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PHOTOOXIDATIVE PRODUCTION OF CARBON MONOXIDE BY PHOTOTROPHIC MICROORGANISMS

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Summary

Net photosynthesis and CO production were measured in cell suspensions of *Chlorella fusca*. The rate of net photosynthesis showed saturation curves with increasing radiation intensities and CO_2-mixing ratios. Maximum rates were found at 35°C with a sharp decrease at higher temperatures. By contrast, the rate of CO production was proportional to the radiation intensity and did not show any saturation up to 1.5 kW · m^{-2} white light. The CO-production rate was higher in blue than in red light and was independent of the CO_2-mixing ratio of the carrier gas within a range of 0—1000 ppmv. We found that the CO-production rate was constant within the physiological temperature range of 10—35° C, but increased considerably at higher temperatures and that CO production by the chlorophyll-deficient mutant of *C. fusca* was 5 times that of the wild type. In addition, we measured CO production in cell suspensions of *Chromatium vinosum*, *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas acidophila*, which were grown either anaerobically in the light or aerobically in the dark. CO production could only be observed when the cells were incubated in the presence of oxygen and light. Under these conditions more CO was produced by aerobically grown cells than by phototrophically grown cells of *R. sphaeroides* and *R. acidophila*. The results obtained indicate that CO was produced by photosensitized oxidations and not by metabolic processes.

Introduction

Recently it has been shown that CO is formed by different C_3-type plants [1—3]. The production was found to be light dependent with production rates of 0.9 $\mu g \cdot h^{-1} \cdot dm^{-2}$ leaf surface for light intensities of 0.5 kW · m^{-2}, resulting

in a global CO production of approximately $0.7 \cdot 10^{14}$ g per annum. The same amount of CO ($0.7 \cdot 10^4$ g per annum) is formed by the oceans, probably by phototrophic microorganisms [4].

The molecular origin of CO and the mechanism of its production by plants and other phototrophic organisms are still a matter of discussion. Troxler and coworkers demonstrated that CO is produced by *Rhodophyceae* and blue green algae as a by-product of the biosynthesis of phycobilins from heme precursors [5,6]. A similar mechanism of CO production has also been observed in animal tissue where hemoglobin is converted by microsomal heme oxygenase to equimolar amounts of biliverdin and CO. Carbon monoxide is formed by the oxidation of the methin bridge of the porphyrin ring [7]. Alternatively other authors [8,9] have suggested that CO is a product of peroxidative degradation of lipids. In addition to that CO has been reported to be formed by other processes [10—15].

Wilks [16] found that the light-dependent CO production by plant material was absolutely dependent on the presence of oxygen. Light was most effective between 480 and 680 nm. He obtained the highest yields of CO using the chlorophyll fraction extracted from the plant material. The plant residue, however, failed to produce CO. More recently, Fischer and Lüttge [17] reported CO formation by *Nerium oleander* as a by-product of the photorespiratory C_1 metabolism.

In the present paper we report studies on the green alga *Chlorella fusca* and on phototrophic bacteria, which are either obligately anaerobic, like *Chromatium vinosum*, or facultatively aerobic, like *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas acidophila*. The results obtained were consistent with a non-metabolic, photooxidative mechanism of CO formation.

Materials and Methods

Microorganisms and growth conditions

Chromatium vinosum strain D (DSM 180), *Rhodopseudomonas sphaeroides* strain 7050 (DSM 137) were obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen. Stock cultures were provided by Dr. H. Biebl and Prof. Dr. N. Pfennig, Göttingen. The bacteria were grown in media which are described elsewhere [18,19]. The *Chlorella fusca* wild type strain C-1.1.10 and the chlorophyll-deficient mutant G 36 strain 1.1.10.36 were obtained from the culture collection at the department of plant physiology of the University of Göttingen [20]. The culture medium was that of Strotmann (Thesis, Frankfurt, 1966; see Ref. 21).

Preparation of cell suspensions

Cultures of *Chlorella fusca* were grown on a rotatory shaker at 23° C and an incandescent light intensity of approximately 2500 lx. After 7 days the cells were harvested, washed and resuspended in 40 ml of mineral medium [21]. The bacteria were either grown under phototrophic conditions in completely filled glass bottles at 30° C and an incandescent light intensity of approximately 1000 lx or under aerobic conditions in the dark in fluted erlenmeyer flascs at 30° C and magnetic stirring of 500 rev./min. At the end of the exponential

growth phase the cells were harvested, washed and resuspended in 40 ml of either 50 mM phosphate buffer pH 7.0 + 10 mM $(NH_4)_2SO_4$ (*Chromatium vinosum*, *R. sphaeroides*) or 50 mM phosphate buffer pH 5.6 + 10 mM NH_4Cl (*R. acidophila*).

The cell suspensions had a density of 1—2 mg dry wt · ml^{-1}. The dry weight was determined after filtering an aliquot of the cell suspension through membrane filters with 0.2 μm pore size (Sartorius, Göttingen, SM 11457). Bacteriochlorophyll and carotenoids were determined from the absorption spectra of cells extracted with acetone/methanol (7 : 2, v/v), using the extinction coefficients indicated by Clayton and Davis [22,23]. Algal chlorophyll was determined as described by Wild and Egle [21].

Measurement of CO production

The scheme of the experimental set-up is shown in Fig. 1. It is similar to that already used and described [2,3]. The cell suspension was filled in a glass cuvette and incubated at 23°C. The cuvette was irradiated by a xenon vapor lamp (Zeiss Icon, Kiel, XBO). The light beam was passed through 20 cm of water, several glass lenses and neutral filters (Schott, Mainz) to filter the ultraviolet and infrared portion of the light spectrum and to adjust the radiation intensity. Red light ($\lambda = 620$ nm) or blue light ($310 \leqslant \lambda \leqslant 480$ nm) were obtained by using the filters RG 2 and BG 3 (Schott, Mainz), respectively. The radiation intensities were measured using a Kettering Radiometer YSI (Yellow Springs Instrument Company, Yellow Springs). The cuvette was flushed with either N_2 (99.9995% purity, Messer, Griesheim, F.R.G.) or air at a flow rate of 450 ml · min^{-1}. These carrier gases were purified by passing them

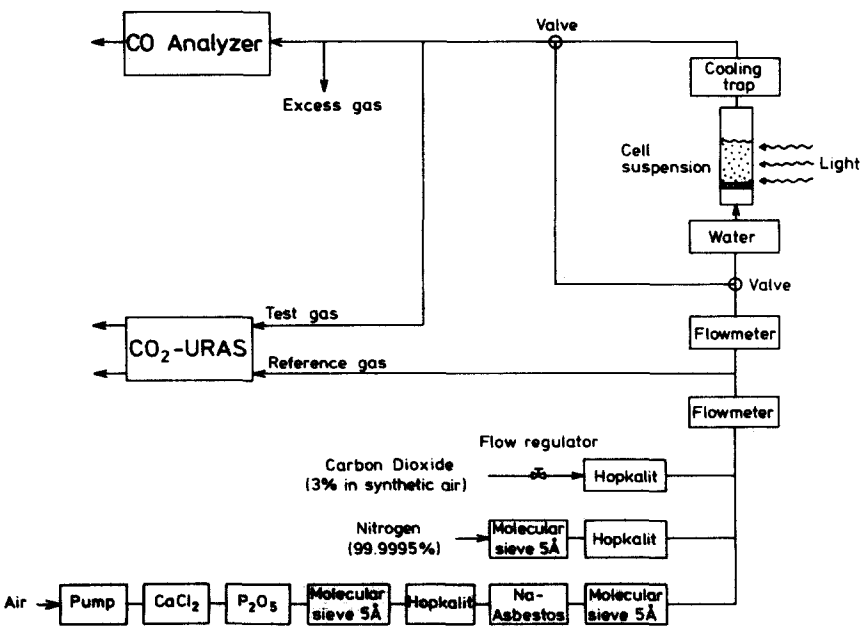

Fig. 1. Scheme of the experimental set-up.

through columns filled with molecular sieve 5 Å (Merck, Darmstadt), Hopkalit (Drägerwerk, Lübeck) and natron asbestos (Merck, Darmstadt), removing CO and CO_2 quantitatively. Before entering the cuvette, CO_2 was again added to the carrier gas in constant amounts. Thus, the CO_2-mixing ratio of the carrier gas could be varied within a range of 0—1000 ppmv and kept constant over the time of experiment. The CO_2- and CO-mixing ratios were measured periodically at the inlet and the outlet of the cuvette by a URAS 2 (Hartman and Braun, Frankfurt) and a continuously working improved CO analyser based on the HgO to Hg vapor conversion [24]. The lower detection limit of CO was 0.2 ppbv, allowing the detection of a CO-production rate of 0.15 $ng \cdot h^{-1} \cdot ml^{-1}$ suspension.

Results

Net-photosynthesis and CO production by Chlorella fusca

The CO formation was measured using cell suspensions of *Chlorella fusca* wild type and of the mutant G 36. The mutant was characterized by its reduced chlorophyll (especially chlorophyll *b*) content, by its smaller photosynthetic units, and by its altered structure of the chloroplast membrane with low concentration of light-harvesting complexes and reduced efficiency for energy transfer from the carotenoids and antennae chlorophyll to the reaction center [25]. The measurements were carried out at different CO_2-mixing ratios, and at various temperatures and radiation intensities up to 1.5 $kW \cdot m^{-2}$. During blank tests the CO- and CO_2-mixing ratios did not show any changes within the cuvette filled with the mineral medium or the buffer used for the experiments. As soon as the cell suspension was added, CO production was observed. In all cases the CO-production rate increased with increasing cell density.

Some of the results are summarized in Figs. 2—4. The results clearly indicate

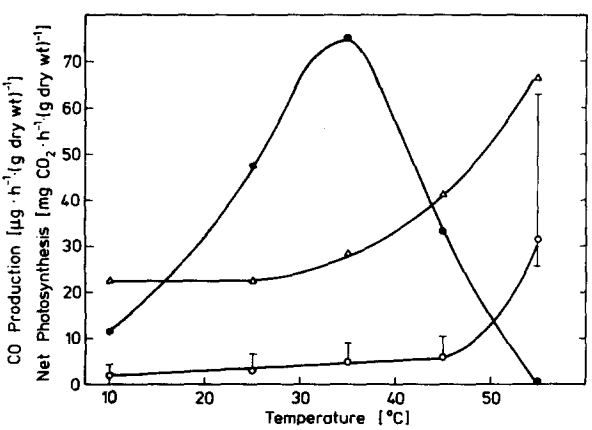

Fig. 2. CO production and net photosynthesis by *Chlorella fusca* in relation to the incubation temperature. The experiments were carried out at 300 ppmv CO_2 and 1.5 $kW \cdot m^{-2}$ white light. The bars represent the range of the CO-production rates when the CO_2-mixing ratio of the carrier gas was varied between 0 and 1000 ppmv. ●———●, net photosynthesis by *C. fusca* wild type; ○———○, CO production by *C. fusca* wild type; △———△, CO production by *C. fusca* mutant G 36.

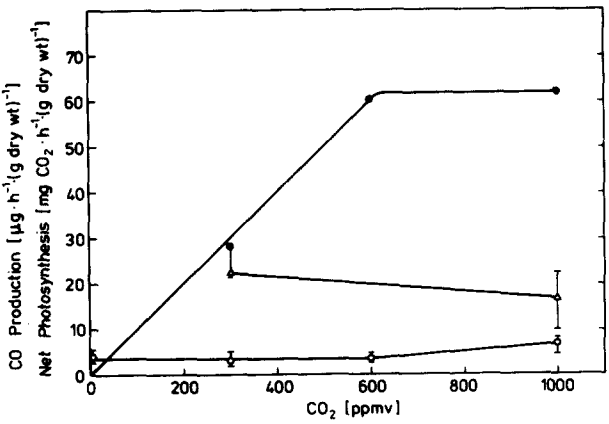

Fig. 3. CO production and net photosynthesis by *C. fusca* in relation to the CO_2-mixing ratio of the carrier gas. The experiments were carried out at 25°C and 1.5 kW · m^{-2} white light. The bars represent the range of the CO-production rates when the incubation temperature was varied between 10 and 35°C. Symbols as in Fig. 2.

that the dependency of the net-photosynthesis rates on temperature (Fig. 2), on the CO_2-mixing ratio (Fig. 3) and on the radiation intensity (Fig. 4) is completely different from that observed for the CO-production rates under the same conditions. For example, the net-photosynthesis rate increased with temperature to a maximum at 35°C and subsequently decreased to nearly zero at temperatures of 55°C. By contrast, the CO-production rate remained constant at temperatures up to 45°C and only increased when the temperature surpassed the physiological level, i.e. $T \geqslant 45$°C.

Although the CO_2 mixing ratios of the carrier gas varied between 0 and 1000 ppmv, the CO-production rate did not show any significant trend. The net-photosynthesis rate, on the other hand, showed the typical CO_2-saturation curve with saturation at 660 ppmv CO_2.

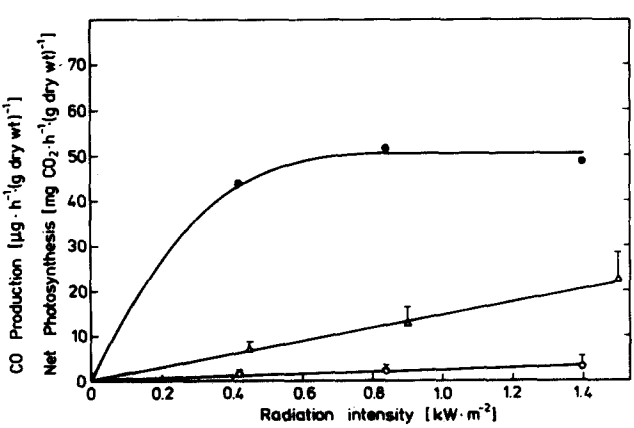

Fig. 4. CO production and net photosynthesis by *C. fusca* in relation to the radiation intensity of white light. The experiments were carried out at 25°C and 300 ppmv CO. The bars represent the range of CO-production rates when the incubation temperature was varied between 10 and 35°C. Symbols as in Fig. 2.

As expected, a saturation curve had also been observed for the net-photosynthesis rate with respect to the radiation intensity of white light. The saturation was reached at a radiation intensity of about 0.7 kW · m^{-2}. In contrast to this observation, the CO-production rates increased linearly with increasing radiation intensities.

It is of interest, that the mutant G 63 showed the same dependencies on temperature, CO_2 and radiation intensity, however, with CO-production rates, which were approximately 5 times higher than those found for the wild type (Figs. 2—4), whereas the net-photosynthesis rate was only half. It is also of interest, that the CO-production rate at a particular radiation intensity was approximately 30 times higher in blue light ($310 \leqslant \lambda \leqslant 480$ nm) than in red light ($\lambda > 620$ nm).

CO production by phototrophic bacteria

The CO-production rates of phototrophic bacteria were measured under anaerobic and aerobic incubation conditions, applying different radiation intensities of white light. The experiments were carried out using cells of *Chromatium vinosum* grown anaerobically in the light and cells of *R. sphaeroides* and *R. acidophila* grown anaerobically in the light as well as

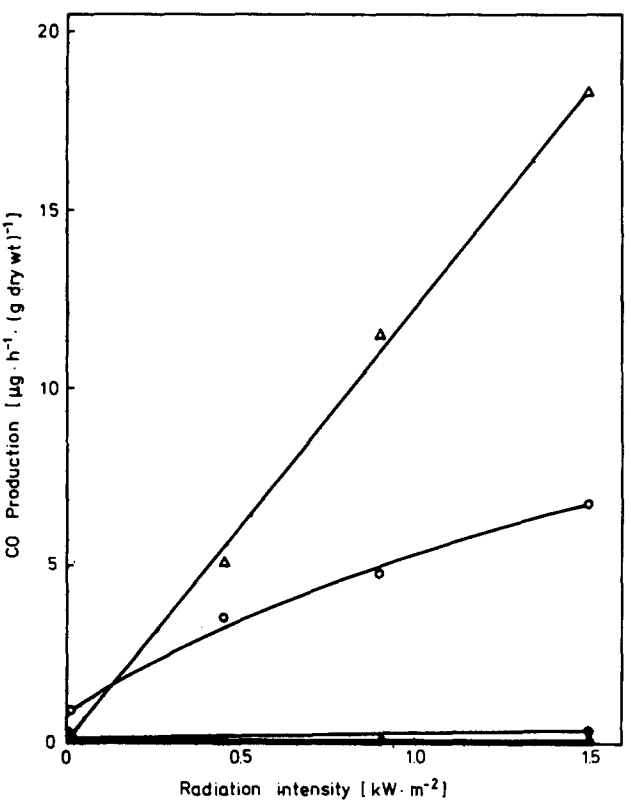

Fig. 5. CO production by *R. acidophila*. Cells grown anaerobically in the light (○,●) and cells grown aerobically in the dark (△,▲) were incubated either under aerobic (open symbols) or anaerobic (closed symbols) incubation conditions at 25°C, 0 ppmv CO_2 and increasing radiation intensities of white light.

TABLE I

CO PRODUCTION BY PHOTOTROPHIC MICROORGANISMS ANAEROBICALLY AND AEROBICALLY IN THE DARK AND IN THE LIGHT *

Microorganism	Growth conditions	Bacteriochlorophyll [$\mu g \cdot (g\ dry\ wt.)^{-1}$]	Carotenoids [$\mu g \cdot (g\ dry\ wt.)^{-1}$]	CO-Production [$\mu g \cdot h^{-1} \cdot (g\ dry\ wt.)^{-1}$]			
				Anaerobic conditions		Aerobic conditions	
				Dark	Light	Dark	Light
Chromatum vinosum	Anaerobic/light	36 470	8080	<0.1	0.4	0.4	5.9
R. acidophila	Anaerobic/light	22 490	5920	<0.1	0.3	0.7	5.7
	Aerobic/dark	1 110	510	<0.1	<0.1	<0.1	18.3
R. sphaeroides	Anaerobic/light	24 240	6960	<0.1	0.3	1.6	4.1
	Aerobic/dark	140	60	<0.1	0.1	0.1	12.1
Chlorella fusca	Aerobic/light	25 130	5600	<0.1	<0.1	<0.1	4.1

* 25°C, 0 ppmv CO_2 and 1.5 $kW \cdot m^{-2}$ white light.

aerobically in the dark. In all the experiments the CO-production rate increased proportionally with the radiation intensity. The results of a typical set of experiments obtained for *R. acidophila* are shown in Fig. 5. The results of the other experiments are compiled in Table I. Under anaerobic conditions the cells did not produce CO even at high radiation intensities. The CO-production rate was also very low under aerobic conditions, when the light had been switched off. A small CO-production rate in the absence of light was observed, however, when cells grown anaerobically in the light were transferred to aerobic incubation conditions. High rates of CO-production were obtained for all microorganisms under test, when the cells were incubated aerobically in the light.

When cells of *R. sphaeroides* and *R. acidophila* were grown under aerobic conditions in the dark, the content of bacteriochlorophyll and carotenoids decreased by a factor of 12—173 due to the repression of the biosynthesis of the photosynthetic apparatus by oxygen. The specific CO-production rate, however, was 3—4 times higher in cells grown aerobically in the dark compared with those grown anaerobically in the light.

Discussion

Our results demonstrate production of CO by a variety of phototrophic microorganisms, when the cells have been exposed to oxygen as well as to light. The rate of CO production was proportional to the radiation intensity and did not show any saturation up to 1.5 $kW \cdot m^{-2}$ white light. Production of CO occurred even under conditions to which the cells were not adapted or even not adaptable. The rate of CO-production by *Chlorella fusca* was not influenced by the CO_2-concentration, did not change within a wide range of physiological temperatures and was not correlated in any form to net-photosynthesis.

In contrast to photosynthesis, blue light was much more efficient for CO formation than red light. Aerobically grown phototrophic bacteria and the chlorophyll-deficient mutant of *C. fusca* showed higher rates of CO production than phototrophically grown bacteria and the photosynthetically more active wild type. CO production increased rapidly when unphysiologically high temperatures (>45°C) were applied. These results together with the strict dependency of CO production on oxygen and light are consistent with the observations by Wilks [16] on plant extracts. They indicate that CO is produced by a photooxidative mechanism and not by a metabolic process. This conclusion is in contrast to a recent study by Fischer and Lüttge [17], who explained the observed CO production by *Nerium oleander* as a by-product of photorespiratory C_1-metabolism, originating either from glycolate via formate or from the tetrahydrofolate-activated C_1-unit. We have to point out that recent field measurements by this laboratory on *Pinus silvestris* did not show any significant change of the CO-production rate, although the net-photosynthesis rate varied considerably due to different physiological conditions of the plant at the different seasons. Similar results were obtained from *Fagus silvatica* in summer when the leaves were green and photosynthetically active as well as in November when the leaves had changed their color from green to brown and were completely photosynthetically inactive [3]. It is, therefore, evident that CO is produced by photooxidation rather than by photorespiration or another metabolic process.

One possible molecular source for the CO production may be the chlorophylls or other porphyrins, which act as photosensitizers and can undergo photobleaching (see Ref. 26). In this case the CO probably would originate from one of the methin bridges of the porphyrins [5—7]. This conclusion is inconsistent with our observations, which show the highest rate of CO production in aerobically grown cells of *R. sphaeroides* and *R. acidophila* with a relatively low bacteriochlorophyll and carotenoid content. It is interesting that the amount of CO formed during the one hour was three times higher than that expected from the total bacteriochlorophyll content, if we assume that one mol of CO is produced for each mol of bacteriochlorophyll. We therefore can assume, that the photooxidation of chlorophylls does not contribute significantly to the observed CO production. This may not be valid for the photooxidation of other porphyrin compounds such as cytochromes.

In addition to the described processes, CO may also be formed by the photosensitization of porphyrins as well as other light absorbing cell compounds, which transfer the energy to other molecules most probably via singlet oxygen ($^1O_2$) which is assumed to be the causative agent of photosensitized oxidations and of photodynamic killing [26]. CO may then be the product of the reaction of $^1O_2$ with a variety of cell constituents, e.g. membrane lipids. Studies on liver microsomes have indeed shown that the peroxidation of membrane lipids is promoted by reactive oxygen species and that CO can be produced during this process [8,9]. It is very likely, that similar processes occur also in plants and phototrophic microorganisms.

The assumption of a photooxidative CO-production by photosensitized reactions and by the involvement of reactive oxygen species is consistent with the results of our experiments. These include: (1) The CO-production rate was

highest at irradiation with blue light ($310 \leqslant \lambda \leqslant 480$ nm) which can sensitize a great variety of membrane compounds in addition to chlorophylls and other photosynthetic pigments.

(2) The CO-production rate was relatively high in cells with an altered arrangement of the photosynthetic pigments within the cell membrane, e.g. in aerobically grown *Rhodospirillaceae* [27] or in the *C. fusca* mutant G 36 [25]. In these cells the efficiency of the carotenoides, which are able to quench photosensitized oxidations [26] is apparently reduced.

(3) A small rate of CO production could even be observed in the absence of light, when phototrophic bacteria grown anaerobically in the light were transferred to aerobic conditions. Under these conditions reactive oxygen species are likely produced in relative high amounts, since the synthesis of superoxide dismutase has not been induced during the previous growth in the absence of oxygen [28].

(4) The rate of CO-production was almost independent of temperature within the physiological range of 10—35° C. Such a temperature independence has also been observed for photodynamic killing of *R. sphaeroides* and *Micrococcus luteus* [29,30] and has been explained by the efficient transfer of energy from the photosensitizer to the molecule which is photooxidized.

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