THE PHOTOREDUCTION OF NICOTINAMIDE-ADENINE DINUCLEOTIDE BY CHROMATOPHORE FRACTIONS FROM *RHODOSPIRILLUM RUBRUM*

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ABSTRACT The photoreduction of nicotinamide-adenine dinucleotide (NAD$^+$), catalyzed by chromatophore fractions from young (1 day) and old (4–5 days) cultures of *Rhodospirillum rubrum*, was measured in the presence of either succinate or 2,6-dichlorophenol indophenol (DPIP) and an excess of ascorbate. The time-course of photoreduction in the succinate system suggested a "reversed electron flow" from the donor to NAD$^+$ mediated by a high energy intermediate produced by a light-induced, cyclic electron transport in the chromatophore fractions. The effects of the uncoupler carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone (FCCP) and of the inhibitors antimycin A and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) were consistent with this interpretation. The time-course of NAD$^+$ photoreduction in the presence of DPIP and ascorbate suggested a direct, light-induced electron transport from the donor to the acceptor. We cannot yet distinguish between a model in which the same reaction center is utilized in the photoreduction by both donor systems (the reaction center component P-870 may relate to two primary acceptors at different redox potential levels) and a model in which each photoreducing system is driven by its own reaction center component.

INTRODUCTION

Fluorometric measurements of the photoreduction of pyridine nucleotide (presumably NAD$^+$) in whole cells of the nonsulfur photosynthetic bacterium *R. rubrum* (R. Govindjee and Sybesma, 1970) have suggested that two different mechanisms for this photoreduction are available to the cells. One is a "reversal" of electron flow mediated by a high energy intermediate produced in a light-induced, cyclic electron transport (Chance and Olson, 1960; Bose and Gest, 1962; Keister and Yike, 1967). The other is a direct, light-induced electron transport from an exogenous or an endogenous electron donor to pyridine nucleotide. The former mechanism seemed to predominate in cells which are in an early stage of development, while the latter was more predominant in older cells. Such a dependency on age was reported also by DeKlerk et al. (1969), who concluded from measurements of the variability of bacteriochlorophyll *a* (BChl *a*) fluorescence in synchronized cultures of various purple bacteria that in young cells light is driving almost exclusively a cyclic electron flow, whereas in older cells a noncyclic, light-induced electron transport is more manifest.

Photoreduction of added pyridine nucleotide (in particular NAD^+) in cell-free preparations from a variety of organisms in the presence of substrates such as succinate or reduced DPIP has been reported by several investigators (cf. Sybesma, 1970). Experiments of Keister and Yike (1967) have indicated that in the presence of succinate, NAD^+ is photoreduced by an energy-linked, reversed electron flow; adenosine triphosphate (ATP) as well as pyrophosphate can cause a reduction of NAD^+ in the dark in such systems. Frenkel (1958) had shown earlier that in the presence of adenosine diphosphate (ADP) and inorganic phosphate, the photoreduction of NAD^+ is inhibited.

A noncyclic electron flow from reduced dyes such as $DPIPH_2$ to NAD^+ has been proposed by Nozaki et al. (1961). Vernon (1963) observed the photooxidation of reduced DPIP and of reduced methylene blue catalyzed by chromatophore fractions from *R. rubrum.*

Recently, Jones and Vernon (1969) reported the photoreduction of NAD^+ by *R. rubrum* chromatophore fractions using succinate or *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) plus ascorbate as substrates. The experiments reported in the present communication do not seem to support their conclusion that in both substrate systems the photoreduction of NAD^+ occurred by energy-linked, reversed electron flow. The time-courses of the photoreduction of NAD^+ in the presence of the different substrate systems and the differential action of inhibitors and the uncoupler FCCP rather suggested different mechanisms for the light-induced reduction of NAD^+.

MATERIALS AND METHODS

R. rubrum strain IV (Giesbrecht) was grown as described previously (Sybesma and Fowler, 1968). Aliquots from 1-day-old cultures (defined as "young") or 5–6-day-old cultures (defined as "old") were taken and "classical" chromatophore fractions were prepared by a method essentially similar to that used by Cusanovich and Kamen (1968). Concentrated suspensions of the chromatophore fractions in sucrose phosphate buffer were stored under vacuum in a refrigerator.

The reaction mixture for the measurements of the NAD^+ photoreduction contained 57 mM Tris buffer, pH 8.0, 0.85 mM $MgCl_2$, 7 mM K_2HPO_4, 6.5 mM NAD^+, and 0.3 mg/ml bovine serum albumin. Either 3.2 mM succinate or 70 μM DPIP in 50 mM ascorbate was added as the substrate system. The added chromatophore fraction suspension resulted in an optical density of approximately 0.8 at 880 nm in a 1 mm path length measuring cuvette.

NAD^+ photoreduction was measured by recording the light-induced increase in absorbance at 340 nm, in a split-beam difference spectrophotometer (Sybesma and Fowler, 1968). The same instrument was used for measuring the light-induced changes in absorbance in the near infrared and the visible region. All measurements were made in stoppered cuvettes; no special effort was made to make the samples anaerobic.

RESULTS AND INTERPRETATIONS

Chromatophores from Young Cultures

Chromatophore fractions from 1-day-old cultures were able to catalyze the photoreduction of NAD^+ with both succinate and DPIP + ascorbate; however, the kinetics in the two systems are very different. With succinate as the electron donor, the light-induced reaction appeared to be reversible, reaching a steady-state level in approximately 30–40 sec (Fig. 1). Such a time-course can be explained by assuming that the photoreduction occurred by an energy-linked, reversed electron flow,

$$\text{succinate} + NAD^+ \xrightleftharpoons{\sim I} \text{fumarate} + NADH, \qquad (1)$$

mediated by a high energy intermediate ($\sim I$) produced during a cyclic, light-induced electron transport (Chance and Hollunger, 1960). From the data of Fig. 1 a difference in free energy change between the succinate-fumarate couple and the NAD^+-NADH couple was estimated to be about 230 mv. Addition of 0.8 mM ADP to the

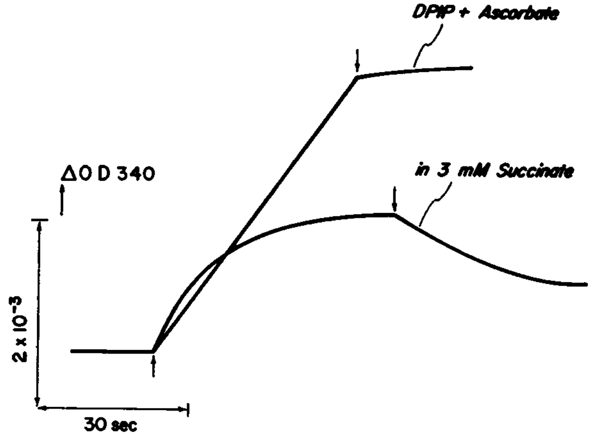

FIGURE 1 Time-course of the light-induced change in the absorption at 340 nm in chromatophore fractions from a 1-day-old culture of *R. rubrum* in the presence of succinate and DPIP + ascorbate. Reaction mixture: see text. The intensity of the actinic light at 880 nm was 3.5 nanoeinsteins $\mathrm{cm}^{-2}\,\mathrm{sec}^{-1}$. ↑ indicates when actinic beam was turned on and ↓ when it was turned off. Rate of NAD^+ reduction for DPIP + ascorbate = 8 μmoles/mg BChl per hr and for the succinate system the initial rate = 10 μmoles/mg BChl per hr.

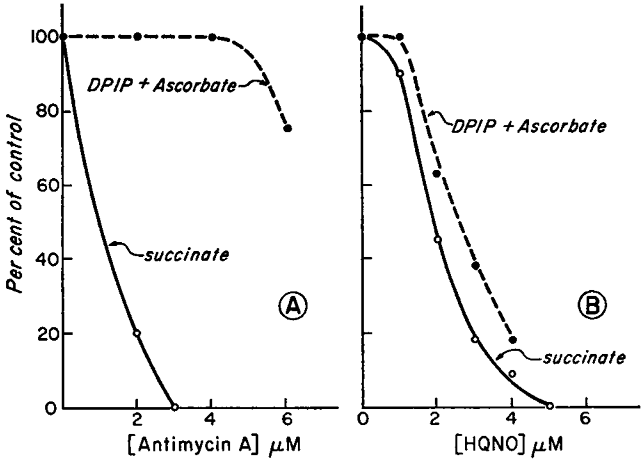

FIGURE 2 The effect on the rate of the light-induced change in the absorption at 340 nm in chromatophore fractions from a 1-day-old culture of *R. rubrum* by (A) antimycin A and (B) HQNO, in the presence of succinate (solid lines) or in the presence of DPIP and ascorbate (dashed lines). Reaction mixture: see text.

phosphate-containing medium resulted in an effect up to about 20% of the control; this indicated that phosphorylation competed with the reduction of NAD^+ in this system. An excess of fumarate drove reaction 1 to the left. Antimycin A inhibited the reaction (Fig. 2 A, solid line) as did HQNO (Fig. 2 B, solid line).

When DPIP and ascorbate were used as the donor system, light-induced NAD^+ reduction proceeded at a constant rate and irreversibly (Fig. 1). The $DPIPH_2$-mediated NAD^+ reduction was far less affected by ADP at the concentration used. (The maximum effect observed was up to about 80% of the control.) Antimycin A was quite ineffective (Fig. 2 A, dashed curve) but HQNO caused an inhibition (Fig. 2 B, dashed curve). The time-course observed in Fig. 1 can be explained by assuming a direct, noncyclic reduction of the nucleotide by $DPIPH_2$. The effects of antimycin A and HQNO with the two-donor systems were consistent with what had been demonstrated in the whole cells of *R. rubrum* (R. Govindjee and Sybesma, 1970); antimycin A affected only the cyclic, light-induced electron transport system while HQNO affected both the systems.

Fig. 3 shows the effect of different concentrations of FCCP on the rate of the NAD^+ photoreduction by the two-donor systems. Although both systems seemed to be affected, the succinate system was inhibited almost completely at concentrations of about 1 μM while the DPIP + ascorbate system remained at a level of about 60% of the control.

No light-induced cytochrome reactions could be detected in the presence of any of the two-donor systems (cf. Fowler and Sybesma, 1970). Fig. 4 shows the light minus dark difference spectra in the near infrared region with the two-donor systems. In the presence of DPIP and ascorbate, the absorption changes were much smaller than in the presence of succinate. The spectral characteristics were the same, in both cases, demonstrating a light-induced oxidation of the reaction center component P-870.

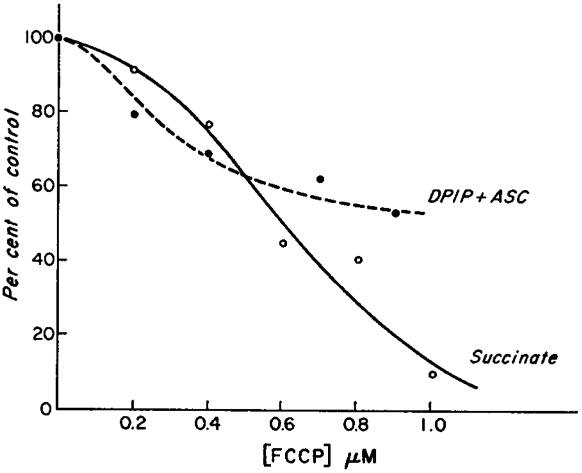

FIGURE 3 The effect of FCCP on the rate of the light-induced change in absorption at 340 nm in chromatophore fractions from a 1-day-old culture of *R. rubrum* in the presence of succinate (solid line) or in the presence of DPIP and ascorbate (ASC) (dashed line). Reaction mixture: see text.

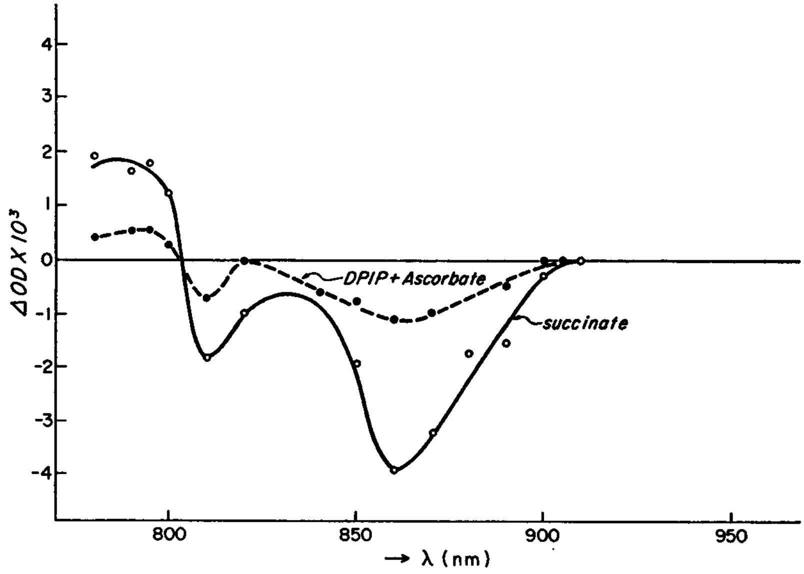

FIGURE 4 Light minus dark difference spectra measured in chromatophore fractions from a 1-day-old culture of *R. rubrum* in the presence of succinate (solid line) or in the presence of DPIP and ascorbate (dashed line). Reaction mixture: see text. The intensity of the actinic light at 586 nm was 1.2 nanoeinsteins cm^{-2} sec^{-1}.

Chromatophores from Old Cultures

In chromatophore fractions from old cultures NAD$^+$ was photoreduced in the presence of DPIP and ascorbate (Fig. 5 A, curve 1) at a constant rate of about 9 μmoles/mg BChl per hr. The kinetics and the responses to antimycin A and HQNO were substantially similar to those described for the chromatophore fractions from

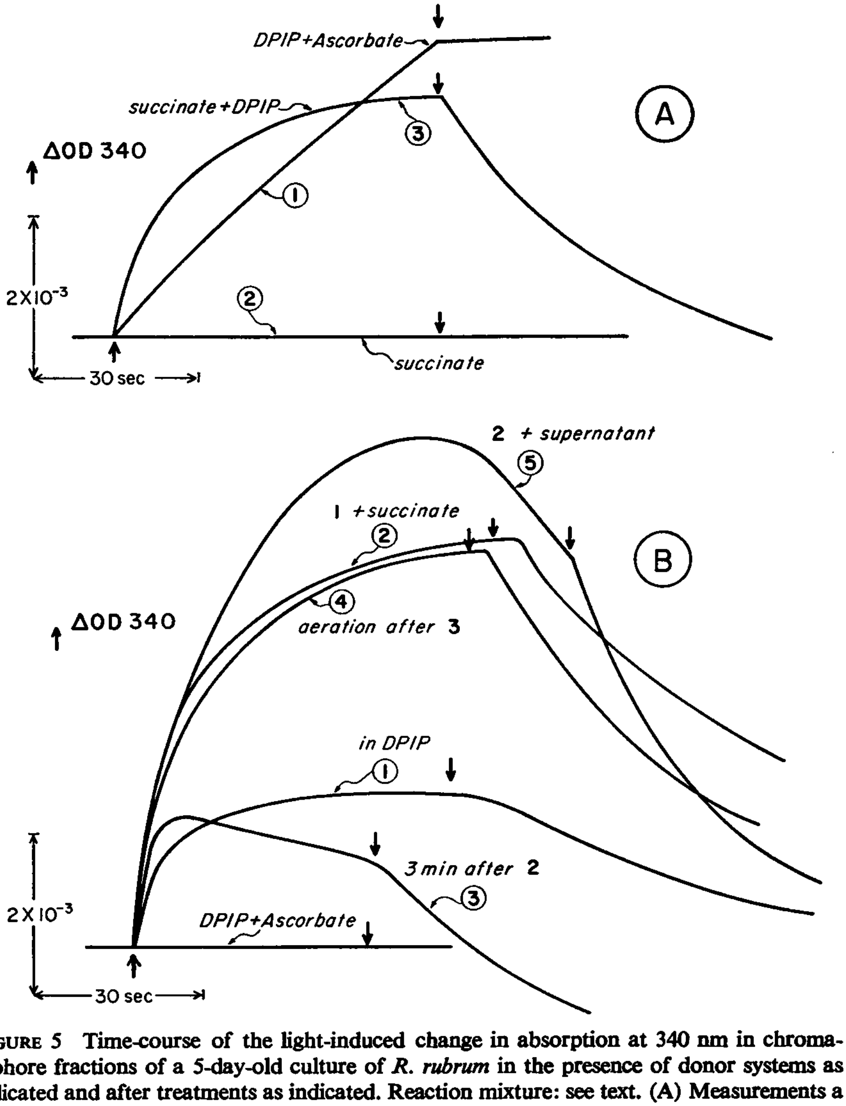

FIGURE 5 Time-course of the light-induced change in absorption at 340 nm in chromatophore fractions of a 5-day-old culture of *R. rubrum* in the presence of donor systems as indicated and after treatments as indicated. Reaction mixture: see text. (A) Measurements a few days after preparation of the chromatophore fractions. (B) Measurements after 3 wk storage of the chromatophore fractions in the dark under vacuum at 3°C. The intensity of the actinic light at 880 nm was 3.5 nanoeinsteins cm^{-2} sec^{-1}. The initial rates varied from about 30 to 40 μmoles/mg BChl per hr.

young cells (cf. Fig. 1). In the presence of succinate alone, hardly any photoreduction of NAD^+ could be observed (Fig. 5 A, curve 2); however, when DPIP (without ascorbate) was added to this system, a reversible, light-induced reduction of NAD^+ seemed to be restored (Fig. 5 A, curve 3).

The ability to catalyze the photoreduction of NAD^+ in the presence of DPIP and ascorbate was gradually lost the longer the chromatophore fractions were stored. No NAD^+ photoreduction could be observed with DPIP and ascorbate after 3–4 wk of storage (at 4°C under vacuum). The addition of DPIP without ascorbate to this "aged" preparation resulted in some reversible photoreduction of NAD^+ (Fig. 5 B, curve 1); the subsequent addition of succinate enhanced this reaction substantially (Fig. 5 B, curve 2). The initial rate of this reaction was 30–40 μmoles/mg BChl per hr. The system was more effective in chromatophore fractions which were stored for 3–4 wk. Subsequent light exposures showed a decreased steady-state level of this photoreduction (Fig. 5 B, curve 3); aeration could bring the level back, almost to its original value (Fig. 5 B, curve 4).

An explanation for these reactions could be that the DPIP substituted for a cofactor for the cyclic electron transport which was lost during the preparation of the chromatophore fractions. Some evidence that DPIP could mediate a cyclic electron transport has been presented before (Bose and Gest, 1962). The addition of DPIP thus could have restored a light-induced electron flow coupled to the production of a high-energy intermediate which could be used to mediate the succinate → fumarate–coupled NAD^+ reduction. The loss of the effectiveness of DPIP to mediate the NAD^+ reduction upon subsequent light exposures and the restoration of this function after aeration, however, would suggest that DPIP acted as an electron sink rather than as a link in a cyclic, light-induced electron transport. It is also possible that aeration restores the redox balance to the proper level for cyclic phosphorylation.

The addition to the succinate-containing reaction mixture of some of the lyophilized supernatant from the centrifugation of the chromatophore fractions resulted in a slight enhancement of the reversible, light-induced reduction of NAD^+; in the presence of DPIP the enhancement was more substantial (Fig. 5 B, curve 5). The chromatophore fraction containing the supernatant showed a light minus dark difference spectrum as illustrated in Fig. 6. The spectrum suggested that cytochrome c_2 may be an active component of the supernatant. The irreversible photoreduction of NAD^+ by chromatophore fractions in the presence of DPIP and ascorbate (Figs. 1 B and 5 A) was inhibited by the supernatant. Under these conditions no light-induced oxidation of cytochrome c_2 or P-870 could be observed unless the sample was made slightly aerobic.

In chromatophore fractions from old cultures, light-induced changes of the absorbance in the near infrared spectral region in the presence of succinate (with or without DPIP) showed the light-induced oxidation of P-870 (Fig. 7). As was the

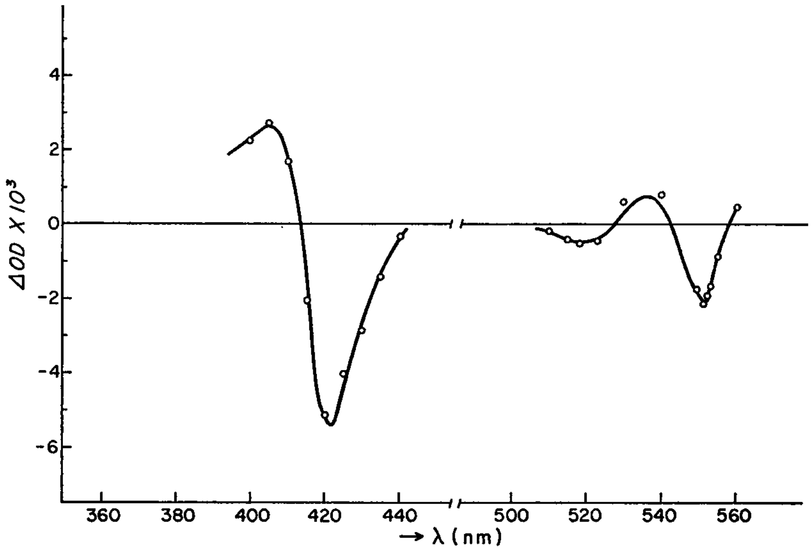

Figure 6 Light minus dark difference spectra measured in chromatophore fractions from a 5-day-old culture of *R. rubrum* after addition of lyophilized supernatant from the final centrifugation step of the preparation of the chromatophore fractions. Reaction mixture: see text. The intensity of the actinic light at 880 nm was 1.4 nanoeinsteins cm^{-2} sec^{-1}.

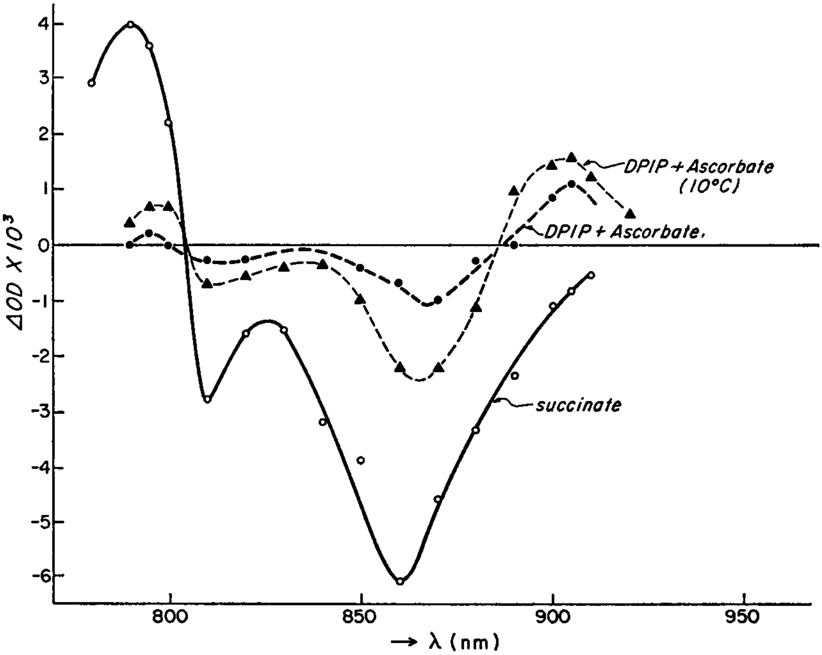

Figure 7 Light minus dark difference spectra measured in chromatophore fractions from a 5-day-old culture of *R. rubrum* in the presence of succinate (open circles), or in the presence of DPIP and ascorbate at room temperature (closed circles), or in the presence of DPIP and ascorbate at 10°C (triangles). Reaction mixture: see text. The intensity of the actinic light at 586 nm was 1.2 nanoeinsteins cm^{-2} sec^{-1}.

case with chromatophore fractions from young cultures, the light-induced absorbance changes in the presence of DPIP and ascorbate were small. The spectral characteristics, however, were different in the chromatophore fractions from old cultures. The ratio between the bleaching in the 860–870 nm region and the blue shift of the 800 nm absorbance band was substantially larger; the maximum bleaching occurred at a wavelength (about 870 nm) slightly shifted to the red as compared with the maximum wavelength (865 nm) of the P-870 bleaching, and an increase in absorbance of wavelengths greater than 890 nm was observed. Lowering of the temperature to about 10°C resulted in a doubling of the magnitude of the absorbance changes and a more pronounced "red shift" (bleaching at about 870 nm and an increase in absorbance at about 905 nm).

DISCUSSION

The difference in kinetics of the NAD^+ photoreduction between chromatophore fractions in a succinate-containing medium and chromatophore fractions in a DPIP- and ascorbate-containing medium clearly suggested different electron transport pathways. The reversible nature of the succinate-mediated photoreduction of NAD^+ indicated a "steady state" in which the forward reaction is driven by a high energy intermediate produced during a cyclic, light-induced electron transport (Chance and Olson, 1960; Keister and Yike, 1967; R. Govindjee and Sybesma, 1970). The difference in free energy change between the fumarate-succinate couple and the NAD^+-NADH couple, although low, is not contradictory to this conclusion.

The irreversibility of the DPIP- and ascorbate-mediated NAD^+ photoreduction suggested a direct, light-induced electron transport from the reduced dye to NAD^+. The indifference to phosphorylating conditions of the latter system indicated that it was not energy linked. This is in accordance with the fact that the uncoupler FCCP affected the DPIP and ascorbate system to a far lesser extent than it did the succinate system. The partial inhibitory effect of FCCP on the DPIP + ascorbate system may be explained by attributing an electron transport–inhibiting effect of this reagent (Sybesma, 1967). Only the cyclic system appeared to be sensitive to antimycin A (R. Govindjee and Sybesma, 1970) and, consequently, only the succinate-mediated NAD^+ photoreduction was inhibited by it. HQNO affected both systems.

Jones and Vernon (1969), using *R. rubrum* chromatophore fractions, have observed similar effects of the antimycin A and HQNO on the succinate system and on the TMPD + ascorbate system (assumed to be similar to the DPIP + ascorbate system). They also observed that antimycin A, even at concentrations lower than those required for the inhibition of the NAD^+ photoreduction in the succinate system, inhibited photophosphorylation; however, based on their observation of the effects of the uncoupler carbonyl cyanide (*m*-chlorophenyl)hydrazone (CCCP) they concluded that with both donor systems NAD^+ is reduced by energy-linked, reversed electron flow. The differential effect of antimycin A on both systems together with the fact that it also inhibited photophosphorylation then is difficult to explain, however.

In chromatophore fractions from older cultures the succinate-mediated photoreduction of NAD^+ seemed to be impaired. In whole cells from older cultures of *R. rubrum*, the noncyclic, light-induced electron transport from exogenous or endogenous substrates to pyridine nucleotide was shown to be predominant (R. Govindjee and Sybesma, 1970). Measurements of the variability of bacteriochlorophyll *a* fluorescence as a function of the age of synchronized cultures were interpreted as demonstrating a predominance of the cyclic electron transport system in young cultures and of the noncyclic electron transport system in older cultures (DeKlerk et al., 1969). The rate of light-induced ATP synthesis was shown to be higher in younger cells than in older cells (Fanica-Gaignier et al., 1971). The present experimental results are consistent with all these observations and seem to indicate that the failure to observe a cyclic electron transport as evidenced by succinate-mediated NAD^+ reduction in preparations from older cultures is due to solubilization of essential components of the cyclic chain. The supernatant after centrifugation of the chromatophore fractions, indeed, contained cytochrome c_2 in a larger amount when the fractions were from older cultures than when they were from younger ones. Adding back the supernatant, however, was far less effective in respect to a reversible NAD^+ photoreduction than was the addition of DPIP. The time of storage of the chromatophore fractions appeared to have a profound influence on the effectiveness of this reaction. These observations would indicate that another component, possibly equivalent to the secondary acceptor Y proposed by Parson and Case (1970), is essential in mediating a light-induced, cyclic electron transport which produces a high energy intermediate necessary to energize the reversed electron flow to NAD^+. DPIP apparently could substitute for Y, not by closing the cyclic chain, however, but by acting as an electron sink. This would mean that in the intact system there is an energy-conserving site between the primary and secondary acceptor. Suggestive in this respect is a recent report of Cogdell and Crofts (1971) which proposed an H^+ binding site between the primary and secondary acceptor in bacterial photosynthesis. No reasons as yet can be given for the inhibitory effect of the supernatant on the DPIP- and ascorbate-supported photoreduction of NAD^+.

In chromatophore preparations from young cultures the presence of succinate resulted in a light minus dark difference spectrum in the near infrared spectral region (Fig. 4, solid line) which indicated the participation of P-870 in the photoreduction of NAD^+. The light-induced absorbance changes observed in the same kind of preparation in the presence of DPIP and ascorbate were much smaller (Fig. 4, dashed curve) but also reflected P-870 oxidation. This could be seen as an indication that both mechanisms for the photoreduction of NAD^+ are driven by the primary photooxidation of P-870. The diminished size of the absorbance changes in the DPIP and ascorbate system could be due to the fact that the rereduction of P-870, either directly by $DPIPH_2$ or indirectly by the reduced dye through a cytochrome, is much faster than the rereduction of P-870 in the succinate system. A cytochrome-like pigment, C-428, having a low redox potential (Sybesma and Fowler, 1968; Fowler and Sybesma, 1970) has been connected with noncyclic photoreduction of pyridine nucleotide in whole cells of *R. rubrum* (R. Govindjee and Sybesma, 1970). A low potential cytochrome (C-552) in *Chromatium*, which may have in this organism a function in the photoreduction of pyridine nucleotide similar to the one of C-428 in *R. rubrum* has been found to react with P-870 at a much higher rate than the high potential cytochrome (C-555) which is involved in light-induced, phosphorylating, cyclic electron transport (Parson and Case, 1970). Thus, according to this interpretation, the cyclic electron transport producing a high energy intermediate used in the reversed electron flow to NAD^+, *as well as* the direct light-induced electron transport from substrate ($DPIPH_2$) to NAD^+, are driven by the primary photooxidation of *one* kind of reaction center component, P-870. The reaction center may contain primary acceptors at different redox potentials; a high potential acceptor may cycle electron back to P-870 and a low potential acceptor may deliver electrons to NAD^+ (cf. Seibert et al., 1971).

A model in which one reaction center component P-870 is driving both electron transport chains was neither supported nor contradicted by the observations of light-induced absorbance changes in chromatophore fractions from older cultures (Fig. 7). In such preparations the presence of succinate resulted in an effective P-870 photooxidation, even though no pyridine nucleotide was reduced. In the presence of DPIP and ascorbate, however, the spectral appearance of the near infrared, light-induced absorbance changes was quite different. These spectral shifts could reflect conformational changes due to changes in the energized state of the membranes (Vredenberg and Amesz, 1966). Questions such as why these changes did not show in membrane fractions from young cultures in which light, in fact, was inducing an energized state of the membranes must then remain unanswered. Alternatively, the changes could reflect the oxidation of a lower potential reaction center component which is more abundant in membranes from older cells than in those from younger ones (cf. Fowler and Sybesma, 1970).

We thank Mrs. Sandra Potter and Miss Nancy Oncken for their capable assistance.

This work was supported by the National Science Foundation, grant GB No. 15777.

Received for publication 1 August 1971.

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