Effect of pH on Glycolate and Ammonia Excretion in L-MSO Treated *Chlorella* Cells

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The effect of pH changes on the excretion of ammonia and glycolate from algal cells into the medium was investigated in L-MSO (final concentration, 0.5 mM) – treated *High-* and *Low* CO_2*-cells* of *Chlorella vulgaris* 211-11h. The excretion was analyzed in the condition in which the cells were continuously gassed with air at 25 °C. At the values tested, generally more ammonia was excreted in L-MSO-treated *Low* CO_2*-cells* than in L-MSO treated *High* CO_2*-cells*. In both kinds of algal cells more ammonia was excreted at low pH-values and absolutely no ammonia was excreted at pH 8. In the dark, no or only slight ammonia excretion was observed in both L-MSO-treated *High* and *Low* CO_2*-cells*. Under all these conditions no or only very low glycolate excretion was observed in both L-MSO treated *High* and *Low* CO_2*-cells*.

In *High* CO_2*-cells* rates of photosynthesis were high at pH 6 and lower at higher pH values. On the other hand *Low* CO_2*-cells* showed practically little dependence of photosynthetic rates on the pH. This result might indicate that the major part of the ammonia excretion observed, was not due to the inhibition of photosynthesis at acid pH values. It is known that ammonia excretion in L-MSO treated algal cells is due to the inhibition of the refixation of ammonia which originates from the glycine-serine aminotransferase reaction in the glycolate pathway. Our results demonstrate that glycolate production and glycolate metabolism are more intense at low pH values when compared to high pH values. This is valid for both *High* and *Low* CO_2*-cells. Low* CO_2*-cells* in *Chlorella vulgaris* 211-11 h exhibit a more active glycolate metabolism than *High* CO_2*-cells*.

Introduction

It is well known that unicellular algae synthesize during photosynthesis glycolate via the glycolate pathway and may excrete an appreciable amount of glycolate into the culture medium [1–4]. It is generally thought that the conditions which favor the production and excretion of glycolate are high O_2 partial pressure, low CO_2 concentration and high light intensity. Moreover, some investigators have reported that ^{14}C-incorporation into glycolate during short-term photosynthetic $^{14}CO_2$ fixation was much higher at high pH values than at low ones. This was valid if the concentration of total inorganic carbon ($NaHCO_3$) was kept constant at all tested pH-values. This was investigated with cells of *Chlamydomonas* and *Scenedesmus* grown under high CO_2 concentrations *(High* CO_2*-cells)* [5, 6]. However, it has been reported later that the *High* CO_2*-cells* of these algae utilized, as the substrate for photosynthesis, only free CO_2 dissolved in the solution and not the bicarbonate ion [7–9]. Therefore, equal concentrations of free CO_2, rather than of bicarbonate should be maintained at the pH values to be tested since the ratio of CO_2 to bicarbonate ions in any solution is strongly dependent on the pH of the solution.

The experiments described in the present paper are made with algal cells that were kept at different pH-values under the same CO_2 tension by bubbling the suspension with ordinary air containing approx. 0.03% CO_2. These experiments showed that in contrast to the general opinion appreciably more glycolate and ammonia is formed at low pH values than at higher ones.

Abbreviations: L-MSO, L-methionine-DL-sulfoximine; *Low* CO_2*-cells,* algal cells grown in ordinary air, *i.e.* with 330 ppm CO_2; *High* CO_2*-cells,* algal cells grown in air supplemented with 3% CO_2; α-HPMS, a-hydroxy-2-pyridyl methanesulfonate; INH, isonicotinyl hydrazide; GS-GOGAT, glutamine synthetase – glutamine – 2-oxo glutarate-aminotransferase.

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Materials and Methods

Chlorella vulgaris 211-11h was obtained from Prof. W. Kowallik (University of Bielefeld, W. Germany but originates from the algal collection of the

University of Göttingen, W. Germany). The algal cells were axenically grown at 25 °C in an inorganic medium according to Hogetsu and Miyachi [10]. The medium did not contain ammonia and was constantly bubbled with ordinary air enriched with 3% CO_2. After several days, the algal suspension was divided into two portions. One was kept at the same conditions *(High CO_2-cells)*, whereas the other portion was bubbled with ordinary air containing ≈ 0.03% CO_2 *(Low CO_2-cells)*. During growth the algae were illuminated by a bank of fluorescent lamps. The cells were harvested and the *High-* and *Low CO_2-cells* were centrifuged at 3000 rpm for 5 min and the pellets resuspended in 100 ml of 20 mM MES (at pH 6.0) or HEPES (at pH 7.0 and 8.0) buffer containing 5% of the culture medium and L-MSO to give a final concentration of 0.5 mM. These suspensions were first kept in the dark for 3 h with constantly gassing the cells with ordinary air containing ≈ 0.03% CO_2. The dark period was supposed to permit incorporation of L-MSO into the cells and to produce full inhibition of glutamine synthetase (GS) [11]. After 3 h dark the cells were illuminated by fluorescent lamps (approx. 10 klux). At chosen intervals, portions of the two types of algal suspensions were harvested and immediately transferred for the measurement of photosynthetic O_2 evolution to the reaction vessel of a Clark type O_2 electrode (Rank Brothers, Co. Ltd., London). After 10 min incubation in the dark the reaction vessel was illuminated by a projector lamp (Leitz Prado Universal) with an intensity of approx. 10 klux. The temperature was kept at 25 °C. Another portion of the harvested suspension was centrifuged at 3000 rpm for 5 min at 4 °C in order to obtain the supernatant of the suspension which was used for ammonia and glycolate determination.

Concentrations of glycolate and ammonia were determined colorimetrically using the methods of Calkins and Weatherburn [12, 13], respectively.

Results

Fig. 1 shows the effect of three different pH values on the time course of ammonia excretion in the presence of a final concentration of 0.5 mM L-MSO in *High* and *Low CO_2-cells* of *Chlorella vulgaris* 211-11h. The experiment has been carried under a condition in which the cells were constantly gassed with ordinary air containing ≈ 0.03% CO_2. In *Low CO_2-cells*, the rate of ammonia excretion was approx. 40% lower at pH 7.0 than at pH 6.0. It is seen that ammonia is excreted without a lag at these pH values. In *High CO_2-cells* the rate of ammonia excretion was approx. 25% lower at pH 7.0 than at pH 6.0. The figure clearly shows that ammonia was excreted with a lag of approx. 1 h at pH 6.0 and a lag of approx. 2.5 h at pH 7.0. No ammonia excretion was observed at pH 8.0 neither in *High* nor in *Low CO_2-cells*. The calculated rates of ammonia excretion at pH 6.0 were 24 and 6 $\mu mol \cdot ml^{-1} pcv \cdot h^{-1}$ for *Low* and *High CO_2-cells*, respectively. During these experiments, no or only a very low amount of glycolate was detected in the supernatant of both *High* and *Low CO_2-cells*. Only in L-MSO treated *Low CO_2-cells* some ammonia was slowly excreted into the medium during the dark period.

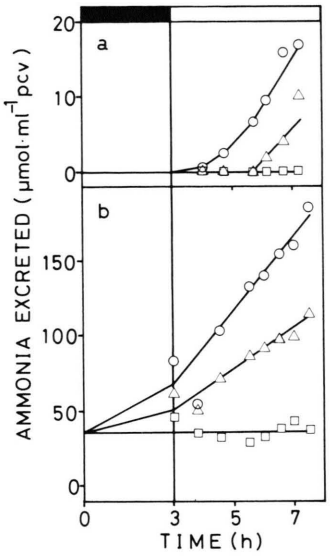

Fig. 1. Effect of pH on the excretion of ammonia in L-MSO treated *High* (a)- and *Low* (b) *CO_2-cells* of *Chlorella vulgaris* 211-11h.
L-MSO (to give a final concentration of 0.5 mM) was added at the onset of the experiment and algal cells were first kept in the dark as shown in the figure. During the experiment, the algal cells were continuously bubbled with air at 25 °C.

Fig. 2 shows the photosynthetic O_2 evolution pattern in cells which were harvested from the same algal suspension as that used in Fig. 1. The algal suspension was quickly transferred to the O_2 electrode vessel which was quickly closed in order to maintain the CO_2 concentration in the suspension at the same level as in the culture medium before the measurement of *photosynthetic O_2 evolution*. After the measurement of the rate of photosynthetic O_2 evolution, without addition of any external inorganic carbon,

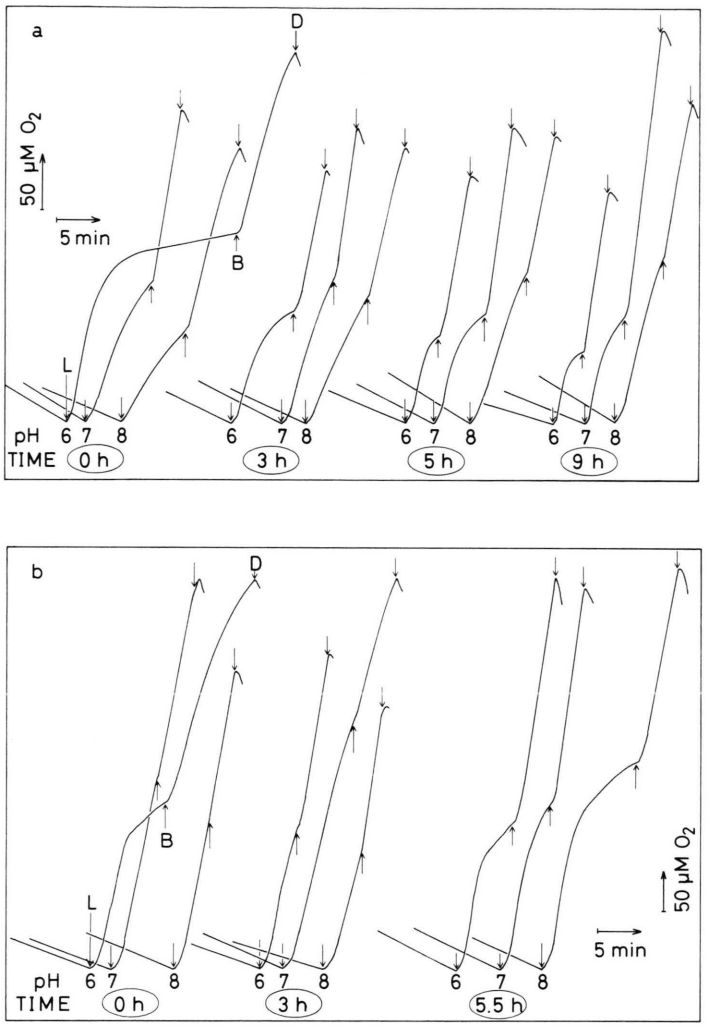

Fig. 2. Influence of the pH value on the photosynthetic O_2 evolution pattern in L-MSO treated *High* (a)- and *Low* (b) *CO_2-cells* of *Chlorella vulgaris* 211-11h.
The algal cells were harvested at the encercled time indicated in the figure (otherwise conditions are as in Fig. 1) and were immediately transferred for the measurement of photosynthetic O_2 evolution to the vessel of the Clark type oxygen electrode; temperature 25 °C.
(L) indicates light on; (B) addition of 10 mM $NaHCO_3$ and (D) light off. This reaction protocol is the same for all curves.

$NaHCO_3$ was injected with a microsyringe in order to measure the maximal rate of photosynthesis. In L-MSO treated *Low CO_2-cells* the rates of photosynthetic O_2 evolution with or without the addition of external $NaHCO_3$ were almost the same at the different values tested and did practically not change in the course of the experiment. On the other hand, actual rates of photosynthesis in the absence of external $NaHCO_3$ were greatly increased in L-MSO treated *High CO_2-cells* at all pH values by the transfer of the algae to the bubbling conditions in which ordinary air, containing $\approx 0.03\%$ CO_2, was used whereas maximal photosynthetic rates increased only slightly. The data shows that due to the transfer to the low CO_2 concentration the affinity characteristics for inorganic carbon were gradually changed to those of *Low CO_2-cells*. The observed rates in the absence of external $NaHCO_3$ were higher at every pH value in L-MSO treated *Low CO_2-cells* (at the time 5.5 h) than those in L-MSO treated *High CO_2-cells* (at 5 h) with the maximal rates of photosynthesis in the presence of external $NaHCO_3$ being almost the same at every pH value in both types of cells.

Discussion

Fig. 1a and b show the time course of ammonia excretion into the medium at the pH values 6, 7 and 8 measured under ordinary air (low CO_2 conditions) in L-MSO treated *High* and *Low CO_2-cells* of *Chlorella vulgaris* 211-11h. L-MSO is a well-known inhibitor

Table I. Effect of pH on changes in the rate of photosynthesis and dark respiration after incubating L-MSO treated *High* (H) and *Low* (L) *CO_2-cells* of *Chlorella vulgaris* 211-11h in ordinary air (0.03% CO_2).

Incubation time [h]	pH	Rate of dark respiration [$\mu mol \cdot ml^{-1} pcv \cdot h^{-1}$]		Rate of photosynthetic O_2 evolution [$\mu mol \cdot ml^{-1} pcv \cdot h^{-1}$] without addition of external $NaHCO_3$ (A)		with 10 mM $NaHCO_3$ (B)		A/B × 100	
		H	L	H	L	H	L	H	L
0[a]	6	77	54	674	727	490	521	138	140
	7	81	52	380	786	776	757	49	104
	8	56	59	206	751	528	798	39	94
3	6	58	60	484	751	702	884	69	85
	7	74	48	467	586	861	635	54	92
	8	61	36	252	524	538	941	47	56
5[b]	6	66	66	661	801	866	900	76	89
	7	86	62	577	790	1069	1044	54	76
	8	88	62	477	836	703	884	68	95
9	6	48		816		1022		80	
	7	58		672		840		80	
	8	90		664		858		77	

Algal cells harvested at intervals were immediately transferred into the Clark type O_2 electrode vessel for measuring dark respiration and photosynthetic O_2 evolution.
[a] Algal cells were first kept in the dark during 3 h.
[b] *Low CO_2-cells* (L) were harvested after 5.5 h.

of glutamine synthetase (GS) [14] and inhibits the assimilation of ammonia via the GS-GOGAT pathway [15]. According to the photorespiratory nitrogen cycle described by Keys *et al.* [16] in photorespiration of higher plants the rate of ammonia release seems to correspond to that of CO_2 release with the ammonia being reassimilated in the reactions of the GS-GOGAT pathway. Ogren showed in his review that ammonia as well as CO_2, is produced by the glycine-serine aminotransferase reaction in the glycolate pathway in mitochondria [17]. From there ammonia is transported into the chloroplast and reassimilated by the GS-GOGAT reactions. This explains why in higher plants ammonia is excreted from the cells when the GS-GOGAT pathway is inhibited by the addition of L-MSO under conditions which are favorable to photorespiration [18]. The same is true for *Chlamydomonas* [11]. Due to these observations the activity of serine formation in the glycolate pathway can be estimated by measuring the rate of ammonia excretion in the presence of L-MSO. This seems to be valid in the light in the presence of L-MSO for *Chlorella vulgaris* 211-11h. The rate of ammonia excretion was the same as that of glycolate excretion in the presence of INH or α-HPMS at pH 6.0 [25 °C] under ≈ 0.03% CO_2 in air (data not shown). It seems as if the rate of ammonia excretion together with that of glycolate excretion observed in the presence of L-MSO was a measure of glycolate synthesis in the cells.

According to Miyachi and Shiraiwa *Chlorella vulgaris* 211-11h cells utilize only free CO_2 as a substrate for photosynthesis [19]. In order to maintain the concentration of free CO_2 constant at the pH-values measured, *High* and *Low CO_2-cells* of *Chlorella vulgaris* 211-11h, suspended in the respective buffers at pH 6 to 8, were constantly gassed with air containing ≈ 0.03% CO_2 at 25 °C during the experiments. Under these conditions, the concentration of free CO_2 is kept at approx. 10.2 μM at the respective pH. As shown in Fig. 1 under these conditions, ammonia formation was higher at lower pH values than at higher pH values. This was valid for both *High* and *Low CO_2-cells*. If one correlates in the present paper ammonia excretion with glycolate formation, the results reported here seem to be in contrast with the known effect of pH on glycolate formation. For *Chlamydomonas* grown under 0.2–0.5% CO_2 Orth *et al.* [5] has reported that the ^{14}C-incorporation into glycolate during photosynthetic $^{14}CO_2$ evolution was higher at high pH values than at low pH values. The measurements of Orth *et al.* [5] have been carried out

at the same concentration of $NaH^{14}CO_3$ at the different pH-values. Under these conditions the concentration of bicarbonate was 1.25 times higher at pH 8.8 than at pH 7, whereas the concentration of free CO_2 was 50-fold higher at pH 7 when compared to pH 8.8. On the other hand, it has recently been reported that high CO_2-grown *Chlamydomonas* cells utilize as a substrate for photosynthesis only free CO_2, dissolved in the solution but not the bicarbonate ion. In contrast to this *Low CO_2-cells* can apparently utilize bicarbonate ions [8, 9] although later it was also reported that *Low CO_2-cells* absorb only free CO_2 by the action of carbonic anhydrase located on the cell surface [20]. From these observations it can be concluded that the data reported by Orth *et al.* [5] are at least partially due to a decrease of the concentration of free CO_2. Therefore, attention should be given to the type of active species of inorganic carbon in photosynthesis and its concentration in the solution when pH and temperature effects are measured.

At every pH measured it appeared that the rate of glycolate formation was higher in *Low CO_2-cells* than that in *High CO_2-cells*. On the other hand, the rate of photosynthetic O_2 evolution without addition of external $NaHCO_3$ is higher in *Low CO_2-cells* than in *High CO_2-cells* at any time (Table I, 0.3 and 5 h) whereas the maximal rate of photosynthesis measured in the presence of external $NaHCO_3$ was almost identical in *High* and *Low CO_2-cells* at all pH values tested. When *High CO_2-cells* are transferred to low CO_2 conditions (0.03% CO_2 in air) the characteristics of *High CO_2-cells* are gradually changed to those of *Low CO_2-cells*. Thus, at pH 8.0 carbonic anhydrase activity and the affinity of algal cells for CO_2 in photosynthesis nearly reaches the level of *Low CO_2-cells* around 3 h after the transfer to the low CO_2 conditions [21]. The ratio of the rate of photosynthetic O_2 evolution under CO_2 limiting and CO_2 saturating conditions which permits to estimate the affinity for CO_2 in *High CO_2-cells*, increases gradually to the values of *Low CO_2-cells* in the course of 9 h, *i.e.* 6 h after the onset of the illumination (Table I). The increase of this ratio, which describes the changes of photosynthetic characteristics of *High CO_2-cells* to those of *Low CO_2-cells* was faster at pH 6 than at pH 7 and 8 (Table I). This is thought to be due to the longer induction time of ammonia excretion in L-MSO treated *High CO_2-cells* at pH 7 when compared to the induction time at pH 6. This data also shows that the low activity of the glycolate pathway in *High CO_2-cells* is not due to an inhibition or low activity of photosynthesis and that the induction of carbonic anhydrase is essential for an active glycolate production and metabolism.

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[1] N. E. Tolbert, in: Encyclopedia of Plant Physiology, **Vol. 6,** Photosynthesis II (M. Gibbs and E. Latzko, eds.), pp. 338–352, Springer Verlag, Berlin 1979.
[2] E. Beck, in: Encyclopedia of Plant Physiology, **Vol. 6,** Photosynthesis II (M. Gibbs and E. Latzko, eds.), pp. 327–337, Springer Verlag, Berlin 1979.
[3] N. E. Tolbert, in: The Biochemistry of Plants, **Vol. 2** (D. D. Davies, ed.), pp. 487–523, Academic Press, 1980.
[4] G. H. Lorimer and T. J. Andrews, in: The Biochemistry of Plants, **Vol. 8** (M. D. Hatch and N. K. Boardman, eds.), pp. 329–374, Academic Press, New York 1981.
[5] G. M. Orth, N. E. Tolbert, and E. Jimenez, Plant Physiol. **41,** 143 (1966).
[6] J. L. Hess, N. E. Tolbert, and L. M. Pike, Planta **74,** 278 (1967).
[7] G. R. Findenegg, Z. Pflanzenphysiol. **79,** 428 (1976).
[8] M. Imamura, M. Tsuzuki, D. Hogetsu, and S. Miyachi, in: Proceedings of the 5. Intern. Congress on Photosynthesis (G. Akoyunoglou, ed.), pp. 471–482, Balaban Intern. Sci. Services, Philadelphia 1981.
[9] J. Berry, J. Boynton, A. Kaplan, and M. Badger, Carnegie Inst. Wash. Year Book **75,** 423 (1976).
[10] D. Hogetsu and S. Miyachi, Plant Cell Physiol. **18,** 347 (1976).
[11] G. Peltier and P. Thibault, Plant Physiol. **77,** 281 (1985).
[12] V. P. Calkins, Ind. Emg. Chem. Anal. (Anal. Chem.) **15,** 762 (1943).
[13] M. W. Weatherburn, Anal. Chem. **39,** 971 (1967).
[14] R. A. Ronzio, W. B. Rowe, and A. Meister, Biochemistry **8,** 1066 (1969).
[15] B. J. Miflin and P. J. Lea, in: The Biochemistry of Plant, **Vol. 5** (B. J. Miflin, ed.), pp. 169–202, Academic Press, New York 1980.
[16] A. J. Keys, I. F. Bird, M. J. Cornelius, P. J. Lea, R. M. Wallsgrove, and B. J. Miflin, Nature **275,** 741 (1978).
[17] W. L. Ogren, Ann. Rev. Plant Physiol. **35,** 415 (1984).
[18] S. G. Platt and G. E. Anthon, Plant Physiol. **67,** 509 (1981).
[19] S. Miyachi and Y. Shiraiwa, Plant Cell Physiol. **20,** 341 (1979).
[20] M. Tsuzuki, Z. Pflanzenphysiol. **110,** 29 (1983).
[21] Y. Shiraiwa and S. Miyachi, Plant Cell Physiol. **26,** 543 (1985).