BBA 3964

FAT METABOLISM IN HIGHER PLANTS

XX. RELATION OF FATTY ACID SYNTHESIS AND PHOTOPHOSPHORYLATION IN LETTUCE CHLOROPLASTS

P. K. STUMPF*, J. M. BOVÉ AND A. GOFFEAU**

Service de Biochimie, Institut Français de Recherches Fruitières, Paris (France)

(Received October 15th, 1962)

SUMMARY

The incorporation of acetate into chloroplast lipids by illuminated chloroplasts in the presence of CoA, HCO_3^-, Mg^{2+}, and Mn^{2+}, is stimulated by TPN when ADP and orthophosphate are present. *p*-Chlorophenyldimethylurea and NH_4^+, inhibitors of photophosphorylation reactions, also decrease acetate incorporation. The system shows a requirement for orthophosphate. These results are interpreted to mean that acetate incorporation into fatty acids of chloroplasts depends on non-cyclic photophosphorylation which results in the formation of $TPNH_2$, ATP, and O_2. Cyclic photophosphorylation alone by which only ATP is photoproduced was not found to stimulate acetate incorporation.

Acetate incorporation is increased when the reaction is carried out in air instead of nitrogen. The effect is more pronounced in the absence of TPN. These results are explained by the ability of oxygen to catalyze a non-cyclic photophosphorylation, and (or) by the role of oxygen as a cofactor in the synthesis of unsaturated fatty acids. Added $TPNH_2$, ATP and O_2 were not found to stimulate acetate incorporation in the dark.

INTRODUCTION

It has been shown recently by SMIRNOV[1], by STUMPF AND JAMES[2,3], and by MUDD[4] that isolated chloroplasts have the ability of incorporating acetate into fatty acids. Although some incorporation takes place in the dark, there is a dramatic increase in fatty acid synthesis when the chloroplast reaction is allowed to proceed in light.

To account for this light effect, SMIRNOV postulated a light-induced acetylation by which reaction a newly activated form of acetate, namely adenosine diphosphoacetate is formed. STUMPF AND JAMES[2,3], however, suggested that the role of light in their chloroplast system was related to the photoformation of ATP and $TPNH_2$ by the mechanism of photophosphorylation.

Abbreviation: PPNR, photosynthetic pyridine nucleotide reductase.

* Present address: Department of Biochemistry and Biophysics, University of California, Davis, Calif. (U.S.A.).

** Permanent address: Service de Biologie, Euratom, Bruxelles (Belgium).

Both ATP and $TPNH_2$ are required in fatty acid synthesis. Known sites for ATP participation are the formation of acetyl-CoA from acetate and CoA, and the formation of malonyl-CoA from acetyl-CoA and HCO_3^-. $TPNH_2$ would be used in the reduction of β-ketoacyl to β-hydroxyacyl intermediates (acetoacetyl reduction), and of α, β-unsaturated derivatives to saturated compounds (crotonyl reduction). It could also presumably participate in oxidative desaturation of saturated fatty acids.

Chloroplasts have two main functions[5]: (a) photophosphorylation by which ATP and $TPNH_2$ are generated in the light, and (b) utilization of these two compounds for endergonic biochemical reactions in the dark, such as the biosynthesis of carbohydrates. It is now well established that the synthesis of sugars from CO_2 by isolated chloroplasts is coupled to, and depends on, the formation of ATP and $TPNH_2$ in the light by the same chloroplasts[6].

It is tempting to consider fatty acid synthesis in the chloroplasts, which, like carbohydrate synthesis, also requires ATP and $TPNH_2$, as part of those biochemical reactions of chloroplasts which are driven by photosynthetically produced ATP and $TPNH_2$. This paper describes experiments that were carried out to study the dependence of fatty acid synthesis on the photoproduction of ATP and $TPNH_2$ by solated chloroplasts and supplements data published elsewhere[2,3].

METHODS

Preparation of lettuce chloroplasts

200 g of lettuce leaves (*Lactuca sativa capitata*) from a local market were homogenized in a chilled MSE Ato-Mix Waring blendor at full speed for 45 sec with 250 ml of a suspending medium of the following composition: 0.5 M sucrose; 0.1 M NaCl; 0.04 M sodium ascorbate; 0.01 M potassium phosphate buffer (pH 7.2); 0.001 M EDTA. The homogenate was first squeezed by hand through eight layers of cheese-cloth, and the dark-green solution filtrated through ten layers of cheese-cloth. The filtrate was centrifuged at 1000 × *g* for 7 min at 2°. The sedimented whole chloroplasts were resuspended in fresh suspending medium and recentrifuged. The sedimented washed whole chloroplasts were resuspended either in fresh suspending medium or in 10 times diluted suspending medium or in 0.01 M sodium ascorbate (pH 7.2). While whole washed spinach chloroplasts disintegrate when resuspended in either one of these hypotonic solutions, lettuce chloroplasts are more resistant to hypotonic disintegration. When viewed under a phase-contrast microscope, the particles looked intact although they had "blebs" on their surface membrane. They resemble in this respect Chinese cabbage chloroplasts which do not disintegrate either when resuspended in hypotonic solutions or in water[7].

Ferredoxin, "photosynthetic pyridine nucleotide reductase"

PPNR was extracted from spinach leaves by the procedure of HILL AND BENDALL[8]. The fraction used was that eluted from the first DE-50 column, and dialyzed against 0.005 M Tris buffer (pH 8.0).

Analytical methods

Chlorophyll content of the chloroplast preparation was determined by the method of ARNON[9]. ^{32}P was used to measure ATP formation[10]. The reaction was stopped with

trichloroacetic acid (final concentration, 2 %). Unreacted inorganic phosphate was converted to magnesium ammonium phosphate with magnesia mixture, and filtered off. Aliquots of the filtrate were evaporated to dryness on shallow plastic cups, and their radioactivity determined by a Geiger–Müller counter of approx. 5 % efficiency with ^{14}C. $TPNH_2$ was measured spectrophotometrically at 340 mμ on aliquots of the reaction mixture. Oxygen evolution was determined manometrically with a Warburg apparatus.

Acetate incorporation into chloroplast lipids was determined by the procedure of STUMPF AND JAMES[2,3].

Experimental conditions

Reactions were run at 20° in Warburg manometer vessels. Two rows of three 150-W incandescent lamps placed under the glass bottom of the Warburg bath provided an illumination of 75000 lux at the bottom of the vessels. In some experiments, before illumination the vessels were flushed for 3 min with nitrogen gas containing less than $20^0/_{00}$ oxygen.

In order to correlate photophosphorylation and lipid synthesis under the same conditions, ATP formation was determined on one reaction mixture, and acetate incorporation on a second reaction mixture. Both reaction mixtures were identical with the exception that in the lipid series [2-^{14}C]acetate was used, and phosphate was unlabelled; in the phosphorylation series, ^{32}P was used, but acetate was unlabelled.

RESULTS

Acetate incorporation by isolated lettuce chloroplasts

Table I shows that in the absence of CoA, HCO_3^-, and ADP, practically no acetate incorporation into chloroplast lipids occurs. However, with all cofactors

TABLE I

ACETATE INCORPORATION INTO CHLOROPLAST LIPIDS BY ILLUMINATED LETTUCE CHLOROPLASTS

The complete reaction mixture contained in a total volume of 1.5 ml: 0.5 ml of washed chloroplasts resuspended in 0.01 M sodium ascorbate (1 mg of chlorophyll/0.5 ml); 675 μmoles of sucrose; 0.2 μmole of [2-^{14}C]acetate (1 μC; 160000 counts/min); 10 μmoles of $MgCl_2$; 100 μmoles of potassium phosphate buffer (pH 8.0); 30 μmoles of $NaHCO_3$; 0.5 μmole of CoA; 2.5 μmoles of ADP. Gas phase, air; incubation time, 60 min at an illumination of 75000 lux at the bottom of the reaction vessels. Temperature, 20°.

Cofactors	*Acetate incorporation (counts/min)*
Complete	2860
Minus CoA, HCO_3^-, ADP	340

added, acetate incorporation varied from day to day; the lowest values obtained were around 2.3 mμmoles (2000 counts/min) of acetate incorporated into total chloroplast lipids in 60 min with 0.5 mg of chlorophyll, under aerobic conditions; the highest value was 20 mμmoles (16000 counts/min) of acetate incorporated.

Addition of the purified PPNR preparation inhibited acetate incorporation;

fortunately however, since isolated lettuce chloroplasts contain a sufficiently high concentration of PPNR, the addition of purified PPNR was not necessary.

Since Tris buffer decreased acetate incorporations it was replaced by phosphate buffer. Veronal buffers were used for phosphate-free experiments and showed no inhibitory effects.

Photophosphorylation by isolated lettuce chloroplasts

Lettuce chloroplasts are capable of active cyclic and non-cyclic photophosphorylation (Table II).

TABLE II

PHOTOPHOSPHORYLATION BY ILLUMINATED LETTUCE CHLOROPLASTS

Both reaction mixtures contained in a total volume of 3.1 ml: 10 μmoles of $MgCl_2$; 80 μmoles of Tris–HCl buffer (pH 8.0); 10 μmoles of ADP; 10 μmoles of potassium phosphate buffer (pH 8.0); 0.6 ml of a washed chloroplast suspension containing 0.5 mg of chlorophyll, 30 μmoles of sucrose, 0.6 μmole of NaCl, 0.6 μmole of sodium ascorbate, 0.06 μmole of EDTA. The reaction mixture for cyclic photophosphorylation contained 30 μg of *N*-methylphenazonium methosulfate; the one for non-cyclic photophosphorylation contained 4 μmoles of TPN, and 0.2 ml of the PPNR preparation. Incubation in light: 15 min for cyclic photophosphorylation and 18 min for non-cyclic photophosphorylation. Illumination, 75000 lux. Gas phase, nitrogen.

Photophosphorylation	*Cofactor*	*Oxygen evolved (μatoms)*	*$TPNH_2$ formed (μmoles)*	*ATP formed (μmoles)*
Cyclic	*N*-Methylphenazonium methosulfate (30 μg)	0.9		8.0
Non-cyclic	TPN (4 μmoles)	4.0	4.0	2.4

In cyclic photophosphorylation ATP is the sole product of the light reaction. The phosphorylation of ADP to ATP is coupled to a cyclic electron flow. Electrons are expelled by light-excited chlorophyll molecules, and return to the now oxidized chlorophyll molecules via a chain of electron carriers[5]. The natural electron carriers of cofactors of cyclic photophosphorylation are yet unknown, but with isolated chloroplasts several compounds are effective in catalyzing cyclic photophosphorylation; namely vitamin K_3, FMN and *N*-methylphenazonium methosulfate.

Non-cyclic photophosphorylation yields three products, namely $TPNH_2$, ATP and O_2. For every mole of TPN reduced, half a mole of O_2 is evolved, and a maximum of one mole of ATP can be formed. In Table II the stoichiometry between TPN reduction and oxygen evolution is good, but only 2.4 μmoles of ATP are formed instead of a maximum of 4.

Non-cyclic photophosphorylation and acetate incorporation into chloroplast lipids

Table III (anaerobic experiments) shows that the presence of substrate amounts of TPN (4 μmoles) in the presence of CoA, HCO_3^-, ADP and orthophosphate markedly increases acetate incorporation into chloroplast lipids, with the participation of an active non-cyclic photophosphorylation. Catalytic amounts of TPN (0.4 μmole) also stimulate lipid synthesis, but to a lesser extent than substrate amounts (4 μmoles). It is of interest that in earlier experiments with lettuce chloroplasts, STUMPF AND JAMES[2,3] were not able to demonstrate a TPN requirement.

In these experiments added TPN is reduced to $TPNH_2$. Formation of ATP is coupled to this reduction (non-cyclic photophosphorylation). Oxidized TPN as such

is not the active component in the stimulation of lipid synthesis since TPN does not stimulate lipid synthesis in the dark[2,3]. Furthermore, when lipid synthesis by illuminated chloroplasts was tested under conditions where $TPNH_2$ and ATP formation by non-cyclic photophosphorylation was inhibited by 3-(*p*-chlorophenyl)-dimethylurea, TPN does not stimulate lipid synthesis in the presence of 3-(*p*-chlorophenyl)-1,1-dimethylurea (Table IV). Therefore, TPN in its oxidized form does not stimulate acetate incorporation into chloroplast fatty acids. $TPNH_2$ alone does not stimulate incorporation of acetate. Thus, when lipid synthesis by illuminated chloroplasts is tested in the presence of TPN, but in the absence of added ADP, $TPNH_2$, rather than ATP, is formed. Under these conditions acetate incorporation is decreased

TABLE III

EFFECT OF TPN ON ACETATE INCORPORATION INTO CHLOROPLAST LIPIDS

All reaction mixtures contained in a final volume of 1.5 ml: 0.2 μmole of [2-^{14}C]acetate (1 μC, 160000 counts/min); 30 μmoles of CO_3HNa; 0.5 μmole of CoA; 2.5 μmoles of ADP; 10 μmoles of $MgCl_2$; 25 μmoles of potassium phosphate buffer (pH 8.0) in expt. No. 9, and 100 μmoles in expt. No. 8; 0.5 ml of washed chloroplasts resuspended in 0.01 M sodium ascorbate, containing 0.3 mg of chlorophyll in expt. No. 9, and 0.5 mg in expt. No. 8. In expt. 9, for every set of conditions, photophosphorylation and acetate incorporation were determined on two separate reaction mixtures. Both reaction mixtures were identical except for acetate and phosphate. [2-^{14}C]-Acetate and unlabelled phosphate were used in the acetate incorporation series; ^{32}P and unlabelled acetate served in the photophosphorylation series. In expt. 8, ATP formation was not measured; $TPNH_2$ formation was determined directly on aliquots of the reaction mixtures used for acetate incorporation. Incubation at 75000 lux for 60 min.

Expt.	*Gas phase*	*TPN added (μmoles)*	*Non-cyclic $TPNH_2$ formed (μmoles)*	*Photophosphorylated ATP formed (μmoles)*	*Acetate incorporation (counts/min)*
9	N_2	0	0	0.1	550
9	N_2	0.4	0.1	0.4	1120
9	N_2	4.0	3.5	2.3	1900
8	N_2	0	0	*	2240
8	N_2	0.4	0.3	*	3300
8	N_2	4.0	3.5	*	6540
8	Air	0	0	*	3940
8	Air	0.4	0.3	*	4940

* Not measured.

TABLE IV

INHIBITION OF ACETATE INCORPORATION BY 3-(*p*-CHLOROPHENYL)-1,1-DIMETHYLUREA, AN INHIBITOR OF NON-CYCLIC PHOTOPHOSPHORYLATION

See Table III expt. 9 for composition of the reaction mixture. Photophosphorylation and acetate incorporation were determined on separate reaction mixtures. Gas phase, nitrogen. Illumination at 75000 lux for 60 min.

Additions		*ATP formed (μmoles)*	*Acetate incorporation (counts/min)*
3-(p-Chlorophenyl)-1,1-dimethylurea (M)	*TPN (μmoles)*		
—	4	2.4	1900
10^{-4}	4	0.2	540
—	0	0.1	550

50 % (Table V). Endogenous ADP present in the chloroplasts probably prevents lipid synthesis from being completely abolished in the absence of added ADP.

Inorganic phosphate, as well as TPN and ADP, is an important component of the lipid synthesis system. Thus when illuminated chloroplasts were permitted to incorporate acetate under optimum conditions and buffered by 0.1 M Veronal (pH 8.2) for 1 h, 1060 counts were incorporated into fatty acids in the absence of phosphate and 3200 counts were incorporated in the presence of 100 μmoles of phosphate.

Thus, acetate incorporation into chloroplast lipids by illuminated chloroplasts, is stimulated by TPN, provided that photophosphorylation of ADP can occur at the same time as $TPNH_2$ is photoproduced. This view is strengthened by the results to be discussed in the next section.

TABLE V

ADP REQUIREMENT FOR ACETATE INCORPORATION IN THE PRESENCE OF TPN

See Table III, expt. 8, for the composition of the reaction mixture. Gas phase, nitrogen. Illumination at 75000 lux for 60 min.

Additions		*Acetate incorporation (counts/min)*
TPN (μmoles)	*ADP (μmoles)*	
0.4	2.5	3300
0.4	0	1760

TABLE VI

EFFECT OF VITAMIN K_3 AND *N*-METHYLPHENAZONIUM METHOSULFATE, COFACTORS OF CYCLIC PHOTOPHOSPHORYLATION, ON ACETATE INCORPORATION INTO CHLOROPLAST LIPIDS

All reaction mixtures contained in a final volume of 1.5 ml: 0.2 μmole of [2-^{14}C]acetate (1 μC, 160000 counts/min); 30 μmoles of CO_3HNa; 0.5 μmole of CoA; 2.5 μmoles of ADP; 10 μmoles of $MgCl_2$; 100 μmoles of potassium phosphate buffer (pH 8.0); 675 μmoles of sucrose, only in expt. 6: 0.5 ml of washed chloroplasts resuspended in 0.01 M sodium ascorbate, containing 1.0 mg of chlorophyll in expts. 6 and 7, and 0.5 mg in expt. 8. No TPN was present. Illumination at 75000 lux for 60 min.

Expt.	*Gas phase*	*Cofactors added*	*Acetate incorporation (counts/min)*
7	N_2	None	1120
7	N_2	Vitamin K_3 (0.3 μmole)	1220
8	N_2	None	2240
8	N_2	Vitamin K_3 (0.015 μmole)	2260
6	Air	None	2860
6	Air	*N*-Methylphenazonium methosulfate (30 μg)	2160
7	N_2	None	1120
7	N_2	*N*-Methylphenazonium methosulfate (30 μg)	1000
7	Air	None	2060
7	Air	*N*-Methylphenazonium methosulfate (30 μg)	1900
8	N_2	None	2240
8	N_2	*N*-Methylphenazonium methosulfate (30 μg)	2220

Cyclic photophosphorylation and acetate incorporation into chloroplast lipids

Table VI shows that vitamin K_3 or *N*-methylphenazonium methosulfate, cofactors for cyclic photophosphorylation, at their optimal concentration for photophosphorylation (or at a lower concentration) do not stimulate lipid synthesis by illuminated chloroplasts. Although the presence of these cofactors permits the formation of a sufficient supply of ATP, ATP alone cannot stimulate lipid synthesis. In the presence of 30 μg of *N*-methylphenazonium methosulfate, conditions that favour a very efficient cyclic photophosphorylation, there is even a decrease in acetate incorporation, consistent with the finding that rather high concentrations of ATP inhibit fatty acid synthesis[3].

When acetate incorporation into chloroplast lipids occurs in the presence of catalytic amounts of TPN (0.4 μmole), only 0.4 μmole of ATP is formed (Table III). In this case ATP could be limiting. Cyclic photophosphorylation could therefore assist in overcoming this ATP deficit. Indeed, if micro-catalytic amounts of *N*-methylphenazonium methosulfate are added to this system, a slight but consistent increase in acetate incorporation is observed (Table VII). The amounts of *N*-methylphenazonium methosulfate used here are micro-catalytic so that they do not suppress appreciably non-cyclic photophosphorylation, *i.e.*, $TPNH_2$ formation[6].

TABLE VII

EFFECT OF *N*-METHYLPHENAZONIUM METHOSULFATE, COFACTOR OF CYCLIC PHOTOPHOSPHORYLATION, ON ACETATE INCORPORATION IN THE PRESENCE AND IN THE ABSENCE OF TPN

See Table VI for details of reaction mixture. 450 moles of sucrose were present in expt. 5; 0.5 ml of washed chloroplasts resuspended in 0.01 M sodium ascorbate contained 1.0 mg of chlorophyll in expt. 5 and 0.5 mg in expt. 8.

Expt.	*N-Methylphenazonium methosulfate added (μg)*	*Acetate incorporation (counts/min)*	
		−TPN	*+TPN (0.4 μmole)*
8	0	2240	3300
8	0.3	2220	3980
5	0		4700
5	0.3		5660

Acetate incorporation into chloroplast lipids in air or nitrogen

In the absence of photophosphorylation cofactors, acetate incorporation into chloroplast lipids is stimulated when the reaction is carried out in air instead of nitrogen (Table VIII), but the relative amount of stimulation is decreased when the reaction is allowed to proceed in the presence of substrate amounts of TPN (4 μmoles) or of catalytic amounts of TPN (0.4 μmole) plus micro-catalytic amounts of *N*-methylphenazonium methosulfate (0.3 μg).

In air, and in the absence of added photophosphorylation cofactors, acetate incorporation is decreased by inhibitors of photophosphorylation. Table IX shows that 3-(*p*-chlorophenyl)-1,1-dimethylurea at 10^{-4} M, concentration at which it inhibits non-cyclic photophosphorylation, also inhibits acetate incorporation. NH_4^+ at 10^{-3}M uncouples ATP formation from electron flow; it also lowers lipid synthesis. DNP at 10^{-4} M does not inhibit photophosphorylation nor lipid synthesis; at 10^{-3} M it inhibits both.

Acetate incorporation experiments in the dark with $TPNH_2$ and ATP produced in a preliminary light reaction

The preceding results indicate that lipid synthesis by illuminated chloroplasts is stimulated by TPN in the presence of ADP and orthophosphate, with non-cyclic photophosphorylation as the generating system in both $TPNH_2$ and ATP. It was therefore of interest to attempt to resolve the lipid synthesis and photophosphorylation system by first permitting photophosphorylation to synthesize ATP and $TPNH_2$ in the light and then determining the effect of subsequent light and dark periods in acetate incorporation. In the following experiments, first $TPNH_2$ and ATP were formed in the light; after 15 min of illumination, CoA was tipped into the reaction mixtures, and acetate incorporation was allowed to proceed either in light or in dark for 60 min. Table X demonstrates that substrate amounts of ATP and $TPNH_2$ which are first formed in light, do not stimulate acetate incorporation in the subsequent dark period. Sufficient amounts of $TPNH_2$ and ATP are formed to supply the needs of the lipid-synthesizing system. The formation of $TPNH_2$ has been measured directly on the reaction mixtures. ATP production has not been determined in these experiments; it has always been found that the reduction of 4 μmoles of TPN is accompanied invariably by the esterification of at least 2 μmoles of ADP.

TABLE VIII

ACETATE INCORPORATION INTO CHLOROPLAST LIPIDS IN AIR OR NITROGEN

See Table VI for details of reaction mixture. Illumination at 75000 lux for 60 min.

Expt.	*Gas phase*	*TPN added (μmoles)*	*N-Methylphenazonium methosulfate added (μg)*	*Acetate incorporation*	
				(counts/min)	*(%)*
6	N_2			580	100
6	Air			2860	500
7	N_2			1120	100
7	Air			2060	180
8	N_2			2240	100
8	Air			3940	180
8	N_2	0.4		3330	100
8	Air	0.4		4940	150
7	N_2	0.4	0.3	2480	100
7	Air	0.4	0.3	3000	120

TABLE IX

EFFECT OF INHIBITORS ON ACETATE INCORPORATION IN AIR

Expt. 7. See Table VI for details of reaction mixture. No TPN was present. Gas phase, air. Illumination at 75000 lux for 60 min.

Inhibitor	*Final concentration (M)*	*Acetate incorporation (counts/min)*
None		2220
3-(*p*-Chlorophenyl)-1,1-dimethylurea	10^{-4}	1120
NH_4^+	10^{-3}	1200
DNP	10^{-4}	2080
DNP	10^{-3}	1280

TABLE X

ACETATE INCORPORATION IN THE DARK WITH $TPNH_2$ AND ATP FORMED IN A PRELIMINARY LIGHT REACTION

Expt. 7. See Table VI for details of reaction mixture. CoA was tipped into the reaction mixture at the end of the 15-min preliminary light reaction. To keep certain vessels dark, they were wrapped with aluminium foil and returned into the illuminated Warburg bath.

Conditions	*Gas phase*	*$TPNH_2$* (μmoles)*	*Acetate incorporation (counts/min)*
15 min light 60 min dark	N_2	2.9	440
15 min light 60 min light	N_2	3.3	2340
15 min light 60 min dark	Air	2.1	740
15 min light 60 min light	Air	3.6	3260

* Measured at the end of the incubation period; TPN added, 4 μmoles.

DISCUSSION

The large stimulation of acetate incorporation into chloroplast lipids, observed when TPN, ADP and orthophosphate are all added to the reaction mixture in an atmosphere of nitrogen and in the light, is best explained by the coupling of lipid synthesis to non-cyclic photophosphorylation. In this system, TPN plays a double role: (a) it acts as the terminal electron acceptor in non-cyclic photophosphorylation; as such it permits photophosphorylation to occur and ATP to be produced; acetate activation by ATP in the presence of CoA may now occur; and (b) concomitantly $TPNH_2$ is formed and becomes available for the reductive steps necessary in fatty acid synthesis. This interpretation is strengthened by the following results: (1) TPN does not stimulate in the absence of added ADP; (2) when non-cyclic photophosphorylation is inhibited by 3-(*p*-chlorophenyl)-1,1-dimethylurea, acetate incorporation is decreased; (3) orthophosphate is required for acetate incorporation; (4) cyclic photophosphorylation, resulting in the formation of only ATP, does not stimulate fatty acid synthesis. The same interpretation applies also to the stimulation of acetate incorporation by air. The oxygen of air can act as a terminal electron acceptor and catalyze an ATP formation by non-cyclic photophosphorylation[11]. In the absence of added cofactors of photophosphorylation, oxygen could thus stimulate lipid synthesis by permitting enough ATP to be formed. In the absence of added TPN, but with O_2 as the terminal electron acceptor, the only $TPNH_2$ available occurs by the reduction of endogenous TPN present in chloroplasts[12]. Fatty acid synthesis would have to be tightly coupled to the reduction of the bound TPN, in order to make full use of the small amounts of $TPNH_2$ available. The reduction of such small amounts of TPN still leaves many electrons available for the photoreduction of O_2, and the coupled formation of ATP. This interpretation is in agreement with the known properties of ferredoxin (PPNR), the electron carrier that mediates the electron trans-

port between chlorophyll and the final electron acceptor, TPN or O_2 (see ref. 15). Reduced ferredoxin is very rapidly oxidized by O_2, but in the presence of TPN and with the aid of chloroplasts, it will preferentially donate its electron to TPN. Only when TPN has been reduced, O_2 can become the electron acceptor. Once formed, $TPNH_2$ is not reoxidized by oxygen, and can thus be used for lipid synthesis. The oxygen-catalyzed photoproduction of ATP is stimulated by ascorbate[13]; ascorbate was present in all the experiments reported here. Thus, when acetate incorporation occurs in air, two types of non-cyclic photophosphorylation can function, the first with bound TPN and the second with O_2 as the terminal electron acceptor. The first type would supply mainly $TPNH_2$ and some ATP, the second would furnish additional ATP. The observed decrease of acetate incorporation with 3-(*p*-chlorophenyl)-1,1-dimethylurea or NH_4^+, in the absence of added TPN but in the presence of air, could of course result from the inhibition of either one or both of these two types of non-cyclic photophosphorylation. Cyclic photophosphorylation could also supply supplemental ATP and would thus be expected to stimulate acetate incorporation in the absence of added TPN. No such stimulation has been observed probably because under those conditions, cofactors of cyclic photophosphorylation even in micro-catalytic amounts deviate non-cyclic electron flow into a cyclic flow, and thus abolish the reduction of the bound TPN. However, in nitrogen micro-catalytic amounts of *N*-methylphenazonium methosulfate (0.3 μg) do stimulate acetate incorporation slightly in the presence of small amounts of TPN (0.4 μmole), suboptimal for fatty acid synthesis. In this case non-cyclic photophosphorylation is not abolished, and yields $TPNH_2$ plus some ATP. *N*-Methylphenazonium methosulfate-stimulated cyclic photophosphorylation supplies more ATP.

Although the effect of air on acetate incorporation is especially significant in the absence of added ATP, there is a stimulation in the presence of TPN. This points towards a second role of oxygen in lipid synthesis by illuminated chloroplasts. In addition stimulating indirectly lipid synthesis by catalyzing a photoproduction of ATP, oxygen could also be a direct cofactor in the synthesis of unsaturated fatty acids. In air, illuminated chloroplasts incorporate acetate into unsaturated acids (palmitoleic, 2.1 %; oleic, 42.5 %) and into saturated acids (palmitic, 48.5 %; stearic, 3.5 %; myristic, 3.3 %)[2] but in nitrogen only saturated fatty acids are formed[3]. It is relevant to note that blue-green algae synthesize the unsaturated palmitoleic and oleic acids predominantly by oxidative desaturation of stearic and palmitic acids, respectively[14]. Both $TPNH_2$ and oxygen are required in oxidative desaturation. Thus the synthesis of unsaturated fatty acids would first require $TPNH_2$ and ATP for the formation of saturated acids, and then O_2 and $TPNH_2$ for the desaturation of these acids.

Since acetate incorporation appears to be coupled to non-cyclic photophosphorylation, it could be concluded that the role of light in fatty acid synthesis is related to the formation of $TPNH_2$, ATP and eventually O_2. Thus fatty acid synthesis in the dark should become as high as in the light if $TPNH_2$, ATP and O_2 are supplied to the reaction mixture. The few experiments carried out to check this hypothesis (Table X) did not yield the expected results. Acetate incorporation into chloroplast lipids in the dark was low even though $TPNH_2$, ATP and O_2 were present. Also the necessity of rather high amounts of TPN (4 μmoles) to obtain a marked stimulation of acetate incorporation with a continuously illuminated chloroplast system, is not

entirely consistent with the above hypothesis. Since in such a system, $TPNH_2$ can continuously be regenerated, catalytic amounts of TPN should be sufficient for the total incorporation of the relatively small quantities of acetate (0.2 μmole) used throughout this work. These results could imply that in fatty acid synthesis by illuminated chloroplasts, light has another role, besides promoting the formation of $TPNH_2$, ATP and O_2 by non-cyclic photophosphorylation. It appears unlikely that photosynthetic acetylation as described by SMIRNOV[1] could represent a second contribution of light to fatty acid synthesis in our system, for the following reasons. With our system a definite requirement of orthophosphate for acetate incorporation is shown. SMIRNOV did not observe a phosphate requirement with his system. In our case, acetate incorporation is inhibited by inhibitors of photophosphorylation; in their presence, photosynthetic acetylation should still continue, and overcome the inhibition of photosynthetic phosphorylation, unless photosynthetic acetylation is inhibited by the same inhibitors as photophosphorylation.

The ineffectiveness of exogenous ATP and $TPNH_2$ to promote acetate incorporation into chloroplast fatty acids in the dark, could also mean that non-cyclic photophosphorylation and fatty acid synthesis are very tightly coupled. Fatty acid synthesis would have to occur concomitantly with photophosphorylation. Only when TPN is being reduced and ADP phosphorylated would acetate be incorporated.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the National Science Foundation (G-14823).

One of the authors (A.G.) is a John Simon Guggenheim Memorial Fellow 1962.

REFERENCES

1 B. P. SMIRNOV, *Biokhimija*, 25 (1960) 545.
2 P. K. STUMPF AND A. T. JAMES, *Biochim. Biophys. Acta*, 57 (1962) 400.
3 P. K. STUMPF AND A. T. JAMES, *Biochim. Biophys. Acta*, in the press.
4 J. B. MUDD AND T. T. MCMANUS, *J. Biol. Chem.*, 237 (1962) 2057.
5 D. I. ARNON, *Bull. Torrey Botan. Club*, 88 (1961) 215.
6 A. V. TREBST, M. LOSADA AND D. I. ARNON, *J. Biol. Chem.*, 234 (1959) 3055.
7 A. GOFFEAU AND J. M. BOVÉ, in preparation.
8 R. HILL AND F. BENDALL, *Nature*, 187 (1960) 417.
9 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
10 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *J. Am. Chem. Soc.*, 76 (1954) 6324.
11 D. I. ARNON, M. LOSADA, F. R. WHATLEY, H. Y. TSUJIMOTO, D. O. HALL AND A. A. HORTON, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1314.
12 A. SAN PIETRO, *Proc. 5th Intern. Congr. Biochem. Moscow*, 1961, Pergamon Press, Oxford, in the press.
13 G. FORTI AND A. T. JAGENDORF, *Biochim. Biophys. Acta*, 54 (1961) 322.
14 K. BLOCH, P. BARONOWSKY, H. GOLDFINE, W. J. LENNARZ, R. LIGHT, A. T. NORRIS AND G. SCHEUERBRANDT, *Federation Proc.*, 20 (1961) 921.
15 K. TAGAWA AND D. I. ARNON, *Nature*, 195 (1962) 534.