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NONCYCLIC PHOTOPHOSPHORYLATION IN PHOTOSYNTHETIC BACTERIA

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Noncyclic photophosphorylation is the formation of adenosine triphosphate in a light-induced electron flow from an external electron donor to an electron acceptor such as pyridine nucleotide. In photosynthesis of green plants the electron donors are the hydroxyl ions of water and hence the over-all reaction also produces, aside from ATP and PNH_2,[1] molecular oxygen, which is liberated when OH^- is photooxidized (see Fig. 1 in preceding article[2]).

Photosynthetic bacteria cannot use OH^- as an electron donor for photosynthesis but use a variety of reductants stronger than OH^-, among them hydrogen gas, succinate, or thiosulfate. With hydrogen gas no input of light energy is required for the reduction of pyridine nucleotide, since hydrogen gas, with the aid of bacterial hydrogenase, can reduce pyridine nucleotide in the dark.[3] The contribution of light to bacterial photosynthesis is then limited to the formation of ATP by cyclic photophosphorylation.[4, 5] With other electron donors such as, for example, succinate, the additional input of energy that is necessary to reduce pyridine nucleotide is provided by light. Thus, in bacterial photosynthesis with succinate as the electron donor, light energy serves a dual purpose: it supplies ATP by cyclic photophosphorylation and, by photooxidizing the electron donor, it also provides electrons for reducing pyridine nucleotides by a noncyclic electron flow mechanism.

Photoreduction of pyridine nucleotide (DPN) by cell-free preparations of *Rhodospirillum rubrum*[6, 7] and *Chromatium*[3] has been observed but attempts to find a simultaneous ATP formation, analogous to the noncyclic photophosphorylation in chloroplasts, led to negative results.[7, 8] As pointed out by Vernon and Ash,[8] a demonstration of ATP formation by a noncyclic electron flow mechanism is more difficult in chromatophores than in chloroplasts because in chromatophores it cannot be experimentally distinguished from a simultaneous ATP formation by cyclic photophosphorylation. In chromatophores, a vigorous cyclic photophosphorylation occurs under anaerobic conditions and without added cofactors,[3] whereas in chloroplasts cyclic photophosphorylation is always under experimental

control since it will not proceed anaerobically to a significant extent without the addition of co-factors.

Evidence for noncyclic photophosphorylation in photosynthetic bacteria, which we now wish to report, has been obtained under conditions when cyclic photophosphorylation was suppressed, thereby making it possible to distinguish the ATP formed by a noncyclic electron flow mechanism from that formed by a cyclic mechanism. In chromatophores of *R. rubrum*, in which cyclic photophosphorylation was made inoperative, ATP formation was obligatorily coupled with a light-dependent electron flow from an external electron donor to DPN as the terminal electron acceptor.

Methods.—Rhodospirillum rubrum (kindly supplied by R. Y. Stanier) was grown anaerobically, at room temperature, under continuous illumination by a bank of fluorescent lights in a medium of Gest *et al.*[9] supplemented with 0.017 *M* NH_4Cl. *Chromatium* cells were grown under similar conditions in a culture medium to be described elsewhere. Two-day-old cultures were centrifuged, the sedimented cells washed with 0.1 *M* tris-HCl buffer, pH 7.8 (used also in subsequent operations) and recentrifuged. A paste of the washed cells was ground by hand, under argon gas, with an equal volume of levigated alumina, the slurry suspended in tris buffer (gassed with argon), and centrifuged at 3,000 × *g* for 15 min. The supernatant fluid was further centrifuged at 12,000 × *g* for 15 min., the residue was discarded, and the supernatant fluid (PS) was used for the isolation of chromatophores. Centrifugations were carried out in the cold and the centrifuge chamber was filled beforehand with argon gas.

The PS preparation was centrifuged in a Spinco, Model L centrifuge, at 144,000 × *g* for 1 hr. The supernatant fluid (S) was frozen for later use. The residue (P), which constituted the chromatophore fraction, was resuspended in tris buffer (gassed with argon) and recentrifuged at 144,000 × *g* for 1 hr. The residue, after suspension in tris buffer, constituted the washed chromatophores (P_1), and the corresponding supernatant fluid (S_1) was saved for later use. In certain experiments the washing procedure was repeated, yielding twice-washed chromatophores (P_2) and a corresponding supernatant fluid (S_2), etc.

The photochemical reactions were carried out in Thunberg-type cuvettes, which were made anaerobic, by five consecutive cycles of evacuation (to 5 mm Hg) followed by flushing with argon gas. The cuvettes were placed in an illuminated glass-walled water bath. DPN reduction was measured periodically (with a Cary recording spectrophotometer) as the difference in optical density at 340 mμ between an illuminated cuvette and a dark control. The reaction was stopped by adding 0.3 ml of 40% trichloracetic acid to each cuvette. The reaction mixture was centrifuged and 1 ml of the supernatant fluid was used for determining the ATP formed,[10] and, where applicable, 0.3 ml of the supernatant fluid was used for determining the lactate formed.[8]

Bacteriochlorophyll was measured at 810 mμ by diluting a suspension of chromatophores with distilled water, as described by Anderson and Fuller.[11]

Results.—Suppression of cyclic photophosphorylation: Cyclic photophosphorylation in cell-free preparations of *R. rubrum* and *Chromatium* was suppressed by the use of antimycin A or 2-heptyl-4-hydroxy-quinoline-N-oxide[12–15] (HOQNO) (a gift of D. P. Hackett). The effect of these inhibitors was most pronounced

with washed chromatophores. As shown in Table 1, unwashed chromatophores had an appreciable photophosphorylation that was resistant to the action of these two inhibitors. This resistant photophosphorylation seemed to depend on factor(s) present in the supernatant fluid as evidenced by the addition of the supernatant fluid to washed chromatophores (Table 1).

TABLE 1

EFFECTS OF INHIBITORS ON PHOTOPHOSPHORYLATION IN CELL-FREE PREPARATIONS OF *Chromatium* AND *R. rubrum*

	Inhibitor added	μmoles of ATP formed/mg chl/hr Chromatium	R. rubrum
Crude extract (PS)	none	103	90
PS	Antimycin A	71	33
PS	HOQNO	33	29
Unwashed chromatophores (P)	none	96	139
P	Antimycin A	42	22
P	HOQNO	13	26
Washed chromatophores (P_1)	none	82	142
P_1	Antimycin A	7	8
P_1	HOQNO	1	15
P_1 + washings (S_1)	none	..	139
$P_1 + S_1$	Antimycin A	..	24
$P_1 + S_1$	HOQNO	..	22

The reaction mixture contained in a final volume of 3 ml: chromatophores, containing 0.1 mg bacteriochlorophyll; hexokinase, 1 mg; and the following in μmoles: tris buffer, pH 7.8, 100; $MgCl_2$, 5; ADP, 0.5; $K_2HP^{32}O_4$, 10; D-glucose 30. Antimycin A (10 μg) or HOQNO (0.03 μmole) was added as indicated. The reaction was run at 20° for 30 min in the light (10,000 Lux).

Aside from inhibiting cyclic photophosphorylation (Table 1), antimycin A and HOQNO were also found to inhibit photoreduction of DPN when succinate was the electron donor (Fig. 1). Succinate could not, therefore, be used as the electron donor for noncyclic photophosphorylation in the presence of either one of these inhibitors. However, antimycin A or HOQNO gave practically no inhibition when ascorbate, together with catalytic amounts of 2,6-dichlorophenol indophenol (DPIP), served as the electron donor for DPN reduction (Fig. 2). It thus became possible to measure ATP formation that is coupled with the electron flow from ascorbate to DPN, under conditions when cyclic photophosphorylation was blocked. Neither the photoreduction of DPN with succinate or ascorbate, nor photophosphorylation, was diminished by repeated washing of chromatophores ($P \rightarrow P_3$). In fact, the once-washed chromatophores (P_1) were more active than the unwashed particles (P).

We have previously reported[20] that photosynthetic bacteria contain a soluble pyridine nucleotide reductase which, when assayed in a chloroplast system, reduced (in the light) triphosphopyridine nucleotide (TPN) in preference to DPN. In the present experiments, however, chromatophores were found to have a tightly bound, DPN-specific pyridine nucleotide reductase. In addition, the chromatophores had a bound transhydrogenase which made possible a light-dependent reduction of TPN in the presence of traces of DPN.

Noncyclic photophosphorylation: As shown in Table 2, a noncyclic photophosphorylation system was functioning with chromatophores from *R. rubrum*. The formation of ATP required the simultaneous presence of an electron donor system (substrate amounts of ascorbate plus catalytic amounts of DPIP) and of an electron acceptor (DPN). The stoichiometry of this reaction appears to be the same

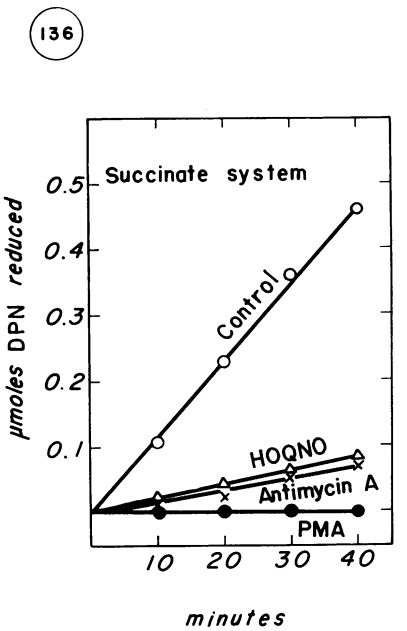

FIG. 1.—Photoreduction of DPN by washed chromatophores of *R. rubrum* (P_1) with succinate. The reaction mixture contained in a total volume of 3 ml, 0.03 mg bacteriochlorophyll, as washed *R. rubrum* chromatophores (P_1), and in μmoles: tris buffer (pH 7.8), 100; $MgCl_2$, 5; DPN, 2; and succinate, 20. The following inhibitors were added as indicated: antimycin A, 10 μg; 2-heptyl 4-hydroxyquinoline N-oxide (HOQNO), 0.03 μmole; or phenyl mercuric acetate (PMA), 0.3 μmole. The reaction was carried out in Thunberg-type cuvettes at 20°C. Gas phase, argon; illumination, 10,000 Lux.

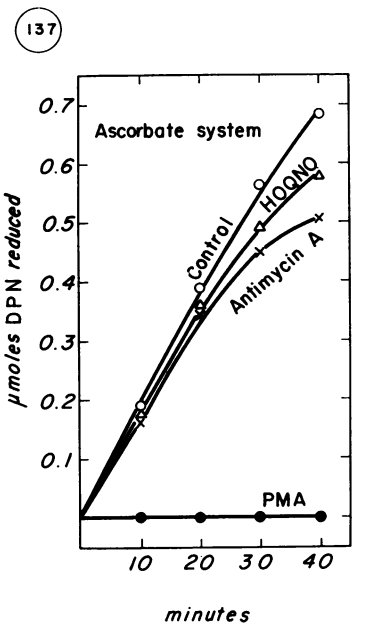

FIG. 2.—Photoreduction of DPN by washed *R. rubrum* chromatophores with ascorbate. The reaction mixture and experimental conditions were the same as described for Figure 1 except that succinate was replaced by 20 μmoles of ascorbate and 0.2 μmole of 2,6-dichlorophenol indophenol.

TABLE 2

NONCYCLIC PHOTOPHOSPHORYLATION IN *R. rubrum*

Components added	Substances added	μmoles ATP formed/mg chl/hr
Electron donor and acceptor	Ascorbate, DPIP, DPN	44.8
Electron donor system	Ascorbate, DPIP	5.4
Electron acceptor	DPN	7.6
None	.....	5.0
Electron donor, acceptor, and inhibitor	Ascorbate, DPIP, DPN, PMA	6.2

All vessels contained antimycin A, 10 μg. The following additions were made where indicated: ascorbate, 20 μmoles; 2,6-dichlorophenol indophenol (DPIP), 0.2 μmole; DPN, 2 μmoles; phenyl mercuric acetate (PMA), 0.3 μmole. Other components of the reaction mixture and conditions were the same as in the control treatment of Table 1.

as in noncyclic photophosphorylation by isolated chloroplasts: one mole of ATP is formed for each mole of pyridine nucleotide reduced (Fig. 3). Experimentally, this stoichiometry was obtained only when the reduced DPN was trapped by an added lactic dehydrogenase system. Without this addition, the reduced DPN was

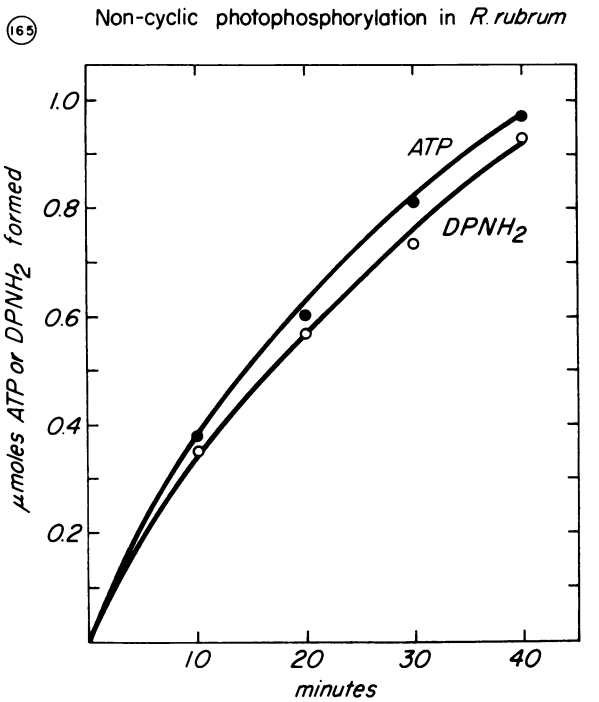

FIG. 3.—Stoichiometry of ATP formation and DPN reduction (as measured by lactate formation) in noncyclic photophosphorylation with *R. rubrum* chromatophores. The reaction mixture contained 0.04 mg bacteriochlorophyll, as washed chromatophores (P_i), and in μmoles: tris buffer (pH 7.8), 100; $MgCl_2$, 5; ADP, 5; $K_2HP^{32}O_4$, 5; ascorbate, 20; 2,6-dichlorophenol indophenol, 0.2; DPN, 0.3; and pyruvate, 10. 25 μg of crystalline lactic dehydrogenase (muscle) and 10 μg of antimycin A were also added. Other experimental conditions were the same as described for Figure 1.

reoxidized in the reaction mixture, resulting in low recoveries of $DPNH_2$ and high $P/DPNH_2$ ratios.

The effectiveness of substrate amount of ascorbate as the electron donor for noncyclic photophosphorylation by *R. rubrum* chromatophores depended on the addition of catalytic amounts of DPIP, which evidently acted as an electron carrier between ascorbate and the electron transport system within the chromatophore. We have also observed a cyclic photophosphorylation, which is resistant to antimycin A (resembling in this respect the cyclic photophosphorylation catalyzed by phenazine methosulfate[13, 15]), when we replaced substrate amounts of ascorbate (20 μmoles) with catalytic amounts (0.5 μmole) and added it jointly with a catalytic amount of DPIP (0.2 μmole).

Phenyl mercuric acetate (PMA) completely inhibited non-cyclic electron flow whether the electron flow was measured as DPN reduction (Figs. 1 and 2) or as ATP formation accompanying the DPN reduction (Table 2). However, in other experiments, we have observed that PMA at this same concentration only partially inhibited the cyclic photophosphorylation. The PMA, therefore, seems to inhibit the step(s) involved between the e^- and the DPN (see Fig. 4).

A diagrammatic representation of noncyclic photophosphorylation in bacteria is given in Figure 4 (compare Fig. 2 in preceding article). It seems likely that

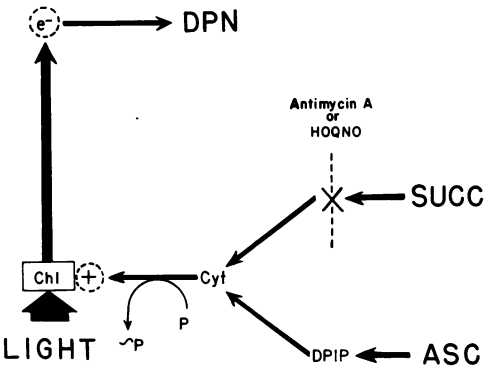

FIG. 4.—Scheme for noncyclic photophosphorylation in *R. rubrum* with either succinate or ascorbate as an electron donor. Details in the text.

electron transport from succinate to DPN shares with the electron transport system of cyclic photophosphorylation, the same step, sensitive to inhibition by antimycin A and HOQNO, that involves electron transfer between a cytochrome of the b type and a cytochrome of the c type.[14, 15] A possible participation of coenzyme Q in this sector of the electron transport chain in bacterial photophosphorylations is discussed elsewhere.[16]

Additional support for noncyclic electron flow in photosynthetic bacteria has come from recent experiments of Losada *et al.*[17–19] on photoproduction of H_2 and photofixation of N_2 by *Chromatium* at the expense of thiosulfate as the electron donor. The results are consistent with the view that electrons expelled from chlorophyll, following the primary photochemical act, are responsible for the reduction of H^+ to H_2 and for the reduction of N_2. The electrons lost by chlorophyll would be replaced by those donated by thiosulfate, via the bacterial cytochrome system. The expected occurrence of photophosphorylation coupled with the electron flow to H_2 or N_2 is now under investigation.

Concluding Remarks.—Since noncyclic photophosphorylation in bacteria (Fig. 4) is analogous to noncyclic photophosphorylation (without oxygen evolution) in chloroplasts (see Fig. 2 in preceding article), photosynthesis in plants and bacteria is now seen as having in common two photochemical processes: cyclic and noncyclic photophosphorylation. In both plants and bacteria light energy, when it supplies ATP by cyclic and by noncyclic photophosphorylation, also brings about a reduction of pyridine nucleotide and oxidation of an exogenous electron donor that is coupled with the formation of ATP.

The differences between bacterial and plant photosynthesis seem to center on the electron donors that are consumed in the reduction of pyridine nucleotide. With some bacterial electron donors, as for example, hydrogen gas, the reduction of pyridine nucleotide requires no input of light energy. With other electron donors, such as thiosulfate or succinate, an input of light energy, in a noncyclic electron flow mechanism, becomes necessary for the reduction of pyridine nucleotide. Thus in bacterial photosynthesis, the reduction of pyridine nucleotide may occur with or without an input of light energy depending on the particular electron

donor used. By contrast, in photosynthesis of green plants, the reduction of pyridine nucleotide invariably requires an input of light energy because the physiological electron donors are hydroxyl ions from water. A comparison of photosynthetic phosphorylation in green plants and photosynthetic bacteria is given in Table 3.

TABLE 3

PHOTOSYNTHETIC PHOSPHORYLATION

GREEN PLANTS	PHOTOSYNTHETIC BACTERIA
noncyclic photophosphorylation:	
a) $2A + 2OH^- \xrightarrow{Cl^-} 2A^- + H_2O + {}^1/_2O_2$	
b) $TPN + 2H^+ + ADP + P + 2A^- \rightarrow TPNH_2 + ADP + 2A$	$DPN + 2H^+ + ADP + P + 2A^- \rightarrow DPNH_2 + ATP + 2A$
Sum:	
$TPN + 2H^+ + ADP + P + 2OH^- \xrightarrow{Cl^-} TPNH_2 + ATP + H_2O + {}^1/_2O_2$	$DPN + 2H^+ + ADP + P + 2A^- \rightarrow DPNH_2 + ATP + 2A$
cyclic photophosphorylation:	
$ADP + P \rightarrow ATP$	$ADP + P \rightarrow ATP$

It now appears that plants, but not photosynthetic bacteria, are able to use hydroxyl ions as electron donors in photosynthesis because plants have evolved an additional pigment system[2] and an auxiliary light-dependent reaction[20] in which the electrons from OH^- are first raised to a potential similar to that of the electron donors in bacterial photosynthesis and are then used in a second photochemical reaction—i.e. the photophosphorylation reaction proper common to plants and bacteria—in which pyridine nucleotide reduction is coupled with the formation of ATP.

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[1] The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P, orthophosphate; PN, PNH_2, DPN, $DPNH_2$, oxidized and reduced forms of pyridine nucleotide and diphosphopyridine nucleotide; DPIP, 2,6-dichlorophenol indophenol.

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