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PHOTOPHOSPHORYLATION AS A FUNCTION OF LIGHT INTENSITY*

S. SAHA**, S. IZAWA AND N. E. GOOD
Department of Botany and Plant Pathology, Michigan State University, Mich. (U.S.A.)
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SUMMARY

1. If there is a critical light intensity below which photophosphorylation cannot occur, it is much lower than heretofore reported. At exceedingly low light intensities phosphorylation does not increase in a linear manner with increasing light intensity. This non-linearity seems to be a property of the electron transport-dependent phosphorylation reaction and not a property of the electron transport itself. The very slight non-linearity is independent of the H^+ concentration of the medium between pH 7.0 and 10.0. The quantum efficiency of photophosphorylation is highest at pH 9 although proton accumulation at this pH is extremely inefficient.

2. Photophosphorylation remains an almost linear function of light intensity in the presence of uncouplers such as methylamine, atebrin, Triton X-100 or Ag^+. On the other hand uncouplers such as carbonylcyanide 3-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol, gramicidin and valinomycin inhibit phosphorylation much more severely at very low light intensities. The marked decrease in the quantum efficiency of phosphorylation with CCCP and 2,4-dinitrophenol at low light intensities is associated with some decrease in the quantum efficiency of electron transport. Nevertheless a part of the decrease in efficiency is probably attributable to the weakness with which very low rates of electron transport are coupled to phosphorylation.

INTRODUCTION

The chemiosmotic mechanism of phosphorylation proposed by MITCHELL[1] demands that a critical proton gradient or charge separation be formed across the thylakoid membranes of chloroplasts before ATP can be synthesized. Since the gradient is thought to result directly from light-driven electron transport, it follows that there should be some light intensity below which photophosphorylation cannot occur; if the critical intensity were not attained the finite permeability of the membranes to ions would prevent a build-up of the requisite electrochemical potential gradient. There is considerable experimental evidence which supports this proposition. Thus TURNER *et al.*[2], DILLEY[3], SAKURAI *et al.*[4] and SCHWARTZ[5] have all reported that photophosphorylation does not increase linearily with increasing light intensities

Abbreviations: CCCP, carbonylcyanide 3-chlorophenylhydrazone; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

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** Present address: Department of Botany, Kalyani University, West Bengal, India

whereas electron transport does. Indeed SCHWARTZ[5] has maintained that there is a pronounced "dead-space" at low light intensities. He found no phosphorylation whatsoever until the level of illumination had reached a significant fraction of the saturating intensity.

The chemiosmotic theory also relates uncoupling to increased ion permeability of membranes. Certainly the action of many of the substances which uncouple electron transport from photophosphorylation could be interpreted in such a manner. For instance, it is known that CCCP and 2,4-dinitrophenol increase the permeability of artificial phospholipid membranes to protons[6], while gramicidin and valinomycin increase the permeability of biological membranes to alkali ions[7]. Moreover almost every amine of appreciable base strength which has a lipid-soluble unionized form is a good uncoupler of electron transport in chloroplasts[8,9]. It is therefore tempting to attribute the uncoupling effect to the ease with which amines can traverse thylakoid membranes. They are, in effect, permeant cations. But, if uncouplers did act in this manner, one might expect them to exaggerate the non-linearity alluded to above.

Finally, if the non-linearity reflects formation of a H^+ gradient, it should be affected by the composition of the medium. In particular, the non-linearity should be modified by the ambient H^+ concentration.

This paper deals with the relationship between photophosphorylation and light intensity at various pH's and in the presence and absence of uncouplers.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach (*Spinacia oleracea* L.) by the following procedure: Washed leaves were ground for about 10 sec in a Waring Blendor in 0.3 M NaCl, 0.003 M $MgCl_2$, 0.01 M KCl and 0.05 M tricine adjusted to pH 7.5 with NaOH. The homogenate was squeezed through 8 layers of cheesecloth then centrifuged at about 2000 × *g* for 5 min. The sediment was resuspended in 0.1 M sucrose, 0.002 M $MgCl_2$, 0.001 M KCl and 0.005 M tricine adjusted to pH 7.3 with NaOH. The suspension was centrifuged briefly (about 15 sec) at 2000 × *g* to remove cell debris and intact cells, and then centrifuged again for 4 min at about 2000 × *g*. The pellet was washed once more in the same medium and was finally taken up in a small amount of the sucrose–tricine medium. Chlorophyll was estimated by the method of ARNON[10].

A stock solution of $Na_2H^{32}PO_4$ was prepared in the following manner: 0.1 ml $H_3{}^{32}PO_4$ in 0.3 M HCl (containing about 5 mC) was taken up in 20 ml of 0.1 M Na_2HPO_4. The resulting radioactive solution was treated with 1 g of acid-washed charcoal (Norit) which had been pre-equilibrated with 0.1 M Na_2HPO_4 solution. The charcoal suspension (about 250 μC/ml) was shaken for 1 h and then filtered through Whatman No. 1 filter paper. An aliquot was checked for contamination with polyphosphates by extracting all of the orthophosphate as phosphomolybdic acid. With some batches of $H_3{}^{32}PO_4$, the solution was still further purified by repeating the Norit treatment. Eventually, in all cases, a reasonably low background radioactivity of 30–40 out of 250000 counts/min was obtained after thorough extraction of the orthophosphate.

Hexokinase (100 mg) was dissolved in 10 ml precooled 0.2 M glucose, 0.001 M tricine (pH 7.0). The solution was dialysed overnight against 1 l of the buffered

glucose at 3–4°. The solution was then stored in small tubes at −15° and thawed immediately before use.

Phosphorylation reaction: Unless otherwise stated all reaction mixtures contained in a total volume of 2.0 ml: 0.1 M sucrose, 0.001 M KCl, 0.003 M $MgCl_2$, 0.01 M $Na_2H^{32}PO_4$, 0.001 M ATP, 1 mg hexokinase, 0.01 M glucose, 0.04 M tricine adjusted to pH 8.4 with NaOH, and chloroplasts with 40 or 20 μg chlorophyll. The electron acceptors, methyl viologen or potassium ferricyanide, were added at the concentrations indicated in the legends of the figures. All reactions were carried out at 19° in a 1-cm cuvette. The cuvette was illuminated from one side by a light beam from a 500-W slide projector passed through a 650-nm interference filter. Combinations of neutral filters (fine mesh screens) were used to control the light intensity, which was measured with a wavelenght-independent radiometer (YSI-Kettering, Model 65). The reaction was run for 2 or 3 min and an aliquot of the reaction mixture was then pipetted into a test tube containing 10 ml of 10% $HClO_4$ saturated with 1-butanol–benzene (1:1, by vol.).

Organic ^{32}P was estimated as residual radioactivity after extraction of the remaining orthophosphate as phosphomolybdate by the method of AVRON[11].

RESULTS

Phosphorylation as a function of light intensity in the absence of uncouplers

Our preliminary experiments did not reveal the previously reported conspicuous non-linearity (see Fig. 1, left). However, when we increased the sensitivity of the method by using low concentrations of orthophosphate having very high specific activities, we did find a slight non-linearity at the very lowest light intensities (see Fig. 1, inset). (In the latter studies it was necessary to prepare the chloroplasts very carefully to avoid contamination with mitochondria and other cell organelles since these catalyzed the incorporation of amounts of phosphate into organic substances which became significant at the extremely low rates of phosphorylation reported here.) Because of the measurable but exceedingly small non-linearity we cannot be sure that there is not some critical light intensity below which no phosphorylation can occur. Nevertheless the critical level, if it exists, must be about 2 orders of magnitude lower than reported by SCHWARTZ[5].

Phosphorylation as a function of light intensity in the presence of inhibitors and uncouplers

It occurred to us that our observations might be reconciled with the observations of earlier workers if the earlier workers had used partially uncloupled and partially inhibited chloroplasts. Accordingly we undertook a study of the effects of inhibitors and uncouplers on the linearity of the phosphorylation reaction. Neither phlorizin which inhibits the phosphorylation reaction nor DCMU which inhibits electron transport had much effect on the linearity; the extent of the inhibition was similar if not identical at all of the intensities studied. The same is true of many uncouplers and uncoupling conditions. Methylamine, atebrin, the non-ionic detergent Triton X-100, and Ag^+ are all effective uncouplers yet they do not introduce the requirement for a critical light intensity others have seen. In contrast the uncoupler CCCP (ref. 12) and the inhibitor–uncoupler 2,4-dinitrophenol do greatly increase the non-linearity.

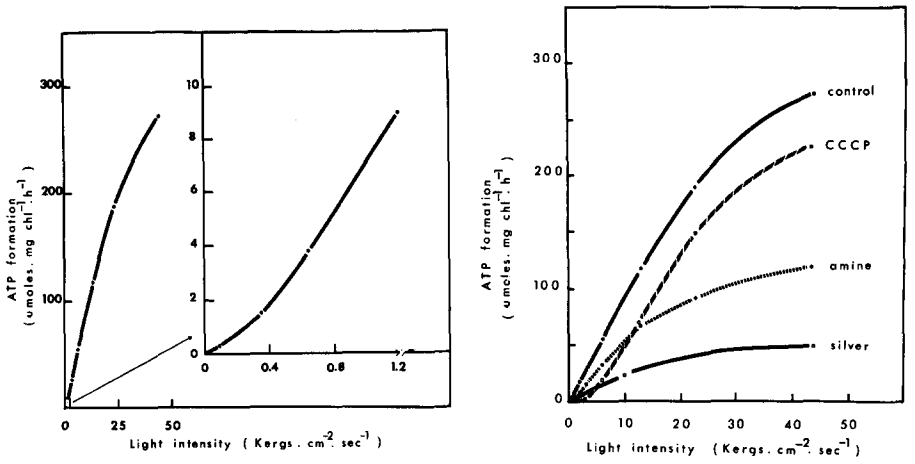

Fig. 1. Photophosphorylation as a function of light intensity. The 2.0 ml reaction mixture (main figure) contained chloroplasts with 40μg chlorophyll, $1 \cdot 10^{-7}$ moles methylviologen and other ingredients as described in MATERIALS AND METHODS. In the experiment illustrated by the inset figure the composition of the reaction was mixture different: Tricine–NaOH, $4 \cdot 10^{-3}$M; KCl, $1 \cdot 10^{-4}$ M; chloroplasts, 20μg chlorophyll. Illumination was by light of 650 nm (interference filter) for 2 min in main figure and 3 min in inset figure. Temperature 19°. pH 8.4.

Fig. 2. Effects of uncouplers on photophosphorylation at low light intensities, I. Reaction conditions were as described for Fig. 1, main figure. Concentrations of uncouplers: CCCP, $1 \cdot 10^{-6}$ M; methylamine·HCl, $2 \cdot 10^{-3}$ M; $AgNO_3$, $2 \cdot 10^{-6}$ M.

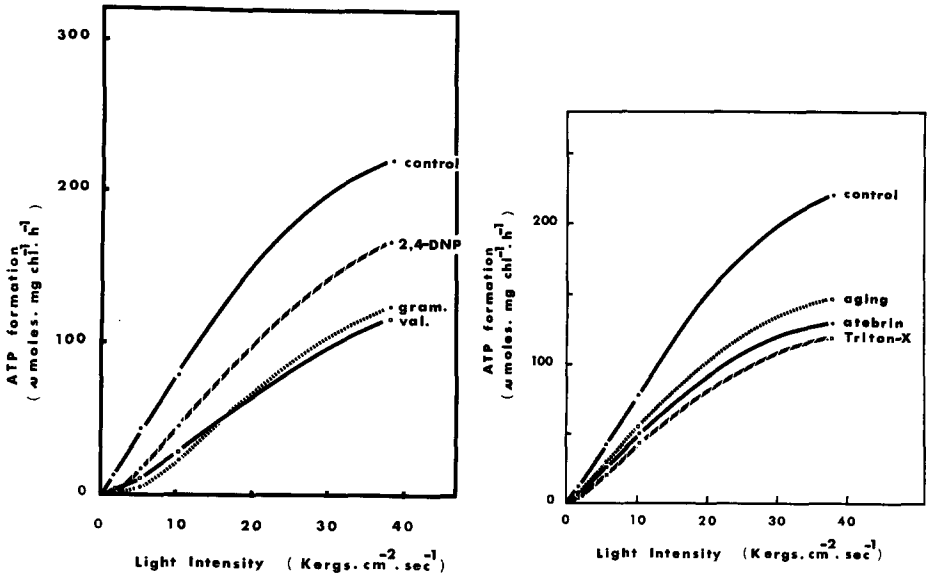

Fig. 3. Effects of uncouplers on photophosphorylation at low light intensities, II. Reaction conditions were as described for Fig. 1, main figure. Concentrations of uncouplers; 2,4-dinitrophenol, $4 \cdot 10^{-4}$ M; gramicidin, $2 \cdot 10^{-8}$ M; valinomycin, $2 \cdot 10^{-7}$ M

Fig. 4. Effects of uncouplers on photophosphorylation at low light intensities, III. Reaction conditions were as for Fig. 1, main figure. Concentrations of uncouplers: Atebrin, $5 \cdot 10^{-6}$ M; Triton X-100, 0.003%. Aging of chloroplasts was at 3–4° for 6h.

In their presence there is a threshold intensity below which little phosphorylation can be detected. The antibiotics valinomycin and gramicidin, which also increase the permeability of membranes to certain ions[7] seem to be intermediate, increasing the non-linearity but not nearly to the same extent as does CCCP (see Figs. 2–4).

These CCCP- and 2,4-dinitrophenol-induced decreases in the low intensity quantum efficiencies of photophosphorylation are associated with appreciable but somewhat smaller decreases in the low-intensity quantum efficiencies of electron transport (*cf.* Fig. 5 with Figs. 2–4).

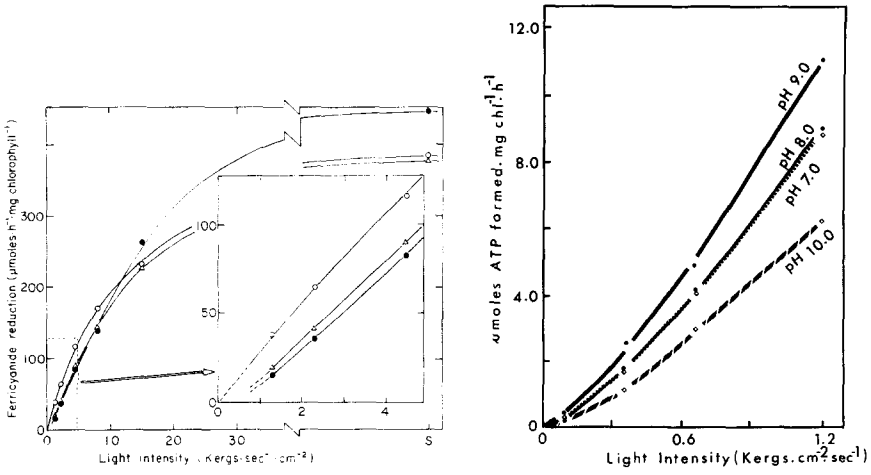

Fig. 5. Electron transport as a function of light intensity. Reaction conditions were as in Fig. 1, main figure except that $4 \cdot 10^{-4}$ M potassium ferricyanide replaced the methylviologen, and the pH was 8.1. "S" represents a very high (saturating) intensity of broad red light. Electron transport was measured as the decrease in absorbance of the reaction mixture at 420 nm. ○, control; ●, + 2 μM CCCP; △, + 130 μM 2,4-dinitrophenol.

Fig. 6. Effects of pH on photophosphorylation at low light intensities. The buffer consisted of $2 \cdot 10^{-2}$ M tricine and $2 \cdot 10^{-2}$ M glycine and the pH was adjusted by additions of NaOH. Otherwise the reaction conditions were as in Fig. 1, inset.

Phosphorylation as a function of light intensity at different pH's

The very small amount of non-linearity shown in Fig. 1, inset seems independent of pH since the shape of the phosphorylation–intensity curve is very similar from pH 7.0 to 10.0 (see Fig. 6). It is interesting to note, however, that the quantum efficiency of photophosphorylation is considerably higher at pH 9 than it is at pH 8.0, the H^+ concentration usually used in photophosphorylation studies.

DISCUSSION

As indicated in the introduction, the chemiosmotic mechanism requires a definite pH gradient and/or electrical potential gradient as a prerequisite for phosphorylation. However, the theory does not predict the amount of proton translocation required to achieve these gradients. For a given amount of proton translocation (without compensating counter-ion movement) there will be a pH gradient and an electrical potential gradient formed. The steepness of the pH gradient will depend

on the size and the buffering capacity of the internal space while the potential gradient will depend on the electrical capacitance of the membrane. Furthermore, even the lowest rates of proton translocation could eventually create sufficient gradients for ATP synthesis, regardless of capacity factors, if the membranes were completely impervious to other ions. For these reasons one would expect the steady-state non-linearity, *i.e.* the non-linearity observed in experiments lasting for minutes or longer, to be very much a function of membrane integrity.

From this point of view a conspicuous non-linearity in the increase of photophosphorylation with increasing light intensities does not seem to be a requirement of the chemiosmotic mechanism. The "critical" intensity might be so low in good chloroplasts as to defy detection. Therefore our failure to find the extent of non-linearity reported by others does not contradict the chemiosmotic theory. However, our results do show that the previously reported large non-linearity is not an inherent characteristic of photophosphorylation and, consequently, such kinds of non-linearities cannot be considered as providing evidence in support of the chemiosmotic hypothesis.

The effects of uncouplers described in this paper are not the effects one would predict from a simple-minded consideration of the chemiosmotic mechanism. If uncoupling results from the dissipation of an electrochemical potential gradient through ion leakage one might expect to find that uncouplers have in common the property of raising the level of the critical light intensity or increasing the non-linearity of the phosphorylation reaction. This is certainly not so. The uncouplers known to make artificial phospholipid membranes permeable to protons (CCCP and 2,4-dinitrophenol) and the uncouplers known to make biological membranes permeable to alkali ions (valinomycin and gramicidin) do act in a manner which could be interpreted in terms of the dissipation of an electron transport-dependent gradient. But even if we attribute some of the non-linearity caused by CCCP and 2,4-dinitrophenol to H^+ leaks in the chloroplast membranes, the fact that Triton X-100 inhibits phosphorylation equally at all light intensities remains to be explained. Triton X-100 certainly destroys H^+ gradients[13], but perhaps it could be argued that the effect of Triton X-100 on given lamella is all-or-none whereas CCCP induces degrees of leakiness However, this interpretation of Triton X-100 uncoupling cannot be plausibly applied to the reversible uncoupling introduced by amines, *etc.* If amines dissipate an essential gradient they must do so at a rate which is both proportional to the rate of gradient formation and independent of the steepness of the gradient. It is not clear how a "leak" could have such characteristics.

At high light intensities the optimum pH for proton uptake is much lower than the optimum pH for phosphorylation[14] but this difference might reflect nothing more than the pH required for maximum activity of some enzyme involved in the phosphorylation reaction. By going to very low light intensities the electron transport itself can be made the rate-determining factor. In this case, if the formation of a proton gradient is a necessary intermediate step and the gradient is less efficiently formed at high pH, one might expect bigger non-linearities in phosphorylation as the pH is raised. Again as can be seen in Fig. 6, this is not so. The efficiency of phosphorylation, unlike the efficiency of proton translocation, is actually highest at high pH but no pH seems to increase or decrease the linearity of the phosphorylation reaction. Unfortunately the relevance of this observation to the chemiosmotic theory is not

as obvious as our superficial analysis implies. Decreased proton translocation does not necessarily mean decreased gradient formation. There is no obvious way of relating proton gradient formation to proton translocation in the absence of any knowledge of the buffering capacity of the inside of the lamellae. Nevertheless one would certainly expect H^+ gradient formation to be influenced in some way by very wide changes in the pH of the medium. The fact that such changes in the medium have no effect on the linearity of the phosphorylation reaction leads us to suspect that the observed slight non-linearity is unrelated to H^+ gradient formation.

ACKNOWLEDGMENT

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