BBA 46037

LIGHT-INDUCED REDUCTION OF PYRIDINE NUCLEOTIDE AND ITS RELATION TO LIGHT-INDUCED ELECTRON TRANSPORT IN WHOLE CELLS OF *RHODOSPIRILLUM RUBRUM*

RAJNI GOVINDJEE AND CHRISTIAAN SYBESMA

Department of Physiology and Biophysics, University of Illinois, Urbana, Ill. 61801 (U.S.A.)

(Received June 15th, 1970)

SUMMARY

Fluorometric measurements of the light induced reduction of pyridine nucleotide (presumably NAD^+) were carried out in living cells of *Rhodospirillum rubrum*.

The rate *vs.* light intensity curves were different in cells from older cultures as compared with cells from young cultures. In young cells pyridine nucleotide was reduced only by higher intensities of the actinic light. The cytochrome oxidized at these intensities was cytochrome c_2 and the light-induced absorbance changes in the near-infrared spectral region showed only the photooxidation of P870. The uncoupler of photophosphorylation carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) inhibited the pyridine nucleotide photoreduction which could be restored partially by cysteine but not by O_2. In older cells pyridine nucleotide was reduced also at low intensities of the actinic light. If such cells were kept in the dark for a couple of days in a substrate-free medium, pyridine nucleotide was reduced efficiently in the light and the cytochrome predominantly oxidized, even at higher intensities, was C428. The light-*minus*-dark spectrum in the near-infrared spectral region showed few signs of the photooxidation of P870. Instead, a red shift of an absorption band at 880 nm was observed. FCCP inhibited the pyridine nucleotide photoreduction also under these conditions but the photoreduction could be restored by O_2.

The results were interpreted as indicating that this organism uses an energy-linked reversal of electron flow mediated by a high-energy intermediate produced during a light-induced cyclic electron transport when in an early stage of their development. Older cells can use an alternative mechanism for the reduction of pyridine nucleotide. This mechanism probably is a direct light-induced electron transport from exogenous as well as from endogenous substrates to the pyridine nucleotide. The switching over from one mechanism to another is related to the internal redox potential.

The present results support a different type of reaction center for each mechanism, although an interpretation based on one reaction center operating both electron transport systems cannot be ruled out.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

INTRODUCTION

Light-induced reduction of pyridine nucleotide, in particular NAD^+, has been observed in living cells of photosynthetic bacteria[1-3] and photoreduction of NAD^+ in cell-free preparations was shown to occur in the presence of substrates, such as succinate or reduced dichlorophenolindophenol[4-6]. There is, however, no general agreement about the mechanism of the photo-induced reaction as it occurs *in vivo*. NOZAKI *et al.*[5] proposed a direct reduction of pyridine nucleotide by non-cyclic light-induced electron flow. Based on studies with *Chromatium*, CHANCE AND OLSON[7] suggested that pyridine nucleotide in photosynthetic bacteria is reduced in an energy-linked reversed electron flow. This was not supported by the experiments of AMESZ[3] with whole cells of *R. rubrum* and *Rhodopseudomonas spheroides*. More recently, however, KEISTER AND YIKE[8] showed that in chromatophore preparations from *R. rubrum*, the succinate-coupled reduction of NAD^+ was an energy-dependent reversal of electron flow in which ATP in the dark could fulfill, at least partially, the function of light as an energy source. Their conclusion, supported by experiments of JACKSON AND CROFTS[9] whith whole cells of *R. rubrum*, was that light-induced pyridine nucleotide reduction is a reversal of electron flow linked to the energy from ATP which is generated in light-induced cyclic electron transport reaction.

Recent experiments of KLEMME AND SCHLEGEL[10] showed that in *Rhodopseudomonas capsulata* chromatophores the light-induced reduction of NAD^+ had different sensitivities to inhibitors of electron transport when different donor systems were used; dichlorophenolindophenol (reduced by an excess of ascorbate) or H_2, mediated a much more resistant NAD^+ reduction than succinate did. Moreover, autotrophically grown cultures showed much higher rates of NAD^+ reduction than heterotrophically grown cultures[11].

Results like these and the apparent functioning of non-cyclic electron transport components in a number of photosynthetic bacteria (*cf.* ref. 12) possibly driven by a separate light reaction[12-15], motivated us to reinvestigate this problem. In the present paper we wish to report some experiments on the light-induced reduction of pyridine nucleotide and its relation to the oxidation of cytochromes and reaction center bacteriochlorophyll. The experiments were carried out under different conditions with living cells of *R. rubrum* from cultures of different ages. The results seem to suggest that the *R. rubrum* cells can make use of either of the two mechanisms, cyclic and non-cyclic, for the light-induced reduction of pyridine nucleotide, dependent on conditions and stage of development (*cf.* ref. 16).

MATERIALS AND METHODS

R. rubrum was grown as described previously[13]. The closed culture bottles were rotated on a roller system below a bank of incandescent lamps, in order to obtain a near homogeneous illumination of the cells. Cells from young (1–2-day-old) cultures or from old (3–5-day-old) cultures were harvested by centrifugation and resuspended in either substrate-(malate and glutamate)-containing or substrate-free media.

The light-induced reduction of pyridine nucleotide was measured by recording the increase of the fluorescence yield at 450 nm upon illumination with (near infrared) actinic light. The fluorescence was excited by 366-nm light from a Xenon–mercury

arc lamp (Hanovia 901B) and a Bausch and Lomb 500-mm, 600 grooves/mm monochomator. The light beam was chopped at a frequency of 150 Hz before it reached the sample. The emitted light was collected from the same surface of the cuvette *via* a filter combination and a Bausch and Lomb 500-mm, 1200 grooves/mm monochromator by an Amperex AVP58 photomultiplier. The entrance and exit slits of the monochromator were set at 10 mm. The output of the muliplier was connected to a PAR Model 120 lock-in amplifier set at the chopping frequency. Actinic light was obtained from a d.c.-fed 300-W projector lamp. Its wavelength was selected by Farrand interference filters.

The light-induced increase of the intensity of the emission at 450 nm excited by the chopped 366-nm light, thus is proportional to an increase of the fluorescence yield. Since reduced pyridine nucleotide is fluorescent and oxidized pyridine nucleotide is not, the yield increase, in this case, is proportional to the increase of the concentration of reduced pyridine nucleotide. Measurements of the spectra of the emission and of the light-induced increase in emission intensity which yielded results similar to the spectra published by OLSON[2] verified this.

Light-induced changes in absorbance were measured by a split-beam differential spectrophotometer described earlier[13]. The same samples were used for the measurements in both the fluorometer and the differential spectrophotometer. All measurements were made at room temperature.

RESULTS

There is a distinct difference in the response to light of *R. rubrum* cells from young (1–2-day-old) cultures as compared to that of cells from older (3–5-day-old) cultures. This is true not only for the light-induced reduction of pyridine nucleotide, but also for the light-induced oxidation of cytochromes and reaction center bacteriochlorophyll.

In cells from young cultures, pyridine nucleotide is reduced only by high in-

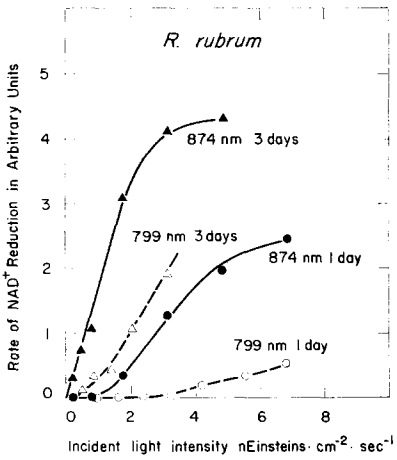

Fig. 1. Rate of light-induced reduction of pyridine nucleotide as a function of the incident light intensity in cell suspensions from 1- and 3-day-old cultures of *R. rubrum*. The absorbance of all suspensions at 883 nm was about 0.8.

tensities of the actinic light while in cells from older cultures, the pyridine nucleotide is reduced effectively also at low intensities. This is illustrated in Fig. 1; the rate of pyridine nucleotide reduction in cells from young cultures, plotted as a function of the intensity of the actinic light followed sigmoid curves, not only for the two wavelengths shown, but for all wavelengths of the actinic light used, ranging from 780 to 930 nm. In cells from older cultures, however, the intensity curves for the rate of light-induced pyridine nucleotide reduction were initially linear, except, perhaps when excited in the 800-nm absorption band.

In fresh cells harvested from young cultures, the absorbance changes observed in the Soret spectral region as a response to low-intensity light (0.5 nEinstein$\cdot$cm$^{-2}\cdot$sec^{-1} at 874 nm) were due to the oxidation of the cytochrome-like pigment C428 (ref. 13). At higher intensities (about 1.5 nEinsteins$\cdot$cm$^{-2}\cdot$sec^{-1} at 874 nm) at which reduction of pyridine nucleotide started to show up, the Soret absorbance changes indicated the oxidation of predominantly one cytochrome with the characteristics of cytochrome c_2 (*cf.* ref. 13). In the near-infrared spectral region, actinic light at such intensities induced a light-*minus*-dark difference spectrum indicative of the oxidation of reaction center bacteriochlorophyll P870. Such spectra are characterized by a bleaching centered at about 870 nm and the blue shift of an absorption band at about 800 nm (ref. 17).

In cells from older (4–5-days) cultures, the light-induced absorbance changes in the Soret spectral region showed the oxidation of only one cytochrome-like pigment, C428, at low intensities ($<$ 0.5 nEinstein$\cdot$cm$^{-2}\cdot$sec^{-1}) of the actinic light. The absorbance changes in the near-infrared spectral region were very small at these intensities. At an increased intensity, the absorbance changes, still for a large part due to the oxidation of C428, started to include the contribution of the oxidation of another cytochrome, presumably cytochrome c_2. The spectral shifts in the near-infrared spectral region induced by such intensities indicated, again, the oxidation of the reaction center component P870.

Cells from older cultures lost all their capability to reduce pyridine nucleotide in the light when left for 1 or 2 days anaerobically in the dark in a substrate-containing medium. Restoration of the light-induced pyridine nucleotide reduction could be obtained only by making the suspensions slightly aerobic. The cytochrome oxidized in the light under these conditions was predominantly C428 as shown in Fig. 2A. The light-induced absorbance changes in the near-infrared spectral region constituted a difference spectrum shown in Fig. 2B; in addition to small changes in the 800-nm band there is a bleaching centered at about 870 nm and an increase in absorption at about 905 nm.

Pyridine nuleotide was reduced very effectively by light in cell suspensions from older cultures which were left for a couple of days in the dark in a substrate-free medium. The drawn lines in Figs. 3A and 3B show the light-*minus*-dark difference spectra measured in these suspensions in the Soret and the near-infrared spectral regions, respectively. The cytochrome oxidized predominantly in the light is C428. The near-infrared spectrum had hardly any shift in the 800-nm spectral region but suggested a red shift of an 880-nm absorption band. If such suspensions were made aerobic no light-induced reduction of pyridine nucleotide could be observed anymore. Illumination immediately after the aeration caused large changes in the Soret spectral region, due to the unspecified pigment or complex of pigments called P435 (refs.

18, 19). About 1 min after the aeration, however, when the light-induced reduction of pyridine nucleotide still was severely inhibited, the light-induced absorption changes in the Soret spectral region had the spectrum given by the dashed line in Fig. 3A. As could be expected, little light-induced oxidation of C428 could be observed because this cytochrome-like pigment is oxidized by O_2. The spectrum did show an oxidation of cytochrome c_2, however. As shown by the dashed line in Fig. 3B aerobic conditions also seemed to have replaced the light-induced red shift of the 880-nm absorption band by a bleaching centered at about 860 nm and a blue shift of an 800-nm band; this difference spectrum has the appearance of an oxidized-*minus*-reduced difference spectrum of the reaction center component P870 (ref. 17). When subsequently the suspension was allowed to become anaerobic again, the ability to reduce pyridine nucleotide by light was restored and the light-induced difference in the absorption showed the spectra given by the drawn lines in Figs. 3A and 3B.

The photophosphorylation uncoupler FCCP had a pronounced effect on the light-induced reduction of pyridine nucleotide. The results of these experiments are illustrated in Fig. 4. In cells from young cultures the uncoupler caused 100% inhibition of the reaction at concentrations as low as 0.5 μM. As shown in Fig. 4IB, O_2

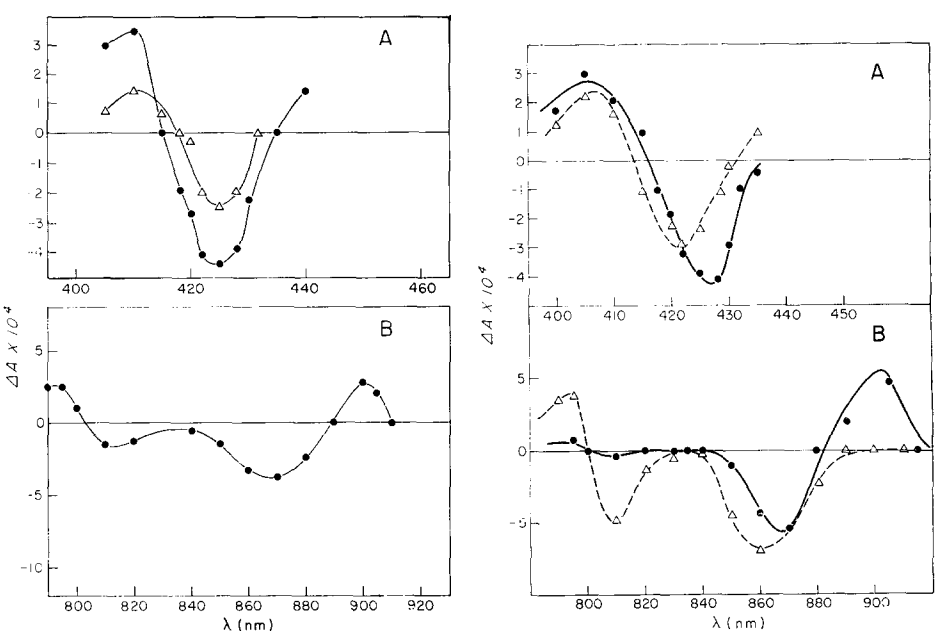

Fig. 2. Light-*minus*-dark difference spectra of absorbance changes in cell suspensions from 4-day-old cultures of *R. rubrum*. A. Absorbance changes induced by 880-nm actinic light: △—△, with an intensity of 0.3 nEinstein·cm^{-2}·sec^{-1}; ●—●, with an intensity of 1.5 nEinstein·cm^{-2}·sec^{-1}. The absorbance of the suspension at 883 nm was about 0.8. B. Absorbance changes in the near-infrared spectral region induced by 586-nm actinic light at an intensity of 1.3 nEinstein·cm^{-2}·sec^{-1} The absorbance of the suspension was about 0.8.

Fig. 3. Light-*minus*-dark difference spectra of absorbance changes in a substrate-depleted cell suspension from a 4-day-old culture of *R. rubrum*. The absorbance of the suspension at 883 nm was about 0.8. A. ●—●, absorbance changes induced by 880-nm light with an intensity of 1.5 nEinstein·cm^{-2}·sec^{-1}; △—△, same 1 min after aeration. B. ●—●, absorbance changes in the near-infrared spectral region induced by 586-nm actinic light at an intensity of 1.3 nEinstein·cm^{-2}·sec^{-1}; △---△, same 1 min after aeration.

had little or no effect on the inhibition. Addition of cysteine, which is known to restore photophosphorylation[20] restored the light-induced reduction of pyridine nucleotide to about 25% (Fig. 4ID). In cells from older cultures (Fig. 4II), FCCP inhibited the light-induced pyridine nucleotide at slightly higher concentrations (about 1 μM) but a partial restoration of the reaction could be achieved by the addition of air (Fig. 4IIC). Although the capability for a restoration diminished in time and disappeared about 10 min after the aeration, restoration could be brought back again by a renewed aeration. Such a repeating restoration of the light-induced pyridine nucleotide reduction in FCCP-containing cell suspensions could not be obtained in cells fresh from 1- or 2-day-old cultures. The light-induced oxidation of cytochrome C428 was little affected by the used concentrations of FCCP in the aged cells.

Antimycin A, in concentrations ranging from 1 to 4 μM had effects very much similar to FCCP; addition to 4 μM antimycin A to aged cell suspensions resulted in a complete inhibition of light-induced pyridine nucleotide reduction under anaerobic conditions. Aeration caused a restoration of the capability for light-induced pyridine nucleotide reduction. In about 5 min, this capability was lost again but could be brought back by renewed aeration. Light-induced absorbance changes in the near-infrared spectral region could be observed only under aerobic conditions; they show a red shift of an 880-nm band as observed in uninhibited aged cells.

2-Heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) had a quite different effect. It inhibited irreversibly the light induced pyridine nucleotide reduction at a concen-

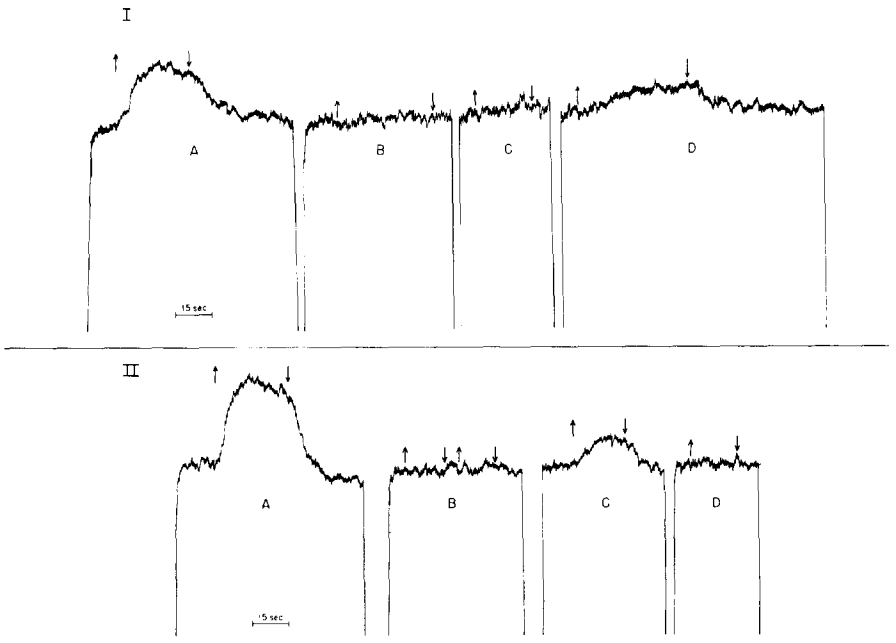

Fig. 4. Time-courses of light-induced reduction of pyridine nucleotide in whole cells of *R. rubrum*. The intensity of the actinic light at 874 nm was 1.5 nEinstein·cm^{-2}·sec^{-1}. I. Cell suspension from a 1-day-old culture; A, without additions; B, 1.2 μM FCCP added; C, the same as B after aeration; D, the same as B with cysteine added. II. Cell suspensions from a 4-day-old culture; A, without additions; B, 1.2 μM FCCP added; C, the same as B after aeration; D, the same as C after 6 min dark.

tration of about 5 μM under all conditions. As shown in Fig. 5, this concentration of HQNO inhibited the light induced oxidation of C428 but enhanced the light induced oxidation of cytochrome c_2. Such phenomena have been reported before[21].

DISCUSSION

The present results seem to indicate that different mechanisms for the photoreduction of pyridine nucleotide are available to *R. rubrum* whole cells and that the predominance of one of these mechanisms depends on the stage of development of the cell. Thus, in younger cells the photoreduction of pyridine nucleotide seemed to be linked to the light-induced oxidation of both, the reaction center component P870 and cytochrome c_2. Following suggestions made earlier[13,14] these components are part of a cyclic electron transport chain and photoreduction of pyridine nucleotide could very well be by a reversed electron flow mediated by the high energy intermediate (or ATP) generated in the light-induced cyclic electron transport[7,8]. This is supported by the effect of uncouplers such as FCCP on the younger cells; pyridine nucleotide reduction is inhibited and can be restored by cysteine but not by O_2.

In older cells another mechanism becomes available. This mechanism, related to the oxidation of the cytochrome like component C428 could be a non-cyclic electron flow leading to a direct light-induced reduction of the nucleotide. The fact that a part of the light-induced pyridine nucleotide reduction could be restored repeatedly in FCCP-inhibited older cells by adding O_2 indicated that FCCP inhibition is, at least in part, indirect. If one assumes that the carbon assimilation in this organism

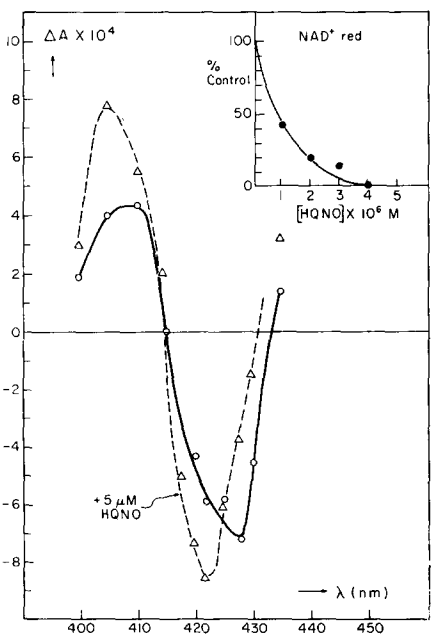

Fig. 5. Effect of HQNO on the lights-*minus*-dark absorption difference spectrum induced by 874-nm actinic light with an intensity of 1.5 nEinstein·cm^{-2}·sec^{-1} in cell suspensions from a 5-day-old culture of *R. rubrum*. ○—○, without addition; △---△, 5 μM HQNO added. Insert: the inhibition of light-induced pyridine nucleotide reduction by HQNO.

requires both ATP and reduced pyridine nucleotide, the inhibition of the ATP formation can lead to an accumulation of the reduced form of the pyridine nucleotide. The aeration then reoxidizes part of the pyridine nucleotide, thus making the light reaction reversible.

When cell suspensions are stored in the dark the latter mechanism for the photoreduction of pyridine nucleotide seems to become an exclusive one. This can be visualized as a result of a lowering of the internal redox potential. There is a recent report[22] about cell suspensions of *R. rubrum* which could develop internal redox potentials as low as −200 mV. If the storage was in a substrate-containing medium, the potential became so low that all light-induced reactions disappeared. Both the light-induced pyridine nucleotide reduction and the light-induced oxidation of cytochromes predominantly the cytochrome-like pigment C428 could be brought back by increasing the potential slightly with air (the addition of more O_2 will oxidize C428 and therefore abolish its light-induced reaction). If the storage was in a substrate-free medium, the redox potential became low, but to a level still high enough to allow both a very efficient light-induced reduction of the pyridine nucleotide, and a light-induced oxidation of C428. Introduction of O_2 in these cell suspensions immediately increased the potential to such a level that C428 became oxidized and the non-cyclic reaction inoperative. However, cytochrome c_2 as well as P870 still could be oxidized by light, under these conditions, although the light-induced reduction of pyridine nucleotide was inhibited. It seems as if these cells were impeded in their capability to reduce pyridine nucleotide by energy-linked reversed electron flow in the light although they still were able to carry out light-induced cyclic electron flow when the internal redox potential was in a proper range of values. The mechanism of the light-induced pyridine nucelotide reduction in these cells, when stored in the dark, therefore seemed to involve almost exclusively a non-cyclic system including the cytochrome-like component, C428.

Recent studies on light-induced reactions of cytochromes and reaction center bacteriochlorophyll in *R. rubrum* whole cells[13–15] have made a case for a model in which the cyclic and the non-cyclic electron transport are driven each by their own reaction center. Studies on bacteriochlorophyll fluorescence induction in synchronous cultures of several species of bacteria including *R. rubrum*[16] also suggested the operation of two photochemical systems, one cyclic and predominant in young cells, the other non-cyclic and predominant in old cells. Thus, it may be that the two mechanisms for the photoreduction of pyridine nucleotide in the cells from the (non-synchronous) cultures used in the present studies are operated by different photochemical systems. In the stored suspensions of cells from older cultures, in which pyridine nucleotide is reduced predominantly by a direct light-induced electron transport, the light-induced near-infrared absorbance changes, instead of showing the characteristics of a P870 oxidation, seemed to include a red shift of an 880-nm absorption band. This red shift, whatever its spectral origin, could reflect the oxidation of a bacteriochlorophyll component P' of a second reaction center. The fact that a similar spectral shift can be induced by chemical oxidation at a lower environmental potential in *R. rubrum* chromatophore fractions[23] supports this. The possibility, however, that this spectral shift is a phenomenon which is not directly related to the primary reaction cannot be ruled out.

Fig. 6 summarizes the interpretation of the present results under the assump-

tion that the non-cyclic flow is mediated by a reaction center different from the one which initiates the cyclic electron flow. The thin lines indicate the electron transport induced by light in cells which are in an early stage of their development. The pyridine nucleotide (presumably NAD^+) is reduced predominantly by a reversal of electron flow mediated by high-energy intermediates produced in a light-induced cyclic electron flow. The internal redox potential is high, probably between + 100 and +300 mV. In a further stage the cells develop a lower internal redox potential which allows a non-cyclic (terminal) flow of electrons to be sustained even in the absence of exogenous substrate. An alternate reaction for the reduction of pyridine nucleotide, indicated by bold lines in Fig. 6 becomes available to the cells. By "forced aging" (leaving the cell suspensions in the dark for a couple of days) the internal redox potential can develop to such low levels that the cyclic electron transport system stops functioning possibly because of the reduction of the primary acceptor X. The non-cyclic flow mediated by a reaction center component P' then becomes the predominant pathway for the light-induced reduction of pyridine nucleotide.

Antimycin A, an electron transport inhibitor, seemed to inhibit predominantly the cyclic electron transport. This is indicated by its indirect effect on (part of) the light-induced pyridine nucleotide reduction. HQNO, however, appeared to inhibit also the non-cyclic electron flow. This inhibitor acted very much like phenyl mercuric acetate[14].

The possibility that electron transport in both pathways is initiated by one type of reaction center including P870 cannot be ruled out. The light-induced oxidation of C428, even at high intensities, in cells fresh from older cultures which occurred concomitantly with P870 oxidation would support that. A very rapid reaction between the non-cyclic electron transport components and P870 then could explain the failure to detect any reaction of P870 at lower redox potentials. Such a system

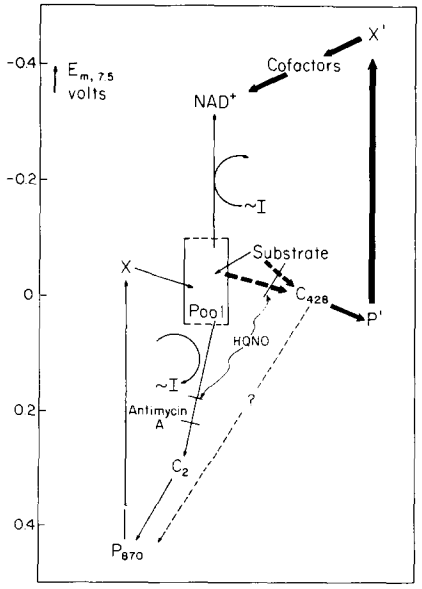

Fig. 6. Simplified diagram of a model for photosynthetic electron transport in *R. rubrum*.

would be consistent with the interpretation which PARSON AND CASE[24] have given to their and other[25] experimental results with *Chromatium*. It would be difficult, however, to visualize in such systems how changes in the internal redox potential can switch the reduction of the pyridine nucleotide from one mechanism involving one cytochrome to another, involving another cytochrome.

ACKNOWLEDGMENTS

We thank Mrs. Beverley Kokjer for her technical assistance and Dr. C. F. Fowler for helpful discussions. This research was supported by National Science Foundation grant GB No. 15777.

REFERENCES

1 L. N. M. DUYSENS AND G. SWEEP, *Biochim. Biophys. Acta*, 14 (1957) 1.
2 J. M. OLSON, *The Photochemical Apparatus, Its Structure and Function, Brookhaven Symp. Biol.*, 11 (1958) 316.
3 J. AMESZ, *Biochim. Biophys. Acta*, 66 (1963) 22.
4 L. P. VERNON AND O. K. ASH, *J. Biol. Chem.*, 235 (1960) 2721.
5 M. NOZAKI, K. TAGAWA AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1334.
6 S. L. HOOD, *Biochim. Biophys. Acta*, 88 (1964) 461.
7 B. CHANCE AND J. M. OLSON, *Arch. Biochem. Biophys.*, 88 (1960) 54.
8 D. L. KEISTER AND N. J. YIKE, *Arch. Biochem. Biophys.*, 121 (1967) 415.
9 J. B. JACKSON AND A. R. CROFTS, *Biochem. Biophys. Res. Commun*, 32 (1968) 908.
10 J. H. KLEMME AND H. G. SCHLEGEL, *Arch. Mikrobiol.*, 63 (1968) 154.
11 J. H. KLEMME AND H. G. SCHLEGEL, *Arch. Mikrobiol.*, 59 (1967) 185.
12 G. HIND AND J. M. OLSON, *Ann. Rev. Plant Physiol.*, 19 (1968) 249.
13 C. SYBESMA AND C. F. FOWLER, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 1343.
14 C. SYBESMA, *Biochim. Biophys. Acta*, 172 (1969) 177.
15 C. SYBESMA AND B. KOK, *Biochim. Biophys. Acta*, 180 (1969) 410.
16 H. DE KLERK, R. GOVINDJEE, M. D. KAMEN AND J. LAVOREL, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 972.
17 L. N. M. DUYSENS, *Transfer of Excitation Energy in Photosynthesis*, Ph. D. Thesis, Utrecht, 1952.
18 L. N. M. DUYSENS, in H. GAFFRON, *Research in Photosynthesis*, Interscience, New York, 1957, p. 164.
19 C. SYBESMA, in H. METZNER, *Progress in Photosynthesis Research*, Göttingen, 1969, p. 1091.
20 D. Y. DE KIEWIET, D. O. HALL AND E. L. JENNER, *Biochim. Biophys. Acta*, 109 (1965) 284.
21 L. SMITH AND M. BALTSCHEFFSKY, *J. Biol. Chem.*, 234 (1959) 1575.
22 W. A. CRAMER, *Biochim. Biophys. Acta*, 189 (1969) 54.
23 C. F. FOWLER AND C. SYBESMA, *Biochim. Biophys. Acta*, 197 (1970) 276.
24 W. W. PARSON AND G. D. CASE, *Biochim. Biophys. Acta*, 205 (1970) 232.
25 M. SEIBERT AND D. DEVAULT, *Biochim. Biophys. Acta*, 205 (1970) 246.