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EVIDENCE FOR INVOLVEMENT OF 2 TYPES OF REACTION IN GLYCOLATE FORMATION DURING PHOTOSYNTHESIS IN ISOLATED SPINACH CHLOROPLASTS

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1. Introduction

It is widely accepted [1–3] that an oxidative process causes glycolate formation in connection with photoassimilation of CO_2 and that sugar phosphates or activated glycol aldehyde are substrates for this reaction. But the nature of the oxidant itself is still contested: Thus H_2O_2 [2, 3] generated by a Mehler reaction [4, 5] during photosynthesis or O_2 [1, 6] itself are frequently thought to act as oxidants. Recently Gibbs and coworkers [3, 7, 8] have concluded mainly from experiments with a reconstituted chloroplast system that 2 oxidation reactions participate in glycolate production, which differ in the nature of the oxidants.

It was assumed by the authors that one of the oxidants is H_2O_2 produced by a Mehler reaction and that the other (unknown one) originates directly by light reaction 2. If this is true, one has to expect glycolate formation even under oxygen concentrations which are too low for direct oxidation of sugar phosphates by oxygen or for generation of H_2O_2 by a Mehler-type reaction. Consequently a 2-phase curve must arise when glycolate production is plotted against oxygen concentrations.

To prove this conclusion glycolate production by isolated intact chloroplasts was measured under controlled low and high oxygen concentrations in the suspension medium.

2. Methods

2.1. *Preparation of chloroplasts and ^{14}C-fixation under controlled oxygen partial pressure*

The chloroplasts were prepared according to the method described earlier [9]. The incubation mixture, consisting of buffer C of Jensen and Bassham [10], chloroplasts (0.14 mg chlorophyll/ml) and non radioactive $NaHCO_3$ (7.9 μmoles/ml) was placed at 20° in the cuvette of an oxygraph, darkened and freed from oxygen by flushing with N_2 by means of a small teflon tube. To achieve controlled low oxygen concentrations in the mixture, the chloroplasts were illuminated (10^4 ft-candles) and bubbling with N_2 was continued. When O_2-evolution by the chloroplasts and O_2-removal by the N_2-stream reached an equilibrium, a constant level of oxygen appeared in the cuvette for about 3–4 min. After this period the O_2-evolution rate of the chloroplasts decreased and the oxygen concentration in the suspension diminished. At the beginning of the oxygen plateau $NaH^{14}CO_3$-solution (100 μCi) was added with a syringe. After a 3 min fixation period the chloroplast suspension was removed and extracted with boiling methanol containing 5% formic acid.

Flushing the chloroplast suspensions with different N_2-flow rates yielded the different O_2-concentration levels shown in fig. 1 and table 1. If higher O_2-levels were needed, exogenous oxygen was introduced (together with nitrogen) into the solution with a second teflon tube. In this case constant higher O_2-concentrations could be achieved by varying the O_2-flow rate (table 2).

2.2. *Measurement of ^{14}C-fixation and analysis of the chloroplast extract*

The boiled chloroplast suspension was separated by centrifugation into a methanol soluble extract and an insoluble pellet. Radioactivity of both fractions was determined with a scintillation counter. The chloroplast extracts were analysed by 2-dimensional paper chromatography using the following solvents: 88%

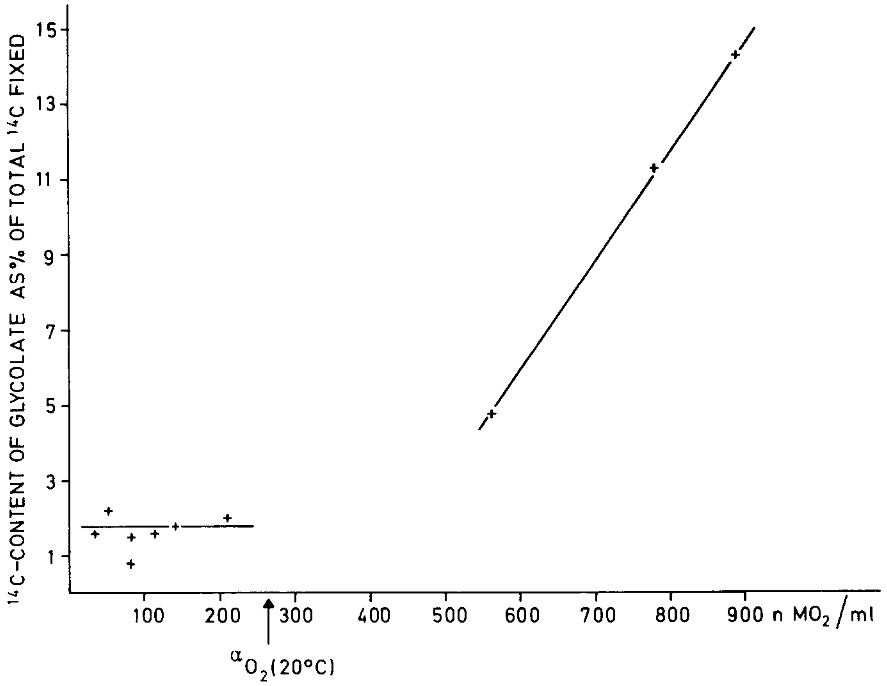

Fig. 1. Formation of ^{14}C-glycolate by isolated spinach chloroplasts fixing ^{14}C under low and high oxygen concentrations.

Table 1
Labelling of fixation products of isolated spinach chloroplasts with ^{14}C under various low oxygen concentrations; 3 min fixation period.

O$_2$-concentration in the medium (nmoles/ml)		35.9	51.5	82.8	114.5	142.6	209.2
% ^{14}C in:	Sugar diphosphates	5.3	7.6	6.5	6.6	6.2	9.1
	Sugar monophosphates + 3-phosphoglycerate	78.6	76.4	78.0	79.6	83.0	84.2
	Insoluble fraction (starch)	2.2	2.0	1.7	1.4	2.0	1.7
	Glycolate	1.6	2.2	1.5	1.6	1.8	2.0

Table 2
Fixation rates and labelling of fixation products of isolated spinach chloroplasts with ^{14}C under different high oxygen concentrations; 3 min fixation period.

O$_2$-concentration in the medium (nmoles/ml)		77.8	563.5	773.0	888.6
Fixation rate (µmoles ^{14}C/mg chlorophyll × hr)		~ 30	27.2	24.3	9.3
% ^{14}C in:	Total sugar-phosphates + 3-phosphoglycerate	93.6	87.8	83.6	80.0
	Insoluble fraction (starch)	3.6	2.3	1.8	1.6
	Glycolate	0.8	4.8	11.3	14.3

(w/w) phenol:water:acetic acid: 1 M Na_2EDTA, 480:160:10:1 and butanol–water (solution I): propionic acid–water (solution II), 1:1; solution I = butanol:water, 750:50; solution II = propionic acid:water, 352:448. Radioautograms were prepared from the chromatograms and the distribution of radiocarbon was investigated with a methane flow counter. The ^{14}C-content of glycolate was confirmed by isolation of glycolic acid from the chloroplast extract with a Dowex 1 X 8 column and liquid scintillation counting.

3. Results

3.1. *Glycolate formation under low oxygen partial pressure*

First glycolate production was studied under various oxygen concentrations lower than oxygen saturation of water under air (table 1 and fig. 1). The data demonstrate that glycolate production was relatively low but independent of the oxygen concentration of the suspension. As the distribution of ^{14}C in the chloroplast extract indicates, there was no Warburg-effect under the O_2-concentrations used, which would be shown by a decrease of ^{14}C in sugar phosphates, PGA and starch. A time course experiment showed that ^{14}C-fixation and glycolate production rates were constant during the incubation period with ^{14}C.

3.2. *Glycolate synthesis under high oxygen pressure*

The reaction which produces glycolate under low oxygen concentrations (reaction "A") cannot be held responsible for the large amounts of glycolate found usually under air. Therefore we tried to demonstrate higher rates of glycolate formation by photosynthesis under high oxygen concentrations which were achieved by flushing the chloroplast suspension with $N_2 + O_2$. The results of this experiment are shown in fig. 1 and table 2. The data demonstrate that under high oxygen concentrations there is a linear relationship between O_2-concentration and the percentage of ^{14}C found in glycolate. This indicates a second type of glycolate synthesis which is oxygen dependent (reaction "B"). Under the high oxygen concentrations used in this experiment, ^{14}C-fixation and the distribution of radiocarbon in the chloroplast extract showed a significant Warburg-effect.

4. Discussion

4.1. *Reaction "A"*

Reaction "A" can be interpreted in a twofold manner: If an enzymic reaction is assumed for which oxygen is a substrate, saturation of this process must occur at lower O_2 concentrations than those reached with our experimental procedure (30 nmoles/ml).

The other possibility suggests a non enzymic reaction in which the concentration of the oxidant is independent of the oxygen concentration in the medium but must have been constant in all 6 samples of the experiment described. As total fixation of ^{14}C indicated an equal rate of the photosynthetic process throughout the whole experiment, it is likely that production of this oxidant is linked more or less directly to this process.

4.2. *Reaction "B"*

The linear dependence of glycolate production on O_2-partial pressure demonstrated in reaction "B" suggests that O_2 itself or a reaction product of oxygen (peroxide) or both are oxidants. As decrease of label in phosphate esters with rising O_2-tensions parallels exactly to the increase of ^{14}C-content of glycolate (table 2), it is obvious that phosphate esters are substrates for this oxidation.

4.3. *Glycolate formation by both reactions*

As dependence of glycolate production on O_2-concentration is linear at higher oxygen tensions, we conclude that under this condition reactions "A" and "B" take place simultaneously. Our results further show the dependence of the ratio of glycolate formation by the 2 reactions on oxygen concentration in the medium. Thus, if oxygen is not currently removed from the suspension, reaction "B" becomes the dominant one in glycolate formation.

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