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THESE

INFLUENCE DE L'ACIDE LINOLENIQUE EXOGENE SUR LA PHOTOSYNTHESE DE CHLOROPLASTES INTACTS D'EPINARD

Présentée à la Faculté des Sciences de l'Université de Neuchâtel (Suisse) pour obtenir le grade de Docteur ès Sciences, option Physiologie Végétale

par

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IMPRIMATUR POUR LA THÈSE

Influence de l'acide linolénique exogène sur
la photosynthèse des chloroplastes intacts
de feuilles d'épinard

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INFLUENCE OF LINOLENIC ACID ON PHOTOSYNTHESIS IN INTACT SPINACH CHLOROPLASTS

Aging of isolated chloroplast lamellae is accompanied by a release of free fatty acids (FFA), especially linolenate ($C_{18:3}$) due to a hydrolysis of membrane lipids. It has been shown that both aging _in vitro_ and exogenous FFA have similar effects on the structure and the main photochemical reactions of thylakoid membrane, namely an inhibition of electron transports and their associated photophosphorylations. In order to elucidate the role played by FFA on photosynthesis, it was important to investigate the action of these compounds not only on thylakoid membranes, but also on intact chloroplasts, which represent a higher level of structural and functional organization: (1) linolenate added to intact chloroplasts inhibits O_2 evolution at concentrations which inhibit neither photoreduction of $NADP^+$ and photophosphorylation, nor the activities of the soluble enzymes of the stroma; this indicates that the inhibition of O_2 evolution by FFA is not related to photochemical reactions; (2) concentrations of Pi and pyrophosphate restore partially O_2 evolution; in the presence of $C_{18:3}$ this activity is optimal for a Pi concentration which is lower than that in the control, i.e. 0.2 instead of 0.5 mM; this is very likely because of a change in the outer envelope permeability; (3) the absorption of Pi by illuminated intact chloroplasts is inhibited by linolenate; knowing that the transport of Pi (inside) and trioses-P (outside) are carried out by the same "translocator" across the outer envelope, we assumed that $C_{18:3}$ may also inhibit the exit of internal metabolites; (4) this hypothesis has been indirectly verified with intact chloroplasts from preilluminated leaves which contain a high pool of metabolites; if $C_{18:3}$ inhibits Pi and trioses-P translocation, one expects that an increase of the internal concentration of triose-P would promote a greater inhibition of O_2 evolution by excess of metabolites in chloroplasts from preilluminated leaves than in those from dark leaves. Indeed, the former chloroplasts are more sensitive to $C_{18:3}$ than the latter; (5) this is also corroborated by the fact that chloroplasts from non preilluminated leaves loaded with exogenous P-glyceric acid have the same behaviour toward $C_{18:3}$ than chloroplasts from preilluminated leaves.

In conclusion, linolenate inhibits O_2 evolution in intact chloroplasts by modifying the structural and functional integrity of the outer envelopes in such a way that the influx of Pi and the efflux of trioses-P are disturbed.

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DETERIORATION PROCESSES IN CHLOROPLASTS : CONTROL OF STRUCTURE AND FUNCTIONS BY UNSATURATED FATTY ACIDS

Deterioration (e.g. aging) of isolated spinach chloroplast leads to an alteration of the structure and functions of thylakoid membranes. These observations were attributed to a release of free fatty acids (FFA), especially linolenate ($C_{18:3}$), following hydrolysis of galactolipids during aging in vitro. This is of great interest since linolenate is the major fatty acid in thylakoid lipids and, depending on the concentration of its free form, seems to control several reactions in photosynthesis.

The similarity between the action of aging and FFA has been verified in many ways : (1) electron micrographs show a marked swelling and disruption of membrane stacking; (2) absorbance and packed volume changes reveal an increase in thylakoid volume, and light scattering indicates an inhibition of light-induced chloroplast shrinkage. These observations indicate that the physicochemical properties of the thylakoid membrane are impaired; (3) this is also corroborated by the inhibition of the pH difference generated across the thylakoid membrane upon illumination (ΔpH) and the concomitant decrease in the proton pump activity and increase in the internal pH (pHi); (4) both aging and an addition of $C_{18:3}$ cause an acid shift of the pH optima for electron flows through both PSs or through either PS alone, this phenomenon being directly correlated with the integrity of the thylakoid membrane and its diminishing ability to create a ΔpH; the electron transports measured in photosystem (PS) II and I and their associated photophosphorylations are inhibited in a sequential fashion showing that electron flow in PS II is much more sensitive than that in PS I; this has been verified with membrane fractions enriched in PS II and PS I activities. The inhibition site of FFA lies on the oxidizing side of PS II, probably between the hypothetical carriers Y_2 and Y_3 and Mn^{2+} ions restore the activity by allowing a shunt between Y_1 and Y_3 ($H_2O \rightarrow Y_1 \rightarrow Y_2 \rightarrow Y_3 \rightarrow$ PS II $\rightarrow$ DCPIP); (5) the activity of o-diphenol oxidase, which seems to be a membrane bound enzyme, is increased by both treatments; (6) in intact chloroplasts, O_2 evolution and phosphate uptake are inhibited indicating the possible action of $C_{18:3}$ on the transport of metabolites across the chloroplast envelope.

In conclusion, the effects of aging and FFA stem from the same mechanism which involves disorganization of the membrane structure with all its consequences on chloroplast functions.

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Effect of linolenate on photosynthesis by intact spinach chloroplasts I.
Inhibition of orthophosphate uptake and of 3-P-glyceraldehyde efflux across the chloroplast envelope

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Linolenic acid ($C_{18:3}$) inhibited photosynthesis by intact spinach chloroplasts. This inhibition was due neither to a lack of NADPH in chloroplasts nor to a direct inhibition of the enzyme activities in the Calvin-Benson cycle. Linolenate inhibited CO_2 fixation and oxygen evolution more effectively than $NADP^+$ photoreduction but did not inhibit the activity of several key enzymes of the Calvin cycle. Linolenate inhibited phosphate influx and 3-phosphoglyceraldehyde efflux across the chloroplast envelope. A hypothesis explaining the inhibition of photosynthesis by linolenate is presented.

Key words: Chloroplasts — Linolenic acid treatment — 3-P-glyceraldehyde efflux — Phosphate uptake — Photosynthesis — Spinach — Transport.

The release of fatty acids during aging processes in vitro has been demonstrated in several cells and organelles such as human erythrocytes (*30*), mitochondria (*10*) and chloroplasts (*8*, *16*, *22*, *39*, *41*). In chloroplasts, the main fatty acids released in the course of aging are C_{18}-unsaturated fatty acids (*8*, *16*, *39*), particularly linolenic acid; these acids are the most abundant in the thylakoid (*8*, *39*) and envelope (*21*) membrane lipids. So far, the action of endogenous (*31*, *38*, *39*) and exogenous (*26*, *35–38*) fatty acids has been mainly studied in relation to the photochemical reactions of the thylakoid. It has been shown that such unsaturated fatty acids inhibit electron flow in photosystems II and I and the associated photophosphorylation (*35*, *37*), light-induced shrinkage in chloroplasts (*35*), and the light-generated ΔpH across thyalkoid membranes in a sequential fashion (*37*). In addition, fatty acids modify the physicochemical properties of the thylakoid membrane in such a way that swelling occurs (*27*, *34*) and that the ΔpH is diminished and pHi increased, inducing a shift of the pH optimum of electron flow towards acidity (*37–39*).

In order to elucidate further the control of photosynthetic activities by these fatty acids, we have now studied the action of linolenate on the photosynthetic apparatus at a higher level of structural integrity. The initial point of interest was: Would free fatty acids influence photosynthetic carbon metabolism in intact chloro-

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[1] This work is part of a doctoral program which is carried out by L. Mvé Akamba in this laboratory.

plasts? Using short chain fatty acids, such as lipoic acid, octanoic acid and methyl octanoate, Pedersen et al. (*20*) have shown with *Chlorella pyrenoidosa* that these acids inhibit photosynthesis by blocking the carboxylation and bisphosphatase reactions and by decreasing the level of ATP. However, fatty acids paradoxically had no effect on ribulose 1,5-bisphosphate-carboxylase. These authors used fatty acids (300–600 μM) which are not representative in chloroplast membrane lipids. At these concentrations, linolenate was shown to inhibit completely all photochemical reactions (*26*, *35–39*).

In view of this, we have investigated the action of linolenic acid on photosynthesis in intact chloroplasts. Our results indicate that linolenic acid inhibits light-dependent oxygen evolution by impairing the exchange of metabolites across the chloroplast envelope. Part of this work has been presented recently (*25*).

Materials and methods

Intact chloroplasts from spinach (*Spinacia oleracea* var. Nobel) were isolated according to Kalberer et al. (*17*) in an isolation medium consisting of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) adjusted to pH 7.6 with NaOH, 330 mM sorbitol, 2 mM EDTA-Na (pH 7.6) and 0.2 mM Na-isoascorbate. Rinsed leaves were placed on distilled water and preilluminated for about 60 min at 4°C with a tungsten 150 w lamp (approx. 2300 lux). About 80 g of leaves, with midribs removed, were then homogenized in a Waring blender for about 5 sec with 100 ml of the isolation medium and filtered through 8 layers of cheesecloth. The filtrate was immediately centrifuged at $2500 \times g$ for 10 sec (excluding acceleration and braking time, using Sorvall RC 2B and rotor SS-34). The supernatant was poured off, and the pellet was resuspended in the same medium. All operations were carried out at 4°C. The chlorophyll content was determined by the method of Bruinsma (*3*). The chloroplast suspension, adjusted to 1.5 mg chlorophyll per ml, was kept at 0–4°C until use. The suspension contained about 70–80% of intact chloroplasts (*13*).

Photosynthetic oxygen evolution was measured with a Clark type oxygen electrode at 20°C in a 5 ml cuvette containing the following reaction mixture: 350 mM sorbitol, 50 mM HEPES-NaOH (pH 7.6), 0.2 mM Na-isoascorbate, 6.6 mM $KHCO_3$, chloroplasts usually at 100 μg chlorophyll/ml and, where indicated, Na-pyrophosphate (pH 7.6), K-phosphate (pH 7.6) and linolenic acid dissolved in ethanol. All reaction mixtures contained less than 0.4% ethanol. The reaction was initiated by white light at an intensity of about 5×10^6 ergs·cm^{-2}·sec^{-1} and monitored with a recorder.

CO_2 fixation was assayed in 5 ml flasks containing 1.5 ml of the same reaction mixture as that for O_2 evolution. The vessels were flushed with N_2 for 4 min and then mounted on an agitator which imparted a swirling motion to the chloroplast suspension. The reaction mixture was preilluminated at about 10^6 ergs·cm^{-2}·sec^{-1} for 5 min before the addition of 6 mM $NaH^{14}CO_3$ (specific radioactivity 0.1 μCi/μmole). After 6 min illumination, 0.1 ml 2 N HCl was added and the mixture was centrifuged. Aliquots of the supernatant were dried on glass wool fiber (*9*) and counted in a Chicago Scintillation counter.

The initial rate of electron transport from H_2O to $NADP^+$ was measured spectrophotometrically in 0.2 mm (0.5 ml) cuvettes by recording the absorbance change at 340 nm in a Zeiss spectrophotometer, modified as described earlier (*24*, *36*). The actinic light (1000 w iodine lamp) was passed through two interference filters (Balzer's Calflex C and Balzer's wide band DT-red) and the intensity at the cuvette was 1.15×10^4 ergs·cm^{-2}·sec^{-1}. The reaction mixture contained 50 mM Tricine (pH 8.4) 35 mM NaCl, 5 mM $MgCl_2$, 0.5 mM K_2HPO_4, 2 mM ADP, 2 mM $NADP^+$, ferredoxin in excess, intact chloroplasts (100 μg chlorophyll/ml) which were broken in this hypotonic medium, and linolenic acid at various concentrations. Ferredoxin was prepared from spinach leaves according to Buchanan and Arnon (*4*).

The uptake of orthophosphate (Pi) by intact chloroplasts (from non-preilluminated leaves) was assayed in a 3 ml O_2 electrode cuvette containing the same reaction mixture as for O_2 evolution, except that pyrophosphate (PPi) and sorbitol were replaced by 0.7 mM Pi, and 350 mM sucrose, respectively. Other conditions were identical to those used for the assay of O_2 evolution. At the beginning and end of the light-reaction (10 min), aliquots of the chloroplast suspension were centrifuged and the Pi concentrations in the supernatants were estimated according to Fiske and Subbarow (*11*).

For the pyrophosphatase assay, a crude enzyme extract was prepared according to Schwenn et al. (*32*) and dialyzed against 50 mM Tris-HCl and 1 mM $MgCl_2$ at pH 8.0. The extract was kept frozen. Pyrophosphatase was assayed as described earlier (*32*) and Pi estimated according to Chen et al. (*6*). Ribulose 5-P-isomerase, ribulose 5-P-kinase and ribulose 1,5-bisP-carboxylase were tested according to Losada et al. (*20*); 3-phosphoglycerate-kinase and glyceraldehyde 3-P-dehydrogenase were also tested (*20*). The activity of phosphotriose-isomerase was measured according to Kelly and Gibbs (*18*).

The efflux of glyceraldehyde 3-phosphate (GAP) was assayed using the shuttle system 3-PGA/GAP as described by Kelly and Gibbs (*19*). The reaction mixture (described in the legend to Fig. 4) contained exogenous 3-PGA which penetrated the intact chloroplast (*40*) and then was reduced to GAP in the stroma. The resulting GAP left the chloroplast and was oxidized to 3-PGA outside the chloroplast by the addition of $NADP^+$ and non-reversible D-glyceraldehyde-3-phosphate dehydrogenase (NR-GAPDH). The enzyme was isolated from the cyotledons of pumpkin grown in the dark according to Kelly and Gibbs (*18*). The assay was carried out in the oxygen electrode chamber for 14 min, so that O_2 evolution in the light could be followed. After 14 min the reaction mixture was centrifuged. The absorbance at 340 nm of the supernatant was determined and the dark "zero time" value was deducted. The amount of NADPH formed corresponded to the efflux of GAP.

Linolenic acid was obtained from Fluka (Switzerland).

Results

The effect of concentrations of linolenic acid on bicarbonate-dependent oxygen evolution by intact chloroplasts in the presence of optimal PPi can be seen in Fig. 1. For the three chlorophyll concentrations used the control rates (100%) were the same on the basis of chlorophyll. The inhibition of CO_2-dependent O_2 evolution by linolenic acid was more effective when a lower concentration of chlorophyll was used. If the chlorophyll concentration was plotted as a function of linolenic acid

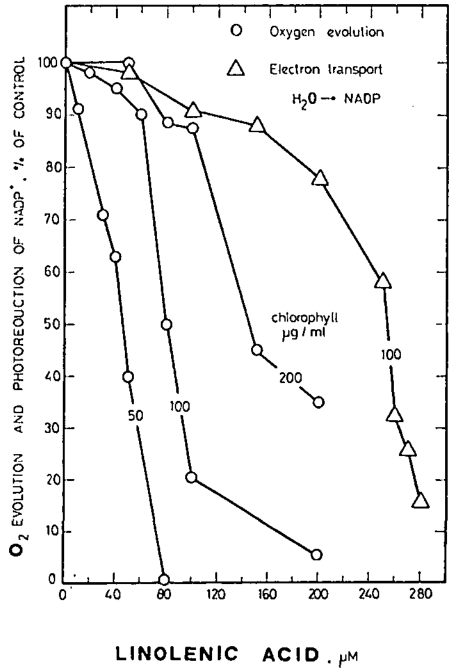

Fig. 1.

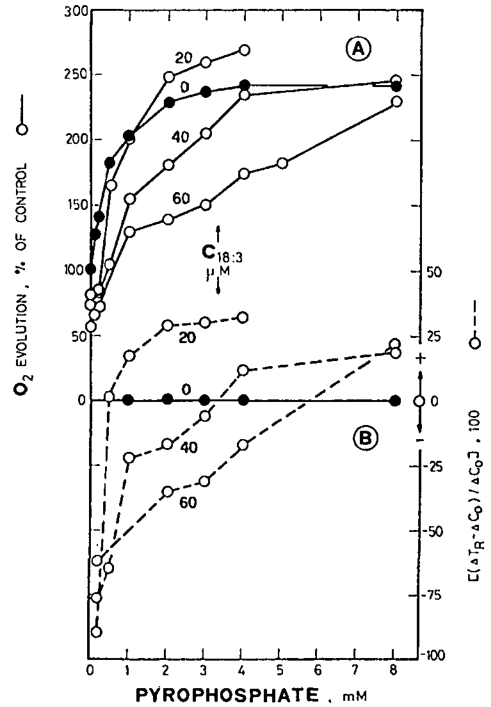

Fig. 2.

Fig. 1. *Effect of linolenic acid on oxygen evolution and eletcron transport at different chlorophyll concentrations.* O_2 evolution and photoreduction of $NADP^+$ were estimated in intact and broken chloroplasts, respectively. Numbers in the figure refer to chlorophyll concentrations (μg/ml). The control rates (100%) were 30–60 μmoles O_2 evolved·mg chl^{-1}·$hr^{-1}$and 155 μmoles NADPH formed·mg chl^{-1}·hr^{-1}. The reaction mixture for O_2 evolution contained 4 mM $Na_4P_2O_7 \cdot 10H_2O$ (pH 7.6); other conditions as described in **Materials and methods.**

Fig. 2. *Pyrophosphate dependence of O_2 evolution by intact chloroplasts in the absence and presence of linolenic acid.* O_2 evolution was expressed in (A) as % of control (in the absence of linolenic acid, pyrophosphate and phosphate, 100% corresponded to 10–17 μmoles O_2 evolved·mg chl^{-1}·hr^{-1}) and in (B) as [Δ treated − Δ control)/Δ control]·100 (where Δ refers to the change in activity due to PPi). Other conditions as described in **Materials and methods.**

concentration, straight lines were obtained for each level of inhibition over most of the range. This indicates that up to 200 μg chlorophyll/ml, identical molar ratios of $C_{18:3}$/chlorophyll inhibit oxygen evolution to a similar extent. Half inhibition occurred at a molar ratio of 0.7. This value was much lower than that for the inhibition of physiological electron flow in thylakoids (*35–37*). The experiments in Fig. 1 were therefore designed to compare oxygen evolution and electron flow from H_2O to $NADP^+$ using the same chloroplast preparation. Electron flow was much less sensitive to linolenic acid than O_2 evolution was, and a $C_{18:3}$/chlorophyll molar ratio of 2.25 was needed for half-inhibition.

Further, the sensitivity to linolenic acid of CO_2 fixation and O_2 evolution was tested under the same conditions. The results in Table 1 indicate that CO_2 fixation was more sensitive than CO_2-dependent O_2 evolution. A 50% decrease in CO_2 fixa-

Table 1 *Comparison of O_2 evolution and CO_2 fixation by intact chloroplasts in the presence of linolenic acid*

Linolenic acid (μM)	% of controls		Ratio O_2/CO_2
	O_2 evolution	CO_2 fixation	
0	100[a]	100[a]	1
40	86	51	1.69
50	80	46	1.88
60	72	41	1.75
80	57	31	1.85

[a] The controls (100%) were 30 and 31 μmoles·mg $chl^{-1} \cdot hr^{-1}$ for CO_2-dependent O_2 evolution and CO_2 fixation, respectively.

tion was observed for a $C_{18:3}$/chlorophyll molar ratio of 0.36. This may be compared to the values of 0.7 and 2.25 for the corresponding inhibitions of O_2 evolution and photoreduction of $NADP^+$, respectively. Moreover, for each concentration of $C_{18:3}$, the ratio of CO_2 fixation to O_2 evolution remained constant at 0.5 to 0.6. Thus, on a concentration basis, $C_{18:3}$ inhibited CO_2 fixation the most, O_2 evolution less and photoreduction of $NADP^+$ the least.

These results suggest that the inhibition of CO_2 fixation and of CO_2-dependent O_2 evolution by linolenic acid is not due to an inhibition of the photochemical reactions but is caused by an impairment of other factors such as the enzymatic steps in the Calvin-Benson cycle or the permeability to reactants of the chloroplast envelope. Experiments with crude extracts from chloroplasts have shown, however, that linolenic acid has no effect on six key enzymes of the Calvin-Benson cycle (*25*): ribulose 5-P-isomerase, ribulose 5-P-kinase, ribulose 1,5-bisP-carboxylase, 3-phosphoglycerate-kinase, glyceraldehyde 3-phosphate dehydrogenase and phosphotriose-isomerase. Similar results were also reported by Pedersen et al. (*29*) using shorter chain fatty acids.

In order to test the second hypothesis that permeability of the envelope to reactants is affected by linolenate, we investigated the action of $C_{18:3}$ on pyrophosphate-dependent oxygen evolution by intact chloroplasts. This activity is an indirect estimation of Pi uptake by intact chloroplasts according to Schwenn et al. (*32*). They have demonstrated that pyrophosphate, together with pyrophosphatase from broken chloroplasts, maintain the external Pi at or near a concentration optimal for photosynthesis, and that phosphate concentration governs the rate of synthesis and

Table 2 *Influence of linolenate on inorganic alkaline pyrophosphatase*

Additions	Ratio mM Mg^{2+}/PPi	μmoles Pi released/mg chl/10 min as a function of linolenate concentration (μM)		
		0	100	300
4 mM $MgCl_2$	2	1.47	1.52	1.43
8 mM $MgCl_2$	4	1.36	1.36	1.31

The reaction mixture contained 100 mM Tricine (pH 8.6), 2 mM pyrophosphate tetrasodium (pH 8.6), $MgCl_2$ as indicated and 0.1 ml enzyme extract corresponding to an original chloroplast suspension containing 100 μg chlorophyll/ml. Reaction time: 10 min at 25°C.

export of photosynthetic intermediates. Fig. 2A shows that in the absence of linolenic acid, O_2 evolution increased with increasing PPi concentration up to 4 mM. In the absence of PPi an increase in linolenic acid concentration decreased the activity. Addition of PPi reversed the inhibition due to low Pi levels in a manner which depended on the concentration of linolenic acid. In the presence of 20, 40 and 60 μM linolenic acid, maximum activity was obtained with 1.5, 4 and 8 mM PPi, respectively. Fig. 2B shows clearly that the inhibition of the PPi-dependent O_2 evolution by linolenic acid was more significant than that of the O_2 evolution not dependent on PPi. The stimulatory effect of PPi at low concentrations is effectively inhibited by linolenate. A very low concentration of PPi, however, was sufficient to reverse the effect of 20 μM $C_{18:3}$ while the effects of 40 and 60 μM $C_{18:3}$ were progressivley less easily reversed by PPi. Linolenate (particularly at 20 μM) increased the final level of O_2 evolution. Since O_2 evolution is an indirect estimation of pohsphate uptake under these conditions (*32*), it may be deduced that $C_{18:3}$ should inhibit both O_2 evolution and Pi uptake. Necessary criteria for this deduction are that pyrophosphate does not permeate the intact spinach chloroplast, as is known to

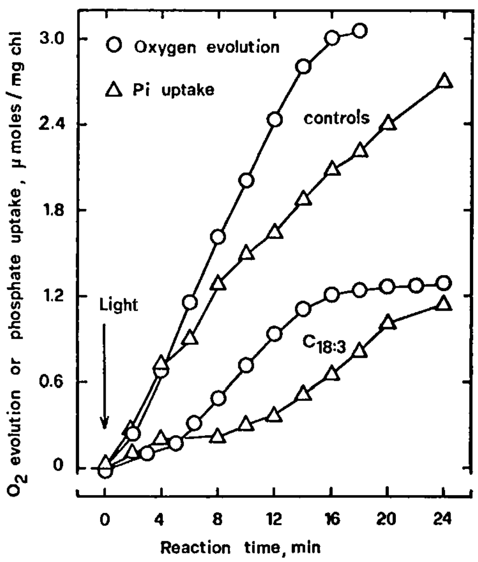

Fig. 3.

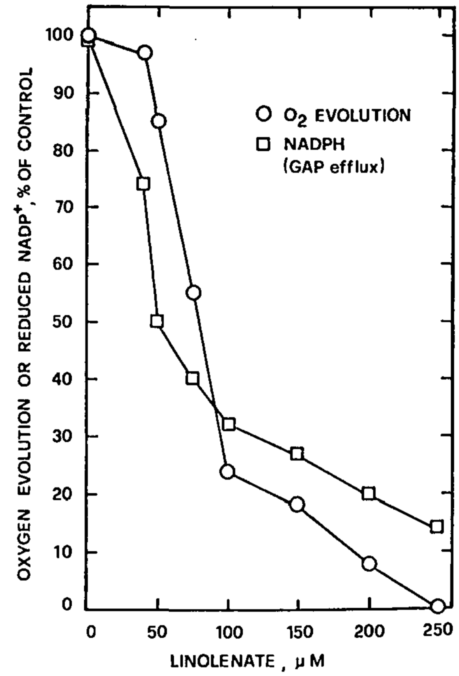

Fig. 4.

Fig. 3. *Effect of linolenate (80 μM) on CO_2-dependent O_2 evolution and on phosphate uptake by intact chloroplasts.*
Fig. 4. *Effect of linolenate on D-glyceraldehyde 3-P (GAP) efflux and CO_2-dependent O_2 evolution in intact chloroplasts.* The reaction mixture contained 350 mM sorbitol, 50 mM HEPES-NaOH (pH 8), 0.2 mM Na-isoascorbate, 6 mM $KHCO_3$, 1 mM $Na_4P_2O_7 \cdot 10H_2O$ (pH 8), 0.5 mM K_2HPO_4, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM 3-PGA, 0.2 mM $NADP^+$, 10^{-4} to 10^{-2} EU/ml of non reversible D-glyceraldehyde-3-phosphate dehydrogenase (NR-DGAPDH) and intact chloroplasts (50 μg chlorophyll/ml). The concentration of ethanol was 0.3% in all reaction mixtures. The control rates (100%) were 5 μmoles NADPH formed/mg chl/hr and 33 μmoles O_2 evolved/mg chl/hr.

be the case (*32*), and that the hydrolysis of pyrophosphate is neither activated nor inhibited by $C_{18:3}$, as shown in Table 2.

Fig. 3 shows a direct measurement of the effect of linolenic acid on CO_2-dependent O_2 evolution and Pi uptake as a function of time, both activities being measured simultaneously in the same vessel. Since no ADP was added, photophosphorylation by broken chloroplasts could not occur and the fall in Pi concentration is the result of Pi uptake by the intact chloroplasts. In the presence of 0.7 mM Pi, the rate of O_2 evolution by the untreated chloroplasts was less than in the standard medium containing 4 mM PPi (legend to Fig. 1). This is in agreement with the data reported by Lilley et al. (*23*). In the controls, the rate of Pi uptake was less than that of O_2 evolution. Both activities were inhibited by $C_{18:3}$ to almost the same extent. It is well established that the absorption of orthophosphate by intact chloroplasts is accompanied by the export of phosphorylated compounds of the Calvin-Benson cycle (*14*, *15*). Since linolenate inhibited the uptake of orthophosphate (Fig. 3), it is to be expected that linolenate also inhibits the efflux of these photosynthetic intermediates. Using cytoplasmic NR-GAPDH, Kelly and Gibbs (*18*) pointed out that the 3-PGA/GAP shuttle is a good simulation of the transport of phosphorylated compounds between the chloroplast and the cytoplasm. Fig. 4 shows that increasing concentrations of linolenate simultaneously decreased O_2 evolution and GAP efflux. One cannot exclude the possibility, however, that linolenate might also inhibit the influx of exogenous 3-PGA (the function of which is to initiate the shuttle). Nevertheless, in both cases the inhibition would occur at the "phosphate translocator" site.

Discussion

In addition to the inhibitory effects of unsaturated free fatty acids on the photochemical reactions in thylakoid membranes (*8*, *26*, *27*, *34–37*, *39*, *41*), we have found that linolenate inhibits photosynthesis by intact chloroplasts. This inhibition was due neither to a lack of NADPH in the chloroplasts nor to a direct inhibition of the enzyme activities of the Calvin-Benson cycle. Indeed, linolenate inhibited CO_2 fixation and oxygen evolution much more effectively than electron transport (Fig. 1 and Table 1). Moreover, in agreement with Pedersen et al. (*29*), we observed that fatty acids did not inhibit six key enzymatic activities of the Calvin-Benson cycle (ref. *25*, cf. also **Results**). Thus, the inhibition of photosynthesis by linolenate is related to the integrity of the chloroplast envelope.

The principal movement of metabolites across the envelope involves influx of Pi and CO_2 and a corresponding efflux of certain sugar-phosphates and especially triosephosphates (*15*). This transport is mediated by a translocator which is located in the inner membrane of the envelope (*15*). The polypeptide nature of this translocator has been characterized recently (*12*). Our results suggest that linolenate disturbs the function of the translocator. We have demonstrated here that linolenate inhibited CO_2-dependent O_2 evolution, phosphate uptake and the efflux of at least one of the phosphorylated compounds, 3-phosphoglyceraldehyde (Fig. 3 and 4). These results suggest that the inhibition of photosynthesis by linolenate is due in the first instance to an inhibition of the phosphate-metabolite exchange across the envelope which in turn causes an accumulation of photosynthetic phosphorylated intermediates in the stroma. Such an accumulation would inhibit by feedback effects several of the key enzymes of the Calvin-Benson cycle (*1*, *5*, *28*). Under these conditions an inhibition of carboxylation has been observed (Table 1). Since electron transport in broken chloroplasts was not affected by low concentrations of linolenate (Fig. 1), it is likely to be the increased ratio NADPH/NADP$^+$ which inhibits electron transport according to the mechanism demonstrated by Arnon and Chain (*2*). This whole sequence of events leading to the increased NADPH/NADP$^+$ ratio could explain why CO_2 fixation is more effectively inhibited than O_2 evolution (Table 1). Moreover, the addition of compounds which affect the exchange of metabolites across the envelope increases the molar ratio of O_2 evolved and CO_2 fixed above unity. For instance, the addition of 3-PGA (*1*, *28*) and high concentrations of orthophosphate (*7*, *28*) inhibited CO_2 fixation more significantly than O_2 evolution. The observation that linolenate has the same effect (Table 1) gives further evidence that $C_{18:3}$ may act on metabolite transport across the envelope.

Recently Purczeld et al. (*31*) have demonstrated that an addition of octanoate (10–20 mM) to intact chloroplasts lowers the stroma pH in the light, resulting in a diminution of the hydrolysis of fructose and sedoheptulose bisphosphates, and in a subsequent inhibition of CO_2-dependent O_2 evolution. The inhibition of CO_2 fixation by linolenate could therefore also be interpreted as due to an alteration of the proton gradient across the envelope and a lowering of the stroma pH. If the very low concentration of linolenate used in our experiments can effectively induce a drop in the pH of the stroma, such a decrease in pH might also alter the function of the "translocator" indirectly. Indeed, preliminary experiments (results to be published) have shown that Pi uptake inhibitited by linolenate was restored at alkaline pH.

It is concluded that linolenate inhibits photosynthesis by impairing the exchange of metabolites across the chloroplast envelope either by direct action on the "phosphate translocator" or by a decrease in the stroma pH.

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Effect of linolenate on photosynthesis by intact spinach chloroplasts II.
Influence of preillumination of leaves on the inhibition of photosynthesis by linolenate[1]

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The inhibitory effect of linolenate on intact spinach chloroplasts depends on the level of the internal pool of metabolites. Chloroplasts from preilluminated leaves or chloroplasts artificially loaded with 3-phosphoglyceric acid required higher concentrations of orthophosphate for maximal rates of CO_2-dependent O_2 evolution than untreated chloroplasts. The loaded chloroplasts were more sensitive to linolenate, and in the presence of linolenate the optimal phosphate concentration was shifted toward lower values. We propose that the inhibition of photosynthesis by linolenate is due to inhibition of the "phosphate translocator".

Key words: Chloroplars — Linolenic acid treatment — Metabolite pool — Photosynthesis — Preillumination of leaves — Spinach.

Linolenic acid is the most abundant free fatty acid released during the deterioration of chloroplast membranes in vitro (*8*, *14*, *18*). We have studied the effect of free fatty acids on spinach thylakoid membranes (*10–14*) and have reported recently that linolenic acid inhibited photosynthesis in intact chloroplasts by impairing phosphate influx and 3-phosphoglyceraldehyde efflux across the envelope of chloroplasts (*15*).

The influx of orthophosphate by intact chloroplasts is accompanied by the efflux of phosphorylated compounds of the Calvin-Benson cycle (*5*, *6*, *15*). The transport of these compounds is mediated by the "phosphate translocator" in a competitive way (*2*, *3*, *5*) and depends on the size of the internal pool of phosphorylated intermediates of the Calvin-Benson cycle. Chloroplasts enriched in internal phosphorylated intermediates ("high pool" chloroplasts) are obtained either by preillumination of leaves prior to isolation (*1*) or by addition of these intermediates to freshly prepared chloroplasts (*3*). "Low pool" chloroplasts can be isolated from leaves stored in the dark. These two types of chloroplasts seem to be ideal material for the study of the effect of an inhibitor on photosynthesis and on the transport of

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[2] This work is part of a doctoral programme carried out by L. Mvé Akamba in this laboratory.

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internal metabolites across the chloroplast envelope. The present investigation shows that the extent of the inhibition of photosynthesis by linolenate depends on the size of the internal pool of metabolites.

Materials and methods

Two kinds of chloroplasts were isolated from spinach leaves (*Spinach oleracea*, var. Nobel): those from preilluminated leaves, and those from leaves stored overnight in the dark. For preillumination, washed leaves floating on distilled water were illuminated with a tungsten 150 w bulb lamp (2300 lux) in a cold room (4°C), for 60 min prior to the isolation of chloroplasts as described earlier (*15*).

Oxygen evolution was measured polarographically in a 5 ml cuvette at 20°C. The reaction mixtures are described in the legends to the figures. In experiments involving the addition of 3-phosphoglyceric acid (3-PGA), chloroplasts were incubated in media containing 3-PGA for 4 min in the dark at about 2°C. The barium salt of 3-PGA was purchased from Calbiochem and converted to its free form by passing it through a Dowex 50 w (H^+) column and the pH was adjusted to about 4 with $NaHCO_3$.

Results

Effect of preillumination of leaves on the inhibition of CO_2-dependent O_2 evolution by linolenate in intact chloroplasts

Fig. 1 shows that the rate of oxygen evolution by intact chloroplasts isolated from preilluminated leaves was higher than the corresponding activity in chloroplasts isolated from leaves stored in the dark (32 vs 21 μmoles·mg $chl^{-1}\cdot hr^{-1}$). In these

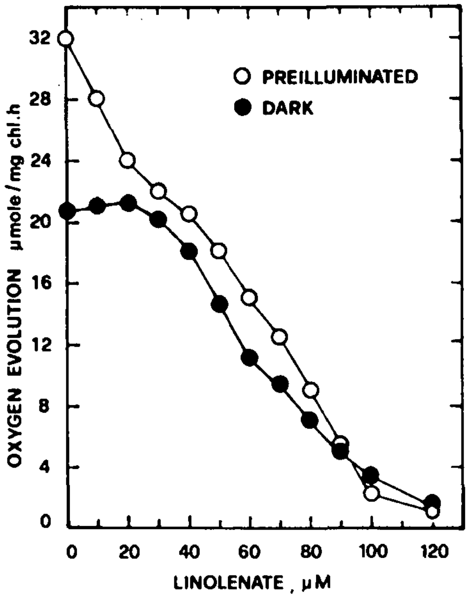

Fig. 1. *Comparison of the effect of linolenate on O_2 evolution in intact chloroplasts prepared from preilluminated and non-preilluminated leaves.* The reaction mixture contained: 350 mM sorbitol, 50 mM HEPES-NaOH (pH 7.6), 0.2 mM Na-isoascorbate, 6.6 mM $KHCO_3$, 4 mM $Na_4P_2O_7\cdot 10H_2O$ (pH 7.6), chloroplasts (100 μg chlorophyll per ml) and linolenic acid (dissolved in ethanol) as indicated. The concentration of ethanol was 0.3% in all reaction mixtures.

experiments about 80% of both types of chloroplasts were intact as estimated by the ferricyanide method (*4*). Addition of low concentrations of linolenate (0–30 μM) had a quantitatively different effect on the two types of chloroplasts: the oxygen evolution was markedly decreased in chloroplasts from preilluminated leaves whereas it was scarcely affected in chloroplasts from leaves maintained in the dark. At concentrations higher than 30 μM, the decrease in both activities was similar. A 50% decrease was observed at 60 to 65 μM linolenate for both types of chloroplasts. Thus, in the first phase of inhibition, chloroplasts from dark-treated leaves appeared to show no response to linolenate, and the inhibition of the activity by linolenate depended on the physiological state of the chloroplasts.

Interaction between the effects of linolenate and of preillumination of the leaves on phosphate-dependent O_2 evolution by intact chloroplasts

Several reports (*6*) have demonstrated that the rate of CO_2-dependent O_2 evolution in intact chloroplasts depends on the exchange between exogenous phosphate and endogenous sugar phosphates, including triose-phosphates and 3-PGA. The amount of exogenous phosphate required to induce the maximal O_2 evolution is an indicator of the pool of these internal metabolites and of the exchange across the envelope. In order to test this hypothesis, we have measured this indicator using "high pool" (leaves preilluminated) and "low pool" (leaves maintained in the dark) chloroplasts. Since linolenate inhibited the absorption of phosphate during photosynthesis in intact chloroplasts (*15*), one would expect that the indicator level would be affected.

Fig. 2A shows that in chloroplasts from preilluminated leaves, oxygen evolution

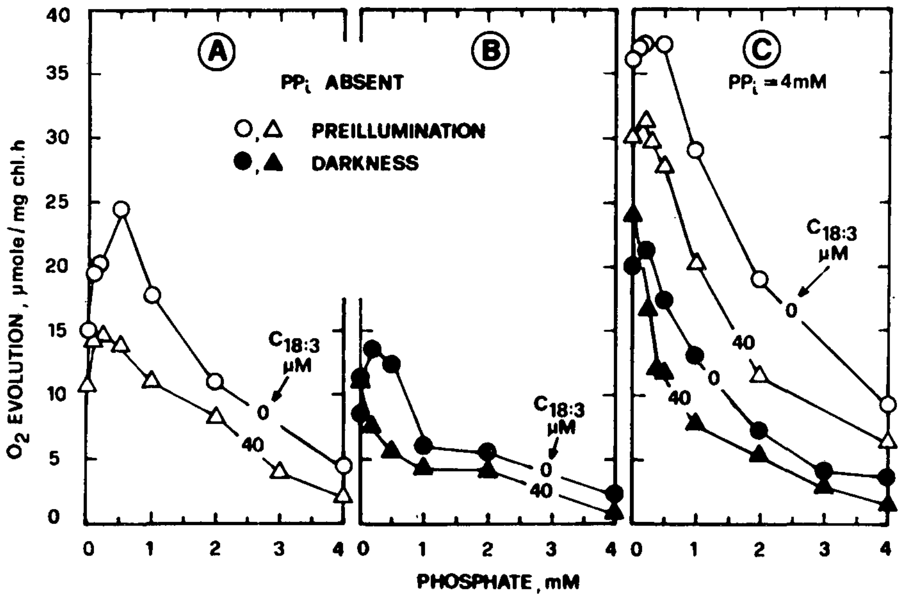

Fig. 2. *The effect of preillumination of leaves and linolenate addition on the phosphate-dependent O_2 evolution by intact chloroplasts.* The basic reaction mixture was as in Fig. 1, except that pyrophosphate was omitted (Fig. A, B) or added (Fig. C).

increased when phosphate was added. The maximum activity was found with 0.5 mM phosphate. Beyond this concentration, the activity diminished markedly and 50% of the maximum value was observed at 1.7 mM phosphate. The inhibition resulted from the exchange of so much external phosphate with internal sugar phosphates, triose-P and 3-PGA that the Calvin cycle was depleted of the latter compounds. In chloroplasts from leaves maintained in the dark (Fig. 2B), the same profile was observed except that O_2 evolution was less and its optimum was attained at 0.2 mM phosphate. The half-inhibition was observed at 0.8 mM phosphate. These results indicate that the concentration of exogenuos Pi which induced the optimal activity of O_2 evolution depended on the pool size of internal metabolites.

The addition of 40 μM linolenate decreased O_2 evolution in both types of chloroplasts (Fig. 2A and 2B), except that it increased the activity in the absence of phosphate for chloroplasts from leaves maintained in the dark. Linolenate also caused a shift to a lower concentration of Pi for the maximum activity, i.e., from 0.5 to 0.2 mM Pi in "high pool" and from 0.2 to 0 mM Pi in "low pool" chloroplasts. The maximum inhibition with 40 μM linolenate occurred at Pi concentrations which enhanced the activities, i.e. from 0 to 0.5 mM (Fig. 2A) and from 0 to 0.2 mM (Fig. 2B). At higher concentrations of Pi, the activity was less affected by linolenate.

The same experiments were performed in the presence of 4 mM pyrophosphate (PPi) (Fig. 2C). In the absence of exogenous Pi, the activities for both types of chloroplasts were higher than in the absence of PPi. This is in agreement with the observation reported by Lilley et al. (*7*) showing the regulatory effect of PPi on O_2 evolution by intact chloroplasts. In the presence of PPi, low levels of Pi increased the activities to a lesser extent; nevertheless, there were optima which were at 0.5 and 0.2 mM Pi for chloroplasts from preilluminated and dark-treated leaves, respectively. These optima were apparently shifted toward lower concentrations of Pi in the presence of linolenate. Contrary to the experiments in Fig. 2A and 2B, the decrease of activity by linolenate was essentially similar with all concentrations of Pi (Fig. 2C).

Influence of linolenate on photosynthesis in the presence of exogenous 3-phosphoglycerate

The results in Fig. 1 and 2 demonstrate that the inhibition of O_2 evolution by linolenate depended on the physiological state of the chloroplasts. In order to confirm this observation, we investigated the influence of linolenate on intact chloroplasts enriched in Calvin-Benson cycle phosphorylated compounds both by preillumination of leaves and, artificially, by incubation of chloroplasts in 3-PGA; 3-PGA is particularly suitable because of its high ability to permeate the envelope of intact chloroplasts (*16*, *17*). In Fig. 3A 3-PGA showed a biphasic effect on light-dependent O_2 evolution: at concentrations up to 1 mM, oxygen evolution decreased considerably in chloroplasts from preilluminated leaves, whereas it was less affected (sometimes stimulated) in chloroplasts from leaves maintained in the dark. At concentrations higher than 1 mM, both activities were similar and decreased more slowly. In the presence of 40 μM linolenate, the activities were essentially the same for both types of chloroplasts. Nevertheless, linolenate more effectively inhibited the activity of chloroplasts from preilluminated leaves than that of the other type of chloroplasts on a percentage basis. Fig. 3B shows more clearly that the inhibition of O_2 evolution by linolenate was not significantly affected by 3-PGA in chloroplasts

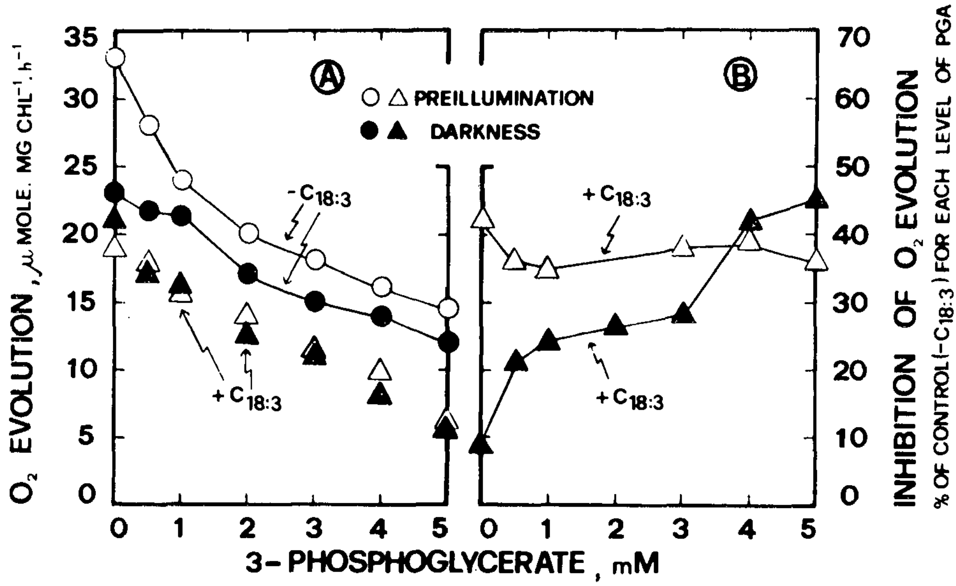

Fig. 3. *Influence of preillumination of leaves and 3-PGA addition on the inhibition of O_2 evolution by linolenate in intact chloroplasts.* The basic reaction mixture was the same as in Fig. 1, except that 3-PGA was added as indicated. Linolenate was at 40 μM.

from preilluminated leaves. With chloroplasts from leaves maintained in the dark, on the other hand, 3-PGA enhanced the inhibition caused by linolenate. Thus, there is an analogy between the effect of leaf preillumination and of exogenous 3-PGA: both treatments make the chloroplasts more sensitive to linolenate.

Discussion

Our results establish a similarity between the effect of preillumination of leaves and the addition of 3-phosphoglyceric acid to intact chloroplasts. Both treatments, which increase the internal pool of photosynthetic phosphorylated compounds (*1*, *3*), have been shown here to make the chloroplasts more active and more sensitive to linolenate (Fig. 1 and 3). Chloroplasts from preilluminated leaves required a higher concentration of Pi than the dark-treated leaf chloroplasts for maximal activity (Fig. 2). This Pi concentration is an indicator of the exportable phosphorylated intermediates. In the presence of linolenate, the indicator was shifted toward lower values of Pi in both types of chloroplasts. Thus, it appears that treatment with linolenate and dark storage of leaves have synergistic effects on the shift of the indicator. This constitutes further indirect evidence that the degree to which internal metabolites are exported has decreased in the presence of linolenate since it diminishes the extent to which Pi releases the internal pool at all levels of the pool. The observation that 3-phosphoglyceraldehyde efflux was inhibited by linolenate (*15*) can now be extended to other phosphorylated internal metabolites.

We reported recently that linolenate inhibited O_2 evolution and CO_2 fixation in intact chloroplasts by inhibiting directly or indirectly the uptake of phosphate and the export of 3-phosphoglyceraldehyde (*15*). This observation and the present results both suggested that linolenate affects the "phosphate translocator" in the chloroplast envelope. This translocator links the uptake of Pi and the efflux of phosphorylated compounds (*6*).

Since the optimal activity of photosynthesis depends on the exchange between Pi and phosphorylated metabolites across the chloroplast envelope (*3*, *6*), we conclude that the inhibition of photosynthesis by linolenate is mainly due to an impairment of this exchange. Such an impairment might result in an accumulation of photosynthetic intermediates and a subsequent inhibition of the carboxylation reaction (*15*). Less likely is an indirect impairment of exchange due to a decrease in the stroma pH as indicated recently (*9*, *15*). In conclusion, it is highly probable that the phosphate-metabolite transporter systems are inhibited by linolenate, but by a mechanism as yet to be established.

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EFFECT OF LINOLENATE ON PHOTOSYNTHESIS BY INTACT SPINACH CHLOROPLASTS

Conditions for the inhibition of orthophosphate uptake

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1. Introduction

Linolenate has been shown [1] to inhibit photosynthesis in intact spinach chloroplasts by inhibiting the influx of orthophosphate and the efflux of 3-phosphoglyceraldehyde. The inhibitory effect of linolenate was shown to depend on the level of the internal pool of metabolites [2] as a result of experiments which on the one hand used chloroplasts from preilluminated leaves or chloroplasts which were artificially loaded with 3-phosphoglyceric acid ('high pool' chloroplasts) and on the other hand chloroplasts from darkened leaves ('low pool' chloroplasts). This suggested that the inhibition of photosynthesis by linolenate is due to an inhibition of the 'phosphate translocator' which is located in the envelope and mediates the exchange of orthophosphate and internal phosphorylated metabolites [3,4]. This investigation is an attempt to elucidate the mechanism by which linolenate inhibits the function of the 'phosphate translocator', in particular with respect to the uptake of orthophosphate.

2. Material and methods

Intact chloroplasts from spinach (*Spinacia oleracea*, var. Nobel) were isolated from leaves as in [1] and the chlorophyll content was determined by the method in [5]. Intact chloroplasts were isolated from preilluminated leaves as in [2]. The orthophosphate concentration was estimated according to [6] and related to curves using readings obtained from standards in the presence of sorbitol or sucrose. Oxygen evolution was measured polarographically and CO_2 fixation was assayed as in [1]. Experimental conditions and reaction mixtures are described in the figure legends.

3. Results and discussion

Figure 1 shows that in the presence of 0.7 mM phosphate in the reaction mixture the rates of O_2 evolution and of phosphate uptake (disappearance of P_i from the medium) with intact chloroplasts were not stoichiometrically related (as is also illustrated in fig.2A). Addition of increasing concentrations of linolenate affected the two activities differently; whilst the rate of phosphate uptake decreased progressively with increasing linolenate concentration, O_2 evolution was first activated and then inhibited. Under the experimental conditions used, the external P_i concentration (0.7 mM) was too high for maximum rates of O_2 evolution as discussed [2,7]. The addition of low concentrations of linolenate inhibited P_i uptake (fig.1 and [1]) and therefore reduced it to a value nearer to the optimum for O_2 evolution: this would explain the stimulatory effect of linolenate on O_2 evolution. With further additions of linolenate chloroplasts were depleted in P_i and consequently O_2 evolution diminished.

Figure 1 shows that the addition of NH_4Cl inhibited

[+] This work is part of a doctoral program which is carried out by L. M. A. in this laboratory

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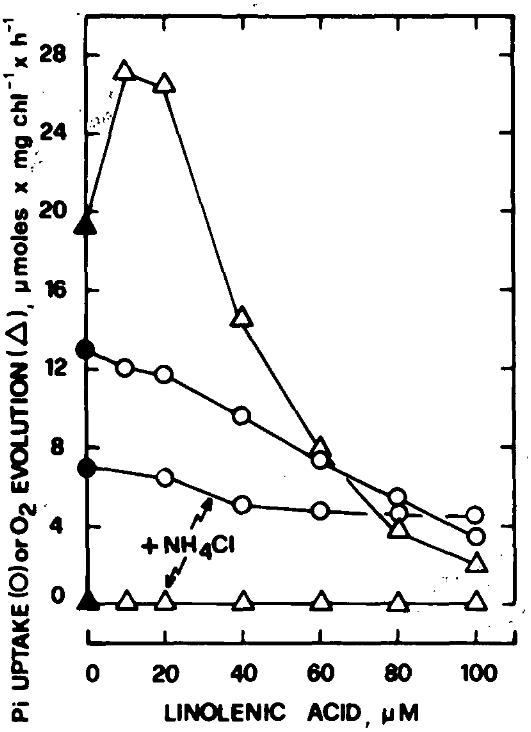

Fig.1. Influence of linolenate on oxygen evolution and phosphate uptake in the light by intact chloroplasts. O_2 evolution and inorganic phosphate uptake were assayed simultaneously in the same vessel containing: 350 mM sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (Hepes) adjusted to pH 7.6 with NaOH, 0.2 mM Na-isoascorbate, 6.6 mM $KHCO_3$, 0.7 mM K_2HPO_4 and chloroplasts (100 μg chl./ml). Linolenic acid (dissolved in ethanol) and 6 mM NH_4Cl were added as indicated. Reactions in the light (white light at ~5 × 10^6 ergs . cm^{-2} . s^{-1}) were carried out at 20°C and stopped after 10 min. The P_i concentrations were estimated in the supernatants, from aliquots removed at the beginning and end of the experiment and centrifuged immediately. Symbols are indicated along the ordinate.

the rate of phosphate uptake by ~50% and completely inhibited O_2 evolution. Added NH_4Cl was shown [8] to affect CO_2-supported O_2 evolution in a complex manner which depended upon the pH and the concentration of phosphate in the chloroplast suspension medium and also on the concentration of NH_4^+ [8]. The inhibition of O_2 evolution by 6 mM NH_4Cl can be explained in two ways:

(i) The uncharged amine (NH_3) penetrates the envelope and with an effectiveness dependent upon the internal pH consumes H^+ to form the charged species (NH_4^+); this would deplete the stromal region of H^+ and raise its pH and this would result in a decrease of CO_2-dependent O_2 evolution by affecting several enzyme activities of the Calvin-Benson cycle [9];

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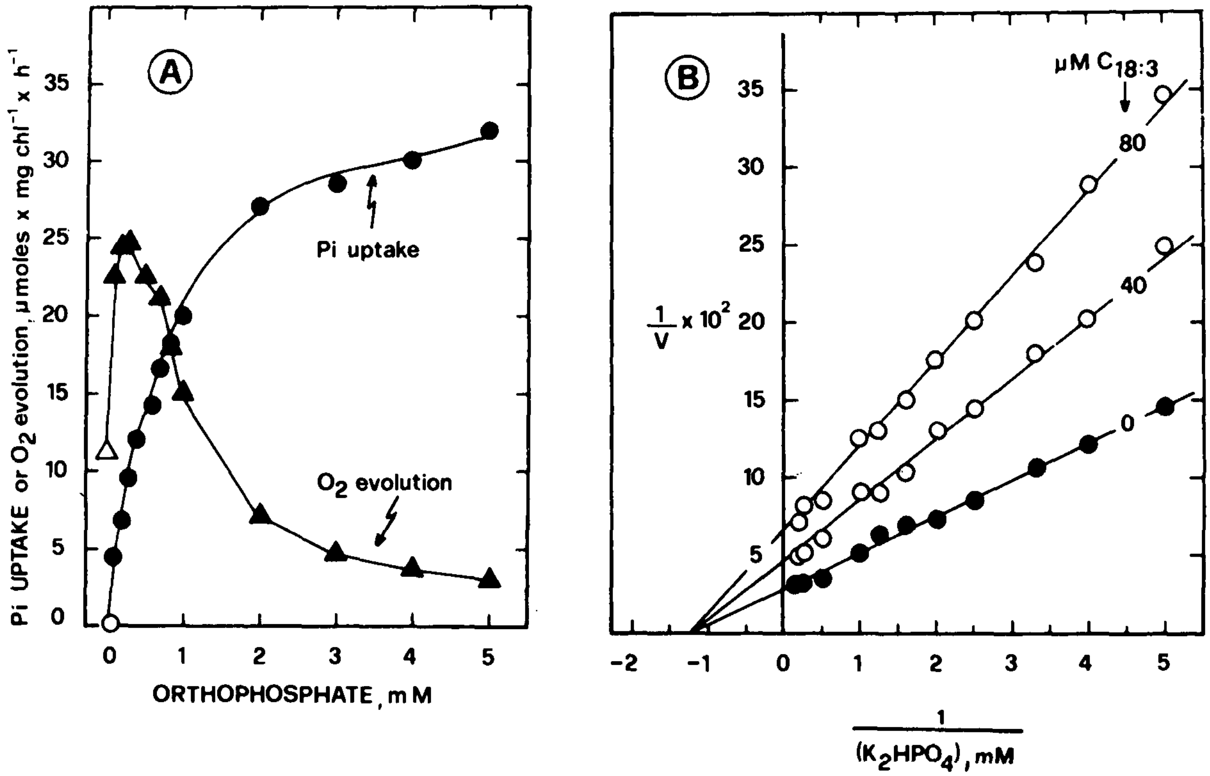
These NH_4Cl-treated chloroplasts were, however, still able to take up P_i and this uptake was only marginally inhibited by linolenate (fig.1). This interesting observation suggests that in the presence of NH_4Cl, phosphate can still partially move across the envelope by a mechanism which is not dependent on its consumption in photophosphorylation.

In fig.2A, O_2 evolution and phosphate uptake by intact chloroplasts were plotted as a function of exogenous phosphate concentration. Whilst the rate of O_2 evolution reached a maximum at about 0.2 mM P_i and decreased rapidly above this concentration, as reported [1,7], phosphate uptake increased significantly up to 2 mM and approached a saturation plateau at higher values of P_i. The latter curve was of the Michaelis type; indeed the double reciprocal plot of the concentration dependence of the rate of phosphate uptake was linear thus indicating an approach to saturation of a carrier mechanism, as illustrated in fig.2B (black symbols). It appears therefore that there is no simple correlation between the rate of P_i uptake and O_2 evolution in the light. The form of the P_i uptake curve, resembling Michaelis-Menten kinetic curves, suggests that P_i is transported inside the chloroplast by a specific carrier named phosphate translocator [3,4].

Addition of linolenate inhibited phosphate uptake and affected the slope and the intercept of the curve with the ordinate (fig.2B). Thus V_m decreased in value with increasing concentrations of linolenate but on the other hand the app. K_m value remained the same. The absolute values of K_m and V_m varied with different market spinach leaves. In spite of this variability, the inhibition by linolenate was always of a non-competitive type. Since it has been demonstrated that the uptake of orthophosphate is mediated by a phosphate translocator located in the envelope of intact chloroplasts [3,4] these results indicated that linolenate did not change the affinity of the translocator for phosphate. The absence of any



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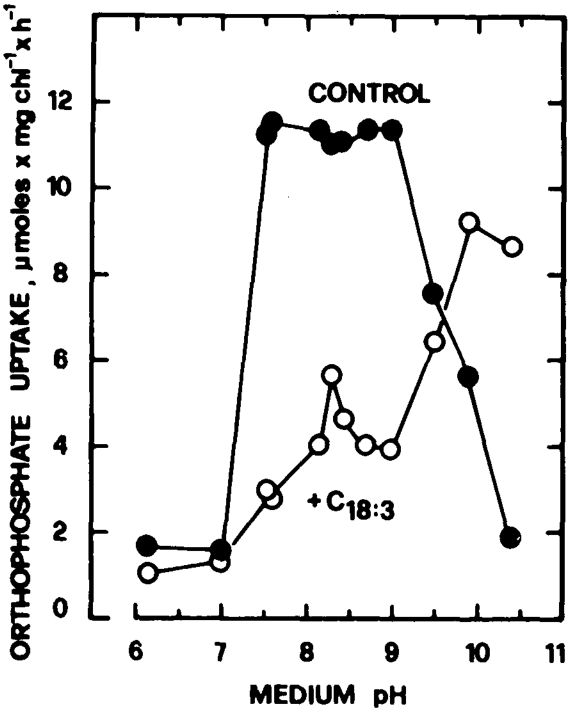

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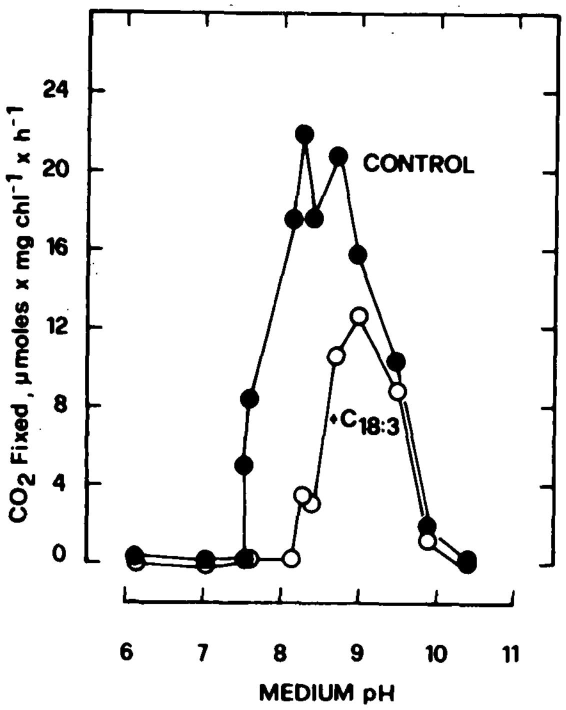

Fig.4. pH-dependence of CO_2 fixation by intact chloroplasts and inhibition by linolenate. Experimental conditions were the same as in fig.3 except that the reaction mixture was supplemented with 6.6 mM $NaH^{14}CO_3$ (0.1 µCi/µmol). The acid-stable labelling was estimated as in [1].

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The results presented give further support to the conclusion [1,2] that linolenate affects the phosphate/triosephosphate translocator in the envelope of chloroplasts. In addition they give evidence relevant to the mode of phosphate binding to the translocator with two possible points of attachment and reveal an additional effect of linolenate on phosphate entry at high pH values.

Acknowledgements

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EFFECT OF LINOLENATE ON PHOTOSYNTHESIS BY INTACT SPINACH CHLOROPLASTS

Conditions for the inhibition of orthophosphate uptake

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1. Introduction

Linolenate has been shown [1] to inhibit photosynthesis in intact spinach chloroplasts by inhibiting the influx of orthophosphate and the efflux of 3-phosphoglyceraldehyde. The inhibitory effect of linolenate was shown to depend on the level of the internal pool of metabolites [2] as a result of experiments which on the one hand used chloroplasts from preilluminated leaves or chloroplasts which were artificially loaded with 3-phosphoglyceric acid ('high pool' chloroplasts) and on the other hand chloroplasts from darkened leaves ('low pool' chloroplasts). This suggested that the inhibition of photosynthesis by linolenate is due to an inhibition of the 'phosphate translocator' which is located in the envelope and mediates the exchange of orthophosphate and internal phosphorylated metabolites [3,4]. This investigation is an attempt to elucidate the mechanism by which linolenate inhibits the function of the 'phosphate translocator', in particular with respect to the uptake of orthophosphate.

2. Material and methods

Intact chloroplasts from spinach (*Spinacia oleracea*, var. Nobel) were isolated from leaves as in [1] and the chlorophyll content was determined by the method in [5]. Intact chloroplasts were isolated from preilluminated leaves as in [2]. The orthophosphate concentration was estimated according to [6] and related to curves using readings obtained from standards in the presence of sorbitol or sucrose. Oxygen evolution was measured polarographically and CO_2 fixation was assayed as in [1]. Experimental conditions and reaction mixtures are described in the figure legends.

3. Results and discussion

Figure 1 shows that in the presence of 0.7 mM phosphate in the reaction mixture the rates of O_2 evolution and of phosphate uptake (disappearance of P_i from the medium) with intact chloroplasts were not stoichiometrically related (as is also illustrated in fig.2A). Addition of increasing concentrations of linolenate affected the two activities differently; whilst the rate of phosphate uptake decreased progressively with increasing linolenate concentration, O_2 evolution was first activated and then inhibited. Under the experimental conditions used, the external P_i concentration (0.7 mM) was too high for maximum rates of O_2 evolution as discussed [2,7]. The addition of low concentrations of linolenate inhibited P_i uptake (fig.1 and [1]) and therefore reduced it to a value nearer to the optimum for O_2 evolution: this would explain the stimulatory effect of linolenate on O_2 evolution. With further additions of linolenate chloroplasts were depleted in P_i and consequently O_2 evolution diminished.

Figure 1 shows that the addition of NH_4Cl inhibited

[+] This work is part of a doctoral program which is carried out by L. M. A. in this laboratory

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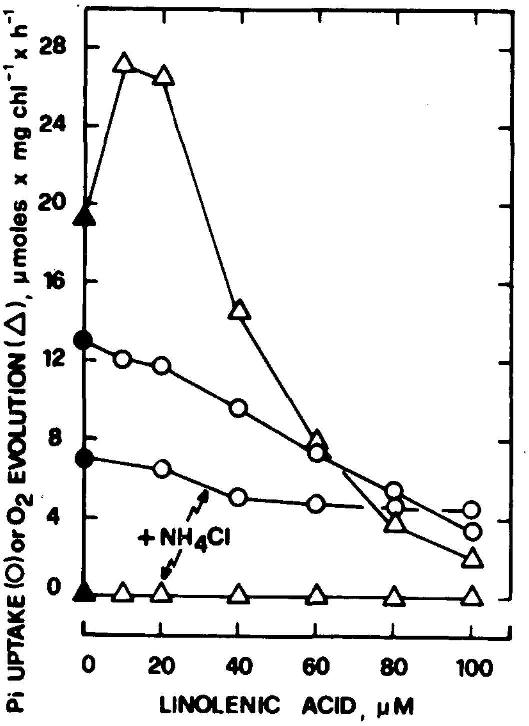

Fig.1. Influence of linolenate on oxygen evolution and phosphate uptake in the light by intact chloroplasts. O_2 evolution and inorganic phosphate uptake were assayed simultaneously in the same vessel containing: 350 mM sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) adjusted to pH 7.6 with NaOH, 0.2 mM Na-isoascorbate, 6.6 mM $KHCO_3$, 0.7 mM K_2HPO_4 and chloroplasts (100 µg chl./ml). Linolenic acid (dissolved in ethanol) and 6 mM NH_4Cl were added as indicated. Reactions in the light (white light at ~5 × 10^6 ergs . cm^{-2} . s^{-1}) were carried out at 20°C and stopped after 10 min. The P_i concentrations were estimated in the supernatants, from aliquots removed at the beginning and end of the experiment and centrifuged immediately. Symbols are indicated along the ordinate.

the rate of phosphate uptake by ~50% and completely inhibited O_2 evolution. Added NH_4Cl was shown [8] to affect CO_2-supported O_2 evolution in a complex manner which depended upon the pH and the concentration of phosphate in the chloroplast suspension medium and also on the concentration of NH_4^+ [8]. The inhibition of O_2 evolution by 6 mM NH_4Cl can be explained in two ways:

(i) The uncharged amine (NH_3) penetrates the envelope and with an effectiveness dependent upon the internal pH consumes H^+ to form the charged species (NH_4^+); this would deplete the stromal region of H^+ and raise its pH and this would result in a decrease of CO_2-dependent O_2 evolution by affecting several enzyme activities of the Calvin-Benson cycle [9];

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These NH_4Cl-treated chloroplasts were, however, still able to take up P_i and this uptake was only marginally inhibited by linolenate (fig.1). This interesting observation suggests that in the presence of NH_4Cl, phosphate can still partially move across the envelope by a mechanism which is not dependent on its consumption in photophosphorylation.

In fig.2A, O_2 evolution and phosphate uptake by intact chloroplasts were plotted as a function of exogenous phosphate concentration. Whilst the rate of O_2 evolution reached a maximum at about 0.2 mM P_i and decreased rapidly above this concentration, as reported [1,7], phosphate uptake increased significantly up to 2 mM and approached a saturation plateau at higher values of P_i. The latter curve was of the Michaelis type; indeed the double reciprocal plot of the concentration dependence of the rate of phosphate uptake was linear thus indicating an approach to saturation of a carrier mechanism, as illustrated in fig.2B (black symbols). It appears therefore that there is no simple correlation between the rate of P_i uptake and O_2 evolution in the light. The form of the P_i uptake curve, resembling Michaelis-Menten kinetic curves, suggests that P_i is transported inside the chloroplast by a specific carrier named phosphate translocator [3,4].

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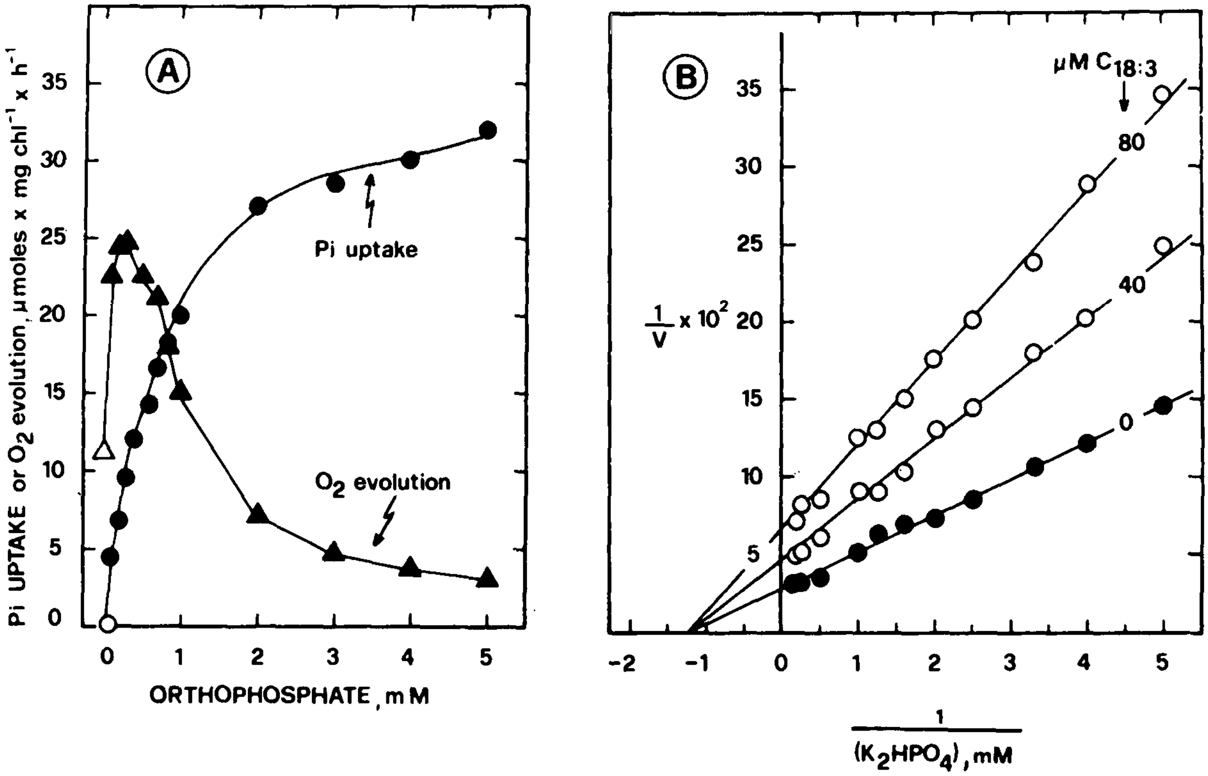

Fig.2. Phosphate uptake and oxygen evolution by intact chloroplasts as a function of external P_i concentration (2A). Conditions and reaction mixtures were the same as in fig.1 except that 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ were added in the reaction medium and chloroplasts were extracted from non-preilluminated leaves. The concentration of inorganic phosphate was varied as indicated and linolenate was at 40 μM. Lineweaver-Burk plots showing the inhibition of inorganic phosphate uptake in intact chloroplasts by 40 μM linolenate (2B). Experimental conditions and reaction mixtures were the same as in fig.2A. The K_m for orthophosphate was 0.8 mM in the presence and in the absence of linolenate. K_i and V_{max} were 60 μM and 39.6 μmol P_i . mg chl.$^{-1}$. h^{-1}.

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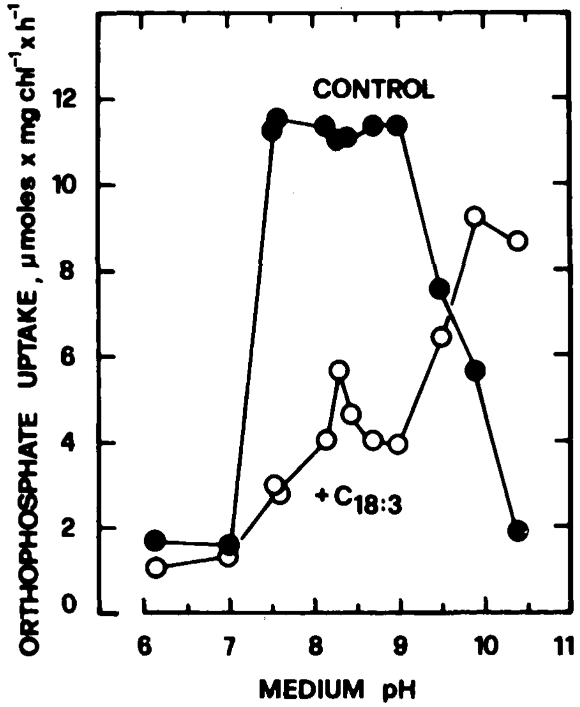
In further experiments phosphate uptake by intact chloroplasts in the light was assayed as a function of external pH (fig.3). The rates of phosphate uptake presented a broad maximum from pH 7.5–9.0. Outside this range, the activities dropped abruptly. Linolenate inhibited phosphate uptake between pH 7.5 and 9.6 (with a peak which was repeatedly observed near pH 8); on the other hand at higher pH values linolenate stimulated phosphate uptake. The pH profile of P_i uptake for the control without linolenate suggests that near the two extremities of the plateau (i.e., pH 7.5 and 9.0) the ionisation constants for the substrate (phosphate) and for chemical groups at the active site of the translocator are favourable for the formation of a translocator–P_i complex. This indicates the possible existence of two sites with different pK values which bind phosphate in the active region of the translocator, as is often the case for an isolated enzyme [10]. The pK_2 of P_i is 7.2, therefore at pH 7.5 the two ion species $H_2PO_4^-$ and HPO_4^{2-} are at about the same concentration, whereas at pH 9.0 the divalent ion species is predominant. Since P_i uptake is not influ-



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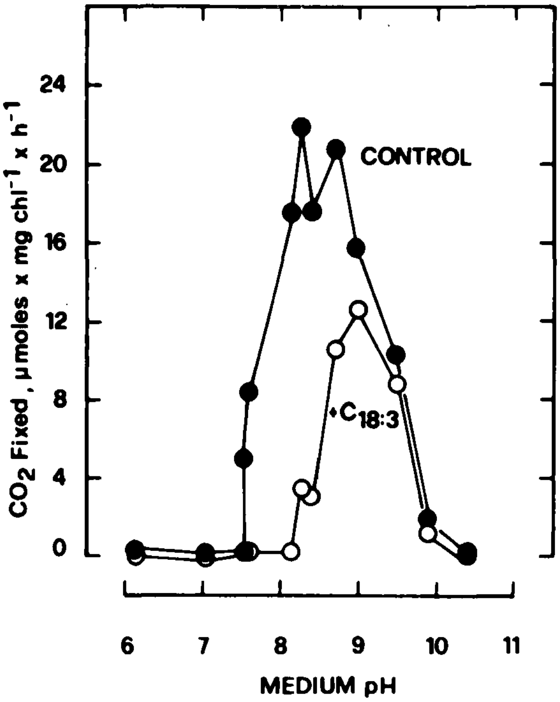

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Effet de l'acide linolénique sur la photosynthèse de chloroplastes intacts de feuilles d'Épinard

IV. Modification de la distribution de l'incorporation de ^{14}C dans les esters phosphates du cycle de réduction du CO_2

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RÉSUMÉ

Mots clés : acide linolénique, photosynthèse, chloroplastes intacts, incorporation de ^{14}C, bisphosphatases.

L'addition d'acide linolénique (rapport molaire $C_{18:3}$/chlorophylle : 0,45) inhibe fortement la fixation de $^{14}CO_2$ par des chloroplastes intacts. L'analyse de la distribution de la radioactivité ^{14}C dans différents esters phosphates révèle une accumulation de bisphosphates d'oses et une diminution de la concentration relative de leurs homologues monophosphates. Ces variations indiquent que l'acide linolénique inhibe les activités des fructoses 1.6-bisphosphatase et sédoheptulose 1.7-bisphosphatase. En outre, en présence d'acide linolénique, la quantité d'acide 3-P-glycérique diminue alors que la quantité de ribulose 1.5-bisphosphate augmente. Ceci suggère un blocage de l'activité de la ribulose 1.5-bisphosphate carboxylase par un excès de bisphosphates d'oses.

Les résultats sont discutés en tenant compte de l'effet de l'acide linolénique sur le transporteur de phosphate/trioses-P et la variation du pH du stroma qui en résulte.

SUMMARY

Key words: linolenic acid, photosynthesis, intact chloroplasts, incorporation of ^{14}C, bisphosphatases.

The addition of linolenic acid (molar ratio $C_{18:3}$/chlorophyll : 0,45) strongly inhibits CO_2 fixation by intact chloroplasts. An analysis of the distribution of ^{14}C radioactivity in different phosphate esters reveals an accumulation of bisphosphate sugars and a diminution of the relative concentration of their monophosphate homologs. These variations indicate that linolenic acid inhibits the activities of fructose 1,6-*bisphosphatase and sedoheptulose* 1,7-*bisphosphatase. In addition, the amount of* 3-*phosphoglyceric acid diminishes whereas the amount of ribulose* 1,5-*bisphosphate increases, in the presence of linolenic acid. This suggests a blockage of ribulose* 1,5-*bisphosphate carboxylase activity due to an excess of bisphosphate sugars.*

These results are discussed with reference to the effect of linolenic acid on the phosphate/triose-phosphate translocator and on the resulting variations of the pH of the stroma.

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I. INTRODUCTION

Au cours de travaux antérieurs nous avons démontré que l'acide linolénique exogène inhibe la photosynthèse de chloroplastes intacts d'Épinard mesurée par la fixation de CO_2 et le dégagement d'oxygène (SIEGENTHALER et MVÉ AKAMBA, 1979; MVÉ AKAMBA et SIEGENTHALER, 1979 *a*, *b*). Aux concentrations qui inhibent la photosynthèse, l'acide linolénique s'est avéré sans action significative sur l'activité des enzymes solubles du stroma et sur le transport d'électrons non cyclique $H_2O/NADP^+$ (SIEGENTHALER et MVÉ AKAMBA, 1979). Par contre, l'inhibition de la photosynthèse s'accompagne d'une diminution des échanges de phosphate inorganique et de glycéraldéhyde 3-phosphate à travers les enveloppes (SIEGENTHALER et MVÉ AKAMBA, 1979; MVÉ AKAMBA et SIEGENTHALER, 1979 *a*). L'acide linolénique affecte donc le fonctionnement du transporteur de phosphate/trioses phosphate (MVE AKAMBA et SIEGENTHALER, 1979 *b*) et, en inhibant l'absorption du phosphate inorganique dans le chloroplaste, a pour effet d'empêcher l'alcalinisation du stroma, normalement liée à ce transport (MVÉ AKAMBA, 1979; SIEGENTHALER, MVÉ AKAMBA et JANECKA (en préparation).

Le but du présent travail est de rechercher le rôle joué par cette acidification dans l'inhibition de la photosynthèse par l'acide linolénique. Une analyse de la distribution du carbone radioactif dans les intermédiaires du cycle de réduction du CO_2 démontre que l'acide linolénique inhibe l'activité des hexose- et sédoheptulose- phosphatases dans le chloroplaste intact.

II. MATÉRIELS ET MÉTHODES

Préparation de chloroplastes intacts

Les chloroplastes intacts sont extraits de feuilles d'Épinard (*Spinacia oleracea* L.) obtenus sur le marché local. Après une préillumination des feuilles durant 60 min, les chloroplastes intacts sont préparés dans un milieu contenant 25 mM d'acide N-2-hydroxy-éthylpipérazine-N'-2-éthane sulfonique (HEPES-NaOH, pH 7.6), 330 mM sorbitol, 2 mM EDTA-Na (pH 7.6) et 0.2 mM Na-isoascorbate, comme il a été décrit antérieurement (SIEGENTHALER et MVÉ AKAMBA, 1979). La concentration en chlorophylle est déterminée selon la méthode de BRUINSMA (1961).

Réaction de fixation de $^{14}CO_2$

La fixation de $^{14}CO_2$ se fait dans un tube à essai contenant 6 ml du mélange réactionnel suivant : 350 mM sorbitol, 50 mM HEPES-NaOH (pH 7.6), 0.2 mM Na-isoascorbate, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA-Na_4 (pH 7.6), 4 mM Na-pyrophosphate, chloroplastes intacts (0.15 mg chlorophylle.ml^{-1}), 6.6 mM $NaH^{14}CO_3$ (radioactivité spécifique : 5 µCi/µmole). Le mélange réactionnel maintenu à 20°C environ est éclairé par une lampe à incandescence de 500 W avec un éclairement dans le mélange réactionnel voisin de 5.10^6 ergs.cm^{-2}.sec^{-1}. Le faisceau lumineux passe à travers un cylindre de 2 l où circule un courant d'eau froide. Avant l'addition de $NaH^{14}CO_3$ les mélanges réactionnels contenant les chloroplastes sont éclairés durant 5 min pour épuiser le CO_2 dissous. Les mélanges réactionnels sont agités et aux temps indiqués, les échantillons de 0,5 ml de suspensions de chloroplastes sont prélevés et mélangés immédiatement à 0,1 ml de HCl (2 N). Après centrifugation (centrifugeuse de table type Vismara), 20 µl de surnageant sont déposés sur une plaque de verre recouverte d'une couche de cellulose MN 300. Les plaques sont ensuite soumises à une électrophorèse dans un système de solvant pyridine/acide acétique/eau (10/35/955 v/v) à 0°C et 900 V (approximativement 40 mA). Les plaques sont séchées et ensuite soumises à une double chromatographie dans la deuxième dimension en présence d'un mélange de solvants 2-butanol/acide formique/eau (6/1/2 v/v). Le front du solvant atteint 14 cm au bout de 4 h 30 min et 17 cm au bout de 6 h, respectivement à partir du point d'origine (SCHÜRMANN, 1969). Après chromatographie, les taches révélées par autoradiographie (films Typox) sont grattées et

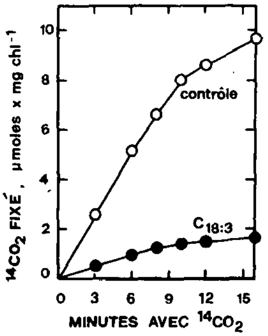

FIG. 1

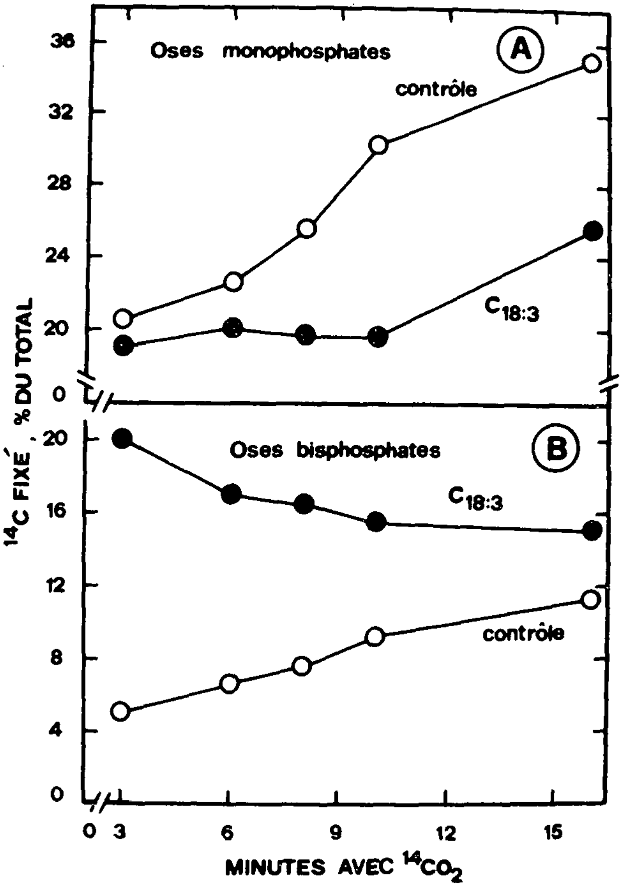

FIG. 2

FIG. 1. — Influence de l'acide linolénique sur la fixation de $^{14}CO_2$ par les chloroplastes intacts à la lumière

— *Influence of linolenic acid on $^{14}CO_2$ fixation in the light by intact chloroplasts*

La concentration finale de $C_{18:3}$ est de 75 µM (0.33 % d'alcool); celle en chlorophylle est de 0.15 mg chl.ml^{-1}. Les mélanges réactionnels contenant les chloroplastes intacts sont illuminés pendant 2 min avant l'addition de 6,6 mM $NaH^{14}CO_3$ (radioactivité spécifique : 0,1 µCi/µmole).

FIG. 2. — Influence de l'acide linolénique sur l'incorporation de ^{14}C dans les phosphates d'oses du cycles de réduction du carbone photosynthétique

— *Influence of linolenic acid on ^{14}C incorporation within phosphate esters of the photosynthetic CO_2 reduction cycle*

Sur les graphiques les symboles représentent (A) l'ensemble des monophosphates d'oses : fructose-, sédoheptulose-, ribose-, ribulose-monophosphates; (B) l'ensemble des bisphosphates d'oses : ribulose-, fructose-, sédoheptulose-, bisphosphates. La valeur 100 % représente les quantités totales de ^{14}C incorporées dans les chloroplastes aux temps indiqués (*fig.* 1). La concentration finale de $C_{18:3}$ est 75 µM.

mélangées à 1,5 ml d'eau auxquels sont ajoutés 10 ml d'un mélange de liquide de scintillation et de Triton X-100 (2 : 1, v/v). Les taches radioactives sont identifiées par comparaison avec des substances authentiques séparées dans les mêmes conditions d'électrophorèse et chromatographie.

Dans les expériences de fixation de CO_2 (*fig.* 1), la radioactivité totale est estimée selon la méthode de DAVIS et COCKING (1966) en déposant des aliquots (0,1 ml) d'extrait acide de surnageant de chloroplastes sur des rondelles de papier en fibre de verre comme décrit précédemment (SIEGENTHALER et MVÉ AKAMBA, 1979).

Tous les mélanges réactionnels contiennent une quantité d'alcool équivalente à celle introduite dans les milieux de réaction contenant de l'acide linolénique (Fluka, Suisse). Dans ce dernier cas, l'acide linolénique (75 µM), fraîchement dissous dans l'éthanol, est ajouté dans le mélange réactionnel avant le $NaH^{14}CO_3$ et mélangé délicatement une dizaine de fois en évitant de briser les chloroplastes intacts.

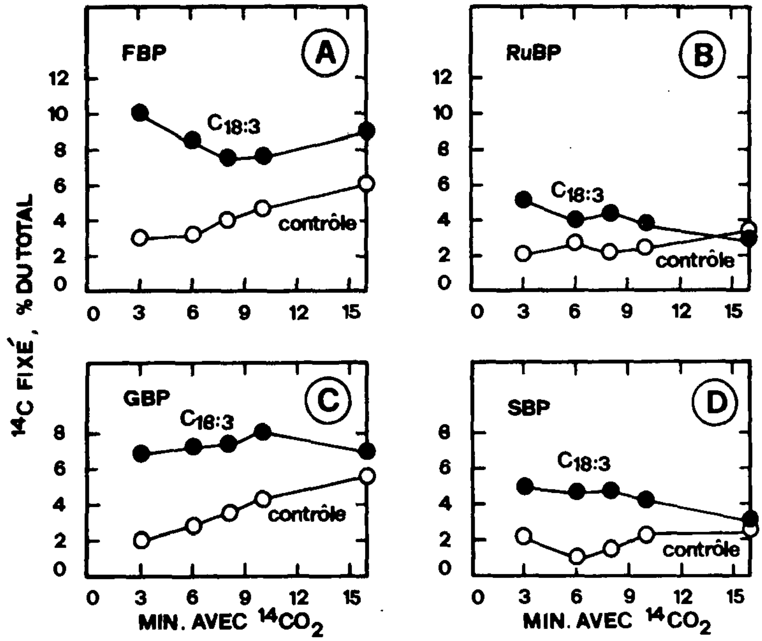

FIG. 3. — Influence de l'acide linolénique sur les cinétiques d'incorporation de ^{14}C dans chaque bisphosphate d'oses du cycle de réduction du carbone photosynthétique

— *Influence of linolenic acid on the kinetics of ^{14}C incorporation within each bisphosphate sugars of the photosynthetic CO_2 reduction cycle*

(A) FBP, fructose 1.6-bisphosphate; (B) RuBP, ribulose 1.5-bisphosphate; (C) GBP, glucose 1.6-bisphosphate; (D) SBP, sédoheptulose 1.7-bisphosphate. La valeur 100 % représente la quantité totale de ^{14}C fixée dans les chloroplastes aux temps indiqués. La concentration de $C_{18:3}$ est de 75 µM.

III. RÉSULTATS EXPÉRIMENTAUX

Les suspensions de chloroplastes utilisées dans ces expériences contiennent environ 80 % de chloroplastes entourés de leur enveloppe externe selon le test au ferricyanure de potassium (HEBER et SANTARIUS, 1970) et présentent des vitesses de dégagement d'oxygène ou de fixation de CO_2 de l'ordre de

40-70 µmoles.mg chl^{-1}.h^{-1}. La figure 1 montre que les quantités de $^{14}CO_2$ fixé augmentent linéairement durant les 10 premières minutes de mesure et aussi que l'addition d'acide linolénique inhibe fortement la vitesse de fixation de $^{14}CO_2$.

Lorsque des échantillons d'extraits acides provenant de chloroplastes intacts (chloroplastes plus milieu extérieur) sont séparés par chromatographie et électrophorèse en couche mince, 12 taches correspondant à des esters phosphates du cycle de Calvin-Benson sont identifiées conformément à la carte chromatographique établie par Schürmann (1969). On peut ainsi suivre la cinétique d'incorporation du ^{14}C dans les différents esters phosphates formés au cours de la photosynthèse. La figure 2 montre que l'incorporation de ^{14}C augmente davantage dans les esters monophosphates (*fig.* 2 A) que dans les esters bisphosphates (*fig.* 2 B). Cette différence entre la teneur endogène des mono- et bisphosphates d'oses est une expression de l'activité enzymatique des hexose-bisphosphatases dans les réactions de la photosynthèse à l'équilibre (Bassham *et al.*, 1968). Si on considère les cinétiques d'incorporation de ^{14}C dans chaque métabolite intermédiaire pris individuellement on remarque que les quantités de fructose-, ribulose-, sédoheptulose- et glucose-bisphosphates augmentent en fonction du temps (*fig.* 3). La présence de glucose 1.6-bisphosphate dans les extraits de chloroplastes isolés a déjà été observée par

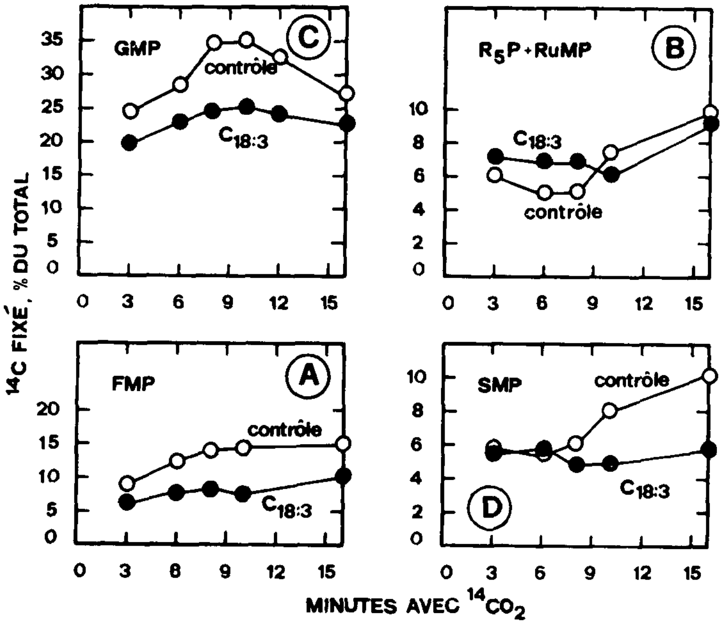

Fig. 4. — Influence de l'acide linolénique sur les cinétiques d'incorporation de ^{14}C dans chaque monophosphate d'oses du cycle de réduction du carbone photosynthétique

— *Influence of linolenic acid on the kinetics of ^{14}C incorporation within each monophosphate esters of the photosynthetic CO_2 reduction cycle*

(A) FMP, fructose monophosphate; (B) R_5P + RuMP, ribose- + ribulose monophosphate; (C) GMP, glucose monophosphate; (D) SMP, sédoheptulose monophosphate. La valeur 100 % représente la quantité totale de ^{14}C fixé dans les chloroplastes aux temps indiqués (voir *fig.* 1). La concentration de $C_{18:3}$ est de 75 µM.

SCHÜRMANN *et al.* (1971). L'incorporation de ^{14}C dans ces métabolites bisphosphates est beaucoup plus faible que l'incorporation de ^{14}C dans les homologues monophosphates (*fig.* 4). Ceci confirme que dans les chloroplastes intacts illuminés l'activité des enzymes phosphatases est très élevée (BASSHAM *et al.*, 1968).

La figure 5 présente les cinétiques d'incorporation de la radioactivité dans l'acide 3-phosphoglycérique (*fig.* 5 A) et le dihydroxyacétone phosphate (*fig.* 5 B). L'APG est le plus abondant des métabolites du cycle de Calvin-Benson (35 % du total après 3 min). La quantité d'APG diminue au bout de 6 min en même temps que la quantité de DHAP augmente, traduisant ainsi une activité croissante des enzymes catalysant la phase de réduction du carbone entre l'APG et le DHAP.

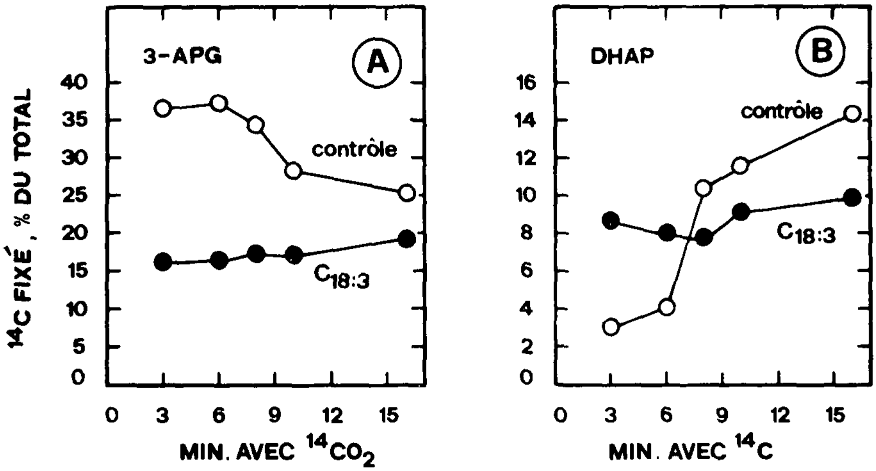

FIG. 5. — Influence de $C_{18:3}$ sur l'incorporation de ^{14}C dans l'acide 3-phosphoglycérique et le dihydroxyacétone phosphate

— *Influence of linolenic acid on ^{14}C incorporation within 3-phosphoglyceric acid and dihydroxyacetone phosphate*

A) 3-APG, acide 3-phosphoglycérique; (B) DHAP, dihydroxyacétone phosphate. La valeur 100 % est la quantité totale de ^{14}C, fixé dans les chloroplastes aux temps indiqués. La concentration en $C_{18:3}$ est de 75 µM.

Le traitement des chloroplastes par l'acide linolénique (rapport molaire $C_{18:3}$/Chl égal à 0,45) provoque une diminution de l'incorporation de la radioactivité dans les monophosphates d'oses et, au contraire, une augmentation de l'incorporation de ^{14}C dans les bisphosphates d'oses (*fig.* 2). Ceci suggère que l'acide linolénique inhibe les activités des enzymes phosphatases. L'examen détaillé des taux d'incorporation de ^{14}C dans les métabolites intermédiaires montre qu'en présence d'acide linolénique l'incorporation de ^{14}C augmente dans chaque bisphosphate d'ose (*fig.* 3), alors qu'elle diminue de façon significative dans chaque monophosphate d'ose homologue (*fig.* 4). Il en est de même pour le DHAP et l'APG (*fig.* 5).

IV. DISCUSSION

Les résultats présentés ici apportent une réponse au problème que pose l'inhibition de la photosynthèse des chloroplastes intacts par l'acide linolénique. Les cinétiques de formation des mono- et bisphosphates d'oses (*fig.* 2) révèlent une intense activité de conversion des bisphosphates d'oses en leurs homologues monophosphates dans les chloroplastes non traités. L'addition d'acide linolénique à des chloroplastes intacts inhibe l'activité des hexose-phosphatases puisque la teneur en esters bisphosphates augmente alors que celle de leurs homologues monophosphates diminue (*fig.* 2). L'acide linolénique inhibe donc l'activité des deux principales phosphatases du cycle de réduction du CO_2 qui catalysent la conversion du fructose 1.6-bisphosphate (FBP) en fructose 6-phosphate et du sédoheptulose 1.7-bisphosphate (SBP) en sédoheptulose 7-phosphate (*fig.* 3 et 4). Une pareille inhibition des enzymes hexose-phosphatases a été observée dans les algues (*Chlorella pyrenoidosa* L.) traitées par un autre acide gras non physiologique, l'octanoate (PEDERSEN *et al.*, 1966). En outre, l'accumulation du ribulose 1.5-bisphosphate (*fig.* 3) dans les chloroplastes traités par l'acide linolénique, et la diminution concomitante des quantités d'APG et de DHAP (*fig.* 5), révèlent que l'activité de l'enzyme ribulose 1.5-bisphosphate carboxylase est également inhibée dans le chloroplaste intact.

Nous avions démontré récemment que l'acide linolénique est sans action sur les enzymes solubles isolés du stroma des chloroplastes et sur le transport des électrons dans les thylacoides isolés (SIEGENTHALER et MVÉ AKAMBA, 1979); or les résultats de ce travail établissent clairement que dans des chloroplastes intacts traités par l'acide linolénique l'activité des phosphatases et de la ribulose 1.5-bisphosphate carboxylase décroît fortement. Il semble donc que l'acide linolénique n'inhibe la photosynthèse que dans la mesure où les chloroplastes sont pourvus de leur enveloppe. Seule la perturbation des échanges entre le stroma et le milieu externe peut expliquer une telle inhibition. En inhibant l'absorption de phosphate inorganique dans les chloroplastes intacts d'Épinard (SIEGENTHALER et MVÉ AKAMBA, 1979; MVÉ AKAMBA et SIEGENTHALER, 1979 *b*), l'acide linolénique empêche l'alcalinisation du stroma qui est normalement liée à ce transport (MVÉ AKAMBA, 1979; SIEGENTHALER, MVÉ AKAMBA et JANECKA, en préparation). L'acidification conséquente du stroma qui d'ailleurs a été observée par PURCZELD *et al.* (1978) en présence d'octanoate, peut rendre compte de l'inhibition des enzymes hexose-phosphatases observée dans nos expériences. Plusieurs auteurs ont montré en effet que l'activité de la fructose 1.6-bisphosphatase était très sensible aux variations de pH (GARNIER et LATZKO, 1972; KELLY *et al.*, 1976). L'inhibition de la RuBP carboxylase peut donc s'expliquer par l'accumulation des bisphosphates d'oses (FBP et SBP), dont le rôle inhibiteur sur la RuBP carboxylase a été largement démontré (BUCHANAN et SCHÜRMANN, 1973; CHU et BASSHAM, 1975; SALUJA et MCFADDEN, 1978).

Le mécanisme de l'inhibition de la photosynthèse par l'acide linolénique ainsi précisé nous semble convaincant dans la mesure où les oses bisphosphates normalement exportés hors du chloroplaste intact (BASSHAM *et al.*, 1968; HELDT et RAPLEY, 1970; HEBER, 1974) sont bloqués dans le stroma (SIEGENTHALER et MVÉ AKAMBA, 1979). En effet, l'inhibition de l'efflux de 3-phosphoglycéraldéhyde par l'acide linolénique (SIEGENTHALER et MVÉ AKAMBA, 1979) et de la diffusion des bisphosphates d'oses hors du chloroplaste intact (MVÉ AKAMBA, 1979; MVÉ AKAMBA et SIEGENTHALER, manuscrit en préparation) en apportent la démonstration.

Remarquons enfin que la radioactivité incorporée dans le glucose 1-phosphate diminue en présence de l'acide linolénique à l'inverse de celle de son homologue bisphosphate qui augmente (*fig.* 3 et 4). Cette observation suggère que la conversion du glucose 1-phosphate en glucose 1.6-bisphosphate catalysée par la phosphoglucokinase est non seulement possible mais que cette réaction, consommatrice d'ATP, serait stimulée dans les chloroplastes traités par l'acide linolénique. Cette stimulation pourrait être due à un excès d'ATP dans les chloroplastes traités par cet acide. Cette hypothèse semble s'accorder avec le fait que la conversion de l'APG en DHAP, elle aussi consommatrice d'ATP, est stimulée au cours des 6 premières minutes en présence de l'acide linolénique (*fig.* 5).

En conclusion, l'acide linolénique semble inhiber la photosynthèse des chloroplastes intacts en modifiant simultanément le transporteur de phosphate, le pH du stroma et l'activité des phosphatases. L'accumulation des bisphosphates d'oses qui en résulte pourrait à son tour expliquer le blocage de la RuBP carboxylase observée dans nos résultats.

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Effet de l'acide linolénique sur la photosynthèse de chloroplastes intacts de feuilles d'Épinard

V. Modification de la distribution des intermédiaires du cycle de réduction du CO_2 entre le stroma et le milieu extérieur

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RÉSUMÉ

Mots clés : acide linolénique, photosynthèse, chloroplastes intacts, mono- et bisphosphates d'oses.

La distribution des métabolites marqués au ^{14}C dans le milieu intérieur (stroma) et le milieu extérieur séparés par centrifugation des suspensions de chloroplastes intacts a été mesurée en cours de photosynthèse. Les cinétiques de répartition des métabolites révèlent que dès les premières minutes de la photosynthèse, l'ensemble des bisphosphates d'oses, notamment le fructose 1,6-bisphosphate, diffusent considérablement vers le milieu extérieur tandis que les monophosphates d'oses restent relativement plus localisés dans le milieu intérieur. Le traitement des chloroplastes intacts par de l'acide linolénique a pour effet de bloquer la diffusion de l'ensemble de ces esters phosphates vers le milieu extérieur. Cependant, la diffusion de l'acide 3-phosphoglycérique, et dans une moindre mesure de la dihydroxyacétone phosphate et du sédoheptulose monophosphate tend à augmenter en présence d'acide linolénique.

Ces résultats s'accordent avec nos travaux antérieurs démontrant que l'acide linolénique inhibe la photosynthèse de chloroplastes intacts en perturbant les échanges de phosphate inorganique et de métabolites phosphorylés au niveau des enveloppes externes de chloroplastes. Le déficit en orthophosphate dans le milieu intérieur et la concentration pléthorique de bisphosphates d'oses dont il s'accompagne expliqueraient l'inhibition de la photosynthèse de chloroplastes intacts d'Épinard par l'acide linolénique. Le modèle d'une telle inhibition est discuté.

SUMMARY

Key words: linolenic acid, photosynthesis, intact chloroplasts, sugar mono- and bisphosphates.

Distribution of ^{14}C-labelled metabolites between the stroma and the external medium of intact spinach chloroplast preparations was analysed during photosynthesis. When kinetics of distribution of metabolites was studied, sugar bisphosphates, namely fructose 1,6-*bisphosphate, appeared to diffuse considerably toward the external medium whereas most of sugar monophosphates remained in the stroma.*

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** A qui les demandes de tirés à part doivent être adressées.

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In linolenate-treated intact chloroplasts diffusion of sugar bisphosphates was impaired but 3-P-glycerate, and to a lesser degree dihydroxyacetone phosphate and sedoheptulose monophosphates appeared to be relatively more exported toward the external medium.

These results substantiate previous reports that linolenate inhibits photosynthesis in intact chloroplasts by impairing the exchange of orthophosphate and internal phosphorylated intermediates across the chloroplast envelope. The shortage in the supply of orthophosphate and the increase in sugar bisphosphates concentration can account for the inhibition of photosynthesis in intact spinach chloroplasts treated with linolenate.

A model explaining this inhibition is discussed.

1. INTRODUCTION

Nous avons démontré que l'acide linolénique inhibe la photosynthèse de chloroplastes intacts (SIEGENTHALER et MVÉ AKAMBA, 1979; MVÉ AKAMBA et SIEGENTHALER, 1979 *a* et *b*). Cette inhibition est liée à un blocage du fonctionnement du transporteur de phosphate/trioses phosphates (MVÉ AKAMBA et SIEGENTHALER, 1979 *b*) localisé dans l'enveloppe du chloroplaste (HELDT et RAPLEY, 1970; HEBER, 1974) et à une modification de la distribution de l'incorporation de ^{14}C dans les intermédiaires du cycle de réduction du CO_2 photosynthétique (MVÉ AKAMBA et SIEGENTHALER, 1979 *c*). Aux concentrations utilisées dans ces expériences, l'acide linolénique n'inhibe ni le transport des électrons $H_2O/NADP^+$ dans les thylacoides isolés (SIEGENTHALER et DEPÉRY, 1976) ni les activités des enzymes solubles isolées du chloroplaste (SIEGENTHALER et MVÉ AKAMBA, 1979). Ces résultats apparemment contradictoires semblent indiquer que l'inhibition de la RuBPcarboxylase pourrait être due à l'accumulation de certains oses, notamment de bisphosphates d'oses comme le suggèrent des expériences relatives à la distribution de la radioactivité dans les intermédiaires du cycle de réduction du CO_2, réalisées avec des extraits totaux de chloroplastes (MVÉ AKAMBA et SIEGENTHALER, 1979 *c*).

Or, dans les conditions normales de la photosynthèse, chaque métabolite du cycle de Calvin-Benson se trouve à une concentration relative donnée, au-dessus de laquelle il peut agir sur le déroulement des autres séquences enzymatiques du cycle (BUCHANAN et SCHÜRMANN, 1973; CHU et BASSHAM, 1975; CHAMPIGNY, 1976). En perturbant les échanges de métabolites et de phosphate au niveau de l'enveloppe (SIEGENTHALER et MVÉ AKAMBA, 1979; MVÉ AKAMBA et SIEGENTHALER, 1979 *a*, *b*) l'acide linolénique devrait avoir pour conséquence une accumulation de certains métabolites dans le stroma. Nous nous proposons de vérifier cette hypothèse en recherchant si les intermédiaires du cycle de réduction du CO_2, dont certains s'accumulent relativement plus que d'autres, restent dans le stroma ou bien sont exportés vers le mélange réactionnel en présence d'acide linolénique.

Le présent travail met en évidence que, sous l'effet de l'acide linolénique, les bisphosphates d'oses s'accumulent préférentiellement dans le stroma tandis que les monophosphates d'oses diffusent plus facilement vers le mélange réactionnel.

2. MATÉRIEL ET MÉTHODES

Préparation de chloroplastes intacts

Les chloroplastes intacts sont extraits de feuilles d'Épinard (*Spinacia oleracea* L.) obtenues sur le marché local. Après une préillumination des feuilles durant 1 h, les chloroplastes intacts sont préparés dans un milieu contenant 25 mM d'acide N-2-hydroxy-éthylpipérazine-N'-2-éthane sulfonique (Hepes-NaOH, pH 7,6), 330 mM sorbitol, 2 mM EDTA-Na_4 (pH 7,6) et 0,2 mM Na-isoascorbate, comme il a été décrit antérieurement (SIEGENTHALER et MVÉ AKAMBA, 1979).

La concentration en chlorophylle est déterminée selon la méthode de BRUINSMA (1961) et le pourcentage de chloroplastes intacts (70-80 %) estimé selon le test au ferricyanure de potassium (HEBER et SANTARIUS, 1970).

Fixation de CO_2

La fixation du CO_2 est déterminée comme il a été décrit antérieurement (MVÉ AKAMBA et SIEGENTHALER, 1979 *c*) dans un tube à essai contenant 6 ml du mélange réactionnel suivant : 350 mM sorbitol, 50 mM Hepes-NaOH (pH 7,6), 0,2 mM Na-isoascorbate, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA-Na_4 (pH 7,6), 4 mM Na-pyrophosphate et des chloroplastes intacts (150 µg de chlorophylle/ml). Ce mélange est préilluminé (10^6 ergs.cm^{-2}.sec^{-1}) à 20°C pendant 5 min avant l'addition de 6,6 mM $NaH^{14}CO_3$ (radioactivité spécifique : 5 µCi/µmole). Aux temps de réaction en présence de bicarbonate radioactif indiqués dans la figure 1, des échantillons de 0,5 ml de suspensions de chloroplastes sont prélevés et centrifugés pendant 20-30 sec (centrifugeuse de table Vismara) puis 0,1 ml de HCl (2 N) est ajouté au culot et au surnageant (milieu extérieur, E). Les culots sont dilués avec 0,1 ml d'eau distillée et homogénéisés par agitation sur Vortex, puis rencentrifugés (env. 5 min); le surnageant clair résultant correspond au stroma du chloroplaste et sera appelé milieu intérieur, I. La comparaison de la quantité de radioactivité présente dans les milieux E et I renseigne sur les flux globaux des métabolites du cycle de réduction du carbone photosynthétique au cours de la réaction lumineuse. La radioactivité totale est estimée selon la méthode de DAVIS et COCKING (1966) en déposant des aliquotes (0,1 ml) d'extrait acide de surnageant de chloroplastes sur des rondelles de papier en fibre de verre comme décrit précédemment (SIEGENTHALER et MVÉ AKAMBA, 1979).

Séparation des métabolites par électrophorèse et chromatographie

Le mélange réactionnel et les conditions expérimentales sont pareils à ceux utilisés pour la fixation du CO_2. Des échantillons d'extraits (généralement 20 µl) provenant du milieu intérieur (I) ou du milieu extérieur (E) sont soumis à une électrophorèse suivie d'une double chromatographie comme il a été décrit (SCHÜRMANN, 1969; MVÉ AKAMBA et SIEGENTHALER, 1979 *c*).

Addition d'acide linolénique

Tous les mélanges réactionnels contiennent 0,3 % d'alcool. L'acide linolénique (Fluka, Suisse) est fraîchement dissous dans l'éthanol, ajouté aux mélanges réactionnels avant le $NaH^{14}CO_3$ puis mélangé délicatement.

3. RÉSULTATS EXPÉRIMENTAUX

La figure 1 présente les taux d'incorporation totale de ^{14}C dans la préparation de chloroplastes intacts. Au cours de la photosynthèse en présence de bicarbonate radioactif, la majeure partie du ^{14}C fixé sous forme acide stable apparaît dans le milieu extérieur aux chloroplastes, comme l'ont également observé BASSHAM *et al.* (1968). Le traitement des chloroplastes intacts par l'acide linolénique diminue l'incorporation de ^{14}C tant dans le milieu extérieur (*fig.* 1 A) que dans le milieu intérieur (*fig.* 1 B).

Lorsque des extraits chloroplastiques acides sont séparés par électrophorèse et chromatographie en couche mince, 12 taches radioactives sont identifiées sur les plaques et correspondent aux principaux esters phosphates du cycle de réduction du carbone photosynthétique (*fig.* 2). La figure 2 A montre que les 12 principaux métabolites sont présents dans le milieu externe aux chloroplastes intacts en l'absence d'acide linolénique. L'examen de la figure 2 C révèle qu'en l'absence d'acide linolénique, seules les taches correspondant à l'acide 3-phosphoglycérique, à la dihydroxyacétone-P, aux monophosphates d'oses, et, dans une moindre mesure, au ribulose 1,5-bisphosphate sont perceptibles dans le milieu intérieur. Les taches correspondant aux bisphosphates d'oses sont peu visibles et souvent disparaissent complètement au cours de la photosynthèse. L'addition de 75 µM d'acide linolénique aux suspensions de chloroplastes intacts a pour effet d'éliminer du milieu extérieur les taches radioactives relatives aux bisphosphates d'oses (*fig.* 2 B) et de diminuer sensiblement les taches correspondant aux dihydroxyacétone-P, ribose 5-P et fructose 6-P. La présence d'acide linolénique renforce de façon très significative les taches des bisphosphates d'oses dans le milieu intérieur (*fig.* 2 D). Ainsi, l'examen de la distribution de ^{14}C entre les milieux extérieur et intérieur révèle que dès les 6 premières minutes de la photosynthèse les bisphosphates d'oses sont abondamment exportés hors du chloroplaste intact et que le traitement de ces chloroplastes par l'acide linolénique supprime cette exportation.

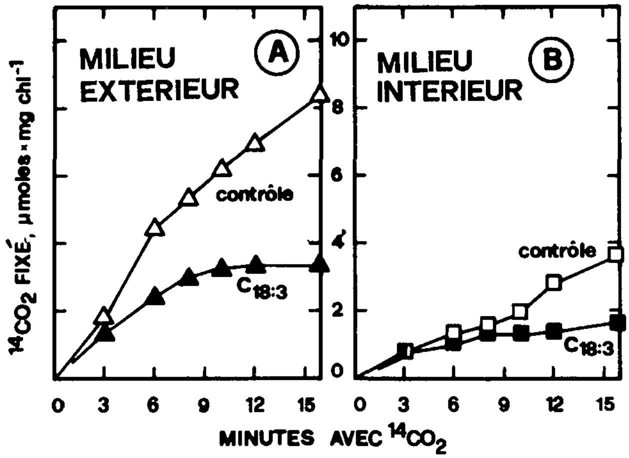

FIG. 1. — Influence du linolénate sur la fixation de $^{14}CO_2$ par des chloroplastes intacts à la lumière et sur la distribution de la radioactivité non volatile entre le milieu extérieur (A) et intérieur (B).

— *Influence of linolenate on $^{14}CO_2$ fixation in the light by intact chloroplasts and on the non volatile radioactivity distribution between the external (A) and internal medium (B).*

La concentration d'acide linolénique dans le mélange réactionnel est de 75 µM (0,3 % d'alcool). Les conditions expérimentales sont décrites dans « Matériel et Méthodes ».

Le marquage des acides aminés apparaît irrégulièrement dans certaines expériences comme par exemple dans les figures 2 B et C. La présence de ces acides dans des extraits chloroplastiques a aussi été observée par SCHÜRMANN (1969). Il n'a pas été possible d'établir une quelconque corrélation entre la présence de ces acides marqués et le traitement des chloroplastes par l'acide linolénique. Des contaminations d'enzymes cytoplasmiques semblent pouvoir expliquer la biosynthèse d'acides aminés à partir des métabolites photosynthétiques comme l'ont suggéré BASSHAM *et al.* (1968).

Le tableau I présente les quantités de ^{14}C fixé dans différents esters phosphates du cycle de réduction du CO_2 photosynthétique trouvés dans les milieux extérieur et intérieur après 3 et 6 min à la lumière. La quantité totale (E + I) de ^{14}C fixé est beaucoup plus petite dans les esters bisphosphates que dans les esters monophosphates, comme nous l'avons démontré par ailleurs (MVÉ AKAMBA et SIEGENTHALER, 1979 *c*). Après 3 et 16 min à la lumière les quantités de ^{14}C fixé

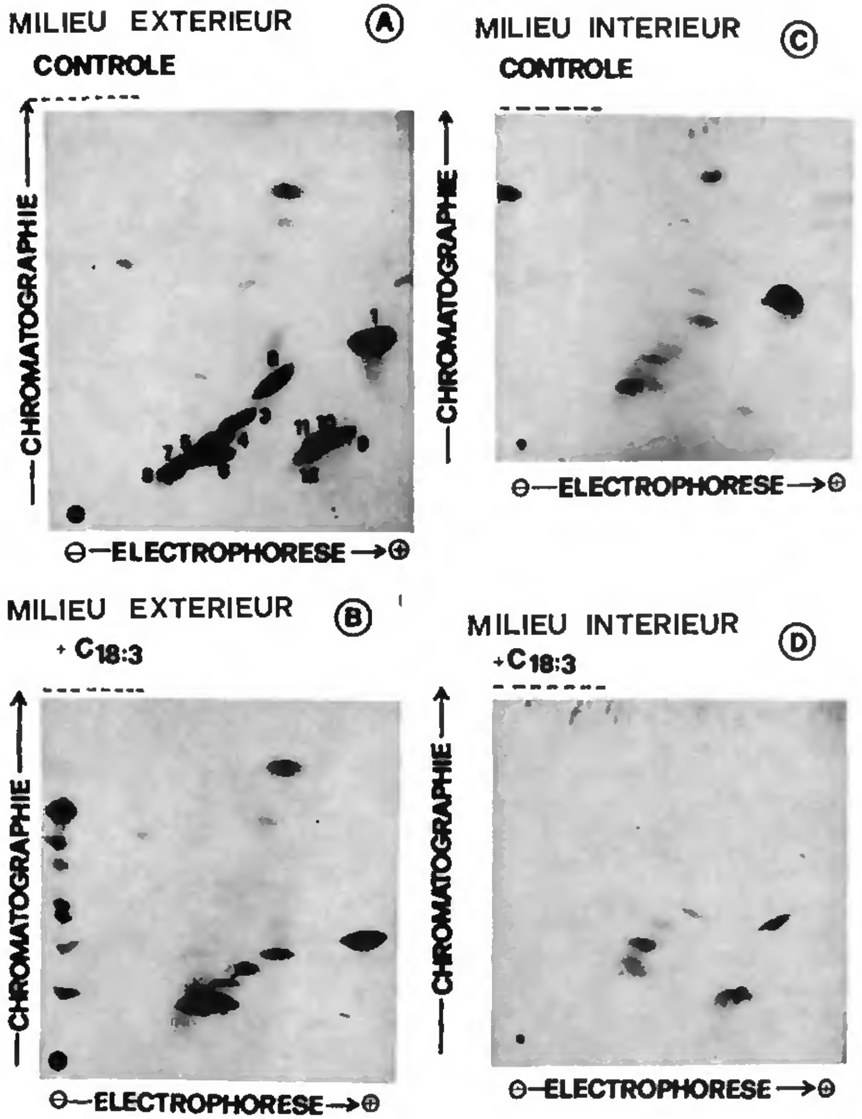

FIG. 2. — Autoradiographie des intermédiaires photosynthétiques présents dans le milieu intérieur du chloroplaste ou dans le milieu extérieur. Influence du linolénate

— *Autoradiography of photosynthetic intermediates present in the internal compartment or in the medium. Influence of linolenate*

La concentration d'acide linolénique ($C_{18:3}$) dans le mélange réactionnel est de 75 µM (0,3 % d'alcool). Temps de la réaction lumineuse : 6 min. Les conditions expérimentales sont décrites dans « Matériel et Méthodes ». (A) Milieu extérieur sans $C_{18:3}$; (B) Milieu extérieur avec $C_{18:3}$; (C) Milieu intérieur sans $C_{18:3}$; (D) Milieu intérieur avec $C_{18:3}$; (1) Acide 3 P-glycérique; (2) Dihydroxyacétone-P; (3) Ribose 5-P et glycéraldéhyde 3-P; (4) Fructose 6-P; (5) Glucose 1-P; (6) Glucose 6-P; (7) Sédoheptulose 7-P; (8) Composé non identifié; (9) Ribulose 1,5-bis-P; (10) Fructose 1,6-bis-P; (11) Glucose 1,6-bis-P; (12) Sédoheptulose 1,7-bis-P.

dans chaque ester phosphate sont plus abondantes à l'extérieur qu'à l'intérieur à l'exception du ribulose 1,5-bis-P (à 3 min) et du sédoheptulose monophosphate et dihydroxyacétone-P (à 16 min). Bien que la quantité globale de fructose 1,6-bis-P marqué soit plus faible que celle de son homologue monophosphate, il est remarquable de constater que le premier ester est relativement plus abondant à l'extérieur que le second. Chez les chloroplastes traités par l'acide linolénique la quantité totale de ^{14}C fixé est plus faible, ce qui est en accord avec nos résultats précédents (MVÉ AKAMBA et SIEGENTHALER, 1979 *c*). D'une façon générale, la quantité de métabolites marqués est relativement plus faible à l'extérieur qu'à l'intérieur (tableau I).

TABLEAU I

Quantité de ^{14}C fixé dans différents métabolites phosphorylés du cycle de Calvin, après 3 et 16 min de photosynthèse (les chiffres représentent des nmoles de $^{14}C.mg$ chlorophylle^{-1})

Fixed ^{14}C amount in different phosphorylated metabolites of Calvin cycle after 3 and 16 min of photosynthesis

Métabolites phosphorylés	Contrôle				75 µM $C_{18:3}$			
	3 min		16 min		3 min		16 min	
	(E)	(I)	(E)	(I)	(E)	(I)	(E)	(I)
Fructose 1,6-bis-P	73	5	738	12	30	30	81	81
Ribulose 1,5-bis-P	26	26	396	79	15	15	45	9
Glucose 1,6-bis-P	45	7	529	96	28	14	78	39
Sedoheptulose 1,7-bis-P	45	7	224	89	21	9	30	38
Glucose mono-P	368	282	2 533	904	40	80	221	184
Fructose mono-P	133	101	1 340	535	14	28	120	60
Sedoheptulose mono-P	128	28	560	690	17	17	81	27
Acide 3-P-glycérique	624	312	2 557	568	64	32	296	46
Dihydroxyacétone P	62	16	875	875	26	25	143	38
Total des métabolites phosphorylés (E + I)	2 288		13 600		433		1 617	
Total de ^{14}C fixé	2 600		14 700		600		1 800	
Autres substances marquées	312		1 100		167		180	

Les rapports des quantités de ^{14}C fixé dans chaque métabolite à l'extérieur et à l'intérieur du chloroplaste intact représentent les taux de diffusion de ces métabolites vers le milieu extérieur. La figure 3 montre que chez les chloroplastes non traités par l'acide linolénique (contrôles) les taux de diffusion ou d'exportation (rapport E/I) de trois catégories de phosphates d'oses augmentent surtout après 9 min de photosynthèse. La valeur E/I des bisphosphates d'oses est beaucoup plus élevée (6 à 8 fois; *fig.* 3 *a*) que celle des oses monophosphates et du glucose monophosphate (1 à 2 fois; *fig.* 3 *b* et *c*). L'exportation de ces composés est inhibée par l'acide linolénique pendant les 16 premières minutes de réaction à la lumière.

La figure 4 rend compte des vitesses d'exportation de chaque bisphosphate d'ose considéré individuellement. Au cours de la réaction lumineuse de fixation de CO_2 par des chloroplastes intacts, on remarque que plusieurs bisphosphates d'oses apparaissent très rapidement dans le milieu extérieur; le fructose bis-P est incontestablement celui qui est le plus abondamment exporté (*fig.* 4 *a*, E/I = 19 au temps 3 min comparé à E/I = 6 pour les sédoheptulose- et glucose-bis-P, *fig.* 4 *b* et *d*). Le taux d'exportation des métabolites varie en fonction du temps (*fig.* 4). Il augmente dans le cas du fructose bis-P et du ribulose 1,5-bis-P et diminue dans le cas des sédoheptulose-bis-P et glucose-bis-P. L'addition de 75 µM d'acide linolénique inhibe généralement l'apparition des bisphosphates d'oses dans le milieu extérieur. En particulier l'exportation du fructose 1,6-bis-P est bloquée complètement (*fig.* 4 *a*), celle du sédoheptulose 1,7-bis-P (*fig.* 4 *b*), du ribulose 1,5-bis-P (*fig.* 4 *c*) et du glucose 1,6-bis-P (*fig.* 4 *d*) diminuent fortement.

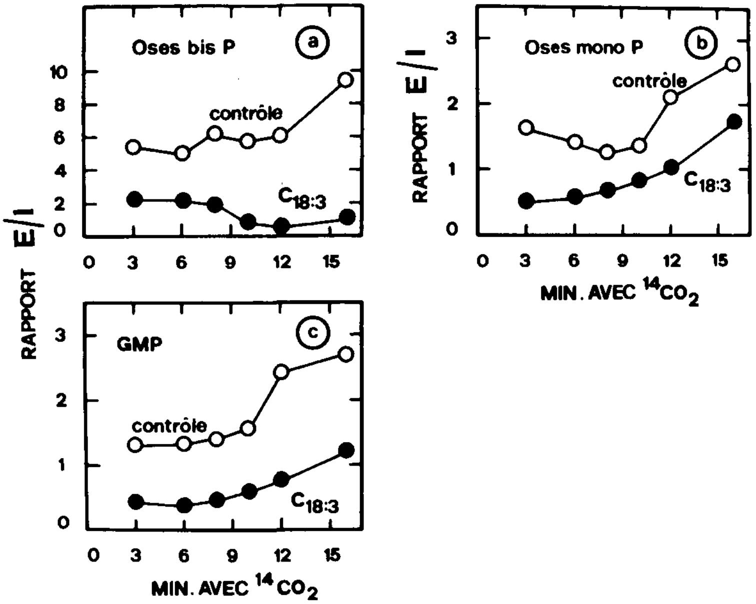

FIG. 3. — Vitesse relative d'apparition des bisphosphates d'oses, des monophosphates d'oses et du glucose monophosphate dans le milieu extérieur aux chloroplastes intacts. Influence du linolénate.

— *Relative rate of appearance of sugar bisphosphates, sugar monophosphates and glucose monophosphate in the chloroplast external medium. Influence of linolenate.*

Les valeurs E/I sont les rapports molaires des quantités de chaque métabolite dans les milieux extérieur (E) et intérieur (I); E/I est considéré comme la vitesse relative d'apparition d'un métabolite dans le milieu extérieur aux chloroplastes intacts. En (*a*) ensemble des bisphosphates d'oses : ribulose-, fructose-, sédoheptulose-, bisphosphates; (*b*) ensemble des monophosphates d'oses : ribose-, ribulose-, fructose-, sédoheptulose-, monophosphates; (*c*) GMP, glucose monophosphate. La concentration d'acide linolénique ($C_{18:3}$) dans le mélange réactionnel est de 75 µM. Autres conditions : *voir* « Matériel et Méthodes ».

En l'absence d'acide linolénique, les trioses-P et les monophosphates d'oses franchissent l'enveloppe du chloroplaste de façon inégale vers le surnageant (*fig.* 5). Le taux d'exportation du fructose 6-P est faible comparé à celui du fructose 1,6-bis-P et croît entre 9 et 16 min à la lumière (*fig.* 5 *c*). Les taux d'exportation des sédoheptulose mono-P (*fig.* 5 *d*) et dihydroxyacétone-P (*fig.* 5 *b*) sont faibles mais décroissent au cours du temps. Le taux d'exportation de l'acide 3-P glycérique varie irrégulièrement et reste faible pendant tout le temps de réaction (*fig.* 5 *a*). L'acide linolénique n'a pas d'effet sur l'exportation du fructose 6-P (*fig.* 5 *c*); à l'inverse, la sortie du sédoheptulose mono-P hors du chloroplaste, fortement inhibée au début, est stimulée après 10 min de photosynthèse (*fig.* 5 *d*). Dans les chloroplastes traités par l'acide linolénique le taux d'exportation de l'acide 3-P glycérique est nettement stimulé (*fig.* 5 *a*); celui de la dihydroxyacétone-P, inhibé au cours des 9 premières minutes, est ensuite stimulé (*fig.* 5 *b*).

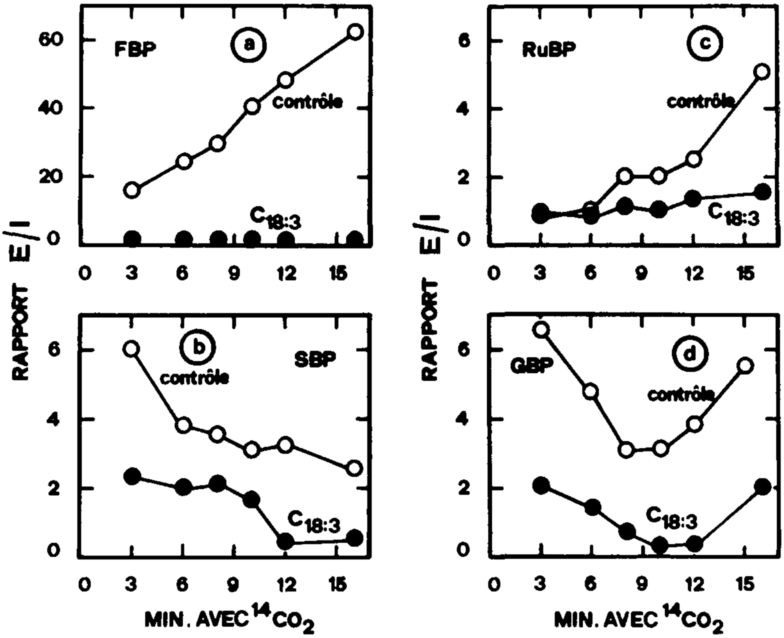

FIG. 4. — Vitesse relative d'apparition des différents bisphosphates d'oses dans le milieu extérieur aux chloroplastes intacts. Influence du linolénate

— *Relative rate of appearance of various sugar bisphosphates in the chloroplast external medium. Influence of linolenate* ($C_{18:3}$, 75 µ*M*)

En (*a*) FBP, fructose 1,6-bis-P; (*b*) SBP, sédoheptulose bis-P; (*c*) RuBP, ribulose 1,5-bis-P; La concentration d'acide linolénique ($C_{18:3}$) est de 75 µM. Autres conditions : *voir* « Matériel et Méthodes ».

4. DISCUSSION

Cette étude révèle que beaucoup de métabolites synthétisés dans le chloroplaste intact sont exportés vers le milieu extérieur au cours de la réaction de fixation du CO_2 à la lumière. Le taux d'exportation de ces métabolites ne semble pas être forcément proportionnel à leurs concentrations dans le stroma. Nos résultats montrent que le taux d'exportation du fructose 1,6-bis-P est très élevé si on le compare à celui du fructose mono-P (*fig.* 4 *a* et 5 *c*); pourtant la quantité totale de fructose 1,6-bis-P radioactif est très faible par rapport à celle de son homologue monophosphate

(Bassham *et al.*, 1968; Mvé Akamba et Siegenthaler, 1979 *c*; *voir* aussi le tableau I). Il en est ainsi des sédoheptulose et glucose dont les formes diesterphosphates sont plus vite exportées, bien que leurs concentrations totales soient faibles (*voir* tableau I). Les métabolites les plus concentrés dans le stroma ne sont donc pas nécessairement ceux qui diffusent le plus vers le milieu extérieur. L'exportation de ces métabolites ne semble donc pas procéder uniquement d'une diffusion passive. D'autre part, le nombre de groupements phosphate estérifiés sur un métabolite photosynthétique augmenterait son aptitude à sortir du chloroplaste intact au cours de la photosynthèse. A nombre égal de groupements esters de phosphate, la longueur de la chaîne carbonée d'un métabolite paraît aussi jouer un rôle dans son aptitude à sortir du chloroplaste intact; la dihydroxyacétone-P et l'acide 3-P glycérique sont ainsi plus vite exportés que le sédoheptulose mono-P, le fructose 6-P et le glucose mono-P (*fig.* 3 et 5).

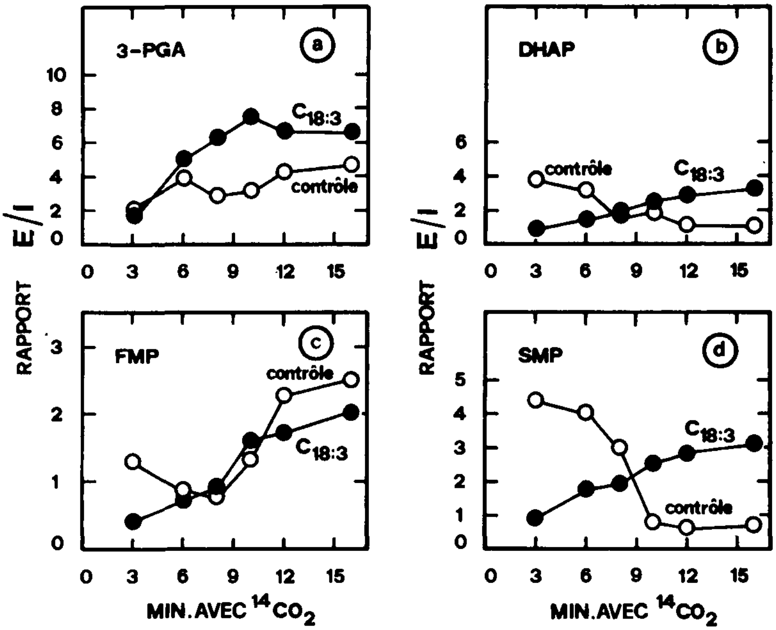

Fig. 5. — Vitesse relative d'apparition de différents trioses-P et monophosphates d'oses dans le milieu extérieur aux chloroplastes intacts. Influence du linolénate

— *Relative rate of appearance of triose-P and various sugar monophosphates in the chloroplast external medium. Influence of linolenate* ($C_{18:3}$, 75 µM)

(*a*) 3-APG, acide 3-phosphoglycérique; (*b*) DHAP, dihydroxyacétone-P; (*c*) FMP, fructose mono-P; (*d*) SMP, sédoheptulose mono-P. La concentration de $C_{18:3}$ est 75 µM. Autres conditions : *voir* « Matériel et Méthodes ».

La présence précoce de plusieurs métabolites phosphates dans le milieu extérieur a été signalée par de nombreux auteurs (Bassham *et al.*, 1968; Lilley *et al.*, 1977). Ceux-ci constatent comme nous d'ailleurs que les trioses-P, l'acide 3-P-glycérique et le fructose 1,6-bis-P sont présents en plus grande quantité dans le milieu extérieur que dans le stroma. En outre, comme Bassham *et al.* (1968), nous observons une quantité de sédoheptulose bis-P plus grande dans le milieu extérieur que dans le stroma, ce qui est en désaccord avec LILLEY *et al.* (1977).

Pour les groupes de BASSHAM et LILLEY, les trioses-P sont les plus abondamment exportés au cours de la photosynthèse; à l'inverse nous n'avons pas observé des taux d'exportation privilégiés pour ces métabolites mais plutôt pour le fructose 1,6-bis-P. Bien qu'observée par de nombreux auteurs (BASSHAM *et al.*, 1968; LILLEY *et al.*, 1977; *fig.* 4 *a*) l'apparition de fructose 1,6-bis-P dans le milieu extérieur est un sujet de controverse. Trois hypothèses peuvent être avancées pour expliquer ce fait. La première est que au moins une partie du fructose 1,6-bis-P pourrait être synthétisée à l'extérieur à partir de trioses-P sous l'effet des activités triose-P-isomérase et aldolase provenant de chloroplastes brisés ou de contaminations cytoplasmiques, argument qui a été avancé par HELDT (1976) pour affirmer que le fructose 1,6-bis-P observé à l'extérieur n'est pas dû à une diffusion de ce composé hors du chloroplaste. Le temps nécessaire à séparer les chloroplastes intacts de leur milieu extérieur est plus long dans notre méthode que dans la méthode de centrifugation-filtration sur huile de silicone adoptée par LILLEY *et al.* (1977). On ne peut donc pas exclure que des réactions secondaires se produisent à l'obscurité dans le milieu extérieur; toutefois, celles-ci ne semblent pas suffire à expliquer les rapports E/I très élevés observés pour le fructose 1,6-bis-P (*fig.* 4 *a*). Une deuxième hypothèse concerne l'activation de la fructose 1,6-bisphosphatase par la lumière (BUCHANAN, 1980). L'enzyme, fortement activée dans le stroma, a pour effet de diminuer la quantité endogène de fructose 1,6-bis-P au profit de son homologue monophosphate. Cette activation de l'enzyme, si elle existe dans le milieu extérieur, est beaucoup plus faible. Ceci pourrait partiellement rendre compte du rapport élevé E/I observé pour le fructose 1,6-bis-P (*fig.* 4 *a*). L'explication la plus vraisemblable de l'apparition de fructose 1,6-bis-P dans le milieu extérieur au cours de la photosynthèse est que ce métabolite est exporté hors du chloroplaste. Notons que l'addition de fructose 1,6-bis-P permet de lever le temps de latence du dégagement d'oxygène dû à un excès de phosphate exogène et que ceci a été considéré comme une preuve que ce métabolite est capable de franchir l'enveloppe du chloroplaste intact (WALKER, 1976).

Nos résultats démontrent clairement que l'acide linolénique inhibe la diffusion des biphosphates d'oses (*fig.* 3 *a* et 4) et dans une moindre mesure celle des monophosphates d'oses (*fig.* 3 *b*) hors du chloroplaste. Nous avons démontré par ailleurs que l'acide linolénique inhibe le transport de phosphate et des trioses-P à travers l'enveloppe du chloroplaste intact (SIEGENTHALER et MVÉ AKAMBA, 1979) en affectant le fonctionnement du transporteur de phosphate/trioses-phosphates (MVÉ AKAMBA et SIEGENTHALER, 1979 *b*). Si les bisphosphates d'oses empruntent le même transporteur, l'inhibition de leur exportation par l'acide linolénique pourrait s'expliquer par le même mécanisme.

Plusieurs auteurs (PEDERSEN *et al.*, 1966; MVÉ AKAMBA et SIEGENTHALER, 1979 *c*) ont suggéré que le traitement des chloroplastes intacts par les acides gras, notamment par l'acide linolénique, inhibe les activités des enzymes hexose-bisphosphatases et RuBP carboxylase dans le cycle de réduction du CO_2 photosynthétique. Cependant l'inhibition de ces enzymes à l'état isolé n'a pu être démontrée ni en présence d'acide linolénique (SIEGENTHALER et MVÉ AKAMBA, 1979) ni en présence d'octanoate (PEDERSEN *et al.*, 1966). L'ensemble de nos travaux (SIEGENTHALER et MVÉ AKAMBA, 1979; MVÉ AKAMBA et SIEGENTHALER, 1979 *a*, *b*, *c*; MVÉ AKAMBA, 1979) suggère que l'inhibition de ces activités enzymatiques se fait de façon indirecte.

La figure 6 résume la séquence des événements qui conduit à l'inhibition de la photosynthèse par l'acide linolénique. Dans un premier temps l'acide linolénique inhibe l'absorption du phosphate exogène et l'exsorption des trioses-P, ce qui devrait empêcher l'alcalinisation du stroma qui a lieu dans les conditions normales de photosynthèse. L'acidification relative qui en résulte inactive à son tour l'activité des enzymes bisphosphatases (Garnier et Latzko, 1972). Cette inactivation enzymatique provoque une augmentation des concentrations en bisphosphates d'oses qui serait sans grande conséquence métabolique si elle ne s'accompagnait d'une inhibition de l'exsorption de ces composés (*fig.* 4). Nos résultats nous permettent d'affirmer que la photosynthèse de chloroplastes intacts traités par l'acide linolénique se déroule en présence d'une concentration pléthorique de bisphosphates d'oses. Or, il a été démontré à plusieurs reprises que des concentrations élevées de fructose 1,6-bis-P (Bowes et Ogren, 1972; Buchanan et Schürmann, 1973; Chu et Bassham, 1975) et de sédoheptulose 1,7-bis-P (Saluja et McFadden, 1978) inhibent l'activité de la RuBP carboxylase isolée. L'inhibition de la RuBP carboxylase (fixation de CO_2) dans les chloroplastes traités par l'acide linolénique est donc très probablement due à l'effet inhibiteur de concentrations trop élevées de bisphosphates d'oses dans

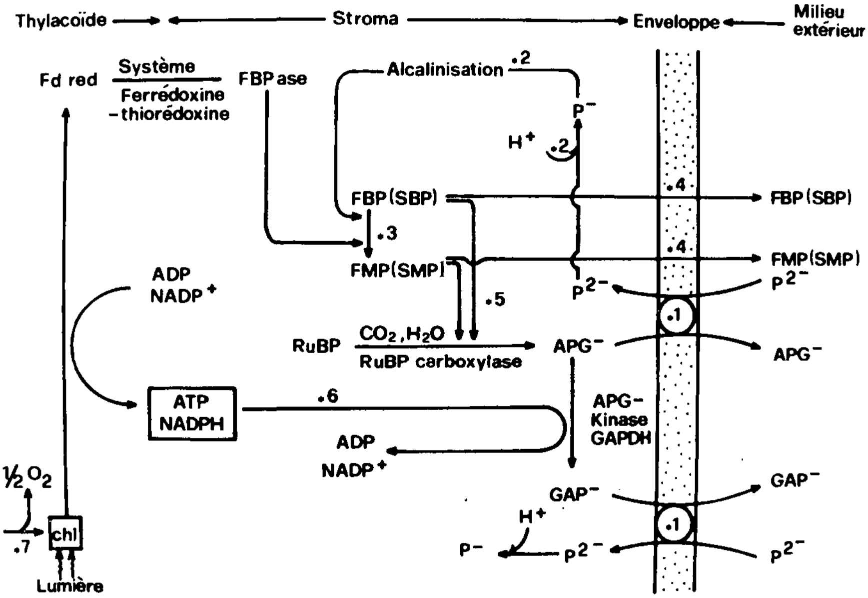

Fig. 6. — Modèle explicatif de l'inhibition de la photosynthèse dans le chloroplaste intact par l'acide linolénique

— *Explanatory model of the inhibition of photosynthesis in linolenate-treated intact chloroplasts*

Inhibition par l'acide linolénique : du transporteur de phosphate/trioses-P, ★ 1; de l'alcalinisation du stroma $HPO_4^{2-} + H^+ \rightleftharpoons H_2PO_4^-$, ★ 2; de la fructose 1,6-bisphosphatase et sédoheptulose 1,7-bis-Pase par diminution du pH provoquant une accumulation pléthorique de FBP (et SBP) et une diminution relative en FMP (et SMP), ★ 3; de l'exsorption de FBP (SBP) et de FMP (SMP), ★ 4; de la RuBP carboxylase par excès de FBP et SBP, ★ 5; de l'utilisation de NADPH par manque d'APG, ★ 6; du dégagement d'O_2 par excès de NADPH, ★ 7 (Siegenthaler et Horakova, 1974).

le stroma. Le NADPH non utilisé par suite de la faible concentration de l'acide 3-P glycérique s'accumule et inhibe le transport d'électrons selon le mécanisme démontré par Arnon et Chain (1975). L'inhibition du transport d'électrons a également pour effet d'empêcher l'activation de la fructose 1,6-bisphosphatase par la lumière par manque de ferrédoxine réduite. Une telle séquence des événements rend compte du fait que l'acide linolénique inhibe les activités de la photosynthèse dans le chloroplaste intact et non dans le chloroplaste reconstitué.

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Linolenate-inhibition of phosphate translocation across the envelope of intact spinach chloroplasts.

Free fatty acids were reported to be released from the membrane lipids during the deterioration process in thylakoids (1). Linolenic acid (C18:3) was shown to be the most abundant of these fatty acids (1-2). Addition of exogenous fatty acids concentrations to freshly prepared chloroplasts could mimic the effect of aging of thylakoid membranes (2-3); namely most of the photochemical activities in the electron transport chain and their associated phosphorylations were impaired in fatty acids-treated chloroplasts (2-3). In our experiments we investigated the effects of linolenate on photosynthesis by intact chloroplasts.

We report here that upon addition of low concentrations of C18:3 to intact spinach chloroplasts the absorption of orthophosphate and the concomitant efflux of triose-phosphates are inhibited. When the effect of pH on the absorption of Pi was studied we could observe a broad optimum for Pi influx ranging from pH 7.5 to 9.0 in the reaction medium. This suggested a possible existence of two essential chemical groups in the active site of the Pi translocator. Linolenate inhibited more efficiently Pi absorption in the neutral range of pH than in the alkaline pH where C18:3 rather enhanced the absorption of Pi. Also the optimal pH for C02 fixation shifted to more alkaline values in C18:3-treated intact chloroplasts. Apparently the internal pH of intact chloroplasts decreased in C18:3-treated chloroplasts although non dissociated C18:3 was unlikely to cross the envelopes of chloroplasts at these pH. When distribution of ^{14}C in the main metabolites was analysed during photosynthesis we could observe an accumulation of radioactivity in sugarbisphosphates in C18:3-treated chloroplasts. The diffusion of sugarbisphosphates out of the stroma was affected by C18:3. The accumulation of sugarbisphosphates suggests an inhibition of sugarbisphosphate phosphatases in treated chloroplasts; but C18:3 could not affect the activity of any of the soluble enzymes isolated from the chloroplasts (5). The high concentrations of sugarbisphosphates in the stroma of linolenate-treated chloroplasts might inhibit the activity of RuBP carboxylase according to a previously reported mechanism (4).

We conclude that linolenate inhibits photosynthesis in intact chloroplasts by impairing the activity of Pi/trioses-P translocator; the consequent shortage of Pi causes acidification of the stroma pH which in turn inhibits the activities of sugarbisphosphate phosphatases. The accumulation of sugarbisphosphates in the stroma could account for the inhibition of C02 fixation in C18:3-treated intact chloroplasts.
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