations by Schlegel and Bartha [28] are in disagreement with these findings. They observed a rate of hydrogen oxidation under "idling" conditions which was less than one-third the rate obtained in the presence

of CO_2. However, a comparison of their results and those reported here may be misleading, since a different phenomenon may be involved. In strain H-1 the enhancement of the oxidative activity due to the presence of CO_2 could not be restored by uncoupling agents in its absence. In H. eutropha the rate of oxygen consumption with H_2 as a substrate was not significantly affected irrespective of the presence of CO_2 or an uncoupler of oxidative phosphorylation. In conclusion, from the observations presented here, and the fact that in complete absence of CO_2 the oxidative activity usually continues at some 40% of the rate attained with an ample supply of CO_2 (see Table 3) it appears that, at least in H. eutropha, there is a limited feed-back control between both activities. However, a good balance between the activities may persist in a gratuitous environment. Apparently under such conditions the energy made available by the oxidative processes is utilized most fully. If the environment imposes restrictions, the energy donating reactions are effected the least. Consequently, more energy is made available than can be utilized effectively and this results in a lower conversion efficiency.

SUMMARY

Energy mobilization in autotrophic-grown hydrogen bacteria derives ultimately from the oxidation of hydrogen. Oxygen consumption is thus a measure of the amount of energy delivered to the biosynthetic process and the ratio of the rate of oxygen consumption over the rate of CO_2 consumption is a measure of the efficiency of the biosynthetic process. The consumption ratio (O/C) of cell suspensions of Hydrogenomonas eutropha has been studied with particular reference to the growth rate.

The best efficiences (O/C between 2 and 3) were observed with cultures growing at the maximum growth rate. When the rate of growth was limited by the supply of inorganic nutrients or by CO_2 a less efficient conversion was obtained (O/C values between 3 and 5).

An evaluation of gas consumption characteristics suggests that under excellent growth conditions the oxidative and assimilatory activities are well balanced, and that under these conditions about 5 moles of ATP are expended in the conversion of 1 mole of CO_2 to new cell material. Under adverse conditions biosynthesis apparently occurs at an appreciably higher ATP expenditure. Evidence is presented which suggests that this low efficiency of energy conversion is due to the circumstance that energy donating reactions of the cell are relatively insensitive to conditions which slow-down the growth rate.

REFERENCES

1. Repaske, R. 1962. J. Biol. Chem., 237: 1351-1355.
2. Peck, H. D. and H. Gest. 1954. Biochim. Biophys. Acta, 15: 587-588.
3. Packer, L. and W. Vishniac. 1955. Biochim. Biophys. Acta, 17: 153-154.
4. Bongers, L. 1967. J. Bacteriol, 93: 1615-1623.
5. W. Ruhland. 1924. Jahrb. f. Wiss. Botanik, 63: 321-389.
6. Packer, L. and Wolf Vishniac. 1955. J. Bacteriol., 70: 216-223.
7. Marino, R. J. and C. E. Clifton. 1955. J. Bact., 69: 188-192.
8. Hernandez, E. and M. J. Johnson. 1967. J. Bacteriol., 94: 996-1001.
9. W. R. Maxberry, G. J. Prochazka and W. J. Payne. 1968. J. Bacteriol., 96: 1424-1426.
10. G. K. Kuehn and B. A. McFadden. 1968. J. Bacteriol., 95: 937-946.
11. Foster, J. F. and J. H. Lichtfield. 1964. Biotechnol. Biorng., 6: 441.
12. Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1964. Manometric Techniques, 4th Ed., Burgess Publishing Company.
13. Bauchop, T. and R. R. Elsden. 1960. J. Gen. Microbiol., 23: 457-469.
14. W. P. Hempfling and W. Vishniac. 1967. J. Bacteriol., 93: 874-878.
15. A. E. Campbell, J. A. Hellebust and S. W. Watson. 1966. J. Bact., 91: 1178-1185.
16. R. Repaske. 1966. Biotechnol. Bioeng., 8: 217-235.
17. Bergmann, F. H., J. Towne and R. H. Burris. 1958. J. Biol. Chem., 230: 13-24.
18. P. Hirsch and H. G. Schlegel. 1963. Archiv für Mikrobiol., 46: 44-52.
19. P. Hirsch. 1963. Archiv für Mikrobiol., 46: 53-78.
20. P. Hirsch, G. Georgiev and H. G. Schlegel. 1963. Archiv für Mikrobiol., 46: 79-95.

21. M. I. H. Aleem. 1966. J. Bact., 91: 729-736.

22. H. G. Schlegel, G. Gottschalk and R. von Bartha. 1961. Nature, 191: 463-465.

23. Pitt, S. J. 1965. Proc. Roy. Soc. (London) Ser. B. 163: 224-231.

24. L. Packer. 1958. Arch. Biochem. Biophys., 78: 54-65.

25. M. C. Evans, B. Bauchanan and D. I. Arnon. 1966. Proc. Natl. Acad. Sci., 55: 928-934.

26. H. L. Kornberg. 1965. Angew. Chem. Int. Ed., 4: 558-565.

27. G. A. Din, I. Suzuki and H. Lees. 1967. Can. J. of Microbiol., 13: 1413-1419.

28. H. G. Schlegel, R. V. Bartha. 1961. Naturwissenschaften, 48: 414-415.

29. H. G. Schlegel and R. V. Bartha. 1961. Z. Naturforschg., 16b: 777-780.

30. R. Bartha. 1962. Arch. Mikrobiol., 41: 313-350.

Figure 1 - Decay of endogeneous activity in H. eutropha immediately after removal of autotrophic atmosphere. Abscissa: time in equibration with air. Polarographic measurements at 33°C.

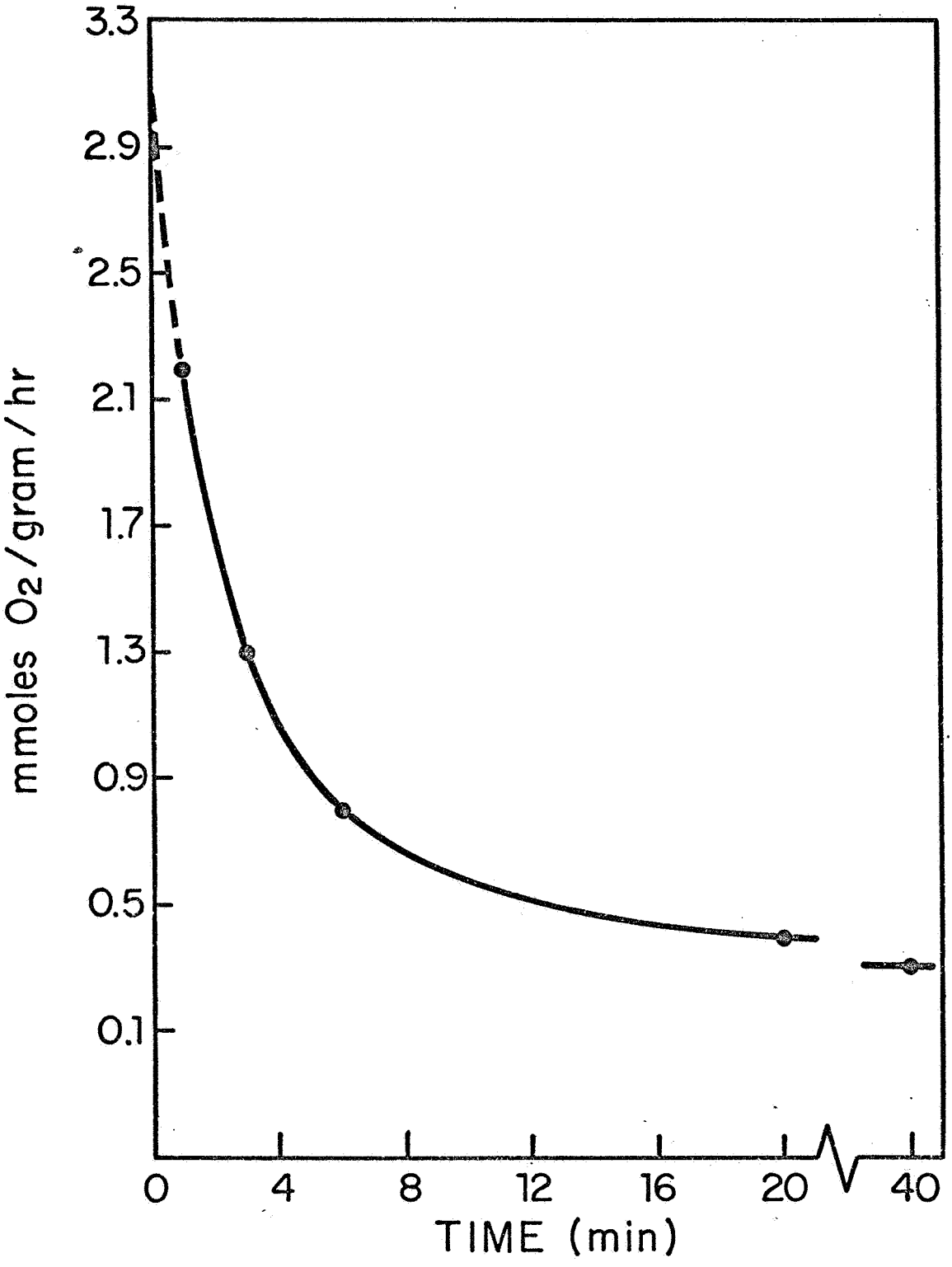

3.3
2.9
2.5
2.1
1.7
1.3
0.9
0.5
0.1
mmoles O2 / gram / hr
0
4
8
12
16
20
40
TIME (min)

Figure 2 - Change in consumption characteristics of H. eutropha after transition from exponential to stationary growth. At 0 hours (after 15 hours steady state culture) suspension dilution was discontinued, and cell concentration allowed to increase. SS-C = steady-state culture. B-C = batch culture. Closed circles: Turbidity (Klett) reading; steady-state cell concentration was 3.5 grams (dry) cell weight per liter; after 4 hours of batch culture cell concentration was 7.5 grams per liter.

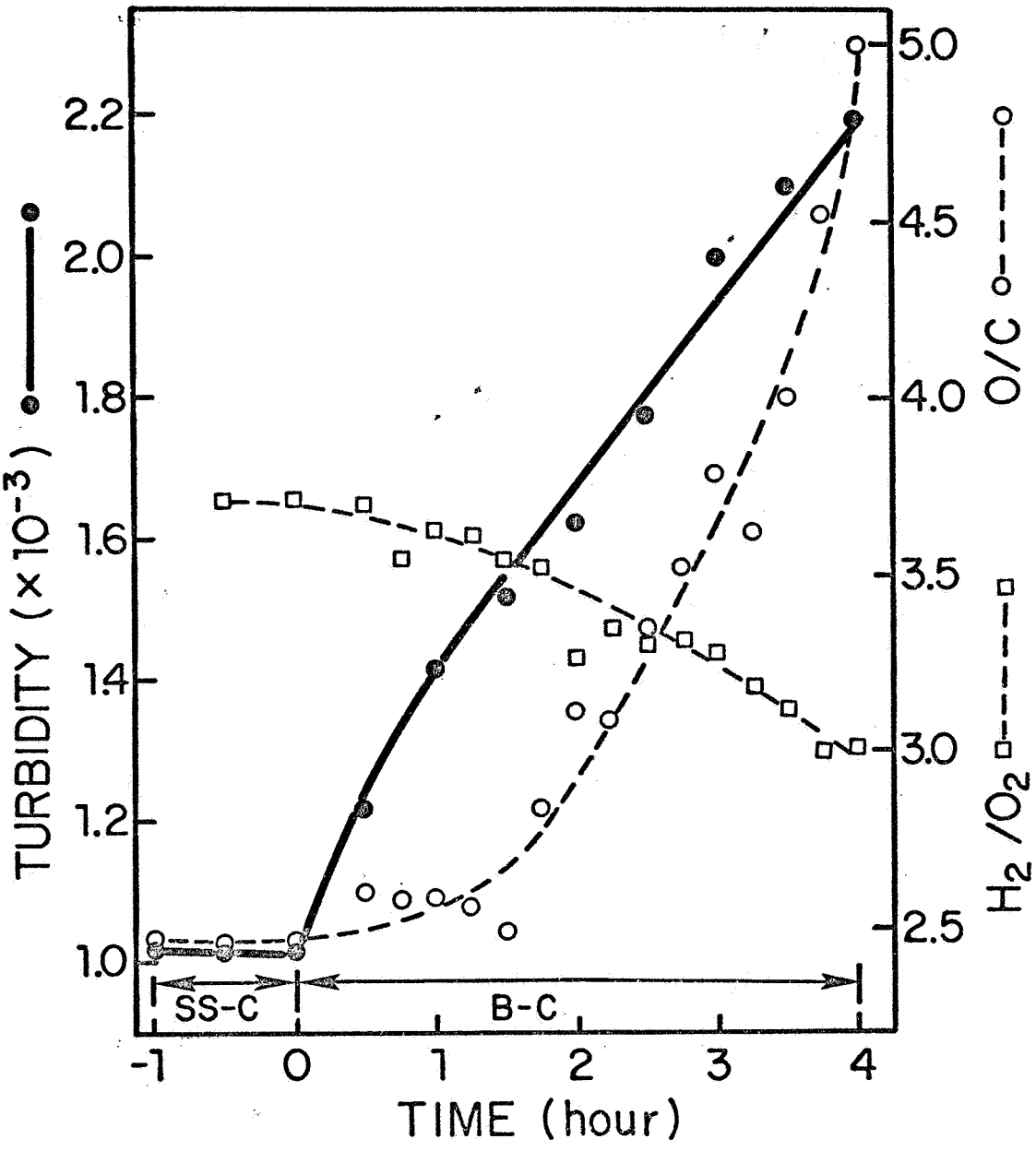

TURBIDITY (×10⁻³)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
5.0
4.5
4.0
3.5
3.0
2.5
O/C
H₂/O₂
SS-C
B-C
-1
0
1
2
3
4
TIME (hour)

Figure 3 - Kinetics of the decline in oxidative and assimilatory activities of H. eutropha during exponential and batch culture. For culture condition see text and legend to Figure 2.

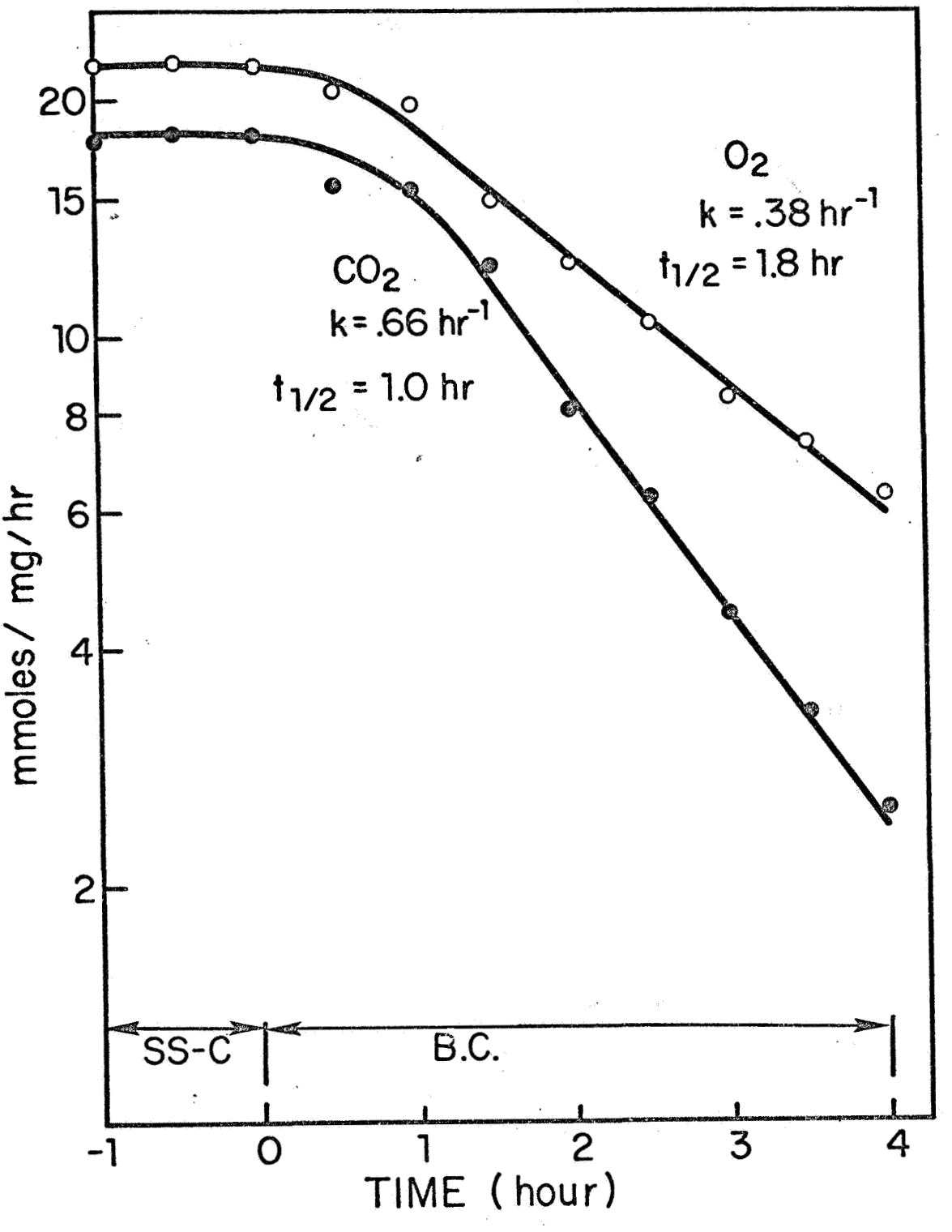
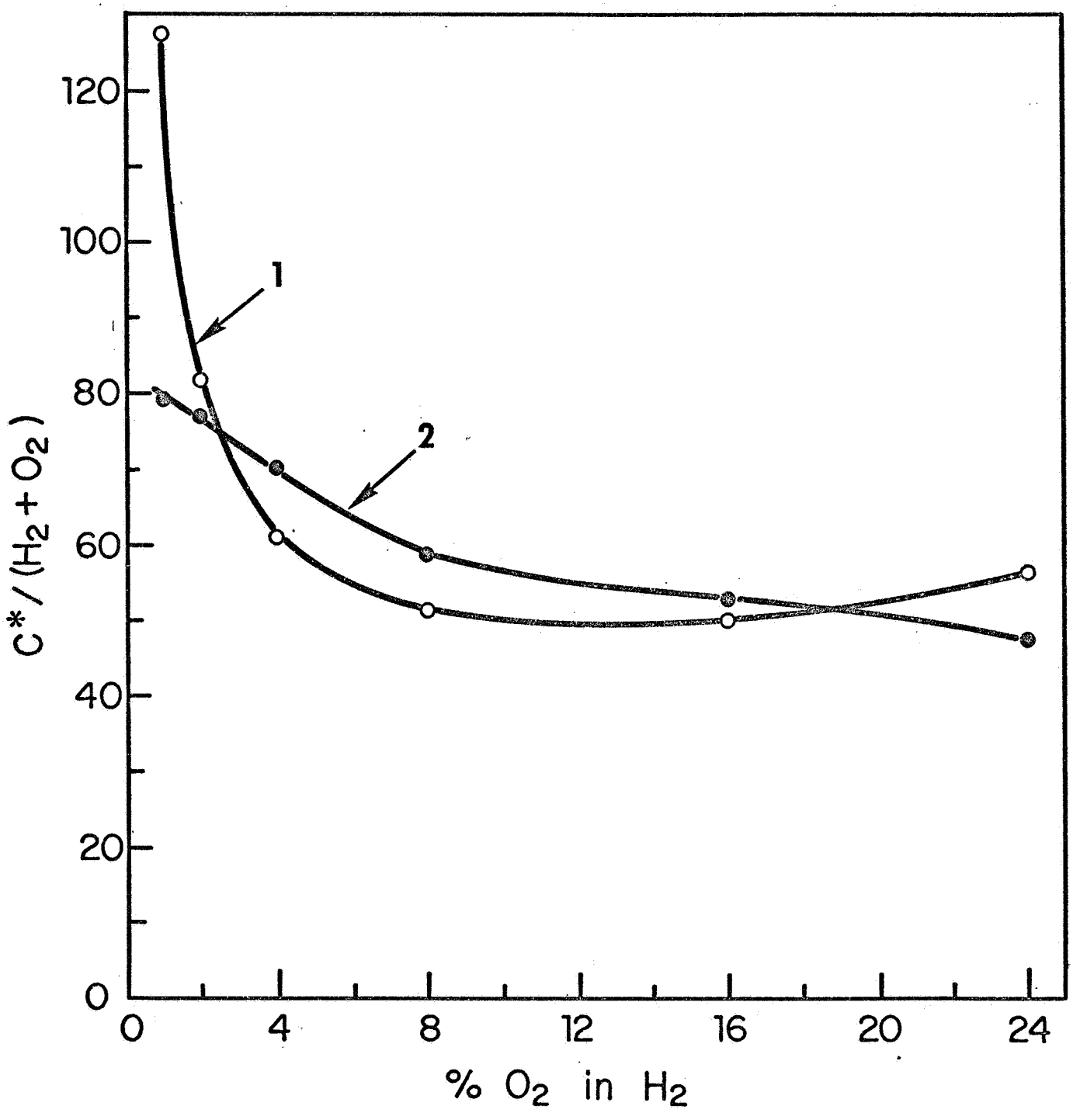

20
15
10
8
6
4
2
mmoles / mg / hr
O_2
k = .38 hr^{-1}
$t_{1/2}$ = 1.8 hr
CO_2
k = .66 hr^{-1}
$t_{1/2}$ = 1.0 hr
SS-C
B.C.
-1
0
1
2
3
4
TIME (hour)

Figure 4 - The ratio of the rate of CO_2 consumption to the rate of total gas consumption as a function of oxygen concentration in hydrogen. Curve 1: low oxygen cells; Curve 2: high oxygen cells. For measuring conditions, see text.


120
100
80
60
40
20
0
C* / (H2 + O2)
1
2
0
4
8
12
16
20
24
% O2 in H2