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Partial Reactions of Photosynthesis in Briefly Sonicated *Chlamydomonas*

II. PHOTOPHOSPHORYLATION ACTIVITIES[1]

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ABSTRACT

Briefly sonicated *Chlamydomonas reinhardi* cells are capable of both cyclic and noncyclic photophosphorylation and, in each case, the maximum rates approach those reported for higher plant chloroplasts. Photophosphorylation coupled to ferricyanide reduction occurs with a P/2e ratio approaching unity.

The conditions for optimum activity are similar to those reported for spinach or swiss chard chloroplasts; the major difference is the extreme sensitivity to salt and relative insensitivity to methylamine. Cell preparation, sonication, and assays were all performed at room temperature under conditions suitable for screening a large number of potential mutants deficient in photophosphorylation activity. This method was easily adapted to *Euglena gracilis* strain Z but not adaptable for *Chlorella vulgaris* or *Scenedesmus obliquus* strain D_3.

Eukaryotic algal systems offer many advantages in the investigation of photosynthetic mechanisms. Algae can be cultured in the laboratory under controlled conditions, subjected to microbiological manipulations, and in some cases even be analyzed genetically. They have been extensively utilized in the analysis of photophosphorylation *in vivo* (18). However, for the analysis of photophosphorylation *in vitro*, algal systems have not been exploited extensively due to the difficulty in obtaining active membrane fractions. Various degrees of success with *in vitro* algal systems have been reported previously with *Chlamydomonas* (10, 13, 17), *Euglena* (7), *Scenedesmus* (16), and *Bumilleriopsis* (4). In this paper the photophosphorylation capabilities of briefly sonicated *Chlamydomonas reinhardi* cells are described. As in the accompanying paper (9), the assay method was developed to analyze large numbers of samples simply and rapidly. The resultant procedure should prove useful for analysis of mutant strains and other suitable algal systems.

MATERIALS AND METHODS

Chlamydomonas reinhardi was grown as described in the companion paper (9). *Euglena gracilis* strain Z was grown either in Hutner's organotrophic medium as described previously (6) or in the autotrophic medium of Böger and San Pietro (5). *Scenedesmus obliquus* strain D_3 was grown as described for *Chlamydomonas*. *Chlorella vulgaris* was grown in Bristol's solution (19) fortified with 0.02 M acetate (8). Cells were harvested and sonicated at a power setting of 80 for 10 sec unless otherwise indicated (see ref. 9). *Scenedesmus* and *Chlorella* cells were sonicated for 1 min at full power prior to Chl extraction and determination spectrophotometrically (1).

Photophosphorylation was assayed by measuring the incorporation of ^{32}Pi into ATP. The reaction mixtures were prepared in a volume of 1.5 ml in 3-ml disposable clear plastic test tubes and illuminated for 1 min with white light (6×10^5 ergs cm^{-2} sec^{-1}). Immediately after illumination, 0.1 ml of 20% trichloroacetic acid was added to each tube and the suspension centrifuged at 6000*g* for 10 min. Aliquots of the supernatant solutions were analyzed for $AT^{32}P$ as described by Avron (2).

Oxygen evolution accompanying ferricyanide or *p*-benzoquinone reduction was performed as described in the earlier paper (9). Phosphorylation accompanying O_2 evolution was assayed as above except that aliquots were removed from the O_2 electrode chamber for analysis of $AT^{32}P$ after 1.5 min illumination.

Carrier free-^{32}Pi was obtained from ICN Corporation; crystalline PMS[2] from Sigma Chemical Co. Other chemicals were as indicated in the previous paper (9).

RESULTS

As noted earlier for photosynthetic electron transport (9), photophosphorylation is conveniently measured in briefly sonicated *Chlamydomonas* cells. The effect of sonication time on the rates of photophosphorylation is shown in Figure 1. Both noncyclic phosphorylation coupled to ferricyanide reduction and PMS-mediated cyclic phosphorylation exhibit optimum activity after only a brief period of sonication. Neither activity is demonstrable without sonication, due to perhaps the impermeability of the cell. In each case, the activity rapidly decreased for sonication times greater than 10 sec and this decrease probably reflected denaturation of some component of the electron transport chain or photophosphorylation apparatus.

The effect of different power settings of the sonicator on the rates of photophosphorylation is given in Figure 2. Using a 10-sec sonication time, it is seen that a power setting of 80 afforded maximal photophosphorylation activity with both

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[2] Abbreviations: PMS: phenazine methosulfate; FCCP: carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone.

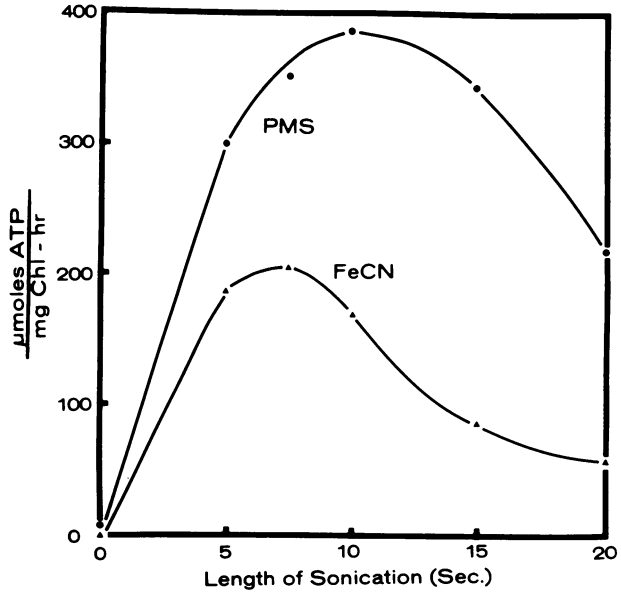

FIG. 1. Effect of sonication time on photophosphorylation. Reaction mixtures contained sonicated cells equivalent to 18 μg of Chl, 50 μmoles of MES-Tricine buffer (pH 7.8), 1 μmole of ADP, 3.75 μmoles of potassium phosphate (pH 7.5), approximately 0.5 μCi of ^{32}Pi, and 0.5 μmole of $MgCl_2$ in a final volume of 1.5 ml. PMS-mediated phosphorylation reactions (●) also contained 0.1 μmole of PMS and 0.01 μmole of DCMU. Ferricyanide-mediated phosphorylation reactions (▲) also contained 1.5 μmoles of $K_3Fe(CN)_6$. Cells were sonicated at a power setting of 80.

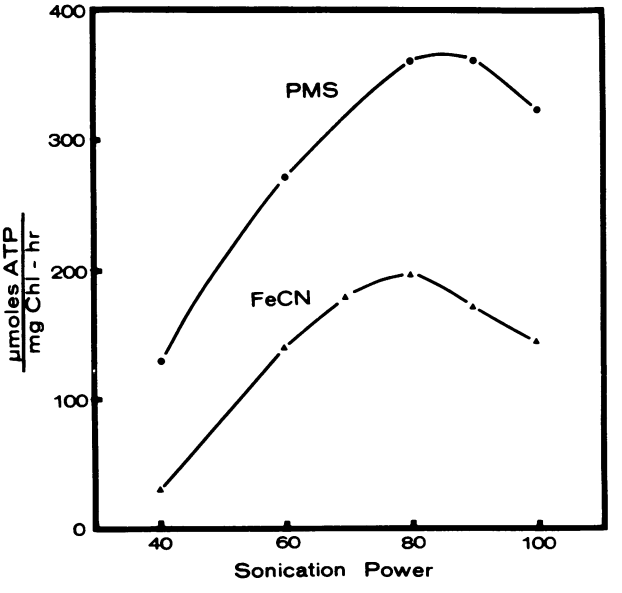

FIG. 2. Effect of sonication power on photophosphorylation. Cells were sonicated 10 sec. Other conditions as described in the legend of Fig. 1.

ferricyanide and PMS. Thus, these conditions were used exclusively in the experiments reported.

The reaction conditions required for maximal photophosphorylation activity in *Chlamydomonas* were extensively investigated. For many reaction components, the results were similar to those reported for higher plant systems. In most instances, typical hyperbolic saturation curves were observed as would be expected. However, several significant differences were observed. For example, the concentration of PMS in the assay mixture is critical. At concentrations significantly different from 0.1 mM, the activity was decreased.

The cofactor for which concentration was most critical was $MgCl_2$. Although photophosphorylation is maximal at 0.3 mM $MgCl_2$, higher concentrations severely inhibit the reaction (Fig. 3). This is true for photophosphorylation mediated by PMS, ferricyanide, or ferredoxin/NADP. In each case, the rate is inhibited 50% at approximately 2 mM $MgCl_2$. Endogenous activity in the absence of added $MgCl_2$ is likely due to that present in the cell preparation, since cell membrane components were not separated from soluble cell sap.

The pH optimum for PMS-mediated phosphorylation occurs at 7.5 to 7.8, whereas that for ferricyanide occurs at a pH of approximately 8.1 to 8.4 (Fig. 4). Both activities are maximal in a pH range somewhat lower than that observed for higher plant chloroplasts as measured in Tricine or MES buffers (12). These data suggest that cell fragments prepared by brief sonication may be somewhat uncoupled.

The effects of several uncouplers on photophosphorylation are shown in Figure 5. FCCP is inhibitory at all concentrations tested; 50% inhibition is noted at about 0.6 μM. Polylysine also inhibits at a low concentration (50% at about 20 μg/ml). However, methylamine, a classical uncoupler of photophosphorylation in higher plant chloroplasts (11), is a relatively poor uncoupler in the briefly sonicated preparation. Even at 40 mM methylamine, the reaction is less than 50% inhibited.

The severe inhibition by $MgCl_2$ and rather weak inhibition by methylamine suggest that salts in general may inhibit photophosphorylation. The results of Figure 6 indicate that this is the case. Both PMS- and ferricyanide-mediated photophosphorylation are 50% inhibited at about 60 mM NaCl; that is, essentially at the same concentration of methylamine which inhibits phosphorylation.

The severe inhibition of net photophosphorylation by salt could be caused by an inhibition of some reaction leading to the formation of ATP, or could be the result of a salt stimulated ATPase reaction. To test these alternatives, assays were performed as usual, except that trichloroacetic acid was added

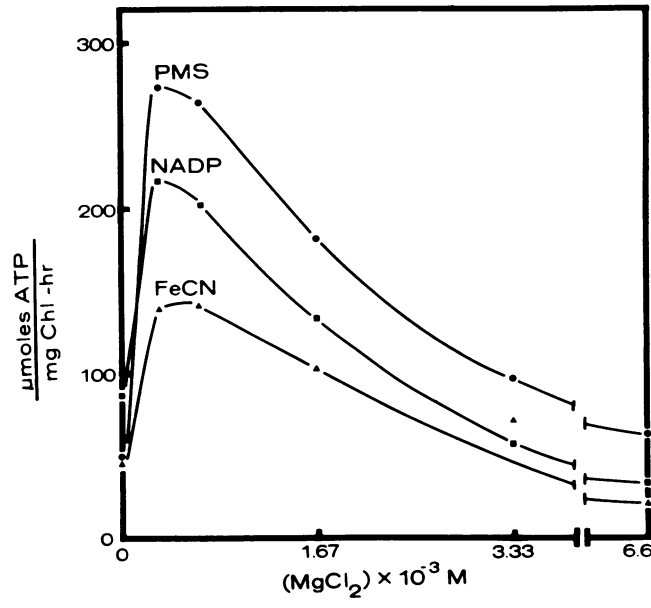

FIG. 3. Effect of $MgCl_2$ on photophosphorylation. PMS- (●) and ferricyanide-mediated reactions (▲) as described in the legend of Fig. 1. NADP-mediated phosphorylation reactions (■) contained 0.5 of μmole NADP and 30 nmoles of spinach ferredoxin in addition to the components given in the legend of Fig. 1.

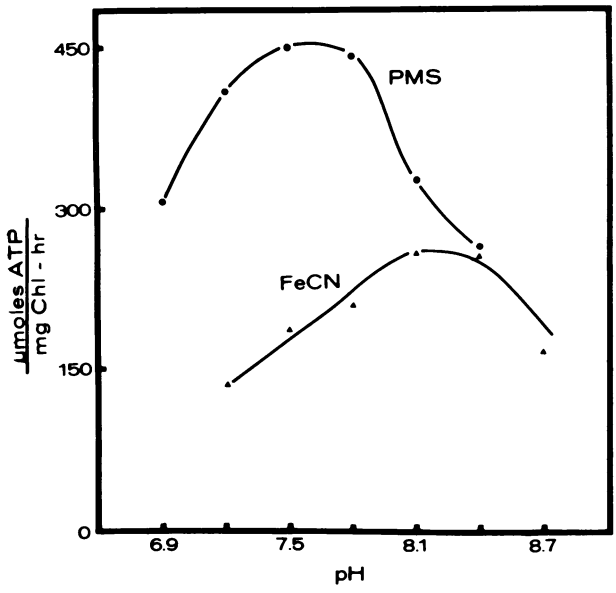

FIG. 4. pH dependence of photophosphorylation. Reaction conditions as described in the legend of Fig. 1.

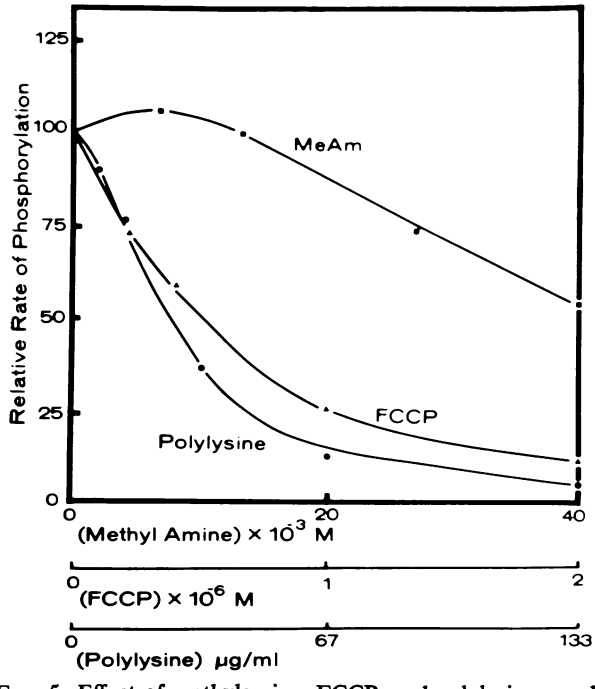

FIG. 5. Effect of methylamine, FCCP, and polylysine on photophosphorylation. Reaction conditions as described in the legend of Fig. 1.

to the reaction vessels at various times after illumination. In the absence of salt, the ATP produced was quite stable, with little loss of AT^{32}P even after 3 min postillumination incubation. When a $MgCl_2$ concentration sufficient to partially inhibit phosphorylation was present, the smaller amount of ATP produced was again stable. Thus, it appears that the salt causes an inhibition of the formation of ATP as opposed to a stimulation of the degradation of ATP.

The rate of ATP formation was linear for about the first 3 min of illumination (Fig. 7). This was true despite the rather rapid conversion of PMS into pyocyanine by white light.

Light intensity curves were determined for both PMS- and ferricyanide-mediated photophosphorylation. The ferricyanide-mediated rate was saturated at 6×10^5 ergs cm^{-2} sec^{-1}, whereas the PMS-mediated rate approached saturation at the light intensity routinely used (1.1×10^6 ergs cm^{-2} sec^{-1}).

A particular advantage of the brief sonication method is the convenience of the system. When the preparation was stored at room temperature after sonication (Fig. 8), activity decreased linearly for the first 30 min, the half-time for both PMS- and ferricyanide-mediated photophosphorylation was about 20 min. However, if the preparation was cooled to 0 C immediately after sonication, photophosphorylation activity was stable for at least 1 hr. Assays were generally performed immediately after sonication, thus eliminating the need for cooling.

Neither BSA nor the reductant sodium ascorbate significantly increased the net rates of photophosphorylation in the sonicated cells, nor did BSA appreciably increase the stability of photophosphorylation. This is in contrast to the observations of Wallach *et al.* (20).

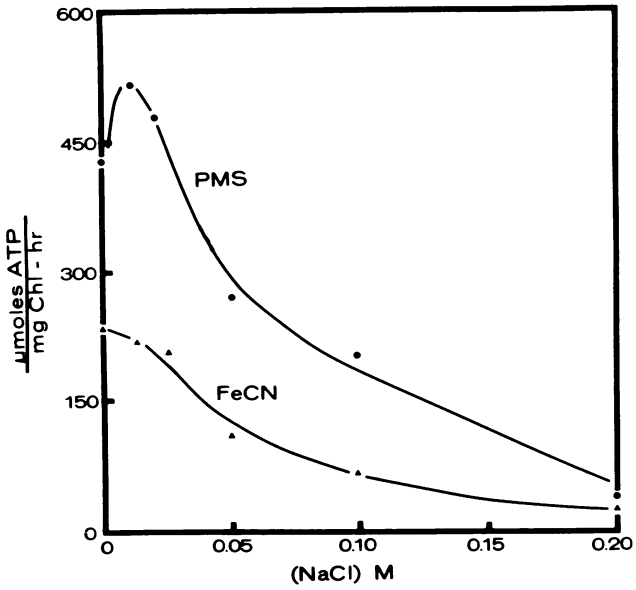

FIG. 6. Effect of NaCl on photophosphorylation. Reaction conditions as described in the legend of Fig. 1.

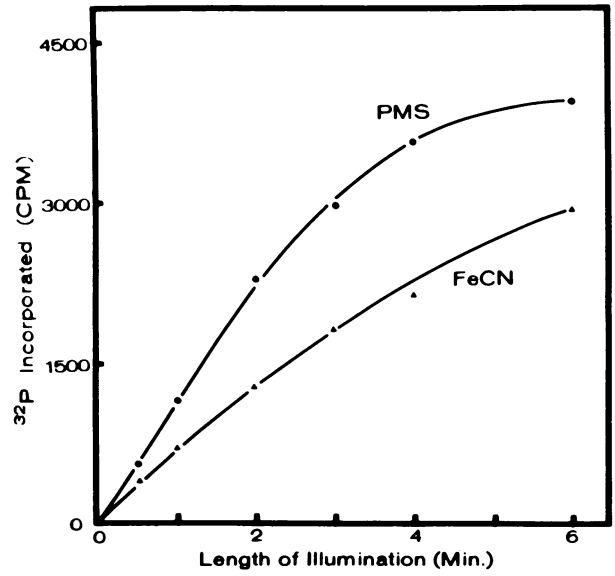

FIG. 7. Photophosphorylation as a function of illumination time. Reaction conditions as described in the legend of Fig. 1.

The P/2e ratios obtained under several experimental conditions are given in Table I. Electron transfer from water to ferricyanide exhibits a P/2e ratio approaching unity; the range in several experiments was from 0.6 to 0.9. Methylamine, at a concentration which would completely uncouple higher plant chloroplasts, decreased this ratio by about 50%. The P/2e ratios observed with *p*-benzoquinone were less than with ferricyanide, although the relative effect of methylamine was the same. The effect of FCCP was also anomalous; the behavior observed was not consistent with classical uncoupling in that very little stimulation of electron transport occurred at any concentration tested. Oxygen evolution was inhibited at concentrations of FCCP greater than 0.2 μM however, phosphorylation was inhibited to a greater degree, thereby resulting in a decreased P/2e ratio.

An attempt was made to extend the brief sonication method to other species of green algae, including *Euglena gracilis* Z, *Scenedesmus obliquus* D_3, and *Chlorella vulgaris*. Heterotrophic *Euglena* cells responded well to sonication and yielded good rates of PMS- and ferricyanide-mediated phosphorylation. Cells grown in autotrophic medium were less active, particularly in ferricyanide-mediated phosphorylation, and presumably require further modification of assay conditions for optimal rates.

The results of experiments performed with *Euglena, Scenedesmus,* and *Chlorella* are given in Table II; for purposes of comparison the data with *Chlamydomonas* are included. The low rates obtained with *Chlorella* and *Scenedesmus* reflect the extreme difficulty in rupturing the cell and thereby permitting access to exogenous electron acceptors and phosphorylation cofactors. Even after 30 sec sonication time which was complicated by heating, poor activities were obtained. Sonication for 1 min at 5 C was equally ineffective. Microscopic observation indicated that neither *Chlorella vulgaris* nor *Scenedesmus obliquus* was broken even after 30 sec sonication, whereas *Chlamydomonas reinhardi* and *Euglena gracilis* were essentially disrupted after 10 sec sonication.

DISCUSSION

This paper demonstrates that stable and reproducible rates of both cyclic and noncyclic phosphorylation can be substantially increased in *Chlamydomonas* preparations, and such increases can be accomplished by a simple and rapid procedure. This method yields results consistent with those reported previously with other preparative procedures and/or other algae (4, 7, 16, 17, 20).

The sonication conditions are extremely critical and should be selected with care, and determined independently for each sonicator used, before performing routine assays (Figs. 1 and 2). Reproducibility can be maintained within 10% provided the proper precautions are taken (9).

Because the broken *Chlamydomonas* preparation used in these assays contained mitochondria and other cell components, the possibility that oxidative phosphorylation might significantly contribute to the over-all phosphorylation capacity of the cells was investigated. However, the rate of phosphorylation in the dark was never greater than 2 or 3 μmoles ATP formed/mg Chl·hr. Thus, there was no apparent interference with phosphorylation activities by other components of the *Chlamydomonas* preparation.

In *Euglena*, however, the rate of phosphorylation in the dark was significant; heterotrophic cells yielded rates of ap-

Table I. *P/2e Ratios for Chlamydomonas*

Reaction	Uncoupler[1]	Oxygen Evolution	Phosphorylation	P/2e
		μmoles O_2 mg Chl·hr	μmoles ATP/mg Chl·hr	ratio
$H_2O \rightarrow FeCN$[2]	None	93	133	0.72
	MeAm	138	112	0.40
	FCCP	84	78	0.46
$H_2O \rightarrow BQ$[3]	None	180	118	0.33
	MeAm	210	95	0.23
	FCCP	156	67	0.21

[1] When present, the methylamine concentration was 6.7 mM; the FCCP concentration was 0.4 μM.

[2] Cells were sonicated 10 sec at a power setting of 80. Other reaction conditions as described in the legend of Fig. 1 except that 1.3 ml of the reaction mixture was placed in the oxygen electrode chamber for illumination.

[3] Conditions were as described in 1 above for ferricyanide reduction except that 0.6 mM *p*-benzoquinone was substituted for ferricyanide.

Table II. *Photophosphorylation in Various Algal Species using Brief Sonication*

Species[1]	Rate of Phosphorylation	
	PMS-mediated	FeCN-mediated
	μmoles ATP/mg Chl·hr	
Chlamydomonas reinhardi[2]	450	225
Euglena gracilis[3]	160	175
Scenedesmus obliquus[2]	32	0
Chlorella vulgaris[4]	40	4

[1] All sonications were at a power setting of 80; *Euglena* was sonicated 5 sec, *Chlamydomonas* and *Scenedesmus*, 10 sec, and *Chlorella*, 15 sec.

[2] Reaction conditions were as described in the legend of Fig. 1.

[3] Heterotrophically grown cells. Reaction conditions were as given in the legend of Fig. 1 except that the pH was 7.5.

[4] Reaction conditions were as described in the legend of Fig. 1 except that assays contained 2 μmoles of $MgCl_2$.

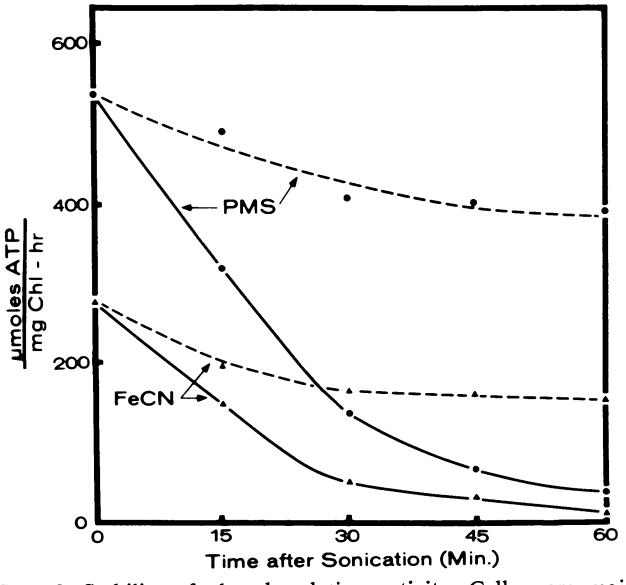

FIG. 8. Stability of phosphorylation activity. Cells were maintained at 25 C (——) or 0 C (– – –) following sonication. Other conditions as described in the legend of Fig. 1.

proximately 80 μmoles ATP/mg Chl·hr and autotrophic cells formed 10 to 15 μmoles ATP/mg Chl·hr. This light-independent phosphorylation was linear for the length of the illumination time and photophosphorylation rates were corrected accordingly.

Although the majority of experiments were performed with either PMS or ferricyanide, it is clear that NADP and *p*-benzoquinone can also mediate phosphorylation in this system (Table I and Fig. 3). Accurate determination of ATP formation directly coupled to NADPH formation was difficult because of the superimposition of significant rates of ferredoxin-mediated cyclic and pseudocyclic electron flow, and corresponding phosphorylation, on NADP reduction. Experiments are currently underway to attempt to resolve this dilemma.

Amines are uncouplers of photophosphorylation in higher plant chloroplasts (11, 14). However, in certain subchloroplast preparations amines do no appear to uncouple (15). The sonicated *Chlamydomonas* system resembles the subchloroplast preparation in its response to methylamine (Fig. 5). The slight inhibition of ATP formation at high methylamine concentration can be attributed to a generalized salt effect in view of the results presented in Figures 3 and 6. In contrast, FCCP markedly inhibits phosphorylation. Polylysine also inhibits phosphorylation; activity is nearly completely inhibited at 130 μg/ml (Fig. 5) while ferricyanide reduction is only slightly over 50% inhibited (unpublished results). However, the uncoupling action of this polyamine is apparently different from that of methylamine since the latter is inert.

The briefly sonicated *Chlamydomonas* system and higher plant chloroplasts show a striking difference in salt effect. The results of Figure 3 indicate that maximum rates of photophosphorylation could be improved with a higher Mg concentration provided the superimposed inhibitory effect above 0.3 mM did not prevail.

From information presently available with three other species of green algae (Table II), it appears that this method has limited application among the green algae, and a correlation between ease of disruption and phosphorylation activity in other algae would be expected.

The results of this and the previous paper (9) demonstrate that *Chlamydomonas* can be conveniently used in a number of the photochemical assays routinely performed with higher plant systems.

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