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Uptake of Carbon Dioxide, Hydrogen and Oxygen by *Hydrogenomonas facilis*

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SUMMARY: The simultaneous uptake of CO_2 , H_2 , and O_2 by non-proliferating cells of *Hydrogenomonas facilis* was studied by means of a new method employing ordinary Warburg vessels each provided with one stopper side-arm and one vent side-arm. *H. facilis* effected the same overall reaction as that carried out by certain anaerobically adapted green algae: $6\text{H}_2 + 2\text{O}_2 + \text{CO}_2 \rightarrow (\text{CH}_2\text{O}) + 5\text{H}_2\text{O}$. In an atmosphere of CO_2 and H_2 , no CO_2 fixation accompanied the quantitative reduction of nitrate to nitrite by molecular H_2 ; nor was there any change in concentration of bicarbonate or in total gas pressure when acetone, pyruvate, or α -ketoglutarate were added.

The interest attached to the comparative biochemistry of CO_2 fixation prompts the description of a technique for investigating the gaseous metabolism of *Hydrogenomonas facilis*, a bacterium which oxidizes molecular H_2 with O_2 , and simultaneously assimilates CO_2 (Schatz & Bovell, 1952). The problem, then, is to measure the simultaneous uptake of CO_2 , H_2 and O_2 . To investigate the metabolism of certain anaerobically adapted green algae which fix CO_2 while carrying out the oxyhydrogen reaction, Gaffron (1942) employed specially constructed Warburg vessels. For the work with *H. facilis*, only the ordinary 2-side-arm (one stopper arm, one vent arm) flasks with centre wells were available. This made it necessary to develop the present method which allowed an analysis of the interrelationships among these three gases in the metabolism of *H. facilis*.

METHODS

The procedure for obtaining autotrophically grown cells of *H. facilis* for investigating its hydrogenase has already been described (Schatz & Bovell, 1952). Similar preparations were used to study fixation of CO_2 . For this, the starting conditions were as follows:

Atmosphere: 10 % air (= 2 % O_2), 1 % CO_2 , and 89 % H_2

Vent side-arm	Main vessel chamber	Stopper side-arm
Loosely rolled 1 × 2 cm. strip of filter-paper; 3-4 cm. of rubber tubing on end of vent	Cells suspended in 2.0 ml. M/30 Sørensen's phosphate buffer (pH 7.17); c. 85 μ l. CO_2 present as dissolved bicarbonate	0.1 ml. freshly prepared aqueous pyrogallol plus 0.1 ml. 2N- H_2SO_4 containing 0.2 % HgCl_2

Volume of the flasks was 18 ml. (± 1 ml.). All experiments were carried out at 30°. Concentrations of O_2 and CO_2 much above those indicated proved impractical; when large residual volumes of unassimilated O_2 and CO_2 were

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absorbed (to be discussed) by the subsequently added alkaline pyrogallol, the Brodie solution was sucked into the main vessel chamber.

After the desired interval, the acid mixture from the stoppered side-arm was tipped in and instantaneously halted metabolism. Equilibration following the release of CO_2 from the bicarbonate usually required 15 min. To determine total CO_2 after tipping in acid, the open end of the manometer was corked and the assembly then removed from the water-bath and set in a rack without danger that the Brodie solution would be sucked over into the flask chilled to room temperature.

With a finely drawn Pasteur pipette, the vent arm channel and the rubber tubing were filled with 50 % KOH. A 0.2 ml. pipette charged with the same solution was firmly inserted in the rubber and the vent cautiously rotated to allow 0.200 ml. (or a similar accurately measured volume close to 0.200 ml.) of alkali to drain into the side-arm where it was immediately absorbed by the filter-paper. It was not necessary to replace all air in the vent arm channel and rubber joint, provided the alkali entered at a rate such that small trapped air bubbles neither rose into the pipette nor were driven down into the side-arm. After return of the assembly to the bath and removal of the cork from the end of the manometer, the absorption of CO_2 was complete in *c.* 15 min.

Mixing the acidified killed cell suspension in the main vessel chamber with the KOH-filter-paper in the vent side-arm produced alkaline pyrogallol which absorbed all residual O_2 within 25 min.

The determination of bicarbonate was direct. The values obtained for total CO_2 and O_2 were obtained indirectly and represented the unconsumed fractions of these gases. The amounts of CO_2 and O_2 actually assimilated by *H. facilis* were calculated by subtraction:

$$\text{total initial gas} - \text{unused or residual gas} = \text{gas consumed by cells.}$$

To determine the consumption of H_2 , the CO_2 , H_2 and O_2 initially present only as gases were considered. Since N_2 was not fixed by *H. facilis* (Schatz & Bovell, 1952) nor absorbed by alkaline pyrogallol, it was inert and disregarded in the computations. However, the increase in volume of liquid resulting from the addition of 0.200 ml. of KOH solution through the vent affected manometric readings as would a non-compressible fluid. For the most accurate work this necessitated the use of corrected values for K_{O_2} and K_{CO_2} (Umbreit, Burris & Stauffer, 1949) in all calculations following the introduction of alkali.

Since the solubilities of O_2 and H_2 are small and numerically close:

$$\alpha_{\text{H}_2}^{30^\circ} = 0.02 \quad \text{and} \quad \alpha_{\text{O}_2}^{30^\circ} = 0.03$$

the oxygen constant K_{O_2} was employed for both gases (as was done by Gaffron, 1942) as well as in the computations for the volume of alkali added through the vent.

In computing the amount of each gas assimilated ($-\Delta\text{CO}_2$, $-\Delta\text{H}_2$ and $-\Delta\text{O}_2$), the following designations were employed:

Time	Mm. manometer reading	Gas
Zero (start of experiment)	<i>a</i>	$C_b = \text{CO}_2$ present as bicarbonate. C_i, H_i and $O_i = \text{total initial CO}_2,$ H_2 and O_2 present as gases
After tipping in acid	<i>b</i>	$C_b = \text{CO}_2$ released from bicarbonate in the control flask. $C_f = \text{total final (unused) CO}_2$
After addition of KOH to vent arm	<i>c</i>	$O_f = \text{total final (unused) O}_2$
After KOH tip	<i>d</i>	$H_f = \text{total final (unused) H}_2$

The bicarbonate content of the control flask was taken as a constant for all vessels since the pH, the volume of liquid, and the partial pressure of CO_2 were identical in all vessels at the beginning of the experiment. The atmospheric concentration in terms of $\mu\text{l.}$ of CO_2 , H_2 and O_2 in all reaction vessels at zero time was calculated by direct proportion from the values for V_p , the gas volume which was known for all flasks, and the atmospheric concentration of the control vessel, which was analysed at the beginning of the experiment. In the control vessel,

$$-\Delta\text{CO}_2 = -\Delta\text{H}_2 = -\Delta\text{O}_2 = 0,$$

so that

$$C_f = C_i + C_b, \quad H_f = H_i \quad \text{and} \quad O_f = O_i.$$

Thus, for computing $-\Delta\text{CO}_2$, let $200/K_{\text{O}_2} = \text{mm. increase in manometric reading due to introduction of } 200 \mu\text{l. of KOH solution into the vent side-arm;}$ and $b + 200/K_{\text{O}_2} = \text{theoretical manometric reading if KOH absorbed no gas.}$ But since C_f is quantitatively removed by KOH,

$$b + 200/K_{\text{O}_2} - C_f/K_{\text{CO}_2} = c,$$

from which

$$C_f = K_{\text{CO}_2} (b - c + 200/K_{\text{O}_2}).$$

Therefore,

$$-\Delta\text{CO}_2 = C_i + C_b - C_f = C_i + C_b - K_{\text{CO}_2} (b - c + 200/K_{\text{O}_2}).$$

In the control vessel,

$$-\Delta\text{CO}_2 = 0,$$

so that

$$C_i = K_{\text{CO}_2} (b - c + 200/K_{\text{O}_2}) - C_b.$$

This expresses the relationship between the concentration of bicarbonate and gaseous CO_2 at zero time.

After mixing the acidified cell suspension in the vessel with KOH on the filter-paper in the vent side-arm, both C_f and O_f are completely absorbed by the alkaline pyrogallol. As a result

$$c - O_f/K_{\text{O}_2} = d, \quad \text{or} \quad O_f = K_{\text{O}_2} (c - d),$$

and

$$-\Delta\text{O}_2 = O_i - O_f = O_i - K_{\text{O}_2} (c - d).$$

To calculate $-\Delta\text{H}_2$, let

$$H_i/K_{\text{O}_2} = a - C_i/K_{\text{CO}_2} - O_i/K_{\text{O}_2},$$

and

$$H_f/K_{\text{O}_2} = d - 200/K_{\text{O}_2}.$$

$$-\Delta\text{H}_2 = H_i - H_f = K_{\text{O}_2} (a - d - C_i/K_{\text{CO}_2}) - O_i + 200.$$

For simplicity and convenience in deriving the above relations, vessel-constant corrections due to the volume of KOH added through the vent have not been introduced. In experiments where close approximations are adequate, it is not necessary to apply the corrections for K_{O_2} and K_{CO_2} .

RESULTS

A typical experiment with 3-day old autotrophically grown cells of *H. facilis* is presented in Table 1. It is evident, disregarding the very low endogenous respiration, that the following reactions are effected by *H. facilis*:

This is the same overall reaction carried out by Gaffron's anaerobically adapted green algae (1942).

Table 1. *Assimilation of CO₂, H₂ and O₂ by Hydrogenomonas facilis*
0.25 mg. dry weight equivalent of cells/vessel. *Q* values calculated from the 60 min. data.

		Time of observation (min.)				
		20	40	60	80	100
		μ l. gas consumed				
Uptake of	KOH in centre well*					
CO ₂ (as HCO ₃ ⁻)	—	11	26	35	48	51
CO ₂ (total)	—	14	26	38	46	55
O ₂	+	27	54	76	102	114
	—	31	56	78	91	118
H ₂	+	63	116	166	200	240
	—	90	164	234	278	335
H ₂ :O ₂	+	2.3	2.1	2.2	1.9	2.1
	—	2.9	2.9	3.0	3.1	2.9
H ₂ :O ₂ :CO ₂	—	6.4:2.2:1.0	6.3:2.1:1.0	6.2:2.0:1.0	6.1:1.9:1.0	6.1:2.1:1.0
<i>Q</i> _{CO₂}	—	.	.	72	.	.
<i>Q</i> _{O₂}	+	.	.	145	.	.
	—	.	.	148	.	.
<i>Q</i> _{H₂}	+	.	.	315	.	.
	—	.	.	446	.	.
Endogenous respiration						
O ₂	+	0	4	6	8	9
<i>Q</i> _{O₂}	+	.	.	12	.	.

* KOH + : atmosphere = 10 % air + 90 % H₂. KOH — : atmosphere = 10 % air + 1 % CO₂ + 89 % H₂.

In other experiments under anaerobic conditions described in Tables 2 and 3, the fixation of CO₂ could not be demonstrated. During the reduction of nitrate to nitrite with molecular H₂, the ostensible fixation of 2 μ l. of CO₂ lies well within the experimental error for Warburg work.

According to calculations from the values cited by Rabinowitch (1945) and Stephenson (1949), both the heat content and free energy from the reduction of nitrate to nitrite by molecular H_2 are approximately two-thirds of the corresponding values for the oxyhydrogen reaction with an equivalent amount of H_2 . For this reason, it was anticipated that during the reduction

of nitrate about two-thirds as much CO_2 would be assimilated per mole of H_2 oxidized with nitrate as compared with O_2 .

Table 2. *Inability of Hydrogenomonas facilis to fix CO_2 during the reduction of nitrate by molecular H_2*

6.0 mg. dry weight equivalent of cells/vessel. Q values calculated from the 50 min. interval required for the actual reduction of nitrate.

	Atmosphere			
	100 % H ₂		1 % CO ₂ +99 % H ₂	
	KOH in centre well		No KOH	
	μ mole KNO ₃ tipped from side-arm			
	0	10.0	0	10.0
Observed μl. gas consumed	0	217	0	225
μl. H ₂ required for reduction of all NO ₃ ⁻ to NO ₂ ⁻	0	224	0	224
Total μl. CO ₂ present at time 0	.	.	295	299
Total μl. CO ₂ present at end	.	.	293	297
μl. CO ₂ fixed	.	.	2	2
Q _{H₂}	.	41	.	43
Q _{CO₂}	.	.	0.4	0.4
Endogenous fermentation				
Total μl. CO ₂ evolved	.	.	-2.0	.
Q _{CO₂}	.	.	-0.4	.

The inability of *H. facilis* to fix CO_2 during the reduction of nitrate was surprising. The organism grows well autotrophically with nitrate as the sole source of nitrogen, and such cultures give an intense test for nitrite (Schatz & Bovell, 1952). This ruled out the possibility that nitrate inhibited the chemosynthetic assimilation of CO_2 by *H. facilis* as it does the photosynthetic fixation of CO_2 by *Chlorella pyrenoidosa* (Warburg & Negelein, 1920), which van Niel (1949) has considered to be a competition between nitrate and CO_2 as H-acceptors in the photolysis of H_2O . Since *H. facilis* failed to apply the energy released from the oxidation of H_2 by nitrate for the fixation of CO_2 anaerobically, it differs from *Thiobacillus thiooxidans*. Vogler & Umbreit (1942) found that *T. thiooxidans* was capable of accumulating in organic phosphate esters the energy derived from the aerobic oxidation of sulphur in the absence of CO_2 , and subsequently utilizing this energy store for the assimilation of CO_2 under anaerobic conditions.

H. facilis also did not consume CO_2 in an atmosphere of H_2 and CO_2 even after 20 hr. (Table 3). With *Rhodospirillum rubrum*, the photoreduction of

CO₂ began only after an adaption period of 8 to 9 hr. (Foster, 1944). With *H. facilis* there was no uptake of CO₂ or H₂ in the presence of pyruvate, α -ketoglutarate, or acetone (Table 3). The assimilation of CO₂ is in considerable measure mediated by a carboxylation of pyruvate and α -ketoglutarate (Ochoa, 1951).

Table 3. *Inability of Hydrogenomonas facilis to assimilate CO₂ and H₂ in the presence of pyruvate, α -ketoglutarate, or acetone*

Supplement tipped from side-arm	Atmosphere	Duration of experi- ment (hr.)	Dry weight equivalent of cells (mg.)	Results
Pyruvate,* 10.0 μ mole	2 % CO ₂ + 98 % H ₂	2.8	16	No change in bicarbonate concentration or mano- metric reading. Rapid gas uptake upon intro- duction of air
α -Ketoglutarate,* 10.5 μ mole	1 % CO ₂ + 99 % H ₂	1.4	13	
Acetone, 10.0 μ mole	1 % CO ₂ + 99 % H ₂	1.8	11	
None	1 % CO ₂ + 99 % H ₂	20	30	38 μ l CO ₂ fixed. (Table 1)
None	1 % CO ₂ + 89 % H ₂ + 10 % air	1	0.25	

* Added as sodium salts.

DISCUSSION

The respirometric method of analysis developed here requires actually only the determination of bicarbonate, and total initial CO₂ and O₂ gases in the control vessel at zero time. With this information plus the values for the gas volume of the filled flask, the constants K_{O_2} and K_{CO_2} , and the manometric readings a , b , c , and d corrected for thermobarometric variation, it is easy to calculate the net exchange of H₂, O₂, and CO₂ independently in flasks gassed from the same mixture. Despite the numerous operations to which each manometer is subjected, the results obtained have been well within the range of experimental error conventionally accepted for simpler operations with the Warburg apparatus.

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