ON THE FUNCTION OF CYTOCHROME f IN PHOTOSYNTHETIC ELECTRON TRANSPORT[1)]

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Cytochrome f, a c-type cytochrome, has been discovered in photosynthetic organisms by Hill and Scarisbrick (Hill and Scarisbrick, 1951) and purified by Davenport and Hill (Davenport and Hill, 1952), who reported the presence and the concentration of the pigment in a number of green plants, including algae. The properties of purified cytochrome f were also studied (Davenport and Hill, 1952). More recent studies have led to the hypothesis (Hill and Bonner, 1961) that cytochrome f is oxidized by the light-oxidized chlorophyll a, and then reduced by the electron provided by water through the exitation of a second pigment. This hypothesis is based on spectroscopic observations on intact cells, and is essentially in agreement with the results of Duysens and Amesz (Duysens and Amesz, 1962).

In the attempt to understand the role of cytochrome f in the phosphorylation-coupled electron transport system of chloroplasts, we have investigated the reduction of added cytochrome f by chloroplasts, and the conditions for its reoxidation.

Cytochrome f was prepared from parsley leaves according to Davenport and Hill (Davenport and Hill, 1952) with minor modifications (G. Forti et Al., unpublished). These include the addition of 1% Triton X-100 to the extraction solvent. This addition allows quite efficient and reproducible extraction of the pigment upon disintegration of leaves in a Waring Blendor. Only the purest cytochrome f (with a ratio: absorbancy at 422/absorbancy at 278

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mμ of 2) was used in the experiments with chloroplasts. Spinach chloroplasts were prepared as previously (Forti and Jagendorf, 1960). Chlorophyll was measured according to Arnon (Arnon, 1949). Photosynthetic pyridine nucleotide reductase (PPNR) and transhydrogenase were prepared as by San Pietro et Al. (San Pietro, 1958; Keister, San Pietro and Stolzenbach, 1960). Glyoxilate reductase was a cristalline preparation (Zelitch, 1955). Cytochrome f was oxidized by dialysis against ferricyanide (Davenport and Hill, 1952), and the excess ferricyanide was removed by extensive dialysis against tris buffer of pH 8. Essentially complete oxidation was obtained. The reduction of cytochrome f by chloroplasts was measured in a Beckman model DB recording spectrophotometer, against a blanck containing the complete reaction mixture except cytochrome f. The absorbancy measurement were made before and after exposing the cuvettes to white light, at 22°C. Calculations of the concentration of cytochrome f were made assuming a molar extinction coefficient equal to the one of cytochrome c, i.e. 2.1×10^4 (Massey, 1958). The difference $O.D._{555}-O.D._{570}$ was taken as the height of the α band of reduced cyt. f, and the displacement of the Soret band from 411 mμ to 422 mμ upon reduction was also checked. Phosphorylation was measured according to Nielsen and Lehninger (Nielsen and Lehninger, 1955).

As can be seen in table I, cytochrome f is rapidly reduced in the light by chloroplasts. The reduction is completely inhibited by 3×10^{-5}M parachlorophenyldimethylurea (CMU), and by oxygen. When the cytochrome is reduced under nitrogen, or when it is added in the reduced form, it is not reoxidized by chloroplasts on readmission of O_2, either in the light or dark. This indicates that the O_2 inhibition is not due to reoxidation of cyt. f, but rather to the oxidation by O_2 of some reductant with more negative potential. The reactivity of such compound with O_2 might be responsible for the well known Mehler's reaction. The inhibition of cytochrome f reduction by CMU indicates that H_2O is the terminal electron donor, and therefore oxygen evolution

should accompany the reaction. Studies are in progress on the stoichiometry of cytochrome f reduction and O_2 evolution.

T A B L E I

Reduction of cytochrome f by chloroplasts

Treatment	Reaction conditions		
	N_2, control	N_2, CMU $3 \cdot 10^{-5}$M	Air, control
2 min., Dark	0.0	0.0	0.0
2 min., Light	15.3	0.0	0.0
2 min., Dark	16.4	0.0	—
1 min., Light	18.7	—	0.0
5 min., Dark	18.7	0.0	—
Air readmitted			
2 min., Dark	18.8	0.0	—
10 min., Light	18.9	0.0	0.0
10 min., Dark	18.9	0.0	0.0

Reaction mixture contained, in micromoles: tris buffer, pH 8.0, 100; $MgCl_2$ 10; ADP 10; Na_2HPO_4 5; cytochrome $f^{ox.}$ 19 millimicromoles; chloroplasts containing 51 micrograms of chlorophyll. Incubation at 22°C. Light: 15,000 lux. Final volume: 3 ml. Incubation was performed in a stoppered spectrophotometer cuvette (1 cm light path). Before the incubation the reaction mixture was flushed with nitrogen (in darkness) by means of a capillary fitted into the stopper of the cuvette. The data are expressed in millimicromoles of reduced cytochrome f.

Table II shows that ATP formation is coupled to cyt. f reduction, and that one mole of ATP is formed per electron pair transferred to the cytochrome. Such finding indicates that a phosphorylating site is situated in the span from H_2O to cyt. f, through the light reaction of the accessory pigment(s).

T A B L E II

Photophosphorylation coupled to cytochrome f reduction

Conditions	mμmoles cytochrome f reduced	mμMoles ATP formed
N_2, 2 min. Dark	0.0	0.0
N_2, 4 min. Light	158.4	—
N_2, 2 min. Light	158.4	79.0
Air admitted		
2 min. Light	158.4	—
(B)°added		
2 min. Dark	158.4	—
5 min. Light	158.4	79.0

Reaction mixture contained, in micromoles: tris buffer, pH 8.0, 100; phosphate 4, containing 100,000 cpm P^{32}; ADP 5; glucose 100; hexokinase 4 mg (type IV Sigma); cytochrome $f^{ox.}$ 160; chloroplasts containing 32 micrograms of chlorophyll.
° Solution (B), added where indicated, contained, in micromoles: TPN 0.4; DPN 4; Glyoxilate 75; PPNR 0.25 units; glyoxilate reductase 0.3 units; transhydrogenase 0.1 units; CMU 0.2. Final volume: 3.8 ml. A sample of the reaction mixture was withdrawn for the measure of phosphorylation when air was readmitted, before addition of (B). The amount of reduced cytochrome f and of ATP formed is referred to the original reaction mixture, being corrected for dilution. Other conditions as in table I. A unit of all the enzymes added is defined as the amount of enzyme which transfers two electron microequivalents per minute in the conditions of the experiment.

The current schemes of photosynthetic electron transport assume that TPN is reduced by light-excited chlorophyll a, through the action of PPNR. We attempted therefore to measure cytochrome f reoxidation upon addition of the system: PPNR-TPN-transhydrogenase--DPN-glyoxilate reductase-glyoxilate. The glyoxilate system was added to provide an efficient TPNH-oxidizing reaction as TPNH reduces non-enzymatically cytochrome f. CMU was added to prevent

reduction of cytochrome f by H_2O. Under these conditions, no reoxidation of cytochrome f could be observed (table II). The possibility that the glyoxilate system added is unable to reoxidize TPNH fast enough to prevent its reaction with cyt. f is made unlikely by the rather strong activity of the added enzymes, which would oxidize TPNH, under the conditions of the experiment, at a rate of 300 millimicromoles per minute. Furthermore, after addition of the electron acceptor system, no more ATP formation could be observed, while one would expect phosphorylation to occur if a cyclic electron transport cytochrome -chlorophyll-TPNH-cytochrome were operating.

In conclusion, our results cast some doubt on the view that TPN is reduced by the same light reaction responsible for the oxidation of cytochrome f. Further studies are in progress on the requirements for cytochrome f reoxidation by chloroplasts, as well as on the mechanism of TPN reduction. We could confirm Horio and Yamashita's report (Horio and Yamashita, 1962) on the ability of chloroplasts to reduce PPNR, and on the lack of reoxidation of this enzyme by TPN, even in the presence of chloroplasts. We found that reduced PPNR reacts readily with cytochrome c, but not with cytochrome f.

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