Mass Spectrometric Analysis of Oxygen Gas Exchange in High- and Low-CO$_2$ Cells of *Ch!orella vulgaris*

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Oxygen gas exchange was monitored in the unicellular green alga *Chlorella vulgaris* 211−11 h by means of a mass spectrometer equipped with a special membrane gas-inlet-system and a photosynthetic reaction vessel. CO$_2$-dependent ^{18}O$_2$-uptake as well as ^{16}O$_2$-evolution were analyzed in both High- and Low-CO$_2$ cells. In High-CO$_2$ cells, the ^{18}O$_2$-uptake in the light (U$_L$) decreased by 65% upon addition of 3 mM NaHCO$_3$, while ^{16}O$_2$-evolution (E) was increased approx. 1.8 times by the same treatment. ^{18}O$_2$-uptake in the dark (U$_D$) was not affected by the addition of external inorganic carbon (Ci). The addition of 3.3 mM NaHCO$_3$ also affected U$_L$ and E in Low CO$_2$-cells, however, to a minor extent. U$_L$ under CO$_2$-saturating conditions was light intensity-independent up to 2 klux and 1.2 klux in High- and Low-CO$_2$ cells, respectively.

Above these light intensities U$_L$ increased approx. 4-fold in High- and approx. 6-fold in Low-CO$_2$ cells. Under CO$_2$-limiting conditions, however, U$_L$ increased in High-CO$_2$ cells even under very low light intensities, showing that photorespiratory oxygen uptake occurred even in the near vicinity of the light compensation point. Under CO$_2$-saturating and strong light conditions U$_L$ represented almost half of E in Low-CO$_2$ cells and about 30% of E in High-CO$_2$ cells. In Low-CO$_2$ cells addition of ethoxyzolamide (EZA), an inhibitor of carbonic anhydrase, enhanced U$_L$ and suppressed E and NET under CO$_2$-limiting conditions, whereas the compound had only a minor effect on High-CO$_2$ cells.

DCMU (3 μM) strongly inhibited E and U$_L$ under CO$_2$-saturating conditions, with the remaining U$_L$ being smaller than U$_D$. KCN (1 mM) and SHAM (1.5 mM) added to DCMU-treated Low-CO$_2$ cells suppressed U$_L$ by approx. 50%. The resulting value corresponded to half of U$_D$. KCN also inhibited E under CO$_2$-saturating conditions, with U$_L$ being strongly enhanced showing a maximal uptake at 0.4 mM KCN. Under these conditions NET was nearly zero. The effect seems to be due to an inhibition of RubisCO and an enhancement of Mehler reactions. At 0.7 mM KCN, DCMU entirely inhibited U$_L$, but oxygen uptake appeared increased after turning the light off. This uptake corresponded to approx. 60% of U$_D$. Whereas KCN and SHAM inhibited approx. 70% of U$_D$, only 16% of U$_L$ was suppressed. These results suggest that the contribution of mitochondrial respiration to U$_L$ was negligeable, since U$_L$ seemed to be suppressed in the light under CO$_2$-saturated conditions. Iodoacetamide, which is an inhibitor of the Calvin cycle and thereby diverts carbon into the respiratory pathway, inhibited E and NET under CO$_2$-saturating conditions, but did not affect U$_L$. This result also shows that U$_L$ is not due to mitochondrial respiration. A hydroxylamine derivative [20, 21] which changes the ratio of the RuBP carboxylation to oxygenation activity in tobacco leaves did not affect this ratio in *Chlorella*.

Introduction

During illumination many plants and algae exhibit the wasteful property to release part of the freshly fixed CO$_2$, at the same time oxygen is taken up [1−3]. The phenomenon is called photorespiration and competes with the CO$_2$-fixation process for reducing equivalents. At the same time other O$_2$-consuming reactions like Mehler type reactions [4] and mitochondrial respiration [5, 6] occur in the cells under illumination. It is obvious that photorespiratory as well as the other O$_2$-consuming reactions counteract photosynthesis. Although many investigations have tried to measure Mehler reactions and mitochondrial respiration under conditions where photosynthesis and photorespiration occur *i.e.* in the light, the extent of their contribution is still an open question [7]. Concerning the Mehler reaction it was

shown that its importance seems to be the supply of ATP for CO_2-fixation in the chloroplasts [8–11]. On the other hand there was no Mehler reaction at all detectable in *Chlamydomonas* [6]. As reviewed by Graham [12], many authors believe that mitochondrial respiration is inhibited in the light in comparison to the rate in the dark, whereas others think that respiration is not affected by light. However, several recent reports using algae showed the persistence of mitochondrial respiration in the light [6, 10, 11].

Only a few methods permit the simultaneous measurement of photosynthetic O_2-evolution and photorespiratory O_2-uptake. The method used in the present study was that of mass spectrometry which permits the discrimination of O_2-uptake in the light measured as $^{18}O_2$ uptake from O_2 evolution measured as $^{16}O_2$ evolution. When algal cells like *Chlorella, Chlamydomonas* etc. are grown in ordinary air (Low-CO_2 cells), it is well-known that a very efficient mechanism for the utilization of Ci is induced (see reviews by [13, 14]). This mechanisms which facilitates the incorporation of Ci seems to disappear, when the algal cells are grown in air enriched with 1–5% CO_2 (High-CO_2 cells). Low-CO_2 cells exhibit a lower K_m (CO_2) for photosynthesis, a lower CO_2 compensation point and a weaker Warburg effect than High-CO_2 cells (see review by [14, 15]). In the case of *Chlorella vulgaris*, used in the present study, it was reported that the properties of Low-CO_2 cells are due to the action of the enzyme carbonic anhydrase, which is synthesized under low CO_2 tension and disappears under high CO_2 tension, facilitating CO_2 transport from the outside to the CO_2 fixation site [16]. Therefore, in the present study, we compared the activity of $^{18}O_2$-uptake in High-CO_2 cells with that in Low-CO_2 cells.

Materials and Methods

Algal cultures

Chlorella vulgaris 211–11 h was obtained from Prof. W. Kowallik (University of Bielefeld, West-Germany) and originates from the algal collection of the University of Göttingen (West-Germany). The algal cells were axenically grown at 25 °C under continuous illumination by incandescent light in an inorganic medium according to Kowallik [17]. The suspension was continuously bubbled with air enriched with 3% CO_2. After several days, the algal suspension was divided into two portions. One was kept in

the same conditions (High-CO_2 cells), whereas the other portion was aerated with ordinary air that is with approx. 0.03% CO_2 (Low-CO_2 cells). These cells were harvested by centrifugation (3000 rpm for 10 min) and the pellets were resuspended in 20 mm MES-NaOH buffer (pH 6.0) to give a final algal concentration of 1 ml pcv per liter.

Measurement of oxygen gas exchange

$^{18}O_2$-Uptake and $^{16}O_2$-evolution was monitored using a mass spectrometer (Finnigan MAT, Bremen, West-Germany) equipped with a special membrane-gas inlet system and a photosynthetic reaction vessel made of stainless steel, fitted with a plexiglass lid. The reaction vessel with a volume of 7.5 ml was used without gas phase. A gas-permeable but water-impermeable polyvinylchloride membrane (size 7 cm^2) was placed on the plastic screen at the bottom of the vessel. During measurements the reaction medium was stirred magnetically. For illumination a projector lamp providing an intensity of 50 klux (162 W × m^{-2}) was used. The temperature was kept at 30 °C. NaHCO$_3$ solutions, as in inorganic carbon source, and inhibitors were injected with a microsyringe through a capillary of the lid. In the system, the sensitivity and the response time are considerably increased by connection of the reaction vessel directly to the ion source. The gas dissolved in the reaction medium is separated by the membrane from the space of the ion source thus by-passing the normal inlet system which permits an only slow and insensitive response due to the large internal volume [18]. Using the mass spectrometer, changes in $^{18}O_2$ (mass 36) and $^{16}O_2$ (mass 32) were simultaneously measured. Experiments were initiated by injection of algae or $^{18}O_2$ gas. After injection of $^{18}O_2$ the algal suspension was kept in the dark until the $^{18}O_2$ and $^{16}O_2$ concentration in the suspension had reached the steady level.

O_2-Uptake rate (U) and gross O_2-evolution (E) were calculated from the following equations as reported by [6].

$$U(mV \times min^{-1}) = (\Delta[M36]_t - k[M36]_t)([M32]_t + [M36]_t)([M36]_t)^{-1}$$

$$E(mV \times min^{-1}) = (\Delta[M32]_t - k[M32]_t) + U([M32]_t)([M32]_t + [M36]_t)^{-1}$$

where k is the rate constant of the O_2-decrease due to oxygen consumption by the mass spectrometer. The rate constant measured in the absence of algae was

0.020 min^{-1}. $[M36]_t$ and $[M32]_t$ represent the signal (mV) of mass 36 and mass 32 at time t, respectively. Δ means the difference of signals between two successive values. The values of signals (mV) were calibrated to the amount of $^{18}O_2$ and $^{16}O_2$ in μmol on the basis of the equilibrium with ordinary air (230 μM at 30 °C).

Results

Fig. 1 shows the effect of the external CO_2 concentration (Ci) on E, U and NET in High-CO_2 cells of *Chlorella vulgaris* 211−11 h. E and NET increased with the CO_2 concentration up to 0.6 mM and saturated above this concentration. In contrast to this, the uptake in the light (U_L) was nearly constant up to 0.1 mM and decreased beyond this concentration by 65% reaching a steady state level at 0.6 mM. In Low-CO_2 cells E, U and NET did not show a clear steady dependence on the concentration of external CO_2 (Fig. 2). To the contrary increasing the external CO_2 concentration rather seems to inhibit E, NET and U_L. This seems to be due to an internal CO_2 accumulation in the cells and to a facilitated CO_2 supply caused by the action of carbonic anhydrase (CA) in Low-CO_2 cells. In both High- and Low-CO_2 cells, U_D was not affected by external CO_2 concentration (Fig. 1 and 2). When light intensity increased in the presence of 3 mM external CO_2 in High-CO_2 cells, U_L was constant up to 2 klux and increased after this lag with light intensity, whereas E and NET had a linear dependency on light intensity up to approx. 3 klux (Fig. 3A) and approached light saturation, that is a steady level above this intensity. Under CO_2-limiting (no addition of external Ci) conditions, a remarkable increase of U_L was observed (Fig. 3B) even under low light intensity. In the light intensity curve E outran U_L at about 1 klux under both CO_2-saturating and -limiting conditions. E reached at 40 klux an almost three times higher value than U_L under CO_2-saturating conditions but was only 10% higher than that of U_L under CO_2-limiting conditions in High-CO_2 cells (Fig. 3). The light intensity curve of Low-CO_2 cells is shown in Fig. 4. Here U_L was nearly constant up to 1.5 klux and increased above this light intensity much steeper in Low-CO_2 cells (Fig. 4) than in High-CO_2 cells

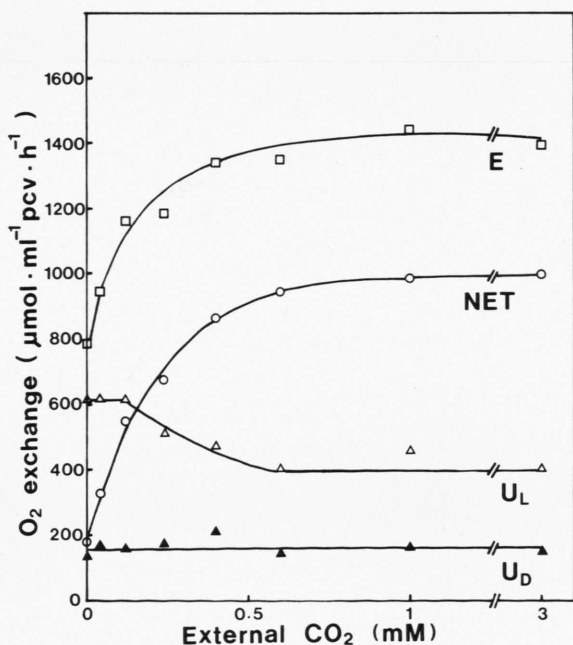Fig. 1. Effect of the external CO_2-concentration on E, U_L and NET in High-CO_2 cells of *Chlorella vulgaris* 211−11 h. The light intensity was kept at 40 klux ($162 \text{ W} \times \text{m}^{-2}$). The concentration of dissolved O_2 ($^{16}O_2 + {}^{18}O_2$) changed from 120 to 164 μM during the experiment.
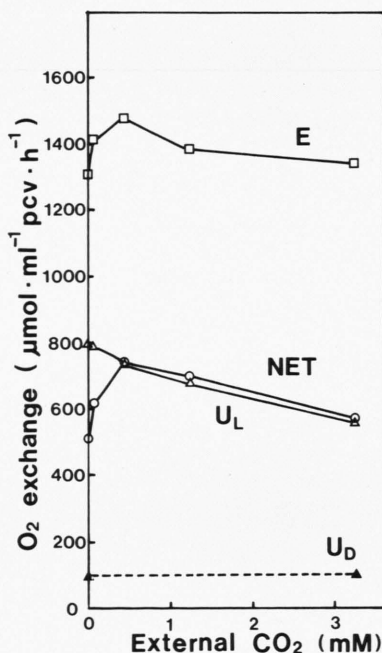
Fig. 2. Effect of the external CO_2-concentration on E, U_L and NET in Low-CO_2 cells of *Chlorella vulgaris* 211−11 h. The light intensity was 40 klux ($162 \text{ W} \times \text{m}^{-2}$). The concentration of dissolved O_2 changed from 307 to 340 μM during the experiment.

Fig. 4. Effect of light intensity on E, U_L and NET measured under CO_2 saturating conditions in Low-CO_2 cells of *Chlorella vulgaris* 211−11 h. The concentration of external CO_2 during photosynthesis was 3.3 mM. The concentration of dissolved O_2 changed from 314 to 332 μM during the experiment.
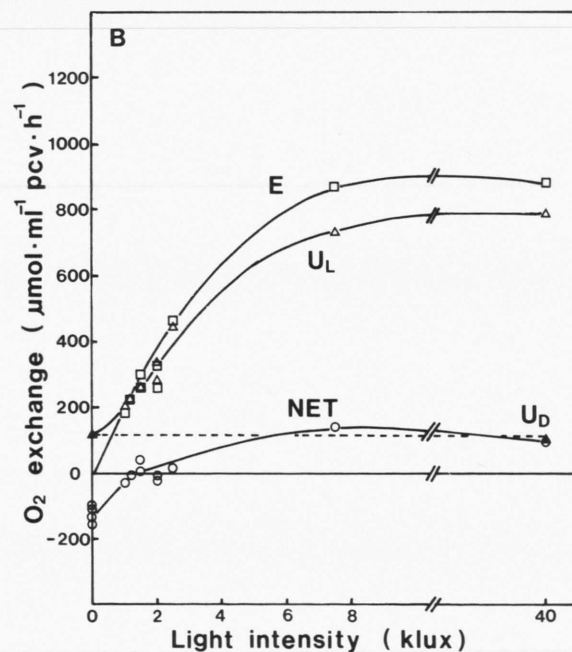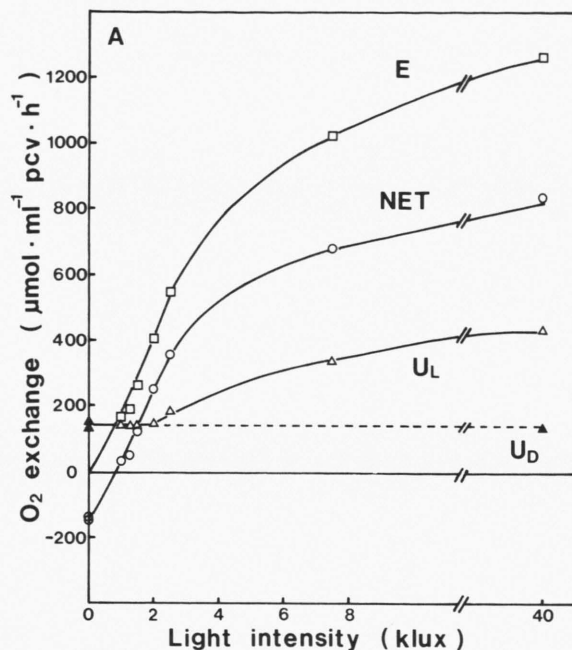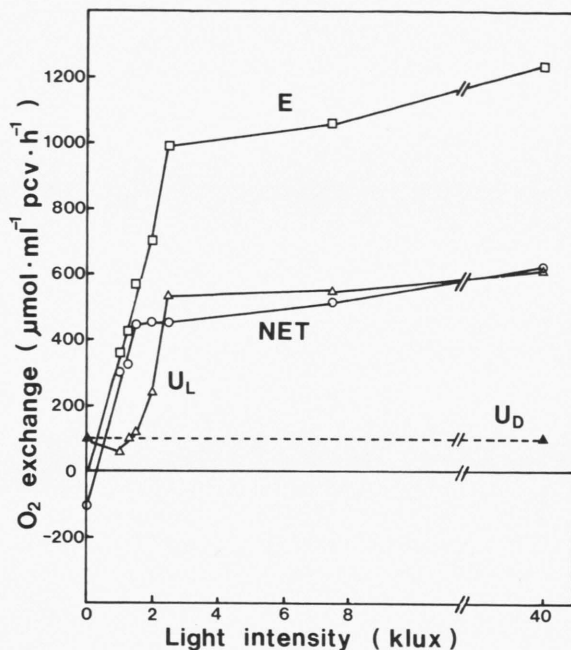

Fig. 3. Effect on light intensity on E, U_L and NET measured under CO_2-saturating (A) and CO_2-limiting (B) conditions in High-CO_2 cells of *Chlorella vulgaris* 211−11 h. The concentration of external CO_2 during photosynthesis was 3 mM. The concentration of dissolved O_2 changed from 319 to 380 μM in Fig. 3A and from 395 to 420 μM in Fig. 3B during the experiment.

(Fig. 3A). A remarkable difference between High- and Low-CO_2 cells was that U_L under both CO_2 and light-saturating conditions reached almost half the value of E in Low-CO_2 cells, while U_L in High-CO_2 cells was always several times smaller than E under the same conditions (Fig. 1−4). Ethoxyzolamide (EZA, 50 μM), an inhibitor of carbonic anhydrase, suppressed NET, E and U_L by 76−78% under CO_2-limiting conditions but the subsequent addition of 10 mM $NaHCO_3$ further decreased U_L and strongly enhanced NET in High-CO_2 cells. In Low-CO_2 cells, EZA (50 μM) suppressed E by 48%, U_L by 74% and NET by 20%. This effect was changed by the subsequent addition of 10 mM $NaHCO_3$ to 68%, 58% and 79% of the control in E, U and NET, respectively (Table I). It should be borne in mind that in these experiments 30% of the inhibition was due to the inhibition of photochemical reactions [19]. The inhibition of E by EZA appeared to be due to the inhibition of the CO_2-supply from the outside to the CO_2-fixation site by inhibition of the enzyme car-

Table I. Effect of Ethoxyzolamide (EZA) on E, U_L and NET measured without addition of external CO_2 in Low-CO_2 cells of *Chlorella vulgaris* 211−11 h.

Material and Condition	Rate of O_2 exchange [$\mu mol \times ml\ pcv^{-1} \times h^{-1}$]		
	E	U_L	NET
A control	1466 (100%)	753 (100%)	713 (100%)
B A + 50 μM EZA	698 (48%)	557 (74%)	141 (20%)
C B + 10 mM NaHCO₃	1004 (68%)	438 (58%)	566 (79%)

bonic anhydrase (CA). E seemed to be completely inhibited by DCMU whereas U_D was not inhibited by DCMU (Fig. 5, Table II). The addition of 1 mM KCN and 1.5 mM SHAM upon this assay suppressed U_D by approx. 70% (Table II). U_L was enhanced by the addition of KCN showing a peak at 0.4 mM (Fig. 6), while E appeared to be inhibited with increasing KCN concentrations. At 0.7 mM KCN, E and U_L showed almost the same rate. The addition of DCMU at this stage lowered U_L to nearly zero. In the presence of 1 mM KCN, U_D still maintained approx. 50% of its original activity (Table II). This data might suggest that mitochondrial respiration is rather suppressed in the light (Table II). Fig. 7 shows the effect of iodoacetamide, which is an inhibitor of triose phosphate dehydrogenase, on E, U and NET under CO_2-saturating conditions in High-CO_2 cells. Approx. 60% of E was inhibited but U_L as well as U_D were not affected by this inhibitor. A hydroxylamine derivative (HA 368) which is an inhibitor of the Warburg effect, observed in tobacco leaves, causes conformational changes of the enzyme RubisCO [20, 21], suppressed both E and U_L, but NET was kept almost constant (Fig. 8).

Fig. 5. Effect of DCMU on E, U_L and NET measured under CO_2 saturating conditions in High-CO_2 cells of *Chlorella vulgaris* 211−11 h. The concentration of external CO_2 during the measurement was 6.4 mM. The concentration of dissolved O_2 changed from 211 to 153 μM during the experiment.
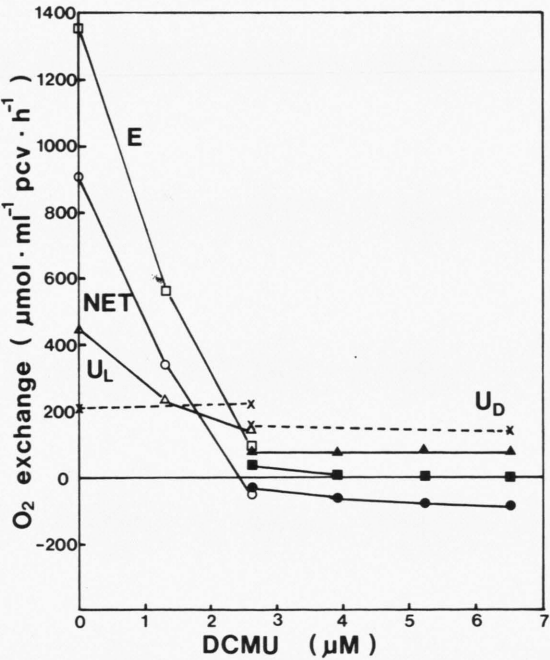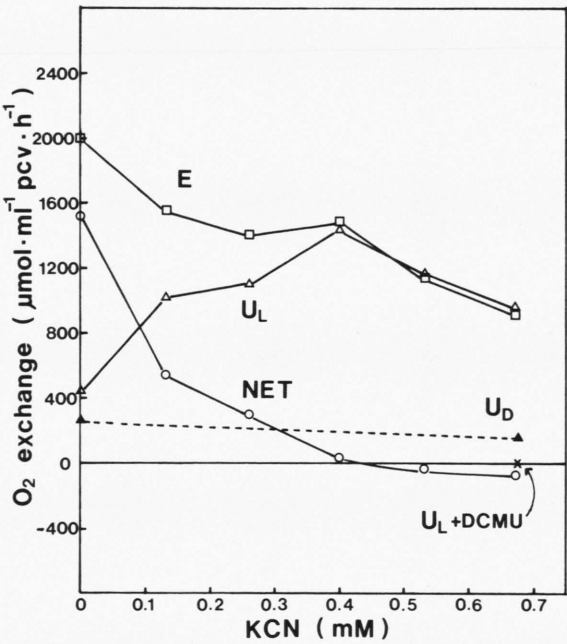

Fig. 6. Effect of KCN on E, U_L and NET measured under CO_2 saturating conditions in High-CO_2 cells of *Chlorella vulgaris* 211−11 h. The concentration of CO_2 during photosynthesis was 3 mM. The concentration of dissolved O_2 changed from 295 to 336 μM.

Table II. Effect of KCN, SHAM and DCMU on U_L and U_D in High-CO_2 cells of *Chlorella vulgaris* 211−11 h.

Exp. No.	Condition	Rate of $^{18}O_2$-uptake [$\mu mol \times ml\ pcv^{-1} \times h^{-1}$] in the	
		light (U_L)	dark (U_D)
I	Control	778 (100%)	117 (100%)
	+ 1 mM KCN		66 (56%)
	+ 1 mM KCN + 1.5 mM SHAM	650 (84%)	37 (32%)
II	Control	456 (100%)	219 (100%)
	+ 2.6 µM DCMU	145 (32%)	229 (105%)
	+ 2.6 µM DCMU		
	+ 1 mM KCN + 1.5 mM SHAM	70 (15%)	148 (72%)

No external CO_2 was added in Exp. I, whereas 6.4 mM $NaHCO_3$ were added in Exp. II. Light intensity was 40 klux. O_2-concentration during the experiment was near the ordinary air-level.
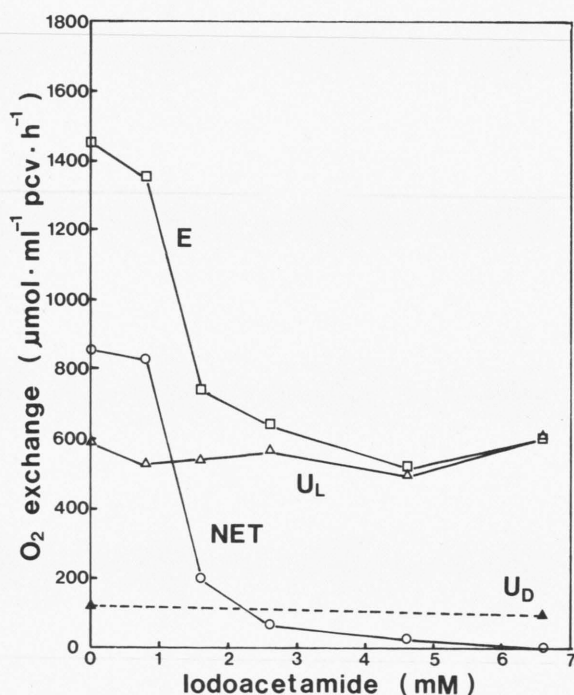
Fig. 7. Effect of iodoacetamide on E, U and NET measured under CO_2 saturating conditions in High-CO_2 cells of *Chlorella vulgaris* 211−11 h. The concentration of external CO_2 during the measurement was 10 mM. The concentration of dissolved O_2 changed from 202 to 180 µM during the experiment.

Fig. 8. Effect of a hydroxylamine derivative (HA 368) on E, U_L and NET measured without addition of external CO_2 in High-CO_2 cells of *Chlorella vulgaris* 211−11 h. The concentration of dissolved O_2 changed from 309 to 248 µM during the experiment. Light intensity was 40 klux (162 $W \times m^{-2}$).

Discussion

In the present paper we show that, when oxygen gas exchange is measured in the light, the ratio of E/U_L is 1.3 under the condition without any addition of e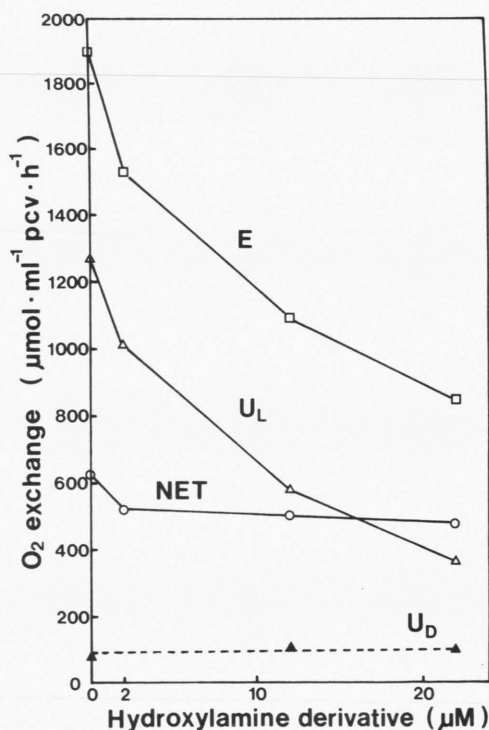xternal CO_2, but 3.5 with 3 mM external CO_2 in High-CO_2 cells. In Low-CO_2 cells, the same ratio is 2.6 without external CO_2 and 2.4 with 3.2 mM external CO_2 (Fig. 1 and 2). If the O_2-uptake, which is dependent on the external CO_2 concentration, is defined as photorespiratory O_2-uptake, it appears that 35% of U_L under CO_2-limiting conditions is due to photorespiratory O_2-uptake in High-CO_2 cells (Fig. 1). In the case of Low-CO_2 cells, it was not possible to exactly calculate the photorespiratory O_2-uptake from Fig. 2, but the extent seems to be smaller than that of High-CO_2 cells. At any rate it appeared that U_L was higher than in High-CO_2 cells, independent on the external CO_2-concentration given during the experiment (Fig. 2), which might be due to the activity of Mehler type reactions. From the literature it is well-known that Low-CO_2 cells exhibit in photosynthesis a high affinity for CO_2 due to the action of the enzyme carbonic anhydrase whereas High-CO_2 cells have only a very low carbonic anhydrase activity [16]. From this it can be expected that E will be suppressed whereas U_L will be increased upon addition of the carbonic anhydrase inhibitor ethoxyzolamide, since the inhibitor is supposed to block the CO_2 supply and as a consequence increases the O_2-supply to RubisCO. Table I generally supports this notion, but it appears that the inhibitory effect of ethoxyzolamide on E was stronger than its stimulatory effect on U_L.

The responses of E, U and NET on the light intensity in High- and Low-CO_2 cells are shown in Fig. 3 and 4 and fit into observations reported in the literature [22]. E measured under CO_2-saturating conditions was very sensitive to light intensity, especially in the weak light region up to 3 klux. In contrast to this, oxygen uptake in the light (U_L) was insensitive to light up to 1.5−2 klux under the same conditions (Fig. 3A and 4), whereas U_L depended on the light intensity under CO_2-limiting conditions (Fig. 3B). This data might mean that CO_2-dependent O_2-uptake in the light, *i.e.* photorespiratory O_2-uptake, also occurs under very weak light conditions even in the vicinity of the light compensation point. The rate of the U_L portion, insensitive to light, was the same as that of U_D. Under CO_2- and light-saturated condi-

tions U_L was 3.3 times higher in High-CO_2 cells and 6 times higher in Low-CO_2 cells than U_D (Fig. 3A and 4). Addition of DCMU, an inhibitor of photosystem II plus KCN plus SHAM, inhibitors of RubisCO and mitochondrial respiration, strongly suppressed U_L whereas the addition of KCN plus SHAM alone did not substantially affect U_L (Table II). The rate of the remaining portion of U_L was enhanced by switching the light off (Table II, Fig. 5 and 6). This data shows that mitochondrial O_2-uptake was inhibited in the presence of these inhibitors in the light, showing that mitochondrial respiration is probably suppressed in the light. From this it is concluded that most of U_L under CO_2- and light-saturating conditions is due to Mehler type reactions, whereas under CO_2-limiting and light-saturating conditions U_L was a mixed composition of Mehler type reactions and photorespiratory O_2-uptake. The stimulatory effect of KCN on U_L seems to be an inhibitory effect on RubisCO, as reported by Siegel *et al.* [23], which means that the stimulation of U_L was mainly due to a Mehler reaction and/or to the reaction which supplies $^{18}O_2$ to glycolate even in the presence of KCN, as reviewed by Beck [24].

The effect of iodoacetamide, an inhibitor of triose phosphate dehydrogenase in the Calvin Benson Cycle, on U_L under CO_2-saturating conditions, as shown in Fig. 7, also suggests that mitochondrial respiration was at least not enhanced in strong light and that most of U_L was due to Mehler type reactions.

As already mentioned above, the occurrence of mitochondrial respiration in the light has been a matter of controversy. Earlier reports have suggested that mitochondrial respiration was inhibited by ATP production in illuminated chloroplasts [12, 25]. In contrast to this, recent reports suggested that mitochondrial respiration is maintained during illumination [6, 11]. The existence of Mehler type reactions, *i.e.* the direct photoreduction of O_2 using energy derived from the photosystems in intact cells also remains a matter of discussion [7], although reports of the literature have shown that Mehler reactions might play an important role for the ATP supply in CO_2-fixation in intact chloroplasts [7, 8]. Recently, for example, Brechignac and André [10] have been able to demonstrate a significant involvement of Mehler type reactions in the red macroalga *Chondrus crispus* whereas Peltier and Thibault [6] reported that no Mehler reactions occurred in

Chlamydomonas. In entire tobacco plants Ishii and Schmid [26] have shown that the $^{18}O_2$-uptake in the light is due to photorespiration and Mehler type reactions in the wild type *N. tabacum* var. John William's Broadleaf, whereas $^{18}O_2$-uptake in the light was exclusively due to photorespiration and contained no Mehler type reactions in the chlorophyll-deficient mutant *Su/su*. As a result, the present study with *Chlorella* subscribes to the opinion that mitochondrial respiration is suppressed in the light, at least under CO_2-saturating conditions, and that

Mehler reactions, whose activity take place in intact cells are enhanced under strong light.

Acknowledgements

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