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AEROBIC AND ANAEROBIC PHOTOPHOSPHORYLATION IN SPINACH-CHLOROPLAST PREPARATIONS UNDER CONTROLLED LIGHT CONDITIONS

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SUMMARY

This report presents an investigation of photophosphorylation in plant-chloroplast preparations under aerobic and anaerobic conditions. The quantum requirement of photophosphorylation at low (rate-limiting) intensities of red light was employed as the assay system. Several catalysts of photophosphorylation, β-naphthoquinone-sulfonic acid, menadione, and phenazine methosulfate showed equal activity under aerobic conditions. The quantum requirement under anaerobic conditions (argon atmosphere) increased (decreased quantum efficiency) with all three catalysts.

Anaerobically, photophosphorylation was inhibited by 0.01 N HCN, regardless of catalyst employed. This inhibition was reversed by aerobic conditions. With 0.01 N HCN, O_2 uptake and H_2O_2 production accompanied photophosphorylation under aerobic conditions. With β-naphthoquinone-sulfonic acid or menadione as catalyst the ratio of peroxide production to O_2 consumption approached the value 2 as did the ratio of ATP production to O_2 consumption.

The compounds *o*-phenanthroline and *p*-chlorophenyldimethyl urea inhibit photophosphorylation under both aerobic and anaerobic conditions. The inhibition of photophosphorylation is found to be related to the inhibition of Hill-reaction activity brought about by these compounds.

Evidence leading to concepts of both cyclic and noncyclic photophosphorylation is discussed. The conclusion is reached that only noncyclic photophosphorylation has been demonstrated in plant-chloroplast preparations.

INTRODUCTION

The light-induced synthesis of ATP catalysed by plant-chloroplast preparations was demonstrated first by ARNON[1] and called photosynthetic phosphorylation. Subsequent work from his laboratory[2-6] led to the conclusion that isolated chloroplasts from spinach catalysed two types of photosynthetic phosphorylation. The type considered to be the most important biologically was designated as "cyclic" photophosphorylation*.

Abbreviations: K_3, menadione; βNQSA, β-naphthoquinone-sulfonic acid; PMS, phenazine methosulfate or *N*-methylphenazonium methosulfate; CMU, *p*-chlorophenyldimethyl urea.

* Cyclic photophosphorylation was observed by FRENKEL[21,22] in bacterial chromatophores before it was considered by ARNON with respect to plant chloroplasts

The mechanism assigned to cyclic photophosphorylation requires that all effective light energy absorbed by plant chloroplasts be employed in the synthesis of ATP. The stoichiometry assigned by ARNON to cyclic photophosphorylation is represented by Eqn. 1.

$$ADP + P \xrightarrow{light} ATP \qquad (1)$$

The type of lesser biological importance was designated as "noncyclic" photophosphorylation. The mechanism assigned to noncyclic photophosphorylation requires that the effective light energy absorbed by the chloroplasts be proportioned between the synthesis of ATP and Hill-reaction activity. The stoichiometry proposed by ARNON to describe noncyclic photophosphorylation is shown in Eqn. 2 with ferricyanide (represented as Fe(III)) employed as the Hill oxidant.

$$4Fe(III) + 2H_2O + 2ADP + 2P \xrightarrow{light} 4Fe(II) + 4H^+ + O_2 + 2ATP \qquad (2)$$

Results from numerous laboratories notably those of JAGENDORF[7-11], VENNESLAND[12-14], WESSELS[15,16], and HILL[17] also demonstrated photophosphorylation with isolated plant chloroplasts either with or without accompanying O_2 production under both aerobic and anaerobic conditions. Thus, ARNON's two hypotheses of cyclic and noncyclic photophosphorylation were accepted as the correct interpretation of the results obtained under anaerobic and aerobic conditions.

A report of experiments from WARBURG's laboratory[18] questioned the concept of cyclic photophosphorylation as the correct interpretation of photophosphorylation obtained under anaerobic or aerobic conditions without an observed production of O_2. WARBURG, employing quinones and naphthoquinones as catalysts for photophosphorylation, concluded from his experiments that photophosphorylation with isolated chloroplast is always coupled to Hill-reaction activity and proceeds with the stoichiometry shown by ARNON for noncyclic photophosphorylation. WARBURG claimed that experiments which could be interpreted as demonstrating cyclic photophosphorylation were, in reality, brought about by a masking of noncyclic photophosphorylation by a reoxidation of the added catalyst which is reduced in the light. The overall stoichiometry for cyclic photophosphorylation according to WARBURG is given in Eqns. 3 through 5.

$$2 \text{ quinone} + 2H_2O + 2ADP + 2P \xrightarrow{light} 2 \text{ hydroquinone} + O_2 + 2ATP \qquad (3)$$

$$2 \text{ hydroquinone} + O_2 \longrightarrow 2 \text{ quinone} + 2H_2O \qquad (4)$$

Balance: $$ADP + P \longrightarrow ATP \qquad (5)$$

The experiments with added catalytic quantities of K_3 or FMN offered by ARNON as evidence for the existence of a cyclic photophosphorylation in plant-chloroplast preparations also were interpreted by WARBURG to be a cyclic light reduction (with concomitant ATP synthesis and O_2 production) and dark reoxidation of the added catalyst according to the stoichiometry of Eqns. 3 through 5. VENNESLAND and co-workers[19] have suggested that their results are a confirmation of WARBURG's interpretation that K_3 and FMN catalyse a noncyclic photophosphorylation. Her

experiments demonstrate that catalytic amounts of K_3 and FMN added to plant-chloroplast preparations undergo a cycle of photoreduction and O_2-dependent re-oxidation which is consistent with the overall stoichiometry suggested by WARBURG. However, in contradiction to WARBURG's generalization, VENNESLAND suggests that a true cyclic photophosphorylation can be demonstrated in plant-chloroplast preparations with either PMS or pyocyanine as catalysts. ARNON[20] in support of his concept of cyclic photophosphorylation with plant-chloroplast preparations cites evidence obtained with the use of the inhibitors CMU, and *o*-phenanthroline, which purportedly show that K_3 and FMN can act as catalysts for a true cyclic photophosphorylation as well as catalysts for the noncyclic type as suggested by WARBURG and VENNESLAND. An experiment from JAGENDORF's laboratory claiming to demonstrate a differential effect of chloride ions on cyclic and noncyclic photophosphorylation with K_3 and FMN as catalysts is cited by ARNON as additional support for the existence of cyclic photophosphorylation.

Because of the importance that has been attached to the concept of cyclic photophosphorylation in the development of the current understanding of the mechanism of photosynthesis, it becomes all the more important to either substantiate or reject this hypothesis. Basically, the evidence obtained with plant chloroplast preparations which is interpreted as demonstrating cyclic photophosphorylation rests on, (1) a reported differential inhibition of Hill-reaction activity and photophosphorylation when the inhibitors CMU and *o*-phenanthroline are employed, and (2) the catalytic activity under anaerobic and aerobic conditions of PMS which is not known to function as a Hill reagent.

A general observation concerning most of the critical experiments is that with few exceptions little or no attention has been paid to the quality of the actinic light employed or the proper control of light intensities. Most of the critical experiments which purportedly demonstrate cyclic photophosphorylation in plant chloroplasts have been performed with white light of high intensities which reportedly saturate the particular chloroplast system under investigation. Consequently, most attempts to correlate data from different experiments obtained with inhibitors do not lead to a quantitative estimation of the phenomenon under consideration.

The experiments reported below were undertaken to examine the relationship between the rate of photophosphorylation and the rate of the Hill reaction with relatively monochromatic light in an intensity range where the rates of reaction are proportional to light intensity. The question which was asked first related to the significance of results which show compounds such as PMS to be more effective catalysts of photophosphorylation than other compounds known to be oxidants for the Hill reaction. The second question related to the effect of inhibitors such as *o*-phenanthroline and CMU on photophosphorylation catalysed by Hill reagents when low intensities of the actinic light were employed.

The results obtained support the conclusion that PMS is not a more effective catalyst of photophosphorylation than other catalysts which are known Hill reagents when the actinic light limits the rate of reaction. Also, the results support the conclusion that photophosphorylation with plant-chloroplast preparations is the direct result of Hill-reaction activity under both aerobic and anaerobic conditions and that PMS acts as a Hill reagent in a manner similar to other quinones. The results of experiments with *o*-phenanthroline and CMU are in agreement with these conclusions.

MATERIALS AND METHODS

The chloroplasts and chloroplast fragments used in these experiments were prepared from young leaves of spinach *America* and New Zealand spinach which were grown in the greenhouse attached to our laboratory.

Spinach leaves were chilled on crushed ice, then deveined and homogenized in their own weight of cold 0.13 M KCl for 40 sec. The suspension was then pressed through four thicknesses of cheese cloth. The suspension thus obtained was centrifuged at 1200 × *g* for 10 min. The supernatant was decanted and, when desired, was centrifuged at 22000 × *g* for 25 min. The sediment from the original centrifugation (1200 × *g*) was resuspended in a small volume of 0.13 M KCl and either washed one time or used directly. This chloroplast fraction is designated in this report as chloroplasts (0/1.2). Microscopic examination reveals that this fraction contains whole chloroplasts and chloroplast fragments. The sediment from the second centrifugation (22000 × *g*) is also resuspended in a small volume of 0.13 M KCl. This fraction is designated chloroplasts (1.2/22). Microscopic examination reveals small chloroplast fragments and grana. All operations concerned with the preparation of the chloroplast fractions were carried out at a temperature of 0°–4°.

The concentration of chlorophyll was calculated in accordance with Beer's law:

$$C_{Chl} = \frac{\log. I_0/I}{\beta_\lambda \cdot d}$$

where C_{Chl} = total chlorophyll concentration (mg/cm³), d = 1.0 cm, and β_{546} = 9.3 cm²/mg (determined by WARBURG for chlorophyll ($a + b$) at wavelength 546 mμ). The method employed for isolating the red light, wavelength 600–670 mμ, and the methods employed for the measurement of light intensities were essentially those which have been developed by WARBURG[21]. In these experiments, two 750-W projection lamps (Sylvania DDB-750 T 12 P) served as the light sources for the two beams of red light. The red band (600–670 mμ) was isolated with the aid of interference filters which were blocked against transmission of radiation between 670 mμ and 1200 mμ. The light intensities of the two parallel beams could be varied with neutral filters. Equal intensities of the two parallel light beams were produced with the appropriate lenses, filters, and diaphragms in much the same way as has already been described[22].

The light intensities of the two beams were equated with the aid of a bolometer (surface area 10 cm²). The bolometer was custom-made by H. Röhrig, Berlin-Schöneberg. The method used for the calibration of the bolometer against a lamp from the Bureau of Standards of the United States has already been described[22, 23]. In some experiments the pheophytin actinometer was employed for the measurement of the quantum intensity[23].

All manometric measurements were made with special Warburg vessels and manometers which have been described by WARBURG[21]. The Warburg bath was a rectangular model with its original bath replaced by a stainless-steel bath containing a large thermopane window mounted on one side. The two parallel actinic lights used in these experiments entered the bath through the thermopane window and were reflected onto the bottom of the Warburg vessel by a mirror mounted at a 45° angle to the incident light beam. Most manometric experiments were performed with

chloroplast preparations with total chlorophyll contents of 0.5 mg chlorophyll per 3.0 ml. This concentration of chlorophyll was found sufficient for the total absorption of the actinic light. Consequently, the light intensities measured with the actinometer or the bolometer were employed in the calculations of the energetic efficiency of photophosphorylation. The method that has been employed in these experiments to report the energetic efficiency of photophosphorylation (Φ_{ATP}) has been to use the reciprocal value ($1/\Phi_{ATP}$) which represents the quantum requirement of photophosphorylation, where

$$\Phi_{ATP} = \frac{\mu\text{moles ATP synthesized}}{\mu\text{moles quanta absorbed}} \quad \text{and} \quad \frac{1}{\Phi_{ATP}} = \frac{\mu\text{moles quanta absorbed}}{\mu\text{moles ATP synthesized}}$$

The unit of light intensity (J) employed in these experiments is the μEinstein/min, where

$$J = \mu\text{Einsteins/min} = \mu\text{moles quanta/min}$$

The synthesis of ATP from ADP and P was determined either by the method of FISKE AND SUBBAROW[24] or by a modification of a titrimetric assay of hexokinase activity[25]. When the latter method was used an aliquot of the perchloric acid extract of the chloroplast suspension is readjusted to pH 7.5 and excess glucose and hexokinase added. The decrease in pH due to the reaction,

$$\text{ATP} + \text{glucose} \longrightarrow \text{glucose phosphate} + \text{ADP} + \text{H}^+$$

is titrated with 0.1 N NaOH with a syringe micrometer which delivers $2 \cdot 10^{-4}$ ml per division. With this method it was found that the formation of 1 μmole ATP could be determined easily since this quantity causes the formation of 1 μmole of glucose phosphate and its equivalent in hydrogen ions whose neutralization measured 50 divisions on the syringe micrometer.

The determination of H_2O_2 production was performed manometrically with the addition of catalase or with the addition of $KMnO_4$ in 0.5 N H_2SO_4.

RESULTS

Quinone catalysis

Table I represents data from an experiment in which βNQSA was employed as a catalyst for photophosphorylation. The experiment was designed to measure the relative energetic efficiency of photophosphorylation under aerobic and anaerobic conditions in red light. According to ARNON[20] the anaerobic conditions which are necessary for obtaining cyclic photophosphorylation with a quinone-catalysed system should show a lower quantum requirement for ATP synthesis (greater energetic efficiency) than is to be expected under aerobic conditions. However, the results presented in Table I show the opposite effect (compare Vessels 1 and 3).

Under anaerobic conditions, a concentration of 0.01 N HCN is found to inhibit completely the quinone-catalysed photophosphorylation whereas photophosphorylation is not affected under aerobic conditions (compare Vessels 2 and 4). WARBURG[18] suggests that the inhibition of the quinone-catalysed photophosphorylation by cyanide under anaerobic conditions is the result of the cyanide inhibition of an enzyme necessary for the reoxidation of the catalytic quantity of quinone reduced by light activity. He proposes that under anaerobic conditions, the catalytic quantity of quinone reduced by Hill-reaction activity is ordinarily reoxidized by the small

quantity of O_2 produced concurrently with the quinone reduction; this reoxidation being catalysed by this cyanide-sensitive phenol oxidase which is active in the absence of cyanide even at such low O_2 tensions. The observation[20] that greater concentrations of quinones are required for maximum rates of photophosphorylation under anaerobic conditions can therefore be explained as simply an O_2-concentration effect on this phenol oxidase.

TABLE I

CATALYTIC ACTION OF βNQSA ON PHOTOPHOSPHORYLATION WITH SPINACH CHLOROPLASTS

The reaction mixture contained in a final volume of 3.0 ml, chloroplasts (0/1.2) containing 0.6 mg chlorophyll, and (in μmoles): Tris–HCl (pH 8.0), 60; $MgCl_2$, 15; ADP, 20; potassium phosphate, 12; KCl, 50; βNQSA, 0.6; and where indicated, KCN, 30. All reactants were adjusted to pH 8.0 before addition. The KCN and βNQSA were added from the side arms of manometer vessels after gas and temperature equilibration. Temperature, 15°; red light, wavelength 600–670 mμ; J = 12 μmoles quanta/min; reaction time was 20 min.

	Vessel			
	1	2	3	4
Catalyst	βNQSA	→	→	→
Inhibitor	—	+HCN	—	+HCN
Atmosphere	Argon	→	Air	→
O_2 (μmoles)	—	—	—	−4.9
H_2O_2 (μmoles)	—	—	—	+9.1
ATP (μmoles)	+3.1	—	+8.1	+7.5
$+H_2O_2/-O_2$	—	—	—	1.86
$+H_2O_2/+ATP$	—	—	—	1.21
$1/\Phi_{ATP}$	77	—	30	32

Under aerobic conditions, the concomitant O_2 uptake (Vessel 4) and H_2O_2 production in 0.01 N HCN was observed by WARBURG[18] under somewhat different conditions of light quality and intensity. The explanation that he proposes requires that the ratio of peroxide production to O_2 consumption equal the value 2. The reaction sequence suggested by him is shown below (Q represents a quinone such as βNQSA, and QH_2 represents its reduced form).

Aerobic photophosphorylation in 0.01 N HCN:

$$2Q + 2H_2O + 2ADP + 2P \xrightarrow{light} 2QH_2 + O_2 + 2ATP \quad (6)$$

$$2QH_2 + 2O_2 \longrightarrow 2Q + 2H_2O_2 \quad (7)$$

Balance: $$2H_2O + O_2 + 2ADP + 2P \xrightarrow{light} 2H_2O_2 + 2ATP \quad (8)$$

This reaction sequence accounts for the experimental values obtained under aerobic conditions and for the ratio of peroxide production to O_2 consumption and the ratio of peroxide production to ATP production when 0.01 N HCN is present in the reaction mixture. In the absence of cyanide and still under aerobic conditions any peroxide formed by the autoxidation of the reduced quinone is decomposed by the catalase of the chloroplasts to H_2O and O_2. Since O_2 production balances O_2 consumption

in the absence of cyanide, the light-induced synthesis of ATP is the only event that is experimentally observed. This sequence of reactions was also suggested by WARBURG and is shown below.

Aerobic photophosphorylation:

$$2Q + 2H_2O + 2ADP + 2P \xrightarrow{\text{light}} 2QH_2 + O_2 + 2ATP \quad (9)$$

$$2QH_2 + 2O_2 \longrightarrow 2Q + 2H_2O_2 \quad (10)$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \quad (11)$$

Balance: $$ADP + P \xrightarrow{\text{light}} ATP \quad (12)$$

It is to be noted that it was photophosphorylation under similar conditions that ARNON observed[5,20] when he developed the theory of cyclic photophosphorylation.

K_3 catalysis

Since K_3 is the quinone employed most often by ARNON, the activity of K_3 and βNQSA were compared as catalysts of photophosphorylation. The results presented in Table II show that the quantum requirement of photophosphorylation ($1/\Phi_{ATP}$) is constant under aerobic conditions which suggests that the catalytic action of K_3 and βNQSA are similar. Since βNQSA is more easily handled in experiments, it was employed more frequently than K_3. The results tabulated in Table II again show the light efficiency of photophosphorylation to be greater under aerobic conditions than anaerobic conditions. The inhibition caused by HCN under anaerobic conditions is observed in both the K_3 and βNQSA-catalysed systems. The peroxide production and O_2 consumption as well as the ATP synthesis are observed aerobically in cyanide as is predicted by WARBURG's theory of quinone catalysis of photophosphorylation.

TABLE II

CATALYTIC ACTION OF βNQSA AND K_3 ON PHOTOPHOSPHORYLATION WITH SPINACH CHLOROPLASTS

The reaction mixture contained in a final volume of 3.0 ml, chloroplasts (0/1.2) containing 0.5 mg chlorophyll, and (in μmoles): Tris–HCl (pH 8.0), 60; $MgCl_2$, 15; ADP, 20; potassium phosphate, 10; KCl, 50; and where indicated, KCN, 30; K_3, 0.3; and βNQSA, 0.3. All reactants were adjusted to pH 8.0 before addition. The catalysts and inhibitor were added from the side arms of manometer vessels after gas and temperature equilibration. Temperature, 15°; red light, wavelength 600–670 mμ; J = 8.9 μmoles quanta/min; reaction time is 20 min.

	Vessel							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Catalyst	K_3	→	→	→	βNQSA	→	→	→
Inhibitor	-	+HCN	—	+HCN	—	+HCN	—	+HCN
Atmosphere	Argon	→	Air	→	Argon	→	Air	→
O_2 (μmoles)	—	—	—	−3.1	—	—	—	−3.4
H_2O_2 (μmoles)	—	—	—	+5.8	—	—	—	+6.2
ATP (μmoles)	+2.4	—	+6.1	+5.2	+2.7	—	+7.4	+6.5
$+H_2O_2/-O_2$	—	—	—	1.87	—	—	—	1.82
H_2O_2/ATP	—	—	—	1.12	—	—	—	0.95
$1/\Phi_{ATP}$	74	—	29	34	66	—	24	27

PMS catalysis

JAGENDORF and co-workers[7] employed PMS as a catalyst for photophosphorylation with chloroplast preparations. Their results and those from other laboratories[6,17] are interpreted as indicating that PMS catalyses only cyclic photophosphorylation under aerobic and anaerobic conditions. Since the usual catalysts of photophosphorylation such as the naphthoquinones and ferricyanide are well-known Hill reagents, the experiments with PMS, which is not recognized as a Hill reagent, raise the question as to whether this compound does catalyse a photophosphorylation in chloroplasts by a mechanism different from the one offered in explanation of quinone catalysis. First, at saturating light intensities catalytic amounts of PMS are reported as supporting greater rates of photophosphorylation than catalytic amounts of the usual quinones such as K_3. Second, at saturating light intensities the inhibition of the PMS-catalysed photophosphorylation by *o*-phenanthroline or CMU has been reported to be negligible at concentrations which block the K_3 and FMN-catalysed photophosphorylation under aerobic conditions[20].

Experiments were initiated in this laboratory to assay the PMS-catalysed photophosphorylation with low intensities of actinic light in order to compare these results with those obtained with the usual quinone-catalysed photophosphorylation.

The results of these experiments reported in Table III present a comparison of the rates of PMS-catalysed photophosphorylation and βNQSA-catalysed photophosphorylation under aerobic and anaerobic conditions. It is seen that at low light intensities no significant difference in rates is observed with PMS catalysis when compared to βNQSA catalysis. The photochemical efficiency of the synthesis of ATP is independent of the catalyst employed under aerobic conditions. However, under anaerobic conditions (Argon atmosphere) the photochemical efficiency of photophosphorylation is greater with PMS than with K_3 or βNQSA as catalyst. Nevertheless, the efficiency of PMS-catalysed photophosphorylation under anaerobic conditions is appreciably less than that observed with PMS, K_3, or βNQSA under aerobic conditions. A further

TABLE III

CATALYTIC ACTION OF PMS AND βNQSA ON PHOTOPHOSPHORYLATION

The reaction mixture contained in a final volume of 3.0 ml, chloroplasts (0/1.2) containing 0.7 mg chlorophyll, and (in μmoles): Tris–HCl (pH 8.0), 60; $MgCl_2$, 15; ADP, 20; potassium phosphate, 15, KCl, 50, and where indicated, PMS, 0.3; and βNQSA, 0.3. All reactants were adjusted to pH 8.0 before addition. The PMS and βNQSA were added from side arms of manometer vessels after gas and temperature equilibration. Temperature, 15°; red light, wavelength 600–670 mμ; Reaction time was 20 min.

	Vessel					
	1	2	3	4	5	6
Catalyst	PMS	→	→	→	βNQSA	→
Atmosphere	Argon	→	Air	→	→	→
J (μmoles quanta/min)	7.1	14.2	7.1	14.2	7.1	14.2
O_2 (μmoles)	—	—	—	−0.3	—	—
ATP (μmoles)	+2.4	+6.3	+4.7	+9.2	+4.9	+9.3
$1/\Phi_{ATP}$	59	45	30	31	29	31

observation to be made from the data in Tables I, II and III, is that the photochemical efficiency of ATP synthesis under anaerobic conditions improves with increasing light intensity whereas no change in efficiency is observed under aerobic conditions within the same range of light intensities. It should also be said that within the range of light intensities employed no observations were made in which the photochemical efficiency of ATP synthesis under anaerobic conditions was as great as under aerobic conditions.

The effect of 0.01 N HCN on PMS-catalysed photophosphorylation as shown in Table IV appears to be similar to the effect of HCN on the quinone-catalysed photophosphorylation. Under anaerobic conditions (Argon atmosphere) the PMS-catalysed photophosphorylation is completely inhibited by HCN, whereas under aerobic conditions the inhibition by HCN is practically absent. As was the observation with quinone-catalysed photophosphorylation, O_2 consumption and H_2O_2 accumulation occur under aerobic conditions with the PMS-catalysed photophosphorylation in 0.01 N HCN. However, in our experiments a consistant value of the ratio of peroxide production to O_2 consumption was not obtained with PMS as catalyst. In other respects, the catalytic action of PMS was observed to be similar to the catalytic action of the quinones K_3 and βNQSA. For example, the photochemical efficiency of ATP synthesis (Table IV) is a constant value under aerobic conditions with either PMS or βNQSA as catalyst. These experiments carried out with low light intensities indicate that the inhibition by HCN of the PMS-catalyzed photophosphorylation does not differ significantly from the effect of HCN on the quinone-catalysed photophosphorylation under similar experimental conditions (Tables I and II).

TABLE IV

EFFECT OF HCN ON PMS-CATALYSED PHOTOPHOSPHORYLATION

The reaction mixture contained in a final volume of 3.0 ml, chloroplasts (0/1.2) containing 0.5 mg chlorophyll, and (in μmoles): Tris–HCl (pH 8.0), 60; $MgCl_2$, 15; ADP, 20; potassium phosphate 10; KCl, 50; and where indicated, KCN, 30; PMS, 0.4; and βNQSA, 0.4. All reactants were adjusted to pH 8.0 before addition. The catalysts and inhibitor were added from the side arms of manometer vessels after gas and temperature equilibration. Temperature, 15°; red light, wavelength 600–670 mμ; J = 9.1 μmoles quanta/min. Reaction time was 20 min.

	Vessel					
	1	*2*	*3*	*4*	*5*	*6*
Catalyst	PMS	→	→	→	βNQSA	→
Inhibitor	—	+HCN	—	+HCN	—	—
Atmosphere	Argon	→	Air	→	Argon	Air
ATP μmoles	+5.4	-	+6.9	+6.4	+2.6	+7.3
$1/\Phi_{ATP}$	54	-	39	28	70	25

The inhibitors, o-phenanthroline and CMU

ARNON[20,28] has also reported that the inhibitors *o*-phenanthroline and CMU can be used to distinguish between a cyclic (anaerobic) and a noncyclic (aerobic) photophosphorylation when K_3 is employed as a catalyst. His results show that at high light intensities the concentration of *o*-phenanthroline or CMU which markedly inhibit the K_3-catalyzed aerobic photophosphorylation does not inhibit the K_3-catalyzed

anaerobic photophosphorylation. He concludes from these experiments that these inhibitors show different effects on the aerobic and anaerobic systems because they are acting on different pathways, one of which is cyclic (anaerobic) and the other noncyclic (aerobic).

The experiments shown in Tables V and VI do not support the conclusions reached by ARNON. When low intensities of actinic light are employed, the inhibition by $3 \cdot 10^{-5}$ M *o*-phenanthroline with either PMS or βNQSA as catalyst does not show a difference between the aerobic and anaerobic phosphorylating systems that can be interpreted in terms of a cyclic and noncyclic mechanism. Under aerobic conditions the inhibition by *o*-phenanthroline is the same in either the PMS or βNQSA system. The data shown in Table V present results which show that at low light intensities the *o*-phenanthroline inhibition is greater under anaerobic conditions than under aerobic conditions.

TABLE V

INHIBITION OF PHOTOPHOSPHORYLATION BY *o*-PHENANTHROLINE

The reaction mixture contained in a final volume of 3.0 ml, chloroplasts (0/1.2) containing 0.5 mg chlorophyll, and (in μmoles): Tris–HCl (pH 8.0), 60; $MgCl_2$, 15; ADP, 20; potassium phosphate, 12; KCl, 50; and where indicated, *o*-phenanthroline, 0.09; PMS, 0.4 and βNQSA, 0.4. All reactants were adjusted to pH 8.0 before addition. The catalysts and inhibitor were added from the side arms of manometer vessels after gas and temperature equilibration. Temperature, 15°; red light, wavelength 600–670 mμ; J = 14.1 μmoles quanta/min. Reaction time was 20 min.

	Vessel					
	1	*2*	*3*	*4*	*5*	*6*
Catalyst	PMS	→	→	→	βNQSA	→
Addition of *o*-phenanthroline	+	−	+	−	+	−
Atmosphere	Argon	→	Air	→	→	→
ATP (μmoles)	+1.1	+5.8	+2.3	+9.7	+2.4	+9.2
$1/\Phi_{ATP}$	256	49	122	29	117	31.0

TABLE VI

INHIBITION OF THE HILL REACTION AND PHOTOPHOSPHORYLATION BY *o*-PHENANTHROLINE

The reaction mixture contained in a final volume of 3.0 ml, chloroplasts (0/1.2) containing 0.5 mg chlorophyll, and (in μmoles): Tris–HCl (pH 8.0), 60, $MgCl_2$, 15; ADP, 20; potassium phosphate, 10; KCl, 50; and where indicated, KCN, 30; *o*-phenanthroline, 0.09, and βNQSA, either 0.5 or 10.0. All reactants were adjusted to pH 8.0 before addition. The inhibitors and βNQSA were added from side arms of the manometer vessels after gas and temperature equilibration. Temperature, 15°, red light, wavelength 600–670 mμ; J = 9.2 μmoles quanta/min. Reaction time was 15 min.

	Vessel			
	1	*2*	*3*	*4*
βNQSA (μmoles)	10	10	0.5	0.5
HCN	−	+	−	−
o-Phenanthroline	−	+	−	+
Atmosphere	Argon	→	Air	→
O_2 (μmoles)	+2.41	+0.67	−1.92	−0.51
ATP (μmoles)	−	−	+4.1	+0.8

Experiments were also designed to find out if the inhibition of photophosphorylation by *o*-phenanthroline could be attributed to its effect on the Hill reaction. When a comparison was made between Hill-reaction activity and aerobic photophosphorylation in $3 \cdot 10^{-5}$ M *o*-phenanthroline, the results as shown in Table VI indicate that the inhibition of photophosphorylation is caused by the inhibition of Hill-reaction activity by *o*-phenanthroline since the concentration effect of *o*-phenanthroline on photophosphorylation parallels the effect of *o*-phenanthroline on Hill-reaction activity. It should be mentioned with respect to Table VI that when substrate amounts of βNQSA (10–20 μmoles) are used as an oxidant in the direct measurement of Hill-reaction activity, no appreciable photophosphorylation can be detected. Further investigations have pointed to the fact that both *p*-benzoquinone and βNQSA inhibit photophosphorylation at concentrations above 0.002 M.

The compound CMU also has been reported to inhibit Hill-reaction activity at concentrations which show little or no effect on photophosphorylation under anaerobic conditions. Investigations in this laboratory have confirmed the finding that CMU is a potent inhibitor of Hill-reaction activity even under light-limiting experimental conditions. A concentration of approx. $1 \cdot 10^{-6}$ M is sufficient to inhibit more than 50% of Hill-reaction activity. However when, CMU was added to the chloroplast photophosphorylation system within this concentration range, no differential effect was observed between the aerobic and anaerobic systems regardless of the catalyst employed. The results shown in Table VII illustrate as with *o*-phenanthroline that CMU inhibition of photophosphorylation does not differentiate between the aerobic and anaerobic system when low light intensities are employed.

TABLE VII

INHIBITION OF PHOTOPHOSPHORYLATION BY CMU

The reaction mixture contained in a final volume of 3.0 ml, chloroplasts (o/1.2) containing 0.6 mg chlorophyll, and (in μmoles): Tris–HCl (pH 8.0), 60; $MgCl_2$, 15; ADP, 20; potassium phosphate, 10; KCl, 50; and where indicated, CMU, 0.003; PMS, 0.4; and βNQSA, 0.4. All reactants were adjusted to pH 8.0 before addition. The inhibitor and catalysts were added from side arms of the manometer vessels after gas and temperature equilibration. Temperature, 15°; red light, wavelength 600–670 mμ; J = 13.7 μmoles quanta/min. Reaction time was 20 min.

	Vessel					
	1	*2*	*3*	*4*	*5*	*6*
Catalyst	PMS	→	→	→	βNQSA	→
Inhibitor	CMU		CMU	–	CMU	–
Atmosphere	Argon	→	Air	→	→	→
ATP (μmoles)	+2.2	+5.2	+3.7	+8.1	+4.1	+8.5
$1/\Phi_{ATP}$	125	53	74	34	67	32

DISCUSSION

What emerges from an analysis of the experiments reported above is an explanation of the mechanism of photophosphorylation uniquely in terms of a Hill reaction associated phosphorylation which was designated by ARNON as noncyclic photophosphorylation. No evidence was obtained in this study that would indicate that cyclic

photophosphorylation could be observed with spinach-chloroplast preparations. Because of the importance that ARNON has attached to the concept of cyclic photophosphorylation as the unifying aspect of both plant and bacterial photosynthesis, it becomes all the more important to analyze the evidence for and against the concept of cyclic photophosphorylation in plant chloroplasts. An analysis of this problem will be made in terms of attempting to answer the three questions which follow:

(1). What experiments can be cited which have not been explained in terms of both noncyclic and cyclic photophosphorylation.

(2). To what extent are the alternate explanations of photophosphorylation attributable to different interpretations of the same experimental observations.

(3). To what extent is the demonstration of cyclic photophosphorylation in plant-chloroplast preparations based on results which are not generally accepted.

With respect to the first question, one may cite the effect of HCN on photophosphorylation which is shown in this report. The results of these experiments as well as those of WARBURG[18] demonstrate that 0.01 N HCN inhibits photophosphorylation under anaerobic conditions but not under aerobic conditions. This result is explained by WARBURG's hypothesis which places the site of the cyanide inhibition on a cyanide-sensitive enzyme system necessary for the reoxidation under essentially anaerobic conditions of the catalyst reduced by Hill-reaction activity (Eqns. 3–5). No explanation of the cyanide inhibition of photophosphorylation under anaerobic conditions and its reversal by O_2 (aerobic conditions) has been offered in terms of a cyclic mechanism of photophosphorylation. However, it is not clear to this investigator that cyanide inhibition of photophosphorylation under anaerobic conditions and its reversal by aerobic conditions are generally accepted observations. In Table XII of a paper by ARNON[26], a cyanide concentration of $2 \cdot 10^{-3}$ M KCN (pH 8.3) is reported to inhibit the K_3-catalysed anaerobic photophosphorylation by 21 %.

With respect to the second question concerning alternate interpretations of identical experimental results, consider ARNON's interpretation of the experimental observation that in the presence of an ethanol–catalase trap, aerobic photophosphorylation with plant chloroplasts is accompanied by O_2 uptake and H_2O_2 production[20]. ARNON explains this observation by introducing a concept which he terms "pseudocyclic photophosphorylation".

This concept explains the O_2 consumption and peroxide production in terms of a photoreduction of O_2. This type of mechanism was first offered by MEHLER[27,28] to explain a similar observation. It requires that O_2 act as a terminal electron acceptor in place of other Hill reagents when only a catalytic amount of FMN or a quinone is added to chloroplast preparations. The mechanism for such a reaction was postulated by MEHLER as follows:

$$4HOH \xrightarrow{light} 4H + 4OH$$

$$4OH \longrightarrow 2H_2O + O_2$$

$$4H + 2O_2 \longrightarrow 2H_2O_2$$

$$2H_2O_2 + 2\text{ethanol} \xrightarrow{catalase} 2\text{ acetaldehyde} + 2H_2O$$

Balance: $$O_2 + 2\text{ethanol} \xrightarrow{light} 2\text{ acetaldehyde} + 2H_2O$$

It is to be noted that both MEHLER and ARNON recognized that substantially increased rates of O_2 consumption and peroxide (acetaldehyde) production were obtained by the addition of catalytic amounts of a quinone.

Experiments from other laboratories[18,29] and our results with respect to photo-induced O_2 consumption and peroxide production in chloroplast preparations do not support MEHLER's interpretation that molecular O_2 can act as a substitute Hill reagent. The explanation offered by both HILL and WARBURG explains the observation in terms of a photoreduction of a Hill reagent and its reoxidation by molecular O_2 (Eqns. 6–8). It is significant that both FMN and K_3 are considered by ARNON to be catalysts of "pseudocyclic photophosphorylation" whereas TPN and ferricyanide are not found to have catalytic activity. The reoxidation by molecular O_2 of the reduced forms of FMN and K_3 lead to the formation of H_2O_2 whereas the reoxidation of the reduced form of TPN or ferricyanide is not catalyzed by molecular O_2 to any considerable extent.

The evidence is more directly interpreted in terms of known chemical reactions by assuming that O_2 does not act as a substitute electron acceptor for Hill reagents but does indeed reoxidize Hill reagents such as the quinones (reduced by light action) and form H_2O_2. However, ARNON's interpretation of these experiments in terms of "pseudocyclic photophosphorylation" requires that the consumption of O_2 be explained by a mechanism which involves the photoreduction of O_2. To the extent that his generalization concerning the concept of cyclic photophosphorylation requires an explanation of these experiments in terms of the photoreduction of O_2 which has not been satisfactorily demonstrated, his concept of cyclic photophosphorylation would appear to be weakened.

With respect to the third question which refers to contradictory results being reported from various laboratories when supposedly investigating the same experimental system, it would seem that such discrepancies would be the easiest to resolve. However, the experimental designs which often depend upon the equipment available at the time when a particular experiment is undertaken are found to differ markedly from laboratory to laboratory. Consequently, the apparent contradictions have to be evaluated not only in terms of a correct and an incorrect result, but also the possibility must be considered that both results may be significant since the experimental systems are sufficiently different. For example, the measurement of aerobic noncyclic photophosphorylation with ferricyanide as the oxidant is often followed by measuring the reduction of ferricyanide spectrophotometrically in place of measuring the concomitant O_2 production with a manometer or polarograph. In our laboratory it has been found that at least at low light intensities the rate of ferricyanide reduction and O_2 production by whole chloroplast suspensions do not agree when the buffer for the reaction mixture is 0.05 M Tris–HCl (pH 8.0).

Another example of contradictory results is seen in experiments in which *o*-phenanthroline is employed as an inhibitor in studies of photophosphorylation in chloroplasts. The results reported in this paper do not show *o*-phenanthroline to differentially inhibit aerobic photophosphorylation as apposed to anaerobic photophosphorylation as reported by ARNON[20,28]. From Tables V and VI of this report, the conclusion is drawn that at low light intensities no differential inhibition of aerobic and anaerobic photophosphorylation by *o*-phenanthroline is demonstrable. An analysis of the published data presented by ARNON to support the claim that

o-phenanthroline does inhibit noncyclic (aerobic) photophosphorylation more strongly than cyclic (anaerobic) photophosphorylation raises questions as to whether such an effect is observed in the experiments presented by ARNON. For example, combining the results from his Tables IX and XI, ARNON[16,30] reports $4 \cdot 10^{-5}$ M *o*-phenanthroline inhibits K_3-catalyzed cyclic (anaerobic) photophosphorylation by 45%, whereas $7 \cdot 10^{-5}$ M *o*-phenanthroline inhibits K_3-catalyzed photophosphorylation [illegible] % Tabulating these results, one obtains the following:

	Concn. of *o*-phenanthroline $\times 10^{-5}$ (M)	% inhibition of K_3 photophosphorylation	Reference
	3	27	Table XI[30]
Anaerobic	4	45	Table IX[26]
	[illegible]	27	Table XIV[26]
Aerobic	3	64	Table XI[30]

The variability shown in these results is sufficient to make one question the important conclusion that is drawn by ARNON, namely, that *o*-phenanthroline inhibits noncyclic (aerobic) photophosphorylation to such an extent that this inhibition is clearly distinguished from that shown in the cyclic (anaerobic) system.

ARNON[20] also reports that CMU inhibits noncyclic (aerobic) photophosphorylation at low light intensities. In Table II of his report, the inhibition of photophosphorylation effected by $2 \cdot 10^{-6}$ M CMU is shown. Whereas the K_3-catalysed system is inhibited 61% by $2 \cdot 10^{-6}$ M CMU under aerobic conditions, the anaerobic system shows only 4% inhibition in the same concentration of CMU. The PMS-catalysed system shows 4% inhibition aerobically and 11% inhibition anaerobically in $2 \cdot 10^{-6}$ M CMU. According to Table III of the same report, at high light intensities the K_3-catalysed system shows 45% inhibition anaerobically and 81% inhibition aerobically in $2 \cdot 10^{-6}$ M CMU. The PMS system shows no inhibition anaerobically or aerobically under similar conditions. ARNON concludes from this data the CMU inhibits the K_3-catalysed "pseudocyclic" (aerobic) photophosphorylation but does not inhibit cyclic (anaerobic) photophosphorylation. He also concludes that the PMS-catalysed photophosphorylation is insensitive to CMU under both aerobic and anaerobic conditions because he considers that PMS is a catalyst for cyclic photophosphorylation only. The experiment presented in Table VI of this report do not show such differential effects on photophosphorylation under aerobic and anaerobic conditions. Since ARNON's results were also obtained with low intensities of actinic light, it would appear that there is a clear experimental discrepancy which should be resolved because of the importance of these results to ARNON's argument for the existence of both a noncyclic and cyclic photophosphorylation. The results reported in this report do not support the experimental claim for *o*-phenanthroline or CMU inhibition, or for the PMS-catalysed photophosphorylation which is supposedly insensitive to these inhibitors.

Several general observations concerning the methodology employed in studying photophosphorylation are perhaps in order. The use of Tris–HCl buffer in chloroplast systems shows some peculiar effects. For example, in our hands, a concentration of 0.05 M Tris–HCl, adjusted to pH 8.0, invariably suppresses O_2 evolution in Hill-

reaction studies under both aerobic and anaerobic conditions. Consequently, the results reported by ARNON[20] in Fig. 6 are surprising to us since the pH–activity curve for O_2 production from the Hill reaction was obtained in 0.067 M Tris–HCl. What is also surprising is that the results shown for Hill-reaction activity as measured by O_2 production with ferricyanide as the oxidant shows a maximum between pH 7.8 and pH 8.0. Our experience with this system invariably shows that Hill-reaction activity decreases above pH 7.4 when O_2 production is assayed under either high or low light intensities. That is, our experiments show that the rate of the Hill reaction at pH 8.0 is approx. 40% of the rate of the Hill reaction of the same preparation when assayed at pH 7.0 with ferricyanide as oxidant. Consequently, no such pH–activity curve as the one shown in Fig. 6 of ARNON's report[20] was obtained in our experiments. Moreover, no effect on Hill-reaction activity was demonstrable when, in addition to phosphate, the entire phosphorylating system was added to the chloroplast preparations when limiting intensities of actinic light were employed and when O_2 production was the assay system.

One further comment concerning the experiments reported in this paper is in order. That is, although the quantum requirement of photophosphorylation was measured routinely and employed as the assay system, no particular importance is attached by us to the absolute magnitude of the numbers obtained under the various experimental conditions. It was noted by us that the minimum quantum requirement ($1/\Phi_{ATP}$) or maximum efficiency (Φ_{ATP}) was independent of the catalyst employed. That is, PMS, βNQSA, and K_3 yielded approximately the same results under aerobic conditions. In all cases, it is observed that the greatest efficiency (minimum quantum requirement) is obtained under conditions which do not support ARNON's cyclic photophosphorylation. If ARNON's concept of cyclic photophosphorylation exists, then one would expect a greater efficiency of photophosphorylation to be observed under anaerobic conditions where accordingly one would be dealing with cyclic photophosphorylation in which all effective light energy is employed for the synthesis of ATP. However, the contrary result is observed in these experiments.

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