LIGHT INHIBITION OF RESPIRATION IN *HALOBACTERIUM HALOBIUM**

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1. Introduction

Some years ago, retinal was discovered in an extremely halophilic organism, *H. halobium* [1]. The pigment is covalently bound to the only protein of the purple membrane fraction which forms patches within the surface membrane of the cells [2]. Because this retinal–protein complex resembles closely the rhodopsins of higher animals – the name bacteriorhodopsin was proposed for it [1].

Recently, a function of bacteriorhodopsin in the purple membrane of *H. halobium* was postulated [3, 4]. Light absorbed by the purple complex band of bacteriorhodopsin causes a cyclic photochemical reaction accompanied by proton release and uptake [5]. Bacteriorhodopsin is oriented in the cell membrane and therefore can act as a light driven proton pump [4]. Protons ejected from the cells apparently do work during their back flow into the cells. This results in competition between the two free energy yielding systems, i.e. the purple membrane system and the respiratory chain. Experimentally a light inhibition of respiration is found. We report here on measurements which allow quantitation of light energy conversion in *H. halobium* on the basis of this light inhibition.

* Dedicated to Professor Dr.-Ing. Karl Winnacker on the occasion of his 70th birthday.

2. Materials and methods

The mutant strain *H. halobium* NRL R_1M_1 was used [6]. Cells were grown in a fermenter as described in [7] or cultured in Erlenmeyer flasks for 4 days at 40°C under illumination and low aeration (final A_{578nm} = approx. 1.5). Cell suspensions were concentrated by low speed centrifugation and careful resuspension in fresh medium. Oxygen consumption was measured in an automatic Warburg apparatus (Mechrolab, Mountain View, California) at 25°C [8] and results calculated according to [9]. Light intensity was measured with a bolometer (Röhrig, Berlin So, Erkelenzdamm 59) as described in [10], absorption in an Ulbricht sphere (Schmidt and Haensch, 1 Berlin 62, Naumannstr. 33). As reference for absorption measurement cells illuminated in the presence of 0.2 M hydroxylamine pH 7, until no further decrease in absorption at 560 nm had occurred, were used. Light from a 250 W projector (Leitz) was filtered through 2 cm 2% $CuSO_4$ solution and an OG 4 filter (2 mm). Optical equipment was obtained from Schott (Mainz) and B. Halle (Berlin). Cell volume was determined in a microhematocrit centrifuge after 100-fold preconcentration.

3. Results

Ten-fold concentrated cell suspensions were used in order to make the experiments less time consuming.

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Table 1
Dependence of respiration in *H. halobium* on cell concentration and oxygen content of the gas phase.

% Oxygen	Cell concentration = 5 X oxygen consumption [μmoles/hr]	Cell concentration = 10 X oxygen consumption [μmoles/hr]
5	2.32	2.95
10	3.31	5.00
20	3.26	6.58
100	3.26	6.70

Measurements were carried out as described under Materials and methods. Concentration (= 1 X) is that of cells in the medium after 4 days growth.

Oxygen consumption is proportional to cell concentration and 20% or higher oxygen content in the gas phase allow maximal respiration (table 1). The cells then consume about 3–4 times their volume of oxygen per hour.

Conditions of maximal respiration were chosen in order to select a metabolic state in which light energy conversion most effectively competes with oxidative phosphorylation. A similar situation is encountered according to Lynen [11] in the competition of glycolysis and respiration for inorganic phosphate/ADP.

Fig. 1. shows schematically the equipment designed for measurement of light inhibition of respiration. It was devised originally by Warburg for photosynthetic studies on *Chlorella* and is described in more detail in [12]. Two manometric vessels each contain 3 ml of the same cell suspension and changes in pressure due to respiration are recorded automatically as changes in volume. Light from a projector is focussed onto the bottom of one of the vessels and differences in oxygen consumption are registered. A combination of filters allow variation of light intensity and selection of different wavelengths. The light intensity is measured after the vessel has been removed and the beam is directed onto a bolometer as shown in fig. 1. The value still has to be corrected for absorption of the additional mirror and lens.

Fig. 2 shows a typical experiment using the arrangement shown in fig. 1. We find values up to 30% inhibition depending on bacteriorhodopsin content of the cells and the light intensity. After turning off the light, cells return to their original respiratory activity. The following four experiments have to be carried out in order to quantitate the relationship between light absorption and oxygen consumption as well as to prove that bacteriorhodopsin mediates light energy conversion:

1) Light absorption measurements of the cells under the conditions of fig. 1;

2) Dependence of light inhibition of respiration on light intensity;

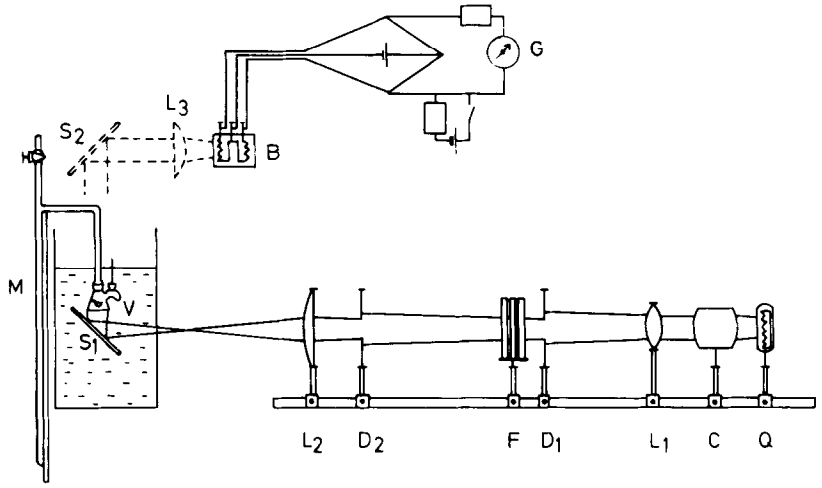

Fig. 1. Experimental equipment for measuring light effects on respiration: Q) quartz lamp; C) condensor; $D_1 D_2$) diaphragms; L_1, L_2, L_3) lenses: F) filters to select desired wavelength; S_1, S_2) mirrors; B) bolometer with Wheatstone bridge; V) manometer vessel; M) manometer; G) galvanometer.

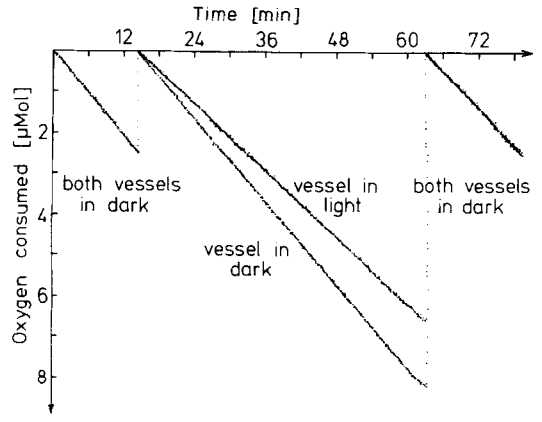

Fig. 2. Light inhibition of respiration in *H. halobium*. Cells were concentrated 10-fold. Gas phase: O_2. Center well contains 0.2 ml 20% KOH. Volume: 3 ml. Illumination with light filtered through 2% $CuSO_4$ (d = 2 cm) and OG 4 (d = 2 mm).

3) Comparison of cells with and without bacteriorhodopsin;
4) The influence of light on growth in comparison with its influence on oxygen consumption.

Absorption of turbid samples with varying thickness can be measured by use of an integrating sphere [13]. The manometric vessels are brought into the sphere and shaken at the same frequency and amplitude as in the respirometer (fig. 3). Photochemically bleached cells serve as reference. Bleaching was achieved in a photochemical reaction of bacteriorhodopsin (λ_{max} = 560 nm) in the presence of hydroxylamine in intact cells leading to the formation of retinaloxime (λ_{max} = 370 nm) as will be described elsewhere [14]. Sample and reference are illuminated with light selected from white light with the same combination of filters as in the experiment of fig. 2. The heat equivalent of this light is equal to that of the wavelength of 570 nm as was measured independently. During absorption measurements both reference and sample are kept in the sphere and absorption is taken as the ratio of the two photomultiplier responses obtained when the positions of the two vessels are exchanged. Thus the result is corrected automatically for multiple absorption.

Table 2 summarizes measurements of absorption and light inhibition at different light intensities and cell concentrations. Increasing light intensity apparently saturates the system. The minimal quantum requirement of the process is therefore obtained from extrapolation to zero light intensity (fig. 4). The data in table 2 indicate that between 25 and 40 quanta have to be absorbed by bacteriorhodopsin in order to prevent consumption of one molecule oxygen. These values will vary somewhat with the physiological state of the cells. Light not absorbed by the purple complex band (λ_{max} = 560 nm) of bacteriorhodopsin does not inhibit oxygen consumption. In addition the only pigment in mutant M_1 cells absorbing in the visible region is bacteriorhodopsin itself. If cells are grown in the presence of 3–5 mM nicotine no more retinal is formed as was found by Reitmeier (unpublished). Fig. 5 demonstrates that in these cells no light inhibition occurs.

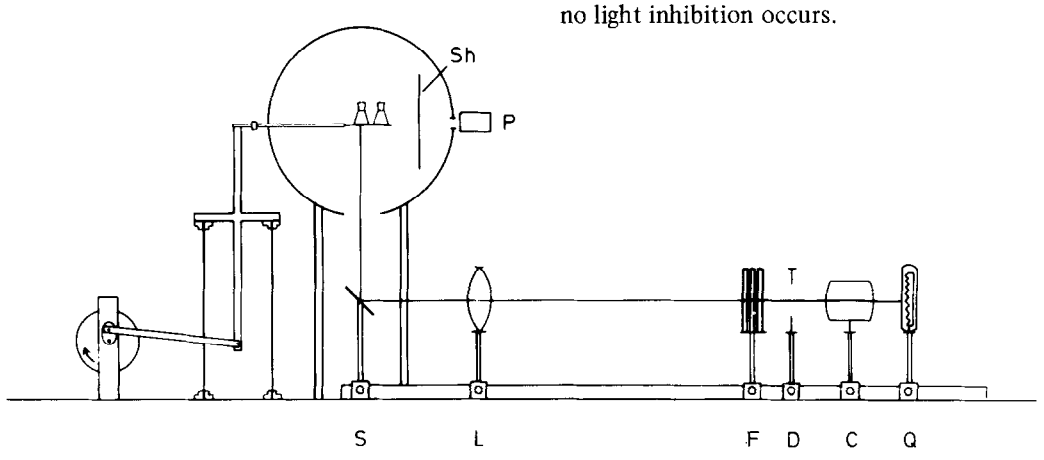

Fig. 3. Ulbricht sphere for absorption measurement of cell suspensions under the conditions of the respiratory experiments. Abbreviations as in fig. 1. P) photomultiplier; Sh) shutter for protection of the photomultiplier against direct incident light.

Table 2
Quantum requirement ($1/\varphi$) of the light inhibition.

Intensity [μmoles quanta/hr]	Quanta absorbed [μmoles/hr]	Oxygen consumption in dark [μmoles/hr]	Oxygen consumption in light [μmoles/hr]	O_2 [μmoles/hr]	$1/\varphi$
2680	308	5.15	3.99	1.16	266
1340	154	5.15	4.20	0.95	162
842	96.5	5.15	4.38	0.77	125
523	60.1	5.08	4.46	0.62	97
421	48.3	5.15	4.59	0.56	86
261	30.0	5.08	4.67	0.41	73
165	19.0	5.01	4.67	0.34	56
83	9.5	5.01	4.81	0.20	47
2680	562	7.48	5.86	1 62	347
1340	281	7.48	6.10	1.38	204
842	177	7.37	6.26	1.11	159
523	110	7.30	6.40	0.90	122
421	88.5	7.37	6.56	0.81	109.5
261	54.8	7.30	6.63	0.67	82
165	34.7	7.08	6.54	0.54	64
82.5	17.4	7.08	6.71	0.37	47

The upper and the lower set of data were obtained with cells at a concentration of 8 X and of 12.5 X respectively. Light intensity was varied by use of neutral glass filters (Schott).

To substantiate further the idea that light inhibition of respiration is in fact due to light energy conversion, cells were allowed to grow at 40°C in the manometric vessel and oxygen consumption as well as cell density increase were measured. Table 3 clearly shows that although cells grow even faster in light than in dark they have a reduced requirement for oxygen. After 12 hr light inhibition ceases under the conditions of the experiment. This is explained by regulation of bacteriorhodopsin synthesis through oxygen content of the culture medium [3,4]. At the beginning of the experiment the cells contain maximal amounts of bacteriorhodopsin due to their previous growth conditions, i.e. low aeration rate. Because

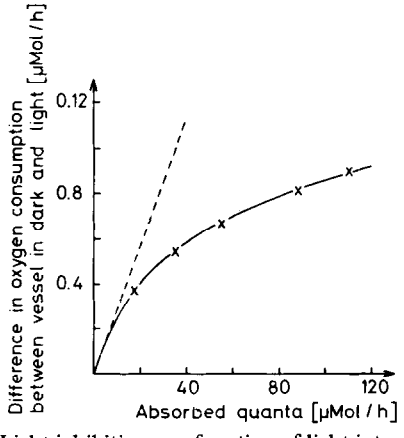

Fig. 4. Light inhibition as a function of light intensity. Data taken from table 2; the slope at the origin is obtained as the normal to the plane of a mirror given a position which allows continuation of the drawn curve without discontinuity into the mirror image.

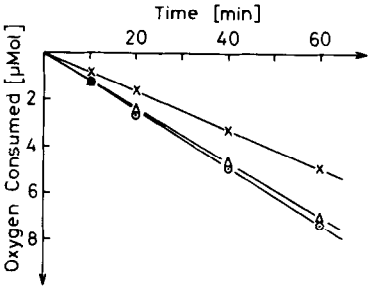

Fig. 5. Comparison of cells grown with and without 3 mM nicotine in the experiment of fig. 2. Growth is not influenced by nicotine. Both samples are then resuspended in fresh medium without addition of nicotine.

Table 3
Growth and oxygen consumption in dark and light.

Time	Cells in dark		Cells in light		% Inhibition
(hr)	A_{578}	Oxygen consumption [μmoles/hr]	A_{578}	Oxygen consumption [μmoles/hr]	
0	0.205	0.513	0.205	0.415	19.1
6	0.255	0.850	0.260	0.690	18.8
12	0.340	1.24	0.355	1.06	14.5
18	0.500	1.76	0.535	1.76	0
24	0.650	2.20	0.710	2.21	0

Four day-old cells were diluted 1:7 with fresh medium and incubated at 40°C in the vessels of the Warburg apparatus. The oxygen consumption of cells growing in light is corrected for their higher cell density.

they are then kept under saturating amounts of oxygen bacteriorhodopsin synthesis not only stops, but in addition the present amount decreases by degradation of bacteriorhodopsin [4].

4. Discussion

Our results suggest that bacteriorhodopsin in *H. halobium* plays a central role in a light energy converting system. The extent of energy conversion can be estimated from known experimental facts. Twelve protons are ejected from mitochondria or bacterial cells during reduction of one molecule of oxygen [15–17]. The cyclic photochemical reaction of bacteriorhodopsin is accompanied by proton pumping in intact cells and a quantum yield of 0.79 for the reaction was found in isolated purple membrane preparations containing bacteriorhodopsin [5]. If one assumes that no more than one proton participates in the primary reaction, 36 absorbed quanta would correspond to 12 protons because they can prevent, on the average, consumption of one molecule of oxygen. The maximal quantum yield of the system in whole cells would be, under these conditions 0.3 with regard to the primary photochemical reaction. Even considerable variation of this quantum yield would still permit to postulate light energy conversion by *H. halobium*.

To the best of our knowledge, bacteriorhodopsin dependent energy conversion is the only photosynthetic system found so far in nature beside the chlorophyll dependent photosynthesis. Further experiments are planned which will compare the efficiency of both systems and will eventually lead to a better understanding of the molecular mechanism of the bacteriorhodopsin system.

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