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AGING OF THE PHOTOSYNTHETIC APPARATUS

IV. SIMILARITY BETWEEN THE EFFECTS OF AGING AND UNSATURATED FATTY ACIDS ON ISOLATED SPINACH CHLOROPLASTS AS EXPRESSED BY VOLUME CHANGES

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SUMMARY

1. The effects of unsaturated fatty acids have been studied and compared with the known effects of aging on volume changes in isolated spinach chloroplasts.

2. C_{18}-unsaturated acids, *i.e.* oleic, linoleic and linolenic acids, and to a lesser extent myristic acid, were the most effective in stimulating swelling and inhibiting light-induced shrinkage. These acids are precisely those which are released in greatest amount from lipids during aging of chloroplast membranes.

3. On the contrary, saturated fatty acids ($C_{8\,0}$, $C_{10:0}$, $C_{16.0}$, $C_{17\cdot0}$ $C_{18:0}$, $C_{20\,0}$), and also $C_{22:1}$, had only slight effects on chloroplast volume changes.

4. Activation of chloroplast swelling and inhibition of light-induced shrinkage by unsaturated fatty acids were found to be time and concentration dependent. Maximum swelling occurred after 20 min with a fatty acid/chlorophyll molar ratio of 9 (200–240 μM per 20 μg chlorophyll/ml) which corresponded to almost full inhibition of light-induced shrinkage. The activity of C_{18}-fatty acids on swelling could be established in the decreasing order: $C_{18\,1} > C_{18.2} > C_{18\,3} > C_{18\,0}$. Although maximum inhibition of light-induced shrinkage was the same after 20 min for the three unsaturated fatty acids, the time required to reach it was inversely related to the number of double bonds.

5. Bovine serum albumin restored light-induced shrinkage inhibited by fatty acids and overcame swelling caused by these compounds.

6. A relationship might exist between the occurrence or the amount of fatty acids in the organelle lipids and their capacity to regulate chloroplast structural changes. Results are compiled in a scheme and discussed as they relate to the known effects of fatty acids and aging on electron flow and energy-linked reactions, on the physicochemical properties and the lipoprotein complexes of the membrane, and on lipid peroxidation.

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INTRODUCTION

We have previously reported that aging *in vitro* of isolated spinach chloroplasts leads to an alteration of the structures and functions of these organelles[1,2]. Indeed, after a few hours of incubation under appropriate conditions (darkness or light, 20 °C, pH 8, *etc.*), chloroplasts undergo irreversible swelling. Simultaneously, the capacity of chloroplasts to carry out light-dependent shrinkage diminished. Aging of chloroplasts is accompanied by uncoupling when ferricyanide is the electron acceptor, by decreases in ATP photohydrolysis, cyclic and non-cyclic ATP synthesis, $NADP^+$ photoreduction and apparent O_2 evolution. In the course of aging, there is also an increase in the *o*-diphenol oxidase activity and a diminution in the latent period which precedes the initiation of the oxidation, phenomena probably depending on the integrity of chloroplast structure[3].

Attempts to duplicate the above changes with fresh (not aged) chloroplasts were made with fatty acids[4]. This working hypothesis[2,4] was suggested by previous studies. First, since the discovery that isolated bean chloroplasts contain a very active enzyme hydrolyzing the galactolipids[5], several authors have characterized the galactolipases in isolated chloroplasts[5–9]. Secondly, it was established that free fatty acids (especially linolenic acid) released by hydrolysis of galactolipids are at least partly responsible for the labile photochemical performances of isolated chloroplasts[10–13]. Thirdly, exogenous fatty acids are known to inhibit photochemical reactions[4,10–16], to have a marked effect on light-induced absorption and fluorescence changes[13,17–19], and to inhibit some of the photosynthetic carbon cycle reactions[20]. Fourthly, some fatty acids induce chloroplast swelling in the dark[13,21,22]. Lastly, lipid peroxidation is involved in chloroplast deterioration and membrane damage[23–27].

In view of these findings which indicate that the action of fatty acids on chloroplast structure and function resembles in many ways the effects of aging, it seemed valuable to investigate a model system in more detail, *i.e.* the influence of fatty acids (one of the end products of lipolytic activity) on the volume changes of chloroplasts. I have focused my attention chiefly on C_{18}-unsaturated fatty acids which are the most representative fatty acids released during aging of chloroplast membranes.

MATERIALS AND METHODS

Spinach chloroplasts were prepared in a medium containing 100 mM Tris–HCl (pH 8) and 175 mM NaCl as described previously[1], and generally, were used unwashed. Chlorophyll was determined by the method of Bruinsma[28]. The chloroplast suspension was then diluted in the same medium to obtain 1 mg chlorophyll/ml and kept at 0–4 °C before use.

Since the action of fatty acids on chloroplast volume changes depended on the molar ratio of fatty acids/chlorophyll, the comparison between the various fatty acids was facilitated by using the same chlorophyll concentration in all reaction mixtures, *i.e.* 20 μg chlorophyll/ml. Therefore, all the results presented in this paper refer to this amount of chlorophyll and any change in fatty acid or chlorophyll concentrations must be evaluated in terms of the fatty acids/chlorophyll ratio. In addition, since volume changes induced by fatty acids were affected by the aging of chloroplasts *in vitro*, great care was taken to insure the use of appropriate controls.

Swelling of chloroplast suspensions was estimated by the decrease in absorbancy at 540 nm ($-\Delta A_{540\ nm}$)[29] in the following reaction mixture: 175 mM NaCl, 100 mM Tris–HCl (pH 8), chloroplasts (20 μg chlorophyll/ml) and, where indicated, fatty acids. Since fatty acids were dissolved in ethanol, all reaction mixtures contained 0.5 % ethanol.

To check the reliability of spectrophotometric measurements as an index of chloroplast swelling, the chlorocrit technique[30] was also employed (only in Fig. 3). The reaction mixture was the same as for the absorbance technique but contained chloroplasts at 1 mg chlorophyll/ml. In this case, more fatty acids were added to maintain a constant fatty acids/chlorophyll ratio (*i.e.* 15 mM fatty acids/1 mg chlorophyll instead of 300 μM/20 μg chlorophyll, *etc.*).

Light-induced shrinkage was estimated by measurement of the light-scattering increase at 90° (546 nm) with a modified[1] photovolt fluorimeter (Model 540). A primary interference filter (Balzers B-40/539) isolated the green light used for the scattering measurements whereas a secondary interference filter (Baird Atomic) prevented interference with actinic light (>600 nm, Kodak filter, wratten N26) emitted from one of the cuvette sides. The temperature was maintained at 25 °C by circulating water around a jacketed 1-cm cuvette, containing the following reaction mixture: 50 mM KH_2PO_4 (pH 6), 35 mM NaCl, 5 mM $MgCl_2$, 20 μM phenazine methosulfate, 0.5 % ethanol, chloroplasts (20 μg chlorophyll/ml) and, where indicated, fatty acids. Increases in scattering intensity following treatment with red light are expressed as percent changes of the initial scattering level (100 % LS).

Fatty acids, bovine serum albumin and all other chemicals (purest form) were purchased from Fluka, except margaric and oleic acids which were purchased from Merck, and phenazine methosulfate from Calbiochem.

RESULTS

A survey of the action of various fatty acids on chloroplast volume changes showed that C_{18}-unsaturated fatty acids, *i.e.* oleic, linoleic and linolenic acids, were the most active. Indeed, they induced swelling and inhibited light-induced shrinkage (Table I). These fatty acids were precisely those which are the most often encountered in chloroplast membrane lipids. On the other hand, saturated fatty acids ($C_{8\ 0}$, $C_{10:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18\ 0}$, $C_{20\ 0}$) and $C_{22:1}$ had only slight effects on chloroplast light-induced shrinkage except for myristic acid ($C_{14\ 0}$), which is found in small amounts in chloroplast lipids. Unexpectedly, an inhibition of dark swelling was caused by some of the saturated fatty acids. Thus, it appears that a relationship might exist between the occurrence or the amount of fatty acids in the organelle lipids and their capacity to regulate chloroplast structural changes. In view of these results, only C_{18}-fatty acids were studied in more detail.

Concentration dependence

Up to a concentration of 240 μM, the three C_{18}-unsaturated fatty acids induced a marked chloroplast swelling (Fig. 1). The stimulation ranged from 200 to 500 %, generally depending on the swelling of the controls. Curves a and b represent typical experiments where the controls were low and high, respectively. Fatty acid-induced swelling was associated with inhibition of light-induced shrinkage. Maximum swelling

TABLE I

SURVEY OF THE ACTION OF FATTY ACIDS ON SWELLING AND LIGHT-INDUCED SHRINKAGE IN CHLOROPLASTS

Fatty acids			*Swelling** (%)**	*Light-induced scattered light**** (%)**	
Name	*Symbol*	*Concn* (μM)		*Rate* §	*Extent* §§
Caprylic	$C_{8\ 0}$	200	−30	−12	− 3
		500	−14		
Capric	$C_{10:0}$	200	− 2	− 8	7
		500	34		
Myristic	$C_{14\ 0}$	200	28	−31	−34
		500	−16		
Palmitic	$C_{16:0}$	200	− 9	3	9
Margaric	$C_{17.0}$	200	−64	− 7	−14
Stearic	$C_{18\ 0}$	200	−80	4	− 2
Oleic	$C_{18\ 1}$	200	363	−68	−78
		500	79		
Linoleic	$C_{18\ 2}$	200	264	−80	−87
		500	11		
Linolenic	$C_{18\ 3}$	200	223	−76	−77
		500	35		
Arachidic	$C_{20\ 0}$	200		0	31
Docosenoic	$C_{22:1}$	200	−11	−18	− 7
		500	−83		

* Incubation: 20 min in the dark.
** %: [(treated−control)/control] × 100.
*** Incubation: 10 min.
§ Rate calculated after 25 s.
§§ Extent calculated after 125 s.

occurred when maximum inhibition of the scattered-light measurements were obtained. With concentrations higher than 240 μM, induced swelling by fatty acids diminished, often reaching, at 500 μM, the same value as for the controls. Compared with these unsaturated acids of the C_{18} series, stearic acid had no significant effect on chloroplast swelling and shrinkage.

Incubation time dependence

Fig. 2 shows time-courses of swelling of isolated chloroplasts in the absence (controls) and presence of two concentrations (200 and 500 μM) of fatty acids. These four experiments were carried out simultaneously. Chloroplast swelling in the control samples was activated in the presence of 200 μM of unsaturated fatty acids. The two swelling curves were time dependent and crossed after a period of time depending on the number of double bonds in the fatty acids. If one considers the time necessary

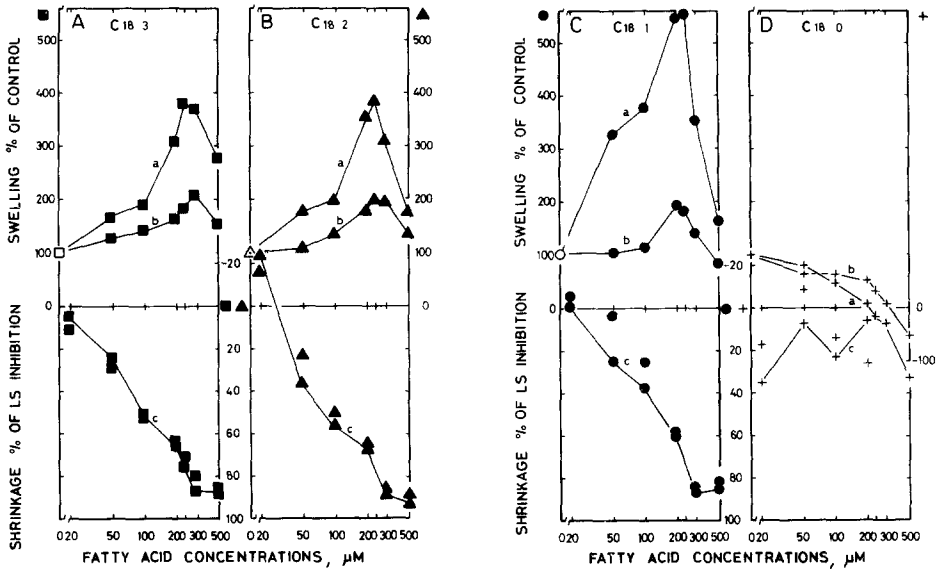

Fig. 1. Influence of various concentrations of C_{18}-fatty acids on chloroplast swelling and light-induced scattered light: *i.e.* linolenic ($C_{18:3}$), linoleic ($C_{18\ 2}$), oleic ($C_{18\ 1}$) and stearic (C_{180}) acids. Incubation times in the presence of fatty acids are 20 min for the swelling and 10 min for the light-scattering (LS) experiments. Results are expressed as a percentage of controls for swelling — (treated/control) × 100 — and as a percentage of inhibition — [(treated–control)/control] × 100 — for light scattering (average of 7 experiments). Curves a and b correspond to experiments where the controls ($-\Delta A_{540\ nm}$) were, respectively, 0.110 and 0.230. Curve c corresponds to the extent (control: 51% ΔLS) and the points which are outside the curve to the rate (control: 35% ΔLS/min) of the reaction.

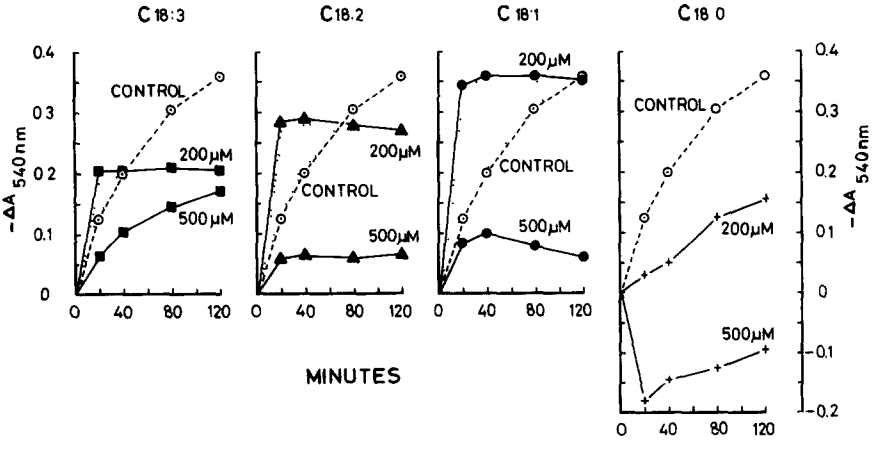

Fig. 2. Influence of incubation time with C_{18}-fatty acids on chloroplast swelling.

for the curves to intersect and the swelling induction capacity of the samples, the activity of the four C_{18}-fatty acids could be established in the decreasing order: $C_{18:1} > C_{18.2} > C_{18\ 3} > C_{18:0}$ (see also the dotted surfaces of Fig. 2). Although this was usually the case, there were several exceptions, possibly due to an aging factor. Maximum swelling was attained after 20 min of incubation. An addition of 500 μM

of unsaturated fatty acids or of 200 μM stearic acid prevented chloroplast swelling almost completely (Fig. 2). But at a concentration of 500 μM, stearic acid ($C_{18:0}$) caused an increase in absorbance.

Fig. 3 showed that swelling curves, with the chlorocrit technique, which measures the chloroplast volumes directly, were quite similar to those obtained with the spectrophotometric technique in the presence and absence of oleic acid (Fig. 1C).

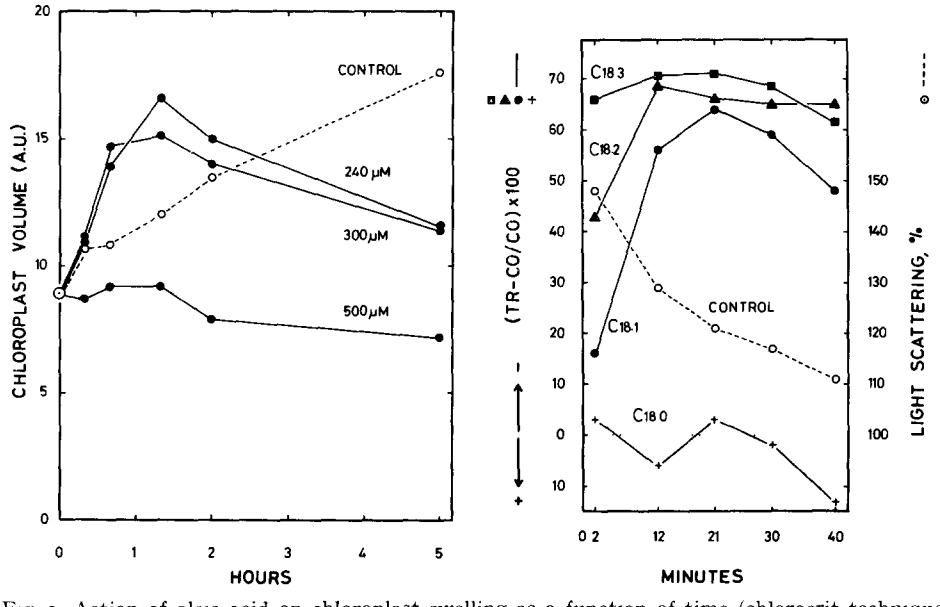

Fig. 3. Action of oleic acid on chloroplast swelling as a function of time (chlorocrit technique). Chloroplast volumes are expressed as arbitrary units (A.U.)[30]. Although the reaction mixtures contained 1 mg chlorophyll/ml, the concentrations of fatty acids indicated on the curves were adapted for 20 μg chlorophyll/ml (see Materials and Methods).

Fig. 4. Influence of C_{18}-fatty acids incubation time on light-induced scattered light changes in chloroplasts. Fatty acid concentrations are 200 μM. Results (average of 5 experiments) are expressed as the inhibition percentage of the light-scattering rate, *i.e.* [(treated–control)/control] $\times$ 100 = (TR–CO/CO) $\times$ 100. The control (% LS) is also given as a function of incubation time.

The action of the C_{18}-fatty acids on light-induced shrinkage is illustrated in Fig. 4. The capacity of the control chloroplasts to undergo light-induced scattering changes decreased rapidly as a function of incubation time at 20 °C. This occurrence was due to an accelerated aging effect in diluted systems (20 μg chlorophyll/ml instead of 1 mg/ml as described previously[1]). The patterns of the scattered-light inhibition by unsaturated fatty acids varied considerably with the degree of saturation of the molecule. Indeed, although maximum inhibition was the same after 20 min for the three fatty acids (60–70 % of inhibition at 200 μM), the time required to reach it was inversely related to the number of double bonds in the molecule. Stearic acid had no effect on light-induced shrinkage.

The inhibition of light-induced chloroplast shrinkage as a function of time and various concentrations of oleic acid was studied in more detail (not shown). A 50 % inhibition of scattered light occurred after 3, 6, 12, 17 and 29 min for, respectively, 500, 300, 200, 150 and 100 μM of oleic acid.

TABLE II

EFFECTS OF SERUM ALBUMIN ON SWELLING INDUCED BY C_{18}-FATTY ACIDS AND ON LIGHT-INDUCED SCATTERED LIGHT INHIBITED BY C_{18}-FATTY ACIDS IN CHLOROPLASTS

Conditions *	*Swelling* (% *of control*) **		*Shrinkage* (% *of scattered light*)	
	$C_{18:1}$	$C_{18:3}$	$C_{18:1}$	$C_{18:3}$
Control	100		165	131
+ Serum albumin (2–5 mg/ml)	103		182	151
+ Fatty acids (200 μM)	320	207		
+ Fatty acids (200 μM) + serum albumin (2 mg/ml)	100	112		
+ Fatty acids (300 μM)			116	100
+ Fatty acids (300 μM) + serum albumin (3 mg/ml)			149	148
+ Fatty acids (500 μM)	146	192		
+ Fatty acids (500 μM) + serum albumin (2 mg/ml)	292	154		
+ Fatty acids (500 μM) + serum albumin (5 mg/ml)	104	108		

* The reaction mixtures for the controls are described in Materials and Methods.

** Controls: $-\Delta A_{540\ nm} = 0.145$ per 20 min.

*** The incubation time in the presence of fatty acids prior to initiation by light is 2 min. % of light scattering correspond to reaction rates.

Effect of serum albumin

Serum albumin was shown to be very effective in preserving the activity of isolated chloroplasts stored *in vitro*[31]. It was postulated that this compound stimulated chloroplast photoreactions by binding unsaturated fatty acids released endogenously during isolation of these organelles[32]. Therefore, it was interesting to test the ability of serum albumin as it relates to the preservation of the chloroplast structure in the presence of exogenous unsaturated fatty acids which behave like swelling agents (see Table I and Fig. 1). Table II refers to experiments involving the combined action of various concentrations of two C_{18}-fatty acids and albumin. The addition of albumin protected chloroplasts against swelling induced by oleic and linolenic acids and restored to the same level the scattering activity which was inhibited by these unsaturated fatty acids. The extent of swelling depended on the delicate control of the fatty acids/bovine serum albumin ratio. Similar results were found with linoleic acid.

DISCUSSION

These results demonstrate that the action of fatty acids can be used as a model system for the aging phenomenon *in vitro* of isolated spinach chloroplasts as expressed by volume changes. Indeed, chloroplast aging *in vitro* was characterized by a slow swelling which reached its maximum after several hours of incubation and by a concomitant inactivation of the organelles to carry out light-induced shrinkage[1,4]. A similar effect has now been observed with fresh chloroplasts treated with increasing concentrations of fatty acids (Fig. 1). In the model system, changes were found to be concentration dependent rather than time dependent. The interesting feature of this phenomenon was that only those fatty acids which are encountered in chloroplast membrane lipids[33,34] (and which are released during aging[10,11,12]) were found to be active. Wintermans *et al.*[12] established that free fatty acids in freshly isolated chloro-

plasts consisted mainly of C_{16}- and C_{14}-saturated acids, with very little C_{18}-acids present. All the saturated fatty acids tested, which have been shown to be absent (or present in small amounts) in chloroplast membrane lipids, have a small effect in stimulating swelling and inhibiting light-induced shrinkage (Table I). According to Wintermans *et al.*[12], and Constantopoulos and Kenyon[11], the acids released during aging consisted mainly of C_{18}- and to a smaller extent of C_{16}-unsaturated acids. These compounds were precisely the most effective swelling agents and inhibitors of light-induced shrinkage. Because of its non-availability at that time, hexadecatrienoic acid, which is also released during aging[11], could not be tested.

It was previously reported that the slow chloroplast swelling occurring in the dark during aging *in vitro* was accelerated in the light with an optimum after 90 min[1]. This phenomenon might now be interpreted as being due to an increase in endogenous unsaturated fatty acids resulting from chloroplast membrane lipid hydrolysis. This interpretation agrees with the observations made *in vivo*[35] showing that upon illumination, the ratio of saturated to unsaturated fatty acids decreased in chloroplasts. Moreover, the diminution of light-induced swelling after 90 min of incubation is probably due to an excess of endogenous fatty acids similar to that causing swelling decrease in the presence of high concentrations (240–500 μM) of unsaturated fatty acids (see Fig. 1) or to a diminution of unsaturated acids as a result of photoperoxidation processes as shown *in vitro*[25]. The volume changes in chloroplasts described thus far are in agreement with previous studies[21,22,13].

Since fatty acids have been shown to inhibit the Hill reaction[4,10–18] and photophosphorylation[4,10,32], they were also expected to alter light-induced shrinkage. This is indeed the case (see Figs 1 and 4). The inhibitory effect of fatty acids on light-induced scattered light can be interpreted by the 2-stage photophosphorylation reaction. This reaction is characterized by a light stage where a high energy intermediate, X_E, is formed and a dark stage, during which synthesis of ATP *per se*[36] occurs. Friedlander and Neumann[32] have demonstrated that linoleic and linolenic acids, when added to the light stage inhibited X_E to the extent of 95 %. When added in the dark, these acids did not inhibit the formation of X_E. Since light-induced shrinkage has been thought to be X_E related, the results thus far are in agreement with the observations of the above-mentioned authors.

Fatty acids released during plastid isolation[32] or in the course of aging *in vitro*[11,12], or exogenous acids added to fresh chloroplasts[21] were shown to inhibit electron flow. An addition of bovine serum albumin largely prevented the loss of Hill activity[31], and also enhanced and stabilized the photophosphorylation efficiency[12,31,32]. When added in the light stage of ATP formation, albumin increased markedly the yield of X_E, but had no effect when added in the dark stage[32]. The fact that fatty acids and albumin act at the same site of the energy-transfer pathway in chloroplasts could explain the restoration by albumin of light-induced shrinkage inhibited by fatty acids (Table II). In addition, bovine serum albumin has been shown to overcome chloroplast swelling caused by fatty acids, in agreement with previous findings[21].

Thus, these results give support to the concept that chloroplast aging *in vitro* is due, at least in part, to a release of free unsaturated fatty acids which induces swelling and inhibits the energy-linked reactions, namely, light-induced shrinkage. Although crucial, the direct effect of unsaturated fatty acids is certainly not the only

one responsible for these phenomena. A number of reactions and factors, summarized in Fig. 5, might also promote these effects. Chloroplast swelling could be due to an interaction of fatty acids with the tertiary structure of protein, inducing a conformational change, as has been established in model experiments[37]. The action of hydrolytic enzymes, namely, galactolipases[5–9,12,38], alters the identity of the lipoprotein

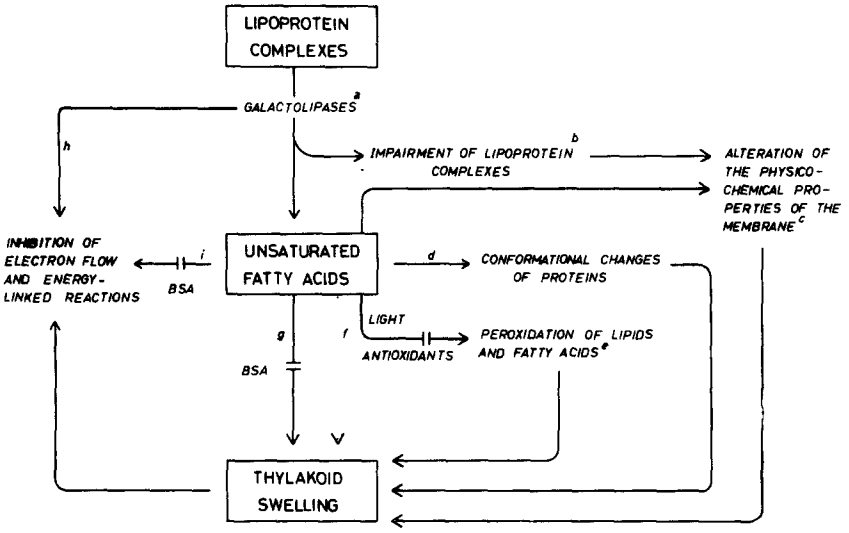

Fig. 5. Reactions which induce thylakoid swelling in the course of aging. [a] *cf.* refs 5–9, 12; [b] *cf.* refs 22, 37, 38; [c] *cf.* ref. 22, [d] *cf* ref. 37; [e] *cf.* refs 23–27; [f] *cf.* ref. 37; [g] *cf.* refs 13, 21, 22 and Figs 1–3 and Table II; [h] *cf* refs 10–12, 38, 40–42; [i] *cf.* refs 4, 10–19, 21 and Figs 1 and 4. BSA = bovine serum albumin.

complexes of the thylakoid[38] which, in turn, modify the physicochemical properties (namely, the osmotic properties) of the membrane and inhibit photochemical reactions in chloroplasts[12]. Galactolipids were indeed shown to have a stimulative effect on the rate of cytochrome *c* photoreduction by intact spinach chloroplasts[44]. Also, triglycerides containing unsaturated C_{18}-fatty acids were able to restore photosystem I activity in heptane-extracted spinach chloroplasts[39]. Consequently, any change in the ratio chlorophyll/galactolipids might greatly effect the photochemical reactions in chloroplasts[43].

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