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A STUDY OF THE CHEMOSYNTHETIC GAS EXCHANGER

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INTRODUCTION

From past research it appears that bioregeneration by hydrogen bacteria such as *Hydrogenomonas eutropha* may be an attractive means of life support for long duration manned space flight.

In previous experiments, exploring the feasibility of this approach, fresh media were used for batch and continuous cultures. For a practical application recycling of liquid fraction of the reactor, with a minimum of processing - such as distillation, filtration or sterilization seems necessary.

One potential problem involved in medium recycling is a gradual build-up of byproducts of metabolism. Undesirable concentrations of such byproducts would result even if only small quantities of intermediary products of metabolism would be added to the liquid each time it is used, since this would eventually create a mixotrophic environment. This must be avoided since it would facilitate the establishment of extraneous organisms and could also lead to phage infection.

To understand the mechanism of formation of excretory products and to provide the necessary background information for design engineering, the phenomenon of leakage of organic products from the cells and reassimilation of such products was investigated in some detail.

In this report the extent of byproduct formation for growth of *H. eutropha* under autotrophic conditions is assessed. In addition, results are presented on the assimilation characteristics of a number of organic acids in a heterotrophic environment by *H. eutropha*.

BYPRODUCT FORMATION AND REASSIMILATION

In the past year attempts were made to simulate closed-cycle operation by reusing the liquid effluent of the reactor. The principle objective was to evaluate the fitness of the reactor fluid for repeated use as a suspending medium for cell growth.

Cell growth and energy utilization in the restocked reactor fluid appeared similar to that observed in freshly prepared medium[1). The results suggested the absence of a build-up of growth-inhibitory substances. These results also indicated that the recycled medium could be properly resupplied with its inorganic nutrients.

Subsequently, attempts were made to determine whether non-toxic products accumulated in the surrounding medium. This could occur during normal growth by excretion of metabolic intermediates or, by cell lysis. To test this assumption the reactor medium was recycled and the concentration of organic compounds in the supernatant fraction of the reactor effluent was determined after each cycle.

In addition, the accumulation of organic carbon in the supernatant fraction of batch cultures was evaluated. The data obtained thus far suggest that under optimum growth conditions, accumulation of leakage products in the growth medium occurs to a very limited extent. It also seems likely that accumulation of *e.g.* organic acids, if excreted to a significant extent will not occur since, according to preliminary observations, such products are readily assimilated again by *H. eutropha*.

[1)] Progress Report on Contract NASW-1596, September 1968.

EXPERIMENTAL METHODS

Culture Techniques. The automated turbidistatic controlled continuous culture with 3 liters of working suspension was used for all autotrophic growth conditions. For the cultivation on organic substrates a chemostat (500 ml working suspension) was employed.

Organic Compounds in the Supernatant of Fraction. Presence of carbonaceous compounds in the spent medium was determined after removal of the cells by filtration (Millipore) or centrifugation. The content of organic carbon in the supernatant fraction was determined by a procedure previously described by Van Hall et al[2]. This procedure permits detection of 2 mg C per liter and has a relative standard deviation of ± 1% at the level of 100 mg C per liter. This method was also employed for the determination of unutilized substrate in cell-yield measurements on added organic substrates. If the effluent of the continuous culture was to be recycled, the suspension was collected and spun (Sharples) in the cold. After addition of nutrients to reestablish the proper nutrient levels the liquid was sterilized and reused.

Excretion products were also evaluated as "combustible substrate", by measuring the amount of oxygen utilized by resting cells for the oxidation of these products. Resting cells of relatively low endogenous activity (*H. eutropha* aerated for 1.5 hours in absence of substrate), were suspended in the supernatant fraction of reactor effluent. The oxidizable substrate was determined polarographically, and the end point of oxidation was considered to occur when the rate of oxygen uptake in the test suspension became equal to the endogenous rate observed in the

[2] C. E. Van Hall, John Safranko and V. A. Stenger, Analytical Chemistry, 35, 315, 1963.

control suspension (resting cells in fresh medium). The concentration of combustible substrate was evaluated by area measurement from a rate vs. time plot.

Excretion of organic compounds in the surrounding liquid of batch cultures was evaluated by means of C^{14}-bicarbonate. For these experiments bicarbonate-C^{14} was added to the growth medium and the C^{14} content of the cell fraction and the supernatant fraction was determined at various phases of cell development. Unutilized C^{14}-bicarbonate was removed by acidification.

Cell Concentration. Cell concentration was determined by turbidity and dry weight measurements.

RESULTS

Excretion Products. Intuitively, one would expect that leakage of intermediary products of metabolism from the cells into the surrounding liquid and also cell lysis occur most prominently after the exhaustion of essential nutrients from the medium, *e.g.* after transition from exponential growth to stationary growth. Indeed, with static cultures, physiologically old cells excrete significant quantities of organic material (see Table 1). Also "deficient growth" leads to significant leakage (see Table 2: -N). On the other hand, the tracer studies showed that rapidly growing (exponential) cells in static culture have a low "steady state" concentration of excretory products. (A similar conclusion is indicated by observations on continuous cultures, see Table 2.) At present, few of the extracellular products are identified - *e.g.* ribose and some amino acids, see Table 2, or their relationship to the overall metabolism defined in any detail.

In Table 2, data are recorded on the steady state concentrations of organic substances in the growth medium of a continuous culture. The data show no significant accumulation, even after the medium is recycled three times. Apparently, under optimal conditions for cell growth and development, the occurrence of autolysis is very limited, or, at least, does not lead to significant levels of free organic substances in the suspending medium. It also appears that under optimal conditions the cells retain intercellularly virtually all products of biosynthesis.

Other possibilities could, however, also account for the virtual absence of extracellular organic products. The possibility that the low level was due to a rapid combustion and reassimilation of excreted material was considered and investigated in two ways. Firstly, the level of organic substances in the supernatant of cultures grown continuously at a relatively low O_2 concentration was compared, the level found in the supernatant of cultures grown at relatively high O_2 concentration. It was assumed that growth under a slight O_2 deficiency would diminish somewhat the combustion of any excreted material and thus allow a small accumulation. With the second method, aliquots were removed from a growing culture, rapidly made anaerobic and subsequently stored at room temperature for given periods of time. It was assumed that cellular excretion would proceed for some time after termination of synthesis, while absence of O_2 would prevent combustion and reassimilation of the excreted material. The amount of oxygen subsequently required for oxidation of the thus accumulated products would be an estimate of its concentration.

The relationship between the level of organic carbon in the supernatant fraction and the O_2 concentration during growth is shown in Table 3. The data show that the concentration of organic carbon in the medium of high-O_2-cells does not deviate significantly from the concentration in medium low-O_2-cells. These results suggest that either no excretion occurs, even at low growth rates (compare expts. 1 and 4 of Table 2 with 2 and 3), or that excreted material is combusted and reassimilated equally well at low O_2 concentrations. The latter possibility appears to be the more reasonable one since the amount of organic carbon excreted during anaerobic incubation increased with time (see Fig. 1). This data shows that the amount of oxygen (area under curves) required for the oxidation of excreted material is a function of anaerobic incubation time. For the oxidation of material excreted during 10 minutes, approximately 1.1 mmoles of O_2 was required, while 2.6 mmoles was needed for materials excreted in 40 minutes of anaerobiosis. Since these materials usually are transient products of metabolism one can assume that in addition to combustion, also reassimilation occurs. If equal amounts are involved in both activities (probably a valid assumption for organic acids, see later) the O_2 values reported here reflect only half the excreted organic carbon.

Although more extensive measurements on excretion are to be made, the present data do permit some preliminary conclusion concerning the steady state rate of organic excretion. From the increase in organic carbon observed immediately after the onset of anaerobiosis a steady state excretion rate of 2 mmoles of organic carbon per gram of cells per hour was estimated. At a growth rate equivalent to a doubling time of 2 hours this excretion rate represents some 10% of the C-turn-over.

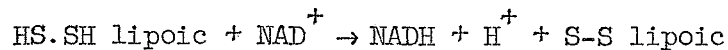
This computation carries the assumption that anaerobic and aerobic excretions are qualitatively and quantitatively similar. The fact that aerated, resting cells are capable of metabolizing the excreted material without a time lag suggests that normal metabolites are involved. The oxidative rates observed in experiments such as illustrated in Fig. 1, were 5 to 6 μmoles O_2/mg cells/hour. These values are nearly similar to oxidation rates observed with lactate pyruvate and acetate, and are about one-third the rate observed at peak growth with H_2 as a sole substrate.

Assimilation of Added Organic Acids. The capability of *H. eutropha* to metabolize lactate, pyruvate, acetate, β-OH butyrate, succinate and α ketoglutarate was tested. It was found that these organic compounds were readily metabolized under aerobic (air) conditions.

The yield of cells per unit of substrate was determined for the lactate-pyruvate-acetate series. The measurements were made in chemostatically controlled continuous culture (500 ml volume). The concentration of the substrate in the feed medium was such that over 90% of the added substrate was utilized by the cells. The dilution rates selected for this experiment were 0.25 hr^{-1} for lactate and pyruvate and .38 hr^{-1} for acetate. Higher dilution rates did lead to culture wash-out and partial utilization of the supplied substrate.

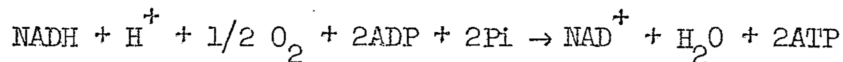
The lactate-pyruvate-acetate series was selected not only because they can constitute part of the excretory material but also since they give us further insight in the energy requisite for cell synthesis.

The results, recorded in Table 4 show that some 20 to 22 grams of cells more are synthesized per mole of pyruvate than obtained with acetate. If, as proposed by Bauchop and Elsden[3] the formation of 10 to 12 grams of cells from "monomers" requires 1 mole of ATP, it appears that pyruvate utilization provides H. eutropha with 2 moles of ATP more than acetate utilization. The results suggest that pyruvate metabolism leads to the reduction of lipoic acid which is reoxidized again by NAD^+ according to:



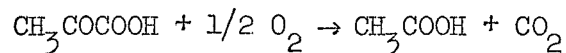
$$\text{HS.SH lipoic} + NAD^+ \rightarrow NADH + H^+ + \text{S-S lipoic}$$

whereby the oxidation energy is converted to ATP formation:



$$NADH + H^+ + 1/2\ O_2 + 2ADP + 2Pi \rightarrow NAD^+ + H_2O + 2ATP$$

The overall reaction of oxidative dicarboxylation of pyruvate yielding carbon dioxide and acetate is:



$$CH_3COCOOH + 1/2\ O_2 \rightarrow CH_3COOH + CO_2$$

The formation of only 2 ATP per gram-atom of oxygen, as observed in heterotrophic cell synthesis, agrees well with the number of phosphorylating sites implied from the ratio of oxygen to CO_2 consumption observed during continuous autotrophic growth[4]. The yield data on pyruvate also suggest that the rates of ATP generation and utilization are equall well matched as in autotrophic growth. A similar energy turn-over is implied by the acetate data.

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[3] Bauchop, T. and S. R. Elsden, J. Gen. Microbiol. 23, 1457, 1960.

[4] Progress Report on Contract NASW-1596, January 22, 1969.

The absence of a growth increment for the lactate-pyruvate difference indicates that the dehydrogenation of lactate to pyruvate supplies H. eutropha no useful energy for cell synthesis. Apparently, as is the case with some other organisms, e.g. E. coli, Aerobacter, Azobacter and yeast, the oxidation of lactate to pyruvate is not linked to NAD. At present it is not known which acceptor functions subsequent to the primary step.

The fact that pyruvate and acetate (and, to some degree also lactate) are utilized relatively efficiently in the presence of air does not necessarily mean that these substrates are also metabolized with similar efficiency if H_2 is supplied simultaneously. The interaction of autotrophic and heterotrophic metabolisms is presently under investigation.

CONCLUSIONS

Provided H. eutropha is permitted to grow at maximum rate, a relatively low steady state level of excreted organic substances is observed in the suspending medium. Approximately 2 to 2.5 mmoles of organic carbon was observed in supernatant fractions of suspensions with a cell concentration of 3.5 grams per liter and a specific growth rate of 0.40 hr^{-1}.

The steady state excretion rate was also determined under these conditions of growth. An excretion rate of approximately 2 mmoles of organic carbon per hour per gram of cells was found. This is some 10% of the carbon turn-over of the cell. Results suggest that under aerobic conditions the excreted products are combusted and/or reassimilated.

Added organic compounds such as lactate, pyruvate, acetate, β-OH butyrate, succinate and α-ketoglutarate are assimilated by *H. eutropha*. Molar growth yield measurements indicate that pyruvate and acetate are efficiently utilized for cell synthesis. The oxidation of lactate to pyruvate does not supply useful energy to the cell. Molar growth yield data suggest that electron transport to oxygen through NADH is coupled to phosphorylation at only two sites.

TABLE 1

Extracellular Product Formation During Batch Growth

Culture Conditions	Extracellular Activ. (% of total)	Extracellular Products
Young Culture[1)]	2	Ribose, Alanine,
Old Culture[1)]	5	Tyrosine, Glutamate
+ N	0-1	--
- N	4-5	--

Extracellular activity represents the amount of organic C^{14} found in the supernatant fluid of suspensions grown in the presence of C^{14}-bicarbonate.

1) L. R. Brown, D. W. Cook, and R. G. Tischer, Dev. Ind. Microbiol., 6, 223-228, 1964.

TABLE 2

Relationship between medium reutilization and the accumulation of soluble organic carbon during continuous growth.

Times Used	Excreted C (mmoles/liter)	Excreted C (% of C-turnover)
1	.9 (± .3)	.7
2	1.3 (± .3)	.9
3	1.4 (± .4)	1.0

H. eutropha was grown in continuous culture (3 liter suspension) at 32°C and a cell concentration of 3.5 grams (dry wt.) per liter. CO_2, O_2, and H_2 partial pressure were maintained at 45 mm, 100 mm and 750 mm respectively. The specific growth rate ($0.40 \pm .02\ hr^{-1}$) was virtually independent of the number of times the reactor fluid was reused. Excreted C was determined as total organic carbon according to method of Van Hall et al.[2)]

TABLE 3

EXCRETION OF ORGANIC CARBON AS A FUNCTION

OF O_2 AND H_2 SUPPLY BY *H. EUTROPHA**

Expt. #	Supply in mm O_2	H_2	Fixation of CO_2 (liter/hr)	Excretion (mmoles/liter)
1	180	High	2.73	2.5
2	54	High	4.36	2.7
3	54	Low	3.70	2.2
4	180	Low	3.15	2.3

*Cells were grown on a 3 liter continuous culture at 32°C at a constant cell concentration of 3.5 grams of cells (dry weight) per liter. CO_2 partial pressure was maintained at 45 mm and total pressure at 900 mm. The dissolved O_2 concentration (measured polarographically) was 0.18 mM at 180 mm while at 54 mm no dissolved O_2 could be detected.

TABLE 4

Molar Growth Yields of H. eutropha

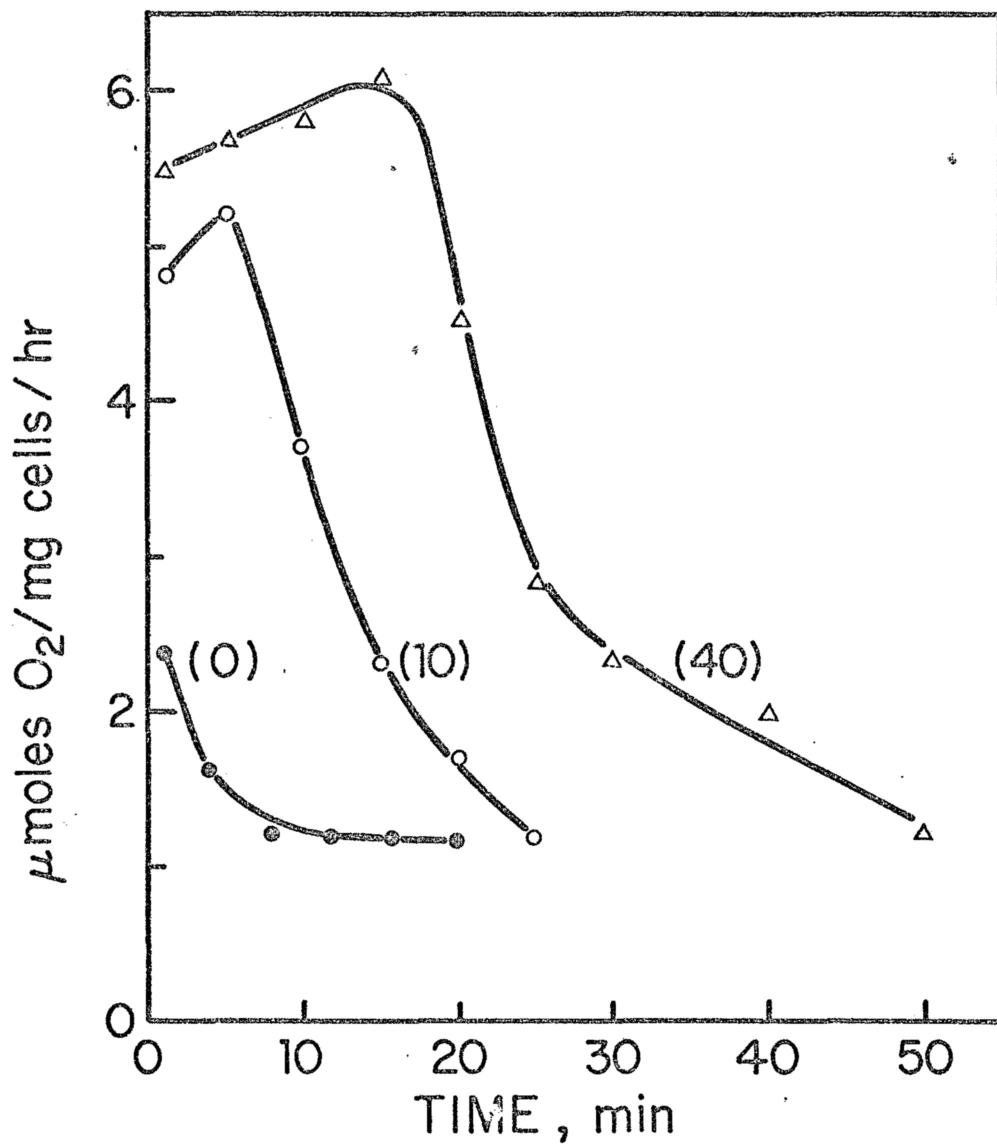
Substrate	D[1]	Molar Growth Yield[2]	Δ	% Combustion
Acetate	.24	22 ± 1		65 ± 3
	.39	21 ± 1		
			21	
Pyruvate	.25	42 ± 1		48 ± 2
			3	
dl-lactate	.25	45 ± 1		43 ± 2

1) D = dilution rate (hr^{-1}).

2) Molar growth yield: grams of cells produced per mole of substrate utilized.

H. eutropha was grown in a chemostat; yield values were determined after the suspension was permitted to reach steady state.

Figure 1 - Oxidation by resting _H_. _eutropha_ of organic excretion products formed under anaerobiosis immediately after cessation of growth. Open circles: supernatant fraction from a suspension (3.5 grams of cells per liter) incubated for 10 minutes under anaerobic conditions. Process: same, but suspension was incubated for 40 minutes. Closed circles: control, no anaerobic incubation. Total oxidizable substrate was calculated from area measurements. The decay constant (first order) for 10 minute-incubation was 0.09 min^{-1} and 0.08 min^{-1} for a 40 minute anaerobic incubation time.


μmoles O_2/mg cells/hr
6
4
2
0
0
10
20
30
40
50
TIME, min
(0)
(10)
(40)