Our present paper deals with the steady-state changes of cytochromes induced by these inhibitors in purple bacteria. The effect of these inhibitors on carotenoid pigments was studied, too. The light-induced absorption change in the presence of these inhibitors showed an interesting blocking phenomenon between cytochromes of *c*- and *b*-types.

METHODS

Culture of bacteria

Photosynthetic non-sulfur purple bacteria, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum*, were cultured anaerobically under illumination as described before.

Spectrophotometric observations

A split-beam spectrophotometer designed in our laboratory[10,11], which has been adapted for cross-illumination of samples, was used for spectrophotometric measurements. Difference spectra at liquid nitrogen temperature were measured with a low-temperature adaptor for the split-beam spectrophotometer[12]. Other experimental procedures are described in a preceding paper[9].

RESULTS

Effect of HOQNO and antimycin A on the absorption spectrum of bacterial suspension in dark

R. spheroides: If HOQNO was added to the aerobic cells, there occurred a shift in the steady state of cytochromes (Fig. 1). This low-temperature difference spectrum

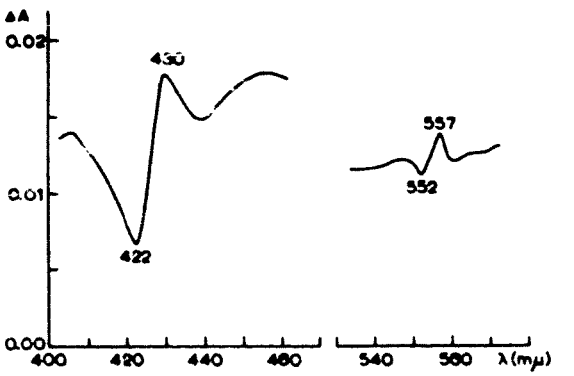

Fig. 1. Difference spectrum of *R. spheroides*, aerobic HOQNO-treated cells minus aerobic cells. HOQNO, $1 \cdot 10^{-4}$ *M*; optical path length, 1 mm; 2.70 mg dry weight/ml; temperature, 77° K.

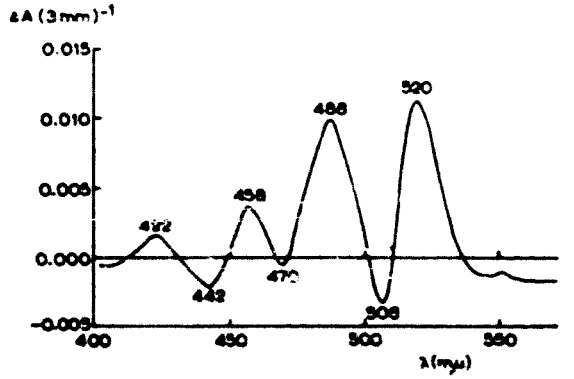

Fig. 2. Absorption spectrum change induced by antimycin A in *R. spheroides* anaerobic cells. Difference spectrum of anaerobic cells minus anaerobic antimycin A-treated cells. [Antimycin A, $3 \cdot 10^{-5}$ *M*; 2.11 mg dry weight/ml; optical path length, 3 mm; temperature, 297° K.

of two "frozen" steady states shows absorbancy increases at 430 mμ and 557 mμ and absorption decreases at 422 mμ and 552 mμ, indicating an increased reduction of a cytochrome of *b*-type and an increased oxidation of a cytochrome of *c*-type. A similar change was also observed in *R. rubrum* aerobic cells when HOQNO was added. It is to be noted that this steady-state change is similar to the response of HOQNO-treated *R. rubrum* aerobic cells to illumination.

After addition of antimycin A ($3 \cdot 10^{-5}$ *M*) or HOQNO ($1 \cdot 10^{-4}$ *M*) to the anaerobic *R. spheroides* cells, carotenoids showed a striking change (Fig. 2). It is like a reversal of the change observed in anaerobic *R. spheroides* cells caused by illumination or oxygenation, suggesting an increase of shorter-wavelength carotenoids[9, 13–15]. At the same time the reduction of *c*-type cytochrome was inhibited and this component remained in a more oxidized state than in untreated cells.

R. rubrum: Reduction of *c*-type cytochrome by anaerobiosis was inhibited by HOQNO as revealed by difference spectra, HOQNO-treated minus anaerobic cells at room and liquid-nitrogen temperatures (Fig. 3). At room temperature, absorption decreases were observed at 422 mμ and 551 mμ; at the lower temperature, the troughs were shifted towards shorter wavelengths and were located at 420 and 548 mμ, with appreciable sharpening of the bands. The band shifts to shorter wavelengths

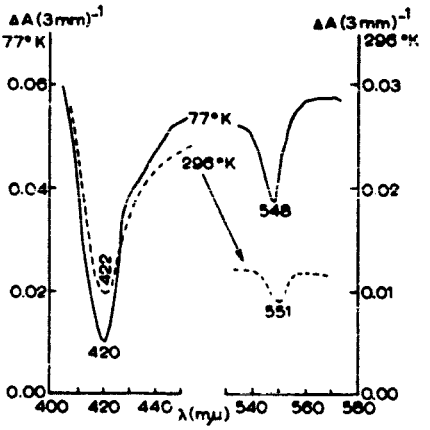

Fig. 3. Steady state change of *c*-type cytochrome in the presence of HOQNO in anaerobic *R. rubrum* cells. [HOQNO], $1 \cdot 10^{-4}$ *M*; optical path length, 3 mm; temperature, 77° K and 296° K.

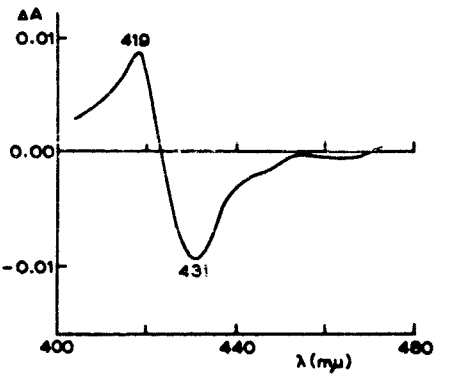

Fig. 4

Fig. 4. Difference spectrum of *R. rubrum*, aerobic minus aerobic HOQNO-treated cells. 2.31 mg dry weight/ml; [HOQNO], $6 \cdot 10^{-5}$ *M*; optical path length, 10 mm; temperature, 297° K.

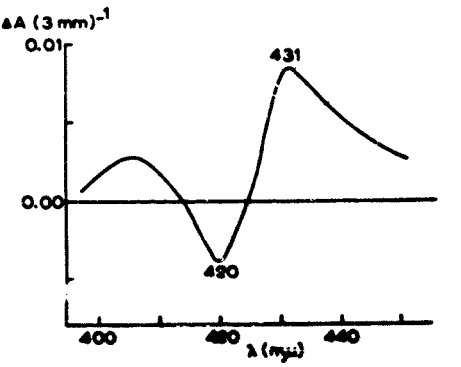

Fig. 5

Fig. 5. Absorption spectrum change by illumination in HOQNO-treated aerobic *R. rubrum* cells (illuminated minus dark difference spectrum). [HOQNO], $1 \cdot 10^{-4}$ *M*; optical path length, 3 mm; temperature, 296° K.

and the sharpening of absorption bands at low temperatures have been observed in heme proteins[13, 16–18]. This result indicates that the reduction of *c*-type cytochrome by anaerobiosis is inhibited by HOQNO.

In the aerobic *R. rubrum* cells, the steady-state changes of cytochromes following the addition of HOQNO were similar to the changes observed in the aerobic *R. spheroides* cells (Fig. 4). This difference spectrum shows that, in the presence of HOQNO, absorption decreased at 419 mμ and increased at 431 mμ, indicating that the steady state of *b*-type cytochrome changed to a more reduced level and cytochrome *c* showed a shift to a more oxidized level, as is the case in *R. spheroides*.

Effect of HOQNO on the light-induced absorption spectrum change

When HOQNO was added to the aerobic suspension of *R. rubrum*, illumination caused an increased absorption at 431 mμ and a decrease at 420 mμ (Fig. 5). This can be interpreted as a reduction of *b*-type cytochrome and an oxidation of *c*-type cytochrome by illumination when HOQNO is blocking the reaction of these two heme components. Cytochrome of *c*-type is largely reduced and *b*-type cytochrome is mainly oxidized in aerobic *R. rubrum* or *R. spheroides* cells in the dark[18,19]. In the presence of HOQNO (in the aerobic cells), *c*-type cytochrome becomes more oxidized and *b*-type more reduced. But there is still enough reduced cytochrome *c* and oxidized cytochrome *b* to permit observation of the light-induced change shown in Fig. 5 in aerobic HOQNO-treated cells.

DISCUSSION

It has been found that HOQNO and antimycin A are effective inhibitors of light-induced phosphorylation of the chromatophores isolated from photosynthetic bacteria[5–7]. Their effects upon the absorption spectrum of cell suspensions were studied in this paper. The inhibition of cytochrome *c* reduction (by anaerobiosis) by the additon of HOQNO or antimycin A to anaerobic suspensions indicates that the electron transfer system is blocked at the dehydrogenase side of cytochrome *c*. It was also shown that cytochrome *c* becomes more oxidized and cytochrome *b* more reduced when HOQNO is added to aerobic suspensions. It was noted that the illumination of an aerobic suspension in the presence of HOQNO induces reduction of *b*-type cytochrome and oxidation of *c*-type cytochrome. This is an interesting phenomenon since it would represent one of the crossover points for the light-induced response observed in intact cells, usually the light response affects the components similarly: they are all oxidized, as in the case of the typical anaerobic response[19]; or if the cells are treated with phenylmercuric acetate, one component, cytochrome *b*, is reduced[9]. Our present results suggest that the cytochromes *c* and *b* are located on the photosynthetic and respiratory electron transport chain and that the *c*-type cytochrome is closer to the photochemical oxidizing site and to the terminal oxidase, while the *b*-type cytochrome is nearer to the photochemical reducing site and to the dehydrogenases. It is indicated, too, that the site of action of these inhibitors is at, or close to, the site of the oxidation–reduction reaction of these two heme protein components.

Our recent data on photophosphorylation under flashing and continuous illumination suggest that electron transport at the site of action of these inhibitors is rate-limiting in the dark process of photosynthetic phosphorylation[8].

To observe the effect of HOQNO or antimycin A on the absorption spectrum change in the dense suspensions of intact cells, higher concentrations of these reagents were necessary than for the inhibition of photosynthetic phosphorylation of the isolated chromatophores. It was, however, noticed in the study of the effects of these reagents on photophosphorylation, that the molar ratio of the reagents to bacteriochlorophyll (or protein) was more important in determining the degree of inhibition than the apparent concentration of these inhibitors[18]. It is not unlikely that in the intact-cell experiments, the local concentrations of these inhibitors in the electron transport system are in a comparable range to those in the phosphorylation experiments with isolated chromatophores, though much larger amounts of the inhibitors are used in the former case.

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The author is grateful to Dr. B. CHANCE for his helpful discussions and criticisms.

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... PHOSPHOPYRIDINE NUCLEOTIDE REDUCTION IN *RHODOSPIRILLUM RUBRUM* AND *RHODOPSEUDOMONAS SPHEROIDES*

J. AMESZ

Biophysical Laboratory, The University of Leiden, Leiden (The Netherlands)*

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SUMMARY

1. By means of fluorescence and absorption spectrophotometry the kinetics and rate of light-induced reduction of phosphopyridine nucleotide reduction were studied in intact cells of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*. The bacteria were grown and studied in organic nutrient media, containing either malate, butyrate, acetate or succinate as substrate.

2. Upon infrared irradiation of moderate intensity ($2-10 \cdot 10^{-9}$ Einstein/sec·cm^2) of wavelength 860 mμ a large pool of pyridine nucleotide was reduced. However, the kinetics indicated a high efficiency only during a short period after onset of illumination. After 5–30 sec the calculated rate of photoreduction of pyridine nucleotide gradually decreased to much lower values. The kinetics and rate of reduction were about the same for different substrates.

3. The lowest quantum requirements were 2–3 quanta per equivalent for pyridine nucleotide reduction; for cytochrome oxidation a quantum requirement of about 3–4 was found in Rhodospirillum.

4. Pyridine nucleotide reduction was either not inhibited, or only partially inhibited, by 2-heptyl-4-hydroxyquinoline-*N*-oxide and fluoroacetate.

5. In Rhodopseudomonas the action spectrum for bacteriochlorophyll fluorescence was proportional to that of pyridine nucleotide reduction, which indicates that only one pigment system is present in purple bacteria.

INTRODUCTION

During the last few years, evidence has accumulated that the reduction of phosphopyridine nucleotide (TPN or DPN) is one of the important reactions in photosynthesis. A light-induced reduction of PN was demonstrated in intact algae and photosynthetic bacteria[1-3] and in cell-free preparations of photosynthetic organisms[4-8].

Abbreviations: PN(H), (reduced) phosphopyridine nucleotide; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

* Postal address: Nieuwsteeg 18, Leiden (The Netherlands).

This reduction was found to proceed, both in intact cells and in extracts, with a high quantum efficiency[7-10].

Recent investigations indicate that the photosynthetic process (the reduction of CO_2 and production of O_2), in chlorophyll *a* containing organisms, involves the cooperation of two photochemical reactions, which are driven by two different photochemical pigment systems[11-14] (for further references see ref. 13).

The action spectrum and kinetics of the light-induced reduction of PN in the blue-green alga, *Anacystis nidulans*, indicate that one of these photochemical reactions results in an efficient photoreduction of PN[9]. This and other evidence indicates that in algae and higher plants the reduction of CO_2 proceeds via PNH.

For photosynthetic bacteria, the available evidence suggests a more complicated picture. Fluorometric and absorptiometric measurements indicated an efficient photoreduction of PN in intact *Rhodospirillum rubrum* and in Chromatium[2,8,15].

A comparison of the initial kinetics of PN-reduction, and of cytochrome-oxidation, indicated a roughly equal rate per equivalent after a short lag period[15]. Like chloroplast preparations, bacterial extracts were able to reduce an appreciable amount of added DPN or TPN upon illumination. FMNH, succinate or cytochrome *c* were simultaneously oxidized[6,16,17]. However, experiments of STANIER *et al.*[18] suggested to these authors the hypothesis that in *R. rubrum* the action of light during photosynthesis in the presence of CO_2 and simple organic acids would, for the main part, be restricted to the generation of ATP. By means of labelled substrates they demonstrated a direct incorporation of acetate, butyrate and succinate into cell material. Acetate (at least in the absence of bicarbonate[19]) and butyrate were converted mainly to poly-β-hydroxybutyrate and most of the succinate to a polysaccharide. In agreement with earlier findings[20,21] only little carbondioxide-fixation was found to occur. On the basis of these findings, the authors proposed a scheme, according to which, for every 9 molecules of acetate which are converted, only one molecule of DPN would be reduced in the light: the major function of the light reaction would be the formation of ATP. LOSADA *et al.*[22], on the basis of experiments with cell-free extracts of Chromatium, similarly concluded that, under certain conditions, the action of light in this bacterium would be restricted to the production of ATP.

Since these authors did not study intermediate catalysts *in vivo*, it remained an open question whether the formation of ATP was preceded by light-induced oxidation–reduction reactions, and, if so, whether reduction of PN was a part of these reactions. In order to obtain direct information about the role of PN in bacterial photosynthesis, we have performed detailed experiments on the kinetics of PN-reduction in living cells of the non-sulphur purple bacteria, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*.

The results of these experiments, which are based on spectrophotometric methods used previously in this laboratory are presented in this paper.

MATERIAL AND METHODS

R. rubrum, strains 1 and 4 was grown either in peptone or in synthetic media.

The bacteria in peptone medium, which contained 1 % peptone (Difco) and 0.5 % NaCl, were grown, either in sterile, completely filled, glass-stoppered flasks, or else with constant bubbling of N_2, at 25°–30° and at a light intensity of about 2000 lux, supplied by incandescent lamps.

The bacteria in synthetic media were grown at 30° and about 4000 lux. These media were basically the same as that described by COHEN-BAZIRE *et al.*[23], except for a higher concentration of NaCl necessary for growing strain 4, and for different concentrations or organic substrates. Stock solution No. 2 of the above authors was replaced by a solution containing 190 g of NaCl and 53.5 g of NH_4Cl/l and adjusted to pH 6.8 with NaOH. Before autoclaving, 1 g of L-glutamic acid and either 1.7 g of sodium acetate, 1.5 g of sodium butyrate, 1.5 g of DL-malic acid or 1 5 g of sodium succinate were added to 1 l of the medium, and the pH was brought to 7.0 with NaOH. These media will be referred to as "acetate", "butyrate", "malate" and "succinate" medium. The bacterial suspensions were gassed usually with N_2–CO_2 (95:5). Strain 1 also grew well in malate and acetate medium gassed with N_2 only.

Rhodopseudomonas spheroides was grown either in malate medium or in the medium of COHEN-BAZIRE *et al.*, unmodified except for a higher concentration of NaCl (3.8 g/l). The casamino acids were replaced by L-glutamic acid and sodium acetate as indicated[23]. The culture was gassed with N_2–CO_2 (95:5).

Unless otherwise stated, the bacteria were harvested by centrifugation at 1000 × *g*, and resuspended, usually in fresh growth medium. The concentration was determined as volume percent wet cells after centrifugation in a Tromsdorff tube.

The suspension was gassed for at least 45 min and before measurement transferred to 1-mm or 1-cm quartz absorption vessels.

Changes in absorption resulting from photosynthetically active (actinic) illumination were measured by means of the same split-beam difference absorption spectrophotometer used in other experiments[24].

In most experiments, PNH fluorescence was measured by means of an apparatus similar to those described earlier[1,2], but equipped with two monochromators in order to select the desired wavelengths for excitation and emission of fluorescence. The fluorescence excitation radiation was provided by a xenon or a mercury arc. Suitable light filters were applied to minimize the effects of false light. In all experiments, actinic illumination was provided by means of a modified slide projector, equipped with a 500-W incandescent projection bulb and with absorption and interference filters (half width 10–15 mμ), or by means of a monochromator and xenon arc. The intensity was measured by means of a calibrated photo-electric cell. The intensities given in this paper are those at the place of the vessel and are expressed in 10^{-9} Einstein/sec·cm²; unless otherwise stated the wavelength was a band around 860 mμ.

The experiments were usually carried out at room temperature (about 22°).

The absorption of the bacterial suspensions was measured with opal glass placed behind blank and sample vessel[25] to minimize the effects of light scattering. A scattering correction was made by subtracting the apparent absorption at 960 mμ (where no absorption by plant pigments occurs) from the measured absorption at other wavelengths.

The quantum requirements reported in this paper were calculated, in a way described previously[24], from the rates of the light-induced absorption changes and from the number of Einsteins absorbed/sec in a given volume. No corrections were applied for optical "flattening" effects or for deviations from Beer's law[9,26,27]; calculation and measurements indicated that these effects together gave a deviation which did not exceed 5–10% for the Rhodospirillum suspensions used.

RESULTS AND INTERPRETATION

The identification of PNH

Intact, living cells of *R. rubrum*, suspended in a suitable medium, show an increase of the blue fluorescence of the cells on illumination with photosynthetically active infrared illumination. This fluorescence was excited by ultraviolet radiation. The emission difference spectrum[1] of this fluorescence and the fluorescence excitation spectrum in the region 260–390 mμ (see ref. 2) were found to be similar to those of enzyme-bound DPNH. This indicated that the fluorescence changes upon infrared illumination of Rhodospirillum were caused by an intracellular reduction of PN.

We found, that the kinetics and the emission spectrum of the blue fluorescence changes in *R. rubrum*, strain 4, were independent of the wavelength of excitation for radiations of 280 mμ and of 340 mμ. This gives further evidence that the changes in fluorescence are caused by PNH.

On the basis of fluorescence measurements, it cannot be decided whether DPN or TPN was reduced: in agreement with earlier experiments of LOWRY *et al.*[28] we found that the fluorescence spectrum and fluorescence yield of a 1-mm layer of a $6.3 \cdot 10^{-4}$ M solution of TPNH were (within the experimental error of a few percent) identical to those for DPNH at the same concentration.

Although the measurement of fluorescence provides a sensitive and specific way of studying the reduction of PN *in vivo*, the method is less satisfactory for quantitative determinations, because the fluorescence yield of PNH, bound to cell constituents, differs by an unknown factor from that of free PNH[29]. The absorption spectrum of PNH in the near ultraviolet appears to be less affected by binding to an enzyme than the fluorescence spectrum: the wavelength of maximum absorption may be somewhat shifted, but the height of the absorption maximum is little changed[29]. Furthermore, the shape of the fluorescence excitation spectrum[2] indicates that in Rhodospirillum no substantial shift of the absorption maximum occurs. However, measurement of PNH by means of its absorption (*cf.* ref. 9) is less specific, because reactions of cytochromes and bacteriochlorophyll also give rise to absorption changes in the ultraviolet.

Fig. 1 A shows some typical recordings of ultraviolet absorption changes occurring upon photosynthetically active illumination. Strain 1 of *R. rubrum* was used in these and the following experiments. The different kinetics at different wavelengths show clearly that these changes reflect a transformation of at least two different compounds. At 380 mμ, and at 315 mμ, a slow phase and a fast one (b, of opposite sign at 315 mμ) are distinguished upon darkening, while at 340 mμ only a slow change is discernible The total absorption difference (a) is the sum of both components.

The difference spectrum of the maximum deflection in the light minus the steady-state in the dark (a) and of the fast change (b) are given in Fig. 2A. The approximate spectrum of the slow absorption change, curve c, is obtained by taking the difference of curves a and b. Fig. 2B shows similar difference spectra measured under different experimental conditions. It can be seen that the spectra marked c of Figs. 2A and 2B are approximately proportional to the absorption spectra of DPNH or of TPNH. The shape of the spectra indicates that, at 330–340 mμ, interfering absorption changes caused by substances other than PNH are small. The fast absorption changes at other wavelengths are probably caused by light-induced

oxidations of cytochromes and of bacteriochlorophyll[20]. They are relatively small at 370 mμ, which wavelength was applied by CHANCE AND OLSON[15] as a reference.

The similarity of the kinetics of the blue fluorescence and of the increase in absorption at 340 mμ gave further indication that the light-induced absorption changes at 340 mμ are mainly due to the reduction of PN. This similarity was

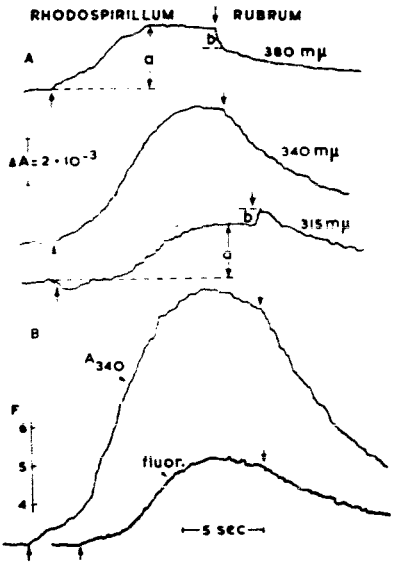

Fig. 1. A, Recordings of the kinetics of changes in absorption in the near ultraviolet upon illumination of 48 h-cultures of *Rhodospirillum rubrum*, grown on malate medium. The bacteria were examined in fresh growth-medium, in a concentration of 2% wet cells in a 1-mm vessel. Actinic illumination was provided by a band around 838 mμ of an intensity of $6.4 \cdot 10^{-9}$ Einstein/sec·cm². Onset of illumination is indicated by an upward pointing arrow and darkening by a downward pointing one. B, A comparison of the kinetics of changes in absorption and fluorescence of a 4% suspension upon illumination. The fluorescence expressed in arbitrary units, was excited by light of 334 mμ and measured at 450 mμ. The other conditions were the same as in Fig. 1A.

observed at high, as well as at low light intensities; and with bacteria which had been grown and suspended in different media. Two typical recordings are given in Fig. 1B. The small differences between the two curves are probably caused by a difference in the optical geometry of the apparatus and by a limited reproducibility of the experimental conditions.

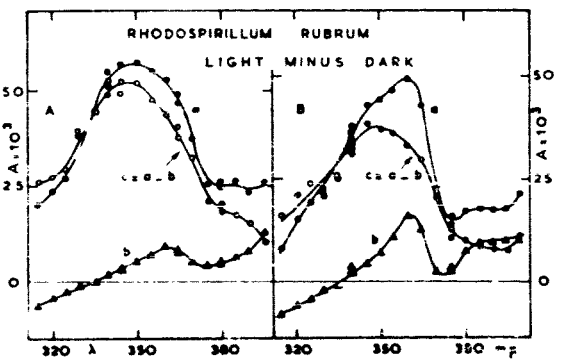

Fig. 2. A, Difference spectra of (a) the maximum, and (b) the rapid, change in absorption of a suspension of *Rhodospirillum rubrum*, strain 1 grown in malate medium. The change c = a − b may be taken to be the magnitude of the slow change. The phases and conditions are the same as those in Fig. 1A. Further details are given in the text. B, The same difference spectra, but obtained with a 2% suspension of a 48 h-culture of *Rhodospirillum rubrum*, grown in peptone and examined in a medium containing butyrate and phosphate, in the presence of N_2-CO_2 (95:5).

The suspension was illuminated by a band around 860 mμ of $6.2 \cdot 10^{-9}$ Einstein sec·cm².

Kinetics of light-induced reduction and oxidation of PN in Rhodospirillum rubrum

Fig. 3 shows typical recordings of the absorption changes occurring at 340 mμ upon infrared illumination of Rhodospirillum grown, and resuspended, in malate medium. Following an induction period of one second or more, a rapid accumulation of PNH occurred. As shown in the top curve, after illumination for about 10 sec the amount of PNH in the cells slowly diminished. Upon darkening, a reoxidation of the accumulated PNH occurred, and gradually the amount dropped to approximately the same level as before illumination. When the suspension was left in darkness for 1 min or more, the same sequence of events was reproduced again. A relatively large amount of PN could be reduced in the light: after a few seconds of illumination of sufficiently high intensity the concentration of photoreduced PNH in the cells was approx. 0.1 of that of bacteriochlorophyll.

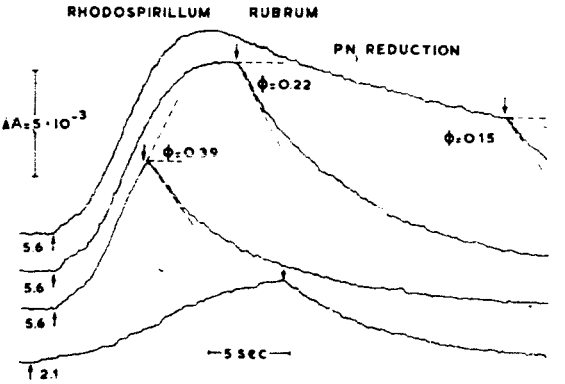

Fig. 3. Kinetics of PN-induced changes in absorbance at 340 mμ in *Rhodospirillum rubrum* upon illumination for different periods of time with light of differing intensity. The bacteria were grown for 48 h and examined in malate medium in a 1-mm vessel, the concentration of wet cells was 4 %. The wavelength of the actinic light was 860 mμ and the intensities applied were 2.1×10^{-7} Einstein/sec·cm² for the lowest curve and 5.6×10^{-9} Einstein/sec·cm² for the other curves. An upward moving trace indicates an increase of absorption and a reduction of PN. The quantum efficiency of the reduction is indicated by Φ (see text).

From the data in Table I it appears that, after illumination periods ranging from 6 to 30 sec, the rate of reoxidation of PNH is, for a given sample of Rhodospirillum, approximately proportional to the amount of light-reduced PNH present in the cells, and is independent of the other experimental parameters, such as the intensity and duration of illumination. Measurement of the oxidation rate at different temperatures indicated a relatively small temperature dependence of the reaction corresponding to an activation energy of about 3000 cal./mole in the range of 1° to 37°. The data suggest that PNH is oxidized in a dark reaction by substances present at an approximately constant concentration (perhaps in large amount). Since the rate of the reoxidation can be measured by shutting off the light, the total rate of photoreduction of PN can be obtained as the difference between the slopes of the curve before and after darkening (see Fig. 3). The three top recordings show that this rate is appreciably higher after 5.7 sec of illumination (third recording) than after 11.7 or 28.5 sec. The small "overshoot" which is observed in the third recording upon darkening is left out of consideration here and will be discussed below.

TABLE I

DARK OXIDATION OF PNH AFTER ILLUMINATION OF *Rhodospirillum rubrum*

The measurements were done with malate-grown *R. rubrum*. Samples No. 1 and No. 3 were taken from two, different, 48-h cultures; No. 2 was taken from a 24-h culture. The experimental conditions were the same as in Fig. 1A. The time required for lowering the concentration of photoreduced PNH to half the value of that upon cessation of actinic illumination is given as the first half-time of the reaction. The second and third half-time indicate the time required to lower the concentration by a factor of 2 again. The wavelength of actinic illumination was 860 mμ for samples No. 1 and No. 2, while for No. 3 it was 838 mμ. The light intensities are given in 10^{-9} Einstein per sec·cm².

Sample No.	*Light intensity*	*Duration of illumination (sec)*	*Half-time for PNH oxidation (sec)*		
			First	*Second*	*Third*
1	10.3	12	5.0	4.8	
1	10.3	27	4.5	4.6	
1	5.6	6	4.2	5.5	
1	2.1	16	4.7	5.0	
1	2.1	30	4.1	4.8	
2	10.3	13	3.6	3.6	3.6
2	5.6	11	3.2	3.5	3.8
3	5.8	9	3.0	3.3	4.0
3	2.1	22	3.8	4.7	

Quantum requirement of PN reduction in different media

The quantum efficiency of the reduction, *i.e.* the number of equivalents of PN reduced upon absorption of one light quantum was calculated from the measured difference between the rates of absorption change before and after darkening, as described in the previous section (see ref. 9). A specific absorption coefficient of 6.22/cm/mM at 340 mμ (see ref. 31) was applied for PNH. Fig. 4 gives the quantum efficiency of the reduction of PN, obtained at three different light intensities, as a function of the time of illumination of the same suspension as that of Fig. 3.

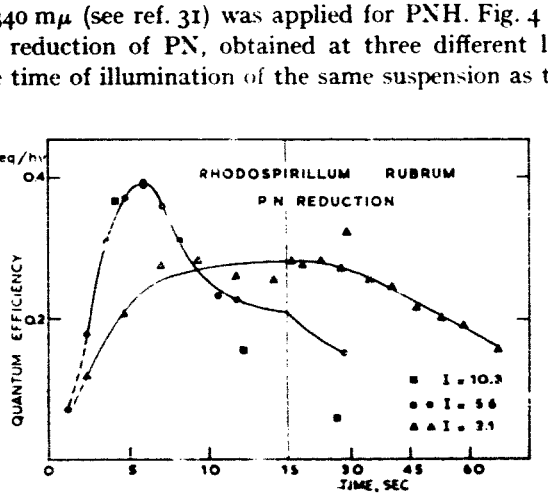

Fig. 4 The quantum efficiency of the reduction of PN in malate-grown *Rhodospirillum rubrum* as a function of the duration of illumination, measured at different light intensities (expressed in 10^{-9} Einstein/sec·cm²). The experimental conditions were the same as in Fig. 3. Solid squares, circles and triangles were obtained by measuring the slope of the recording before and after darkening as described in the text. O—O, and △—△, calculated for two recordings from the measured slope during illumination at the time indicated and from the rate-constant of the dark-oxidation of PNH.

The light-induced changes in absorption and fluorescence, of bacteria grown and suspended in acetate, butyrate or succinate medium were partly similar to those grown in malate. Like with bacteria grown in malate medium the rate of reduction of PN, and the amount of reduced PN, reached a maximum after some seconds of illumination and then gradually dropped to much lower values. However, for bacteria grown in butyrate medium a rapid reduction was observed immediately after onset of illumination. Bacteria grown in butyrate, transferred to an inorganic medium and supplied with H_2–CO_2 (95:5) for at least 1 h, reduced PN at a rate which was only little lower than that observed in nutrient medium.

The oxidation of PNH in the dark followed approximately the kinetics of a first order reaction with Rhodospirillum grown in acetate and succinate media. For bacteria grown in butyrate medium a higher order (between 1 and 2) was usually observed. However, the course of the reaction may have been influenced by a lack of homogeneity in the bacterial population.

The quantum efficiency observed was of the same order of magnitude for differently grown bacteria: quantum requirements for Rhodospirillum grown in malate and butyrate are summarized in Table II. Table II and Fig. 4, which show a decrease of the quantum efficiency after prolonged illumination, give data obtained for relatively short periods of illumination (of the order of 1 min or less). Experiments of longer duration suggested a much lower rate of light-induced reduction of PN after prolonged illumination of moderate intensity. After several minutes of illumination, in many experiments, no measurable absorption decrease was observed upon darkening. Sometimes small absorption shifts were observed, but it is uncertain whether these were caused by PNH or by another compound.

TABLE II

QUANTUM REQUIREMENT AT DIFFERENT LIGHT INTENSITIES OF LIGHT-INDUCED REDUCTION OF PN ON *Rhodospirillum rubrum*

Bacterial cultures grown in butyrate or malate medium for periods of 24 h or 48 h were used in a 4% suspension contained in a 1-mm vessel. The light intensities are given in 10^{-9} Einstein per sec·cm². The numbers between parentheses in the last three rows indicate the duration of the preceding illumination. As described in the text, the quantum requirements were calculated from the slope of the curves of absorption at 340 mμ against time just before, and after, darkening.

Sample	*Light intensity*	*Quantum requirement (hv/equiv.) and duration of illumination (sec)*		
1, Butyrate, 2 d.	9.1	7.0 (0)	5.5 (15)	
1, Butyrate, 2 d.	5.3	4.3 (0)	2.9 (3)	9.2 (92)
1, Butyrate, 2 d.	0.69	1.7 (0)	1.5 (4)	3.5 (197)
1, Butyrate, 2 d.	4.7	4.6 (0)	2.5 (6)	4.3 (18)
2, Butyrate, 2 d.	0.87	7.3 (0)	3.5 (10)	2.9 (45)
3, Malate, 1 d.	9.6	4.0 (5)	5.3 (7)	
3, Malate, 1 d.	2.5	5.1 (7)	3.7 (12)	4.4 (26)
3, Malate, 1 d.	0.76	9.5 (13)	13 (24)	
4, Malate, 2 d.	5.6	2.5 (6)	4.5 (12)	6.4 (24)

Reduction of PN by Rhodopseudomonas spheroides

Experiments on changes in fluorescence and absorption of *R. spheroides* upon illumination indicated a similar mechanism of PN-reduction as occurred with *R. rubrum*.

The kinetics of this reduction (Fig. 5) followed in principle the same pattern as and the maximum efficiency observed was about the same as, with Rhodospirillum. We did not measure precisely whether the rate of dark reoxidation depended only on the concentration of PNH as in Rhodospirillum. Measurements with bacteria, grown on the modified medium of COHEN-BAZIRE for 2 days (see METHODS) indicated a quantum requirement of 2.2 and 3.1 per equivalent after illumination for 11 sec and 36 sec respectively. The light intensity was $2.7 \cdot 10^{-9}$ Einstein/sec·cm^2 and the wavelength of illumination was 838 mμ in these experiments. Experiments with bacteria grown on malate medium indicated only a relatively low rate of light-induced reduction of PN after a prolonged period of illumination. Upon illumination with light of 860 mμ wavelength, and of an incident intensity of $3.8 \cdot 10^{-9}$ Einstein sec·cm^2, a maximum rate of reduction, as indicated by the rate of reoxidation upon darkening, was observed at about 7 sec. after the onset of illumination. After 1 min the rate was 27 %, and after 55 min 15 %, of this maximum rate.

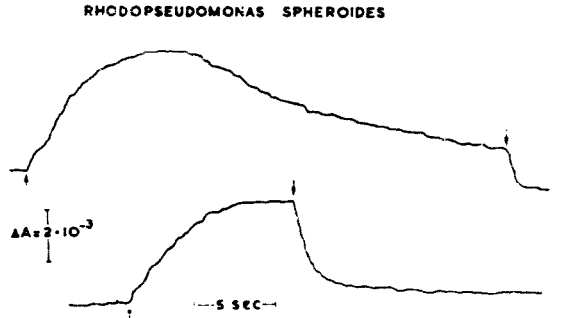

Fig. 5. Kinetics of light-induced changes in absorption at 340 mμ in *Rhodopseudomonas spheroides*. The bacteria were grown and examined in the modified medium of COHEN-BAZIRE *et al.*, as indicated in the text. Illumination was effected by a band around 838 mμ. The intensity was $10.9 \cdot 10^{-9}$ Einstein/sec·cm^2. Other experimental conditions were the same as those in Fig. 3.

The quantum requirement of cytochrome oxidation

Measurement of the initial rate of light-induced absorption decrease at 428 mμ in Rhodospirillum indicated a quantum requirement for cytochrome oxidation of about 3 to 4 quanta per equivalent upon illumination with light of 860 mμ. This number is only an approximate one: the measurements were less accurate than those obtained on reduction of PN because the absorption changes were smaller and more rapid than those at 340 mμ. The quantum requirement was independent of light intensity in the range of 0.3 to $3 \cdot 10^{-9}$ Einstein/sec·cm^2. A specific absorption coefficient[22] of 66 l/cm/mequiv. (reduced minus oxidized) for the cytochrome was used: the bacteria, taken from the same culture as sample 3 of Table II, were grown and examined in malate medium. With butyrate-grown Rhodospirillum, the same as sample 2 of Table II, roughly the same quantum requirement was indicated. At $1.3 \cdot 10^{-9}$ Einstein/sec·cm^2, the initial rate of increase in absorption at 428 mμ upon darkening after 2 sec to 30 min of illumination was found to be about twice as low as the initial rate of absorption decrease at this wavelength upon onset of illumination; at 420 mμ it was 1.2 times higher than the initial rate.

An interpretation of these results is difficult because of the complicated reaction

kinetics[33,34] and would require more experimental data. However, the experiments indicate that, in contrast to the reduction of PN, an efficient oxidation of cytochrome occurs immediately upon illumination, and that a high turnover-rate of *c*-, and *b*-type cytochromes is maintained during continued illumination.

Inhibition by HOQNO and fluoracetate

HOQNO (kindly given by Dr. J. W. LIGHTBOWN, London), an inhibitor of cyclic photophosphorylation[35,36], was found not to inhibit, or to inhibit only in part the light-induced reduction of PN in Rhodospirillum. Fig. 6 shows the rather complex results of some measurements with butyrate-grown bacteria. At 4 sec after the onset of illumination, the highest concentration applied ($1.2 \cdot 10^{-5}$ M) gave about 60% inhibition; after 15 sec no substantial effect was caused by any concentration of inhibitor. After 30 sec, HOQNO caused partial inhibition at low concentration and slight stimulation at high concentration.

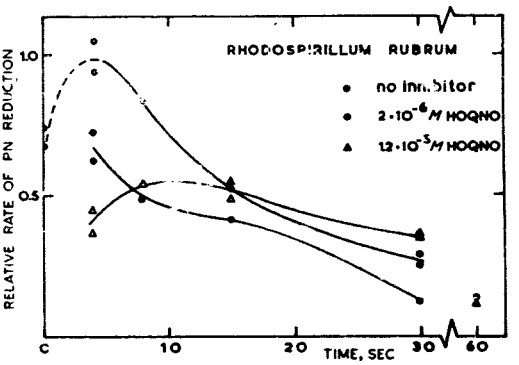

Fig. 6. Relative rates of light-induced reduction of PN in a 72-h culture of Rhodospirillum grown in butyrate medium and measured in the presence of different concentrations of HOQNO. The 0.3% suspension was contained in a 1-cm cuvette; the light intensity was $4.5 \cdot 10^{-9}$ Einstein/sec·cm^2. PNH was measured by the blue fluoresence, excited by light of 340 mμ.

In the presence of inhibitor, the reduction was less reproducible and more strongly dependent upon the preceding light–dark regime than with unpoisoned bacteria. However, in experiments with other samples of malate and butyrate-grown bacteria at most a partial inhibition was observed even at a concentration of 10^{-5} M or higher.

Fluoroacetate, an inhibitor of the aconitase reaction of the tricarboxylic acid cycle, has been reported to inhibit the photosynthetic assimilation of acetate and butyrate by *R. rubrum*, but only slightly that of malate and succinate; the oxidative dark assimilation of all these acids, however, is almost completely inhibited[37].

Measurements on the light-induced reduction of PN revealed only a slight inhibition by fluoroacetate in the presence of malate, acetate or butyrate. For example, a concentration of $1.6 \cdot 10^{-3}$ M gave about 30% inhibition of the maximum rate of PN-reduction by malate-grown *R. rubrum* and did not inhibit acetate-grown bacteria; a concentration of $5 \cdot 10^{-3}$ M fluoroacetate inhibited the reduction of PN by butyrate-grown bacteria by about 20%. The experiments were performed with a 48-h culture of bacteria, at a light intensity of $3.0 \cdot 10^{-9}$ Einstein/sec·cm^2. The bacterial suspension was incubated for at least 40 min with fluoroacetate before measurement.

Action-spectrum of the reduction of PN and of bacteriochlorophyll-fluorescence

In red and blue-green algae it was found that the action-spectrum of chlorophyll-fluorescence[38] is different from that of PN-reduction[9] or of cytochrome-oxidation[12,13,24]. It was concluded that the fluorescent chlorophyll *a* belongs mostly to a photosynthetic pigment system which is different from that causing both the reduction of PN and cytochrome oxidation. To determine whether the active absorption by the different types of bacteriochlorophyll and by the carotenoids is the same in effecting light-induced PN-reduction as in exciting bacteriochlorophyll fluorescence, points of the action spectra for both light processes in *R. spheroides* were determined. In order to minimize uncertainties due to light absorption by the pigments and to keep self-absorption of chlorophyll-fluorescence small, a dilute suspension (0.7 %) contained in a 1-mm vessel was used and the fluorescence was filtered by a filter transmitting above about 900 mμ. The detecting photomultiplier faced the same side of the vessel as the incident beam.

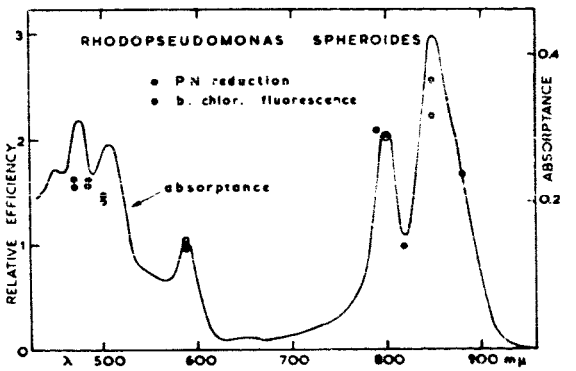

Fig. 7. A comparison of the efficiency of light of different wavelengths in causing the reduction of PN and bacteriochlorophyll-fluorescence in *Rhodopseudomonas spheroides*. The bacteria, grown and examined in butyrate medium, were in a 0.7 % suspension, contained in a 1-mm quartz vessel. The half-width of the exciting beams was about 7 mμ. The fluorescence points are the mean of at least two measurements. The relative efficiencies for both light processes are given in arbitrary units; the mean values at 590 mμ have been made to coincide.

PN-reduction was measured as absorption increase at 340 mμ. A monochromator with a xenon arc provided photosynthetic illumination. The same illumination was applied for exciting bacteriochlorophyl-fluorescence but in these experiments the light was chopped at 50 Hz.

The results are given in Fig. 7. The points are plotted as the reciprocal of the number of incident quanta per second required to bring about, in a certain time, the accumulation of a certain amount of PNH, or to excite a certain amount of fluorescence. It appears that the action-spectra of both processes are approximately proportional to each other in the spectral regions were bacteriochlorophyll and the carotenoids absorb. This indicates that in purple bacteria the same photochemical system of pigments is responsible for both processes.

The relatively high efficiency of carotenoids in effecting bacteriochlorophyll-fluorescence is in agreement with earlier measurements by GOEDHEER on the same species[39].

DISCUSSION

The results reported in this paper indicate that, in *R. rubrum* and *R. spheroides*, a large pool of PN is reduced upon illumination. The reduction is observed in bacteria, which have been grown and examined in organic media in the presence of various organic acids acids as substrate. However, a high efficiency of the reduction is only observed during a short period (about 0.5 min) at the beginning of an illumination period. The available evidence indicates that, after longer illumination, a considerable decrease of the efficiency occurs. In many experiments no indication for a light-induced reduction of PN was found after a few minutes of illumination.

The observation that in intact cells the reduction of PN also occurs in the presence of HOQNO indicates that the observed reduction is not an indirect effect caused by a light-induced ATP generation, but that the reduction results from a photochemical oxidation–reduction process in the chromatophore. An indirect reduction of PN, *e.g.* effected by ATP and a succinate–fumarate couple, discussed previously as a possibility[15,40] would be severely affected by HOQNO, which inhibits cyclic phosphorylation. Further indication that the reduction of PN is closely connected with the primary photochemical process is given by the observation that the rate and kinetics of the reaction are similar for bacteria grown and examined in the presence of different organic acids; the assimilation products[18], however depend upon, the kind of substrate used. Also the observation that the rate of reduction is about the same in the presence of fluoroacetate in either acetate or butyrate medium and in the presence of H_2, CO_2 and inorganic salts provides additional evidence.

It is possible that the light-induced reduction of PN, observed in intact cells, proceeds by means of the same mechanism as the reduction of added DPN in a cell-free preparation of chromatophores. The reduction by a preparation of chromatophores is also thought to result directly from a light-induced oxidation–reduction reaction[6,17,41].

The lowest quantum requirements for the reduction of PN, which have been observed during a period of illumination, were found to be about 2–3 per equivalent for different substrates. This quantum requirement is of the same order of magnitude as that observed for the reduction of 1 equivalent of CO_2 in the presence of various hydrogen donors by various photosynthetic bacteria including *R. rubrum*[42]. Also the quantum requirement found for the oxidation of a cytochrome in Chromatium[43] (which was about 1); for the oxidation of bacteriochlorophyll[44] (3.2 or less); and for the oxidation of cytochrome in Rhodospirillum (about 3–4, as reported in this paper) are of the same order of magnitude. This indicates that, during the period of maximum efficiency, a large part of the reducing equivalents or electrons which are produced by the primary photochemical reaction, reacts with PN.

The kinetics of absorption- and fluorescence-changes upon darkening, as illustrated in the third curve of Fig. 3, show that the reduction of PN goes on during a fraction of a second after the light is shut off. This indicates that the reduction of PN is not a primary photochemical process but that PN is reduced in a "dark" reaction with an unknown, reduced intermediate (*cf.* ref. 15). The same is also indicated by the temperature-dependence[45] of the rate of reduction at high light intensity.

In connection with the observation that HOQNO only partially inhibits the

reduction of PN in intact cells, recent findings of NOZAKI *et al.*[41] are of interest. These authors report that the reduction of DPN in a chromatophore preparation of *R. rubrum* was inhibited by HOQNO in the presence of succinate but not in the presence of a small amount of the "unphysiological" cofactor 2,6-dichlorophenol indophenol, and of ascorbate (as oxidizable substrate). This suggests that in the cell a compound other than succinate is oxidized, or that the preparation of the chromatophore was damaged or was deficient in some catalyst or substrate.

After prolonged illumination of intact cells, little or no reduction of PN was observed, in spite of the fact that the bacteria were suspended in media in which rapid growth, and thus photosynthesis, occurred in continuous light, and that the turnover of cytochrome still occurred at a high rate. This does not contradict the hypothesis that the major product of the light reaction is ATP, generated by cyclic phosphorylation. It is remarkable that such a considerable amount of PNH accumulates during the first seconds of illumination. For *R. rubrum*, the amount of PNH present in the cells, after a few seconds of illumination at a high intensity, was approx. 0.1 of the amount of bacteriochlorophyll (on a molar basis). Reduction-rates of about $5 \cdot 10^{-2}$ to $10 \cdot 10^{-2}$ μmoles of PNH/μmole of bacteriochlorophyll/sec were observed; these rates are about 10 times higher than those reported in cell-free preparations ($0.5–1.5 \cdot 10^{-2}$ μmoles DPNH/μmole bacteriochlorophyll/sec)[6,17,41].

The mechanism which slows down the reduction of PN after a short time of illumination is difficult to understand. It has been stated that the bacteria "need" only little light-driven reduction of PN for photosynthesis in the presence of organic substrate[18]. One would expect PNH to accumulate in the light, so the reduction of PN would be stopped by lack of PN and the utilization of light energy would be directed to cyclic ATP-production. However, the amount of PNH in the cell decreases after prolonged illumination, so that this simple explanation does not hold. It might be speculated that illumination causes an exhaustion of ATP in the cell, and an accumulation of ADP, which stimulates cyclic phosphorylation, and decreases the rate of reduction of PN. That cyclic phosphorylation and DPN reduction may be competitive reactions was demonstrated by FRENKEL in a cell-free extract[6]. It is also possible that the rate of cyclic phosphorylation is increased, and that of reduction of PN decreased, because the first reaction is stimulated by an accumulation of oxidized compounds, produced in the light. This accumulation could be caused by a relatively too slow dark reaction of, *e.g.*, an oxidized cytochrome. Some support for this hypothesis is given by the gradual accumulation of oxidized cytochromes which was observed upon continued illumination of intact *R. rubrum*[33,34], and which we found also under the conditions of our experiments. A similar effect was observed for the light-induced oxidation of bacteriochlorophyll[44].

Another possibility could be that there is a second, much smaller, pool of PN in the bacteria, which, unlike the large pool, has a high turnover-rate after a long period of illumination. This pool might cause changes in absorption which are too small to be identified and measured by the present method. This point is still being studied; at present the results do not yield convincing evidence for, or against, the hypothesis.

Since the fluorescence- and absorption- spectra of TPNH and DPNH are the same, it cannot be decided, from our experiments, whether TPN or DPN is reduced in the intact cell. In cell-free systems DPN is reported to be reduced[6,16,17,41] but there is some conflicting evidence on this point[41,44].

As shown in Fig. 7, in *R. spheroides* the activities of light, at various selected wavelengths, in effecting the reduction of PN, and in exciting bacteriochlorophyll-fluorescence, are approximately proportional to each other. This indicates that one photochemical pigment system is responsible for both light processes. This is in agreement with the hypothesis[13,46] that in purple and green bacteria, which unlike algae and higher plants are unable to evolve oxygen, only one pigment system is active in photosynthesis.

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