ENERGY-LINKED REDUCTION OF NICOTINAMIDE ADENINE DINUCLEOTIDES IN CELLS OF RHODOSPIRILLUM RUBRUM.

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The light driven, succinate dependent reduction of NA-dinucleotides by chromatophores from photosynthetic bacteria has been extensively investigated (Frenkel, 1958; Vernon & Ash, 1959; Nozaki, Tagawa & Arnon, 1961; Bose & Gest, 1963). Duysens & Sweep (1957), Chance & Olson (1960), and Amesz (1963) have measured the light induced reduction of NA-dinucleotides in cells of Rhodospirillum rubrum, Rhodopseudomonas spheroides and Chromatium by spectrophotometric and fluorimetric techniques. Direct reduction by non-cyclic electron flow (Nozaki et al, 1961) and reduction by energy-linked reversed electron transport (Chance & Olson, 1960) have been suggested as mechanisms for NAD(P)H production by R. rubrum. Keister & Yike (1967) have shown from the sensitivity to inhibitors and uncouplers, that the light dependent reduction of NAD by chromatophores with succinate as substrate was by energy dependent, reversed electron flow, and they showed that ATP in the dark was largely able to replace the light reactions as an energy source.

The present paper reports a study of changes in the oxidation-reduction state of NA-dinucleotides in intact cells of R. rubrum during aerobic-anaerobic transitions and in the light and dark. The reduction in the light was sensitive to uncoupling agents and to inhibition of cyclic electron flow, while the anaerobic reduction was not. The results suggest that the light dependent reduction of NA-dinucleotides in cells of R. rubrum is by energy dependent, reversed electron flow.

RESULTS AND DISCUSSION

Changes in the oxidation-reduction state of NA-dinucleotides and cytochrome c_2 of cells of R. rubrum during light-dark and aerobic-anaerobic transitions are shown in Fig. 1A. On illumination the cytochrome was rapidly oxidised while the NA-dinucleotide was reduced. The changes were reversed in the dark. On aeration, oxidation of both the cytochrome and the nucleotide occurred and both became reduced when the suspension became anaerobic. The steady state level of oxidation of the cytochrome was lower in the aerobic suspension than in the light. Aeration of the suspension in the light gave rise to a partial reoxidation of the NA-dinucleotide but to no change in the cytochrome. On switching off the light, the level of cytochrome oxidation fell to the aerobic steady state, while the nucleotide became further oxidised. Similar changes were observed in a substrate free medium (0.1M Choline chloride, 5mM MES, pH 6.5) showing that the bacteria contained sufficient endogenous substrate to support electron flow.

The kinetics of the changes can be seen more clearly in Fig. 1B. As shown by Chance & Olson (1960) and Amesz (1963) the onset of NA-dinucleotide reduction on illumination showed a lag and followed behind the oxidation of cytochrome, which occurred very rapidly. The delay before onset of reduction was ~1 sec. the half-time for reduction after this was ~3 sec. and the initial rate ~5.5×10^{-3} moles/mole B Chl/sec. In the aerobic-anaerobic transitions, both oxidation and reduction of NA-dinucleotide lagged behind that of the cytochrome and both changes were slower than those occurring in the light.

Effect of 2-Heptyl-4-hydroxy-quinoline-N-oxide (HOQNO) on NA-dinucleotide Reduction

The electron transfer inhibitor HOQNO has been shown to inhibit cyclic phosphorylation in chromatophores but has no effect on the dark respiration of whole cells of R. rubrum (Baltscheffsky & Baltscheffsky, 1958; Nishimura, 1963). In the presence of HOQNO the light inhibition of respiration in whole cells was prevented (Ramirez & Smith, 1968). These and similar experiments

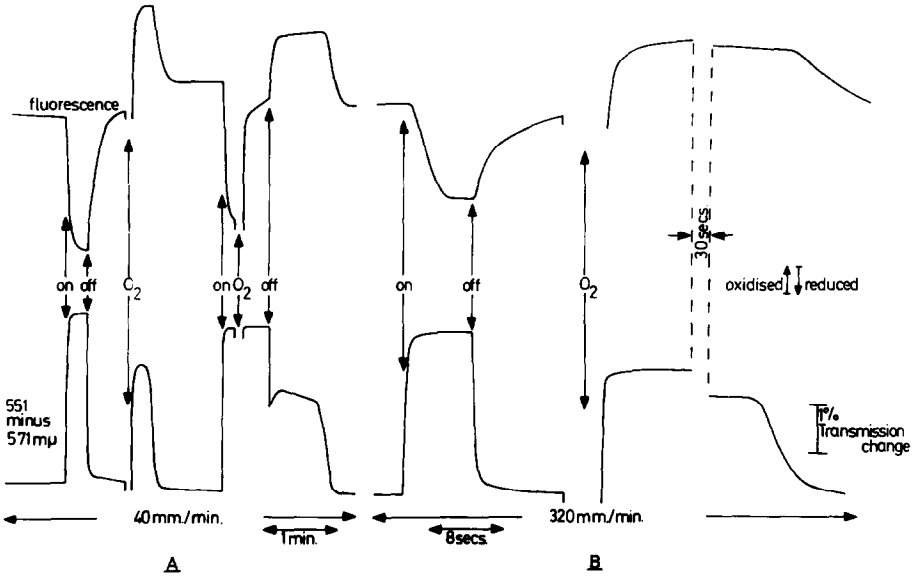

Figure 1. Kinetics of oxidation and reduction of NA-dinucleotides and cytochrome c_2 in cells of R. rubrum.

Cells of the blue-green mutant (Strain G-9) were grown for 48 hr. in the light in the medium of Sistrom (1960), washed and resuspended in a minimal volume of 0.1 M-choline chloride, 5 mM 2[N-Morpholino]ethane Sulphonic Acid (MES), pH 6.5. Reduction of NAD(P) was followed fluorimetrically. A quartz-iodine lamp (100W.) screened by a Balzers R-UV filter (T_{max}=339 mμ) and a Wratten 18 B filter was used for the exciting beam, and the fluorescence was measured at 60° by a photomultiplier covered with Wratten 98 and 2 B filters, together with 1 cm. of sat. $CuSO_4$ in a perspex cell. Cytochrome c_2 changes were followed in the same cuvette by double-beam spectrophotometry (Chance & Olson, 1960) using 551 and 571 mμ as measuring and reference wavelengths. This wavelength pair compensated the large broad-banded absorbancy change due to cytochrome cc' on illumination, and gave minimal interference from cytochrome b changes during aerobic-anaerobic transitions (A. R. Crofts & J. B. Jackson, unpublished observations). The measuring photomultiplier was screened by a Wratten 8 filter and 1 cm. sat. $CuSO_4$. Illumination was from a quartz-iodine lamp (100W.) screened by a Wratten 88 A filter. There was no interference between beams, and none from the actinic light.

The reaction mixture contained 0.1 ml. cell suspension in 2.5 ml. growth medium ($O.D._{880}$= 14.4) at pH 6.5 and 30°. Oxygen was added by vigorously stirring in air. The upper trace shows NA-dinucleotide and the lower trace cytochrome c_2 changes. A downward deflection indicates reduction. Fig. 1A, - changes measured at chart speed of 40 mm/min. Fig. 1B, - chart speed 320 mm/min.

(Smith & Baltscheffsky, 1959) have led to the conclusion that HOQNO inhibits cyclic electron flow without inhibiting respiratory electron flow. Amesz (1963) suggested from the incomplete inhibition of NA-dinucleotide reduction in cells of R. rubrum by HOQNO that cyclic electron flow and phosphorylation

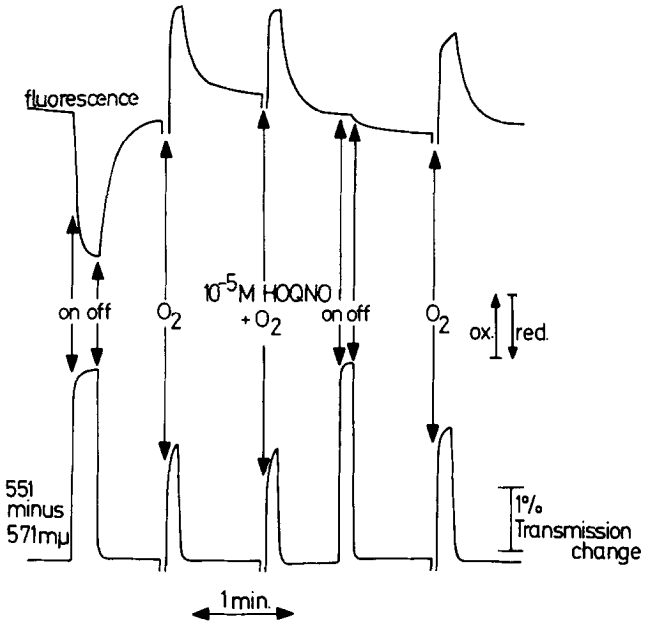

Figure 2. Effect of HOQNO on NA-dinucleotide and cytochrome c_2 changes. Conditions as for Fig. 1A. The change on addition of HOQNO, due to fluorescence of the inhibitor, has been subtracted. Additions were as indicated.

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were not essential for reduction and that reduction was by direct non-cyclic electron flow.

The effects of HOQNO on NA-dinucleotide and cytochrome c_2 changes in aerobic-anaerobic and light-dark transitions in cells of R. rubrum are shown in Fig. 2. In contrast to the observation of Amesz (1963), HOQNO almost completely inhibited the light induced reduction of NA-dinucleotide. The inhibitor was without effect on the oxidation and reduction accompanying the aerobic and anaerobic changes. The steady state level of cytochrome oxidation in the light and also in the aerobic state was slightly increased in the presence of HOQNO. The results suggest that cyclic electron flow was necessary for NA-dinucleotide reduction in the light.

Effect of Carbonyl-cyanide-p-trifluoromethoxy-phenylhydrazone (FCCP) on NA-dinucleotide and cytochrome c_2 changes

A clear distinction between direct reduction and energy-linked reversed electron flow as mechanisms for NA-dinucleotide reduction in R. rubrum

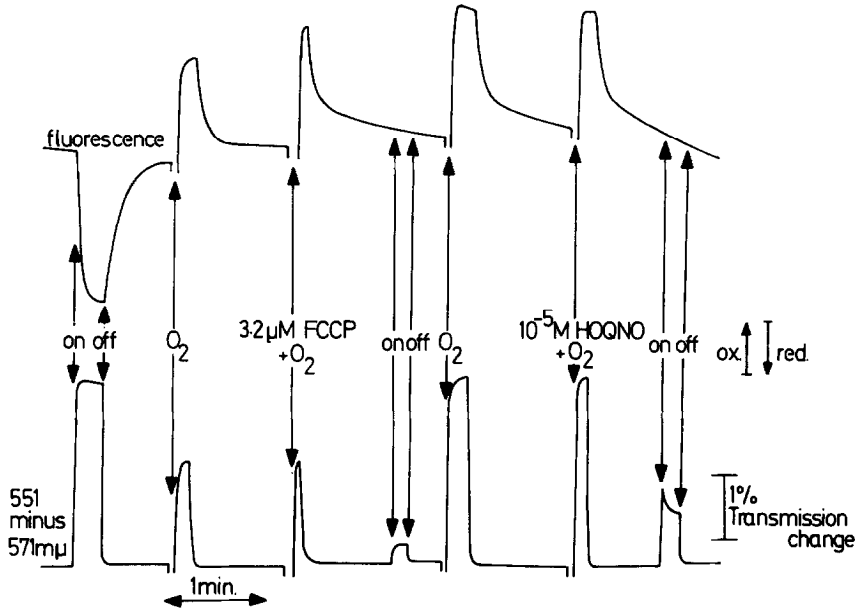

Figure 3. Effect of FCCP on NA-dinucleotide and cytochrome c_2 changes. Conditions as for Fig. 2. Additions were as indicated.

may be shown by the effect of the uncoupler FCCP. Ramirez & Smith (1968) have shown that FCCP inhibited ATP formation in cells of R. rubrum and prevented the light inhibition of respiration. FCCP stimulated the endogenous dark respiration (J. B. Jackson, unpublished observation). In the presence of FCCP, the light induced reduction of NA-dinucleotide was completely inhibited (Fig. 3). The aerobic level of oxidation was somewhat higher in the presence of uncoupler and the rate of reduction on anaerobiosis slightly increased. The effects of FCCP on the cytochrome changes were also of interest. The light induced oxidation of cytochrome c_2 was largely eliminated in the presence of uncoupler, while the steady state level of oxidation on aeration and the rate of reduction on anaerobiosis were markedly increased and reached a level comparable to that in the light in the absence of FCCP. The light induced oxidation of the cytochrome was partially restored by addition of HOQNO, suggesting that the reduced steady state in the presence of FCCP was a consequence of the removal of an inhibitory step in electron flow leading to cytochrome c_2.

TABLE I

Direct estimation of NA-dinucleotides in cells of R. rubrum

Conditions	NADH	NAD^+	TOTAL NAD	NADPH	$NADP^+$	TOTAL NADP
	% TOTAL		mμmoles	% TOTAL		mμmoles
Light anaerobic	69	31	25.4	100	0	2.5
Dark anaerobic	9.5	90.5	30.1	55	65	2.3
Dark aerobic	0	100	29.9	0	100	1.0
Light anaerobic + FCCP	17.5	82.5	31.3	40	60	3.2
Dark aerobic + FCCP	0	100	27.2	0	100	0.1
Light anaerobic + HOQNO	23	77	37.4	93	7	1.4
Dark aerobic + HOQNO	1	99	28.9	0	100	0.1

NA-dinucleotides were estimated spectrophotometrically using the extraction procedures of Estabrook, Williamson, Frenkel & Maitra (1967). Reaction mixtures contained growth medium and bacteria together with FCCP (8.5×10^{-6} M) or HOQNO (1.7×10^{-5} M) where indicated. Final vol. 2.9 ml., temp. 22°, $O.D._{880}$=45, pH 6.5. Suspensions were allowed to become anaerobic under N_2. Light samples were illuminated with white light for 25 sec.; aerobic samples were bubbled with air for 30 sec. before killing.

The results indicate that the reduction of NA-dinucleotide in the light was energy dependent and that FCCP released an inhibition due to coupling in cyclic electron flow prior to cytochrome c_2, and also in respiratory electron flow between cytochrome c_2 and oxygen.

Direct Estimation of NA-dinucleotide changes in R. rubrum

Direct estimation of the oxidation state of the nucleotides showed changes which were qualitatively similar to those shown by fluorimetry (Table I). The majority of the nucleotide present was NAD, with only 5-10% as NADP. This difference in levels probably reflects the plentiful supply of substrate in the growth medium. The total NA-dinucleotide present was about 30 mμmoles/ $O.D._{880}$=45 (or 0.03 moles/mole B Chl using the extinction coefficient of Clayton, 1963). The total change in Figs. 1, 2 and 3 would be 10 mμmoles NADH.

Energy-linked Steps in R. rubrum Electron Flow

The effects of FCCP and HOQNO on the changes in the oxidation-reduction level of NAD and cytochrome c_2 suggest the presence of at least three sites of energy conservation in the electron flow pathways in cells of R. rubrum.

i) In cyclic electron flow, between the light reaction and cytochrome c_2 reduction.

ii) In respiratory electron flow between cytochrome c_2 and oxygen.

iii) Between NAD and the site at which the electron donor substrate for energy-linked NAD reduction interacts with electron flow.

The failure of HOQNO to inhibit respiratory electron flow from NAD to oxygen, or to inhibit cytochrome c_2 oxidation in the light, localizes its site of action in the cyclic electron flow pathway prior to the reduction of the cytochrome. Aeration in the light gave rise to no increase in the level of oxidation of cytochrome c_2 and, in the presence of FCCP, the aerobic level of oxidation was comparable to that achieved in the light in the absence of uncoupler. These results suggest that a major proportion of the cytochrome c_2 in the cells was able to interact with both cyclic and respiratory electron flow pathways.

Acknowledgements

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