SPECTROPHOTOMETRIC STUDIES ON PRIMARY AND ASSOCIATED REACTIONS IN PHOTOSYNTHESIS

W. J. VREDENBERG

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKS-UNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR D. J. KUENEN, HOOGLERAAR IN DE FACULTEIT DER WISKUNDE EN NATUURWETENSCHAPPEN, TEN OVERSTAAN VAN EEN COMMISSIE UIT DE SENAAT TE VERDEDIGEN OP WOENSDAG 26 MEI 1965 TE 16 UUR

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PROMOTOR PROF. DR L. N. M. DUYSENS

STELLINGEN

I

De argumentatie die Chance gebruikt voor zijn hypothese, dat niet geoxideerd P 890 maar geoxideerd cytochroom de primaire fotooxidant van de fotosynthese is, is onjuist.

B.Chance en B.Schoener, in: Abstracts 8th Annual Meeting Biophys.Soc., Chicago, 1964 n^{r}.FD 9
Dit proefschrift, hoofdstuk III

II

In algen is er in het pigmentsysteem 1 geen rechtstreekse energieoverdracht van fluorescerend chlorofyl *a* naar het fotochemisch actieve pigment P 700.

Dit proefschrift, hoofstuk V

III

De speculaties van Kok *et al.* en van Bassham, dat in algen in pigmentsysteem 2 per geabsorbeerd quantum 2 electronen worden getransporteerd, zijn aanvechtbaar.

B.Kok, B.Cooper en L.Yang, Microalgae and Photosynthetic Bacteria, Special Issue of Plant and Cell Physiol., 1963 p.373
J.A.Bassham, J.Theoret.Biol., 4 (1963) 52
Dit proefschrift, hoofdstuk V

IV

Voor een quantitatieve bepaling van indolazijnzuur, na extractie en zuivering uit plantenmateriaal verkregen, is de "biotest" minder betrouwbaar dan een fluorimetrische methode.

D.Burnett en L.J.Audus, Phytochemistry, 3 (1964) 395
L.E.Powell, Plant Physiol., 39 (1964) 836

V

De hypothese van Franck en Rosenberg, dat bij de fotosynthese in algen en hogere planten de primaire reacties plaatsvinden in één reactiecentrum, is in strijd met de waargenomen toename van de chlorofyl-fluorescentie bij belichting met licht van langere golflengte bij 2°C.

J.Franck en J.L.Rosenberg, J.Theoret.Biol., 7 (1964) 276
Dit proefschrift, hoofdstuk V, figuur 5.12

VI

Teneinde verwarring te voorkomen, is het gewenst om de relaxatietijden die in de vergelijkingen van Bloch voor de paramagnetische resonantie zijn ingevoerd niet de spin-spin- en spin-rooster relaxatietijd, maar de transversale en longitudinale relaxatietijd te blijven noemen.

VII

De conclusie van Sistrom dat er in de purper-bacterie Rhodopseudomonas spheroides slechts 2 verschillende bacteriochlorofyl typen zijn, in plaats van 3, zoals in de meeste andere bacteriën, berust op onvoldoend nauwkeurige meetresultaten.

W.R.Sistrom, Biochim.Biophys.Acta, 79 (1964) 419
J.Crounse, W.R.Sistrom en S.Nemser, Photochem.Photobiol., 2 (1963) 361

VIII

Mendelssohn negeert in zijn beknopte verklaring van de afwezigheid van een tripelpunt in helium ten onrechte de attractie tussen de moleculen in de vloeistof, en legt te veel nadruk op de rol van deze aantrekking bij de vorming van de vaste stof.

K.Mendelssohn, M & B Laboratory Bull. vol. IV no.4

IX

Bij vacuümpompen, waarvan de werking berust op persorptie, heeft de pompsnelheid, bij constante druk, als functie van de hoeveelheid reeds geabsorbeerd gas een onverwacht verloop.

X

Het valt sterk te betwijfelen of in algen gereduceerd chlorofyl *b*, zoals voorgesteld door Rumberg, de primaire fotoreductant van pigmentsysteem 2 is.

B.Rumberg, Nature, 204 (1964) 860

XI

Bij de aanschaf van gecompliceerde wetenschappelijke apparatuur dient, naast de kwaliteit en de prijs van de verschillende handelsmerken, de service door fabriek of leverancier een belangrijk punt van overweging te zijn bij de keuze.

XII

Om te komen tot een betere en snellere uitwisseling van wetenschappelijke informatie in internationaal verband, is het dringend gewenst om in het studieprogramma voor het doctoraal examen de eis op te nemen, dat bij het houden van voordrachten en het schrijven van wetenschappelijke verslagen en scripties gebruik wordt gemaakt van de engelse taal.

XIII

Gezien de ontwikkeling in de toenadering der kerken, verdient het ten zeerste aanbeveling om in nieuwe woonwijken zoveel mogelijk tot gemeenschappelijke kerkbouw te komen; in reeds gevestigde wijken dient, voordat een kerkgemeenschap overgaat tot eventuele kerkbouw, de mogelijkheid onder ogen te worden gezien om te komen tot gemeenschappelijk gebruik en beheer van een bestaand kerkgebouw.

26 mei 1965

W. J. Vredenberg

Aan mijn ouders
Aan Regina

CONTENTS

ABBREVATIONS

DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea
HOQNO - 2-heptyl-4-hydroxy-quinoline-N-oxide
NADP - nicotinamide-adenine dinucleotide phosphate
PMA - phenyl mercuric acetate

Temperatures are expressed in degree Celsius. The various cytochromes are designated by the symbol C, followed by the number indicating the wavelength, in mμ, of the maximum of the difference spectrum (oxidized minus reduced form) in the blue wavelength region.

CHAPTER I

INTRODUCTION

The overall reaction of photosynthesis can be written as

$$CO_2 + 2\ H_2A \xrightarrow{h\nu} \frac{1}{n}\ (CH_2O)_n + H_2O + 2\ A \quad \text{(van Niel (104))}$$

In green plant and algal photosynthesis the hydrogen donor (H_2A) for the light-driven reduction of carbon dioxide is water. In addition to the chlorophyllous pigments chlorophyll *a* (algae and higher plants), bacteriochlorophyll (purple bacteria) and bacterioviridin (green bacteria), photosynthetic organisms contain various other, so-called accessory pigments such as carotenoids, chlorophyll *b* (green algae and higher plants) and the phycobilins phycoerythrin and phycocyanin (blue green and red algae). It has been shown (43) that light energy ($h\nu$) absorbed by the chlorophyllous and accessory pigments is transferred to (bacterio-)chlorophyll. Results of measurements of the average oxygen production in periodic short light flashes have led to the concept of the so-called "photosynthetic unit" (58). Amongst the various models which have been proposed (43, 27, 34, 62; see also refs. 118 and 56), we will use the one (43, 56) which has proved to be consistent with experimental results and theoretical considerations.

The photosynthetic unit is defined as an assembly of a number of (bacterio-)chlorophyll molecules and a photoreactive molecule. This photochemically active molecule and the primarily associated reactant(s) compose the "reaction center". Light energy transferred to or absorbed by one of the chlorophyll molecules of the unit is transferred from molecule to molecule and is finally captured by the reaction center which upon excitation reacts chemically. It was proved theoretically possible that an efficient transfer of energy from several hundred (bacterio-)chlorophyll molecules occurs to the reaction center by a mechanism of inductive resonance (43).

This thesis deals with results of measurements of light-induced changes in absorbancy and fluorescence which occur in intact cells of photosynthetic bacteria and algae. Part of these changes are probably due to primary photochemical reactions associated with the reaction center.

Observations of Wassink *et al* (146) on the fluorescence yield of bacteriochlorophyll *in vivo* and of Duysens (43, 46) on light-induced absorbancy changes in the infrared in photosynthetic bacteria suggested an experimental method for the possible identification of the reaction center of bacterial photosynthesis. The fluorescence yield of bacteriochlorophyll was found to increase

at intensities at which photosynthesis is saturated (146). These data were interpreted as evidence that photochemical energy conversion and fluorescence were competitive processes (117, 146): If photosynthesis is saturated or inhibited, the excess absorbed energy comes partly out as fluorescence. In intact cells of the purple bacteria Chromatium and Rhodospirillum rubrum the spectra of light-induced absorbancy changes in the infrared wavelength region, where the bulk, if not all, of the absorption is due to bacteriochlorophyll, suggested that these changes reflect the photoconversion (bleaching) of a bacteriochlorophyll-like pigment present in a small concentration (43, 46, 35). This pigment, called P 890, was suggested to be part of the photosynthetic reaction center(43). In chapter III experimental evidence is presented that bleaching of P 890 is quantitatively correlated with an increase in the fluorescence yield of bacteriochlorophyll. Similar experiments suggested that in green bacteria a pigment P 840 is part of the reaction center.

In 1954 Duysens (44) gave spectral evidence that in R. rubrum a cytochrome is oxidized upon illumination with photosynthetically active light. Many experiments carried out since then have shown that in all photosynthetic organisms studied cytochromes are oxidized upon illumination (28, 45, 109, 50, 2, 113, 101). Chance and Nishimura (29) found that cytochrome C 423.5, which is one of the cytochromes involved in the light reactions of Chromatium (109), can be photooxidized at a temperature of -196° with an efficiency equal to that at 20°. This was taken as evidence that in bacterial photosynthesis cytochrome oxidation is a primary temperature independent reaction (29, 30, 33). In chapter IV results of measurements on kinetics and spectra of light-induced absorbancy changes at room and low temperatures are presented which support the hypothesis that oxidized P 890 rather than oxidized cytochrome is the primary photooxidant.

During the last years evidence has been obtained that algal and higher plant photosynthesis is sensitized by two different light reactions (50, 51, 86, 150), which were found to be driven by different pigment systems, called systems 1 and 2, with different absorption characteristics (51). Both pigment systems contain chlorophyll *a* and accessory pigments (phycobilins and chlorophyll *b*), but system 2 contains relatively more of the accessory pigments (51). System 1 activates the reduction of NADP (2) and the oxidation of a pigment P 700 (86, 142), cytochrome (50, 51) and a plastoquinone (5); system 2 effects, via a primary reactant Q (53), the reduction of the substances oxidized by system 1, and brings about the evolution of oxygen. The current hypothesis on the primary act(s) of algal photosynthesis is that in each of the two light reactions energy is transferred to the reaction center of the pigment system, in analogy with the concept of bacterial photosynthesis. It has been proposed (53) that the primary oxidant Q, which quenches the fluorescence yield of chlorophyll *a* excited by pigment system 2 (chlorophyll a_2), is part of the reaction center of light reaction 2. Because the absorption band of

P 700, which undergoes an oxidative bleaching upon illumination with light mainly absorbed by system 1, is located near by the fluorescence band of chlorophyll *a in vivo*, it was suggested that P 700 is part of the reaction center of light reaction 1 (87, 89). In chapter V light-induced changes in the fluorescence yield of a pigment, called H 720, are reported which are sensitized by light reaction 1. H 720 is probably identical with a chlorophyll *a* excited by pigment system 1 (chlorophyll a_1). However, because of lack of correlation with absorbancy changes due to P 700, it is suggested that the fluorescence yield of chlorophyll a_1 is associated with the quenching of the as yet unidentified oxidant X of system 1. This implies that X is a part of the reaction center of light reaction 1.

It is beyond the scope of this thesis to discuss photosynthetic reactions which are not directly associated with the reaction center. For a discussion of other photosynthetic reactions we refer to recent reviews on photosynthesis (75, 56) and to the proceedings of recent symposia on photosynthesis ("La Photosynthèse", Colloques Internationaux du C. N. R. S., Paris, 1963; "Photosynthetic Mechanisms of Green Plants", Publ. 1145, Natl. Acad. Sci., Natl. Research Council, Washington D.C., 1963).

Part of the results discussed in this thesis have been published elsewhere (133, 134, 139-142).

CHAPTER II

MATERIALS AND METHODS

2.1 *Algae and Bacteria*

2.1.1 Culturing of organisms

The algae and bacteria used in the experiments were grown in liquid culture media.

Rhodospirillum rubrum (von Esmach) Molisch, strains 1 and 4, and *Rhodopseudomonas spheroides* van Niel were cultured in a synthetic medium, similar to that used by Cohen-Bazire *et al* (40), with modifications as described by Amesz (3).

Chromatium spec., strain D, was grown in a medium given by Hendley (71).

Chloropseudomonas ethylicum (127), strain 2K, was grown in a medium given by Kondrat'eva and Moshentseva (93, 14).

Anacystis nidulans (Richt.) Drouet and Daily was cultured in medium C of Kratz and Myers (95), modified as described by Hoogenhout and Amesz (72).

Schizothrix calcicola (Ag.) Gom., strain TX 27, was cultured in a medium given by Hoogenhout and Amesz (72).

Cultures were obtained by inoculation from agar slants. A culture was diluted with fresh growth medium when it had reached its maximal density, usually after a few days. Cultures of purple bacteria and algae were contained in cylindrical glass vessels of about 200 ml. The cultures were bubbled with a gas mixture consisting of air or N_2, both with 5% CO_2, to prevent exhaustion of CO_2 and settling of the suspension, and, for purple bacteria, to maintain anaerobiosis. Green bacteria were cultured in stoppered bottles of about 50 ml. Culturing tubes and bottles were thermostated in perspex water baths, which were placed in a light box, providing illumination of the cultures from aside (72). Algae were illuminated by fluorescent lamps, bacteria by incandescent lamps.

2.1.2 Preparation of suspensions for measurement

The organisms were harvested by centrifugation, resuspended at a higher concentration of cells, usually in fresh growth medium, and gassed with the same gas mixture as applied in the culture. For some experiments (see sections 3.3 and 3.4) bacteria were washed with and after centrifugation resuspended in water and bubbled with 5% CO_2 to maintain aerobic conditions. For part of the low temperature experiments (see section 4.3) bacteria were resuspended in a medium which did not crystallize and which remained transparent upon freezing. This medium was a solution

consisting of a mixture of glycerol and a 50% solution of potassium glycerophosphate at a radio of 1:3. A similar mixture, consisting of glycerol and sodium sorbitol borate, was used for measuring derivative absorption spectra of several algae at low temperatures (63). For Chromatium the essential photosynthetic substrates such as thiosulfate, sulfide and bicarbonate were added to this "non-crystallizing" medium.

2.2 *Apparatus for Measuring Changes in Absorbancy and Fluorescence*

Light-induced changes in absorbancy were determined with a "split-beam" differential spectrophotometer, described in detail by Amesz (4). A monochromatic light beam of weak intensity alternately is reflected and transmitted by a synchronously rotating (50 cps) quartz disk half of which is aluminized (see fig. 2.1). This results in two alternately chopped beams which are called the measuring beams, I_m. These are focussed on two reaction vessels which are filled with samples of the photosynthetic material. One of the vessels can be illuminated with one or more continuous light beams ("actinic beams"), I_a, while the other remains in the dark. The measuring beams hit the photocathode of a photomultiplier. The anode signal is fed to an a.c. amplifier, rectified by a phase sensitive 50 cps chopper, and registrated by a registrating d.c. millivolt meter. The intensities of the two measuring beams transmitted by the vessels can be equilibrated by adjusting optical wedges which are placed in the light paths of the two separate beams. A change in absorption of the suspension brought about by actinic light causes a deflection on the recorder. This deflection is proportional to the difference between the intensities (ΔI) of the two measuring beams transmitted by the illuminated and the dark suspension.

By means of this apparatus the kinetics and spectra of the absorbancy changes occurring upon illumination and darkening were studied. Kinetics of absorbancy changes occurring within 0.25 sec. could not be measured because of the response speed of the recorder. Spectra of the light-induced changes in absorbancy were measured by determining the steady state deflection (light minus dark) of the recorder as a function of the wavelength of the measuring beam. The change of absorption (ΔA) at a given wavelength in a first approximation is proportional to log ($\Delta I/I$) (c.f. ref. 43, p.70), in which I is the intensity of the measuring beam transmitted by the dark vessel; ΔI is small compared to I. When I is kept constant, the deflection shown by the recorder is proportional to ΔA at each wavelength. In experimental practice the d.c. output of the photomultiplier, which is proportional to I, was kept constant at each wavelength by adjusting the voltage across the dynodes of the photomultiplier. For the automatic recording of spectra of light-induced absorbancy changes in the blue wavelength region in purple bacteria (see section 4.3), we

used an electronic device with which the overall sensitivity of the apparatus was kept constant, and which provided a recorder deflection of zero at each wavelength if both vessels were in the dark. This device will be described in section 2.3.1.

The instrument also could be adapted to measurements of changes in fluorescence. This was done for experiments on purple and green bacteria in which light-induced absorbancy changes in the infrared wavelength region were compared with light-induced changes in bacteriochlorophyll fluorescence. The measuring beams were cut off and one of the actinic beams was chopped at 50 cps; this modulated beam thus functioned as an excitation beam. Light-induced changes in fluorescence were determined either by measuring the fluorescence as a function of the intensity of the excitation beam (see section 3.3), or by measuring the change in fluorescence yield brought about by a second non-modulated actinic beam (see section 3.5). The electronics of the apparatus were not modified.

Light-induced changes in the fluorescence of chlorophyll *a* in algae were measured in a fluorescence apparatus similar to the one described by Duysens and Sweers (53). Fluorescence is excited by means of a chopped beam of weak intensity, additional actinic illumination causes changes in the fluorescence yield. Kinetics and spectra of light-induces changes in fluorescence excited by short flashes and caused by one or more actinic flashes given previous to the excitation flash, were measured in the same apparatus, equipped with a flow attachment which will be described in section 2.4.

2.3 *Attachments to the Absorption Difference Spectrophotometer*

2.3.1 Automatic recording of absorption difference spectra

A block diagram of the recording absorption difference spectrophotometer is shown in fig. 2.1. The d.c. signal of the photomultiplier is compared to a d.c. reference voltage. The difference signal is amplified by a d.c. amplifier (Atlas, Bremen), and fed into a high voltage control system (h.v.c.) which controls the output of the high voltage supply (h.v.c.) of the photomultiplier (p.m.) in such a way that the d.c. output of the multiplier is kept close to the reference signal. The feedback system only responds to low frequency changes in the multiplier signal; 50 cps a.c. signals are cut off by a RC filter.*

Due to small differences in lenses, mirrors and reaction vessel in the two measuring beams, the dark signal of the apparatus was not constant when the wavelength was varied. Automatic compensation for this wavelength dependency was achieved as follows: The wavelength drive attached to the monochromator is coupled to the slide of a one turn wire wound loaded potentiometer by means of two selsyn control transformers (General Electric). The potentiometer is loaded at taps which are located at distances corre-

* I wish to thank Mr. F.de Haan for his contribution to the development of this device.

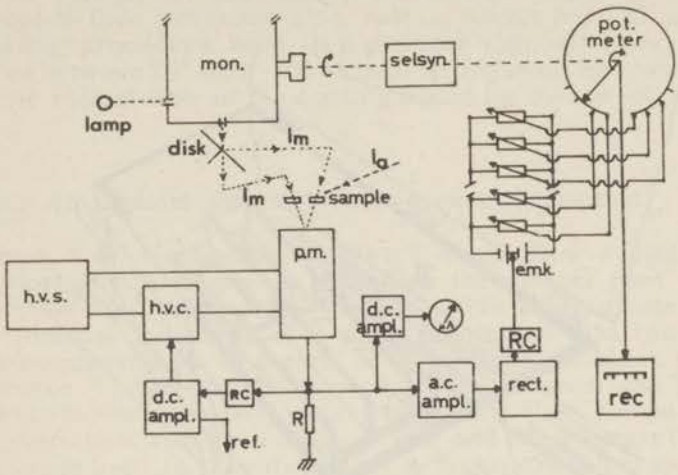

Figure 2.1 Block diagram of the recording attachment of the absorption difference spectrophotometer. The optical part of the apparatus is schematically drawn. Lenses and mirrors in the light paths of the measuring beams (I_m) and the actinic beam (I_a) have been omitted. Further details are described in the text (see also section 2.2).

sponding to 5 mμ turns of the wavelength drive of the monochromator. The d.c. load source is in series with a set of parallel shunted potentiometers. The variable voltage of each potentiometer is supplied to the corresponding tap. Since the resistance of the loading circuit was low compared to that of the potentiometer segments, the voltage at each tap was only slightly affected by that of the adjacent ones. The voltage of the potentiometer slide is fed into the recorder and adjusted at each wavelength interval to compensate the dark signal of the apparatus. This device enabled us to record in the dark a nearly flat base line over a wavelength region of 100 mμ with two vessels of approximately the same absorbancy. It was only used in the experiments with bacteria (see section 4.3).

2.3.2 Low temperature cell compartment

Kinetics and difference spectra of light-induced changes in absorbancy at temperatures down to -210° were measured in a specially constructed sample holder, a simplified sketch of which is given in fig. 2.2. The cuvettes are placed on a horizontal flat brass plate (1). The plate is cooled by liquid nitrogen (or an other coolant), which flows through cylindrical tubes (2) soldered against it. The plate is equipped with heating elements (100 watt) for warming up the material after the cooling. By means of perspex strips (3)

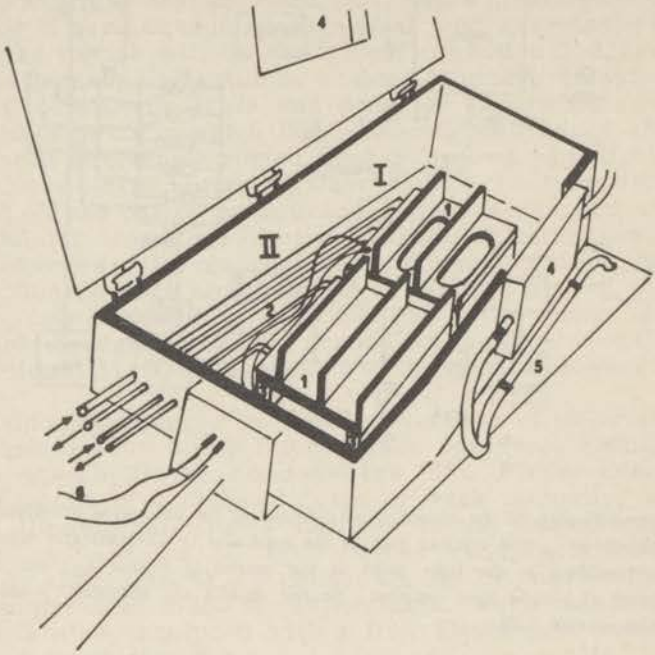

Figure 2.2 The low temperature cell compartment used for the measurement of light-induced changes in absorbancy between 20° and -210° in algal and bacterial suspensions. The lid of the compartment has been opened to show the essential parts of the cooling units I and II. The meaning of the numbers and further details are given in the text.

the cooling unit is mounted on the bottom of a rigid box. The cooling unit is divided into two nearly identical parts, I and II, which are isolated from each other, and which can be cooled and heated separately. Part I of the unit contains holes for the measuring beams. The box is equipped with perspex double windows (4). Fogging on the sample vessels is prevented by minimizing leakage of air from the surroundings into the interior of the box. Fogging of the windows on the outside is prevented by blowing dry heated air in between the perspex plates (5). The temperature is measured by a resistor of negative temperature coefficient (type Philips 05P/1k) which is mounted on top of the cooling unit (6). The resistor is calibrated against a thermocouple in a water filled cuvette placed at location I of the sample holder.

The liquid nitrogen was contained in a 5 liter Dewar flask, equipped with a safety valve adjusted at an internal pressure of 1.3 kg/cm^2. The lowest temperature which could be obtained was about -210°, when liquid hydrogen, and about -170°, when liquid air was used. In most experiments the suspensions were

precooled rapidly in part II of the sample holder. Cooling of the samples then occurred at a rate of about 8-10°C per sec. The heating procedure went in a similar (opposite) way. Temperatures between 20° and -120° could be adjusted by manual control of the rate of flow of the cooling liquid by means of a needle valve.

2.4 *Flow Attachment for the Fluorescence Apparatus*

Figure 2.3 is a schematical sketch of the flow system added to the fluorescence apparatus, of which the optical part was similar to that described earlier (108, 53). The flow cuvette, which has an internal thickness of 3 mm, is divided into two identical flow compartments by means of a partition of black perspex of thickness 1 mm, which is mounted in between and parallel to the transparent (perspex) side walls. The flow compartments are in connection with each other at one end of the cuvette. The suspension is kept in circulation by a transparent perspex centrifugal pump. The temperature of the suspension was kept constant by circulating thermostated water around the wall of the cylindrical glass reservior. The speed of flow was measured by a calibrated flow rate meter and could be varied by a needle valve or by a variable clamp on one of the silicon tubes in the circuit. The exit slit of a Bausch and Lomb monochromator equipped with a water cooled super high pressure Hg lamp (Philips SP 1000), or a diafragm placed in the slideholder of 500 W projector, was perpendicularly focussed on the cuvette. The projector was used with the same filter combinations as used by Duysens and Sweers (53). The fluorescence exciting beam was interrupted at 50 cps, and the fluorescing spot was imaged by means of a lens upon the entrance slit of an analyzing monochromator. In other experiments the fluorescence was concentrated directly on the photomultiplier, and interference filters in combination with suitable cut-off filters were used to isolate narrow wavelength regions. Actinic monochromatic light from a 500 W slide projector was focussed upon the rear side of the cuvette. The actinic beam also was perpendicular on the surface of the cuvette. The image spot could be varied in width from 1 to 10 mm. The black wall between the two compartments prevented any spurious effect from actinic or fluorescence light on the photomultiplier, even if the wavelength of the actinic light was the same as that of the measuring light.

For the automatic recording of fluorescence difference spectra a "double flow" cuvette was used, as shown in the right hand part of fig. 2.3. The fluorescence exciting beam alternated at mains frequency between the upper and lower flow compartments. This was achieved by placing a disk, driven by a synchronous motor, directly in front of the entrance slit of the monochromator. If the flow speed and the intensity of the actinic light were equal in both compartments, the fluorescence signal was zero. If in one of the compartments the (flowing) suspension was illuminated with

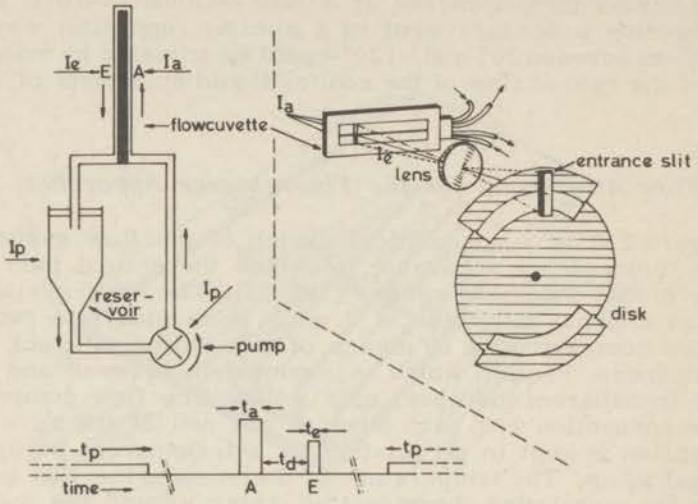

Figure 2.3 Left hand side of the figure.
A schematic picture of the flow attachment of the fluorescence apparatus used for the measurement of fluorescence in a flowing algal suspension.

The actinic beam I_a changes the fluorescence yield of the cell suspension at A. The fluorescence is measured by means of an exciting beam I_e at E. Scattered light from I_a, or the fluorescence excited by I_a, does not reach the measuring photomultiplier. The reservoir and the transparent pump can be "preilluminated" by relatively weak light I_p. The periodic pattern of illumination "seen" by a single cell is shown at the bottom part of the figure. The times are inversely proportional to the variable flow rate. Independently the dark time t_d can be varied by moving the cuvette in the direction of flow, and the times t_a and t_e of the actinic and exciting illumination are changed by varying the width of the illuminated areas. The shortest time (at maximum flow rate) for t_e, t_a and t_d was 4 msec, and for t_p 4 sec; the dark times between t_p and t_a and between t_e and t_p then were 0.5 and 1.0 sec respectively.

Right hand side of the figure.
In some experiments instead of a single cuvette a "differential" one was used which consists of an upper and lower flow compartment separated by a thin partition parallel to the direction of flow. Both the flow rate and the actinic illumination could be varied independently for these compartments. By means of the slotted disk shown in the figure, that rotated with mains frequency, the two compartments were alternately illuminated at E; the phase and frequency sensitive apparatus then records the difference in fluorescence intensities between the two compartments.

actinic light, the difference in fluorescence between upper and lower compartment was recorded as a function of the wavelength by driving the wavelength drive of the monochromator by means of a synchronous motor.

When it is assumed that all cells in the suspension move at

the same rate, the pattern of illumination is the same as that in which a stationary suspension is illuminated at periodic intervals, as shown in the bottom part of fig. 2. 3.

About 40 ml of an algal suspension was used. At the maximum speed of the pump the circulation time of the suspension was about 6 sec, and the minimum value of t_e, t_a, and t_d (for definition see fig. 2. 3) was 4 msec, that of t_p was 4 sec. t_e and t_a could be varied by a factor of 10 by changing the geometry of the optics, t_d by a factor of about 50 by moving the cuvette. Higher values of these times could be obtained by reducing the speed of flow. The intensity of the exciting beam usually was taken as low as possible in order to minimize any influence of this light upon the fluorescence yield.

2.5 *Experimental Methods*

2.5.1 Absorbancy measurement

The absorbancy of an algal or bacterial suspension was measured in a Zeiss PMQ II spectrophotometer or in a Unicam SP 700 recording spectrophotometer. Opal glass was placed behind both the blank and sample vessel in order to minimize the effect of scattering of the suspension (128). An approximate correction for scattering was made by subtracting from the measured absorbancy at each wavelength the apparent absorbancy at a wavelength at which no intrinsic absorption occurs (i. e. 740 mμ in algae, 850 mμ in green bacteria, and 950 mμ in purple bacteria). It was shown (4) that for algae and purple bacteria this method provides a good approximation of the true absorbancy of the suspension near the absorption maximum of chlorophyll, respectively bacteriochlorophyll, in the long wavelength region. The spectra recorded on the Unicam spectrophotometer were replotted on a linear wavelength scale, and corrected for scattering.

2.5.2 Measurement of changes in absorbancy and fluorescence

Measurements of small changes in absorbancy and fluorescence were carried out in the apparatus described in section 2.2. Such measurements require a careful selection of the intensity and the wavelength bands of the measuring or fluorescence exciting beams with respect to those of the usually much stronger actinic beams (43, 4).

In the differential spectrophotometer the intensity of the measuring beam usually was of the order of 10^{-11} einstein/cm^2 sec in the blue wavelength region and about 5.10^{-11} einstein/cm^2 sec in the infrared, which in our experiments was sufficiently low as not to cause a change in absorbancy. Since the measuring beams in principle cause photochemical reactions (i. e. absorbancy changes), the response brought about by additional actinic light might be influenced by the wavelength and the intensity of the measuring

beams. This effect was especially important in experiments in which kinetics and intensity curves of light-induced changes in absorbancy occurring in different wavelength regions were compared (see sections 3.4 and 5.3.1). For the fluorescence experiments described in sections 3.5 and 5.3.3, the same applied for the intensity of the fluorescence exciting beam.

Suitable filter combinations, placed in front of the detecting multiplier and in the actinic beams, were used to prevent spurious transients on the recording apparatus upon switching on and off of the actinic light.

When changes in absorbancy are measured in spectral regions in which fluorescence emission occurs (i.e. around 685 mμ in algae, 780 mμ in green bacteria, and 890 mμ in purple bacteria), the observed signals may (partly) be due to a light-induced change in fluorescence excited by the measuring beams (121, 77, 73). In our experiments at the intensities used this effect was checked to be too small to disturb the measurements appreciably: the signals were not affected by placing, between the vessels and the photomultiplier, suitable narrow band pass filters which transmitted only part of the fluorescence. In a similar way it was checked that the signals were not due to fluorescence excited by the actinic light.

2.5.3 Measurement of actinic light intensities

Intensities of the actinic light (expressed in einstein/cm^2 sec) were measured by calibrated photocells, and are given per cm^2 of the illuminated surface of the cuvette, correcting for the reflection at the wall. This means that for the absorbancy and fluorescence measurements carried out in the absorption difference spectrophotometer, in which the actinic beams hit the cuvette at an angle of 22° (see ref. 4, p. 23), the values of the intensities given have to be multiplied by a factor 1.4 and 3.1 to obtain the intensity of the actinic light inside and outside the cuvette, respectively. The intensity inside the cuvette is the intensity "seen" by the cells.

2.5.4 Measurement of quantum requirements and action spectra

For some of the photoreactions studied the quantum requirement and the action spectrum were determined in a way as described by others (4, 43, 49). At an intensity of actinic illumination at which the rate of the reaction was proportional to the intensity, the initial rate of the absorbancy change occurring upon onset of the illumination was measured at a wavelength of maximum absorbancy change of the reactant studied (i.e. 420 - 422 mμ for cytochrome oxidation in algae and bacteria, 890 mμ for the pigment P 890 in purple bacteria, and 435 and 700 mμ for P 700 in algae). The number of moles converted per unit of time was calculated by dividing the inital rate by the specific molecular extinction coefficient, which for various compounds is

known from biochemical estimates (10, 78, ref. 118, pp. 606, 1807). By dividing this number by the number of absorbed quanta, the quantum efficiency of the reaction was calculated. The number of absorbed quanta was calculated from the absorbancy of the suspension (see 2.5.1) and the intensity of the actinic light (2.5.3). Corrections were applied for reflections at the cuvette walls and for inhomogeneities of the actinic beam. The accuracy with which quantum requirements were measured was limited by the accuracy with which the rate of the reaction and the number of absorbed quanta could be determined. The rate of the reaction was estimated as an average of about three measurements with a mean error of 5 - 10%. Moreover, a systematical error is introduced by neglecting "optical flattening" effects and possible deviations from Beer's law for the absorption of the suspension. It was estimated that for a 1.5% suspension of Anacystis these effects cause a deviation from the "true" rate by a factor of about 0.8 (ref. 4). For Schizothrix this value may roughly be the same: for Chromatium this factor is probably nearer to 1. The number of absorbed quanta was established with a precision not better than 15%, mainly because of an inaccuracy in the measurement of the intensity of the actinic beam and of the inhomogeneity of this beam. Thus, regardless of the uncertainty introduced by optical flattening, the error in the calculated quantum requirements is about 15 - 20%.

An action spectrum, giving activities for quanta of different wavelengths, was measured by determining, under conditions at which light was not saturating, the number of quanta at each wavelength which bring about a certain absorbancy or fluorescence change or rate of reaction. From the activity versus intensity curve, measured at one wavelength, the relative activity was calculated and plotted relative to that at the most active wavelength.

CHAPTER III

PRIMARY PHOTOCHEMICAL REACTIONS IN PURPLE AND GREEN BACTERIA

3.1 *Introduction*

In intact cells and cell-free preparations of purple bacteria light-induced reversible changes in absorbancy have been found in the near-infrared region, where the bulk, if not all, of the absorption is due to bacteriochlorophyll (43, 46, 6, 35). In Rhodospirillum rubrum the absorption difference spectrum shows a decrease in absorbancy of about 2%, with a maximum at about 880 mμ, and an increase around 795 mμ. Since in R. rubrum bacteriochlorophyll has a major absorption maximum at 880 mμ and a minor one at 800 mμ, the changes in absorbancy were attributed to a small fraction of the bacteriochlorophyll (46), which we call P 890. The light-induced reversible "bleaching" at 880 mμ was more rapid and occurred at lower intensities of exciting light in the absence of hydrogen-donors under oxidizing conditions (46). A similar bleaching could be brought about in aqueous extracts on addition of mixtures of potassium ferri- and ferrocyanide with oxidation reduction potential of about 0.5 volt (48, 65). It was suggested (46, 48, 65, 35) that the light-induced absorbancy change around 880 mμ reflects the oxidative bleaching of P 890. The energy of light absorbed by the bacteriochlorophyll type B 890 may be transferred to P 890 (c.f. ref. 43), which becomes oxidized on receiving energy. P 890 may be the photochemically active molecule, and thus be part of the "reaction center" (43). Oxidized P 890 might oxidize the hydrogen donor, possibly by way of one or more cytochromes (44, 46, 47, 37).

If the transfer from B 890 to P 890 occurs from the lowest excited (fluorescing) singlet state, the efficiency of the transfer to P 890 is lowered upon bleaching of this pigment and the fluorescence yield of B 890 will increase. If was found for Chromatium that the fluorescence yield of bacteriochlorophyll increased at higher intensities of exciting light (146). The increase in fluorescence yield was higher and occurred at lower intensities in the absence of hydrogen donors and under oxidizing conditions (146). Bacteriochlorophyll fluorescence was found to originate from the bacteriochlorophyll type B 890 (ref. 43).

In this chapter it will be shown that the increase in bacteriochlorophyll fluorescence is correlated with the bleaching of P 890, and that this correlation can be explained quantitatively by the hypothesis of energy transfer mentioned before. It will also be shown that the bleaching occurs with high quantum

efficiency. Results of similar experiments done with the green bacterium Chloropseudomonas ethylicum will be presented, which indicate that in these species a pigment P 840 presumably acts as a reaction center.

3.2 *Methods*

Measurements were carried out in the split-beam difference spectrophotometer as described in section 2.2. In R. rubrum changes in absorbancy were brought about by the 577 and 579 mμ lines of a GEC ME/D 250 W Hg lamp. For fluorescence measurements this light beam was chopped. For the experiments with Cps. ethylicum a non-modulated actinic light beam of a wavelength band around 430 mμ was used which was provided by a d.c. fed slide projector, equipped with filters. Fluorescence was excited by a second, modulated beam of low intensity of the same wavelength. In all experiments suitable filter combinations were placed in front of the multiplier (DUMONT 6911) for cutting off the excitation and actinic light and for selecting the wavelength of fluorescence. Experiments at low temperature were carried out in the low temperature cell compartment described in section 2.3.2. Spectra of intact cells of R. rubrum in the long wavelength region at -170° were recorded in the same apparatus by measuring the difference signal between a blank and a sample vessel as a function of the wavelength. These spectra are not true absorption spectra, but they were adequate to identify the location of the absorption maxima at 20° and -170°.

3.3 *Evidence for a Reaction Center P 890 in Rhodospirillum rubrum*

3.3.1 Kinetics and light curves of the absorbancy changes at 880 mμ and of bacteriochlorophyll fluorescence

Fig. 3.1 shows recordings of the changes in absorbancy at 880 mμ occurring upon actinic illumination, and of the infrared fluorescence excited by light of the same intensity. The absorbancy and fluorescence measurements were carried out shortly after each other and at the same sequence of light and dark periods. The fluorescence increased, starting from an initial level f. Under the conditions used at least two distinct phases could be distinguished in both the absorbancy change (a, b) and the fluorescence increase (a_f, b_f); the phases a and b appear to be correlated with a_f and b_f, respectively.

In fig. 3.2 the steady state absorbancy difference in light and darkness, measured at 880 mμ, and the steady state level of the fluorescence are plotted as a function of the intensity of the actinic light. The changes in absorbancy and in fluorescence

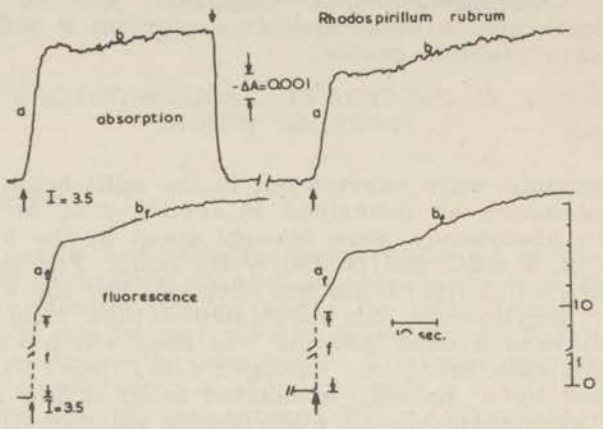

Figure 3.1 Rhodospirillum rubrum (strain 1), suspended in growth medium.
The top tracings are time recordings of the light-induced absorbancy changes at 880 mμ. The bottom tracings show recordings of the total fluorescence, $\lambda > 850$ mμ. The switching on and off of the actinic and fluorescence exciting light, 590 mμ, is indicated by upward and downward pointing arrows. The intensity of the light was 3.5×10^{-9} einstein/cm^2 sec. The actinic and fluorescence exciting light was given after 10 sec darktime (left hand tracing) and after 15 sec darktime (right hand tracing) for both absorbancy change and fluorescence.

yield (upward curvature of the fluorescence versus intensity curve) both occurred at actinic intensities above 5.10^{-10} einstein/cm^2 sec. The initial fluorescence level f also is plotted as a function of the intensity. Since f was proportional to I, the fluorescence yield at the start of the illumination apparently is independent of the intensity. This means that under these conditions the change in the fluorescence is slow, as compared to the response time of the apparatus.

Figs. 3.1 and 3.2 show that in intact cells of Rhodospirillum rubrum suspended in fresh growth m dium a decrease in absorbancy at 880 mμ, caused by the photobleaching of P 890, is qualitatively correlated with an increase in the fluorescence yield of bacteriochlorophyll. For cells which were washed and suspended in aerobic distilled water similar results were found (139); the changes occurred at lower intensities.

At -170° the changes in absorbancy at 880 and 795 mμ still occurred upon illumination, which indicates that the bleaching of P 890 occurs at this low temperature. The reactions were reversible: in the dark the original steady state was reached again. For dried bacterial extracts this reversibility has been observed at 1°K by Arnold and Clayton (6).

At -170° both the absorption spectrum and the absorbancy difference spectrum showed a shift of the 880 mμ maximum of about 20 mμ to longer wavelength, both in R. rubrum and Chromatium. This shift provides an additional argument for the

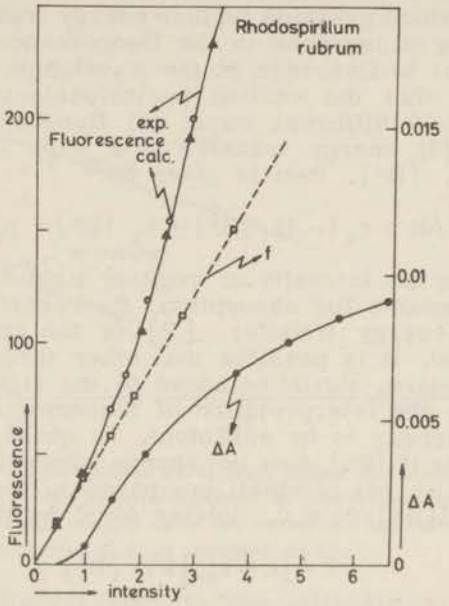

Figure 3.2 Rhodospirillum rubrum (strain 1), suspended in growth medium.
The steady state absorbancy difference light minus dark at 880 mμ, respectively the steady state level of fluorescence is plotted as a function of the monochromatic (590 mμ) actinic, respectively fluorescence exciting intensity. The intensity curve of the initial fluorescence intensity f is also shown. The calculated fluorescence curve (see text) is given by open circles. The amount of fluorescence (left scale) is expressed in arbitrary units. The absorbancy difference (right scale) is given in optical density units.

assumption that the change in absorbancy around 880 mμ is due to the bleaching of a pigment similar to B 890. It was already known that in Rhodospirillum rubrum the fluorescence band of B 890, which has a maximum at about 900 mμ, shifts about 20 mμ to longer wavelength upon cooling to liquid nitrogen temperatures (16).

In addition to the infrared absorbancy changes, a light-induced increase in absorbancy was observed at -170° in the blue wavelength region, with a broad absorbancy band around 435 mμ. As will be discussed in section 3.4, this change is not correlated with those occurring in the infrared.

3.3.2 A quantitative relation between the fluorescence yield of B 890 and the photobleaching of P 890

The observed correlation between the increase in B 890 fluorescence and P 890 bleaching can be quantitatively described by the following hypothesis: Excitation energy is transferred

from B 890 to P 890. Upon excitation of P 890 this component is bleached, which prevents further energy transfer from excited B 890, causing an increase in the fluorescence of B 890; P 890 is assumed not to fluoresce at the wavelength of measurement. We postulate that the excited bacteriochlorophyll, B*, looses its energy in 3 different ways: (1) fluorescence, (2) internal conversion, (3) energy transfer to P 890. The change in B*-concentration, [B*], then is given by:

$$d[B^*]/dt = c_1 I - (k_1 [B^*] + k_2 [B^*] + k_3 [B^*] [P]) \qquad 2.1$$

in which I is the intensity of exciting light and c_1 and the k's are rate constants for absorption, fluorescence, internal conversion and energy transfer. [P] is the concentration of unbleached P 890. It is possible that other terms, containing variable parameters, should be added to the right side of eq. 2.1. However, for the interpretation of the present experiments the terms given appear to be sufficient. At quasi steady state conditions, that is if [B*] does not change appreciable in 10^{-8} sec. ($k_1 \approx 10^8$), $d[B^*]/dt$ is small compared to the other terms and we may put $d[B^*]/dt = 0$. Solving for I we obtain:

$$I = (k + k_3' [P]) [B^*] \qquad 2.2$$

where $k = (k_1 + k_2)/c_1$ and $k_3' = k_3/c_1$.

The intensity of fluorescence is given by:

$$I_f = k_1 [B^*] \qquad 2.3$$

The fluorescence yield is

$$\varphi_f = I_f/I = k_1/(k + k_3' [P]) \qquad 2.4$$

At zero intensity, all P 890 is in the non-bleached form: $[P] = P_o$: At high intensities, at which all P 890 is bleached: $[P] = 0$. At intermediate intensities $o<[P]<P_o$. The change in absorbancy is equal to:

$$\Delta A = c_2 (P_o - [P]) \qquad 2.5$$

in which c_2 is a constant, equal to the difference in absorption coefficient of the unbleached and bleached forms of P 890. Eliminating [P] from eqs. 2.4 and 2.5, we get:

$$1/\varphi_f = (k + k_3' P_o)/k_1 - (k_3'/k_1 c_2) \Delta A \qquad 2.6$$

According to eq. 2.6, $1/\varphi_f$ and ΔA are linearly related. Fig. 3.3 shows that $1/\varphi_f$ is in first approximation linearly dependent on ΔA, which supports our hypothesis. The values of $1/\varphi_f$ and ΔA for the same intensities were obtained from the experimental curves given in fig. 3.2. The value of $1/\varphi_f$ at $\Delta A = 0$ was taken

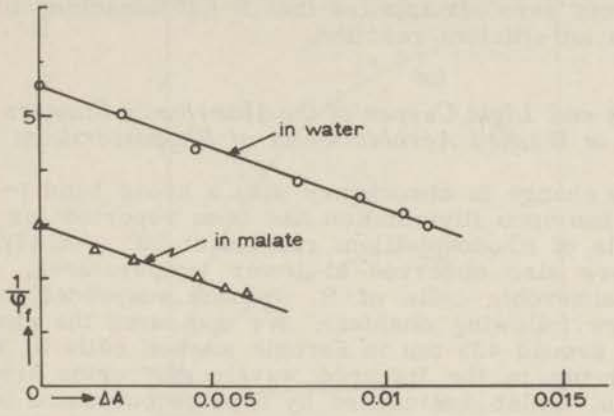

Figure 3.3 The inverse of the fluorescence yield, $1/\varphi_f$, plotted as function of the absorbancy change, ΔA, for bacteria suspended in growth medium (open triangles), and in water (open circles). The two curves are not comparable, since the fluorescence intensities were not measured absolutely.

as the slope of the fluorescence-versus-intensity curve at $I = 0$. In fig. 3.2 calculated intensities of fluorescence are also given. These were calculated from the ΔA curve by substituting the experimental values of ΔA in eq. 2.6. The two constants k'_3/k_1c_2 and $(k + k'_3P_o)/k_1$ in the right side of eq. 2.6 were obtained from the corresponding line of fig. 3.3. The first constant is equal to the slope of the line, the second is equal to $1/\varphi_f$ at $\Delta A = 0$. The experimental and calculated curves of fluorescence appear to be similar. For an aerobic aqueous suspension the results are also shown in fig. 3.3.

3.3.3 The quantum requirement for P 890 bleaching

The quantum requirement for the bleaching of P 890 was measured for an aerobic aqueous suspension, to which 10^{-5} M phenyl mercuric acetate was added, which was found to minimize the rate of P 890 restoration in the dark. From the initial rate of the absorbancy change at 880 mμ, the intensity of the actinic light (590 mμ) and the absorbancy of the suspension, the quantum requirement for P 890 bleaching was calculated. It was assumed that the specific molecular extinction coefficient of P 890 was equal to that of bacteriochlorophyll (ϵ = 96 mM^{-1} cm^{-1}) (ref. 118, p. 1807) and that that of oxidized P 890 was zero. The quantum requirements, calculated from the initial rates measured at two intensities of actinic light, for samples taken from the same culture were 3.3 and 3.1 quanta per molecule for bacteria suspended in water and PMA added, 3.1 and 3.6 for bacteria washed and suspended in water, and 3.2 for bacteria washed and suspended in water and PMA added. These numbers will be

lower, if the molar extinction coefficient of oxidized P 890 is different from zero. It appears that P 890 bleaching in photosynthesis is an efficient reaction.

3.4 *Kinetics and Light Curves of the Absorbancy Changes around 435 mμ in Washed Aerobic Cells of Rhodospirillum rubrum*

A positive change in absorbancy with a broad band (~ 40 mμ) around 435 mμ upon illumination has been reported for aerobic washed cells of Rhodospirillum rubrum at 20° (ref. 47). These changes were also observed at lower temperatures, even at -170°, in anaerobic cells of R. rubrum suspended in growth medium (see following chapter). We compared the changes in absorbancy around 435 mμ in aerobic washed cells at 20° with those occurring in the infrared wavelength region around 880 and 795 mμ, which are caused by a photoconversion of P 890 (section 3.3). It has been suggested (47, 35) that the positive changes around 435 mμ were correlated with the P 890 changes. However, Olson and Kok (107) concluded that the time course of the changes in the blue wavelength region was different from that in the infrared, and that consequently the changes were caused by at least two different reactions. Since in Olson and Kok's experiments the intense measuring light might have affected the kinetics, and since Clayton (35) reported that the kinetics were the same in his experiments, we compared the kinetics at 435, 795 and 866 mμ, using actinic light of the same wavelength, 590 mμ. As fig. 3.4 shows, the absorbancy change at 435 mμ was saturated at lower intensities of actinic light than that at 795 and 866 mμ. The light curves of the changes at 866 and 795 mμ were found to be proportional. In these experiments it was verified that the measuring beams were of too low intensity to cause a measurable change in absorbancy (see section 2.5.2). We found in addition the decay time of the dark reaction at 435 mμ to be smaller than at 795 mμ, which is in agreement with the results of Olson and Kok (107) and with recent results obtained by Kuntz *et al.* (96), who found in addition that the decay kinetics of the changes at 792 mμ were slower than those of the long wavelength bands.

Apparently the absorbancy change around 435 mμ thus is not caused by the oxidation of P 890. The spectrum of the changes differs from that reported for carotenoid changes (130, 131, 106), and from that ascribed to cytochrome *b* reduction (106). Thus probably the changes have to be attributed to an as yet unknown pigment. The fact that the pigment reacts at -170° suggests that the change is associated with a primary photochemical process. For cell-free extracts of R. rubrum it was reported

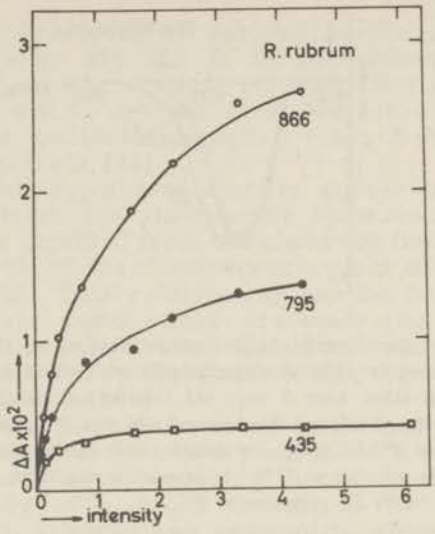

Figure 3.4 Rhodospirillum rubrum, washed aerobic sample, suspended in distilled water. The steady state absorbancy differences ΔA at 435 mμ (squares), 795 mμ (solid circles) and at 866 mμ (open circles) in light and darkness are plotted as a function of the intensity of the actinic light. The actinic light was of a wavelength band around 590 mμ; the intensity is expressed in 10^{-9} einstein/cm^2 sec. The figure shows that the 435 mμ curve "saturates" at a lower intensity than the 866 and 795 mμ curves.

that the light and dark kinetics of the absorbancy changes at 435 mμ were the same as those of the light-induced E.S.R. signals (122).

3.5 *Evidence for a Reaction Center P 840 in Green Photosynthetic Bacteria*

In the green photosynthetic bacteria Chloropseudomonas ethylicum and Chlorobium limicola a decrease in absorbancy upon illumination was observed in the infrared region (133, 134). Fig. 3.5 shows the spectrum of these changes for Cps. ethylicum. The spectrum has a peak around 840 mμ and therefore was attributed to that of a pigment P 840 which is bleached upon illumination (133).

It has been shown (132) that in Cps. ethylicum there is a substantial energy transfer from the main chlorophyll, chlorobium chlorophyll, to B 810 (formerly denoted as chlorophyll-770 (111, 112)), and that light absorbed exclusively by B 810 is effective in oxidizing a c-type cytochrome, C 425 (ref. 113). It was found that at high intensities of exciting light the fluorescence yield of B 810 was higher than at low light intensity,

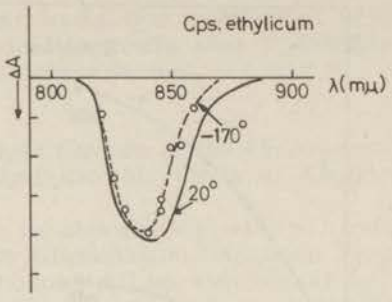

Figure 3.5 Spectra of the reversible light-induced changes in absorbancy in the infrared region in whole cells of Chloropseudomonas ethylicum at 2° and -170°. The cells were taken from 3 days old cultures and resuspended in water. Actinic light was of a wavelength band around 436 mμ. The solid curve is the spectrum obtained at 2° (ref. 133), the dotted curve was obtained for another sample at -170°. The spectrum at -170° is adjusted to the same height in the maximum near 840 mμ as the spectrum at 2°.

while the fluorescence yield of chlorobium chlorophyll was independent of the intensity (132, 113). These data can be interpreted as evidence that P 840 receives energy from B 810. We obtained evidence for this hypothesis from measurements with Cps. ethylicum in which the changes in absorbancy at 840 mμ and the changes in fluorescence at 838 mμ (at which wavelength presumably all fluorescence is due to B 810) were measured at several intensities of actinic light. It was found (133) that the inverse of the fluorescence yield of B 810 was proportional to the bleaching of P 840, which (c.f. eq. 2.6) argues in favor of the hypothesis. Reversible bleaching of P 840 was found to proceed at -170°. (fig. 3.5). Photooxidation of cytochrome C 425 did not occur at temperatures below -45° (134). This result qualitatively is similar to that found in purple bacteria, and indicates (see section 4.3) that light-induced cytochrome oxidation is not a temperature insensitive reaction.

3.6 *Discussion*

The results reported in this chapter indicate the existence of reaction centers P 890 in purple bacteria and (less conclusively) P 840 in green bacteria. The experiments were performed with the purple bacterium R. rubrum and the green bacterium Chloropseudomonas ethylicum. Difference spectra of the light-induced changes in absorbancy in the near-infrared wavelength region in other purple bacteria (43, 46, 35, 38) and in the green bacterium Chlorobium limicola (134) indicate that also in these species a bacteriochlorophyll-like pigment is bleached upon illumination. Although the location of the long wavelength peak

in the difference spectrum of various purple bacteria is not quite the same (e.g. 870 mμ in R. spheroides (35), 880-890 mμ in R. rubrum and Chromatium (43, 46)), the reaction center in purple bacteria will be denoted as P 890, in analogy with the designation of the bacteriochlorophyll types B 800, B 850 and B 890 in these bacteria (43).

Evidence for the hypothesis that in purple bacteria energy transfer occurs from the fluorescent bacteriochlorophyll type B 890 to P 890 was obtained from the observed linear relationship between the inverse of the fluorescence yield of B 890 and the bleaching of P 890. This relationship can be derived theoretically, when certain assumptions are made (eq. 2.6). Also in the green photosynthetic bacterium Cps. ethylicum such a relation was demonstrated between the fluorescence yield of B 810 and the bleaching of P 840 (133).

In "normal" bacteria under normal conditions the light-induced absorbancy changes at 890 mμ are about 1-2% of the absorption at this wavelength. Clayton reported that in an aqueous extract of an aged culture of a green mutant of R. spheroides the optical density at 870 mμ was reduced to about one half upon illumination (35); the light minus dark difference spectrum, however, was essentially similar to that of "normal" purple bacteria. This indicates that upon illumination the specific absorption of P 890 is reduced to at least one half and hence that in normal bacteria probably an appreciable change in absorption occurs in a relatively small number of (P 890) molecules, instead of a small change in all B 890 molecules. This is consistent with the observation that the general shape of the difference spectrum is about the same for different species (43) and also for aqueous extracts of different mutants (35, 96) and detergent treated fragments of extracted chromatophores (35) which show marked differences in the relative absorption of the various bacteriochlorophyll types. The same applies to chromatophores of various bacteria, in which nearly all B 890 was bleached by a strong oxidant (96), and for intact cells of R. spheroides with an artificially induced low bacteriochlorophyll content (39).

The observations that the long wavelength maximum of the P 890 difference spectrum coincides with the maximum of B 890 absorption and that this maximum shifts upon cooling similar to the maxima of B 890 absorption and fluorescence strongly suggest that P 890 is a pigment similar to B 890, but photochemically active. To explain the decrease in absorbancy at about 800 mμ and the increase at 795 mμ, which are found in addition to the decrease in absorbancy around 890 mμ, it was proposed (54) that P 890 is an aggregate consisting of one molecule similar to B 890 and one molecule similar to B 800 in addition to certain bearer molecules. Upon illumination the absorption maximum of the B 890-like molecule is decreased and that of the B 800-like one is shifted. It was argued (15, 41, 147), contrarily to other suggestions (11, 25, 34, 74, 69), that the

in vivo bands of bacteriochlorophyll presumably are due to a binding of bacteriochlorophyll to protein bearer molecules.

Evidence has been given that the light-induced bleaching of P 890 is due to an oxidation; addition of mixtures of ferri- and ferrocyanide to bacterial extracts causes similar, although not completely identical changes in absorbancy in the infrared (48, 65, 36, 96). From experiments of this kind it was estimated that the oxidation reduction potential of P 890 is 0.44 volt (55, 96).

It was suggested that oxidized P 890 in turn may oxidize a cytochrome (47). Light-induced cytochrome oxidation was observed in various photosynthetic bacteria (44, 47, 28, 109, 113, 101). The quantum requirement of the oxidation of P 890 in intact cells of R. rubrum was about 3 or less. In chromatophores of Chromatium, R. spheroides and R. rubrum the quantum requirements for P 890 bleaching were reported to be of the same order of magnitude, varying from 1.5 to 4.5 (ref. 36). For R. rubrum the quantum requirement of P 890 oxidation is of the same order of magnitude as that reported for cytochrome oxidation and for NAD reduction in this species (3); for the cytochromes C 423.5 and C 422 in Chromatium the quantum requirement was found to be 1.0 or even lower (section 4.3.2). In the green bacterium Cps. ethylicum the quantum requirement of P 840 bleaching (oxidation) could not be measured with good precision, due to the relatively small concentration of P 840. However, the quantum requirement does not appear to differ very much from that found for the oxidation of the cytochrome, which was 2-3 (ref. 113).

The primary act of photosynthesis has been postulated to be the light-induced generation of a photooxidant and a photoreductant (105). The physical mechanism of this reaction is still unknown and has been subject of several speculations (26, 37, 47, 62, 76).

Our results strongly suggest that P 890 in purple bacteria, and possibly P 840 in green bacteria, is the primary reductant in bacterial photosynthesis, which is oxidized (bleached) upon excitation by a reaction which proceeds independently of the temperature, as observed in intact cells at -170°, and in films of dried chromatophores at 1°K (ref. 6). From lifetime studies of bacteriochlorophyll fluorescence in intact cells of Chromatium and R. spheroides at 20° and -196° it has been concluded that at low temperature energy is trapped by a primary photochemical reaction (120, 138). Excitation of the primary reductant P 890 occurs by energy transfer from bacteriochlorophyll B 890 to P 890, presumably by a mechanism of inductive resonance (43). A transfer of this kind was shown to be theoretically possible with good efficiency, if one takes into account the concentration of P 890 and the fluorescence yield of bacteriochlorophyll *in vivo* (43, 46).

The primary photoreductant of bacterial photosynthesis has not been identified as yet. Recently Clayton reported in R. spheroides light-induced absorbancy changes in the infrared region, different from those of P 890, which were ascribed to

a primary photoreduction of bacteriochlorophyll (38). We confirmed Clayton's observation and observed similar absorbancy changes in Chromatium and in the mutant strain EMS 65 of R. spheroides (Vredenberg, Amesz and Duysens (143)). The evidence, however, suggests that the changes were caused by a conformational change of the lipoprotein bearer molecule of bacteriochlorophyll, rather than by a reduction of bacteriochlorophyll (143). We did not observe changes of this kind in R. rubrum.

The observed correlation between fluorescence increase and P 890 bleaching might be explained by a different hypothesis: If the reduced form of the unidentified photoreducant X is present in the same concentration as oxidized P 890, both in the light and in the dark, then equation 2.6 can be derived by assuming that in the dark the (oxidized) photoreducant quenches the fluorescence of bacteriochlorophyll, but that in the light the reduced form does not. However, a priori it is unlikely that the concentration of oxidized P 890 would be the same as that of reduced X under different conditions.

In algae it was found that the fluorescence yield of chlorophyll *a* excited by the photochemical system 2 is controlled by a quencher Q which is reduced on illumination (53); there is also weak evidence that the fluorescence of chlorophyll *a* excited by system 1 is controlled by the photoreductant of the photochemical system 1 (see section 5.3.4).

CHAPTER IV

CYTOCHROME REACTIONS AT ROOM AND AT LOW TEMPERATURE IN PURPLE BACTERIA

4.1 *Introduction*

Cytochromes are oxidized upon illumination in all species of photosynthetic organisms studied (44, 28, 47, 109, 102, 50, 2, 113, 101). It has been shown that in the purple bacterium R. rubrum at least two different cytochromes, C 428 and C 422, are oxidized (47), the first one mainly by low intensity light and the second one in rather strong light. Kinetic experiments indicated that upon illumination of an anaerobic sample of Chromatium at least 4 different cytochromes are oxidized, namely the cytochromes C 423.5, C 426, C 422, and C 430 (ref. 109).

In the preceding chapter evidence was given that in purple and green bacteria photosynthetic energy is transferred from the bulk of the light absorbing pigments to a reaction center P 890 which becomes oxidized upon excitation. The reaction has been shown to be temperature independent. It has been suggested that cytochromes were oxidized directly or indirectly by oxidized P 890 (ref. 47).

Chance and Nishimura made the important observation that in Chromatium upon infrared illumination cytochrome C 423.5 was oxidized at -196° with an efficiency roughly equal to that at room temperature; after darkening the cytochrome remained oxidized (29). Although they did not observe a light-driven oxidation of the other cytochromes in Chromatium and of the cytochromes in R. rubrum at -196°, the authors suggested that also for these cytochromes photooxidation would have taken place at this temperature if the cytochromes would have been in the reduced state (29, 30); they believed to have indications that in R. rubrum the cytochromes became oxidized in the dark upon cooling to -196° and thus could not be oxidized by light (30, 106). They suggested that generally in bacterial photosynthesis cytochrome oxidation is a temperature independent photoreaction (29, 30).

In this chapter it will be shown that the photooxidation of all cytochromes in various purple bacteria studied so far, except C 423.5 in Chromatium, is stopped on lowering the temperature, while no dark oxidation takes place on cooling. The temperature at which the photooxidation stops is different for the various cytochromes. For cytochrome C 422 in Chromatium this temperature was between -110 and -120°, for the other cytochromes it was between -25° (C 422 in R. rubrum) and -50° (C 420 in R. spheroides).

4.2 *Methods*

Measurements of absorbancy changes were carried out in the split-beam difference spectrophotometer, equipped with the low temperature cell compartment (see 2.3.2). For recording the spectra of the difference in absorbancy of the reference and the measuring cuvette we used the device described in 2.3.1.

4.3 *Results and Interpretation*

4.3.1 Cytochrome oxidation in Chromatium between 20 and -210°

Photooxidation of cytochrome C 423.5 was studied in anaerobic samples at 20, -170 and -210°. The results of Chance and Nishimura (29) on the efficient photooxidation of C 423.5 at -170° were confirmed, but only for low intensities of actinic light (140). We found that the apparent initial rate of oxidation at -170° caused by a high intensity of actinic light was lower than at 20° and, for the same intensity, was still lower at -210° (ref. 140). These results suggested that upon lowering the temperature the photooxidation of C 423.5 was saturated at lower intensities of actinic light. The experiments at -170° were extended to higher light intensities than those used before. For these experiments the recording apparatus was adjusted at a response time of 0.1 sec. Fig.4.1 shows that the efficiency for the oxidation of C 423.5 at intensi-

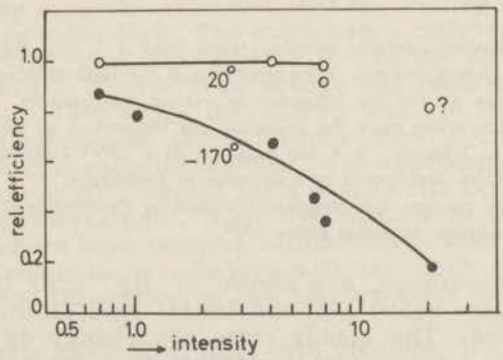

Figure 4.1 The relative efficiency for cytochrome C 423.5 oxidation in Chromatium at 20° and -170°, plotted as a function of the intensity of actinic light of 590 mμ. At each intensity the efficiency is calculated by dividing the initial rate of the absorbancy change at 423.5 mμ by the intensity of the actinic light (expressed in 10^{-10} einstein/cm^2 sec). The measurements at 20° were done in the same sample. At intensities above 10^{-9} einstein/cm^2 sec the reaction at 20° occurred within the response time of the recorder. The efficiencies at -170° are given as the ratio of reaction rates at -170° and 20° and were determined at each intensity in a fresh sample. The figure shows that at higher intensities the efficiency for the oxidation at -170° decreases.

ties above 10^{-10} einstein/cm^2 sec is appreciably lower at -170° than at 20°. However, the rate of the reaction at -170° did not appear to be light saturated at the highest intensity used. Consistent with earlier suggestions (140), the results indicate that the rate of oxidation of C 423.5 is dependent on the temperature, although the mechanism of the reaction cannot satisfactorily be understood from the results obtained so far.

Photooxidation of the other cytochromes in Chromatium was not observed at -170° (29, 140). At room temperature these cytochromes were oxidized at high light intensity. By aeration in the dark, according to Olson (109), C 423.5 and C 426 are oxidized, but not C 422 and C 430.

The kinetics of C 422 photooxidation were studied at various temperatures in aerobic samples. Time courses of the light-induced spectral changes at 422 mμ, measured in growth medium at

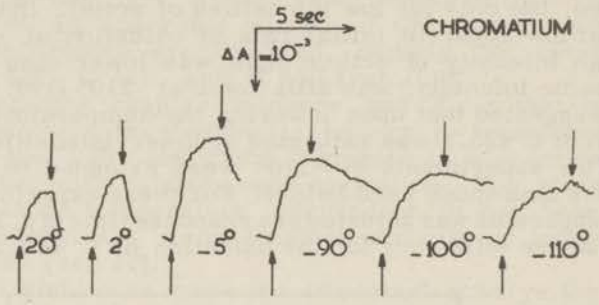

Figure 4.2 Chromatium, aerobic sample, taken from a 2 days old culture, suspended in fresh growth medium. Time recordings of the light-induced reversible absorbancy changes at 422 mμ measured at various temperatures. Upward and downward pointing arrows mark the beginning and the end of an illumination period. The actinic intensity, λ = 840 mμ, was 23 x 10^{-11} einstein/cm^2 sec. An upward deflection corresponds to a decrease in absorbancy.

The changes are presumably caused by cytochrome C 422. The initial rate of oxidation decreases below -90°.

various temperatures, are shown in fig. 4.2. Upon decreasing the temperature below -75° the initial rate of the photooxidation of C 422 drops. The steady state absorbancy difference ΔA also decreases, but to a smaller extent than the initial rate. This is shown more directly in fig. 4.3, in which the initial rate and the steady state absorbancy difference, measured at high intensity, are plotted on a relative scale as a function of the temperature. This figure indicates that below -75° the initial rate of C 422 photooxidation is limited by a "dark reaction". This means that C 422 oxidation is temperature-dependent. At temperatures below -110° the rate of C 422 oxidation becomes unobservably low. In the glycerol-glycerophosphate medium (section 2.2) the rate of the dark reduction of C 422 at 20° was much lower than in growth medium, whereas the rate of photooxidation was higher.

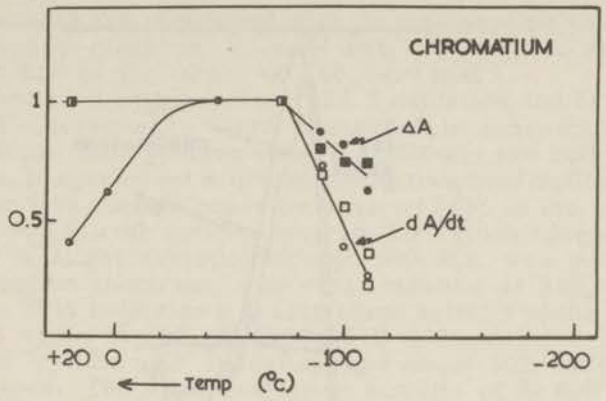

Figure 4.3 Chromatium, different aerobic samples, taken from 2 days old cultures, suspended in fresh growth medium (circles), respectively in "non-crystallizing medium" (squares). The steady state absorbancy difference (black symbols) and the initial rate of the absorbancy change at 422 mμ are plotted as a function of the temperature (°C). Coinciding values are represented by half-blackened symbols. Values for the absorbancy change and the initial rate are given in relative units, the maximum value being put equal to 1. Actinic intensity (λ = 840 mμ) was 23 x 10^{-11} einstein/cm^2 sec.

Upon lowering the temperature the initial rate of C 422 oxidation decreases more rapidly than the steady state change.

The following experiment shows that on cooling C 422 did not become oxidized in the dark. The absorbancy difference spectrum, light minus dark, was recorded at 20° in the non-crystallizing medium (fig. 4.4). Then both reference and sample cuvette were cooled down to -170°, while the sample cuvette remained continuously illuminated and the reference cuvette remained in the dark. The spectrum recorded at -170° was approximately the same as that obtained at 20°, except for a 2 mμ shift of the absorption band to shorter wavelength. If the cytochrome in the dark reference cuvette would have become oxidized upon cooling, the recording at -170° would have coincided with the dark reference line, since then the absorption in the two cuvettes would have been the same.

Using bacteria suspended in growth medium, we obtained similar results. The spectra at -170° were, however, less precise due to increased scattering caused by cracks or microcrystals of the frozen suspension.

4.3.2 Quantum requirements and action spectrum of cytochrome oxidation in Chromatium

Only for one cytochrome, C 423.5, the quantum requirement has been determined with some precision. In his most recent experiments, Olson (110) calculated a quantum requirement from

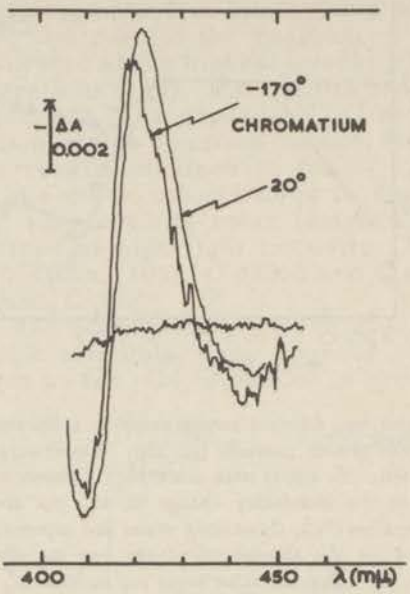

Figure 4.4 Chromatium aerobic sample suspended in the "non-crystallizing" medium. The difference in absorption at 20° and -170° between two suspensions, of which one was continuously illuminated (also during cooling) and the other was kept in the dark, is recorded as a function of the wavelength. The reference line is the tracing at 20°, which is recorded when both suspensions are in the dark. The intensity of the actinic light of 840 mμ was 7.2×10^{-11} einstein/cm^2sec.

the initial rate of oxidation varying from 0.7 to 1.4 for light of 860-890 mμ, assuming a change in specific extinction of 62.3 $cm^{-1}\ mM^{-1}$, which was based on estimates of the specific extinction of a cytochrome extracted from Chromatium (10). Using the same specific absorbancies, we calculated from our measurements in anaerobic samples, taken from various cultures, requirements for C 423.5 oxidation varying from 0.7 to 0.9, for light of 840 mμ. The requirement for C 422 oxidation, measured in the aerobic "non-crystallizing medium", was calculated from measurements at 20° and was found to be about 0.6. The quantum requirement for C 422 oxidation in bacteria suspended in aerobic complete medium was established from the initial rate of the absorbancy change occurring upon illumination at -45°. At this temperature the reduction of oxidized C 422 upon darkening was rather slow, and the initial rate of the oxidation was optimal. It was calculated that about 0.75 quanta per electron were needed.

The assumed specific absorbancies for the cytochromes are uncertain, because of uncertain identification (10). Therefore it cannot be concluded that the quantum requirements for the oxidation of these cytochromes are smaller than 1. It is likely, however, that the photooxidations are very efficient reactions.

The experiments are consistent with the assumption that the requirements are close to 1, and that the specific absorbancy differences are of the order of 100 $cm^{-1}\,mM^{-1}$.

Action spectra of cytochrome C 423.5 oxidation and of bacteriochlorophyll fluorescence were measured in anaerobic samples of Chromatium. Both spectra showed relatively low activity of the carotenoids, in agreement with earlier observations on fluorescence efficiency in this species made by Duysens (43). In the wavelength region 580-950 mμ the action spectrum for cytochrome oxidation, except for a slight deviation around 800 mμ, was proportional to the absorption spectrum, with clear maxima at 590, 800, 850, and 890 mμ. This indicates a nearly equal activity of the bacteriochlorophyll types B 800, B 850 and B 890; only for B 800 the activity for cytochrome oxidation was about 20% less than for the other types. The apparent lower activity of B 800 might be due to an inhomogeneity of the culture which may have contained old, less active, cells with a relatively high absorption at 800 mμ. Centrifugation of the culture yielded fractions with different infrared absorption spectra. The action spectrum of bacteriochlorophyll fluorescence showed a complete proportionality to the absorption spectrum in the wavelength region 580-820 mμ. The fluorescence experiments could not be extended to wavelengths above 820 mμ, because of spurious effects of the actinic light (section 2.5.2).

4.3.3 Cytochrome oxidation in Rhodopseudomonas spheroides and Rhodospirillum rubrum

Fig. 4.5 shows the kinetics of light-induced cytochrome oxidation in R. spheroides at various temperatures. The absorban-

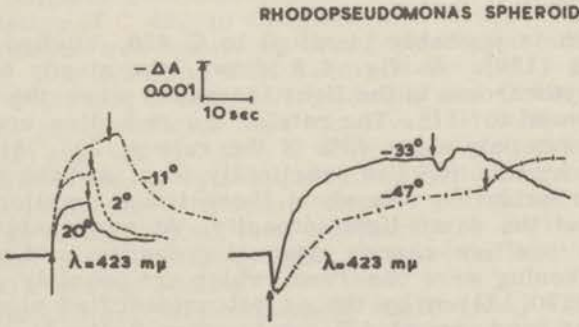

Figure 4.5 Rhodopseudomonas spheroides, aerobic sample, suspended in non-crystallizing medium; time recordings of the light-induced absorbancy changes at 423 mμ, measured at various temperatures. The intensity of the actinic light (λ = 860 mμ) was 1.5×10^{-9} einstein/cm^2 sec. The rate of reduction of the cytochrome upon darkening decreases upon lowering the temperature.

cy difference spectra at 20° (fig. 4.6), measured at different intensities of actinic light, indicate the photooxidation of a cyto-

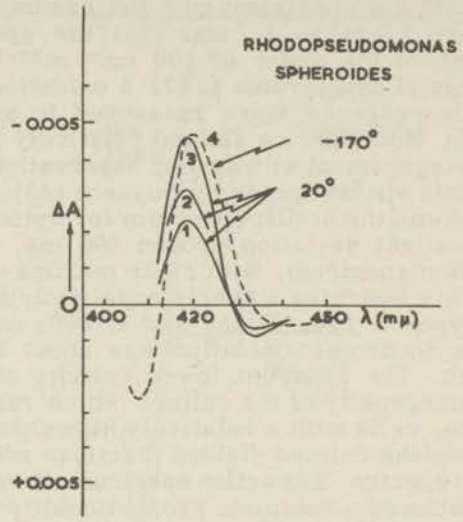

Figure 4.6 Rhodopseudomonas spheroides, aerobic sample, taken from a 3 days old culture, suspended in non-crystallizing medium. The solid lines, indicated by the numbers 1, 2 and 3, are the absorbancy difference spectra light minus dark measured at the relative intensities of actinic light 50, 100 resp. 300 (arbitrary units). The dashed line is the absorbancy difference spectrum measured of two samples at -170° of which one was continuously illuminated during cooling with a relative intensity of 50 and the other was kept in the dark. The spectra were redrawn from recordings as shown in fig. 4.4 and corrected for a small bending of the dark zero lines.

chrome which is probably identical to C 420, studied by Smith and Ramirez (130). As fig. 4.5 shows, the steady state level of oxidized cytochrome in the light increased when the temperature was lowered to -11°. The rate of the reduction upon darkening at -11° was only about 40% of the rate at 20°. At -47° the rate of the dark reduction was practically zero, and the rate of the light-induced oxidation was about three times smaller than the rate at 20° at the same light intensity. At temperatures below -11° also a positive change upon illumination and a negative one upon darkening were observed, which are possibly caused by carotenoids (130, 131) or by the as yet unidentified pigment with the absorption band around 435 mμ (section 3.4). At -170° only this positive reversible change in absorbancy was observed. At -170°, C 420 was in the reduced state in the dark, as indicated by the difference spectrum at -170° of two frozen suspensions from which one was kept in the dark and the other was continuously illuminated during cooling. Except for a 2 mμ shift of the maximum, this spectrum resembled both in shape and height the difference spectrum at 20°. At the intensity used, the positive change was too small to distort the spectrum at -170° appreciably (see fig. 4.6).

In Rhodospirillum rubrum we obtained similar results. C 248 could be oxidized down to about -50°. Upon addition of 3 x 10^{-6} M HOQNO, which inhibits cyclic photophosphorylation in extracts (9), the photooxidation of C 428 was inhibited and another cytochrome, C 422, became oxidized upon illumination, in agreement with results of others (129). In the presence of HOQNO, photooxidation of C 422 was observed down to about -25°. After cooling in the dark to -170° C 428, and possibly C 422 also, were in the reduced state, as was indicated by the difference spectrum at -170° measured by the method mentioned above. Only a positive change in absorbancy was observed upon illumination at -170° with a spectrum similar to that found in washed aerobic cells at 20° (ref. 47) that was ascribed to an as yet unknown pigment (section 3.4).

4.4 *Discussion*

The results reported in this chapter show, contrarily to Chance's suggestion (29, 33), that in bacterial photosynthesis cytochrome photooxidation is not a primary temperature insensitive reaction. This suggestion was mainly based on the observation that in Chromatium cytochrome C 423.5 was oxidized at -196° with an efficiency that was the same as at 20° (29). In our experiments this observation was confirmed, but only for low intensities of actinic light. Our data indicate that for higher intensities the efficiency for the photooxidation at -170° is lower than at 20° and becomes still lower upon a further lowering of the temperature to -210°. The limitation of the rate of oxidation at decreasing temperatures was demonstrated more precisely for the photooxidation of C 422 in Chromatium. Below -75° the rate of C 422 oxidation was saturated at a lower light intensity than at 20°. No C 422 oxidation still occurred at -120°. For the cytochromes C 422 and C 428 in R. rubrum and C 420 in R. spheroides no photooxidation was observed at temperatures below -25° (C 422) and -50° (C 420, C 428). As reported (section 3.5.), the photooxidation of cytochrome C 425 in the green bacterium Chloropseudomonas ethylicum stopped at temperatures below 50°. It was further shown that, contrarily to earlier suggestions (29, 30, 106), the bacterial cytochromes are not oxidized when kept in the dark during cooling.

The cytochrome reactions appear qualitatively similar to "conventional" dark reactions, with the difference that for cytochromes the temperature has to be much lower in general, before a limitation of the reaction rate becomes apparent. The occurrence of these reactions at relatively low temperatures suggests that the cytochromes are located very close, if not adjacent, to the primary oxidant P 890. Between such closely connected or adjacent molecules electron or hydrogen transfer may occur even at low temperatures (126). However, direct evidence for the mechanism of cytochrome oxidation is not available at present.

Recently Chance and DeVault reported that high intensity monochromatic light flashes (694 mμ) of 100 nsec duration brought about changes in absorbancy at 422 mμ in anaerobic samples of Chromatium, which were attributed to a photooxidation of cytochrome C 423.5 (ref. 33). The changes at 20° occurred within 20 μsec and the authors stated: "..... our results at temperatures of liquid nitrogen suggest that no decrease in the rate occurs ..." (33). However, the conclusion that at liquid air temperature the oxidation goes with an equal efficiency as at room temperature (33) contradicts our observation on the relatively low efficiency of high intensity light for causing C 423.5 oxidation at -170°.

Because of the rapid accumulation of oxidized C 423.5 in Chromatium upon illumination, and because C 423.5 accumulates at intensities at which no accumulation of P 890 is observed, it was postulated that C 423.5, instead of P 890, is the primary reductant of photosynthesis in Chromatium (32, 33). However, for a primary photoproduct of photosynthesis which reacts in the main electron transport chain one may predict a high turn-over rate at high light intensities. The low rate of reduction of oxidized C 423.5 in the dark under all conditions applied so far suggests therefore that C 423.5 is not in the main electron transport chain of photosynthesis. On the contrary, it was found that for P 890 the turn-over rate was high (e.g. fig. 3.1), which supports the hypothesis that P 890 is the primary photooxidant. Moreover, a high turn-over rate of the primary photoreactant of photosynthesis includes the possibility to observe an accumulation of the dark products of photosynthesis (e.g. cytochromes, NADH) without observing any accumulation of the primary reactant(s) (e.g. P 890). This point sometimes has been overlooked in the interpretation of experimental data on the presumed primary photooxidation of cytochrome C 423.5 (ref. 32, 33).

The possibility cannot be excluded that in the intact cell, as a consequence of the cooling procedure, a structural change occurs in the configuration of the protein bearer molecule, to which P 890 and cytochrome may be attached, in such a way that no electron or hydrogen transfer can take place between these molecules at low temperatures. However, the observation that after warming up of the frozen samples the cytochrome photoreactions at 20° were restored indicates that at least no permanent damage was effected.

The low quantum requirement of cytochrome oxidation, which for C 423.5 and C 422 in Chromatium was close to 1, and which for C 428 in R. rubrum was reported to be 3-4 (ref. 3), indicates that these reactions are efficient photosynthetic processes. In the preceding chapter the quantum requirement of P 890 oxidation was reported to be of the same order of magnitude. These findings are in agreement with the hypothesis that cytochromes are oxidized by P 890. The light and dark kinetics of the absorbancy changes in an aqueous suspension of Chromatium chromatophores, ascribed to P 890 and cytochrome oxidation, were consistent with the hypothesis mentioned above (37).

The action spectra of cytochrome oxidation and of bacteriochlorophyll fluorescence were found to be nearly proportional, except for a slight deviation at 800 mμ. In R. spheroides the observed proportionality between the action spectra of NAD reduction and of bacteriochlorophyll fluorescence was interpreted as evidence that in bacterial photosynthesis only one pigment system is responsible for the light reactions (3). Additional evidence was given by Blinks and van Niel (13).

Recently it was suggested (38) that in purple bacteria two photochemical reactions function, of which one activates a primary oxidation of cytochrome and a reduction of bacteriochlorophyll, and the other brings about a primary oxidation of P 890 and a reduction of ubiquinone. As discussed in this chapter and in the preceding one, our results give no support for a primary photooxidation of cytochrome or for the suggested photoreduction of bacteriochlorophyll.

CHAPTER V

PRIMARY PHOTOCHEMICAL REACTIONS IN ALGAE

5.1 *Introduction*

It is well established that algal photosynthesis is driven by two different photochemical reactions (50, 51, 86, 150), photoreactions 1 and 2 (ref. 51), which are sensitized by the pigment systems 1 and 2. Experiments on light-induced changes in absorbancy (50, 51, 86, 150, 2, 5) and in fluorescence (52, 53) in algae have given evidence for the following (simplified) scheme (55):

NADP ← X ← (system 1) ← P 700 ← cytochrome ← PQ ← Q ← (system 2) ← ZH ← H_2O.

Biochemical experiments also have supported the evidence for this scheme (99). The arrows indicate the direction of hydrogen or electron transport. Phosphorylation reactions have been omitted. NADP is nicotinamide-adenine dinucleotide phosphate (2), P 700 is the primary reductant of photoreaction 1, which upon illumination shows a decrease in absorption around 705 mμ (83, 87, 89), PQ is a plastoquinone (5), Q is the primary oxidant of system 2 which quenches the fluorescence of chlorophyll a_2 (ref. 53), and X and ZH are the hypothetical primary oxidant of reaction 1 and the reductant of reaction 2.

The two photochemical reactions have different action spectra (51). Light of such a wavelength that it is mainly absorbed by system 1 is called light 1, light 2 is analogously defined. In blue-green and red algae, red and blue light in general is light 1, and green or orange light, which is absorbed mainly by the phycobilins, is light 2. In the absence of poisons light 1 causes an oxidation of the redox couples located between the systems 1 and 2, and light 2 a reduction. DCMU, which was known as a potent inhibitor of the Hill reaction (148) and of photosynthesis (12), was found to inhibit the electron transport chain probably between plastoquinone and Q (53, 5).

The scheme, as well as the evidence for it, is not complete since it cannot easily explain all aspects of the light and dark kinetics of certain intermediates. Further details will be discussed in section 5.4.

In this chapter experiments are described on the photoreactions of the pigment P 700 and of cytochrome, and on the chlorophyll fluorescence in the near infrared wavelength region. The experiments were done mainly with intact cells of the blue-green algae Schizothrix calcicola and Anacystis nidulans at various temperatures and at different regimes of pre-illumination, and also in the presence of various inhibitors. Quantum requirements were determined for the photooxidation of P 700 and cytochrome

and (for the first time) also for the photoreduction of oxidized P 700.

Although many papers have been published about red and near infrared fluorescence attributed to various types of chlorophyll (42, 43, 52, 53, 21, 22, 97, 17, 18, 66, 67, 68, 91, 24, 119, 115), *changes* in fluorescence of chlorophyll a_1, which is the chlorophyll *a* belonging to the pigment system 1, have not been studied in too much detail. We will describe experiments on the (relatively) small infrared fluorescence changes of a pigment, called H 720, which is probably a chlorophyll *a*-like pigment excited by the photochemical system 1.

5.2 *Methods*

Absorbancy measurements were carried out in the split-beam difference spectrophotometer, when necessary, equipped with the low temperature cell compartment described in 2.3.2. Fluorescence measurements were done in the fluorescence apparatus, equipped with the attachment for measuring fluorescence in a flowing suspension described in 2.4. In addition to a greater precision attainable, a further advantage of this attachment was the possibility of studying the kinetics of the fluorescence from 0.005 sec onwards after the application of an actinic flash. In experiments in which the kinetics of the fluorescence decay in the dark after an actinic illumination, or the relative activity of one or more actinic illuminations of different wavelengths were studied, the "single" flow cuvette was used. In these experiments the excitation light was provided by a 500 W projector with suitable filter combinations to isolate narrow wavelength regions for the excitation light. In the experiments in which the emission spectra of fluorescence changes were measured the "double flow" cuvette was used. In these experiments the excitation light was provided by a monochromator, equipped with a Hg lamp. The fluorescence emission was analyzed by a second monochromator and detected by an EMI 9558 QC photomultiplier. The spectra were recorded at a slidwidth of the analyzing monochromator which corresponded to a bandwidth of about 3-5 mμ.

5.3 *Results and Interpretation*

5.3.1 Kinetics, action spectrum and quantum requirement of P 700 and cytochrome oxidation

The spectrum of the difference in absorption of the steady states in light and darkness in Schizothrix calcicola shows (fig. 5.1), in addition to the main peak at 420 mμ which indicates the oxidation of a cytochrome, a shoulder around 435 mμ. This shoulder has also been found in Anacystis nidulans (4) (see also

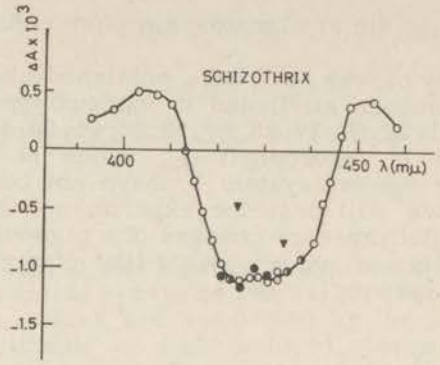

Figure 5.1 Difference spectrum, light minus dark, of the steady state absorption at 2^{o} for a suspension of Schizothrix, taken from a 3 days old culture, in the presence of 1.1×10^{-6} M DCMU. The open circles were measured for actinic light of 680 mμ and 2.8×10^{-10} einstein/cm^2sec, and the solid ones for actinic light of 560 mμ and 6×10^{-10} einstein/cm^2sec. The triangles represent the changes at -170^{o} in actinic light of 680 mμ.

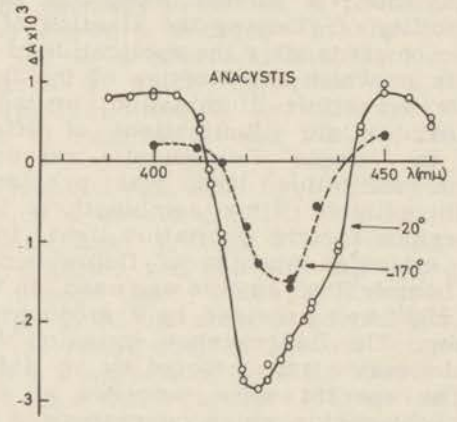

Figure 5.2 Difference spectra, light minus dark, of the absorbancy changes, ΔA, for a suspension of Anacystis, taken from a 2 days old culture. The spectrum of the reversible changes at 20^{o} (open circles) was measured for one sample. At -170^{o} the back reaction upon darkening was slow and perhaps not completely reversible. The changes in absorbancy occurring upon first illumination at -170^{o}, ΔA (-170), was taken from the time course. At each wavelength ΔA (20) and ΔA (-170) were measured for a fresh sample. The difference spectrum at -170^{o} was obtained by multiplying at each wavelength the ratio ΔA(-170)/ΔA(20) with the difference spectrum at 20^{o}. The intensity of the actinic light of a wavelength band around 680 mμ was 4×10^{-10} einstein/cm^2sec. The figure shows that at -170^{o} less (if any) cytochrome than at 20^{o} is oxidized upon illumination, but that the absorbancy changes with a peak around 430 mμ still occur.

fig. 5.2) and in other blue green algae (84). It has been shown that in an acetone extracted suspension of chloroplasts particles a photooxidation of P 700 occurs, which gives rise to the disappearance of absorption bands around 700 and 432 mμ (85). We found that for Schizothrix at 2°, in the presence of DCMU, the half times of the light-off reactions at 435 and 705 mμ after turning off the actinic light were the same and about 2-2.5 times smaller than that at 420 mμ. The estimated half times at the conditions used were found to be independent of the concentration of light oxidized P 700 and of the wavelength of the actinic light. Absorbancy difference spectra of Anacystis are shown in figs. 5.2 and 5.3. The spectra show that at -170° light causes absorbancy changes with maxima around 435 and

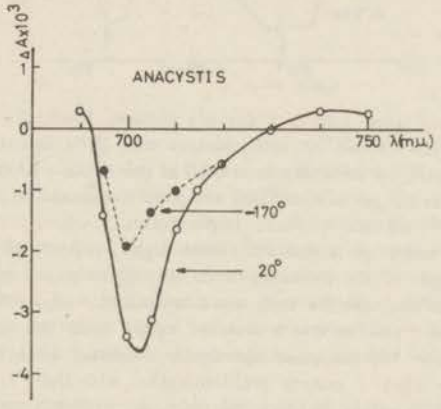

Figure 5.3 Difference spectra, light minus dark, of the absorbancy changes in the near infrared for a suspension of Anacystis, taken from a 3 days old culture. The spectrum of the reversible changes at 20° is given by the curve through the open circles. The spectrum at -170° (solid symbols) represents the steady state absorbancy changes occurring after a number of light and dark intervals. The absorbancy change upon first illumination was much greater but was not completely reversible. The spectrum of the changes which were reversible is plotted. The intensity of the actinic light of a wavelength band around 430 mμ was 5×10^{-10} einstein/cm^{2}sec. The data show that at -170° P 700 is oxidized upon illumination.

705 mμ. These experiments suggest that at -170° the cytochrome is not photooxidized (or to a lesser extend), and support the proposal (85, 124, 151, 81) that the absorbancy change at 435 mμ is caused by or is closely associated with the photoconversion of P 700.

Fig. 5.4 shows the kinetics of cytochrome oxidation and reduction at 20°, occurring upon switching from light 2 or from darkness to light 1, and after switching from light 1 to darkness or to light 2. The initial rate of cytochrome oxidation in red actinic light (light 1) is lower after green light (light 2) than shortly after red light, but the rate of the oxidation after a

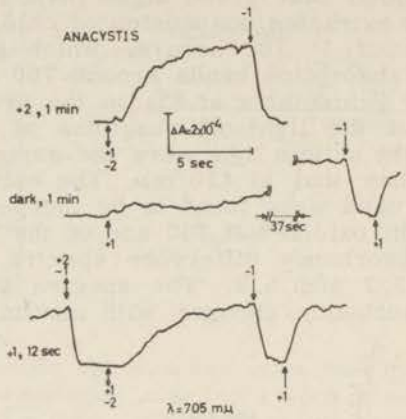

Figure 5.4 Anacystis, taken from a 1 day old culture. Kinetics of the absorbancy changes at 705 mμ caused by an oxidation of P 700. Upward and downward pointing arrows mark the switching on and off of the actinic illuminations 1 and 2, which are of wavelength bands around 430 and 560 mμ and of intensities 8×10^{-10} and 6×10^{-10} einstein/cm^{2}sec, respectively.

Upon onset of illumination with light 1, 2 sec after an illumination period with light 1, the oxidation starts immediately and at a high rate, however if instead of darkness the cells are illuminated with light 2 during 2 sec, a delay and a much smaller rate is observed for the oxidation in light 1. After 1 minute darkness P 700 becomes but slowly oxidized after about 40 sec in light 1, however after 1 minute preillumination with light 2, P 700 becomes oxidized in light 1 within a much shorter time, although there is a pronounced delay after the onset of the illumination.

relatively long dark period is still lower. Similar time courses were found for the absorbancy changes at 420 mμ. At 2° the effects were much less pronounced, and the initial rate of the light-on reaction after preillumination with light 1 was about 3 times higher than at 20°, when measured under the same conditions. These results suggest that there is a pool which reduces oxidized P 700 and cytochrome in a dark reaction, which is slowed down upon lowering the temperature. In light 1 the pool is oxidized and in light 2 it is reduced, which suggests that the pool reacts in between the two pigment systems.

It has been shown that the photooxidation of cytochrome and P 700 by light 1 is reversed by additional illumination with light 2 (50, 142). The reduction by light of system 2 was demonstrated to occur via a dark reaction, which is slowed down by a lowering of the temperature (142).

Fig. 5.5 shows the action spectrum of P 700 oxidation in Schizothrix, as measured by the absorbancy changes at 705 and 435 mμ in the presence of 1.1×10^{-6} M DCMU. The relative heights of the bands at 680, 620 and 560 mμ suggest that the spectrum is proportional to that of pigment system 1.

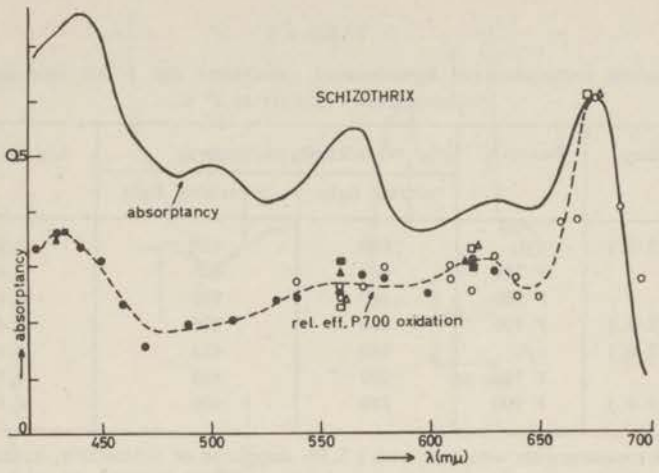

Figure 5.5 Spectrum of the relative activities of light of different wavelengths in initiating P 700 oxidation in a suspension of intact cells of Schizothrix, taken from a 2 days old culture, in the presence of 1.1×10^{-6} M DCMU. The open and solid circles represent the activities of actinic light in initiating absorbancy changes at 435 and 705 mμ, respectively. The action spectrum with open circles was arbitrarily adjusted to the same height as the absorbancy spectrum at 680 mμ, and the spectrum of the open circles was made to coincide at 620 mμ with that of the solid ones. In the same figure relative activities for P 700 oxidation in other samples of different cultures are represented by squares and triangles.

Results of measurements of the quantum requirements of P 700 and cytochrome oxidation are summarized in Table 5.1. The change in the molar extinction coefficient of P 700 at 705 mμ was assumed to be equal to that of chlorophyll *a*, which at 675 mμ is 80 $cm^{-1}\,mM^{-1}$, (ref. 118 pg. 606), and at 435 mμ was taken to be about 40 $cm^{-1}\,mM^{-1}$, which was based on the observation that at saturating actinic intensities of light of 560 mμ, in the presence of DCMU, the steady state absorbancy difference at 435 mμ was about one half of that at 705 mμ. This is in accordance with the spectra reported by Kok (85) and Witt (152, 123). In Anacystis the quantum requirement of cytochrome oxidation at 2° was calculated to be 2.7, whereas at 20° it was found to be 8. The latter value is in the same range as those reported by Amesz and Duysens (2).

5.3.2 Quantum requirement for the photoreduction of P 700

Fig. 5.6 shows that in the presence of 10^{-4} M PMA P 700 in Schizothrix is in the oxidized state in the dark. This oxidized P 700 could be photoreduced by light 2 and oxidized again by additional light 1. PMA had a similar effect upon the absorbancy changes in the blue wavelength region. These experiments show

TABLE 5.1

Quantum requirements of light-induced cytochrome and P 700 oxidation in Schizothrix calcicola at 2° C.

Culture	Reactant	Wavelength, λ (mμ)		1/φ (hν/eq.)
		actinic light	measuring light	
1 (3 d.)	cyt.	680	423	3.5
1	P 700	680	435	2.0
1	P 700	430	705	5.6
2 (2 d.)	P 700	430	705	6.4
3 (2 d.)	cyt.	680	423	3.0
3	P 700	680	435	2.2
4 (2 d.)	P 700	680	435	2.6

The measurements were done with a 1.5% suspension of Schizothrix, contained in a 1 mm vessel, in the presence of 1.1×10^{-6} M DCMU, and carried out at 2°. The initial rates of the absorbancy changes upon actinic illumination were plotted as a function of the intensity. The quantum requirements were calculated for the regions of low intensities in which the initial rate was approximately proportional to the intensity. The difference in specific extinction between oxidized and reduced cytochrome at 423 mμ was assumed to be 70 cm^{-1} mM; the molar extinction coëfficient of P 700 at 705 and 435 mμ was assumed to be 80 respectively 40 cm^{-1} mM^{-1} (see text). For the calculations corrections were applied for the relatively weak scattering of the suspension, but not for the flattening of the absorbancy spectrum.

that PMA does not markedly affect the functioning and interaction of the two pigment systems, but causes a shift of the oxidation reduction level of P 700 and cytochrome towards the oxidized state. In Anacystis PMA had the same effect.

The quantum requirement of the photoreduction of dark-oxidized P 700 by light 2 (560 mμ) was estimated in intact cells of Schizothrix in the presence of 10^{-4} M PMA. On basis of the assumptions discussed in the previous section, this quantum requirement was estimated to be about 3 from the initial rate of the increase in absorbancy at 435 mμ upon illumination with light of 560 mμ. Since part of the energy incident at 560 mμ is absorbed by system 1 and thus is used for photooxidation, the requirement for reduction by quanta absorbed by system 2 is lower than 3.

5.3.3 Kinetics and spectra of changes in the near infrared fluorescence in Schizothrix calcicola

Light-induced changes in the fluorescence spectrum in the red and near infrared wavelength region were measured in Schizothrix by means of the differential flow apparatus (see 2.4). A fluorescence difference spectrum will be denoted as "spectrum (n, x-f)". This should be read as follows: The spectrum gives

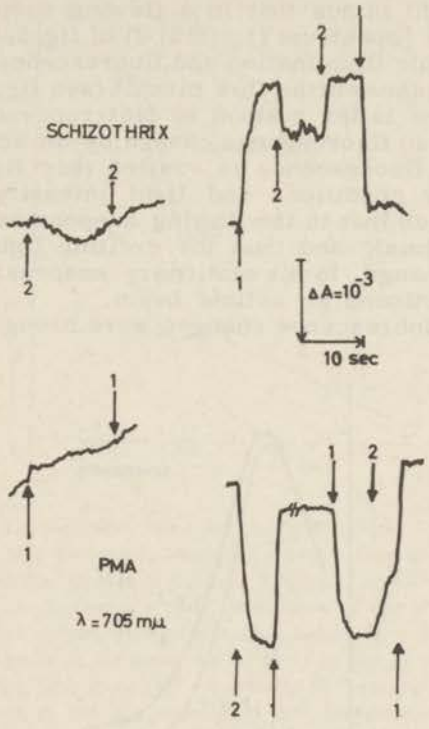

Figure 5.6 Schizothrix, taken from a 2 days old culture. Recorder tracings of the light-induced absorbancy changes at 705 mμ in an unpoisoned suspension (upper part of the figure), and in a suspension with 10^{-4} M PMA (lower part). An upward deflection indicates an oxidation of P 700, a downward deflection means a reduction. The meaning of the symbols is the same as in figure 5.4. The intensities of the actinic light of 430 (1) and 560 mμ (2) were 12 x 10^{-10} respectively 3.1 x 10^{-10} einstein/cm^2 sec. The tracings show that in the presence of PMA, but not in its absence, P 700 is in the oxidized state in the dark; reduction occurs by light 2 and oxidation by light 1.

the difference in fluorescence of two states x and f of the suspension, in exciting light n (light 1 or light 2), as a function of the wavelength. The state f denotes a flowing suspension without actinic illumination, the state x denotes either a stationary suspension without additional actinic illumination (s), or a flowing suspension with actinic illumination [(f+1) or (f+2)]. Spectra of changes in fluorescence, brought about by light 1 (430 mμ) or by light 2 (560 mμ), were measured by recording as a function of the wavelength the difference in fluorescence of a stationary and a flowing suspension [spectra (1, s-f) and (2, s-f)], or the difference in fluorescence emission, excited by light 1 (or light 2), in a flowing suspension which was illuminated

with actinic light minus that in a flowing suspension which was kept in the dark [spectrum (1, (f+2)-f) of fig. 5.8]. In the flowing suspension actinic illumination and fluorescence excitation occurred at different places in the flow circuit (see fig. 2.3). A spectrum measured by the latter method in fact represents the spectrum of the increase in fluorescence caused by an actinic flash, given shortly before fluorescence is excited (see fig. 2.3). In all experiments flow conditions and light intensity of exciting light were chosen such that in the flowing suspension the fluorescence yield was minimal, and thus the exciting light did not cause a fluorescence change. In the stationary suspension the measuring beam also functioned as actinic beam.

Since these fluorescence changes were brought about by actinic

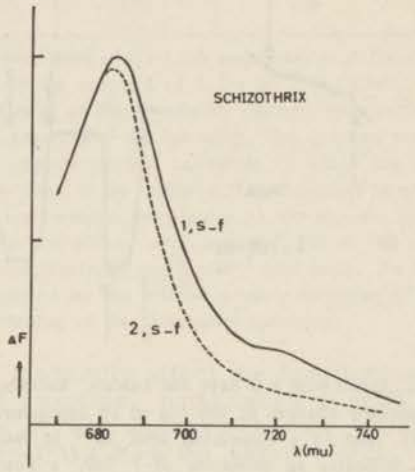

Figure 5.7 Schizothrix, sample taken from a 3 days old culture. Measurements with the differential flow cuvette, in which in one compartment the suspension was stationary, (s-f). Only illumination with exciting light was used; the time of excitation in the flow cuvette was 0.004 sec. Difference spectrum (1,s-f) is for exciting light of 436 mμ of an intensity of the order of 10^{-9} einstein/cm^2sec, and spectrum (2,s-f) for exciting light of 547 mμ of an intensity which was about 2.5 times lower than that of the blue light. The intensity of light 2 was adjusted so that the spectra were made to coincide at 685 mμ. For curve 1 the fractional increase in fluorescence at 685 mμ was about 25%, for curve 2 this was about 15%. The difference spectra (and those of figure 5.8) are as recorded and thus not corrected for the wavelength dependent sensitivity of the photomultiplier and of the monochromator. Neither was a correction applied for the self-absorption which, around 685 mμ, may have lowered somewhat tracing 2 with respect to tracing 1. Nevertheless there is a distinct difference in shape between these two spectra, which shows that at least two different substances are present, which cause the changes in fluorescence. In light 1 the fluorescence increase at 720 mμ relative to that at 685 mμ is more than twice as high as in light 2.

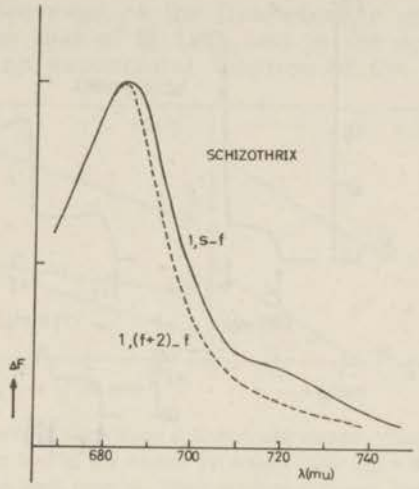

Figure 5.8 Schizothrix, sample taken from a 3 days old culture. Fluorescence difference spectra, for blue excitation, which are made to coincide at 685 mμ. Tracing (1, s-f) is similar to that of fig. 5.7. Tracing (1, (f+2)-f) is the spectrum of the increase in fluorescence upon illumination of one of the compartments of the differential cuvette with green actinic light; the suspension was flowing in both compartments at the same rate of flow, t_e and t_d were 0.04 sec, t_a was 0.4 sec. I_a and I_e were about 10^{-8} respectively 10^{-9} einstein/cm^{2}sec. The changes in fluorescence at 685 mμ were about 30% for the solid curve, and 20% for the broken one. The fluorescence increase in the flowing suspension is apparently little affected by the short flash of exciting light 1. The figure shows that also in blue excitation green actinic light activates relatively less the fluorescence around 720 mμ than blue actinic light.

illumination with light 2 (560 mμ), the spectra (2, s-f) of fig. 5.7 and (1, (f+2)-f) of fig. 5.8 are predominantly due to chlorophyll a_2: the first one is that for green, the second one for blue exciting light. However, we found that the much smaller changes in fluorescence occurring upon blue actinic illumination with blue exciting light had a distinctly different spectrum (tracings (1, s-f) of fig. 5.7 and 5.8). As shown by Duysens and Sweers (53), blue light largely, if not completely, suppresses changes in the fluorescence of chlorophyll a_2. Figures 5.7 and 5.8 show that at least two different pigments are present which show changes in fluorescence upon illumination. One of these pigments (presumably chlorophyll a_2) is primarily excited by green, the other, which the hump at 720 mμ, primarily by blue or (as we will see) by red light. We call the substance responsible for the latter fluorescence spectrum, H 720. Additional evidence showing that the fluorescence changes at 718 and 685 mμ are caused by at least two substances is given in fig. 5.9. If only one pigment would be responsible, the time courses should be

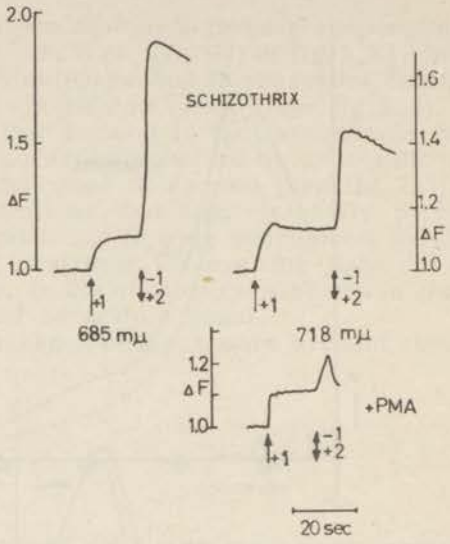

Figure 5.9 Schizothrix, sample taken from a 3 days old culture. Measurement in a stationary suspension in the fluorescence apparatus as used by Duysens and Sweers (53). The recorder only responds to the weak, modulated, exciting light but not to the strong, unmodulated, actinic light. The recorder tracings represent the fluorescence yield at 685 and 718 mμ in weak blue exciting light. Increases in the fluorescence yield occur upon illumination with blue actinic light (+1) and upon switching from blue to green light (-1+2), which is mainly absorbed by pigment system 2. The lower tracing at 718 mμ was measured in the presence of 10^{-4} M PMA. The intensity of the exciting light was 7 x 10^{-11} einstein/cm^2sec, those of the blue and green actinic light were 72 respectively 66 (x 10^{-11} einstein/cm^2sec). The figure shows that the ratio 718/685 of the increase in the fluorescence yield caused by light 1 is about twice as high as that caused by light 2. It follows that the fluorescence increases are due to at least two different substances. The substance contributing mainly to the increase in light 1 fluoresces predominantly at 718 mμ, and that contributing to the increase in light 2 fluoresces more strongly at 685 mμ. In the presence of PMA the fluorescence caused by light 1 is about the same, but that caused by light 2 is much less.

mutually proportional for a given pattern of illumination, which is not so.

As shown in the bottom part of fig. 5.9, the changes in fluorescence due to chlorophyll a_2 (occurring upon switching from light 1 to 2) are largely suppressed after the addition of 10^{-4} M PMA. The (1, s-f) spectrum measured in the presence of PMA was similar to the (1, s-f) spectra of figs. 5.7 and 5.8. This strongly suggests that these spectra approximate the fluorescence spectrum of H 720. H 720 probably is one of the chlorophylls of system 1.

Fig. 5.10 shows that after a short period of actinic illumina-

tion the rate of decrease of the fluorescence of chlorophyll a_2 is more rapid than that of H 720, and in the rather restricted range observed is an exponential function of the time. Fig. 5.11

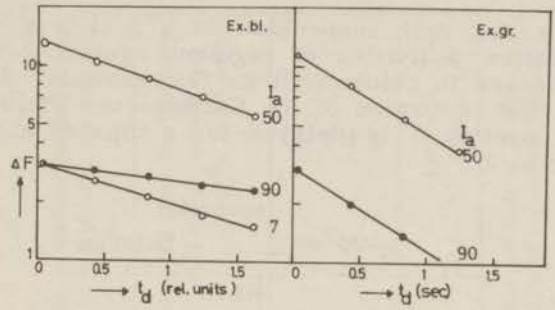

Figure 5.10 Schizothrix, sample taken from a 2 days old culture. The increase in the yield of fluorescence at 718 mμ caused by actinic light (t_a = 0.4 sec), plotted as a function of the dark time t_d. Open symbols represent measurements with green (560 mμ) actinic light, solid symbols refer to red (685 mμ) actinic light. Fluorescence was excited by blue light (left hand side of the figure) and by green light (right hand side of the figure) both of low intensity; t_e was 0.4 sec. The actinic intensities I_a are expressed in 10^{-9} einstein/cm^{2}sec. From the figure it may be concluded that the decay of the fluorescence increase in the dark is exponential and that for blue excitation the increase in fluorescence yield caused by red light decays slower than that caused by green actinic light; in green exciting light the decay is the same both after green and red actinic light. Unfortunately the flow rate in the experiments of the left hand part and thus the time scale was not checked absolutely. It is possible that the slopes of the lines with open circles are the same in the left and right hand parts.

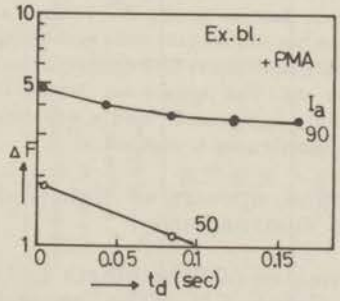

Figure 5.11 Schizothrix, sample taken from a 2 days old culture. The increase in fluorescence at 685 mμ, excited by weak blue light, and caused by green and red actinic light, in the presence of 10^{-4} M PMA, is plotted as a function of the dark time between actinic and exciting light. The meaning of the symbols is the same as in figure 5.10. t_e and t_a are 0.04 sec. Actinic intensities are expressed in 10^{-9} einstein/cm^{2}sec. In the presence of PMA red actinic light causes a much higher increase in fluorescence after a darktime of 0.01 sec than green actinic light. The decay in the dark after red is much slower than after green actinic light.

qualitatively shows that in the presence of PMA the rate of decrease of H 720 fluorescence is nearly the same as in non-treated cells (note the different time scales of figs. 5.10 and 5.11).

By cooling the cell suspension to 2°, it was possible to measure relative activities of pigment system 2 in bringing about the increase in chlorophyll a_2 fluorescence. As is shown in fig. 5.12, the reversion of the fluorescence yield of chlorophyll a_2 by system 1 is delayed for a time of about 0.3 sec upon cooling to 2°.

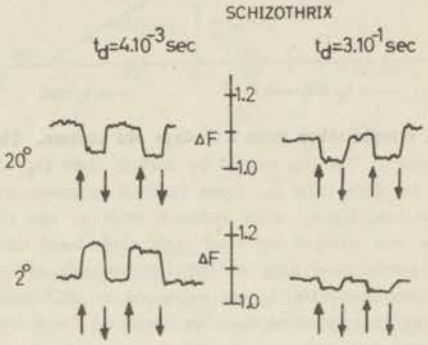

Figure 5.12 Schizothrix, sample taken from a 3 days old culture. Recorder deflections due to changes in the fluorescence yield, measured at 685 mμ, in blue exciting light, occurring upon illumination with red (685 mμ) actinic light in cells which were preilluminated with green light. t_e and t_a were 0.04 sec, t_d was 0.004 and 0.3 sec; the time between t_p and t_a was about 0.5 sec. The measurements were done at 20 and 2°. The red actinic intensity was about 70 x 10^{-9} einstein/cm^{2}sec, the intensity of the exciting light was 10^{-10} einstein/cm^{2}sec. An upward pointing arrow marks the beginning of actinic illumination, a downward pointing one the end. The figure shows that red light causes a decrease of the fluorescence yield, except at 2° after a dark time of 0.004 sec at which an increase in the fluorescence is observed.

5.3.4 Points of action spectra of light-induced changes in the near infrared fluorescence

Points of action spectra of Schizothrix for a number of activities are given in Table 5.2. The second column shows the activity of light flashes of different wavelengths in enhancing chlorophyll fluorescence, measured after a short dark time at 2°. The relative activity at 560 mμ compared to that at 685 mμ indicates an activation by pigment system 2. The third column shows the activity in depressing chlorophyll fluorescence, measured after a long dark time, and indicates an activation mainly by system 1. The too low values at 560 and 620 mμ may be caused by a simultaneous increase in fluorescence caused by system 2. Experimental details of the experiments

TABLE 5.2

Relative activities of actinic light of different wavelengths for causing changes in the yield of fluorescence at 685 mμ in blue excitation, and for activating P 700 oxidation. Maximum activities were arbitrarily put equal to 100.

λ_a (mμ)	Increase in fluorescence at 2°; t_a = 0.04 sec; t_d = 0.004 sec	Decrease in fluorescence at 2°; t_a = 0.04 sec; t_d = 0.3 sec	Increase in fluorescence 10–4 M PMA added; t_a = 0.04 sec t_d = 0.3 sec	Decrease in absorbancy at 700 and 435 mμ; 1.1x10^{-6}M DCMU added
680	55	100	100	100
620	68	15	25 (46)	46
560	100	20	45 (43)	44
430	32	65	70 (70)	60
activation	system 2 chlorophyll a_2	system 1 chlorophyll a_2	system 1 chlorophyll a_1	system 1 P 700

The measurements were done with samples, taken from different cultures of Schizothrix calcicola of 2 days age. The changes in fluorescence and absorbancy were measured and plotted as a function of the actinic intensity. The relative change per incident quantum was calculated for the regions of lower intensities in which the changes were proportional to the actinic intensity. The values in parentheses in the fourth column were obtained from measurements in a stationary suspension and were carried out in modified fluorescence apparatus (see text).

shown in the second and third column are given in the legend of fig. 5.12. The fourth column gives activities in enhancing fluorescence (of H 720) in the presence of 10^{-4} M PMA, which minimizes fluorescence changes due to chlorophyll a_2. The activating reaction seems to be photoreaction 1. The relatively low activity of quanta of 620 mμ probably is due to leakage of phycocyanin out of the cell, caused by the relatively strong stirring of the suspension in the centrifugal pump during the flow experiments. The cell suspension showed, after standing for about one hour after the completion of the experiments, an orange color due to phycocyanin leakage. The values in parentheses in the fourth column give activities for enhancing fluorescence in the presence of PMA in a stationary suspension and show an activity of quanta of 620 mμ which is "normal" for system 1. These experiments were carried out in a modified fluorescence apparatus (Duysens, unpublished), in which fluorescence emission is measured immediately after the actinic light is shut off. The fifth column gives the activities for P 700 oxidation in the presence of DCMU, which are proportional to pigment system 1.

One might speculate that an increase in the fluorescence yield of H 720 (chlorophyll a_1) is correlated with the bleaching of P 700, in a similar way as the increase in bacteriochlorophyll B 890 fluorescence is correlated with P 890 bleaching in photosynthetic bacteria (section 3.3). However, we found that in weak light 1, in the presence of 10^{-4} M PMA, when the fluorescence yield of H 720 was minimal, P 700 was completely in the oxidized (bleached) form. Furthermore, in the presence of PMA light 2 restored P 700 absorption, but did not cause a decrease in the fluorescence yield of H 720, even not when this fluorescence was enhanced by prior illumination with blue light. Thus the bleaching of P 700 and the increase in the fluorescence yield of H 720 were not correlated. This shows that H 720 does not transfer electronic excitation energy from its fluorescent singlet state to P 700. It may be associated with another substance (perhaps X) which is not located in the electron transport chain between the two primary light reactions.

5.4 *Discussion*

Semi-quantitatively the results of the measurements reported in this chapter are consistent with the simplified scheme mentioned in the introduction of this chapter:

$$\text{NADP} \leftarrow \text{X} \leftarrow (\text{system 1}) \leftarrow \text{P 700} \leftarrow \text{cyt} \leftarrow \text{PQ} \leftarrow \text{Q} \leftarrow (\text{system 2}) \leftarrow \text{ZH} \leftarrow \text{H}_2\text{O}$$

Recent experiments have given indications that other compounds have to be inserted in the scheme. There is evidence that ferredoxin is the electron donator of the photoreduction of NADP by pigment system 1 (135). Ferredoxin probably reacts at a site between X and NADP (8). A photosynthetic role of the chlo-

roplast redox compound plastocyanin, isolated by Katoh (79), has been suggested (80, 92, 94). It has an oxidation-reduction potential of about + 0.39 volt, and might react in the chain between the two primary light reactions (94). Rumberg (125) concluded from the spectrum of light-induced absorbancy changes around 650 mμ that in chloroplasts chlorophyll *b* is photoreduced by pigment system 2; it was suggested that chlorophyll *b* reacts in the chain between system 1 and 2. The site of phosphorylation reactions has been subject of several discussions (51, 88, 7). Recent experiments of Trebst (137) and Wessels (149) support the hypothesis (51) that "non-cyclic" phosphorylation occurs in the reaction chain between plastoquinone and cytochrome.

If the scheme is correct, the minimal number of quanta absorbed by system 1 necessary to oxidize P 700 or cytochrome should be 1 (ref. 51). The minimal requirement for quanta absorbed at 680 mμ will be between 1 and 2, since also system 2 (chlorophyll a_2) absorbs at 680 mμ. We found at 2° values varying from 2.0 to 2.6 for the initial apparent rate of P 700 oxidation. It is possible that the somewhat too high values in these cultures are caused by failure of some chlorophyll molecules to transfer energy to the reaction center of photoreaction 1. If this would have been the case, also the quantum requirement of photosynthesis should have been too high for the same culture. It was reported by Kok that in broken spinach chloroplasts the photooxidation of P 700 by light mainly absorbed by pigment system 1 proceeds with an efficiency approaching 1 eq./hν (88, 90). The fact that the estimated quantum requirements for cytochrome oxidation are somewhat higher than those for P 700 oxidation may be caused by incorrect estimates of specific absorbancies of these pigments.

More difficult to explain is the observation that the quantum requirements for cytochrome and P 700 oxidation are three times higher at room temperature than at 2°. In our experimental set up only the sum of the rates of oxidizing and reducing reactions is observed. The quantum requirements computed are those of the initial total rate. It is conceivable that the true quantum requirements for P 700 and cytochrome oxidation by photoreaction 1 are the same at room temperature and at 2°, but that during the first seconds of illumination a (dark) reduction occurs, caused by a pool which contains reduced substances, and that the rate of this reduction is much lower at 2° than at room temperature. The striking effect that the initial rate of oxidation of P 700 and cytochrome is affected by preillumination can be interpreted by the assumption that the pool contains catalysts which react in between the two pigment systems. After red (or blue) light the pool is partly oxidized, and therefore is less active in reducing P 700 and cytochrome during the first seconds of actinic illumination than after preillumination with green light, in which the pool is reduced. The observation (see fig. 5.4) that after a preillumination with light 2 P 700 becomes oxidized in light 1 at a higher rate than after a dark

period of the same duration remains to be explained. One might speculate that light of system 2 causes, in addition to a reduction, a partial phototransformation of the pool into a form which is inactive in reducing the oxidized photoproducts of photoreaction 1. It was suggested by Olson and Smillie (114) that the low initial rate of cytochrome oxidation in Anacystis and Euglena at 20° is due to a "cyclic" electron flow in reaction 1. However, the high initial rate of the reduction of NADP in Anacystis at 20°, as observed by Amesz and Duysens (2), is not consistent with their suggestion.

The estimated quantum requirement of 3 for the reduction of oxidized P 700 suggests that the requirement of quanta of 560 mμ absorbed by pigment system 2 probably is between 1 and 2 per equivalent. No further approximation of the true requirement can be made, because of an uncertainty about the relative absorbancies of the two pigment systems at 560 mμ. Our data give no support for the hypothesis (88) that the quantum requirement for the photoreduction of oxidized P 700 is lower than 1 per equivalent. A quantum efficiency of 1 eq./hν for both light reactions would imply an overall requirement of photosynthesis of 8 quanta per molecule of CO_2 reduced or of O_2 evolved, and probably somewhat more than 8 if additional light quanta are needed for cyclic phosphorylation (46). A quantum requirement of 8, except for those found by Warburg and coworkers (144, 145), is not inconsistent with experimental data (59, 60).

In contradistinction to the conclusions of other authors, who reported photooxidation of a cytochrome at -150° in a chloroplast preparation (102, 151), and in spinach and Swiss chard leaves at -196° (ref. 31), we did not observe an appreciable photooxidation of cytochrome at -170° in the red and blue-green algae Porphyridium cruentum, Schizothrix calcicola and Anacystis nidulans. All these results, if correctly interpreted, indicate that, just like in photosynthetic bacteria (see section 4.3), light-induced cytochrome oxidation at low temperature is not a general phenomenon in oxygen evolving photosynthetic organisms. It may be that in algal photosynthesis for some cytochromes the temperature has to be lowered below -150° (c.f. ref. 151) or even below -196° (c.f. ref. 31) before a limitation of the rate of cytochrome oxidation occurs. Our results are consistent with the hypothesis that not cytochrome but P 700 is the primary reductant of pigment system 1 in algal photosynthesis. Photooxidation of P 700 occurs at -170° with good efficiency (87, 142).

It has been shown (1, 19, 22, 64, 70, 85, 136) that in algae and green leaves chlorophyll *a* has different absorption bands in the infrared region, which are probably due to different modifications (forms) of chlorophyll *a* (20, 100). Evidence for a participation of the chlorophyll *a* forms in one of the two pigment systems has been deduced from action spectra of photosynthesis (51, 61) and of reactions of certain intermediates in the electron transport chain (103). Results of fluorescence measurements indicated (52, 53) that at least two different forms

of chlorophyll *a* exist, of which one is excited by light absorbed by system 2 (chlorophyll a_2) and the other by light absorbed by system 1 (chlorophyll a_1). Changes in the yield of fluorescence of chlorophyll a_2 were studied and discussed by Duysens (53). They were attributed to the photoreduction of a hypothetical primary oxidant Q, which in its oxidized form is a quencher of the fluorescence of chlorophyll a_2. The values in the second column of table 5.2 show that the action spectrum of the increase of the fluorescence of chlorophyll a_2 (i.e. the photoreduction of Q) is proportional to the spectrum of pigment system 2. Results of Butler and Bishop (23) on changes in fluorescence in a wild and a mutant strain of Scenedesmus also confirm these conclusions. The difference spectrum of the fluorescence of chlorophyll a_2 does not show a pronounced shoulder around 720 mμ. This is in accordance with results of Duysens and Sweers (53), Lavorel (97), and of Rosenberg *et al* (119) with other species. The third column of table 5.2 indicates that the reversion of the increase in the fluorescence of chlorophyll a_2 (i.e. the oxidation of QH) is brought by system 1. The photooxidation of QH by light of system 1 was delayed upon lowering the temperature which indicates that the reaction goes via a dark reaction. Light-induced changes in fluorescence excited by blue light occurring upon blue or red illumination both in normal and PMA treated cells of Schizothrix, and in Pophyridium cruentum and Porphyra sp. (140, 142), strongly suggest the existence of a pigment, called H 720, which fluoresces relatively more strongly at 720 mμ than chlorophyll a_2. The fluorescence yield of H 720 increased upon illumination with light 1 (table 5.2 column 4). It seems that H 720 is a chlorophyll *a* belonging to, and receiving energy from the other pigments of system 1.

In green leaves and several algae a weak fluorescence band around 720 mμ in addition to the main chlorophyll band has been reported (42, 22, 97). At low temperature the fluorescence band at 720 mμ was found to be intensified manifold with respect to the band at 685 mμ (17, 21, 67, 68, 91). It has been suggested (68, 91) that at least part of the emission around 720 mμ orginates from a pigment which is excited more efficiently by light 1. It remains to be established whether this pigment is identical or related to H 720, or not. The same applies for the pigment of which the fluorescence at 720 mμ shows a relatively higher degree of polarization than the bulk of the chlorophyll *a* fluorescence around 685 mμ, as reported by R.A. Olson and co-workers (115, 116) and by Lavorel (98). It was suggested that this pigment is excited by light 1 (ref. 98, 24).

An unexpected result is the lack of correlation between the increase in H 720 fluorescence and the decrease in P 700 absorption. If H 720, which is primarily excited and activated by light 1, is identical with chlorophyll a_1, then there is no evidence for a direct transfer of energy from the fluorescent excited singlet state of chlorophyll a_1 to P 700. As demonstrated for P 890 in purple bacteria (chapter III), this would have implied

a correlative increase in the fluorescence yield of chlorophyll a_1 upon bleaching of P 700. Possibly, the fluorescence of chlorophyll a_1 increases upon the reduction of the primary photoreductant X of photoreaction 1. This would be analogous with the increase of the fluorescence of chlorophyll a_2 upon reduction of the primary photoreductant Q of pigment system 2 (ref. 53). However, the possibility cannot be excluded that there are light-induced changes in fluorescence of a chlorophyll *a*, excited by system 1, which is different from H 720. These changes, which are too small to be observed with the technique applied so far, may be associated with the photobleaching of P 700. An alternative interpretation of the primary reactions in light reaction 1 was given (57), which suggests that energy transfer occurs from the bulk of the fluorescent chlorophyll a_1 molecules to intrinsically weakly fluorescent chlorophyll a_1' molecules present in a relatively small concentration and associated with P 700.

In conclusion the results reported in this chapter are not inconsistent with the hypothesis that in algal photosynthesis the primary photoreactions take place at two different reaction centers 1 and 2, belonging to the pigment systems 1 and 2, respectively. It is suggested that in each of these reaction centers the primary photoreductants X and Q act in the oxidized form as a quencher of the fluorescence of chlorophyll *a*. At present X and Q and also the primary photooxidant Y of system 2 have not been identified by absorption difference spectrophotometry. There is strong evidence that P 700 is the primary photooxidant of pigment system 1 (87, 89). However, it must be concluded that the primary reactions of pigment system 1 in algae are of a more complicated structure than those of the bacterial system.

SUMMARY

Evidence has been obtained that in photosynthesis conversion of light energy into chemical energy takes place by the cooperation of a large number of light absorbing (bacterio-)chlorophyll molecules. A model system of such an assembly of molecules, which is called the "photosynthetic unit", has been proposed, in which a photochemically active molecule present in a small concentration receives energy by inductive resonance transfer from the bulk of the light absorbing, non-photochemically active molecules. The photochemically active molecule and the primarily associated reactant(s) compose the so-called photosynthetic reaction center.

The main subject of this thesis concerns an experimental identification of the reaction center, and of reactions closely associated with it. The experiments are performed with intact cells of photosynthetic bacteria and algae. Sensitive absorption and fluorescence difference spectrophotometry is used as the basic technique.

Chapter I gives an introduction to the subject. In chapter II the apparatus and experimental methods are briefly described. For measuring light-induced changes in absorbancy at lower temperatures down to -210°C a low temperature sample holder was constructed fitted for use with a differential split-beam absorption spectrophotometer. A device for automic wavelength scanning of the difference in absorbancy of two reaction vessels with photosynthetic material, and a "differential flow" attachment with two separated flow compartments for the fluorescence apparatus, by means of which small changes in fluorescence could be measured with increased precision, are described.

Evidence for bacteriochlorophyll-like pigments, called P 890 and P 840, to be part of the photosynthetic reaction center in purple and green bacteria respectively, is presented in chapter III. The efficient photobleaching of these pigments, which occurs independently of the temperature, was correlated with an increase in the fluorescence yield of bacteriochlorophyll. For the purple bacterium Rhodospirillum rubrum this correlation is quantitatively explained by energy transfer from bacteriochlorophyll to P 890, which is present in a concentration of about 1-2% of the bulk of the bacteriochlorophyll. In chapter IV results of experiments on the kinetics and spectra of light-induced cytochrome oxidation in various bacteria at temperatures between 20 and -210°C are discussed. Of all cytochromes studied only C 423.5 in Chromatium was photooxidized at -210°. The photooxidation of the other cytochromes was stopped at a temperature between -25 and -120°, specific for each of them. The quantum requirement for the oxidation of two different cytochromes in Chromatium was determined and found to be close to one. The results are interpreted as evidence that the cytochromes are closely attached to the photosynthetic reaction center by which, upon excitation, they are oxidized in a temperature dependent

reaction. It is argued that C 423.5 is not an intermediate in the main electron transport chain of photosynthesis in Chromatium. The earlier hypothesis, based on the observed efficient photo-oxidation of C 423.5 at liquid nitrogen temperature, that in bacterial photosynthesis cytochrome oxidation is a primary temperature insensitive reaction is critisized.

In chapter V experiments with algae are described. The results are interpreted in terms of two different light reactions 1 and 2 which drive algal photosynthesis. The quantum requirements for the oxidation of the pigment P 700 and of cytochrome by light mainly absorbed by the photochemical system 1 and for the reduction of oxidized P 700 by light of system 2 were determined to be 2 - 2.6, 3 - 3.5 and about 3 respectively, for the blue-green alga Schizothrix calcicola. The results are not inconsistent with the assumption that the true quantum requirement for the photochemical transport of 1 electron is 1 for each of the two pigment systems. Changes in the fluorescence excited by system 1 were found in the wavelength region 670 - 750 mμ and attributed to a photoactive pigment H 720, which is probably a chlorophyll *a* excited by system 1. Because of lack of correlation of fluorescence changes of H 720 with redox changes of the primary reductant P 700 of system 1, in a similar way as found in bacteria between bacteriochlorophyll fluorescence and P 890 bleaching, it is suggested that the fluorescence yield of H 720 is controlled by the redox level of the primary oxidant X of system 1. The results suggest that in algal photosynthesis the primary photoreactions take place at two different reaction centers 1 and 2, in which the primary oxidants X and Q, respectively act as a quencher of the fluorescence of chlorophyll *a*.

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Teneinde te voldoen aan het verzoek van de faculteit der Wiskunde en Natuurwetenschappen, volgt hier een kort overzicht van mijn academische studie.

In 1954, na het behalen van het eindexamen H. B. S. B aan het Baudartius Lyceum te Zutphen, begon ik mijn studie in de faculteit der Wiskunde en Natuurwetenschappen aan de Rijksuniversiteit te Utrecht. Het candidaatsexamen, letter d, legde ik in november 1956 af. De studie voor het doctoraalexamen experimentele natuurkunde geschiedde onder leiding van de hoogleraren Dr. J. A. Smit en Dr. P. M. Endt. Het experimentele onderzoek werd gedaan in het Fysisch Laboratorium. Bij de werkgroep Optica werden door mij aanslag functies van helium gemeten, met behulp van een kwantenteller. Bij de F. O. M. werkgroep Ms IIa deed ik een onderzoek naar de discriminatie effecten die optreden bij de bepaling van concentratie verhoudingen in gasmonsters met behulp van een massaspectrometer. Het doctoraalexamen werd afgelegd in januari 1960. Gedurende de jaren 1958 tot 1960 was ik als assistent verbonden aan het practicum voor praecandidaten natuurkunde.

In 1960 werd ik benoemd tot wetenschappelijk ambtenaar bij de Biofysica aan de Rijksuniversiteit te Leiden, en begon ik aan het onderzoek dat geleid heeft tot dit proefschrift. In januari 1964 werd ik benoemd tot wetenschappelijk ambtenaar 1e klas.