SEPARATION BY MONOCHROMATIC LIGHT OF PHOTOSYNTHETIC PHOSPHORYLATION FROM OXYGEN EVOLUTION

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Although photoproduction of oxygen is an essential part of the over-all photosynthetic process in plants, it does not appear to be essential for the formation of ATP,[1] a key step in the conversion of light energy into chemical energy.[2–4] Upon illumination, isolated chloroplasts form ATP by two photochemical reactions, cyclic and noncyclic photophosphorylation.[4, 5] In neither of these is photoproduction of oxygen essential: it is either absent, as in the case of cyclic photophosphorylation,[4, 6] or, when present, as in the case of noncyclic photophosphorylation,[7, 8] it can be experimentally separated[9] without impairing ATP formation (and TPN reduction). From this, it was suggested[10, 11] that the photosynthetic energy conversion process consisting of cyclic and noncyclic photophosphorylation is basically the same in oxygen-evolving green plants as in the strictly anaerobic photosynthetic bacteria, the photosynthesis of which is never accompanied by oxygen evolution. Oxygen evolution would, in this view, be an accessory photochemical reaction[10] dependent mainly on an accessory pigment system, which like chlorophyll *b* or the phycobilins, is found only in oxygen-evolving photosynthetic cells.

Other investigators have suggested, however, that ATP formation by chloroplasts *in vivo* is always accompanied by oxygen evolution. In this view, the experimentally demonstrable cyclic photophosphorylation without oxygen evolution would be an artifact resulting from the use of such added cofactors as methyl phenazonium methosulfate (phenazine methosulfate); in endogenous cyclic photophosphorylation, it was held,[12] oxygen evolution would always occur but would be masked by a simultaneous and equal oxygen consumption, the over-all result being an oxygen exchange, i.e., a pseudocyclic[6] type of photophosphorylation.

It seemed desirable, therefore, to investigate the relation of ATP formation to oxygen evolution in the recently discovered endogenous type of cyclic photophosphorylation by chloroplasts,[13] the type dependent on the native chloroplast protein, ferredoxin. This cyclic photophosphorylation is free from the objection that it is catalyzed by exogenous catalysts, some of which, like phenazine methosulfate, are clearly nonphysiological.

The findings reported here indicate that the ferredoxin-dependent cyclic photophosphorylation by isolated chloroplasts is independent of photoproduction of oxygen; it occurs in far-red monochromatic light (708 mμ) at which only little oxygen evolution by isolated chloroplasts has been observed. The experimental unmasking of ferredoxin-dependent cyclic photophosphorylation in chloroplasts was greatly facilitated by the prevention of photochemical oxygen evolution; nevertheless, this type of photophosphorylation is facilitated, not impeded, by the presence of oxygen gas, which always surrounds functioning chloroplasts *in vivo*. These findings are consistent with the central role which is envisaged[13] for ferredoxin in photosynthetic phosphorylation (cyclic and noncyclic) by chloroplasts in intact cells.

Methods.—The source of light was an incandescent 1200-watt General Electric projection lamp, cooled by a combination of rapid forced air circulation, circulation of cold water through copper coils wound around the metal lamp housing, and by passing the light beam through a glass-enclosed circulating cold water filter, 5 cm thick. The monochromatic spectral bands were isolated with interference filters (made by Balzers Aktiengesellschaft, Balzers, Liechtenstein) and focused on the bottom of the reaction vessel by a combination of lenses and a 45° mirror, an arrangement similar to that described by Warburg and Krippahl.[14] The spectral characteristics of interference filters were checked with a Cary Model 14 recording spectrophotometer. The purity of the isolated monochromatic band was spectroscopically monitored. In using monochromatic light at 708 mμ (or longer) it was important to select filters with sharp cutoff characteristics toward radiation on the shorter wavelength side.[15] Without these precautions even a relatively small admixture of shorter wavelength radiation may nullify the effects of far-red monochromatic light.

The intensity of the incident monochromatic light was measured by Warburg's bolometric method,[16] with the use of a Lumner-Kurlbaum bolometer. The bolometer was calibrated against a standard lamp from the U.S. Bureau of Standards. The incident light intensity in all experiments was greater than 3 μeinsteins ("μmoles" of quanta) per min. At lower light intensities little photophosphorylation was observed. The light intensity used was adequate to give significant but not high photophosphorylation rates.

The photophosphorylation reaction was carried out at 15°C in rectangular Warburg vessels, which were continuously shaken in a rectangular constant temperature waterbath with a glass side wall through which the light beam entered to fall on the 45° mirror that was mounted in the bath below the reaction vessels. Broken chloroplasts[17] (P_{18} or C_{18}) were used in all experiments. The ATP formed was estimated by the method of Hagihara and Lardy,[18] including the step with the addition of K_2HPO_4 as a carrier. Oxygen evolution was measured polarographically with a platinum electrode modeled after that of Hagihara.[19]

Results and Discussion.—In previous experiments[13] with white light under anaerobic conditions, the ferredoxin-dependent cyclic photophosphorylation was experimentally demonstrated by omitting TPN and by using the inhibitor CMU,[1] noted for its effectiveness in inhibiting oxygen evolution by chloroplasts. In other experiments, not reported here, the omission of chloride gave results similar to those obtained by the addition of CMU. (Without chloride, chloroplasts cannot evolve oxygen.[20, 21]) These findings indicated that other suitable experimental methods for suppressing photoproduction of oxygen by chloroplasts should also be effective in unmasking the ferredoxin-dependent cyclic photophosphorylation.

In photosynthesis by intact cells, far-red monochromatic light does not support oxygen evolution[22] and, at wavelengths longer than 680 mμ, is associated with a decline in quantum efficiency.[23] In our earlier experiments with isolated chloroplasts, in which the oxygen evolution reaction was chemically separated from ATP formation and TPN reduction, there were indications that these partial reactions may lend themselves to separation by monochromatic light.[9] It seemed possible therefore that far-red light of a wavelength that would not support oxygen evolution would still support the ferredoxin-dependent cyclic photophosphorylation and do

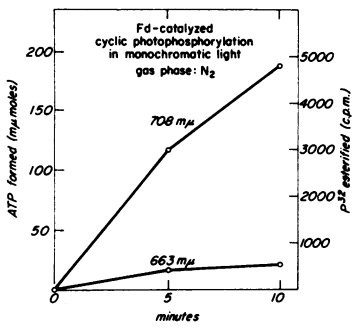

FIG. 1.—Ferredoxin-catalyzed cyclic photophosphorylation in monochromatic light. The reaction mixture (final volume of 3.0 ml) included broken chloroplasts containing 400 μg of chlorophyll, 2 mg of spinach ferredoxin, and the following in μmoles: tris buffer, pH 8.3, 100; $MgCl_2$, 5; ADP, 2; $K_2HP^{32}O_4$, 2. Prior to adding $K_2HP^{32}O_4$, each reaction mixture was preilluminated for 5 min by its respective monochromatic light. Light intensity was 5.8 and 4.1 μeinsteins per min at 708 mμ and 663 mμ, respectively. The reaction vessels were capped with rubber serum bottle stoppers through which, at the indicated times, 0.5 ml aliquots of the reaction mixture were withdrawn with a syringe, for ATP determination. The ATP formed is plotted as μmoles ATP in 1.5 ml of reaction mixture. The reaction vessels were continuously flushed with N_2, first for 5 min in the dark, and then during the entire period of exposure to light.

so without the addition of a chemical inhibitor of oxygen evolution.

These considerations were sustained by experiments represented by Figure 1 and Table 1. Table 1 shows that red monochromatic light effectively supported oxygen evolution by isolated chloroplasts at wavelengths below but not above 700 mμ. Conversely, as shown in Figure 1, ferredoxin-dependent cyclic photophosphorylation, under anaerobic conditions and without added CMU, occurred at 708 mμ but was greatly reduced at 663 mμ. In other experiments, similar to those with white light,[13] ferredoxin-dependent anaerobic cyclic photophosphorylation was strikingly increased at 663 mμ by the addition of CMU which abolished photoproduction of oxygen at this wavelength (Table 1).

TABLE 1

EFFECT OF RED MONOCHROMATIC LIGHT ON PHOTOPRODUCTION OF OXYGEN BY ISOLATED CHLOROPLASTS

Wavelength (mμ)	O_2 evolved (μmoles/min)
663	0.14
672	0.12
696	0.11
708	0.03
718	0.01
663, CMU	0.00

The reaction mixture (final volume 2.7 ml) included chloroplasts (containing 180 μg of chlorophyll), 150 μg of spinach ferredoxin, and the following in μmoles: tris buffer, pH 8.3, 125; $MgCl_2$, 5; TPN, 1. 0.5 μg of CMU was added where indicated. The same reaction mixture was successively illuminated by each respective beam of monochromatic light for about 30 sec. The optical system was adjusted to give the same intensity of monochromatic light (5.2 μeinsteins/min) at each wavelength of light. Temperature was 17°C. The initial concentration of O_2, dissolved in the reaction mixture, was 6.1×10^{-6} M.

The inability of monochromatic light at 663 mμ to support *anaerobic* cyclic ATP formation (Fig. 1) was observed after preilluminating the chloroplast system at this wavelength for 5 min under nitrogen and in the absence of CMU. The significance of these observations, which led to another series of experiments, will be discussed elsewhere.

Since reduced ferredoxin is nonenzymatically oxidized by molecular oxygen,[24] it might be argued that photochemically reduced ferredoxin would constitute an electron leak to oxygen and thus be ineffective in catalyzing cyclic photophosphorylation under aerobic conditions, that is, under conditions which normally exist around chloroplasts *in vivo*. This possibility was tested by investigating cyclic photophosphorylation at 708 mμ, under aerobic conditions. Figure 2 shows that at 708 mμ ferredoxin catalyzed ATP formation in an atmosphere of air. In fact, under aero-

bic conditions, low concentrations of ferredoxin were more effective than under anaerobic conditions in catalyzing ATP formation (compare Fig. 2 here with Table 1 in ref. 13, which gives the ATP formed in 15 min of illumination).

Evidence that the photophosphorylation at 708 mμ shown in Figure 2 was indeed of the ferredoxin-dependent cyclic type was provided by experiments with antimycin A. Sensitivity to low concentrations of antimycin A was found to distinguish the ferredoxin-dependent cyclic photophosphorylation from other types of photophosphorylation by chloroplasts: cyclic photophosphorylation catalyzed by phenazine methosulfate or menadione, and the ferredoxin-dependent noncyclic photophosphorylation coupled with TPN reduction. Figure 3 shows that aerobically at 708 mμ, as under N_2 in white light,[13] antimycin A inhibited ATP formation.

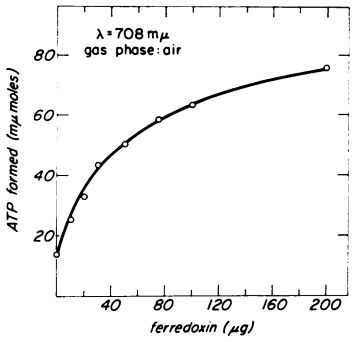

FIG. 2.—Effect of ferredoxin on cyclic photophosphorylation in air at 708 mμ. The reaction mixture contained (in a final volume of 1.5 ml) spinach ferredoxin as indicated, broken chloroplasts containing 200 μg of chlorophyll, and the following in μmoles: tris buffer, pH 8.3, 50; $MgCl_2$, 2.5; ADP, 1; $K_2HP^{32}O_4$, 1. The reaction was carried out for 4 min under air. The light intensity was 4.9 μeinsteins per min.

Heretofore, a notable distinction between oxidative phosphorylation by mitochondria and photosynthetic phosphorylation by chloroplasts was the insensitivity of the latter to low concentrations (5×10^{-5} *M*) of 2,4-dinitrophenol (cf. review[25]). However, as shown in Figure 4, ferredoxin-dependent cyclic photophosphorylation was strongly inhibited by low concentrations of dinitrophenol. Fifty per cent inhibition was given by about 3×10^{-5} *M* dinitrophenol.

Apart from sensitivity to antimycin A and dinitrophenol, ferredoxin-dependent cyclic photophosphorylation differed from other photophosphorylations by our chloroplast preparations in that it occurred at 708 mμ. The ferredoxin-dependent noncyclic photophosphorylation (with water as the electron donor) failed to take place at this wavelength. Under our experimental conditions, we also found no cyclic photophosphorylation at 708 mμ in the presence of phenazine methosulfate with the same monochromatic beam which was effective for the ferredoxin-dependent cyclic photophosphorylation. However, a cyclic photophosphorylation at 710 mμ, catalyzed by phenazine methosulfate, has been reported by Kok and Hoch.[26]

Summary.—To unmask the ferredoxin-dependent cyclic photophosphorylation and to demonstrate its independence from photoproduction of oxygen it was necessary to use special experimental devices such as far-red monochromatic light, anaerobic conditions, and inhibitors of oxygen evolution. However, if ferredoxin-dependent cyclic photophosphorylation is to be considered the cyclic photophosphorylation *in vivo*, then it must be shown that this type of photophosphorylation will also occur under the natural conditions of photosynthesis in leaves, i.e., in white light and in the presence of oxygen. The present findings and those reported earlier[13] provide evidence that ferredoxin-dependent cyclic photophosphorylation by isolated chloroplasts is indeed compatible with these natural conditions of photosynthesis in leaves. Evidence for cyclic photophosphorylation in intact leaves, as distinguished from isolated chloroplasts, has recently been reported by Forti and Parisi.[27]

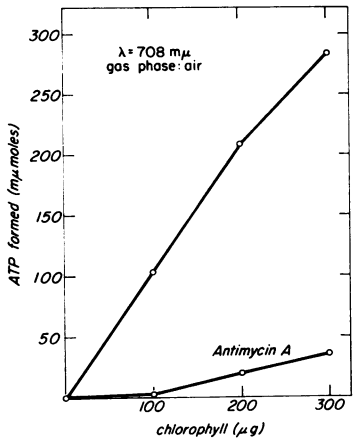

FIG. 3.—Effect of antimycin A on ferredoxin-catalyzed cyclic photophosphorylation at 708 mμ under air. The reaction mixture was as given for Fig. 2, except that 300 μg of spinach ferredoxin was used throughout and the amount of chlorophyll varied as indicated. 10 μg of antimycin A were added where indicated. The reaction was carried out for 10 min. Light intensity was 3.8 μeinsteins per min.

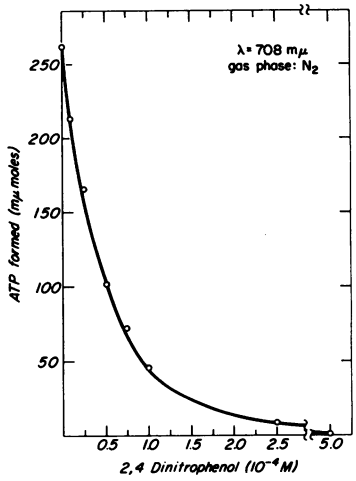

FIG. 4.—2,4-Dinitrophenol inhibition of ferredoxin-catalyzed cyclic photophosphorylation. The reaction mixture was as given for Fig. 2, except that 1.5 mg of spinach ferredoxin was used throughout, and a variable amount of 2,4-dinitrophenol as indicated. The reaction was carried out for 5 min under nitrogen. Light intensity was 4.1 μeinsteins per minute at 708 mμ.

Work from several laboratories (most recently reviewed by Smith and French[28]) has led to a now widely held view that photosynthesis in green plants involves the cooperation of at least two pigment systems, each of which carries on separate partial reactions essential to the over-all process. There is little biochemical evidence, however, as to which partial photochemical reaction is associated with which specific pigment system. The occurrence of ferredoxin-dependent cyclic photophosphorylation at 708 mμ and its independence from oxygen evolution is consistent with a hypothesis that, in chloroplasts, photophosphorylation (and TPN reduction[9, 10]) is dependent mainly on the chlorophyll *a* pigment system and hence can be experimentally separated from the photochemical activity of chlorophyll *b*, which is associated with photoproduction of oxygen.

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[1] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, orthophosphate; TPN, $TPNH_2$, oxidized and reduced forms of triphosphopyridine nucleotide; CMU, *p*-chlorophenyldimethyl urea.

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*CONFORMATIONAL CHANGES IN MYOSIN B AND ACTOMYOSIN INDUCED BY ATP**

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The ATP superprecipitation of "myosin B" suspended in neutral 0.1 *M* KCl depends on many conditions in just the same way that muscle contraction depends on the same conditions; the phenomenon is therefore considered to be the simplest model of contraction. Our investigations seek to ascertain the molecular nature of this primitive "contraction," as well as of other ATP-myosin B reactions. The materials we have used—myosin, actin, myosin B—were extracted from rabbit muscle and purified (more stringently than usual) by standard procedures.[1] Also the methods employed—light scattering, viscosity, electron microscopy (chiefly by the mica replica technique[2, 3])—were employed in conventional fashion. It should be noted, however, that while most physical studies on muscle proteins employ KCl solutions, it was necessary for electron microscopy to use NH_4HCO_3 solutions;