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Fatty-acid-induced release of manganese from chloroplasts *

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The effect of both endogenous and exogenous unsaturated free fatty acids on manganese release from chloroplasts of chill-resistant (spinach) and chill-sensitive (tomato, bean) plants was studied. The level of endogenous free fatty acids increased 2–3-fold during cold and dark storage of leaves of chill-sensitive plants and was accompanied by depletion of about 60% of total chloroplast manganese content. Similar effects were observed when accumulation of free fatty acids in chloroplasts was achieved by storage of growing tomato plants for a few days in the dark at room temperature. In contrast, the cold and dark treatment of leaves of chill-resistant plant (spinach) affected neither free fatty acid, manganese levels nor Hill-reaction activity in chloroplasts. Incubation of chloroplasts of both chill-sensitive and chill-resistant plants with bean leaf galactolipase resulted in an accumulation of free fatty acids and a release of approx. 60% of total manganese content. The same amount of total manganese content was released following 3 h incubation of chloroplasts with linolenic acid at fatty acid/chlorophyll ratio (w/w, 2:1–10:1). The efficiency of C_{18} unsaturated fatty acids/linolenic, linoleic, oleic on manganese release from chloroplasts was established in decreasing order $C_{18:3} > C_{18:2} > C_{18:1}$. The results indicate that the inhibitory effect of both endogenous and exogenous fatty acids on Hill reaction depends on the release from chloroplasts of functionally active, loosely bound manganese. Thus, similarly to both Tris and hydroxylamine treatments of chloroplasts, the incubation of chloroplast preparations with unsaturated fatty acids may be a useful tool for manganese depletion of chloroplasts.

Introduction

Krogman and Jagendorf [1] were the first who observed inhibition of photosynthetic electron transport by unsaturated C_{18} fatty acids. Since that time numerous studies were carried out on the effects of exogenous [2–10] and endogenous [11–15] free fatty acids released from membrane lipids either during aging of isolated chloroplasts [19–14] or following treatment of chloroplasts with lipolytic enzymes [16–21] on the structure and function of chloroplasts. Much attention was payed to elucidate both the site and mechanism of photosynthetic electron-transport inhibition by free fatty acids and its reversibility by bovine serum albumin and manganese [7,9,16,22].

The cold and dark-induced inactivation of PS II electron flow in chloroplasts of chill-sensitive plants [10] appeared to be a common effect of galactolipid hydrolysis providing inhibitory fatty acids [23,24] and manganese depletion from chloroplast [25]. In addition, these studies established that both cold and dark treatment of leaves of

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Abbreviations: PS II, Photosystem II; Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

chill-sensitive plants and Tris washing of chloroplasts by the method of Yamashita and Butler [26] affect electron-transport chain of chloroplast at a common site [10,25], sensitive to free fatty acids [24]. Thus, it is likely that there is a correlation between the increased level of free fatty acids in chloroplasts and the loss of manganese. The results presented in a preliminary report [27] are in agreement with this suggestion.

Data presented in this paper indicate that free fatty acids, released from chloroplasts either by endogenous galactolipase or by digestion of chloroplasts with bean leaf galactolipase as well as exogenous free fatty acids, induce release of manganese from chloroplasts resulting in an inhibition of both Hill reaction activity and O_2 evolution. Thus, like Tris or hydroxylamine washing of chloroplasts, fatty-acid-induced release of manganese from chloroplast appears to provide a new tool for studying the function of this metal in photosynthetic oxygen evolving systems. A preliminary report of this work has appeared in abstract form (Ref. 27).

Materials and Methods

Plant material. Leaves of tomato (*Lycopersicon esculentum*, Mill., cv. Norton) were harvested from the plant grown under greenhouse conditions used for commercial purpose. Leaves of bean (*Phaseolus coccineus*, L. cv. Piękny Jaś) were harvested usually between the 12th and 16th day after planting the bean seeds. Conditions of both seed germination and growing of bean plant have been described in the previous paper [10]. Spinach leaves (*Spinacia oleracea*, L. cv. Matador) were purchased in the local market.

Temperature treatment of leaves and plants. Tomato, bean and spinach leaves were stored at 0–4°C in the dark for 4 days as described previously [10]. Tomato plants were grown in flower pots under normal conditions of greenhouse. When plants had 5–8 leaves and were 30–40 cm in height they were placed in darkness at 25°C for 5–7 days.

Isolation of chloroplasts. Chloroplasts were isolated as described previously [10] except Tris buffer instead Hepes was used and bovine serum albumin was omitted from isolation solutions.

Isolation of galactolipase. Galactolipase (lipid acyl hydrolase) cf. Ref. 28 preparations were isolated from bean leaf chloroplasts as described by Anderson et al. [29] omitting the inactivation step of protein inhibitor, since we did not observe its presence. Fractions of the enzyme after ammonium sulphate treatment were used for chloroplast digestion.

Activity of the enzyme preparations was estimated following incubation for 15 min at 30°C of lyophilized spinach chloroplasts used as substrate [29]. No detergent was added, since unsaturated free fatty acids at low concentrations are the most effective surface-active agent [30]. The reaction mixture for enzyme activity measurements contained 20 mM potassium phosphate buffer (pH 7.0), 2 μg enzyme preparation and substrate equivalent to 100 μg chlorophyll. Incubation was terminated by the addition of 1 ml of 0.1 M HCl in 96% ethanol. The activity of galactolipase preparations was about 3 μmol free fatty acid liberated/min per mg protein.

Incubation of chloroplast with galactolipase. Tomato and spinach chloroplasts (3–5 mg Chl) at a concentration of 0.4 mg Chl/ml were incubated with galactolipase at a protein/Chl ratio of 5 : 1 (w/w) at 30°C in the presence of 20 mM potassium phosphate buffer (pH 7.0). After appropriate time of incubation samples containing 100 μg of chlorophyll were withdrawn for fatty acid determinations, while the remaining sample was span down and after washing with phosphate buffer and centrifugation the pellet was used for measurements of manganese content.

Incubation of chloroplasts with fatty acid. Chloroplast preparations (3–5 mg Chl) were incubated with fatty acid at 25°C at fatty acid/Chl ratio 1 : 1–10 : 1 (w/w). Incubation medium contained 20 mM potassium phosphate buffer (pH 7.0). After 1–3 h of incubation chloroplasts were precipitated by centrifugation and after washing the pellet with phosphate buffer and repeated centrifugation, manganese content was measured in the pellet.

Analytical methods. Hill reaction activity was determined by the reduction of DCIP measured at 620 nm as described previously [10] as well as in the presence of 1 μM DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone). In the presence of this inhibitor the activity of electron trans-

port from water to DCIP was about 5–15% lower than in its absence in chloroplast preparations from both control and cold and dark stored leaves.

Oxygen evolution was followed polarographically at 25°C with a Clark type electrode (Yellow Springs Instrument Co.) using reaction mixture as in Ref. 31. Light intensity applied was equal to $1.35 \cdot 10^3$ J · m^{-2} · s^{-1}. The effect of cold and dark treatment of leaves on the activity of oxygen evolution was approximately the same as that of Hill reaction in the presence of DBMIB. Free fatty acid content in chloroplast particles was determined by colorimetric method using Rhodamine 6G [32]. Manganese content was estimated by atomic absorption spectroscopy using an Instrumentation Laboratory Model 551 video I apparatus. Chloroplasts containing 3–5 mg chlorophyll were wet-ashed prior to analysis with 2 ml $HNO_3 + HClO_4$ at a 3:1 ratio according to Blankenship and Sauer [31]. Chlorophyll and protein content were determined as in Refs. 33 and 34, respectively.

Chemicals.

Unsaturated fatty acids: oleic (*cis*-9-octadecanoic), linoleic acid (*cis,cis*-9,12-octadecadienoic) and linolenic acid (*cis,cis,cis*-9,12,15-octadecatrienoic) were purchased from Sigma Chemical Co. and Fluka. Rhodamine 6G was obtained from BDH and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) was kindly provided by Prof. A. Trebst. The other chemicals of analytical grade were from P.O.Ch. Poland.

Results

Effect of free fatty acids accumulated in chloroplast on manganese release during cold and dark storage of leaves

It was found that the level of free fatty acids in chloroplasts of bean and tomato increases almost two-fold following 4 days of cold and dark storage of detached leaves, whereas it is practically constant in chloroplasts of spinach leaves stored under the same conditions. The stability of free fatty acid level in spinach chloroplasts is accompanied by stability of both manganese level and Hill reaction activity. Slight decrease (for about 15% of total manganese) of manganese is due to the loss of functionally inactive fraction of this metal as

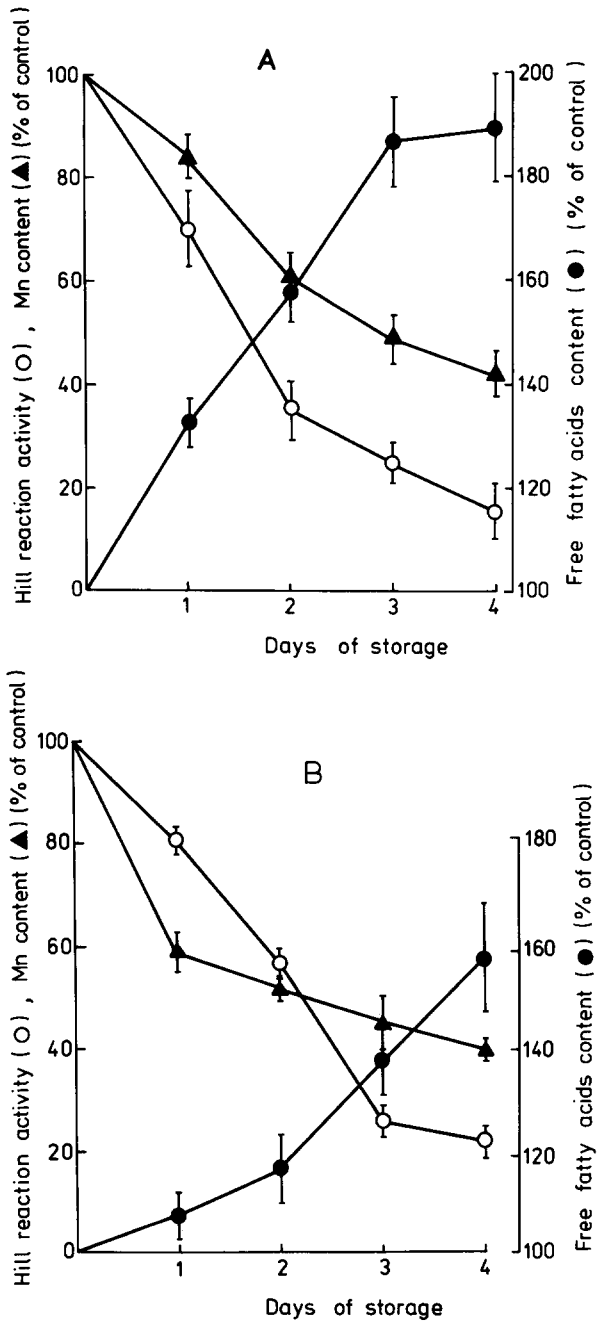


Fig. 1. Time-course of free fatty acid accumulation, manganese depletion and loss of Hill-reaction activity in chloroplasts following cold and dark storage of bean (A) and tomato (B) leaves. The 100% values corresponded to 30–48 and 65–140 μmol DCIP reduced/mg Chl per h, 2.3–2.7 and 1.8–2.0 μmol free fatty acid/mg Chl and 5–8 and 5–7 gatom Mn/400 mol Chl for bean and tomato chloroplasts, respectively. Vertical lines represent the means of ±S.E. of 3–5 and 4–7 experiments for bean and tomato, respectively.

observed previously with tomato [25] and spinach chloroplasts [35].

Accumulation of free fatty acids in chloroplasts of bean and tomato (Fig. 1AB) during cold storage of detached leaves is accompanied by decrease of about 2/3 of total manganese content and loss of Hill reaction activity. Release of free fatty acids in bean chloroplasts is much faster than in tomato chloroplasts although galactolipase activity in chloroplasts from both plants is almost the same [36].

Effect of dark storage at room temperature of tomato plants on fatty acid accumulation and manganese release

When growing tomato plants were stored for few days at 25°C in dark both decrease of Hill reaction activity and enhanced sensitivity of this reaction to exogenous linolenic acid were observed [10] suggesting an accumulation of endogenous

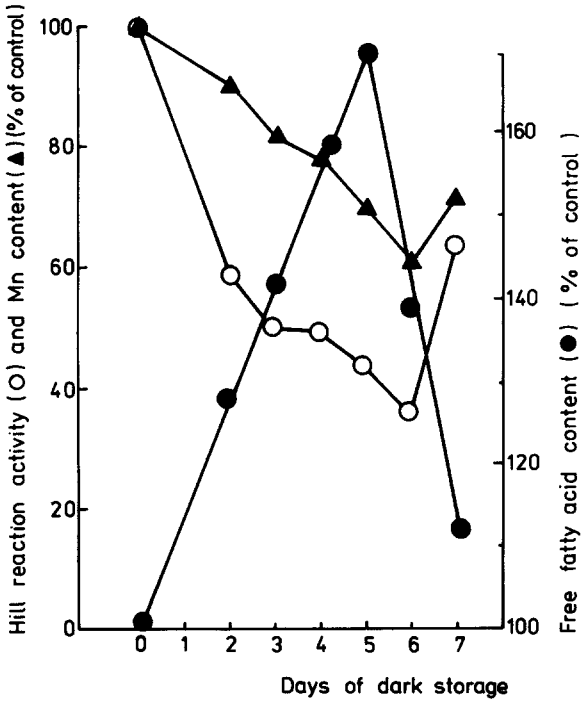


Fig. 2. Time-course of free fatty acids accumulation, manganese depletion of chloroplasts and decrease of Hill-reaction activity during dark storage of tomato plants at room temperature. The 100% values were: 1.2 μmol free fatty acid/mg Chl 94 μmol DCIP reduced/mg Chl per h and 7 gatom Mn/400 mol Chl.

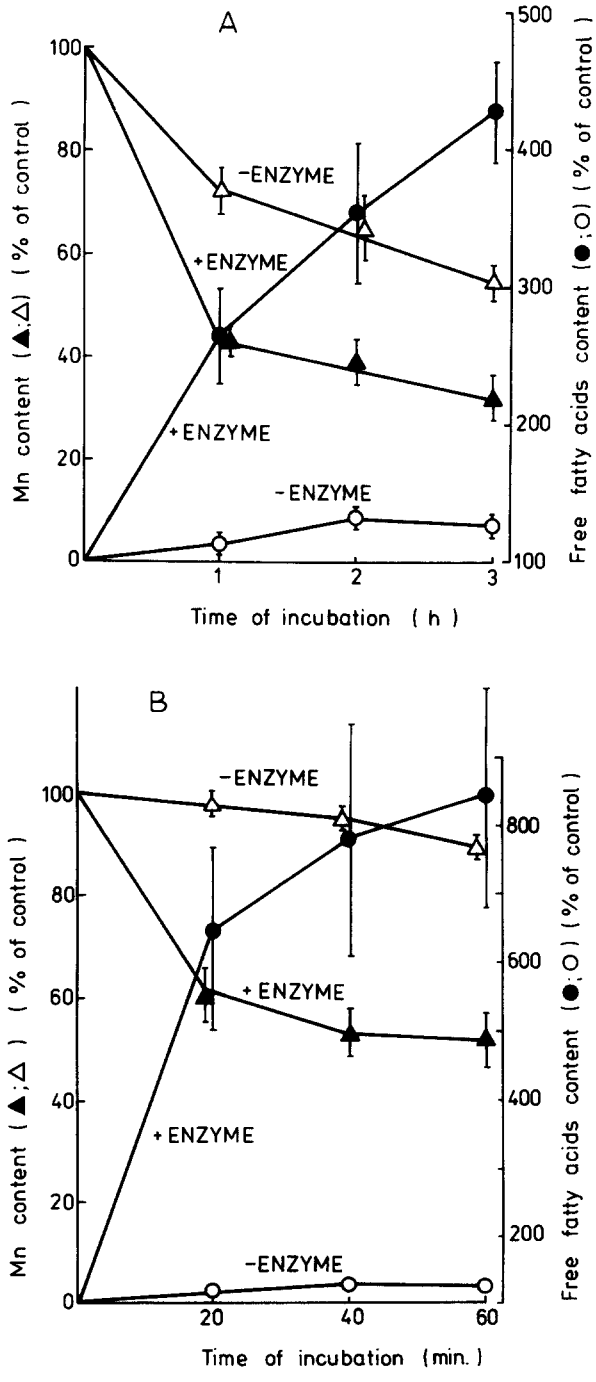
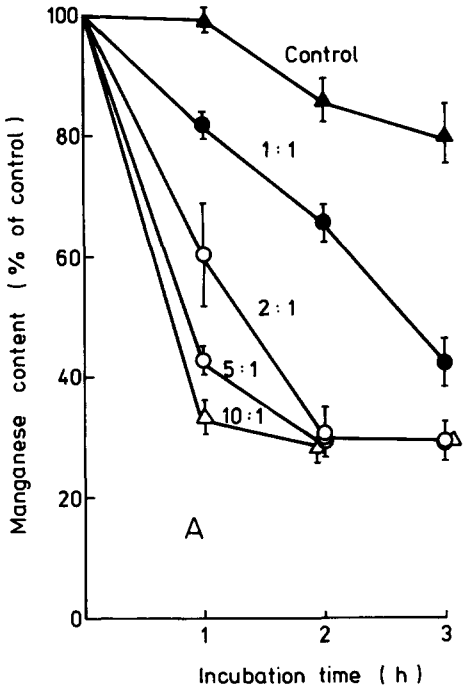
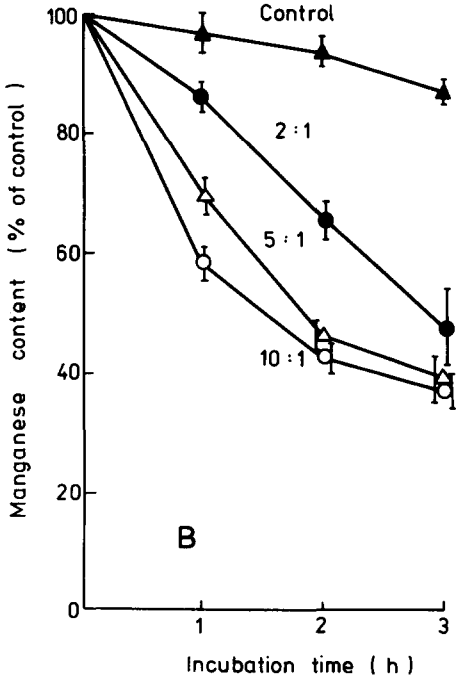


Fig. 3. Time-course of free fatty acid accumulation and manganese release from tomato (A) and spinach (B) chloroplasts incubated with bean leaf galactolipase. The 100% values were: 1.8–2.0 and 0.9–1.1 μmol free fatty acid/mg Chl and 8–9 gatom Mn/400 mol Chl for tomato and spinach chloroplasts, respectively. Vertical lines represent the means ± SE of 4–6 and 3–5 experiments for tomato and spinach, respectively.

free fatty acids in chloroplasts. In order to check this supposition we have studied increase of free fatty acids in chloroplasts of dark-stored tomato plants with respect to loss of Hill-reaction activity





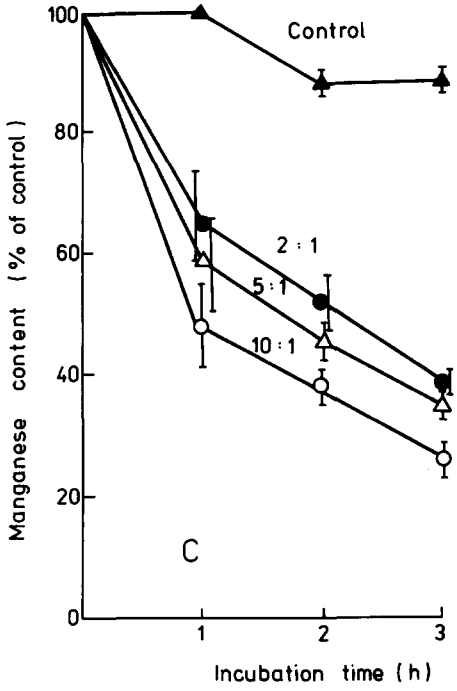


Fig. 4. Effect of incubation of bean (A), tomato (B) and spinach (C) chloroplasts with various fatty acid/Chl ratio (w/w).

and manganese level. As can be seen in Fig. 2 during the first few days of dark storage of plants accumulation of free fatty acids and decrease of both Hill-reaction activity and manganese contente occur. During the second phase, which takes place 1–2 days after the first one, a decrease of free fatty acids level and small increase of both Hill reaction activity and manganese level become apparent. Further dark storage of plants for 1–2 days does not change essentially the levels of these parameters. The maximal increase of free fatty acids and the maximal decrease of both manganese content and Hill reaction activity occur between 3 and 5 days of dark storage depending on plant conditions, i.e., age of plants their growth conditions and illumination. Following 7–8 days of dark storage of young tomato plants discoloration of leaves is observed suggesting that some degenerative changes occur, so plants were not stored longer. All these changes reflect probably response of the plant to dark treatment at room temperature. Similar behaviour of Hill reaction activity

following dark storage of tomato plants both at 0°C and room temperature have been reported previously [10].

Effect of galactolipase treatment of chloroplasts on fatty acid accumulation and manganese release

Elevated levels of endogenous free fatty acids in chloroplasts could also be achieved by digestion of chloroplasts with bean leaf galactolipase. Time-course of release of both free fatty acids and manganese from tomato and spinach chloroplasts is shown in Fig. 3A and B, respectively. It is interesting that under the same experimental conditions extent of release of both free fatty acids and manganese is the same in spinach chloroplasts (Fig. 3B) after 1 h, while in tomato chloroplasts it is after 3 h. Moreover, manganese depletion of spinach chloroplasts in the absence of added galactolipase is much slower than that in tomato chloroplasts, probably due to lower activity of endogenous galactolipase in the former plant [36].

Effect of exogenous unsaturated free fatty acids on manganese release from chloroplasts

Unsaturated C_{18} fatty acids are effective agents in destroying structure and photochemical properties of chloroplasts [3,6–10,15]. Studies on the free fatty acid composition of chloroplasts of cold and dark stored leaves indicate that the most significant changes occur in the level of linolenic acid which increses by 5.5, 7.7 and 12.7% of the total free fatty acids in bean, cucumber and tomato chloroplasts respectively, while essentially no changes were observed in spinach chloroplasts [23]. Incubation of bean, tomato and spinach chloroplasts with linolenic acid at a free fatty acid/chlorophyll ratio 1:1–10:1 (w/w) (Fig. 4A, B and C) results in a release of manganese from chloroplasts especially effective at higher fatty acid/chlorophyll ratio. After 3 h of incubation about 60% of total manganese content is released from chloroplasts. Under these conditions in control experiments (i.e., in the absence of linolenic acid) about 15% of functionally inactive manganese is lost.

Not only linolenic acid, but also other C_{18} unsaturated fatty acids are known as deletorious agents of photochemical and structural properties of chloroplasts. In order to compare effectiveness

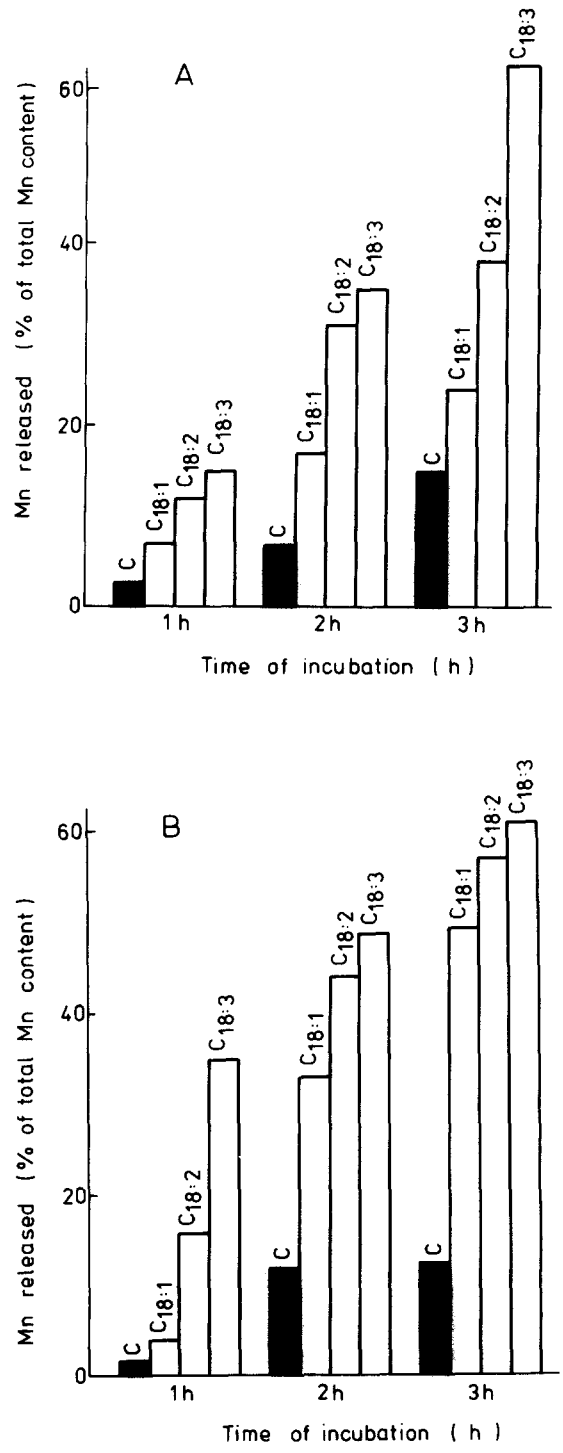


Fig. 5. Comparison of the effect incubation of chloroplasts with oleic, linoleic and linolenic acids on manganese release from spinach (A) and tomato (B) chloroplasts. Fatty acids were used at 2:1 free fatty acid/Chl ratio (w/w). (C), control.

of other C_{18} unsaturated fatty acids on manganese release from chloroplasts experiment presented in Fig. 5AB was carried out. As can be seen, in both tomato and spinach chloroplasts linolenic acid is the most effective in releasing of manganese, regardless the time of incubation.

Discussion

In the studies of the inactivation of Hill reaction and oxygen evolution by free fatty acid three interrelated aspects should be considered: (i) an inhibitory effect of free fatty acids, (ii) the damage of the membrane structure due to lipid hydrolysis and (iii) the depletion of chloroplast manganese.

Effect of free fatty acids

Although an inhibitory effect of free fatty acids on Hill reaction activity is known for a long time [1], data concerning the level of free fatty acids in chloroplasts are rare and essentially restricted to either fresh or aged spinach chloroplasts in which they amount to about 0.3–2 μmol/mg Chl [12,13,15,29]. Chloroplasts of chill-sensitive plants, i.e., tomato and bean (Fig. 1) and maize [36], contain 2–3 μmol free fatty acid/mg Chl. Both ageing of isolated chloroplasts [23] as well as cold and dark treatment of leaves of chill-sensitive plants (Fig. 1A and B) or dark treatment of tomato plants at room temperature (Fig. 2) result in an increase of free fatty acid level in chloroplasts of tomato and bean up to about 3 and 5 μmol/mg Chl, respectively. It is concomitant to a decrease of manganese content and Hill-reaction activity. In contrast, cold and dark treatment of spinach leaves does affect neither free fatty acid and manganese level nor Hill-reaction activity.

The inhibitory effect of exogenous free fatty acids on PS II activity is easily reversed by addition of bovine serum albumin [16,19,23,37]. Albumin has also a protecting effect on ageing of chloroplasts or chloroplasts treated with lipolytic enzyme [12,13,29], and this effect is usually considered as due to fatty acid binding. However, in aged chloroplasts [22] or after prolonged incubation of chloroplasts with linolenic acid [8] or following cold and dark storage of leaves of chill-sensitive plants [24], the effectiveness of bovine serum albumin in restoration of PS II activity was low. This may be explained now by free fatty acid-induced release of manganese from its site of action.

Two inhibitory sites in PS II affected by free fatty acids have been distinguished [9,24]. The first one, affected also by Tris washing of chloroplasts [26] or by cold and dark storage of leaves of chill-sensistive plants [24], seems to be irreversible due to the loss of manganese [25,38]. However, both manganese content and Hill-reaction activity are restored upon illumination of cold and dark stored leaves of chill-sensitive plants [25,24,38]. The second inhibitory site is reversible after washing of free fatty acid-treated chloroplasts with bovine serum albumin, so the electron flow from 1,5-diphenylcarbazide to DCIP can be completely restored in both fresh spinach [9] and tomato chloroplasts [24]. Recently, some new sites of linolenic acid inhibition have been discovered. Vernotte et al. [39] have found linolenic acid inhibition sites on the reducing site of PS II, while Goldbeck and Warden [40] located one site between pheophytin and Q_A on the reducing side of PS II and the other between electron donor Z and P-680 on the oxidizing side of PS II.

Hydrolysis of membrane lipids

The inactivation of Hill reaction and O_2 evolution due to damage of thylakoid membranes by lipid acyl hydrolases were studied by several authors. They applied pancreatic [18,21,41,42] and snake venom phospholipases [18,21,43], bean leaf galactolipase [16,24,29] and potato acyl hydrolase [17,20]. In this type of experiment it is difficult to differentiate the effect of lipid depletion from the inhibitory effect of released free fatty acid on PS II activity. Lipolytic acyl hydrolases have a broad substrate specificity and various activities towards individual membrane lipids. Thus, potato enzyme isolated by Galliard [44] or Hirayama et al. [45] is 2–3-times less active against mono- and digalactosyldiacylglycerol than that from bean leaves [46]. It is also 2.5–4-times less active with respect to phosphatidylcholine than to digalactosyldiacylglycerol [46]. This may explain the very small effect of potato lipolytic acyl hydrolase on both Hill reaction activity and lipid content of subchloroplast particles II and therefore the lack of protective affect of added bovine serum albumin in this process (cf. Fig. 2C in Ref. 20). Different

results were obtained when pancreatic phospholipase A_2 was used (cf. Fig. 4D of Ref. 21).

Since glycolipids (mono- and digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol) amount about 80% of the total lipids in thylakoid and envelope membranes [47]; in the present study galactolipase was used. Large contents of galactolipids ensure appropriate level of fatty acids released by galactolipase, either endo- or exogenous, to affect PS II and manganese content. Manganese release following galactolipase treatment (Fig. 3A and B) can be solely ascribed to free fatty acid effect and not to deletorious action of lyso compounds [48], since the same result is observed with exogenous free fatty acids (Fig. 4A, B and C).

When phospholipase A_2 was used by various authors the results disagree in respect to inhibition of PS II electron flow [18,21,43] and contribution of phosphatidylglycerol [43] or phosphatidylcholine [21] hydrolysis to inactivation of Hill reaction.

Large amounts of galactolipids might be removed from chloroplasts without any marked effect on the electron flow if defated bovine serum albumin was present. It prevents the binding of free fatty acid to chloroplasts [16,24,29]. However, the inhibitory effect of fatty acids released during lipolytic acyl hydrolase digestion of chloroplasts on PS II electron flow is sometimes neglected [17,21]. Linolenic acid [40] or phospholipase A_2 treatment of chloroplasts [42,43] influence also fluorescence yield and Signal II. These effects may be due to the action of liberated free fatty acid rather than to lipid depletion from chloroplasts.

Manganese depletion

Data presented in this report appear to indicate that an inhibitory effect of free fatty acids on Hill-reaction activity and oxygen evolution is due to the depletion of a large portion of chloroplast manganese which is the constituent of two pools *: the weakly bound and the strongly bound. Weakly bound manganese is removed either by EDTA [35,50–52] or by incubation of chloroplasts with low concentration of divalent cations [50,51] as well as following the cold and dark storage of tomato [25] and spinach leaves. The size of weakly bound manganese pool is variabale [50–52]: very low in lettuce and spinach chloroplasts, and prominent in pea, white clover and some pokeweed chloroplasts [51]. Depletion of weakly bound manganese does not inhibit Hill reaction and O_2 evolution, indicating that this pool is non-functional in electron transport [25,35,53].

Strongly bound manganese pool, which is believed to play a functional role in oxygen evolution and Hill-reaction activity amounts about two-third of total manganese content according to Chaniae and Martin [35] and is usually called Tris extractable. It is also affected by NH_2OH treatment [50,54–56], chaotropic agents [57,58] heat treatment [58], divalent cations [50,51,59] and as shown in the present work by endogenous or exogenous free fatty acids. Tris-acetone [60] or Tris-hydroxylamine [61] extraction of chloroplasts lead to more extensive depletion of chloroplasts Mn than Tris extraction alone does. There are, however, discrepancies in the amount of Mn release by Tris washing. Some authors have reported that this treatment removes from chloroplasts only 10–20% [31,63,64] or 30% [65] of total Mn content, while from EDTA/Ca^{2+}-treated chloroplasts 25% of total Mn was released [50]. Even in Tris-washed inside-out vesicles about 50% of total Mn content still remains [66]. Thus, the presence of as much as 50–80% of original Mn content in chloroplasts preparations or inside-out vesicles, may explain the easiness of O_2-evolving system reactivation in light or dark (+DCIP/ascorbate) [62–64,66]. Similarly, upon illumination of cold and dark stored tomato leaves an increase of Mn content up to 80% of initial level and a concomitant restoration of Hill reaction activity were observed [25].

Very strongly bound pool of manganese in chloroplasts, which comprises about one third of total Mn [35], is not affected by either endogenous (Figs. 1A and B and 3A and B) or exogenous (Fig. 4A, B and C) free fatty acids. Thus, the extend of free fatty acid-induced release of strongly bound chloroplast manganese is similar to that released by Tris or hydroxylamine washing. This offers a new tool for studies of Mn contribution to the

* According to a recent proposal of Amesz [49] different pools of manganese in chloroplasts could be called: the weakly bound, the strongly bound and the very strongly bound.

structure of the oxygen-evolving system. At present, we have applied free fatty acid-induced removal of strongly bound Mn to check whether depletion of this metal is accompanied by a release from PS II particles of some polypeptide: especially that of 33 kDa.

Note added in proof (Received June 9th, 1986)

In the course of our recent studies concerning galactolipase activity and free fatty acid levels in chloroplasts of several chill-sensitive and chill-resistant plants we have compared both Rhodamine 6G and gas-liquid chromatography methods for free fatty acid determination in the same sample of chloroplasts. It appeared that, depending on plant species, values found by the former method are 2–3-times higher than those obtained by the gas-liquid chromatography method.

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