PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

VII. VITAMIN K AND RIBOFLAVIN PHOSPHATE AS COFACTORS OF CYCLIC PHOTOPHOSPHORYLATION*

F. R. WHATLEY, M. B. ALLEN** AND DANIEL I. ARNON

Laboratory of Plant Physiology, Department of Soils and Plant Nutrition, University of California, Berkeley, Calif. (U.S.A.)

(Received August 12th, 1958)

SUMMARY

Vitamin K substances and FMN appear to catalyze separate pathways of cyclic photophosphorylation. The FMN pathway shows a dependence on added TPN and greater sensitivity to inhibition by dinitrophenol and *o*-phenanthroline than the vitamin K pathway. Both pathways are inhibited by *p*-chloromercuribenzoate, gramicidin and methylene blue but not by arsenite or antimycin A.

Cyclic photophosphorylation catalyzed by phenazine methosulfate resembled the vitamin K pathway in its independence from added TPN and resistance to inhibition by dinitrophenol and *o*-phenanthroline.

The role of vitamin K in phosphorylations by plant and animal tissues is reviewed.

A possible physiological role for cyclic photophosphorylation in photosynthesis of green plants is suggested.

INTRODUCTION

Isolated chloroplasts are capable of carrying out complete photosynthesis[1-3] but when conditions are so arranged that CO_2 fixation does not occur, they can use the absorbed light energy for the generation and accumulation of adenosine triphosphate. This process, which has been termed photosynthetic phosphorylation[1], has been observed outside the living cell, in 2 photochemical reactions represented by Eqns. 1 and 2.

$$2\,ADP + 2\,P + 2\,TPN + 4\,H_2O \xrightarrow{light} 2\,ATP + 2\,TPNH_2 + O_2 + 2\,H_2O \quad (1)$$

$$n\,ADP + n\,P \xrightarrow{light} n\,ATP \quad (2)$$

The following abbreviations will be used: ATP, ADP, adenosine triphosphate and diphosphate, respectively; FMN, riboflavin phosphate (flavin mononucleotide); TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; tris, tris(hydroxymethyl)aminomethane.

* A preliminary report of this work was given to the midwestern Section of the American Society of Plant Physiologists at Ann Arbor, Michigan in June, 1957.

** Present address: Division of Comparative Biology, Kaiser Foundation Research Institute, Richmond, California.

References p. 46.

Eqn. 1 shows the formation of ATP as a component of "assimilatory power"[4,5]. In this reaction ATP formation is coupled with the reduction of TPN and evolution of oxygen; the generation of the energy-rich pyrophosphate bonds of ATP accounts for only a portion of the light energy captured by the chloroplast. Another portion is used for the formation of $TPNH_2$[5]. Eqn. 2 represents what is now called "cyclic photophosphorylation"—a photochemical reaction in which all of the trapped light energy is converted into ATP[5]. Previously[1,3-8], cyclic photophosphorylation has been described under the name of photosynthetic phosphorylation—a general term now being reserved for the light-induced formation of ATP whether by reaction 1 or 2.

Until recently the catalysts of photosynthetic phosphorylation have been investigated solely in relation to cyclic photophosphorylation, since this was the only known type of light-dependent phosphorylation in chloroplasts. The first factors which were found to stimulate cyclic photophosphorylation without being themselves consumed in the reaction were magnesium ions and ascorbate[1,9]; the next to be recognized were riboflavin phosphate[10] (FMN) and vitamin K compounds[11].

A working hypothesis was formulated by visualizing the light reaction as resulting in the simultaneous generation, through a photolysis of water, of a reducing agent [H] and an oxidizing agent [OH] at opposite ends of an electron-transport chain; the cofactors of cyclic photophosphorylation would then bring about, as members of the electron-transport chain, a stepwise recombination of these products of photodecomposition of water. It was suggested that the energy liberated in this stepwise reconstitution of water becomes available for ATP formation. The role of the first electron or hydrogen acceptor was tentatively assigned[3,6,8] to either FMN or, as proposed by WESSELS[12], to vitamin K.

Further studies of photosynthetic phosphorylation made it apparent that the initially suggested chain of electron carriers required revision. TPN, not included in earlier formulations, was identified as a catalyst of cyclic photophosphorylation[13,14] and as a hydrogen acceptor in the generation of assimilatory power (Eqn. 1). Moreover, evidence was obtained under modified experimental conditions[15] that FMN and vitamin K may be active in separate pathways of cyclic photophosphorylation rather than in one. This article presents this evidence in detail and discusses the role of these and other cofactors in photophosphorylation.

METHODS

Broken chloroplasts were used in all the experiments described herein. The broken chloroplasts were prepared by two different methods and bear the corresponding designations of P_{1s} and C_{1s}. The P_{1s} particles were prepared by suspending isolated whole chloroplasts (P_1) in a dilute salt solution (0.035 *M* NaCl) as previously described[16]. The C_{1s} particles were prepared in the same manner as P_{1s}, except that ascorbate was added to all solutions used in the isolation, washing and disruption of whole chloroplasts[8], *i.e.*, the procedure was as follows: leaves were ground in 0.35 *M* NaCl containing 0.02 *M* Tris buffer, pH 8.3, and 0.01 *M* sodium ascorbate. The whole chloroplasts (C_1) were washed in 0.35 *M* NaCl containing 0.01 *M* sodium ascorbate, and then disrupted by suspending in ice-cold 0.035 *M* NaCl containing 0.01 *M* sodium ascorbate.

In certain experiments washed particles, designated C_{1s_3}, were used. These were

References p. 46.

prepared by suspending the C_{1s} particles in 0.035 M NaCl containing 0.01 M sodium ascorbate and collecting the particles by centrifugation at 18,000 $\times$ g for 10 min. The washing and centrifugation procedure was repeated 3 times.

Chloroplast extract (CE) was prepared as previously described[17] except that dilute NaCl (0.035 M) was substituted for water as the extractant. In some experiments the CE was dialyzed overnight at 3° (the same temperature as was used in making all other preparations) against 10^{-3} M Tris, pH 7.4, containing $5 \cdot 16^{-5}$ M sodium ethylenediaminetetraacetate, pH 7.4, and 10^{-2} M sodium ascorbate.

Chlorophyll determinations and measurements of cyclic photophosphorylation were carried out by methods previously reported[8]. Unless otherwise specified, the reactions were carried out for 20 min, with a cerise fluorescent light[2], at 15°, under nitrogen, and at pH 8.3.

RESULTS

FMN and vitamin K as alternate pathways in cyclic photophosphorylation

Fig. 1 shows that maximal rates of cyclic photophosphorylation were obtained with either FMN or vitamin K as catalysts. At a saturating concentration of one catalyst, the addition of the other gave no further increase in photophosphorylation, but little phosphorylation occurred unless either FMN or vitamin K was added. In the experiments represented by Fig. 1, vitamin K_3 (menadione) was used, but similar results were also obtained with vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride).

The FMN and vitamin K pathways or "systems", as they will now frequently be referred to, were compared with respect to their dependence on other cofactors and sensitivity to various inhibitors.

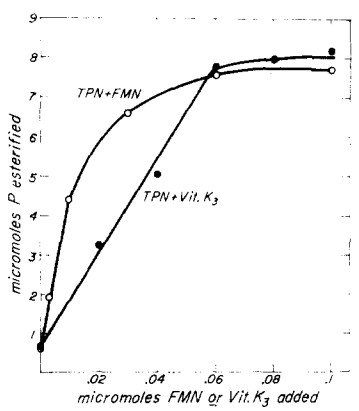

Fig. 1. FMN and vitamin K_3 as independent catalysts of cyclic photophosphorylation. The reaction mixture contained 0.1 mg chlorophyll in chloroplast fragments (C_{1s}), and, in μmoles: Tris, 80; $[^{32}P]K_2HPO_4$, 10; $MgCl_2$, 5; ADP, 10; TPN, 0.3; FMN or vitamin K_3, as indicated. Total vol. 3 ml.

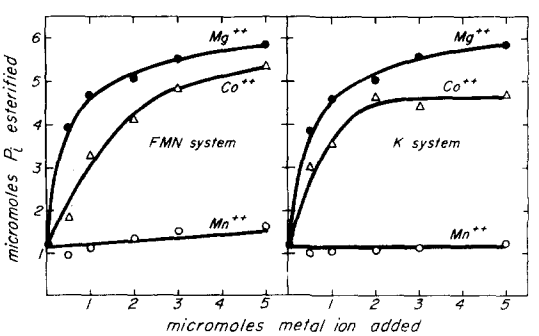

Fig. 2. Metal requirements of cyclic photophosphorylation catalyzed by FMN and by vitamin K. The reaction mixture contained 0.1 mg chlorophyll in "broken" chloroplasts (C_{1s}) and, in μmoles: Tris, 80; $[^{32}P]K_2HPO_4$, 10; ADP, 10; TPN, 0.3; FMN, 0.1 *or* vitamin K_5, 0.6; Mg, Mn and Co as indicated. Total vol., 3 ml.

References p. 46.

Magnesium requirement

As in other reactions involving the transfer of a phosphate group (*cf.* ref.[18]) Mg^{++} was required for phosphorylation by chloroplasts in either the FMN or vitamin K system. Mg^{++} was replaceable by Co^{++} but not by Mn^{++} (Fig. 2). Zn^{++}, Ni^{++} and Fe^{++} gave little or no stimulation at 10^{-3} *M*, whereas calcium, vanadium, molybdenum and copper were more or less inhibitory.

The role of ascorbate

The strikingly beneficial effect[10] of added ascorbate on what is now called cyclic photophosphorylation led to the tentative inclusion of this substance as a cofactor in the previously proposed schemes for photosynthetic phosphorylation[11,3,6,8]. At low concentrations of vitamin K and FMN ascorbate greatly increased photophosphorylation by either pathway (Figs. 3a and 3b). In our experiments, ascorbate increased cyclic photophosphorylation under both aerobic and anaerobic conditions (*cf.* ref. [19]).

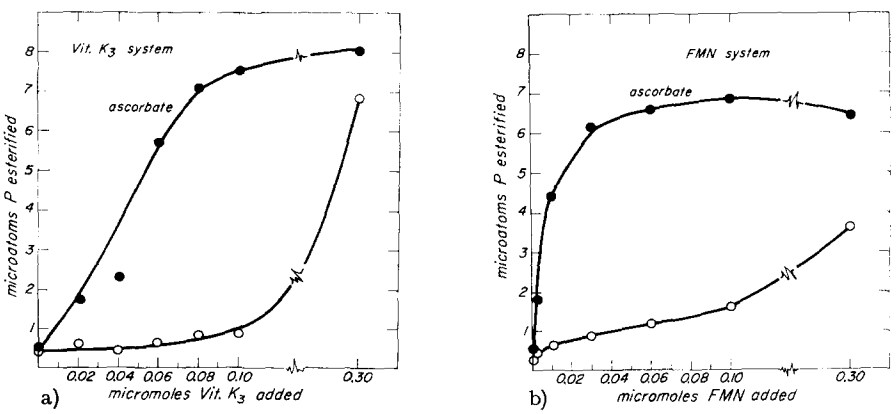

Fig. 3. a) Effect of ascorbate on cyclic photophosphorylation with vitamin K_3. b) Effect of ascorbate on cyclic photophosphorylation with FMN. The reaction mixture (final volume, 3 ml) contained broken chloroplasts prepared without ascorbate (P_{1s}), tris, $MgCl_2$, $[^{32}P]K_2HPO_4$, and ADP as described under Fig. 1, FMN or vitamin K_3 as indicated. The FMN vessels also contained 0.3 μmole TPN. Experimental conditions were as described in METHODS.

More recent evidence suggests that the beneficial effect of ascorbate on photophosphorylation may result from protecting some essential components of chloroplasts against inactivation rather then from acting as a cofactor of photophosphorylation. A serious loss of phosphorylating capacity occurred in isolated chloroplasts on standing even at low temperatures. This loss was prevented for a period of several hours[13] if the solutions used for grinding the leaves and washing the isolated chloroplasts contained 0.01 *M* ascorbate (Table I). We are therefore now inclined to assign to ascorbate a protective action, perhaps as a poising agent, rather than as a catalyst in the electron-transport chain. Seasonal and environmental fluctuations in the ascorbate content of leaves and chloroplasts could conceivably account, at least in part, for the observed variations in the activity and stability of chloroplasts isolated at various times from leaves of different origin.

References p. 46.

TABLE I

STABILIZATION OF PHOSPHORYLATING CAPACITY IN CHLOROPLASTS BY ASCORBATE

Experimental conditions as described under Fig. 1 except that in the "control" series P_{18} particles, prepared without ascorbate were used instead of C_{18} particles prepared with ascorbate (see METHODS), 0.1 μmole FMN and 0.1 μmole vitamin K_5 were added to the reaction mixture. Temperature 20°; 30 min illumination.

Storage time at 0° (h)	*μmoles P esterified*	
	Control	*ascorbate treated*
0	5.5	7.7
0.5	2.4	7.1
1	1.7	8.1
2	—	8.0
4	—	8.0
24	—	3.0

TPN requirement

The original identification of TPN as a catalyst of cyclic photophosphorylation was made using a reaction mixture in which full phosphorylating activity was brought about by supplying low concentrations of both FMN and vitamin K[13]. Rigid experimental proof was therefore lacking for the participation of TPN as a catalyst in the separate FMN and vitamin K phosphorylation pathways. Experiments to test this point have shown a consistent catalytic effect of TPN on the FMN pathway (at low concentrations of FMN) but only a smaller and less consistent effect on the vitamin K pathways of cyclic photophosphorylation (Fig. 4). However, even in the FMN system no increased photophosphorylation resulted from adding TPN if the FMN concentration was high—several times higher than that needed to give maximum phosphorylation in the presence of TPN. The same maximal photophosphorylation was obtained with low concentrations of FMN supplemented with TPN as with high concentrations of FMN with or without added TPN.

Reduced TPN can be oxidized by either FMN or vitamin K, and an enzyme catalyzing this reaction, TPNH diaphorase, was found in chloroplasts by AVRON AND JAGENDORF[20]. It is possible, however, that TPN is not normally a component

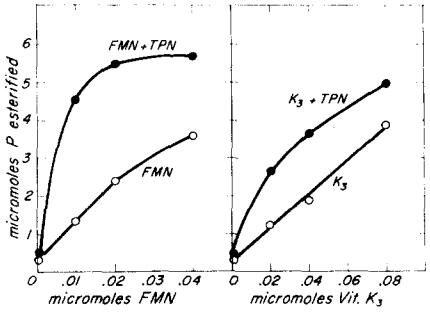

Fig. 4. Effect of TPN on cyclic photophosphorylation catalysed by FMN and by vitamin K. The reaction mixture contained 0.1 mg chlorophyll in washed chloroplast fragments (C_{183}), dialyzed *CE* equivalent to 0.2 mg chlorophyll, and, in μmoles: Tris, 80; [^{32}P]K_2HPO_4, 10; $MgCl_2$, 5; ADP, 10; TPN, 0 or 0.3; FMN or vitamin K_3 as indicated. Total vol., 3 ml.

References p. 46.

of the vitamin K pathway but rather of the FMN pathway of cyclic photophosphorylation. This conclusion is supported by the data discussed below.

The TPN-reducing factor

The photochemical reduction of TPN was found to be mediated by a specific TPN-reducing factor[13] which has recently been extensively investigated by SAN PIETRO AND LANG[21]. The TPN-reducing factor is present in chloroplasts, from which it can be readily extracted with water or dilute salt. An aqueous extract of chloroplasts (*CE*) has served in previous experiments[13] as the source of the TPN-reducing factor.

It would be expected that a photophosphorylation system which depended on the reduction of TPN would also show a dependence on the TPN-reducing factor present in *CE*. Photophosphorylation by the FMN and vitamin K pathways was compared using broken chloroplasts preparations (C_{1s3}) from which the water-soluble components found in *CE* were removed by repeated washings (see METHODS). As shown in Table II the omission of *CE* markedly reduced photophosphorylation in the FMN system but had little effect on the vitamin K system.

TABLE II

EFFECT OF CHLOROPLAST EXTRACT (*CE*) ON CYCLIC PHOTOPHOSPHORYLATION BY THE FMN AND VITAMIN K PATHWAYS

Experimental conditions as described under Fig. 1 except that washed chloroplast particles (C_{1s3}) and dialyzed *CE* (See METHODS) were used. 0.3 μmole vitamin K_5 or 0.03 μmole FMN were added to the reaction mixture.

Treatment	*μmoles P esterified*	
	FMN system	*Vit. K_5 system*
Complete	6.5	6.3
CE omitted	2.7	5.9
Chloroplasts omitted	0.2	0.3

Effect of inhibitors

Apart from the different requirement for TPN, support for the view that FMN and vitamin K catalyze alternative pathways for cyclic phosphorylation was found in their response to inhibitors. To certain inhibitors their response was similar but to others it was different. Both systems were inhibited by *p*-chloromercuribenzoate—an inhibition which was prevented by glutathione (Table III). Both systems were also inhibited by gramicidin and methylene blue (Table IV) but not by arsenite (Table V) or antimycin A (Table IV). The resistance of photosynthetic phosphorylation to arsenite inhibition argues against the participation of lipoic acid as an electron carrier in this process[5].

The FMN and vitamin K pathways differed in their sensitivity to dinitrophenol and *o*-phenanthroline. With respect to dinitrophenol neither the FMN nor the vitamin K system was significantly inhibited by a dinitrophenol concentration (10^{-4} *M*) which uncouples oxidative phosphorylation by mitochondria. But as shown in Table VI the vitamin K system differs from the FMN system in being relatively insensitive

References p. 46.

TABLE III

EFFECT OF *p*-CHLOROMERCURIBENZOATE (CMB) AND GLUTATHIONE ON CYCLIC PHOTOPHOSPHORYLATION

Experimental conditions as described under Fig. 1. The reaction mixture containing chloroplasts was incubated with the inhibitor for 20 min before starting the reaction. 0.1 μmole FMN *or* 0.3 μmole vitamin K (as vit. K_3 or vit. K_5) was added to the reaction mixture.

	µmoles phosphate esterified		
	FMN system	*Vitamin K system*	
		Vit. K_3	*Vit. K_5*
Control	4.8	5.3	4.4
10^{-3} glutathione	5.9	5.7	4.4
$1.5 \cdot 10^{-4}$ *M* CMB	0.7	0.9	1.1
Glutathione + CMB	5.2	5.5	4.4

TABLE IV

EFFECT OF ANTIMYCIN, GRAMICIDIN AND METHYLENE BLUE ON CYCLIC PHOTOPHOSPHORYLATION

Experimental conditions as described under Fig. 1. 0.1 μmole FMN, *or* 0.3 μmole vitamin K_3 or K_5 was added to the reaction mixture.

	µmoles phosphate esterified		
	FMN systen	*Vitamin K system*	
		Vit. K_3	*Vit. K_5*
Control	8.3	7.0	6.7
Antimycin A, 10 μg	7.4	7.1	6.5
Gramicidin, 40 μg	1.4	2.4	0.9
Methylene blue, $5 \cdot 10^{-3}$ *M*	0.5	0.3	0.6

TABLE V

EFFECT OF ARSENITE ON CYCLIC PHOTOPHOSPHORYLATION

Experimental conditions as described under Fig. 1. 0.1 μmole FMN *or* 0.3 μmole vitamin K_3 was added to the reaction mixture.

Treatment	*µmoles P esterified*	
	FMN system	*Vit. K_3 system*
Control	4.6	9.9
10^{-4} *M* arsenite	3.9	9.3
$5 \cdot 10^{-4}$ *M* arsenite	3.9	9.0
10^{-3} *M* arsenite	4.0	8.6

TABLE VI

DINITROPHENOL INHIBITION OF CYCLIC PHOTOPHOSPHORYLATION

Experimental conditions as described under Fig. 1. 0.1 μmole FMN *or* 0.3 μmole vitamin K (as vitamin K_3 or vitamin K_5) was added to the reaction mixture.

Dinitrophenol (M)	*μmoles phosphate esterified*		
	FMN system	*Vitamin K system*	
		Vit. K_3	*Vit. K_5*
0	7.7	8.8	7.1
$1 \cdot 10^{-4}$	6.8	8.6	7.4
$2 \cdot 10^{-4}$	6.6	8.2	7.3
$4 \cdot 10^{-4}$	5.8	7.8	7.1
$6 \cdot 10^{-4}$	4.2	6.9	6.9
$8 \cdot 10^{-4}$	3.2	6.7	6.4
$10 \cdot 10^{-4}$	1.6	5.6	6.7

TABLE VII

o-PHENANTHROLINE INHIBITION OF CYCLIC PHOTOPHOSPHORYLATION

Experimental conditions as described under Fig. 1. 0.1 μmole FMN, or 0.3 μmole vitamin K (as vitamin K_3 or vitamin K_5) was added to the reaction mixture.

o-Phenanthroline (M)	*μmoles phosphate esterified*		
	FMN system	*Vitamin K system*	
		Vit. K_3	*Vit. K_5*
0	6.6	7.3	6.2
$2 \cdot 10^{-5}$	3.0	5.5	6.5
$4 \cdot 10^{-5}$	1.5	4.0	5.8

TABLE VIII

CYANIDE INHIBITION OF CYCLIC PHOTOPHOSPHORYLATION

Experimental conditions for the pH 8.3 experiment were as described under Fig. 1. In the pH 7.4 experiment, the tris buffer, cyanide, and radioactive phosphate were adjusted to pH 7.4 and the chloroplast addition increased to give 0.2 mg chlorophyll per vessel. 0.1 μmole FMN *or* 0.3 μmole vitamin K (as vitamin K_3 or vitamin K_5) was added to the reaction mixture.

KCN (M)	*μmoles phosphate esterified* pH 8.3			*μmoles phosphate esterified* pH 7.4		
	FMN system	*Vitamin K system*		*FMN system*	*Vitamin K system*	
		Vit. K_3	*Vit. K_5*		*Vit. K_3*	*Vit. K_5*
0	7.1	7.9	6.9	8.1	5.3	7.6
$5 \cdot 10^{-4}$	4.5	8.0	4.4	4.0	5.1	6.0
10^{-3}	1.9	8.1	3.9	3.6	5.2	5.4
$2 \cdot 10^{-3}$	0.8	6.2	1.8	3.2	6.2	5.6

to dinitrophenol even at a concentration of 10^{-3} *M*. The vitamin K system is also less sensitive than the FMN system to inhibition by *o*-phenanthroline (Table VII). The resistance of the vitamin K system to inhibition by dinitrophenol and *o*-phenanthroline was observed with two forms of vitamin K: menadione (vitamin K_3) and 2-methyl-4-amino-1-naphthol hydrochloride (vitamin K_5).

A similar conclusion about FMN and vitamin K catalyzing alternative pathways for electron transport in photosynthetic phosphorylation was reported recently by WESSELS[19] but on the basis of findings which in some respects do not agree with our own. In his experiments, the addition of 10^{-3} *M* KCN increased phosphorylation with vitamin K_3 by over 100% but severely inhibited phosphorylation with FMN. We have also observed inhibition of the FMN system by cyanide but no significant stimulation of the K system. In fact, depending on pH and the form of vitamin K used, cyanide inhibited also the K system (Table VIII). This inhibitor does not appear therefore to differentiate clearly between the two cyclic photophosphorylation systems.

In our previous inhibition experiments[6,8] the reaction mixture contained both FMN and vitamin K, but the latter was added in a low concentration, insufficient to sustain a full rate of phosphorylation without the participation of the FMN pathway. Inhibitors to which both FMN and vitamin K are sensitive, for example methylene blue, gave, as would be expected, complete inhibition of the mixed system but with inhibitors like dinitrophenol, the inhibitory effect on FMN was partially obscured by the presence of vitamin K in the reaction mixture. Other differences in experimental conditions between the recent and the previous experiments: pH 8.3 versus 7.4, lower concentration of chlorophyll (0.1 mg versus 0.5 mg), ADP versus AMP as the phosphate acceptor, and higher rates of phosphorylation have also altered the previously observed effect of some inhibitors, for example cyanide and gramicidin.

Effect of non-physiological cofactors: phenazine methosulfate

SINGER AND KEARNEY[22,23] found that succinic dehydrogenase as well as other "cytochrome reducing dehydrogenases" react faster with the dye phenazine methosulfate than with any other known hydrogen acceptor. Likewise in cyclic photophosphorylation by bacterial particles[24,25] and by isolated chloroplasts[26], phenazine methosulfate gave higher rates of phosphorylation than were obtained with the other cofactors.

In experiments with isolated chloroplasts phenazine methosulfate was found to abolish the requirement for other cofactors of cyclic photophosphorylation[26]. The question then arises whether the action of phenazine methosulfate represents a special type of cyclic photophosphorylation. Little can be said about this now except to point out that in one respect at least, namely, in its resistance to certain inhibitors, the phenazine methosulfate pathway resembles the vitamin K pathway. As shown in Table IX, cyclic photophosphorylation catalyzed by phenazine methosulfate was resistant to inhibition by dinitrophenol and *o*-phenanthroline.

DISCUSSION

The mechanisms of photosynthetic phosphorylation are as yet so imperfectly understood that little beyond working hypotheses can be usefully advanced as a guide to

References p. 46.

TABLE IX

DIFFERENTIAL SENSITIVITY OF PHENAZINE METHOSULFATE (PMS) AND FMN PHOSPHORYLATING SYSTEMS TO DINITROPHENOL AND *o*-PHENANTHROLINE

Experimental conditions as described under Fig. 1 except that illumination was for 10 min; 20 μg PMS (0.07 μmole) *or* 0.1 μmole FMN was added to the reaction mixture.

	μmoles phosphate esterified	
	PMS system	*FMN system*
Control	6.8	4.6
$6 \cdot 10^{-4}$ *M* Dinitrophenol	6.0	3.2
10^{-3} *M* Dinitrophenol	6.2	2.6
$2 \cdot 10^{-5}$ *M* *o*-phenanthroline	7.5	1.8
$4 \cdot 10^{-5}$ *M* *o*-phenanthroline	6.8	0.9

future work. We find it helpful at present to distinguish between two possible pathways of cyclic photophosphorylation: one catalyzed by FMN and one by vitamin K. The non-physiological catalyst which has been most widely investigated, phenazine methosulfate, appears to act in a manner resembling the vitamin K pathway.

The results obtained distinguish between the FMN and vitamin K pathways of cyclic photophosphorylation on the basis of their differential dependence on TPN and sensitivity to dinitrophenol and *o*-phenanthroline. The conclusion that TPN is a component of the FMN pathway but not necessarily of the vitamin K pathway of cyclic photophosphorylation is also supported by another line of evidence. It was shown previously[5] that isolated chloroplasts can, when illuminated, reduce substrate amounts of TPN with an accompanying formation of ATP. Table X shows that inhibition by dinitrophenol and *o*-phenanthroline of this "primary" phosphorylation in which TPN is the terminal hydrogen acceptor was similar to that of the cyclic photophosphorylation with TPN and FMN. On the other hand, cyclic photophosphorylation with TPN plus vitamin K is only slightly depressed by dinitrophenol or *o*-phenanthroline.

On the basis of these findings an FMN pathway of cyclic photophosphorylation is tentatively proposed as shown in Fig. 5.

In the proposed scheme it is visualized that FMN, in conjunction with a cytochrome

TABLE X

DIFFERENTIAL SENSITIVITY OF PHOSPHORYLATING SYSTEMS TO DINITROPHENOL AND *o*-PHENANTHROLINE

Experimental conditions as described under Fig. 1 except as follows: the reaction mixture had P_{1s} chloroplast particles (see METHODS) containing 0.25 mg chlorophyll, 4 μmoles TPN, *CE* derived from a quantity of chloroplasts containing 2 mg chlorophyll and, when indicated, 0.1 μmole FMN or 0.3 μmole vitamin K_3.

	μmoles P esterified		
	TPN	*TPN + FMN*	*TPN + Vit. K_3*
Control	5.4	13.0	15.1
$1.5 \cdot 10^{-3}$ *M* dinitrophenol	1.1	1.4	12.2
Control	5.2	14.3	15.7
$7 \cdot 10^{-5}$ *M* *o*-phenanthroline	1.6	3.2	15.6

References p. 46.

system mediates in the transfer of hydrogen between TPN and [OH], the oxidized product of the photodecomposition of water. No attempt will be made at this time to propose a mechanism for cyclic photophosphorylation by the vitamin K pathway.

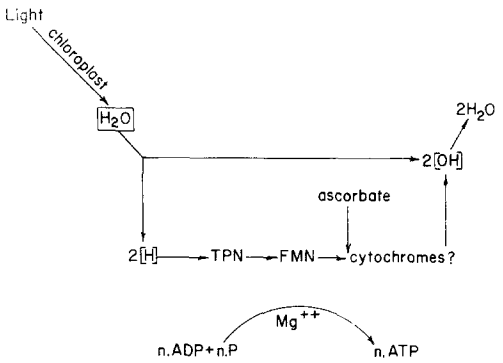

Fig. 5. Diagram representing cyclic photophosphorylation by the FMN pathway.

It seems reasonable to assign tentatively to the cytochrome system of chloroplasts the role of catalyzing, in cyclic photophosphorylation, the terminal recombination of the products of water photolysis. (Compare NIEMANN AND VENNESLAND[27] and MARRÈ AND SERVETTAZ[14].) The cytochrome system of chloroplasts differs from that of mitochondria in being apparently devoid of a conventional cytochrome oxidase[28,29] which could oxidize cytochromes by molecular oxygen in the dark. This may account for our failure to demonstrate phosphorylation by chloroplasts supplied with reduced pyridine nucleotides and molecular oxygen in the dark[5]. In the scheme given in Fig. 5 ascorbate is shown as affecting the span between FMN and [OH], possibly at the cytochrome level. The addition of ascorbate was not required for the primary phosphorylation with TPN but was important for cyclic photophosphorylation with FMN (Fig. 3b).

The evidence now available permits us to view the FMN pathway as analogous in some respects to the electron-transport system and its coupled phosphorylations in mitochondria. Apart from other possible differences which still remain unknown, the FMN pathway of cyclic photophosphorylation is known to differ from that of oxidative phosphorylation in that TPN rather than DPN is the pyridine nucleotide concerned in the electron transport and, most notably, that not molecular oxygen but [OH], some as yet unidentified oxidant, formed ultimately at the expense of photodecomposition of water, is the terminal electron acceptor. As for the vitamin K pathway of cyclic photophosphorylation it is also independent of molecular oxygen but little else can be usefully said at this time about its probable mechanism.

The outstanding fact about photosynthetic phosphorylation is its universal occurrence among all photosynthetic organisms tested so far, whether higher plants or bacteria[5]. The conversion of light energy into phosphate bond energy seems to be more intimately related to early photosynthetic events than CO_2 fixation as evidenced by the close structural association in both chloroplasts and bacterial chromatophores, of phosphorylating activity with the chlorophyll pigment system. Unlike the CO_2-fixing enzymes, which in chloroplasts are water-soluble and readily dissociable from

References p. 46.

the chlorophyll pigment system[6,7,17] and in the case of bacterial chromatophores[32] apparently not even structurally joined together, the enzymes of photosynthetic phosphorylation are always tightly bound to the particles containing the chlorophyll pigments. The possible implications of photosynthetic phosphorylation for biochemical evolution are discussed elsewhere[5].

The effects of phenazine methosulfate on cyclic photophosphorylation have been variously interpreted. JAGENDORF AND AVRON[26] regard them as casting doubt on the specificity of vitamin K and flavins as the natural cofactors of photosynthetic phosphorylation. GELLER's interpretation[24] is that phenazine sulfate "serves as a fast "bypass" or "short circuit" for electron transport around the site which is rate limiting in the system".

Although we are inclined toward Geller's interpretation we still consider as tentative[3,8] our formulations in which FMN and vitamin K are shown as cofactors of photosynthetic phosphorylation. These substances fulfill so far only the first requirement that natural cofactors must satisfy: they are normal constituents of chloroplasts and green leaves. Magnesium and ascorbate have long been known to be present in chloroplasts[30]. FMN is widely distributed in green leaves[31]; OHTA AND LOSADA in our laboratory (unpublished data) have found FMN to be a regular constituent of chloroplasts. Of unusual interest, however, is the anti-hemorrhagic factor, vitamin K, which occupied, since its discovery in plants, a unique position among other vitamins in being specifically associated with chloroplasts. Because of this striking association and the recent work linking vitamin K to phosphorylation systems in green plants, animals, and bacteria, the literature pertaining to vitamin K will be reviewed later in more detail.

Possible physiological significance of cyclic photophosphorylation

In previous discussions[3,4,6–8] cyclic photophosphorylation was regarded as the sole photosynthetic phosphorylation and its physiological significance was sought in its contribution of ATP to CO_2 assimilation. The identification of a second type of photosynthetic phosphorylation, as a component of assimilatory power[5], renders the earlier interpretation of cyclic phosphorylation too narrow.

It is tentatively proposed that another physiological role of cyclic photophosphorylation might be the conversion of light energy into ATP under conditions when CO_2 assimilation is, for one reason or another, decreased or even stopped altogether. This might arise during the well-known midday closure of stomata in leaves of higher plants[33,34] which restricts the supply of CO_2. The midday closure of stomata often occurs as a result of water deficit in the plant. Cyclic photophosphorylation, unlike CO_2 assimilation, provides a mechanism for the utilization of light energy without the consumption of water, which, it is visualized, undergoes in this process successive photodecomposition and reconstitution.

It is conceivable that devices other than stomatal closure are available, both to higher plants, and to lower plants without stomata, for curtailing CO_2 assimilation when the normal photosynthetic products accumulate in the cell. Under such conditions it would greatly benefit the cell to have a supply of ATP generated at the expense of light energy. This ATP of photochemical origin could then be used to drive the many ATP-dependent reactions as for example, the incorporation of ammonia through glutamine formation[35] and protein and fat synthesis (KREBS AND KORNBERG[36]).

References p. 46.

One is tempted to suggest that cyclic photophosphorylation itself may, under certain conditions, be a device for diverting light energy into channels other than CO_2 assimilation. Cyclic photophosphorylation would thus represent a pattern evolved by photosynthetic cells to use light energy for accomplishing cellular work independently of CO_2 assimilation.

Vitamin K in green plants and its role in phosphorylations

The association of vitamin K with photosynthetic tissues was apparent when it was first discovered. The green tops of carrots were found by ALMQUIST[37] to be a good source of vitamin K but carrot root contained little or none; DAM *et al.*[38-42] found that the outer, green leaves of cabbage had four times as much vitamin K as the inner leaves. Moreover, they found vitamin K in the chloroplasts but not in the cytoplasm of green leaves; in spinach leaves, for example, chloroplasts contained all the vitamin K present in the pressed leaf juice. DAM[39] also demonstrated the presence of vitamin K in *Chlorella vulgaris* and, in smaller concentrations, in four photosynthetic bacteria including *Rhodospirillum rubrum*.

The form of vitamin K found in green plants, vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), partially resembles chlorophyll in containing the phytyl side chain. The synthesis of vitamin K_1 in germinating seedlings parallels that of chlorophyll, as was shown by DAM and his coworkers. Yet, despite this striking association with chlorophyll the function of vitamin K remained a mystery. DAM[38], reviewing this subject, concluded that "the significance of vitamin K to the function of chloroplasts is just as unknown as the significance of the carotenoids". Later, in an attempt to test directly the effect of vitamin K substances on photosynthesis, he found that the addition of vitamin K_3 (2-methyl-1,4-naphthoquinone) completely inhibited photosynthesis in *Chlorella* whereas dicumarol, a known vitamin K antagonist, had no effect on either photosynthesis or growth. GAFFRON[43] who investigated the subject more extensively found that photosynthesis in *Scenedesmus* was inhibited by low concentrations of vitamin K_3 and phthiocol (2-methyl-3-hydroxyl-1,4 naphthoquinone). The inhibiting effect of the vitamin K substances appeared from the results of GAFFRON[44] to center on the oxygen evolution reactions in photosynthesis.

Since the active nucleus of vitamin K compounds is the naphthoquinone ring it was natural to suspect from an analogy with the known effectiveness of quinone as a Hill reagent that vitamin K could in fact, be the hydrogen acceptor in a physiological equivalent of that reaction in chloroplasts. But the experiments of ARNON AND WHATLEY with vitamin K_3 as a Hill reagent led to negative results (*cf.* ref. [6], p. 502). Moreover, WESSELS[12] in an independent investigation, found that low concentrations of vitamin K_3, phthiocol and dicumarol inhibited the Hill reaction. In the animal the administration of vitamin K_3 produces as great an anti-hemorrhagic effect as that of the natural vitamin K_1; both vitamin K_3 and K_1 are inhibited by dicumarol. Nevertheless, WESSELS concluded that in his experiments vitamin K_3 acted like dicumarol as an "anti-vitamin K" substance, inhibiting the natural vitamin K_1 in chloroplasts. This interpretation of his results, as well as his comparison of the redox potentials of Hill reagents and vitamin K, and other theoretical considerations, led him to suggest that the natural vitamin K_1 in chloroplasts "is involved in the transfer of hydrogen to Hill oxidants".

Direct experimental evidence for the role of vitamin K_3 and related naphtho-

References p. 46.

quinones in *stimulating* a photosynthetic reaction was first found in experiments on photosynthetic phosphorylation[11]. Here, as was later also observed by WESSELS[19], vitamin K_3 acted not as an inhibitor, but as a powerful catalyst; its catalytic activity in photosynthetic phosphorylation was inhibited by dicumarol, as was its blood clotting activity in animals.

The first suggestion that vitamin K may be a factor in phosphorylation came from the work of MARTIUS AND NITZ-LITZOW[45] with liver mitochondria. They noted that in low concentrations dicumarol acted as an uncoupler of oxidative phosphorylation is a manner similar to dinitrophenol. In subsequent experiments they found in mitochondria isolated from livers of vitamin K-deficient chicks a 30% diminution of oxidative phosphorylation which could be almost completely overcome *in vitro* by the addition of vitamin K_1. MARTIUS[46–48] concluded from this and other evidence that vitamin K occupies, in the chain of oxidative phosphorylation, a role similar to that usually assigned to flavoproteins: it mediates the hydrogen transfer between DPN and cytochromes. It is significant in this connection that according to a recent report from DAM's laboratory vitamin K in the liver appears to be associated primarily with mitochondria[49].

The experiments with chloroplasts yielded parallel evidence for the role of vitamin K in ATP synthesis by photosynthetic phosphorylation[11]. The effect of vitamin K substances on phosphorylation by chloroplasts had certain distinctive features: (a) Chloroplasts were less exacting with regard to the side chain on the third carbon of the naphthoquinone ring. In the experiments of MARTIUS with mitochondria the phytyl side chain was required; vitamin K_3, in which the phytyl is replaced by H, was inhibitory. Chloroplasts could use vitamin K_3, phthiocol (side chain = OH) and other naphthoquinone derivatives. If a phytyl side chain was required chloroplasts were evidently able to add it to the naphthoquinone ring. (b) The increase in phosphorylation caused by the addition of catalytic amounts of one of the naphthoquinones was very large, of the order of twenty-fold[11]. Although the main catalytic effect on photosynthetic phosphorylation was the same with different forms of vitamin K, certain differences among them were observed with respect to sensitivity to inhibitors and dependence on cofactors.

Experimental confirmation of the role of vitamin K in both oxidative phosphorylation and photosynthetic phosphorylation has recently come from several laboratories. DALLAM AND ANDERSON[50] and BEYER[51] observed an effect of vitamin K_1 on oxidative phosphorylation by rat liver mitochondria, and BRODIE *et al.*[54] by extracts of *Mycobacterium phlei*. COLPA-BOONSTRA AND SLATER[52,53] showed that reduced vitamin K_3 could be oxidized by heart-muscle mitochondria in a reaction accompanied by a dinitrophenol-sensitive oxidative phosphorylation. GELLER[24] found that vitamin K_3 catalyzed photosynthetic phosphorylation by particles of *Rhodospirillum rubrum* while AVRON AND JAGENDORF[55], CHOW AND VENNESLAND[56] and WESSELS[19] observed similar effects with isolated chloroplasts.

ACKNOWLEDGEMENT

This investigation was aided by grants from the National Institutes of Health and Office of Naval Research.

References p. 46.

REFERENCES

1 D. I. ARNON, M. B. ALLEN AND F. R. WHATLEY, *Nature*, 174 (1954) 394.
2 M. B. ALLEN, D. I. ARNON, J. B. CAPINDALE, F. R. WHATLEY AND L. J. DURHAM, *J. Am. Chem. Soc.*, 77 (1955) 4149.
3 D. I. ARNON, *Science*, 122 (1955) 9.
4 D. I. ARNON, *Ann. Rev. Plant Physiol.*, 7 (1956) 325.
5 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *Science*, 127 (1958) 1026.
6 D. I. ARNON, in O. H. GAEBLER, Ed. *Enzymes: Units of Biological Structure and Function*, Academic Press, New York, 1956.
7 D. I. ARNON, M. B. ALLEN, F. R. WHATLEY, J. B. CAPINDALE AND L. L. ROSENBERG, *Proc. 3rd Int. Congr. Biochem.*, Brussels, 1955, p. 227.
8 D. I. ARNON, M. B. ALLEN AND F. R. WHATLEY, *Biochim. Biophys. Acta*, 20 (1956) 449.
9 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *J. Am. Chem. Soc.*, 76 (1954) 6324.
10 F. R. WHATLEY, M. B. ALLEN AND D. I. ARNON, *Biochim. Biophys. Acta*, 16 (1955) 605.
11 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *Biochim. Biophys. Acta*, 16 (1955) 607.
12 J. S. C. WESSELS, *Rec. trav. chim.*, 73 (1954) 529.
13 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *Nature*, 180 (1957) 182.
14 E. MARRÈ AND O. SERVETTAZ, *Archiv. Biochem. Biophys.*, 75 (1958) 309.
15 F. R. WHATLEY, M. B. ALLEN AND D. I. ARNON, *Plant Physiol.*, 32 (Supplement) (1957) iii.
16 M. B. ALLEN, F. R. WHATLEY AND D. I. ARNON, *Biochim. Biophys. Acta*, 27 (1958) 16.
17 F. R. WHATLEY, M. B. ALLEN, L. L. ROSENBERG, J. B. CAPINDALE AND D. I. ARNON, *Biochim. Biophys. Acta*, 20 (1956) 462.
18 W. D. MCELROY AND A. NASON, *Ann. Rev. Plant Physiol.*, 5 (1954) 1.
19 J. S. C. WESSELS, *Biochim. Biophys. Acta*, 25 (1957) 97.
20 M. AVRON AND A. T. JAGENDORF, *Arch. Biochem. Biophys.*, 65 (1956) 475.
21 A. SAN PIETRO AND H. M. LANG, *J. Biol. Chem.*, 231 (1958) 211.
22 T. P. SINGER AND E. B. KEARNEY, *Biochim. Biophys. Acta*, 15 (1954) 151.
23 T. P. SINGER AND E. B. KEARNEY, in *Methods of Biochemical Analysis*, 4 (1957) 307.
24 D. M. GELLER, Doctoral Dissertation, Div. Med. Sci., Harvard Univ. 1957.
25 M. KAMEN AND J. W. NEWTON, *Biochim. Biophys. Acta*, 25 (1957) 462.
26 A. T. JAGENDORF AND M. AVRON, *J. Biol. Chem.*, 231 (1958) 277.
27 R. H. NIEMAN AND B. VENNESLAND, *Science*, 125 (1957) 353.
28 R. HILL AND R. SCARISBRICK, *New Phytologist*, 50 (1951) 98.
29 W. O. JAMES AND V. S. DAS, *New Phytologist*, 56 (1957) 325.
30 E. I. RABINOWITCH, *Photosynthesis and Related Processes*, 1, New York, Interscience, 1945.
31 W. H. SEBRELL AND R. S. HARRIS, *The Vitamins*, 3 vols., Academic Press, New York, 1954.
32 R. C. FULLER AND E. C. ANDERSON, *Plant Physiol.*, 32 (Supplement) (1957) xvi.
33 M. G. STALFELT, *Physiol. Plantarum*, 8 (1955) 572.
34 O. V. S. HEATH AND B. ORCHARD, *Nature*, 180 (1957) 180.
35 W. H. ELLIOTT, *Biochem. J.*, 49 (1951) 106.
36 H. A. KREBS AND H. L. KORNBERG, *Ergeb. Physiol.*, 49 (1957) 212.
37 H. J. ALMQUIST, *Nature*, 140 (1937) 25.
38 H. DAM, *Advances in Enzymol.*, 2 (1942) 317.
39 H. DAM, *Am. J. Bot.*, 31 (1944) 492.
40 H. DAM, *Biochem. J.*, 32 (1938) 485.
41 H. DAM, E. HJORTH AND I. KRUSE, *Physiologia Plantarum*, 1 (1948) 379.
42 H. DAM, J. GLAVIND AND N. NIELSEN, *Z. physiol. Chem.*, 265 (1940) 80.
43 H. GAFFRON, *J. Gen. Physiol.*, 28 (1945) 259.
44 H. GAFFRON, *J. Gen. Physiol.*, 28 (1945) 269.
45 C. MARTIUS AND D. NITZ-LITZOW, *Biochim. Biophys. Acta*, 12 (1953) 134.
46 C. MARTIUS, *Biochem, Z.*, 326 (1954) 26.
47 C. MARTIUS, *Proc. 3rd Inter. Congr. Biochem.*, Brussels (1955) 1.
48 C. MARTIUS AND D. NITZ-LITZOW, *Biochem. Z.*, 327 (1955) 1.
49 J. P. GREEN, E. SONDERGAARD AND H. DAM, *Biochim. Biophys. Acta*, 19 (1956) 182.
50 R. D. DALLAM AND W. W. ANDERSON, *Biochim. Biophys. Acta*, 25 (1957) 439.
51 R. E. BEYER, *Biochim. Biophys. Acta*, 28 (1958) 663.
52 J. P. COLPA-BOONSTRA AND E. C. SLATER, *Biochim. Biophys. Acta*, 23 (1957) 222.
53 J. P. COLPA-BOONSTRA AND E. C. SLATER, *Biochim. Biophys. Acta*, 27 (1958) 122.
54 A. F. BRODIE, M. M. WEBER AND C. T. GRAY, *Biochim. Biophys. Acta*, 25 (1957) 448.
55 M. AVRON AND A. T. JAGENDORF, *Biochim. Biophys. Acta*, 26 (1957) 262.
56 C. T. CHOW AND B. VENNESLAND, *Plant Physiol.*, 32 (Supplement) (1957) iv.