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Reflections on the early studies of the mechanism of photophosphorylation in isolated chloroplasts: a commentary by

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on 'The relation of photosynthetic phosphorylation to the Hill reaction'
by M. Avron, D.W. Krogmann and A.T. Jagendorf
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When I joined Dr. A.T. Jagendorf's laboratory as a post-doctoral fellow in 1955, the report by Arnon and his colleagues of the discovery of photophosphorylation in chloroplasts [1] was a subject of heated debate [2]. Several arguments led to the then widely accepted conviction that the interpretation of Arnon and colleagues that they had discovered a new type of phosphorylation was incorrect. Among these were the following arguments. (a) Their method of isolation of chloroplasts in 0.35 M NaCl must have led to a very impure preparation highly contaminated with other organelles, notably mitochondria. (b) Chloroplasts were well known, by then, to be capable of photoreducing a variety of electron acceptors, by the Hill reaction [3]. A previous report, by a highly respected laboratory [4], clearly demonstrated ATP formation by a reconstituted system where NAD^+ was photochemically reduced by chloroplasts and the product NADH oxidized by mitochondria through the then established process of oxidative phosphorylation. It was widely believed that this was the most likely explanation for the 'apparent photophosphorylation' reported by Arnon and colleagues. (c) Although coupling to electron transport was postulated, no demonstration of electron flow accompanying the phosphorylation was presented. (d) The rates of ATP synthesis observed were very low, 2–5 μmol ATP/mg Chl per h, particularly when compared with the well-established rates of photosynthesis of the Hill reaction around 200–400 μequiv./mg Chl per h. They could thus easily be due to an irrelevant side-reaction.

My doctoral research dealt with oxidative phosphorylation in plants [5,6]. I was therefore well acquainted with the techniques of measuring mitochondrial activity and phosphorylation. Dr. Jagendorf was interested at

(Left to right) Mordhay Avron, David Krogmann and André Jagendorf

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the time in developing techniques for isolation of highly purified chloroplasts, free of other intracellular organelles [7,8], and was therefore well versed with the techniques of chloroplast isolation and assay of their photochemical activity. He therefore suggested that, in parallel with my initial project, which involved the identification, isolation and characterization of the enzyme which is known today as ferredoxin-NADP reductase [9,10], we try to resolve the controversy on the newly reported photosynthetic phosphorylation. In the 2 years which had passed since the original report of Arnon and colleagues not a single confirmatory report of the finding had appeared. With our respective backgrounds it seemed relatively trivial to prove unquestionably that Arnon's results were due to a mixture of the two well-know reactions, the Hill reaction catalysed by chloroplasts and oxidative phosphorylation catalysed by the contaminating mitochondria. Of course, as soon as we started experimenting it became clear that Arnon and collaborators were right. His 'dirty' or our 'clean' chloroplast preparations had insignificant mitochondrial activity, but indeed possessed a new kind of phosphorylation reaction, light-driven and specific to thylakoids [11]. We were still worried by the low rates of phosphorylation, but a detailed study of the optimal conditions for the reaction soon resulted in 'respectable' rates of around 200 μmol ATP formed/mg Chl per h [12].

It may be of interest to pause here and describe a small 'faux-pas' which happened to us during these investigations, and may have a take-home lesson for the younger scientist who reads this account. In the original work of Arnon and collaborators, AMP was used as the phosphate accepter. Since we were very careful to use well-washed chloroplasts in our experiments, we noticed that the more we washed, the lower was the activity of the preparation. Furthermore, the activity could be fully restored by adding back to the washed thylakoids the soluble supernatant from the washing which contained a heat-labile factor. We immediately suspected that the factor could be adenylate kinase and the real substrate for photophosphorylation ADP rather than AMP. However, to our disappointment, a sample of 'authentic' ADP, which at that time was hard to get and which we received from a colleague, proved to be no better than AMP, thus 'proving' to us that we must have found a new 'extractable factor' (which we promptly named EF) necessary for photophosphorylation [11]. As we entered into the study of optimizing the conditions for photophosphorylation, we purchased a new bottle of ADP, and the effect of EF became considerably smaller [12]. When we eventually purified out ADP (and found that it contained a considerable amount of AMP), the pure ADP was equally effective with or without EP. Thus, EF was indeed adenylate kinase; ADP, and not AMP, was the real substrate of photophosphorylation and the 'authentic' ADP in the bottle which we first used must have completely hydrolysed to AMP and P_i [13]. Dr. Jagendorf, in his 'Homage to the Helpful Chloroplast' [14], noted that, since AMP was the sole substrate used in the early experiments, "If adenylate kinase had not been present (in the isolated chloroplasts) the rate of photophosphorylation in all the initial experiments would most likely have been 0.0".

Photophosphorylation was now an established fact. However its relation to electron transport and photosynthesis was still tenuous. The heated arguments regarding the rate of phosphorylation were fully abated when we found [13] that when phenazine methosulfate was used as the electron carrier, the chloroplasts could support photophosphorylation at rates exceeding 700 μmol/mg Chl per h. Later experiments [15] demonstrated rates exceeding 2500 μmol/mg Chl per h.

To convince ourselves that photophosphorylation was an integral part of the process of photosynthesis, we felt it imperative to prove that the action spectrum of photophosphorylation was identical to that of photosynthesis, and thus that chlorophyll served as the major light-absorbing pigment in both. To do that, the only device then available was the high-irradiance carbon-arc spectrograph constructed by Dr. Sterling B. Hendricks at the USDA laboratories at Beltsville, Maryland, which provided the complete visible spectrum at sufficient intensities spread over a meter of length. With Dr. Hendrick's help we set-up our photophosphorylating mixtures across his spectrograph and indeed found that the action spectrum of photophosphorylation was the same as that for photosynthesis [16].

Finally, the relation of photophosphorylation to electron transport had to be clarified and Dr. David W. Krogmann, a graduate student at the time, joined us in obtaining the necessary evidence. In the original work by Arnon et al. [1], no evidence for electron transport was provided. Subsequently [17] Arnon and colleagues demonstrated that ATP synthesis can accompany the reduction of ferricyanide and evolution of oxygen. However, it seemed to us necessary to establish the dependence of phosphorylation on electron transport, and vice-versa, before the process could be considered a truly coupled mechanism. The paper chosen for inclusion in this selection [18] demonstrated that not only was photophosphorylation completely dependent on electron transport, but the rate of electron transport was considerably enhanced (about 3-fold) when, and only when, all the reagents necessary for phosphorylation were provided. Phosphorylation ceased when the electron acceptor was exhausted, and the rate of electron flow decreased when the phosphate acceptor was exhausted. Krogmann also observed that this coupling was related to the integrity of the chloroplasts. When chloroplasts were mistreated, for example by high dilution in 0.35 M NaCl at pH 6.2, they became 'uncou-

pled', exhibiting high rates of electron transport, unable to catalyse photophosphorylation, and totally unresponsive to the addition of the reagents necessary for photophosphorylation. Thus, the first procedure to uncouple chloroplasts was established. Many of these observations may seem self-evident to the modern student, but one should remember that in the context of the time, when 'coupling' was a process just postulated and 'chemiosmosis' was still in its preconception period, many of these were startling observations which brought great excitement to their discoverers.

Finally, it is interesting to note a peculiar 'side-effect', which was observed and reported in these studies. It was noticed that, while the addition of optimal ADP (1 mM) to a complete system lacking ADP indeed stimulated the rate of electron transport, the addition of suboptimal amounts of ADP (3 μM) inhibited it. When ATP was substituted for ADP, full inhibition was observed at all concentrations exceeding 3 μM. These observations were the forerunner of a long series of studies on the regulatory effect of nucleotide binding to the ATP synthase complex on ATP synthesis in photophosphorylation [19–21].

The process of photophosphorylation is today a subject of investigation by many laboratories utilizing a great variety of approaches [22,23]. Some of the basic tenets of the chemiosmotic hypothesis, which serves as the present day basis of our understanding of the bioenergetics of coupled ATP synthesis, were first and most convincingly illustrated in this system [14]. Nevertheless, there is still much to be done before we can say that we have uncovered the secret of how several protons, travelling through the ATP synthase complex down an electrochemical gradient of protons, drive the esterification of ADP to form ATP.

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THE RELATION OF PHOTOSYNTHETIC PHOSPHORYLATION TO THE HILL REACTION*

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INTRODUCTION

Oxidation–reduction reactions in chloroplasts were first discovered quite unrelated to phosphorylation, by R. HILL[1]. When light-driven phosphate esterification was discovered subsequently by ARNON *et al.*[2] there was no direct demonstration of oxidative or reductive steps accompanying the phosphorylation. A series of oxidative steps was, however, postulated as the basis for ATP*** formation in the absence of oxygen uptake or evolution[3].

It was not until the discovery by ARNON *et al.*[4] that phosphate esterification could accompany ferricyanide reduction and the evolution of oxygen, that oxidative

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*** Abbreviations to be used in this paper are as follows: ADP, adenosine-diphosphate; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; FMN, flavin mononucleotide; TPN, triphosphopyridine nucleotide.

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and phosphorylative reactions could directly be related in chloroplasts. This finding was later extended to TPN as net electron acceptor. Phosphorylation accompanying both types of reduction has been confirmed in this laboratory[5].

ARNON *et al.* also observed some stimulation of the rate of reduction of ferricyanide if the reagents necessary for phosphorylation (phosphate magnesium and ADP) were added to the reaction. Since ferricyanide reduction in the HILL reaction without phosphorylating reagents had been known for a number of years, it seemed to us that the relation of the HILL reaction to phosphorylation deserved further clarification.

METHODS

HILL reactions were measured with trichloroindophenol or ferricyanide as electron acceptors. Indophenol assays were run by exposing a cuvette containing buffer, sodium chloride, 0.08 μmoles dye, and chloroplasts containing 5 to 15 μg chlorophyll to saturating light for 30 sec and measuring the change in optical density to 620 mμ[6].

Ferricyanide assays were done by (a) a procedure similar to the indophenol assay except for tripling the chloroplast concentration, increasing the exposure time to 2 min, and substituting 1.5 μmoles of ferricyanide for indophenol. The change of optical density at 400 mμ was followed. (b) The procedure of KROGMANN AND JAGENDORF[7] where ferrocyanide formed during the reaction reduces ferric iron to ferrous iron and the latter is measured as its orthophenanthroline complex at 510 mμ. The greater sensitivity of this assay permits one to use the same low chloroplast concentrations (5 to 15 μg chlorophyll) and short illumination times (30 sec) as used in the indophenol assay.

Where phosphorylating reagents were used, they were added to the above reaction mixture in the cuvette.

Activation of chloroplasts by sodium chloride dilution[8] was carried out by diluting the chloroplast suspension containing 60 to 100 μg chlorophyll per ml 100 fold in ice-cold 0.35 *N* NaCl buffered with 0.02 *M* Tris maleate at pH 6.2.

Phosphate uptake accompanying ferricyanide reduction was measured by the method of BERENBLUM AND CHAIN[9].

Spinach chloroplasts were prepared in 0.4 *M* sucrose, 0.05 *M* Tris buffer pH 7.8, 0.01 *M* NaCl and washed once as previously described[10].

RESULTS

Effects of phosphorylating reagents on ferricyanide reduction and accompanying phosphorylation

It was shown previously that in order for phosphorylation accompanying ferricyanide reduction to proceed at optimal rates, the addition of ADP, Mg, and inorganic phosphate was necessary[4]. The effect of these reagents on the reduction of ferricyanide is shown in Table I. It is evident that the presence of ADP, Mg and phosphate brings about a marked increase in the rate of ferricyanide reduction in addition to allowing phosphorylation to proceed. For maximal rates, all three factors are necessary.

A study was made of the effect of the three reagents, ADP, phosphate, Mg, each varied independently with the other two at optimal concentration. Both the rate of reduction and that of phosphorylation were observed.

Fig. 1 illustrates the effect of Mg concentration on the reduction of ferricyanide and the accompanying phosphorylation. Both can be seen to be greatly affected. Phosphorylation proceeds only to a small extent in the absence of Mg, as reported previously for cyclic phosphorylation using FMN as a catalytic cofactor[11]. Reduction, on the other hand proceeds to a significant extent in the absence of Mg; this reduction

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is equivalent to that observed in a conventional HILL-reaction assay with ferricyanide as an electron acceptor. It is evident, though, that the addition of Mg brings about a very large increase in the rate of reduction. Phosphorylating and reducing activity show a similar response, suggesting that the two are interrelated, or "coupled".

TABLE I

EFFECT OF PHOSPHORYLATING REAGENTS ON FERRICYANIDE REDUCTION

Assay as described under METHODS (a). A value of 100 corresponds to a reductive activity of 290 μmoles ferricyanide reduced per mg chlorophyll/h.

Additions	*μmoles*	*Ferricyanide reduced*
None	—	100
Mg	10.0	116
ADP	1.5	90
Phosphate	50.0	140
Mg + ADP	10.0 + 1.5	110
Mg + phosphate	10.0 + 50.0	149
ADP + phosphate	1.5 + 50.0	156
Mg + ADP + phosphate	10.0 + 1.5 + 50.0	286

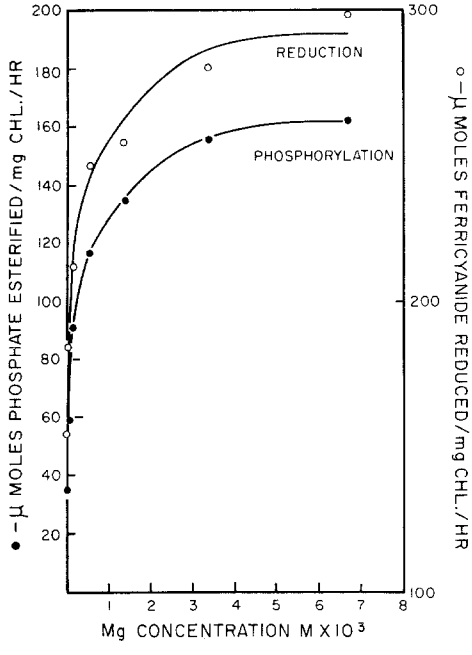

Fig. 1. Effect of Mg concentration on ferricyanide reduction and accompanying phosphorylation. Reaction mixtures contained in μmoles: Tris, pH 7.8, 10; NaCl, 17; ferricyanide, 2.5; phosphate, pH 7.8, 1.25; ADP, 1.5. Also, chloroplasts containing 37 μg chlorophyll, Mg as shown and water to a total volume of 0.75 ml. Reactions were run for 8 min, under N, in the light (about 5,000 f.c.) at 15°, in 10-ml Erlenmeyer flasks in an illuminated Warburg bath.

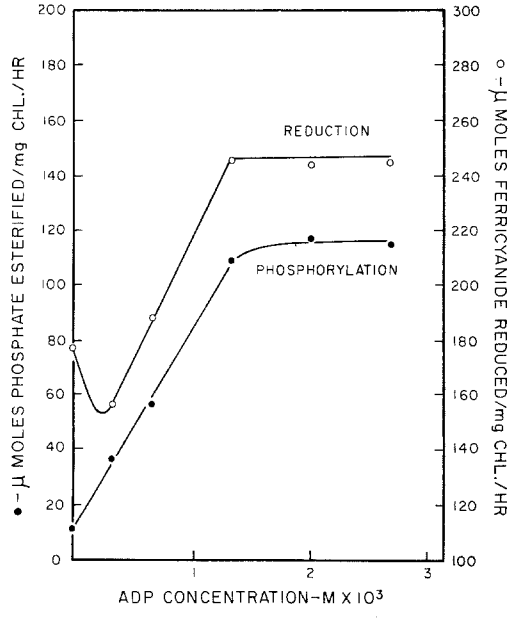

Fig. 2. Effect of ADP concentration on ferricyanide reduction and accompanying phosphorylation. Reaction mixture contained in μmoles: Tris pH 7.8, 20; NaCl, 35; ferricyanide, 5; phosphate, pH 7.8, 2.5; Mg, 5. Also, chloroplasts containing 103 μg chlorophyll; ADP as shown and water to a total volume of 1.5 ml. Reactions were run for 12 min, under N, in the light (about 5,000 f.c.) at 15°, in 25-ml Erlenmeyer flasks, in an illuminated Warburg bath.

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The requirement for ADP in both phosphorylative and reductive activity is illustrated in Fig. 2. It can be seen that phosphorylation is strictly dependent upon the ADP supply until saturation is reached. Reduction, on the other hand, is inhibited by low concentrations of ADP, and stimulated by higher concentrations. The inhibitory action of low ADP concentration will be dealt with in a later section. The stimulatory effect of ADP is similar in both processes, supporting the idea that the two effects stem from the same basic reaction of ADP.

The dependence of the two reactions on the availability of ADP can also be seen by following the time course of ferricyanide reduction and phosphate esterification in the presence of different ADP concentrations. Such an experiment is shown in Fig. 3. In the absence of ADP, no phosphate is esterified, while ferricyanide is reduced at a slow rate. In the presence of non-saturating ADP concentrations (see Fig. 2) phosphate is esterified until the ADP supply is exhausted. Ferricyanide reduction proceeds at a very rapid rate only as long as phosphorylation proceeds

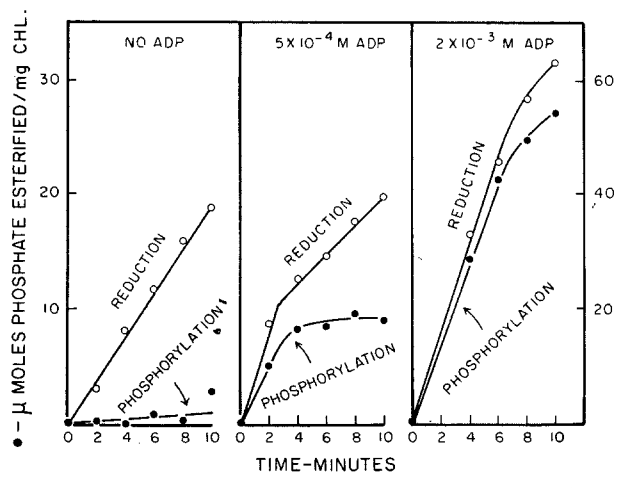

Fig. 3. Time course of ferricyanide reduction at different ADP concentrations. Reaction mixtures contained in μmoles: Tris pH 7.8, 80; NaCl, 140; ferricyanide, 20; phosphate, pH 7.8, 10.0; Mg, 20. Also, chloroplasts containing 260 μg chlorophyll, ADP as shown and water to a total volume of 6.0 ml. Reactions were run under N, in the light (about 5,000 f.c.), at 150, in 25-ml Erlenmeyer flasks in an illuminated Warburg bath. Samples for phosphate and ferricyanide analyses were withdrawn at the times indicated with a hypodermic syringe.

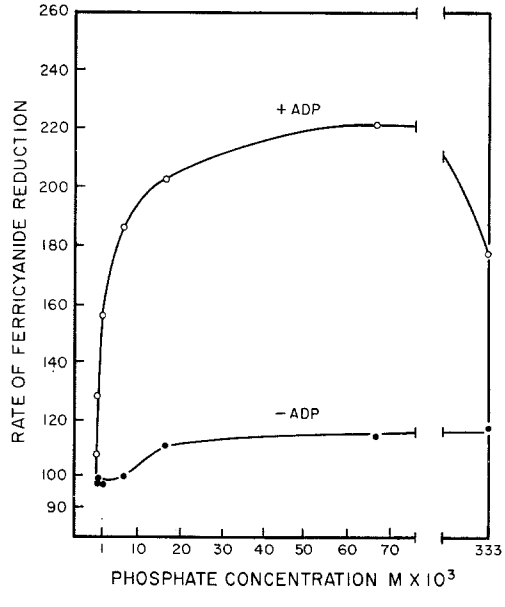

Fig. 4. Effect of phosphate concentration on ferricyanide reduction. Reaction mixtures contained in μmoles; Tris, pH 7.8, 40; NaCl, 70; ferricyanide, 1.5; Mg, 10; ADP, where present, 1.5. Also, chloroplasts containing 34 μg chlorophyll, phosphate as shown and water to a total volume of 3.0 ml. Reactions were run for 2 min, under air, in the light (about 5,000 f.c.) at room temperature (about 25°), in a cuvette, as described under METHODS (a). A rate of 100 corresponds to a reductive activity of 380 μmoles ferricyanide reduced per mg chlorophyll/h.

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and then slows down. At saturating ADP concentrations both reactions proceed at optimal rates. The phosphorylative activity and the stimulation of ferricyanide reduction are both dependent to a similar extent on the supply of ADP.

The effect of phosphate concentration on the reduction of ferricyanide is shown in Fig. 4. It is evident that the large stimulation of ferricyanide reduction is dependent upon phosphate concentration. Saturation is reached at about 10^{-2} M. This is approximately twenty-fold higher concentration than that quantitatively required for the accompanying phosphorylation. A similar phosphate concentration curve, though, has been shown in the case of cyclic photosynthetic phosphorylation[11]. As indicated in Table I, phosphate by itself has a small stimulatory effect. The -ADP curve in Fig. 4 illustrates this effect. In contrast with the effect in the presence of ADP, here, high concentrations are necessary before any stimulation can be detected due to phosphate alone.

Effect of phosphorylating reagents on indophenol reduction

Ferricyanide reduction by intact chloroplasts proceeds at a slower rate than does the reduction of some other electron acceptors. The best electron acceptor in terms of its rate of reduction is di- (or) tri-chloroindophenol. It seemed of interest to see whether this faster rate of reduction (approximately three-fold faster than the ferricyanide reduction rate) could also be stimulated by the addition of phosphorylating reagents. Table II illustrates that some stimulation can be observed here, too. The stimulation is again dependent upon the presence of all three components for maximal activity. It is evident, though, that in contrast with ferricyanide reduction (see Table I) where the stimulation is of the order of 300 %, the maximal stimulation observed with indophenol is about 35 %. It is of interest to note that the stimulated rate is very similar in both cases, approximately 800 to 900 μequiv. of acceptor reduced per mg chlorophyll/h, while the unstimulated rates are markedly different.

TABLE II

EFFECT OF PHOSPHORYLATING REAGENTS ON TRICHLOROINDOPHENOL REDUCTION

Assay as described under methods. A value of 100 corresponds to 680 μequiv. of indophenol reduced per mg chlorophyll/h.

Additions	*µmoles*	*Indophenol reduced*
None	—	100
Mg + ADP + phosphate	10 + 1 + 50	135
Mg + ADP	10 + 1	102
ADP + phosphate	1 + 50	105
Mg + phosphate	10 + 50	105

Stimulation of ferricyanide reduction by other means

It was previously found by KROGMANN AND JAGENDORF[8] that ferricyanide reduction can be stimulated to a large extent by dilution of chloroplasts 100-fold in a NaCl solution buffered at pH 6.2. Table III illustrates the stimulation of ferricyanide and indophenol reduction by the addition of Mg, ADP and phosphate and by the dilution technique. It is evident that either method causes a large stimulation in the rate of ferricyanide reduction. In the particular experiment cited, larger

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TABLE III

STIMULATION OF REDUCTION BY PHOSPHORYLATING REAGENTS AND BY A DILUTION TECHNIQUE

Indophenol assays as described under METHODS. Ferricyanide assays as described under METHODS (b). Total volume 3.0 ml. Reaction mixture contained 0.2 *M* sucrose, 0.025 *M* phosphate pH 7.8, 0.005 *M* NaCl. Mg and ADP were added at 10.0 and 0.5 μmoles, respectively.

Electron acceptor:	*Ferricyanide*		*Trichloroindophenol*	
Addition	*None*	*Mg, ADP*	*None*	*Mg, ADP*
	microequivalents reduced/mg chlorophyll hour			
Untreated chloroplasts	254	538	540	665
"Activated" chloroplasts	870	860	983	995

stimulation was obtained by the dilution technique than by addition of the phosphorylating reagents. This, however, is not always the case and the ratio of activation by the two techniques has varied considerably in different experiments. An important point to be noted, though, is that once the chloroplasts were "activated" by the dilution technique, no further stimulation is obtained by the addition of phosphorylating reagents. Similar results were obtained in the case of indophenol reduction (Table III). As previously noted, the initial activity here is higher and the effect of the phosphorylating reagents smaller.

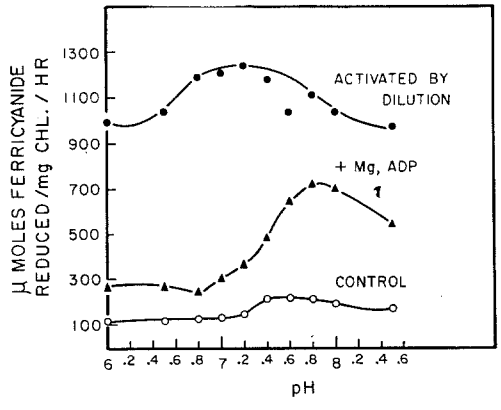

Fig. 5. Relation of pH to activation. Reaction mixtures contained in μmoles: sucrose, 600; phosphate at the pHindicated, 75; NaCl, 15; where added, Mg, 10; ADP, 0.5. The total volume was 3 ml. Reactions were run as described under METHODS (b). The chloroplasts used contained the following amounts of chlorophyll as μg/3 ml; control, 39; plus Mg, ADP, 16; activated by dilution, 7.

In order to differentiate further the stimulatory effect of the dilution technique and the phosphorylating reagents, a pH curve was run for both effects. It was shown previously that cyclic photosynthetic phosphorylation proceeded optimally at pH 7.8 to 8.0. It would thus be expected that the "activation" of ferricyanide reduction by the phosphorylation reagents would possess a similar pH optimum. Fig. 5 illustrates that this indeed is the case. After "activation" by the dilution technique, a different pH curve is obtained with a broad optimum between pH 6.8 and 7.6.

It is of interest to note, in this connection, that KROGMANN AND JAGENDORF had previously shown[8] that chloroplasts "activated" by the dilution technique had lost most of their ability to perform photosynthetic phosphorylation.

The relation of light intensity and the stimulatory effect

The stimulation caused by the presence of the phosphorylating reagents may be thought of as being brought about by allowing the reactions necessary for phos-

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phorylation to proceed. Since these are, most probably, not light dependent, one would expect the presence of Mg, ADP and phosphate to be affecting only the "dark reaction".

It has been shown[12] that by investigating the relation of light intensity to the rate of the HILL reaction, one could differentiate between effects related to the "dark reaction" and ones related to the "light reaction". The HILL reaction follows the kinetics described by the equation

$$V = \frac{Kd\ I}{I\ (Kd/Kl)}$$

where V = the rate of the reaction; I = light intensity; Kd = a constant related to the "dark reaction"; Kl = a constant related to the "light reaction". By rearrangement, the equation

$$1/V = 1/Kl\ (1/I) + 1/Kd$$

is obtained. Thus, if one plots $1/V$ *vs.* $1/I$, a straight line should be obtained. Conditions affecting the "dark reaction" will be reflected in the change in the intercept of the line, while conditions affecting the "light reaction" will be reflected in changes in the slope of the line.

Fig. 6 illustrates such a plot. It is evident that the effect of Mg and phosphate

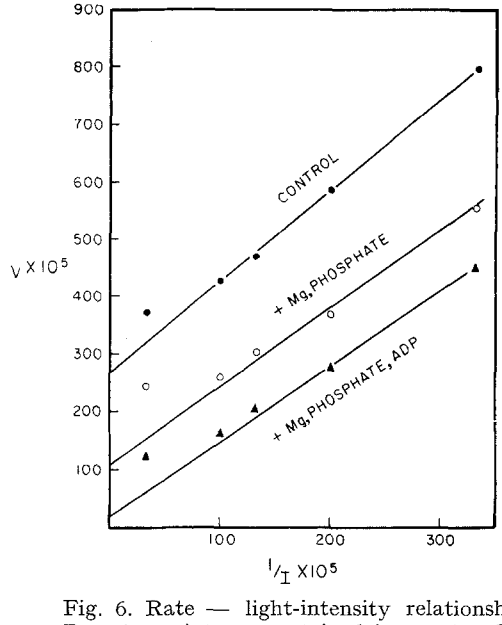

Fig. 6. Rate — light-intensity relationships. Reaction mixtures contained in μmoles: Tris, pH 7.8, 40; NaCl, 70; ferricyanide, 1.5; where used Mg, 10; phosphate pH 7.8, 100; ADP, 1.5. Also chloroplasts containing 32 μg chlorophyll in a total volume of 3.0 ml. Light intensity was varied by changing the distance between the light source and the cuvette. Assays were performed as described under METHODS (a).

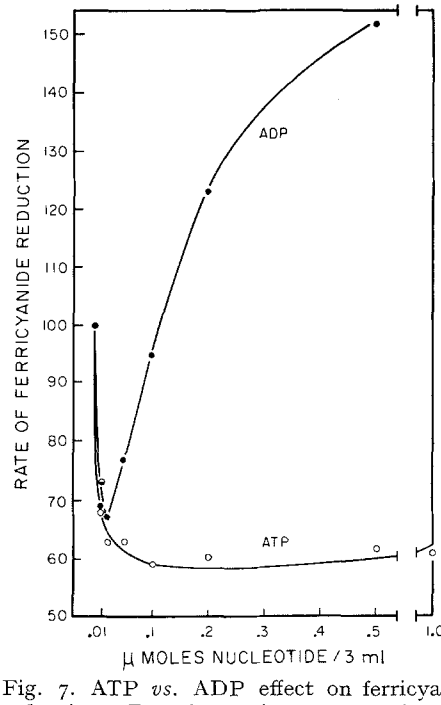

Fig. 7. ATP *vs.* ADP effect on ferricyanide reduction. Reaction mixture contained in μmoles: Tris, pH 7.8, 40; NaCl, 70; ferricyanide, 1.5; Mg, 10; phosphate, 10; where used, ADP and ATP as indicated. Also, chloroplasts containing 20 μg chlorophyll per cuvette. The final volume was 3 ml. Assays were performed as described under METHODS (a). A rate of 100 corresponds to a reductive ability of 350 μmoles ferricyanide reduced per mg chlorophyll/h.

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and of Mg, ADP and phosphate is to change the intercept of the line with only a very small effect, if any, on the slope of the line. Thus, the effect of the phosphorylating reagents can be interpreted as relating to the dark, rather than the light reaction.

ATP inhibition

It was shown in Fig. 2 that low concentrations of ADP inhibited, rather than stimulated the reaction. Since the reaction produced ATP in the light, the very small amount of ADP present would be esterified in a very short period and thus the inhibitory agent should be ATP. Fig. 7 shows that this is the case. In the presence of increasing concentrations of ADP, an inhibition is obtained followed by a large stimulation as previously shown. In the presence of ATP, only the inhibition is produced. It can thus be concluded that ATP, rather than ADP is the actual inhibitory agent.

It was previously shown that for maximal stimulation of the reductive ability of chloroplasts, all three of the phosphorylating reagents had to be present. This is not the case for the inhibitory action of ATP. As seen in Table IV, ATP causes a similar percent inhibition independent of the presence or absence of Mg and/or phosphate.

TABLE IV

RELATION OF ATP INHIBITION TO Mg AND PHOSPHATE

Assay as described under METHODS (a). A value of 100 corresponds to a reductive activity of 310 μmoles ferricyanide reduced per mg chlorophyll/h.

	µmoles	*Ferricyanide reduced*	*Per cent inhibition by ATP*
None	—	100	
ATP	0.2	66	34
Mg	10	134	—
Mg + ATP	10 + 0.2	72	46
Phosphate	100	163	—
Phosphate + ATP	100 + 0.2	103	37
Mg + phosphate	10 + 100	163	—
Mg + phosphate + ATP	10 + 100 + 0.2	93	43
Mg + phosphate + ADP	10 + 100 + 2.0	320	—

DISCUSSION

It is evident from the above that the rate of the HILL reaction, as standardly measured, can be greatly increased by the addition of phosphorylating reagents. The rate of reduction of an electron acceptor measured in a HILL reaction without phosphorylation, then, can be looked upon as related to the degree to which the chloroplasts are "uncoupled" under the particular conditions of measurement. The extent to which Mg, phosphate and ADP stimulate the reaction would similarly be related to the degree of "coupling" in the chloroplasts. We thus emerge with a situation similar in some respects to that previously found in oxidative phosphorylation, where the rate of electron transport is limited by the rate at which phosphorylation can proceed.

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It is not yet clear whether the similarity extends to the basic mechanisms responsible for this behavior.

In postulating a mechanism for a coupled "HILL reaction", we have tried to formulate the minimum number of reactions which seem to be necessary to explain the presently available evidence. Similar, but more involved mechanisms, have been postulated for oxidative phosphorylation[13].

$$\text{I.}\quad H_2O \longrightarrow XH + YO$$
$$\text{II.}\quad XH + I + A \longrightarrow X + I^* + AH$$
$$\text{III.}\quad I^* + P_i \longrightarrow I \sim P$$
$$\text{IV.}\quad I \sim P + ADP \longrightarrow ATP + I$$
$$\text{V.}\quad AH + Fe(CN)_6^{-3} \longrightarrow A + H^+ + Fe(CN)_6^{-4}$$
$$\text{VI.}\quad YO \longrightarrow Y + 1/2\ O_2$$

Overall:

$$H_2O + 2Fe(CN)_6^{-3} + ADP + P_i \xrightarrow{\text{light}} 2Fe(CN)_6^{-4} + ATP + 1/2\ O_2 + 2H^+$$

Reaction I is the light reaction, consisting of the splitting of a water molecule into an oxidizing (YO) and a reducing (XH) portion. Electron-transport steps follow, in one of which energy is transferred to a compound I, transforming it to I* (represented in reaction II). I* or a product derived from it or which reacts with it, will transfer the phosphate to ADP forming ATP (represented in reactions III and IV). The same net effect could be achieved by ADP reacting with I* and the combination reacting with inorganic phosphate. The reduced product produced in reaction II, (AH), or some other acceptor to which the electron has been transferred, reduces ferricyanide to ferrocyanide (represented in reaction V). The oxidized product of the splitting of water (YO) reacts to produce as a final product free oxygen and free acceptor (represented in reaction VI). The overall reaction involves, as observed, light-dependent reduction of ferricyanide, esterification of inorganic phosphate into ADP, evolution of oxygen and production of hydrogen ions. It should be emphasized that the above formulation is not intended to represent the particular reactions which take place, but only the minimal possible number of reasonable reactions which must be assumed to take place. In all probability, many more reactions are involved, and each reaction above may be a summation of several others.

The observed phenomena can be related to the above formulation in the following manner. I* can be considered as an inhibitor of electron transport, as suggested in the case of oxidative phosphorylation[13]. The rate of ferricyanide reduction is slow because of the presence of high concentrations of I* produced during electron transport (reaction II). The addition of ADP, phosphate and Mg lowers the I* concentration by reacting with it to produce ATP (reactions III, IV) and thus permits a faster rate of electron transport to proceed. This, of course, is one of the observations reported in this paper. Phosphate and Mg were shown to cause small stimulations in electron transport. This is consistent with the expected effect in reaction III. The inhibition caused by ATP may be brought about by shifting the equilibrium of reaction IV to the left. It is of interest, in this connection, that chloroplasts "activated" by dilution, do not seem to be inhibited by ATP.

"Uncoupling" may be defined as a process by which electron transport is made to proceed at maximal rates in the absence of concomitant phosphorylation. It can be conceived of as a process which, in some manner, causes the conversion of I* to I

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or allows XH to reduce A in the absence of I. From the data presented earlier, treatment of the chloroplasts by the dilution technique can be considered as such an uncoupling process. The fact that the addition of the phosphorylation reagents causes no increase in activity beyond that induced by the dilution technique is consistent with this interpretation. It could also, however, be due to a shift in the site from which electrons are transferred to ferricyanide. Thus, after "activation", ferricyanide might be capable of receiving electrons from a site preceding the first electron-transport step which is "coupled" to phosphorylation. Reduction of indophenol dye appears to be coupled to phosphorylation to only a negligible extent. This might be due either to uncoupling action of trichlorophenol-indophenol, or to a difference in the site of electron transport to the two acceptors.

In conclusion, it would seem that the HILL-reaction concept should be modified to include the coupling of phosphate esterification to the electron-transport reactions involved. This seems to hold true at least where ferricyanide is used as an electron acceptor. Indications are however, that even with the electron acceptor permitting the fastest electron transport in the absence of phosphorylating reagents, trichloroindophenol, the same basic phenomena exist.

SUMMARY

Ferricyanide reduction and accompanying phosphorylation were shown to be increased similarly by the addition of Mg, phosphate and ADP. The presence of all three components is necessary for maximal reductive and phosphorylative activity. Similar increases in the rate of ferricyanide reduction can also be induced by a dilution technique. The addition of Mg, ADP and phosphate to a chloroplast suspension treated by the dilution technique does not cause any further increase in the rate of ferricyanide reduction.

The reduction of trichloroindophenol dye shows similar, but much smaller, effects.

From light intensity studies, it is concluded that the effect is largely on the non-light-dependent steps of the HILL reaction.

ATP was shown to inhibit the rate of ferricyanide reduction.

The observations are explained in terms of a proposed scheme in which phosphate is esterified during electron transport reactions leading to ferricyanide reduction, and the rate of electron transport is limited by the rate of phosphorylation.

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