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Effect of Oxygen on the Growth (Yield) of *Chlorella vulgaris*

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Abstract. The growth yield of *Chlorella vulgaris*, Y_{kJ} defined as g cells harvested per kJ of light energy absorbed by the cells, was assessed in a turbidostat culture by varying CO_2 and O_2 partial pressures (p_{CO_2} and p_{O_2}). The value of Y_{kJ} ranged from 3.1×10^{-3} to 5.0×10^{-3} g cells/kJ under light-limited conditions [$p_{CO_2} = 1.0 \sim 2.4\%$, $p_{O_2} = 0 \sim 65\%$; total pressure of gas (composed of CO_2, O_2 and N_2) = 1 atm]. In the light-limited environment, the algal specific growth rate μ deteriorated appreciably with the increase of p_{O_2}. The deterioration accounts for the above range of Y_{kJ} observed. The growth inhibition due to oxygen that was defined by subtracting from 1.0 the ratio of μ at given values of p_{O_2} to that at $p_{O_2} = 0\%$ extended from 0.07–0.30 (7–30%). However, glycolate could not be detected in the turbidostat culture. Isotopic experiments on the specific rate of $^{14}CO_2$ uptake also revealed that the inhibition due to oxygen was from 22–38% when p_{O_2} was varied from 0 to nearly 100%. These effects of oxygen were discussed, referring to the activity of ribulose-1,5-bisphosphate carboxylase that is inhibited competitively by oxygen.

Key words: *Chlorella vulgaris* – Turbidostat culture – Growth yield (Y_{kJ}) – Oxygen inhibition in *Chlorella* growth – RuP_2 carboxylase/oxygenase – Carbonic anhydrase.

The growth yield Y_{kJ} (or Y_{kcal}) of the blue-green algae *Spirulina platensis* and *Microcystis aeruginosa* was assessed previously (Aiba and Ogawa, 1977; Ogawa et al., 1978, 1979; Ogawa and Aiba, 1978). In the present communication, the experimental material will be extended from the blue-green algae to the green alga *Chlorella* to secure a broader spectrum of data on the light-energy conversion. The question of whether or not the difference in their degree of organelle differentiation affects the energy-conversion efficiency was the basis of this work.

In spite of the classical finding, i.e., inhibitory action of O_2 on photosynthesis of green algae (Warburg, 1920), the effect of O_2 on algal growth (yield) has not been made clear. However, in pursuit of the biochemical mechanism of CO_2-fixation in plants, Decker (1955) disclosed a specific phenomenon designated as photorespiration. Since then, several research workers have been paying their attention to photorespiration to elucidate the O_2 effect that was discovered originally by Warburg (1920) (Turner and Brittain, 1962; Tolbert, 1974). Recently, Zelitch (1975, 1979) and Oliver and Zelitch (1977) claimed a possibility to improve the efficiency of photosynthesis by warding off the effect. Indeed, ample room is left open for examining the possibility of enhancing algal growth (yield) by minimizing the photorespiration. Accordingly, it is deemed worthwhile conducting further experiments on the O_2 effect with respect to the growth of *Chlorella* cells.

Materials and Methods

Microalga and Culture Medium. The microalga used was *Chlorella vulgaris* 211/8 K strain, solicited from the Culture Centre of Algae and Protozoa, Cambridge, England. The composition of the culture medium used throughout was (g/l): KH_2PO_4 1.25, KNO_3 1.25, $MgSO_4 \cdot 7H_2O$ 1.25, $CaCl_2 \cdot 2H_2O$ 0.04, $FeSO_4 \cdot 7H_2O$ 0.002, H_3BO_3 2.86×10^{-3}, $MnSO_4 \cdot 7H_2O$ 2.5×10^{-3}, $ZnSO_4 \cdot 5H_2O$ 0.222×10^{-3}, $CuSO_4 \cdot 5H_2O$ 0.079×10^{-3}, Na_2MoO_4 0.021×10^{-3}.

Cultivation Procedure. A turbidostat culture was used to assess the growth yield (Y_{kJ}) of this alga. A schematic diagram of the turbidostat

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Non-Standard Abbreviations. INH = isonicotinic acid hydrazide; PPO = 2,5-diphenyloxazole; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimetylurea; CA = carbonic anhydrase; RuP_2 = ribulose-1,5-bisphosphate

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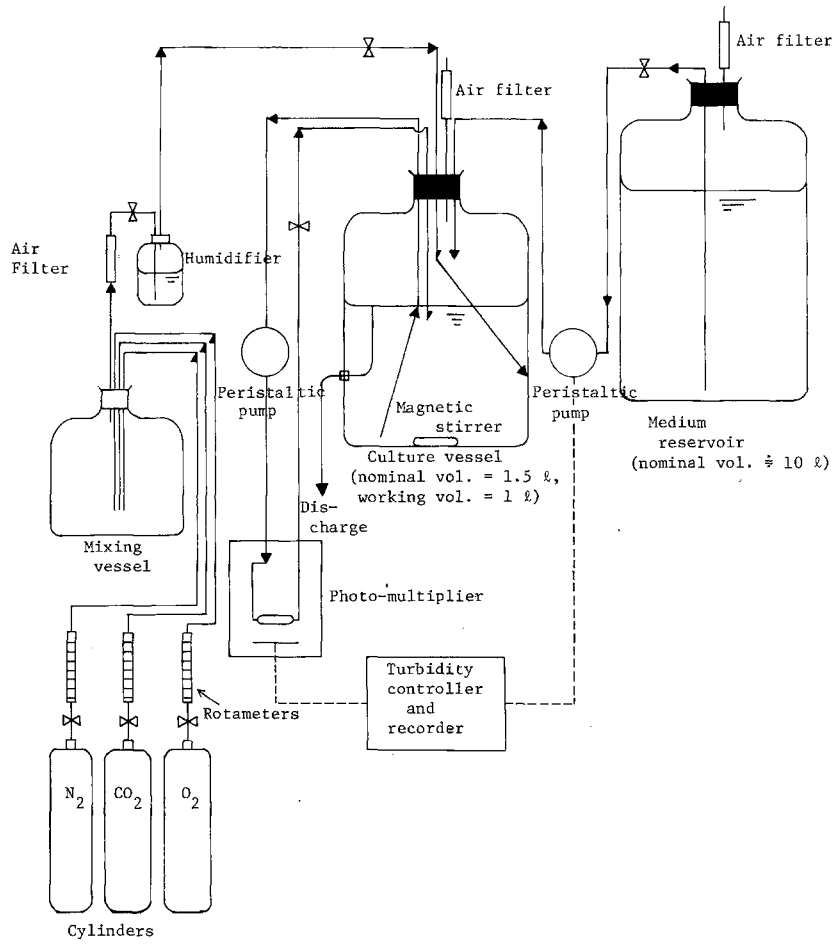

Fig. 1
Schematic diagram of turbidostat culture

used is shown in Fig. 1. The culture vessel (Roux bottle, 1.5 l capacity, working volume $\simeq$ 1 l), charged with the medium (made sterile at 120° C for 10 min) and inoculated (from a preculture in shaken flasks) was agitated with a magnetic stirrer at 30° C. The front surface of the vessel was irradiated by a halogen lamp through an infrared cut-filter. The lamp and the filter were those which were used previously in the cultivation of *Spirulina platensis* (Aiba and Ogawa, 1977; Ogawa and Aiba, 1978). The composition of the gas mixture used was changed by adjusting the flow rate of either N_2, CO_2 or O_2 from each cylinder via precision rotameters (Type KG-2 or KG-3, Kusano Sci. Inst. Co., Tokyo) into the mixing chamber (see Fig. 1). p_{CO_2} ranged from 1.0–2.4%, p_{O_2} from 0–65%, and p_{N_2} from 33–99%, provided: p = volume percentage of each gas (total pressure = 1 atm).

The accuracy of p_{CO_2} values in the mixed gas was checked by using an infrared gas-analyzer (Type AIA-21, System-600, Horiba Seisaku-sho, Tokyo). The gas mixture was passed into the Roux bottle through a sterile air-filter of glass fibers and a humidifier (a glass vessel partially filled with deionized water). In some runs, ambient air diluted with N_2 gas was also used to realize p_{CO_2} = 0.015%.

The set of devices used to control the turbidity was Model TD100, Turbidity Controller, New Brunswick Sci. Co., Inc., New Brunswick, N.J. During a transient (unsteady) period which lasted for nearly 48 h, fluctuations in the flow rate F of the fresh medium into the culture vessel attenuated. The steady state of the turbidostat culture was established when the cumulative volume of the fresh medium charged into the vessel was confirmed to be linear against time after 48 h. Then, the specific growth rate μ was determined as $\mu = F/V$, where V = working volume of the vessel.

Incident Light Energy and Absorbance I_a/I_o of the Algal Suspension. The intensity of light I_o transmitted through the vessel filled with the culture medium was measured with a bolometer (Kipp and Zonen, Delft, The Netherlands). The absorbance I_a/I_o with regard to the algal cells suspended in the medium was measured either with the opalescent-plate method (Shibata, 1958) or by an integrating sphere spectrophotometer (UV-210, Shimazu, Kyoto) used previously (Aiba and Ogawa, 1977; Ogawa et al., 1978). The intensity of light I_o employed in this work was 1.7×10^{-3} or 2.7×10^{-3} J/cm^2 · s (5.3 or 8.4 klux).

These intensities were far from saturation ($\simeq$ 15 klux) which was confirmed by preliminary experiments on the specific rate of oxygen evolution vs. light intensities. Consequently, the turbidostat culture here was apparently "light-limited".

Glycolate Excreted. Chlorella cells were cultured in shaken flasks [4 to 6 klux (tungsten lamp); air or CO_2-enriched gas (p_{CO_2} = 5%)] at 30° C for 7 days. Cells were harvested by centrifugation (about 1,800 × g for 7 min) and rinsed once with a potassium-phosphate buffer solution (pH 8.0). A cell suspension (OD_{550} = 0.76; $X \simeq$ 0.25 g/l) in the same buffer solution was obtained. 12 ml of the suspension was transferred into an Erlenmeyer flask (100 ml), and the flask was shaken in the dark at 30° C for 10 min after adding either deionized water (0.15 ml) or isonicotinic acid hydrazide (INH) solution (0.15 ml, 0.1 M).

This pretreatment was followed by an addition of a sodium bicarbonate solution (1.5 ml, 0.1 M) into the flask (p_{CO_2} = 0.7%). Right after the addition, each flask was exposed to a tungsten lamp (about 3 klux) and was gassed with pure O_2 for 3 h. 1 ml of the suspension was withdrawn every 45 min. The supernatant liquid

secured by centrifugation of the sample at 1,000 × *g* for 10 min was subjected to colorimetric analysis of glycolate (Calkins, 1943).

Uptake of $^{14}CO_2$. About 40 – 50 ml of the culture medium in which algal cells had been grown up to $X \simeq 0.03$ g/l at 30°C and 3 klux (fluorescent light) were distributed into Erlenmeyer flasks (100 ml). The algal suspension was gassed with CO_2 ranging from $p_{CO_2} = 0.4 - 2.3\%$, and the rest component of the gas was either pure N_2 or pure O_2. The gassing continued for more than 1 h in the dark to warrant an equilibrium between the gas and the liquid. A rectangular vessel of glass (35 × 35 × 50 mm) was then filled with the cell suspension and the glass vessel was sealed with a rubber plug, leaving no free space.

Immediately after an addition of 200 µl or 50 µl of $NaH^{14}CO_3$ solution with a microsyringe, the glass vessel was exposed to various intensities of light (6.5 – 9.5 klux; halogen lamp). 200 µl of the reactant was sampled every 1.5 min with another microsyringe into a vial, and each run at 30°C and pH ≃ 6.0 was terminated at 7.5 min after irradiation. The withdrawal of the reactant was followed by an addition of an aqueous solution of hydrochloric acid (6 N, 1 ml) into the vial. The suspension withdrawn was dried on a boiling waterbath and deionized water (0.1 ml), 99.5% v/v ethanol (3 ml) and a toluene solution of PPO (2,5-diphenyloxazole) (5 g/l, 10 ml) were added into the vial to observe the decomposition rate (dpm) of the acid-stable fraction of the cells. A liquid-scintillation counter (LS-250, Model 1969, Beckman Instruments Inc., Fullerton, Calif.) was used. The specific uptake rate of $^{14}CO_2$ could be assessed from the decomposition rate and the data on specific radioactivity of $NaH^{14}CO_3$, dpm/total carbonate in the sample (Ogawa and Aiba, 1978).

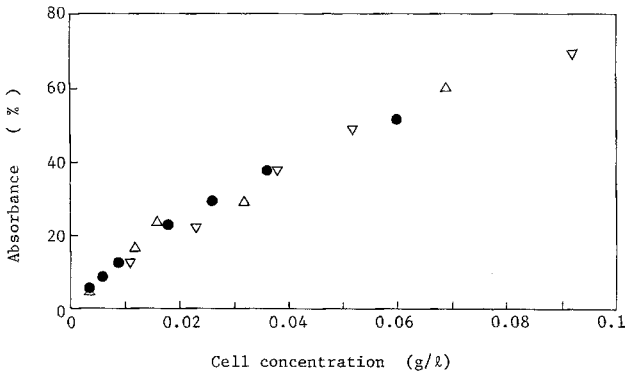

Fig. 2. Absorbance vs. cell concentration

Symbol	Method	Culture conditions
●	Integrating sphere spectrophotometer	air
△	Opalescent plate	$p_{O_2} = 97.6\%$, $p_{CO_2} = 2.4\%$
△	Opalescent plate	$p_{N_2} = 97.6\%$, $p_{CO_2} = 2.4\%$

Results

Absorbance as a Function of Cell Concentration. The absorbance I_a/I_0 of *Chlorella vulgaris* was plotted against the cell concentration in Fig. 2. It is clear that this correlation was consistent, irrespective of the difference in the measurement procedure (either the opalescent-plate method or the integrating sphere spectrophotometer) and regardless of the difference in culture conditions.

Growth (Yield). Experimental data on the turbidostat culture of *Chlorella vulgaris* are summarized in Table 1. Smaller values of specific growth rate for Run Nos. 10 to 12 are apparently ascribed, in addition to the "light-limited" environment, to the fact that CO_2 also limited the growth because of the use of gas fairly low in p_{CO_2} (about half of the value in air). When incident light intensity and p_{CO_2} values were kept unchanged, respectively, the increase of p_{O_2} in the supply gas resulted clearly in the decrease of μ values. Experimental data in Table 1 and the light absorbance data in Fig. 2 could determine Y_{kJ} values as shown in the last column of the table by the same method as reported previously (Aiba and Ogawa, 1977; Ogawa et al., 1979).

It can be seen from Table 1 that an average of Y_{kJ} values taken as 4.3×10^{-3} g cells/kJ at $p_{O_2} = 20\%$ was of the same order of magnitude as those assessed previously with *Spirulina platensis* (Aiba and Ogawa, 1977) and *Microcystis aeruginosa* (Ogawa et al., 1979).

Y_{kJ} values were apparently affected adversely by p_{O_2} values. Subtracting from 1.0 the ratio of μ value at p_{O_2} in Table 1 to that at $p_{O_2} = 0\%$ (and/or $p_{O_2} = 10\%$ exceptionally for Run Nos. 10 to 12) as the degree of growth inhibition due to O_2, the inhibition ranged from 0.07 – 0.30 (7 – 30%; see Table 2 later on). Glycolate could not be detected at all in the effluent from the turbidostat culture.

Respiratory Activities. A cell suspension ($X \simeq 0.22$ g/l) containing DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea, 8.5×10^{-5} M] was irradiated in the glass vessel at various intensities of the halogen lamp (see Fig. 3). Clearly, the respiration in the dark was hardly affected by the light up to 20 klux. This is in sharp contrast with *Spirulina platensis* studied previously; the respiration of *Spirulina platensis* faded conspicuously when the light intensity exceeded 4.7 klux (Ogawa and Aiba, 1978).

Glycolate Excretion. Figures 4a and b show that glycolate was excreted from the algal cells when an inhibitor (INH) was added to the suspension. It is interesting to note from Fig. 4a that the rate of glycolate excretion was virtually zero in the absence of INH, provided the values of p_{CO_2} in the cultures were low [air (preculture) and 0.7% in the experiment]. It is also significant to remark from Fig. 4b that glycolate excretion was observed regardless of the presence or absence of INH, provided p_{CO_2} values were changed abruptly from 5% (preculture) to 0.7% (in the run).

Table 1
Experimental data on turbidostat cultivation and determination of Y_{kJ}

Run No.	Light intensity I_0 (J/cm² s)	Partial pressure p_{CO_2} (%)	Partial pressure p_{O_2} (%)	Specific growth rate, μ (h^{-1})	Glycolate (mg/l)	Y_{kJ} (g/kJ)
1	1.7×10^{-3}	1.0	0	0.060	0	5.0×10^{-3}
2	1.7×10^{-3}	1.0	20	0.052	0	4.3×10^{-3}
3	1.7×10^{-3}	1.0	65	0.050	0	4.1×10^{-3}
4	2.7×10^{-3}	1.0	0	0.077	0	4.3×10^{-3}
5	2.7×10^{-3}	1.0	20	0.067	0	3.8×10^{-3}
6	2.7×10^{-3}	1.0	65	0.054	0	3.1×10^{-3}
7	2.7×10^{-3}	2.4	0	0.083	0	5.0×10^{-3}
8	2.7×10^{-3}	2.4	20	0.077	0	4.8×10^{-3}
9	2.7×10^{-3}	2.4	65	0.069	0	3.6×10^{-3}
10	2.7×10^{-3}	0.015	10	0.034	0	1.9×10^{-3}
11	2.7×10^{-3}	0.015	35	0.029	0	1.7×10^{-3}
12	2.7×10^{-3}	0.015	60	0.028	0	1.4×10^{-3}

Cell concentration = 38.6 ± 9 mg/l

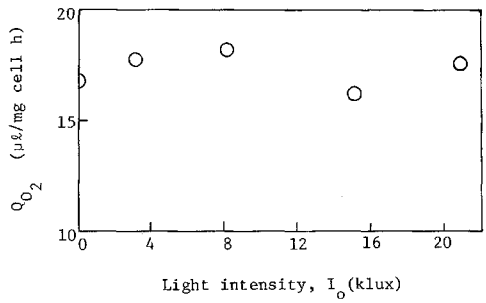

Fig. 3. Respiratory activities under light. DCMU was added to the cell suspension at a concentration of 8.5×10^{-5} M

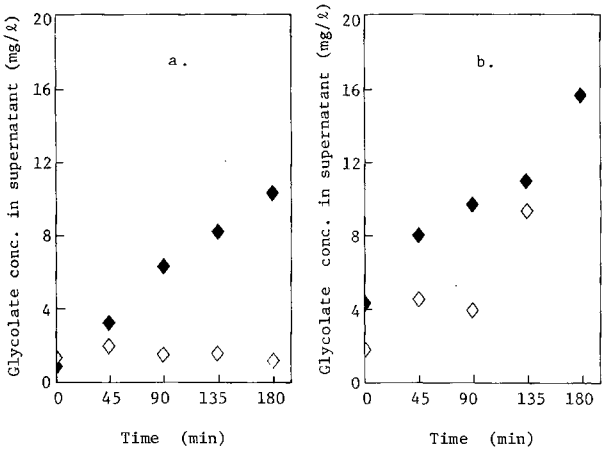

Fig. 4a and b. Glycolate excretion from *Chlorella vulgaris*. **a** Deals with algal cells precultured with CO_2 in air, while **b** pertains to cells precultured in CO_2-enriched gas (p_{CO_2} = 5%). Symbols ◆ and ◇ are for the presence or absence of INH, respectively

$^{14}CO_2$ Uptake. It is evident from Fig. 5 that anaerobic runs exhibited larger uptake rates of $^{14}CO_2$ than the aerobic ones. Solid lines were assumed through solid and/or open triangles in Fig. 5 by the method of least squares.

The linearity, as noted from the figure, between $^{14}CO_2$ uptake and time is considered to justify the assumption that the amount of $NaH^{14}CO_3$ used in each run did not limit the uptake rate of $^{14}CO_2$. It must be mentioned that dissolved O_2 in the culture medium for anaerobic runs in Fig. 5 should have increased slightly (several % of the saturation; data not shown here) because of photosynthesis which continued for 7.5 min. Thus, the anaerobic runs have not been literally so.

The degree of O_2 inhibition of photosynthesis was defined here as the subtraction of the ratio of $^{14}CO_2$ uptake rate for aerobic to that of for anaerobic conditions from 1.0. So far, the inhibition extended from 0.22 – 0.38 (22 – 38%; see Table 2 later).

Discussion

According to recent reports (Ingle and Colman, 1976; Hogetsu and Miyachi, 1979), an appreciable activity of carbonic anhydrase (CA) was induced when *Chlorella* cells had been grown in ordinary air ($p_{CO_2} \simeq 0.03\%$), whereas no induction was noted in cells precultured in an environment of $p_{CO_2} \simeq 2\%$. The experimental observation in Fig. 4a that the rate of glycolate excretion was actually zero in the absence of INH could then be attributed to the existence of CA, because the cells (in Fig. 4a) precultured in air might have induced CA. The

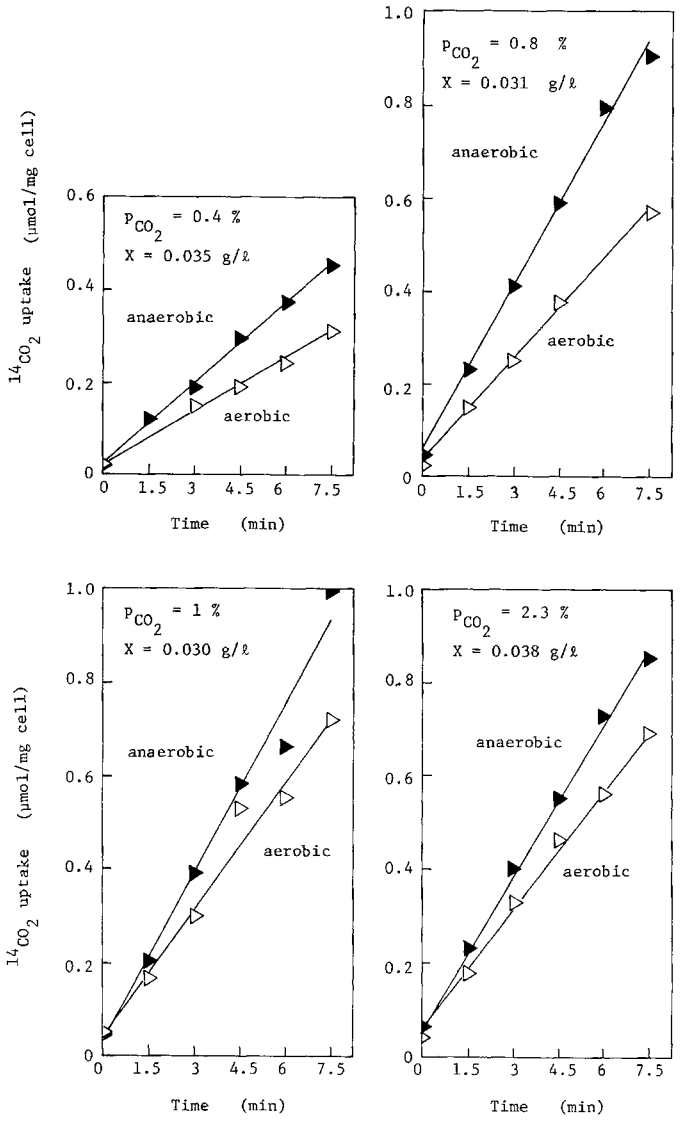

Fig. 5. $^{14}CO_2$ uptake. "Anaerobic" and "aerobic" imply the absence and presence of oxygen in *Chlorella* suspension (cell conc., X given in each diagram). Values of p_{CO_2} in the figure are those under which the suspension was prepared separately

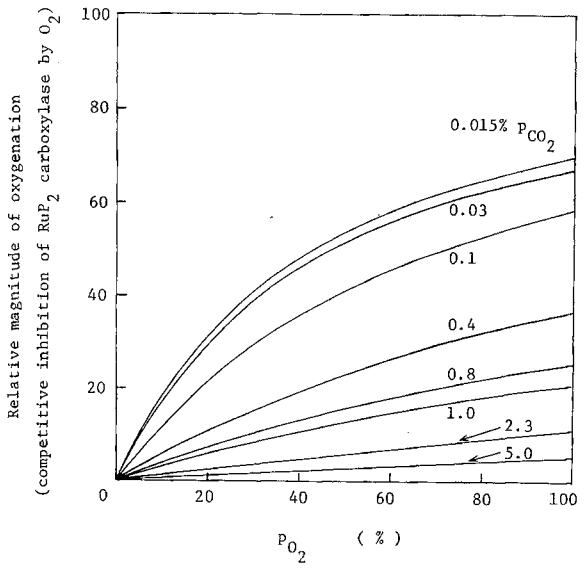

Fig. 6. Relative magnitude of oxygenation reaction of RuP_2 carboxylase. $K_m = 3.4\times10^{-5}$ M, $K_i = 3.7\times10^{-4}$ M

presence of CA could have alleviated oxygenation of RuP_2 carboxylase; in other words, enhanced carboxylation could have minimized the rate of glycolate excretion.

The above argument might be supported by the data in Fig. 4b that an appreciable amount of glycolate was excreted even in the absence of INH when the cells precultured in $p_{CO_2} = 5\%$ were transferred to $p_{CO_2} = 0.7\%$. Apparently, CA could not have been induced in the cells (in Fig. 4b). The uninduced cells of CA might have failed to enhance carboxylation, resulting, thus, in the dominance of oxygenation in RuP_2 carboxylase. Consequently, glycolate was excreted even in the absence of INH.

This sort of unusual operation that cells precultured in high values of p_{CO_2} are intentionally transferred to an atmosphere of fairly low value of p_{CO_2} is not common practice in *Chlorella* cultivation. As a corollary, no glycolate was excreted in the turbidostat culture in this work (see Table 1). However, this inference does not negate photorespiration *per se* in *Chlorella* cells, and indeed, glycolate was excreted into the medium in the particular instance mentioned above.

Here, the following equation on the competitive inhibition of enzyme kinetics was assumed to assess a relative magnitude of oxygenation reaction of RuP_2 carboxylase (Ku and Edwards, 1977a, b).

$$1 - \frac{v}{v_{max}} = \frac{[CO_2]}{[CO_2] + K_m(1 + [O_2]/K_i)} \quad (1)$$

provided: v = rate of carboxylation, wherein $[CO_2]$ (CO_2 concentration in liquid, M) and $[O_2]$ (O_2 concentration in liquid, M) are in equilibrium with p_{CO_2} and p_{O_2} in gas, respectively; for simplicity, liquid was taken as water and Bunsen coefficient α_{CO_2} and α_{O_2} at 30° C were taken (see later)

v_{max} = maximum value of carboxylation;
K_m = Michaelis constant with respect to $[CO_2]$, (M);
K_i = equilibrium constant between enzyme (RuP_2 carboxylase) and inhibitor (O_2), (M).

A graphical representation of Eq. (1) is shown in Fig. 6, provided: $K_m = 3.4\times10^{-5}$ M, $K_i = 3.7\times10^{-4}$ M (Laing et al., 1974), $\alpha_{CO_2} = 0.665$ ml/ml H_2O, and $\alpha_{O_2} = 0.026$ ml/ml H_2O (Umbreit, 1957).

Although the relative magnitude of oxygenation ($1 - v/v_{max}$) was affected by $[CO_2]$ and $[O_2]$ as apparent

Table 2
O_2 inhibition (observation and calculation); data are cited from Table 1 (turbidostat culture) and Fig. 5 ($^{14}CO_2$ uptake)

Experiments	Run No.	Partial pressure		Inhibition (%)	
		p_{CO_2} (%)	p_{O_2} (%)	Observed	Calculated
Turbidostat culture	1	1.0	0	0	0
	2	1.0	20	13	5
	3	1.0	65	17	15
	4	1.0	0	0	0
	5	1.0	20	13	5
	6	1.0	65	30	15
	7	2.4	0	0	0
	8	2.4	20	7	3
	9	2.4	65	17	8
	10	0.015	10	0	0
	11	0.015	35	15	45
	12	0.015	60	18	49
$^{14}CO_2$ uptake	1	0.4	0	0	0
	2	0.4	99.6	35	37
	3	0.8	0	0	0
	4	0.8	99.2	38	25
	5	1.0	0	0	0
	6	1.0	99.0	26	22
	7	2.3	0	0	0
	8	2.3	97.7	22	11

from Eq. (1), the value of $(1-v/v_{max})$ was plotted against p_{O_2} in Fig. 6, parameter being p_{CO_2} principally for the sake of convenience. Needless to say, curves drawn in the figure should be dependent on K_m and K_i values, respectively. Consequently, other values of K_m and K_i than those used in Fig. 6 such as $K_m = 1.75 \times 10^{-5}$ M, $K_i = 3.54 \times 10^{-4}$ M (Badger and Andrews, 1974) or $K_m = 4.90 \times 10^{-6}$ M, $K_i = 1.90 \times 10^{-4}$ M (Ku and Edwards, 1977b) were checked separately. However, the change in values of $(1-v/v_{max})$ in Fig. 6, depending on K_m and K_i values available from the references, was not serious enough to prohibit the following discussion on the degree of oxygenation.

Experimental data on O_2 inhibition regarding the rates of specific growth of *Chlorella vulgaris* and $^{14}CO_2$ uptake by the cells are reproduced from Table 1 and Fig. 5 into the 5th column of Table 2. At this point it is deemed necessary to mention the respiration in the dark as shown earlier in Fig. 3. Respiration in the dark of *Chlorella* cells studied here was least susceptible to change in light intensity as contrasted with a blue-green alga, *Spirulina platensis*, for instance (Ogawa et al., 1978). In addition, $p_{O_2} = 0\%$ in the table was actually far from anaerobic due to the photosynthetic liberation of O_2 and also owing to the well-known high affinity of cytochrome oxidase to O_2 in the mitochondria (Lloyd, 1974). Hence, decarboxylation in the dark need not be considered in the discussion on Table 2 which follows.

By and large, the effect of O_2 on the rates of specific growth and $^{14}CO_2$ uptake of *Chlorella* cells could be represented by the competitive inhibition of O_2 in RuP_2 carboxylase (see Table 2). The last column in the table pertains to the competitive inhibition by O_2 which is evaluated from Eq. (1). It can be noted from comparison of the data in the last two columns that O_2 inhibition observed with respect to $^{14}CO_2$ uptake was mostly explicable from competitive inhibition.

A marked difference between observation and calculation with regard to Run Nos. 11 and 12 could be partly accounted for by the induction of CA in low p_{CO_2} values, the rationale of which was referred to earlier. In other words, the degree of O_2 inhibition (calculated) seems to have been overestimated without consideration of CO_2 that is most probably made available by the action of CA. The disagreement of data between the last two columns in Table 2 with respect to O_2 inhibition of the specific growth rate is deemed to be plausible from another viewpoint, because the growth inhibition due to oxygen (calculated) deals exclusively with O_2 inhibition in RuP_2 carboxylase. In fact, a complicated network of biochemical reactions that follows, culminating in the algal growth, could not necessarily be reflected by the performance of the single enzyme RuP_2 carboxylase.

However, in view of the deviation of O_2 inhibition observed from that calculated in Table 2 (upper half, except for Run Nos. 11 and 12), the competitive inhibition might also be largely responsible for the algal growth deteriorated by the presence of O_2.

Lastly, it is urged that the difference in values between the last two columns in Table 2 could not simply lead to an argument on the presence and/or

absence of photorespiration in *Chlorella vulgaris* studied in this work.

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