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Uncouplers Stimulate Photosynthesis in Intact Chloroplasts by Enhancing Light-Activation of Enzymes Regulated by the Ferredoxin-Thioredoxin System

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ABSTRACT

Some uncouplers stimulate CO_2-dependent O_2 evolution by intact spinach chloroplasts at pH 8.6. This effect is not due to alkalinization of the stroma. The stimulation is observed only when photosynthesis has been partly inhibited by the presence of H_2O_2, generated in a Mehler-type reaction by the broken chloroplasts which always contaminate the intact chloroplast preparations. The addition of methyl viologen increases the Mehler-type reaction and results in greater inhibition of photosynthesis. The addition of excess catalase stimulates photosynthesis by preventing accumulation of H_2O_2. The uncouplers stimulate photosynthesis primarily by enhancing the light-activation of enzymes that are regulated by the ferredoxin-thioredoxin system, and this effect results from the influence of the uncouplers on the redox poising of the ferredoxin in the intact chloroplasts.

Photosynthesis in intact chloroplasts is known to be stimulated by the addition of either NH_4^+ or $CH_3NH_3^+$ (8). Two possible explanations for this effect have been offered: first, that NH_4^+, as a weak base, causes the pH of the stroma to rise to the pH optimum for the operation of the reductive pentose phosphate cycle (11, 15, 23); and second, that NH_4^+, as an uncoupler, decreases the ATP:2e ratio so that ATP and NADPH are now produced in the proportions in which they are required for CO_2 assimilation, and in this way NADP reduction is not restricted by a deficiency of ADP (20, 21). An alkalinization of the stroma by NH_4^+ may be of importance when the stroma becomes acidified by the addition of acetate or NO_2^- (11, 18), but it seems unlikely that NH_4^+ stimulates photosynthesis in this way in the absence of these weak acids since it has been shown that $CH_3NH_3^+$ (which is presumed to act exactly like NH_4^+) can stimulate photosynthesis under conditions where it causes no significant change in the pH of the stroma (25).

In the present work we have reinvestigated the mode of action of NH_4Cl in stimulating photosynthesis, as part of a wider study of the ATP:2e ratio in intact chloroplasts during photosynthesis. We find that, under appropriate conditions, FCCP[3] can also stimulate photosynthesis and that both NH_4Cl and FCCP stimulate photosynthesis primarily by enhancing the light-activation of those enzymes that are regulated by the ferredoxin-thioredoxin system (5). The present findings show that the stimulation by NH_4Cl of photosynthesis first is only partly explained in terms of a decreased ATP:2e ratio and, second, can be independent of any alkalinization of the stroma.

MATERIALS AND METHODS

Intact chloroplasts were isolated from leaves of outdoor-grown spinach (*Spinacia oleracea* L.) essentially according to Heldt and Sauer (14). About 50 g of leaves were homogenized with 200 ml of a medium containing 0.33 M sorbitol, 1 mM $MgCl_2$, 20 mM Mes adjusted to pH 6.2 with NaOH. The chloroplast pellet was finally resuspended in a medium containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 50 mM Hepes adjusted to pH 7.6 with NaOH. Chl was estimated according to Arnon (3). O_2 evolution was measured in twin electrodes as described by Allen and Whatley (2). For measurements of photosynthetic O_2 evolution, the reaction medium contained, in a final volume of 3 ml: 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 10 mM $NaHCO_3$, and 50 mM Hepes adjusted to the pH indicated with NaOH. For measurements of catalase activity, 3 mM H_2O_2 was also present. Whenever catalase addition is indicated, it was added to a final concentration of 1,300 U/ml. A unit of catalase activity is defined as the quantity of catalase activity required to liberate O_2 at a rate of 1 μmol/min. The percentage of intact chloroplasts was measured according to Heber and Santarius (12) and it was routinely between 60 and 80%.

For the assays of the light-activated enzymes, chloroplasts were incubated in the O_2 evolution reaction mixture, and aliquots were removed at various times and injected directly into the appropriate enzyme assay mixture (1 ml final volume). Enzyme activity was followed spectrophotometrically at 340 nm at 22 C. Enzyme activity is expressed in $\mu mol \cdot h^{-1} \cdot mg^{-1}$Chl. MDH was assayed in a medium containing: 100 mM Tris-HCl (pH 7.9), 0.15 mM NADPH, and 2 mM oxaloacetate. FBPase was assayed in a medium containing: 100 mM Tris-HCl (pH 7.9 or 8.7 as indicated), 5 mM $MgCl_2$, 2 mM EDTA, 2 mM fructose bisphosphate, 1 mM NADP, 2 U phosphoglucose isomerase, and 1 U glucose 6-P dehydrogenase. Ru-5-P kinase was assayed in a medium containing: 100 mM Tricine-NaOH (pH 8.0), 10 mM $MgCl_2$, 2 mM ATP, 2 mM P-enolpyruvate, 0.15 mM NADH, 6 U pyruvate kinase, and 10 U lactate dehydrogenase. Following the addition of the sample there was an initial fast oxidation of NADH which soon reached a slower linear rate. At this point 1 mM Ru-5-P was added and a new linear rate of NADH oxidation was observed. The activity of the Ru-5-P kinase was determined from the difference between these two rates of NADH oxidation. PGA kinase was assayed in

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[3] Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MDH, NADP-malate dehydrogenase; FBPase, fructose 1,6-bisphosphatase; Ru-5-P, ribulose 5-phosphate; PGA, 3-phosphoglycerate; GAPDH, NADP-glyceraldehyde-3-phosphate dehydrogenase; MV, methyl viologen; SBPase, sedoheptulose 1,7-bisphosphatase.

a medium containing: 100 mM Tricine-NaOH (pH 8.0), 10 mM $MgCl_2$, 5 mM ATP, 2 mM PGA, 0.2 mM NADH, and 1 U glyceraldehyde-3-P dehydrogenase. GAPDH was assayed in a medium containing 100 mM Tricine-NaOH (pH 8.0), 10 mM $MgCl_2$, 5 mM ATP, 2 mM PGA, 0.15 mM NADPH, and 2 U PGA kinase.

All enzymes, biochemicals, and 3-amino-1,2,4-triazole were purchased from Sigma.

RESULTS

The Enhancement by Uncouplers of CO_2-Dependent O_2 Evolution. The optimum pH of the stroma for CO_2-dependent O_2 evolution is around pH 8.1 (25). This pH is attained in isolated intact chloroplasts when they are illuminated in media buffered within the pH range 7.6 to 8.0 (25). The optimum pH of the suspending medium for a particular chloroplast preparation varies with the condition of the chloroplasts: the more "leaky" the chloroplasts the higher the optimum pH of the medium (11, 25). It has been generally assumed (11, 15, 23) that NH_4^+ and $CH_3NH_3^+$ stimulate photosynthesis by increasing the stromal pH to the optimum, although it has been observed (25) that $CH_3NH_3^+$ can stimulate photosynthesis without a significant alkalinization of the stroma. Figure 1A shows that a low concentration of NH_4Cl stimulated photosynthesis when measured in a range of media with pH values which extend both below and above the optimum pH of the stroma. Thus, the stimulation by NH_4Cl cannot be due to an alkalinization of the stroma, inasmuch as it occurs when the pH of the medium exceeds the optimum pH of the stroma. Earlier (8, 15), NH_4^+ had been shown to inhibit photosynthesis when the medium pH was greater than pH 7.8. However, the concentrations of NH_4^+ used in this earlier work were much higher than those used in the present study, and the observed inhibition was probably due to an excessive degree of uncoupling. FCCP was also shown (23, 25) to be inhibitory for CO_2-dependent O_2 evolution when the reaction medium was at pH 7.6, primarily because of an acidification of the stroma (25). However, Figure 1A shows that when the pH of the medium was raised to pH 8.6, FCCP stimulated O_2 evolution. When the medium was set at pH 8.6 (which corresponds to the observed stromal pH of 8.6 [25]) then both NH_4Cl and FCCP stimulated to the same extent. This indicates that it is the shared ability of these reagents to uncouple that is responsible for this stimulation rather than any effect which these reagents might have on the pH gradient across the chloroplast envelope.

A stimulation by FCCP of CO_2-dependent O_2 evolution in intact chloroplasts had been observed previously only under anaerobic conditions suboptimal for photosynthesis (21). Figure 1B shows that a somewhat higher concentration of NH_4Cl was required at pH 7.6 than at 8.6 for the maximum stimulation of O_2 evolution, and that while 4 mM NH_4Cl inhibited at pH 8.6, it stimulated at 7.6. These differences in the sensitivity to NH_4^+ at the two pH values can be attributed to the greater accumulation of NH_3 from the more alkaline medium (8). Figure 1C shows that concentrations of FCCP which are inhibitory at pH 7.6 (23, 25), stimulated O_2 evolution at pH 8.6.

The Relationship Between the Uncoupler-Induced Stimulation of Photosynthesis and the Presence of H_2O_2. It has been shown (6) that the addition of catalase stimulates photosynthesis in isolated chloroplasts. Table I shows that the enhanced O_2 evolution observed in the presence of added catalase was not increased further by an addition of either NH_4Cl or FCCP, which can even become inhibitory in the presence of added catalase (Rosa, unpublished data). Table I also shows that the percentage of stimulation of O_2 evolution observed on addition of catalase increased about 8-fold when the pH of the medium was raised from pH 7.6 to 8.6. Under experimental conditions similar to those of Table I, but with the addition of ferricyanide to act as an electron acceptor for the broken chloroplasts present, the rates of electron transport observed in the absence of uncoupler or of additional catalase were stimulated 3- to 4-fold when the pH of the medium was raised from pH 7.6 to 8.6 (*cf.* 9). Thus, it seems likely that, under the conditions of the experiment shown in Table I, more H_2O_2 would have been produced at pH 8.6 than at 7.6 by the broken chloroplasts present via a Mehler-type reaction. It is probably for this reason that the added catalase gave a greater stimulation at pH 8.6 than at 7.6.

It is generally accepted that catalase is absent from chloroplasts. However, as observed by previous authors for other preparations

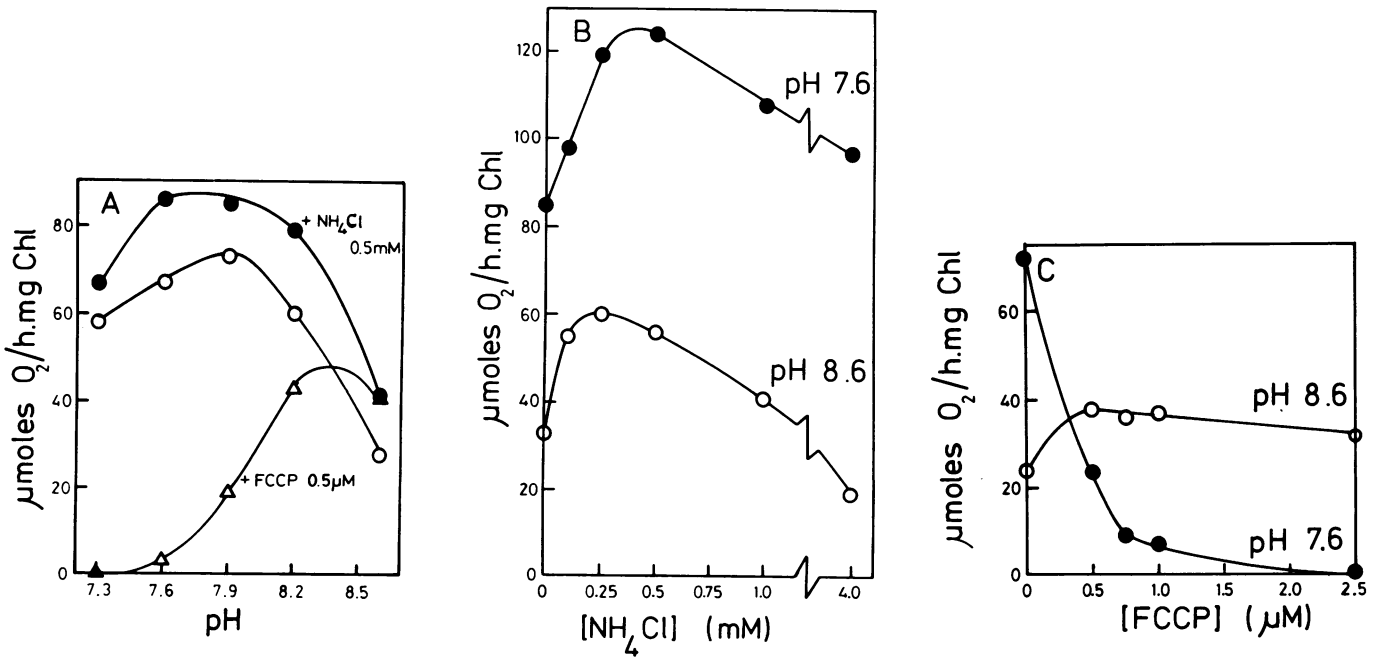

FIG. 1. Effect of NH_4Cl and FCCP on photosynthetic O_2 evolution measured at different pH values. The reaction mixtures were as described under "Materials and Methods." Chl concentrations were 45 μg/ml (A), 33 μg/ml (B), and 27 μg/ml in (C).

Table I. *Effect of Catalase (cat) and Uncouplers on Photosynthetic O_2 Evolution*

The Chl concentration was 15 μg/ml. Chloroplasts were 78% intact. The reaction media were buffered to the pH shown. The rates of O_2 evolution were calculated from the O_2 electrode traces soon after the maximal rates were attained.

Additions	Rate of O_2 Evolution					
	pH 7.6		pH 8.2		pH 8.6	
	− cat	+ cat	− cat	+ cat	− cat	+ cat
	$\mu mol \cdot h^{-1} \cdot mg^{-1} Chl$					
	145	165	85	110	39	89
0.5 mM NH_4Cl	155	166	107	96	54	57
0.5 μM FCCP	6	0	61	18	91	75

Table II. *The Relationship Between the Catalase Activity and Photosynthetic O_2 Evolution in Intact Chloroplasts*

The Chl concentration was 29 μg/ml. Chloroplasts were 73% intact. The reaction medium was at pH 7.6.

Additions	Catalase Activity	Rate of O_2 Evolution
	$U \cdot mg^{-1} Chl$	$\mu mol \cdot h^{-1} \cdot mg^{-1} Chl$
	20	66
0.5 mM NH_4Cl	20	92
50 mM Aminotriazole	2	44
50 mM Aminotriazole + 0.5 mM NH_4Cl	2	75
Catalase	>1,000	90
50 mM Aminotriazole + catalase	105	62
Catalase + 0.5 mM NH_4Cl	>1,000	90

(26), a measurable catalase activity was found to contaminate the preparation of isolated chloroplasts used in the present study (Table II). When this endogenous catalase activity was decreased to 10% of its original activity (Table II) by an addition of the specific catalase inhibitor aminotriazole, the rate of photosynthetic O_2 evolution continued at 70% of its maximum (see also [2]). Table II also shows that the degree of stimulation observed on addition of NH_4Cl was greatest when the catalase activity was least and vice versa. Aminotriazole presumably inhibits O_2 evolution by inhibiting endogenous catalase. This inhibition of O_2 evolution is largely reversed by adding NH_4Cl or catalase, which produce an equivalent stimulation of the control rate. The same reversal is produced by the addition of catalase + NH_4Cl together. The stimulation brought about by NH_4Cl in these experiments depends upon the prior depression of the rate of O_2 evolution by H_2O_2 produced in the medium by contaminating broken chloroplasts.

Since the catalase activity in the chloroplast preparations used in the present study is of extrachloroplastic origin, it may be removed by washing (26). Table III shows that, in direct contrast to the effect produced by decreasing the catalase activity with aminotriazole, decreasing the catalase activity by washing the chloroplasts stimulated O_2 evolution. Addition of either NH_4Cl or of an excess of catalase increased the rate of O_2 evolution of the unwashed chloroplasts to that of the washed chloroplasts, but neither NH_4Cl nor catalase significantly affected the rates of O_2 evolution of the washed chloroplasts. The washing procedure did not appreciably alter the proportion of broken and intact chloroplasts in the preparation (Table III), but washing results in the loss of ferredoxin from broken chloroplasts (4). Since it is reduced ferredoxin which reacts with O_2 to form H_2O_2 it can be inferred that the washed chloroplasts had a lower rate of H_2O_2 production than did the unwashed. This suggestion is supported by the absence of any significant effect on an excess of catalase on the O_2

Table III. *The Effect of Washing the Chloroplasts on the Catalase and Photosynthetic Activities*

Where indicated, the chloroplasts were washed once in the resuspending medium. The Chl concentration was 16 μg/ml. The chloroplasts were not broken by the washing procedure (73% intact before and 76% intact after washing); the reaction medium was at pH 7.6.

Additions	Type of Chloroplasts	Catalase Activity	Rate of O_2 Evolution
		$U \cdot mg^{-1} Chl$	$\mu mol \cdot h^{-1} \cdot mg^{-1} Chl$
	Not washed	20	58
	Washed	11	77
0.5 mM NH_4Cl	Not washed	20	77
	Washed	10	71
Catalase	Not washed	>1,000	78
	Washed	>1,000	75

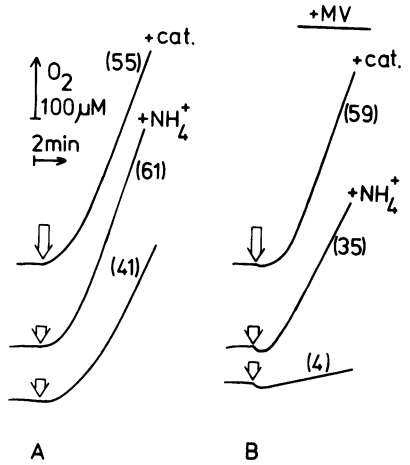

FIG. 2. O_2-electrode traces showing effect of MV on photosynthetic O_2 evolution. Chl concentration was 47 μg/ml. The reaction medium was at pH 7.6. Where indicated, 0.5 mM NH_4Cl was added. The numbers in parentheses refer to the rates of O_2 evolution expressed in $\mu mol \cdot h^{-1} \cdot mg^{-1} Chl$.

evolution of the washed preparations (Table III). Similarly, the absence of any significant effect of NH_4Cl on the O_2 evolution of the washed preparations (Table III) is consistent with our conclusion that stimulation of photosynthetic O_2 evolution by NH_4^+ depends on the presence of H_2O_2.

If this conclusion is correct then an increased rate of H_2O_2 production by the broken chloroplasts should enhance the stimulation of photosynthesis by NH_4^+. The experiment of Figure 2 was carried out to test this prediction. In this experiment, MV was added at a low concentration (0.5 μM) to accelerate the production of H_2O_2 by the broken chloroplasts through a Mehler-type reaction. At this low concentration, MV is unable to enter the intact chloroplasts (1). The production of H_2O_2 in the presence of MV is demonstrated by the O_2 uptake observed immediately after switching on the light (Fig. 2B). This initial O_2 uptake was soon followed by a net O_2 evolution dependent on CO_2 fixation. Inclusion of MV resulted in a considerable inhibition in the rate of O_2 evolution (Fig. 2, bottom traces). Since addition of excess catalase after 4 min of illumination gave a burst of O_2 evolution similar in amount to the O_2 consumed in the initial O_2 uptake (data not shown), we conclude that the MV-dependent O_2 uptake observed soon after the light was switched on did not continue at the same rate during the subsequent net O_2 evolution, and thus

did not result in a significant underestimation of the actual rate of CO_2-dependent O_2 evolution. In the presence of MV, a measurable pool of H_2O_2 was formed initially, which was maintained at a steady-state level during the subsequent O_2 evolution by a balance between its production by the MV-enhanced Mehler-type reaction and its breakdown either by the endogenous, contaminating catalase, or by other peroxidases present in the chloroplasts (10, 27). The low affinity of catalase for H_2O_2 explains both how the small pool of H_2O_2 is maintained and why such a large amount of added catalase is necessary to remove it. The inhibitory effect of MV on O_2 evolution is clearly attributable to the H_2O_2 formed, since MV had no significant effect in the presence of added catalase (Fig. 2, top traces). The increased level of H_2O_2 due to the presence of MV has the predicted result of enhancing the stimulatory effect of NH_4^+. Thus, in the presence of MV, NH_4Cl stimulated O_2 evolution about 9-fold (Fig. 2B). NH_4^+ was able to restore only some 60% of the rate of O_2 evolution observed in the absence of MV.

Figure 2B shows that addition of NH_4^+ did not appreciably affect the concentration of H_2O_2 maintained in the presence of MV, although addition of NH_4^+ did increase the initial rate of O_2 uptake, presumably acting here simply as an uncoupler of electron transport in the broken chloroplasts. In our experiments, we have added MV to increase the H_2O_2 rather than add H_2O_2 directly, since the direct addition of H_2O_2 does not lead to the constant levels of H_2O_2 that are maintained in the presence of MV (about 12 μM in the experiment of Fig. 2). During the preparation of the present paper, a report appeared (19) which showed that H_2O_2 at a concentration of about 10 μM inhibited CO_2-dependent O_2 evolution in intact chloroplasts. This H_2O_2 level was maintained by increasing the proportion of broken chloroplasts present.

The present finding of an H_2O_2-requirement for the stimulation of O_2 evolution on addition of NH_4^+ prompted us to enquire whether there was a similar requirement for the stimulation of O_2 evolution on addition of FCCP observed at pH 8.6 (Table I). Figure 3 shows that FCCP behaved very much like NH_4^+ (Fig. 2) in that the maximum stimulation of O_2 evolution by FCCP was raised from about 70 to about 450% by the addition of MV. Furthermore, as with NH_4^+, FCCP was able to restore only a part of the activity observed in the absence of MV (Fig. 3).

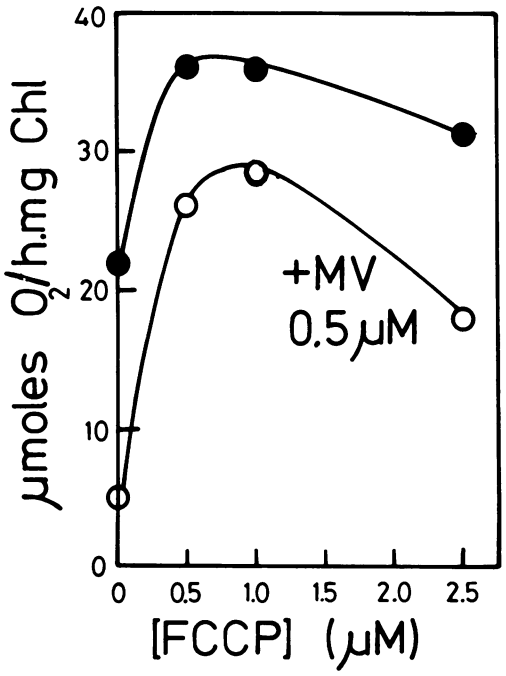

FIG. 3. Effect of FCCP on O_2 evolution in the presence of MV. Conditions were as in Figure 2 except that the reaction medium was at pH 8.6.

H_2O_2 as an Inhibitor of the Light Activation of the Ferredoxin-Thioredoxin Regulated Enzymes. The results of the experiments reported above indicated that the uncouplers NH_4^+ and FCCP stimulated photosynthesis by counteracting the inhibitory effect of H_2O_2. Thus, the site of inhibition of H_2O_2 was sought so as to establish whether uncouplers could remove this inhibition and in this way stimulate photosynthesis. The inhibition by H_2O_2 of photosynthesis in intact chloroplasts has been extensively studied, and a variety of proposals have been made for the site of action of H_2O_2. Thus, the inhibitory effect of H_2O_2 on photosynthesis has been variously ascribed to its action as an oxidant of the SH-groups of enzymes (6), as an inhibitor of PGA reduction (7), or as an inhibitor of the reductive pentose phosphate cycle mostly at the level of the FBPase and SBPase activities (13, 16). This previous work suggested to us that it might well be profitable to explore the possibility that the inhibitory action of H_2O_2 was specifically upon those enzyme that are light-activated via the ferredoxin-thioredoxin system (5).

Table IV shows the results of experiments in which the activities of four of the six enzymes known to be light-activated via the ferredoxin-thioredoxin system (5) have been measured under conditions where the steady-state H_2O_2 level was varied. The rates of O_2 evolution measured during the light-activation are also given, and can be observed to parallel the changes in enzyme activity. Three of the enzymes assayed play a part in the reductive pentose phosphate cycle; the fourth, MHD, does not. With all four enzymes, the light-activation was virtually abolished under conditions where the H_2O_2 level was high (presence of MV), and the light-activation was increased under conditions where the H_2O_2 level was lowered (presence of added catalase). It seems that the light-activation of these enzymes is sensitive to inhibition by H_2O_2. The activities of these light-activated enzymes in the dark were unaffected by the presence of either catalase or MV.

Uncouplers and the Light-Activation of Enzymes. When the effect of uncouplers on the light-activation of these enzymes was investigated, it was found that the light-activation of the enzymes was enhanced by addition of uncouplers, together with a stimulation of O_2 evolution. Table V shows the enhancement of the light-activation of MDH, FBPase, Ru-5-P kinase and GAPDH by the addition of NH_4^+ (either at pH 7.6 or 8.6) and by the addition of FCCP at pH 8.6. In the presence of MV, the relative effectiveness of the uncouplers was enhanced. Thus, in the absence of MV, addition of NH_4^+ resulted in a 60% increase in the light-activated FBPase activity, but in the presence of MV, the increase due to NH_4^+ was some 650%. Of the enzymes tested, FBPase was stimulated to the greatest extent. At pH 7.6, the experiment was repeated with an enzyme, PGA kinase, known not to be light-activated by the ferredoxin-thioredoxin system. Table V shows that this enzyme was affected neither by NH_4^+ nor by MV. It can also be seen from Table V that catalase, like FCCP or NH_4Cl, enhanced the light-activation of all the enzymes examined. Figures 4, A and B show that the concentrations of NH_4Cl required to obtain the maximum enhancement of the light-activated FBPase and MDH activities were similar to those required to obtain the maximum stimulation of O_2 evolution, either in the absence (Fig. 4A) or in the presence (Fig. 4B) of MV. It has been shown previously (17) that FBPase appears in a completely activated form when assayed at pH 8.7 rather than at the physiological pH of 7.9, whether or not the chloroplasts from which it is taken have been illuminated. Figure 4 shows, first that NH_4Cl had no effect on this fully activated FBPase activity (pH 8.7) and, second, that the FBPase activity observed on illumination in the presence of NH_4Cl reaches about 80% of the fully activated activity (*cf.* 17).

DISCUSSION

The first conclusion to be drawn from our work is that under optimal concentrations of HCO_3^-, Pi, and $MgCl_2$, the stimulation

Table IV. *Effect of Catalase and MV on the Light-Activation of Enzymes Regulated by the Ferredoxin-thioredoxin System*

The illuminated reaction mixture was at pH 7.6 in experiments A and B and at pH 8.6 in experiments C and D. In experiment A, the Chl concentration was 83 μg/ml; after 7 min of illumination 0.1 ml samples were taken and assayed for MDH activity. In experiment B, the Chl concentration was 103 μg/ml; after 7 min of illumination 0.1 ml samples were taken and assayed for FBPase activity. In experiment C, the Chl concentration was 53 μg/ml; after 5 min of illumination 15 μl samples were taken and assayed for Ru-5-P kinase activity. In experiment D, the Chl concentration was 65 μg/ml; after 5 min of illumination 50 μl samples were taken and assayed for GAPDH activity.

Conditions	Additions	Activities							
		A		B		C		D	
		O_2	MDH	O_2	FBPase	O_2	Ru-5-P kinase	O_2	GAPDH
		$\mu mol \cdot h^{-1} \cdot mg^{-1} Chl$							
Dark			20		2		430		77
Light		45	28	27	24	46	602	28	137
	Catalase	58	48	50	41	70	645	38	164
	0.5 μM MV	10	21	9	8	15	408	6	86
	0.5 μM MV + Catalase	58	44	51	34	67	602	37	122

Table V. *Effect of NH_4Cl and FCCP on the Light-Activation of Enzymes Regulated by the Ferredoxin-thioredoxin System*

The illuminated reaction mixture was either at pH 7.6 or 8.6 as indicated. In experiment A (Chl concentration 103 μg/ml), 0.1 ml samples were taken after 7 min of illumination. In experiment B (Chl concentration 53 μg/ml), 15 μl samples were taken after 8 min of illumination. In experiment C (Chl concentration 65 μg/ml), 30 μl samples were taken after 7 min of illumination. In experiment D (Chl concentration 82 μg/ml), 0.1 samples were taken after 6 min of illumination. In experiment E (same chloroplast preparation as experiment B), 15 μl samples were taken after 6 min of illumination. In experiment F (Chl concentration 65 μg/ml), 50 μl samples were taken after 5 min of illumination.

Conditions	Additions	Enzyme Activities				
		MDH (A)	FBPase (A)	Ru-5-P kinase (B)	GAPDH (C)	PGA kinase (C)
		$\mu mol \cdot h^{-1} \cdot mg^{-1} Chl$				
pH 7.6 Dark		5	2	408	228	1000
Light		20	37	665	298	1042
	1 mM NH_4Cl	25	58	752	357	1080
	0.5 μM MV	10	8	430	89	1012
	1 mM NH_4Cl + 0.5 μM MV	18	49	624	258	1100
		(D)	(D)	(E)	(F)	
pH 8.6 Dark		6	4	430	77	
Light		19	33	602	137	
	0.5 mM NH_4Cl	23	45	731	149	
	0.5 μM FCCP	24	45	665	125	
	0.5 μM MV	11	15	408	86	
	0.5 μM MV + 0.5 mM NH_4Cl	18	42	624	131	
	0.5 μM MV + 0.5 μM FCCP	19	39	560	100	
	Catalase	20	52	650	164	
	0.5 μM MV + Catalase	19	44	602	122	

of photosynthesis in intact chloroplasts elicited by addition of uncouplers is observed only if H_2O_2 is present. This conclusion is based on the following lines of evidence: (a) the relative stimulation by uncouplers was increased when the normally low level of H_2O_2 was increased by addition either of MV, to enhance a Mehler-type reaction, or of aminotriazole to inhibit the endogenous catalase; and (b) the stimulation by uncouplers was abolished when the H_2O_2 normally present was virtually removed from the system either by the addition of a large excess of catalase, or by inhibiting the Mehler-type reaction of the broken chloroplasts by washing off their ferredoxin.

During the preparation of the present paper a report appeared (22) which confirms some of our present findings. Slovacek and Hind (22) agree with us that NH_4Cl stimulates photosynthesis in intact chloroplasts by restoring the rate of photosynthesis when this rate has been diminished by the presence of H_2O_2. Their work extends to the effects of antimycin and to the effects of limiting factors other than the presence of H_2O_2.

The second conclusion to be drawn from the present work is that H_2O_2 inhibits the light-activation of those enzymes regulated by the ferredoxin-thioredoxin system, and that uncouplers effectively abolish this inhibition. In support of this conclusion, it has

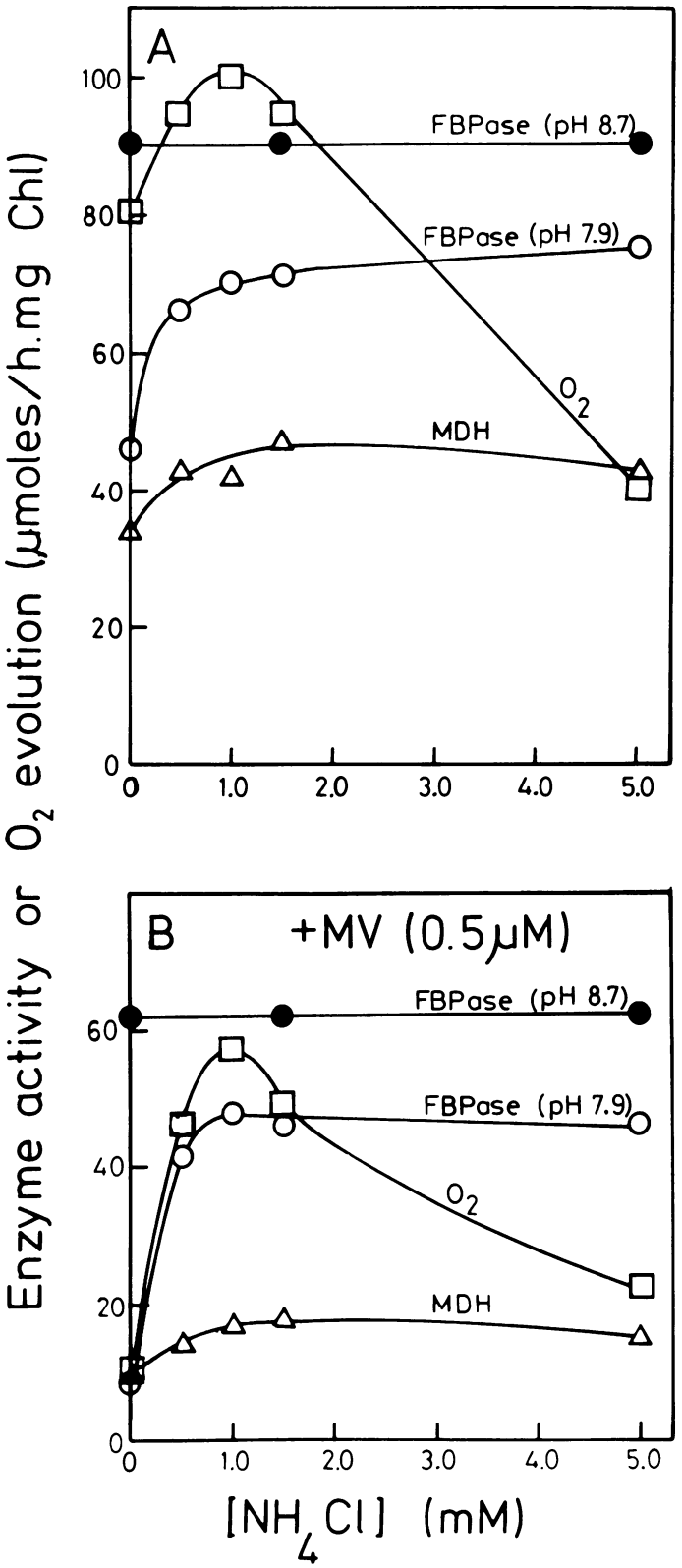

FIG. 4. Effect of NH_4Cl on the light-activation of MDH and FBPase in the absence or presence of MV. The pH of the reaction medium was 7.6. After 7 min of illumination, 0.1 ml samples were taken and assayed for MDH and FBPase activities. FBPase activity was assayed both at pH 7.9 and 8.7. A, Chl concentration was 97 μg/ml. B, Chl concentration was 103 μg/ml. Before the light was switched on the FBPase activity (at pH 7.9) was 5 $\mu mol \cdot h^{-1} \cdot mg^{-1}$Chl (A), and 2 $\mu mol \cdot h^{-1} \cdot mg^{-1}$Chl (B); the MDH activity was 19 $\mu mol \cdot h^{-1}$Chl (A), and 5 $\mu mol \cdot h^{-1} \cdot mg^{-1}$Chl (B).

been observed that when the H_2O_2 level is increased by addition of MV, light-activation is not observed; and conversely, when the H_2O_2 is decreased by the addition of a large excess of catalase, the light-activation is increased. Furthermore, inclusion of either NH_4Cl or of FCCP at the appropriate pH allows the enzymes to become light-activated even in the presence of H_2O_2. The light-activated enzyme activity that was most sensitive to inhibition by H_2O_2 was FBPase. This observation is in agreement with the finding (13, 16) that fructose bisphoshate accumulates when the reductive pentose phosphate cycle of intact chloroplasts is inhibited by the presence of H_2O_2. Our observation of an inhibition by H_2O_2 of the light-activation of GAPDH is also in agreement with the finding (7) that the PGA reduction in a reconstituted system was inhibited by H_2O_2.

There are a number of possible ways in which H_2O_2 could inhibit the enzymes that are light-activated via the ferredoxin-thioredoxin system (5, 6, 10, 13, 16, 27). Whatever the mechanism, it is clear from the present results that such an inhibition could account for the inhibition of photosynthesis by H_2O_2, and that the uncouplers NH_4^+ and FCCP release the H_2O_2-induced inhibition both of the light-activated enzymes and of photosynthesis. Generally, the same conditions are required for uncouplers to stimulate photosynthesis as are required for uncouplers to enhance the light-activation of the enzymes. However, it is of interest to note that even where the uncoupler concentration is high enough to inhibit photosynthesis (presumably, because of an excessively decreased ATP:2e ratio) uncouplers are still effective in counteracting the inhibition by H_2O_2 of light-activation (Fig. 4). Thus, at the higher uncoupler concentrations, an enhancement of enzyme activity is observed even when photosynthesis is inhibited. Hence, the enhancement of enzyme activity cannot result directly from a stimulation of photosynthesis.

A possible explanation of the way in which uncouplers release the H_2O_2-induced inhibition of the enzyme activation and thus stimulate photosynthesis may be offered. When H_2O_2 is added, the resulting inhibition of those enzymes of the reductive pentose phosphate cycle that are light-activated via the ferredoxin-thioredoxin system (particularly FBPase) causes a decreased turnover of the reductive pentose phosphate cycle with a corresponding decrease in the rate at which ATP and NADPH are utilized. This results in a deficiency of ADP, which limits the rate of electron-transport along the noncyclic pathway so that there is insufficient reduced ferredoxin available to activate the enzymes via the ferredoxin-thioredoxin pathway. The primary effect of NH_4^+ or FCCP may, therefore, be to liberate noncyclic electron-transport from the restriction imposed upon it by the lack of ADP, which would result in the ferredoxin becoming more reduced. A related explanation has been offered previously for the observation that antimycin stimulates both FBPase activity and photosynthesis (24). It has been suggested that because antimycin inhibits cyclic electron-transport on the oxidizing side of ferredoxin, it increases the reduction state of ferredoxin and thereby enhances the activity of FBPase. It is well to remember, however, that NH_4^+ uncouples cyclic electron-transport as readily as it uncouples noncyclic. Therefore, under conditions where the availability of ADP limits cyclic electron-transport, NH_4^+ should increase the rate of cyclic electron-transport and should do so by removing a block on the oxidizing side of the ferredoxin. The action of NH_4^+ in this case would be expected to lead to a decrease in the reduction state of ferredoxin. Since we believe that in intact chloroplasts under the conditions of the present experiments the rate of cyclic photophosphorylation is much less than of noncyclic (20), the net effect of the addition of uncoupler will be an increase in the reduction state of the ferredoxin. The explanation offered here for the stimulation of photosynthesis by uncouplers depends primarily upon the ability of uncouplers to increase electron-transport rather

than upon their ability to decrease the ATP:2e ratio *per se* (20–22).

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