CYCLIC AND NON-CYCLIC PHOTOPHOSPHORYLATION IN CHLOROPLASTS DISTINGUISHED BY USE OF LABELED OXYGEN [1, 2]

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Since it was first reported that isolated chloroplasts utilize light energy in the formation of ATP[5] (2) a number of catalysts of the reaction have been described (3). It was thought that these catalysts functioned as electron carriers in a cyclic process which does not involve molecular oxygen. The proposed process consisted of their reduction by a photochemically produced reductant and their subsequent reoxidation, in a series of phosphorylation linked reactions, by the associated photochemically produced oxidant (1). Phosphorylation was attributed to the reoxidation of the reduced catalyst on the basis of an analogy to oxidative phosphorylation in mitochondria. The non-participation of molecular oxygen in the cycle was postulated partly on the basis of an analogy to photophosphorylation in the chromatophores of Rhodospirillum (9) and partly on the basis of two experimental observations. These observations were A: the fact that phosphorylation by chloroplasts was independent of exogenous oxygen, especially at high concentrations of catalysts such as FMN (21), and B: the fact that experiments with labeled oxygen revealed none of the exchange which would result from its continuous production and consumption (14).

When it was discovered that phosphorylation also could be coupled to non-cyclic reactions such as the photochemical reduction of ferricyanide and TPN^+ by chloroplasts (3, 4) a re-evaluation of the earlier concept of cyclic phosphorylation became necessary. In the absence of any reoxidation process phosphorylation still occurred and consequently the site of phosphorylation (if only one) had to be assigned to the reduction process. Moreover, for a number of reasons enumerated below, the apparently settled matter of the non-participation of molecular oxygen in the FMN catalyzed system had to be reconsidered:

I. It had been shown that FMN is reduced by illuminated chloroplasts with the concomitant production of oxygen (10, 19). It seemed odd that the phosphorylation intermediates, phosphate and ADP, which stimulate rather than inhibit oxygen production in the presence of ferricyanide, should have abolished oxygen production with FMN.

II. Photophosphorylation with FMN was dependent on oxygen pressures at lower concentrations of the catalyst (17).

III. One of us (N.E.G.) showed that FMN reduction, whether measured spectrophotometrically in the absence of oxygen or indirectly by the method of Good and Hill (10), could be stoichiometrically related to ATP formation, the yield of ATP per mole of electrons transferred being the same as that observed in the reduction of ferricyanide (unpubl). This identity of stoichiometry seemed to preclude any unaccounted-for electron flux such as that involved in the catalysis of a cyclic process.

IV. Finally, an examination of the data obtained from the preliminary experiments with oxygen isotopes (14) revealed that the system used was probably diffusion limited. Because of a rather high rate of net oxygen uptake, caused presumably by the riboflavin sensitized photooxidation of ascorbic acid, the liquid phase oxygen concentration was vanishingly low. Since detection of an exchange reaction depends on the simultaneous movement of one isotope into the liquid and a different isotope out of the liquid, it is obvious that a diffusion limited system with its one way oxygen movement gives no information on exchange.

Meanwhile Jagendorf and his associates (13) and Hill and Walker (11) had shown that N-methylphenazonium ions (phenazine methosulfate or PMS) or pyocyanine catalyzed high rates of ATP formation by illuminated chloroplasts. Jagendorf was also able to show that PMS catalyzed phosphorylation was very little affected by 3-(4-chlorophenyl)-1,1-dimethylurea (CMU), a substance which inhibits both photosynthesis and the Hill reaction probably by inhibiting the mechanism of oxygen production (20, 8); photophosphorylation in the presence of ferricyanide or FMN was, on the other hand, completely inhibited by CMU.

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[5] The following abbreviations and trivial names have been employed: ADP, adenosine diphosphate; ATP, adenosine triphosphate; TPN^+, triphosphopyridine nucleotide; FMN, flavin mononucleotide; menadione, 2-methyl-1, 4-naphtho-quinone; CMU, 3-(4-chlorophenyl)-1, 1-dimethylurea; PMS (Phenazine methosulfate), N-methylphenazonium ion; pyocyanine, 1-hydroxy-5-methylphenazonium ion.

Moreover, earlier unpublished work by Hill, Davenport, and Good had suggested strongly that although pyocyanine did not undergo reduction by chloroplasts and reoxidation by molecular oxygen (10) it was nevertheless photochemically reduced by chloroplasts as judged by its catalysis of methaemoglobin reduction. Consequently the concept of a cyclic process not involving oxygen, which was rapidly becoming untenable with regard to the FMN system for which it was originally proposed, seemed to fit the pyocyanine and PMS systems very well.

To differentiate between these two mechanisms, that is to differentiate "dye" reduction followed by its reoxidation by molecular oxygen from dye reduction followed by reoxidation by some oxidized precursor of oxygen, the oxygen isotope exchanges in the various systems were investigated by means of the recording mass spectrometer described elsewhere (6, 12). In this report full appreciation has been accorded to the disequilibria associated with diffusion barriers. All isotope ratios, and consequently the calculated exchange rates, have been corrected for the effects of diffusion barriers on the isotope ratio of dissolved oxygen by the method of Brown and Weis (7). To keep such corrections small, the measured rates were kept as low as practicable by the use of minimal amounts of chloroplasts and the experimental vessel was shaken vigorously to minimize disequilibria across the gas-liquid interface. Moreover, the large net uptake of oxygen which characterized the earlier experiment (14) was almost completely abolished, even in the presence of FMN and ascorbate, by interposing an FMN filter between the light source and the reaction vessel. Under these revised conditions the amounts of ATP formation, oxygen production, and oxygen consumption were determined with FMN, menadione, pyocyanine, and PMS as catalysts.

Methods

Chloroplasts were obtained from market spinach. The leaves were ground in a chilled mortar with ice-cold buffer (0.35 M sucrose, 0.01 M NaCl, 0.05 M tris (hydroxymethyl) aminomethane, pH adjusted to 7.8 with H_2SO_4). The larger leaf fragments were removed by passing the brei through glass wool and smaller fragments were removed by centrifuging for 1 minute at 200 × *g*. The supernatant was then spun at 1000 × *g* for 7 minutes. The chloroplasts were resuspended and washed once with buffer. They were finally resuspended in buffer, filtered through glass wool to remove any clumps and stored briefly in an ice bath.

The reaction mixture consisted of chloroplasts containing from 30 to 155 μg chlorophyll, buffer, radioactive K_2HPO_4 (0.01 M), either ADP (10 μmoles) or ATP (1.0 μmoles) with hexokinase (0.6 mg) and glucose (30 μmoles), and $MgSO_4$ (24 μmoles). The various catalysts were added in the amounts indicated in the tables. The total volume was 3.0 ml. It was necessary to add catalase (highly purified from horse liver) to the PMS system in order to hasten the decomposition of hydrogen peroxide formed in the dark by PMS catalyzed oxidations of substances present in the chloroplast preparations.

Reactions were run at 14° C in a 15.8 ml rectangular vessel attached to the leak inlet to the mass spectrometer. The shaking rate was *236* oscillations per minute at a vessel excursion of *2* cm. Vessels were flushed with helium in darkness while they were being shaken. The oxygen labeled with a very large excess of O^{18} was admitted and the vessel was closed. Shaking was continued in the dark until the gas and liquid phases had completely equilibrated. At this point the light was turned on. About 15 minutes elapsed between the mixing of the reaction components and the beginning of illumination. An 18 minute light period was usually employed during which time the changing levels of oxygen isotopes were measured. After each run the mass spectrometer was calibrated using gas with a known oxygen concentration. In most experiments the very intense light was filtered through a concentrated solution of FMN before it impinged on the reaction vessel.

Following the period of illumination the reaction mixture was added to 10 % perchloric acid and the organic phosphate (ATP or glucose-6-phosphate) was measured as the radioactivity remaining after extraction of the orthophosphate as phosphomolybdate (18).

Results & Discussion

These results support unambiguously the reasoning outlined in the introduction: Phosphorylation with FMN was associated with the production and consumption of the expected amount of oxygen (table I). On this point our data agree with similar data of Nakamoto et al (16). On the other hand, pyocyanine and PMS catalyze a mechanism of phosphorylation in which molecular oxygen seems not to be involved (table II). Thus there is no longer any reason for retaining the original concept of cyclic phosphorylation with FMN since molecular oxygen is certainly involved in the cycle. The apparent oxygen independence of systems with high FMN concentrations is not really at variance with these findings since these concentrations of FMN would allow a fairly high concentration of $FMNH_2$ to accumulate without depleting FMN to a suboptimal concentration. This high concentration of $FMNH_2$ would react so promptly with the oxygen produced in situ that very little could escape to the gas phase. Consequently a closed system could be maintained indefinitely under a nitrogen atmosphere. At low concentrations of FMN, however, it would be impossible to build up enough $FMNH_2$ to trap all of the produced oxygen. Some oxygen would escape, more FMN would be reduced, and soon the remaining level of electron acceptor would be sub-optimal, resulting in severely limited electron transport and a correspondingly limited phosphorylation. It also seems unlikely that phosphorylation accompanies the reoxidation of $FMNH_2$

TABLE I

NON-CYCLIC PHOTOPHOSPHORYLATION*

CATALYST	CHLOROPHYLL CONC μg/REACTION	OXYGEN CONC %	OXYGEN EXCHANGE μATOMS		ATP FORMED μMOLES
			UPTAKE	PRODUCTION	
FMN (3.3×10^{-5} M)	31	0.87	1.75	1.7	1.85
"	85	0.28	2.7	2.6	2.6
Menadione (1.5×10^{-4} M)	40	0.45	2.45	1.65	0.57
"	42	0.32	1.25	0.72	0.33
"	42	0.35	1.00	0.70	0.31
FMN (3.3×10^{-5} M) + Ascorbate (3.3×10^{-3} M)	56	0.48	0.55	0.44	0.40
FMN (3.3×10^{-5} M) Menadione (1.0×10^{-5} M)	54	0.42	0.29	0.24	0.07
Ascorbate (3.3×10^{-3} M)	54	0.21	0.40	0.17	0.14

* The reaction mixture consisted of spinach chloroplasts, catalyst, tris buffer pH 7.8, radioactive K_2HPO_4 (0.01 M), ADP (10 μmoles) and $MgSO_4$ (24 μmoles), total volume 3.0 ml. Temperature 14° C. Light from a 1,000 w incandescent projection lamp was filtered through about two centimeters of a strong FMN solution. Time of illumination 18 to 37.8 minutes. The vessel was shaken at 236 cycles per minute with an excursion of 20 mm. Oxygen concentration is that of the liquid phase and is referred to the concentration in equilibrium with pure oxygen gas.

since this reoxidation is probably a spontaneous, nonenzymatic process. The accumulated evidence therefore suggests that phosphorylation with FMN as electron acceptor is in no way different from the process with ferricyanide as acceptor, save that in the reduced form of FMN is autoxidizable. Rates of electron transport, sensitivity to inhibitors, and the efficiency of phosphorylation in terms of the electron flux apparently are the same with either acceptor.

Photophosphorylation with menadione as electron acceptor has not yet been investigated thoroughly but the reactions catalyzed by this quinone probably are very similar to those catalyzed by FMN. However, menadione reduction in our experiments was not efficiently coupled to phosphorylation; oxygen production was greater than would have been predicted from the amount of ATP formed. This may be, in varying degrees, a property common to quinoid oxidants since the reduction of indophenol dyes by chloroplasts is completely dissociated from phosphorylation (15).

It is obvious (table II) that the methylphenazonium ion (PMS) and pyocyanine catalyze a reaction which does not involve the production and reutilization of molecular oxygen. From 5 to 15 μmoles of ATP were formed for each μatom of oxygen produced. Moreover, it has long been known that illuminated spinach chloroplasts in the absence of exogenous electron carriers exchange the oxygen of water with mo-

TABLE II

CYCLIC PHOTOPHOSPHORYLATION*

CATALYST	CHLOROPHYLL CONC μg/REACTION	OXYGEN CONC %	OXYGEN EXCHANGE μATOMS		ATP FORMED μMOLES
			UPTAKE	PRODUCTION	
N-methylphenazonium ion (PMS)	155	0.31	0.31	0.34	4.6
(3.3×10^{-5} M)	56	0.56	0.22	0.25	1.45
Pyocyanine	56	0.53	0.10	0.15	1.75
(3.3×10^{-5} M)	83	0.54	0.20	0.18	1.08
	66	0.52	0.16	0.13	1.55
	155	0.73	0.49	0.49	4.2

* The reaction mixture consisted of spinach chloroplasts, catalyst, tris buffer pH 7.8, radioactive K_2HPO_4 (0.01 M), ATP (1.0 μmole), hexokinase Sigma type II (0.6 mg), glucose (30 μmoles) and $MgSO_4$ (24 μmoles). Horse liver catalase was added with PMS. Total volume 3.0 ml. Temperature 14° C. Incandescent (white) light was used. Time of illumination 18 to 37.8 minutes, vessel shaken as in table I. Oxygen concentration expressed as above.

lecular oxygen at a slow rate (5) and consequently even the small amount of exchange observed in the presence of PMS and pyocyanine may be unrelated to their catalytic function.

The exclusion of molecular oxygen from the PMS and pyocyanine catalyzed electron transport chains is of some importance in fixing the site of CMU action. Bishop concluded that the phenyl-dimethylureas inhibit the mechanism which produces molecular oxygen because they inhibit photosynthesis but not photoreduction in Scenedesmus (8). Our results, coupled with the observations of Jagendorf cited above, provide further evidence correlating oxygen production and CMU sensitivity. Our evidence, in this case from in vitro studies, lends further support to Bishop's hypothesis.

Summary

Oxygen isotope exchanges during photophosphorylation by spinach chloroplasts have been followed with a mass-spectrometer. Oxygen exchange is equivalent to or greater than phosphate uptake when FMN or menadione is used as the catalyst if one assumes a P/O ratio of 1.0. With PMS or pyocyanine as the catalyst, phosphate uptake is from 5 to 15 times as great as the oxygen exchange. It is concluded that FMN and menadione catalyze a non-cyclic phosphorylation in which molecular oxygen is the final electron acceptor and that PMS and pyocyanine catalyze a cyclic phosphorylation in which molecular oxygen plays no part.

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