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Polyunsaturated Fatty Acid Biosynthesis in Cotyledons from Germinating and Developing *Cucumis sativus L.* Seedlings[1]

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ABSTRACT

Etiolated *Cucumis sativus L.* cotyledons preferentially catabolized exogenous [1-^{14}C]oleic acid and [1-^{14}C]linoleic acid with relatively little incorporation into complex lipids or desaturation of the ^{14}C-labeled fatty acids. Following a 16-hour exposure to light, the greening cotyledons efficiently desaturated the exogenous ^{14}C-labeled fatty acids. A small amount of oleate desaturation to linoleate was observed in etiolated tissue, but hardly any linoleate desaturation to α-linolenate was detected. Both oleate and linoleate desaturation showed diurnal variations with maxima at the end of light periods and minima at the end of dark periods. Illumination of etiolated tissue by flashing light, as opposed to continuous light, failed to stimulate either chlorophyll or α-linolenic acid biosynthesis, and both processes could be halted or reversed by 10 micrograms per milliliter cycloheximide. Production of polyunsaturated fatty acids from [1-^{14}C]acetate, [1-^{14}C]oleic acid, and [1-^{14}C]linoleic acid, by greening cucumber cotyledons, was markedly affected by tissue integrity with finely chopped cotyledons having very little capacity for their synthesis and intact seedlings showing the highest rates.

Polyunsaturated fatty acids are major components in the membranes of most photosynthetic and nonphotosynthetic plant tissues. The degree of desaturation of the membrane lipids may be associated with both chilling and freezing resistance of the plant, but the overwhelming preponderance of polyunsaturates in photosynthetic and many seed tissues implies that they may also serve other roles (6). Cucumber tissues present a useful model system for studying the metabolism of polyunsaturated fatty acids during seed germination, growth of cotyledons, and the onset of photosynthetic competence. All of these processes can be studied in one tissue, the cucumber cotyledon. We have previously reported that linoleic acid accounts for 70% of the fatty acid of mature cucumber seed (9). Following greening of germinated cotyledons, the bulk of this pool of linoleic acid was further desaturated to α-linolenic acid, which comprises 60 to 70% of the fatty acid of mature photosynthetic tissue. To further elucidate the metabolism of these two long-chain polyunsaturated fatty acids, we have extended the earlier *in vivo* studies. Here differences in fatty acid metabolism in etiolated and greening cotyledons are reported. The oleate and linoleate desaturation activities are also characterized with respect to a diurnal light/dark cycle, intermittent *versus* continuous illumination, tissue integrity, and a range of inhibitors, all of which affect the extent of desaturation occurring in the cotyledon.

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MATERIALS AND METHODS

Plant Material. Seeds of *Cucumis sativus L.* var Alpha green were supplied by the Niagara Chemical Division, F. M. C. Corporation, Modesto, Calif. Etiolated seedlings were grown as previously described (9). Seedlings were greened for 16 h in the presence of white light of intensity (quantum flux) <200 μE m^{-2} s^{-1}.

Substrates and Chemicals. [1-^{14}C]Oleic acid (56 Ci mol^{-1}) and [1-^{14}C]linoleic acid (60 Ci mol^{-1}) were purchased from Amersham/Searle. Ethylene glycol monoethylether was obtained from Sigma. All solvents were reagent grade.

Incubation of Tissue. Cucumber cotyledons were excised from greening seedlings and dried on a glass plate. ^{14}C-Labeled fatty acids were applied to the upper surfaces in the form of microdroplets (<10 μl/cotyledon) of ethylene glycol monoethylether solution. The microdroplets were allowed to be absorbed by the tissue before being placed in a 2-ml incubation mixture containing a 0.1 M phosphate buffer (pH 6.5). All incubations were performed under aerobic conditions with gentle shaking in a water bath at 25 C. Illumination was provided by fluorescent white light (General Electric F15T8D, Daylight, 15 w) of intensity <200 μE m^{-2} s^{-1}.

Lipid and Pigment Analysis. Total lipid mixtures were extracted in hexane-isopropyl alcohol (3:2, v/v) and Chl was extracted in 80% acetone as previously described (9). Chl was determined according to the method of Arnon (1). Lipid analyses were performed and the identity of fatty acid products were verified as in reference 9.

RESULTS

Metabolism of Exogenous Fatty Acids in Etiolated and Greening Cotyledons. A comparison of the metabolism of exogenous [1-^{14}C]oleic acid in etiolated and greening cucumber cotyledons is shown in Figure 1. In the greening cotyledons, there was no conversion of the ^{14}C-labeled fatty acid into non-lipid material and essentially all of the ^{14}C was recovered in the fatty acid methyl ester fraction after lipid extraction. The added [1-^{14}C]oleate was desaturated at a constant rate throughout the 32-h incubations, first appearing as [1-^{14}C]linoleate and subsequently as [1-^{14}C]linolenate, in which form about 40% of the label eventually accumulated. In contrast, the etiolated tissue, which also rapidly absorbed exogenous [1-^{14}C]oleate, apparently utilized most of the fatty acid as a substrate for β-oxidation. The total ^{14}C label in the tissue declined by >90% during the incubations, with most of it lost to the atmosphere as $^{14}CO_2$, as measured by trapping in KOH. The small amount of [1-^{14}C]oleate remaining in the tissue was desaturated as far as [1-^{14}C]linoleate, albeit at a much lower rate than in the greening tissue, but only trace quantities of the final desaturation product, [1-^{14}C]linolenate, were detected.

The results of a similar experiment in which [1-^{14}C]linoleate was added to etiolated and greening cotyledons are depicted in

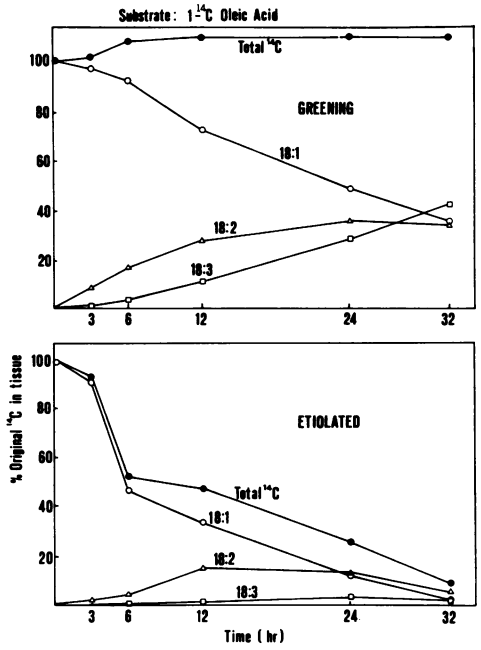

FIG. 1. Metabolisms of [1-^{14}C]oleic acid by greening and etiolated cucumber cotyledons. Ninety-five to 100% of the ^{14}C-labeled fatty acids applied as an ethylene glycol monoethylether solution had penetrated the cuticle of the tissue within 5 min of application and could not be washed off; this figure represents the 100% original ^{14}C in the tissue at the start of each incubation. All plants were 7 days old and the greened cotyledons had been illuminated for the previous 16 h. All incubations with etiolated cotyledons were performed in darkness, whereas greening cotyledons were incubated in the light.

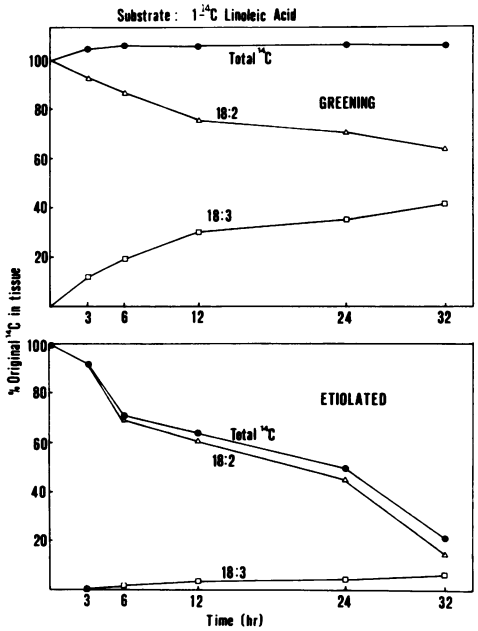

FIG. 2. Metabolism of [1-^{14}C]linoleic acid by greening and etiolated cucumber cotyledons. Conditions for the experiments are the same as described in legend of Figure 1.

Figure 2. Once again, the exogenous ^{14}C-labeled fatty acid was utilized as a substrate for acylation of lipids and for desaturation to [1-^{14}C]linolenate in the greening tissue. The etiolated tissue catabolized most of the added [1-^{14}C]linoleate, although at a somewhat slower rate than when [1-^{14}C]oleate (Fig. 1) was the substrate, and, after 32 h, >20% of the added ^{14}C was still recovered as fatty acid. Whereas the greening tissue had desaturated >40% of the exogenous [1-^{14}C]linoleic acid after 32 h, the etiolated tissue had only desaturated about 6% after the same time.

Uptake of [1-^{14}C]Oleic Acid into Glycerolipids by Etiolated Cotyledons. Most of the [1-^{14}C]oleic acid added to etiolated cucumber cotyledons was broken down into non-lipid products, although a small proportion of the ^{14}C was found in complex lipids (Fig. 3). However, even this lipid pool was eventually broken down since the ^{14}C in all the labeled glycerolipids declined after 12 to 32 h. Unlike the illuminated cotyledons (10), there was no net accumulation of ^{14}C in any of the glycerolipids of the etiolated tissue. The major labeled glycerolipid was the phospholipid phosphatidylcholine which was also the principal site of linoleate accumulation. Each of the labeled glycerolipids contained a large proportion (>50% total ^{14}C) of the first desaturation product of oleate, *i.e.* linoleate, but only trace amounts of the subsequent product, α-linolenate, were detected in most of the lipids (Fig. 4).

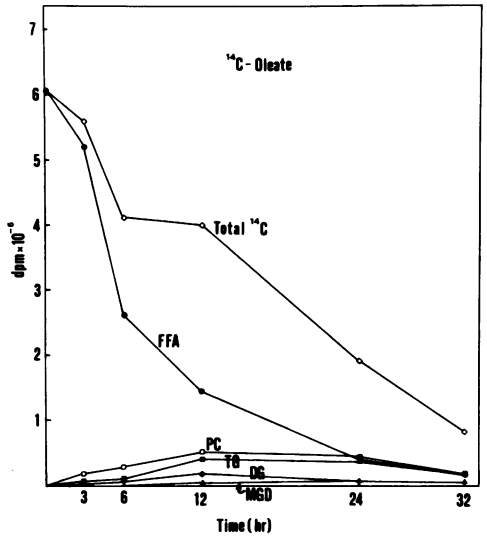

FIG. 3. Time course of the incorporation into lipid classes of ^{14}C label from [^{14}C]oleate by etiolated cucumber cotyledons. Only the principal ^{14}C-labeled lipids are shown. FFA, free fatty acid; PC, phosphatidylcholine; TG, triacylglycerol; DG, diacylglycerol.

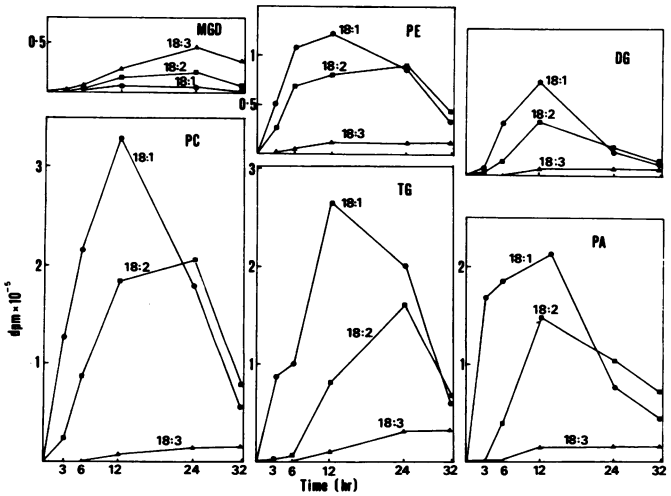

FIG. 4. Time course of the incorporation of the desaturation products of [1-^{14}C]oleate into lipid classes by etiolated cucumber cotyledons. PE, phosphatidylethanolamine; DG, diacylglycerol; PC, phosphatidylcholine; TG, triacylglycerol; PA, phosphatidic acid.

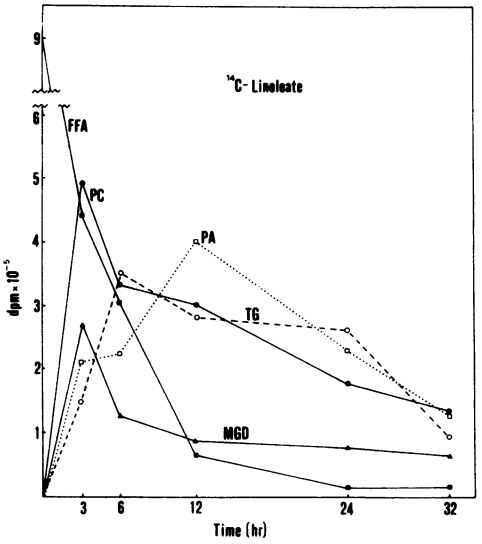

FIG. 5. Time course of the incorporation into lipid classes of ^{14}C label from [1-^{14}C]linoleic acid by etiolated cucumber cotyledons. Only the principal ^{14}C-labeled lipids are shown. FFA, free fatty acid; PC, phosphatidylcholine; PA, phosphatidic acid; TG, triacylglycerol.

Only MGD[2] contained α-linolenate as its principal labeled fatty acid and the galactolipid accounted for no more than 3% of the total ^{14}C after 24 h. Although both [^{14}C]oleate and [1-^{14}C]linoleate declined rapidly after 12 to 24 h, as the lipids were being broken down, the small amount of ^{14}C found in α-linolenate stayed fairly constant. This implies a differential turnover rate for α-linolenate, even in a tissue which is rapidly catabolizing fatty acids as a major energy source.

Uptake of [1-^{14}C]Linoleic Acid into Glycerolipids by Etiolated Cotyledons. The overall breakdown of exogenous [1-^{14}C]linoleic acid by etiolated cotyledons initially proceeded at a slower rate than did the breakdown of [1-^{14}C]oleic acid (compare Figs. 1 and 2). This was reflected in a much increased rate of acylation of linoleic acid compared to oleic acid (Figs. 5 and 3, respectively). All of the major glycerolipids readily incorporated [1-^{14}C]linoleic acid in the initial 3 to 12 h of the experiment and showed a rapid loss of ^{14}C thereafter. Analysis of the fatty acids of these lipids (Fig. 6) demonstrated that little or no [1-^{14}C]linoleate was desaturated over the course of the study. The galactolipid, MGD, accumulated a small amount of [^{14}C]α-linolenate, but the flux of unchanged [^{14}C]linoleate through MGD was more than 5-fold in excess of this. [1-^{14}C]Linoleate was turned over at a much more rapid rate than [1-^{14}C]oleate and may be a preferred substrate for both acylation to complex lipids and oxidative breakdown during seed germination. This would be reasonable since the major fatty acid in the mature seed is indeed linoleic acid.

Diurnal Variation in Desaturating Activities. The variation in desaturating activities of both greening and light-grown cucumber cotyledons during a 16-h dark/8-h light regime is given in Figure 7. All desaturating activities tended to increase during the light period and decrease during the dark period. Following the first four light periods, light-grown cotyledons were able to desaturate over 3 times the amounts of [^{14}C]oleate compared with plants grown for a similar time in the dark (Fig. 7, bottom). However, the rise in [^{14}C]oleate desaturation in the illuminated cotyledons during each light period was more than offset by the decline during the dark period after day 6 and the daily maxima showed a progressive decline thereafter. Dark-grown tissue showed limited ability to desaturate [^{14}C]oleate as previously noted. However, this activity was dramatically stimulated when the cotyledons were

[2] Abbreviation: MGD, monogalactosyl diacylglycerol.

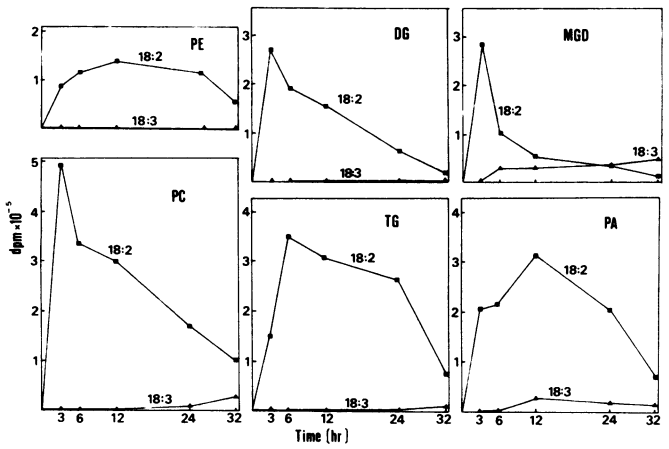

FIG. 6. Time course of the incorporation of the desaturation products of [1-^{14}C]linoleate into lipid classes by etiolated cucumber cotyledons. PE, phosphatidylethanolamine; DG, diacylglycerol; PC, phosphatidylcholine; TG, triacylglycerol; PA, phosphatidic acid.

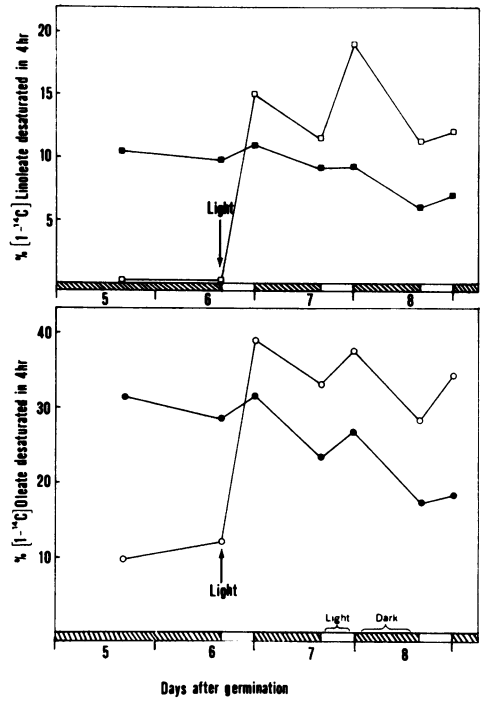

FIG. 7. Diurnal variation in [1-^{14}C]oleate and linoleate desaturation in light-grown and greening cucumber cotyledons. Cucumber seedlings were germinated as previously described (9) and grown either in complete darkness (○—○, □—□) or in a 16-h dark/8-h light regime (●—●, ■—■). After the dark period on day 6, dark-grown plants were placed in the 16-h dark/8-h light regimen. Assays were performed on both greening (*i.e.* previously dark-grown) plants and light-grown plants at the start and finish of each light period from day 6 until day 8. Excised cotyledons were incubated with the appropriate ^{14}C-labeled fatty acid for 4 h in the dark. The results are average values of two duplicate experiments.

illuminated on day 6 and was some 25% higher than the activity of the light-grown tissue after 8 h illumination. On subsequent days, the maximum activities declined as the fall during darkness was greater than the rise during the light period. But the overall decline in this greening tissue was slower than that in the light-grown tissue.

[^{14}C]Linoleate desaturation was seen in the light-grown cotyle-

dons and showed a similar consistent, although much less marked, decrease with time (Fig. 7, top). There was only a minimal increase in the activity over the course of the light periods with larger decreases during the dark periods. Dark-grown cotyledons exhibited no detectable capacity for [1-^{14}C]linoleate desaturation until they were illuminated on day 6. After 8 h in the light, there was a sharp rise in [1-^{14}C]linoleate desaturation to a value 36% higher than that of the corresponding light-grown tissue. In contrast to [^{14}C]oleate, [1-^{14}C]linoleate desaturation in greening cotyledons only achieved its maximum value after two light periods, when it was over twice as high as in the light-grown tissue. During the third day of greening, there was a steep fall in activity during the dark period and only a very small rise during the light period, with the over-all maximum activity showing a sharp decline.

Effect of Mode of Illumination upon Chl Biosynthesis and Linoleic Acid Desaturation. The greening response of etiolated cucumber cotyledons was found to differ with the nature of the illumination given to the tissue (Fig. 8). Chl biosynthesis proceeded most rapidly in the presence of continuous light from a 15-w fluorescent lamp (Fig. 8b) and, after 48 h, the Chl *a*/*b* ratios were of the order of 1.8 to 2.3. Intermittent illumination for 2 min/h with a 15-w lamp drastically curtailed Chl biosynthesis compared with the continuous-light regimes. In addition, the Chl *a*/*b* ratio was abnormally high in these cotyledons (of the order of 5.0–8.0).

The results of parallel analyses of the α-linolenic acid levels in these treatments are shown in Figure 8a. The effect of continuous illumination was broadly similar to that on Chl levels with the intermittent-light regime causing only a slight rise in α-linolenate levels. The more dramatic rises in α-linolenate levels in response to continuous illumination were accompanied by concurrent declines in the levels of linoleic acid in the cotyledons with the sum of the linoleic + α-linolenic acids remaining almost constant. These results, therefore, would suggest that (*a*) a brief exposure to light does not trigger the formation or activation of a linoleate desaturase; and (*b*) the formation of Chl and of linolenate are in some manner coordinated.

Effect of Cycloheximide upon Chlorophyll Biosynthesis and Fatty Acid Desaturation. The changes in Chl concentration during greening of cucumber cotyledons and the effects of cycloheximide are shown in Figure 9. The addition of cycloheximide at 10 μg ml^{-1} at zero greening time had little effect on the initial rate of greening (<3 h) but had significantly inhibited it after 6 h, and the Chl concentration then started a progressive decline until most of the cotyledons had lost all their Chl and had taken on the yellow color of etiolated plants after 18 h.

In the absence of cycloheximide, excised cucumber cotyledons were able to desaturate progressively more exogenous [1-^{14}C]linoleic acid with increasing hours of greening, although there was an initial lag period of about 3 h before desaturation was observed. This increasing capacity for linoleate desaturation levelled off between 14 to 18 h after the onset of illumination. The addition of 10 μg ml^{-1} cycloheximide to the greening cotyledons after 6 h in the light had no immediate effect on the rate of linoleate desaturation. However, after 18 h of greening, the cycloheximide-treated cotyledons showed a sharp decline in this activity compared to the untreated controls. The lag time, before cycloheximide at this concentration affected linoleate desaturation, was thus 8 to 12 h, whereas Chl biosynthesis was affected 4 to 8 h after the inhibitor was applied. In contrast, the cycloheximide completely abolished the light-stimulated increase in linoleate desaturation, if given immediately prior to greening, but did not affect Chl accumulation for 3 to 6 h.

Effect of Tissue Integrity upon Polyunsaturate Formation. The extent to which label from the added ^{14}C-labeled substrates was accumulated by cucumber cotyledons in polyunsaturated fatty acid varied according to the nature and extent of damage inflicted upon the tissue. In Figure 10, the formation of polyunsaturated fatty acid from [1-^{14}C]acetate, [1-^{14}C]oleic acid, and [1-^{14}C]linoleic acid is compared in four treatments of varying tissue integrity. In all cases, the actual uptake of labeled substrates by the tissues did not vary significantly. Finely chopped cotyledons were unable to produce more than trace amounts of polyunsaturated fatty acids, whatever the substrate used. Cotyledons excised from the hypocotyl, but otherwise undamaged, were unable to convert [1-^{14}C]acetate into polyunsaturated fatty acids in a 6-h incubation, although they were able to form palmitate and oleate from this precursor. They were also able to desaturate both

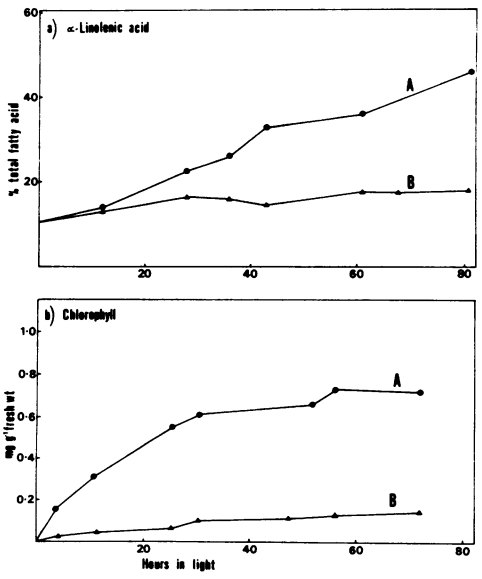

FIG. 8. Comparison of (a) α-linolenic acid and (b) Chl biosynthesis in etiolated cucumber cotyledons subjected to (A) continuous and (B) intermittent illumination. Seven-day-old dark-grown seedlings were illuminated either continuously or by a 2-min pulse of light every 2 h.

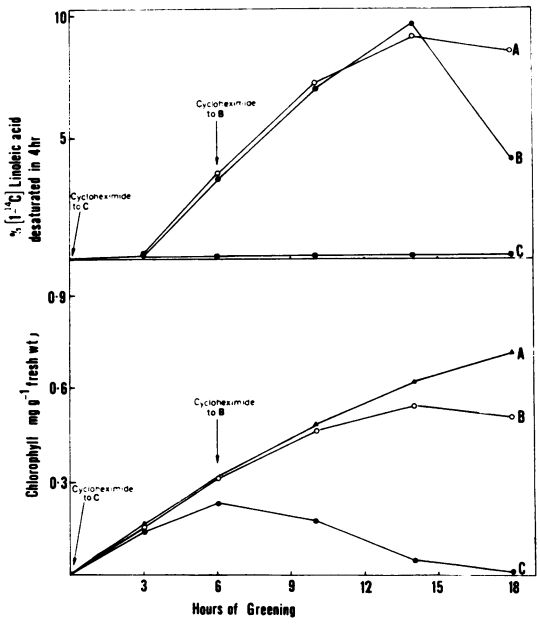

FIG. 9. The effect of cycloheximide upon Chl concentration and linoleic acid desaturation in greening cucumber cotyledons. A, detached cotyledons, no additions; B, detached cotyledons + 10 μg ml^{-1} cycloheximide after 6 h; C, detached cotyledons + 10 μg ml^{-1} cycloheximide after 0 h.

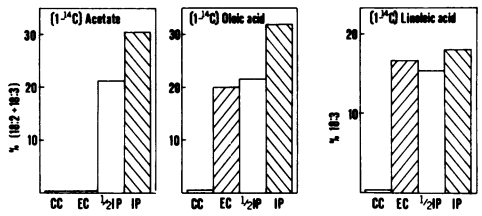

FIG. 10. The effect of tissue integrity upon fatty acid desaturation in greening cucumber cotyledons. ^{14}C-labeled fatty acids were applied to the tissue which was then incubated for 6 h in the light. IP, intact plant; ½ IP, seedling with one cotyledon half removed; EC, excised cotyledon; CC, chopped cotyledon.

[1-^{14}C]oleic and linoleic acids. Removal of one-half of the cotyledon pair led to a diminished capacity of the remaining half-cotyledon for polyunsaturated fatty acid formation from both [1-^{14}C]acetate and [1-^{14}C]oleic acid, compared to intact plants with both cotyledons present. The fully intact plants consistently formed a greater proportion of polyunsaturated fatty acids from all three substrates although the difference was only small in the case of [1-^{14}C]linoleic acid. Analysis of the lipid products of these incubations showed that the amount of labeled monogalactosyl diacylglycerol was proportional to the amount of [^{14}C]α-linoleate acid formed and that most of the label in this galactolipid was due to [^{14}C]α-linolenate.

DISCUSSION

Fatty acid metabolism in a germinating oil-seed tissue, such as cucumber, is a complex process. The major energy reserve of the tissue is linoleic acid esterified to triacylglycerols and phospholipids. During germination, the bulk of this lipid is broken down to free fatty acid, which is then β-oxidized in the glyoxysomes and finally converted to sucrose via tricarboxylic acid cycle and gluconeogenic enzymes located in the mitochondria and cytosol (11). It is apparent that etiolated cucumber seedlings maintain this heterotrophic metabolism since they rapidly catabolized both of the exogenous ^{14}C-labeled fatty acids supplied to them. Although the breakdown of fatty acid was the principal process occurring in the tissue, a proportion of the added [1-^{14}C]oleic acid was desaturated as far as [1-^{14}C]linoleate. This implies that active synthesis of linoleate may also be occurring in the tissue, at least if the substrate is present. However, the subsequent desaturation step to α-linolenate occurred at very low rates and reflects the poor capacity of etiolated tissue to synthesize this fatty acid (9). [1-^{14}C]Linoleic acid served as a much more efficient substrate for acylation than did [1-^{14}C]oleic acid, and parallel experiments showed that [1-^{14}C]stearic and [1-^{14}C]palmitic acids were only very slowly acylated to complex lipids. There may be a relationship between the degree of unsaturation and rapidity of acylation, although this may be a simple scavenging mechanism for preferentially removing the highly reactive free polyunsaturated fatty acids for conversion into a less disruptive form (13, 14).

Following 16 h greening, the fatty acid metabolism of the cotyledons had undergone a dramatic change. We have previously discussed the light-dependent induction of the enzymes responsible for polyunsaturated fatty acid biosynthesis (9). The 16 h illumination given to the cotyledons in the present study was sufficient to halt the breakdown of exogenous ^{14}C fatty acids. This breakdown was probably due to glyoxysomal activity (4, 15). Following greening, the tissue had substantially changed to an autotrophic metabolism as evidenced by an increase in dry weight and the onset of CO_2 fixation, O_2 evolution, and light-dependent *de novo* fatty acid synthesis. Since etiolated cucumber cotyledons preferentially catabolized exogenous ^{14}C-labeled fatty acids, rather than accumulating or desaturating them, it was decided to use only greening cotyledons for the subsequent investigation of possible glycerolipid intermediates in polyunsaturated fatty acid biosynthesis (10).

Fatty acid desaturation in developing cucumber cotyledons can be modulated by a variety of natural and artificial effects. We have already noted dramatic effects of both growth temperature and illumination upon these processes (9). Diurnal variation in the levels of starch, NADPH, and ATP in plants can be readily observed and are directly attributable to the absence of photosynthesis in the dark. Thus, it is to be expected that other processes that depend upon ATP and NADPH will also show such daily fluctuation. In particular, fatty acid desaturation, which requires O_2 and reduced electron donors, *e.g.* reduced ferredoxin, would presumably not be present in large amounts during the dark period. Diurnal variations in the levels of monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol, and phosphatidylglycerol have been observed in the leaves of *Spinacia oleracea* (5) and in the fronds of the fern *Pteridium aquilinum* (7), which suggest a high rate of turnover of these lipids.

The gradual decline in the maximal desaturation achieved during each day/night cycle indicates that the tissue synthesizes less polyunsaturated fatty acids as it matures and achieves full photosynthetic competence. This implies that turnover of polyunsaturated fatty acids is not rapid in mature tissue since the desaturating system only functions fully when required for *de novo* synthesis. Similarly, the sensitivity to the light/dark cycle probably falls off as the overall activities decline with age. The diurnal variations in complex lipids found in spinach (5) and bracken (7) thus are probably due to turnover of glycerol or headgroup moieties rather than of the polyunsaturated acyl chains.

Bahl *et al.* (2) have reported that 1-ms light flashes alternating with 15-min dark periods result in normal greening of etiolated wheat leaves and the usual stimulation of acetyl lipid synthesis, in particular the polyunsaturated glycolipids. Subsequently, they (2) found that the lipid composition and ultrastructure of isolated organelle and sub-organelle fractions were the same in plants subjected to either continuous or intermittent illumination (3). More recently, Arntzen's group (personal communication) have noted that a 2-min light + 118-min dark regime (similar to the one used in this study) caused drastically reduced levels of Chl and, in particular, Chl *b*. Some photosynthetic electron transport could be detected, but granal stacking did not occur until continuous illumination was provided. Here, similar reductions in Chl levels and a deficiency in Chl *b* after intermittent-light treatment compared to continuously illuminated controls were found, but it was also observed that α-linolenic did not rise in intermittently illuminated plants. The lack of granal stacking in such tissue may be due to the absence of the light-harvesting Chl-protein but the dearth of α-linolenic acid may also be a contributory factor.

The light-induced rise in desaturation activity can be totally abolished by the addition of cycloheximide to the tissue immediately prior to illumination. Cycloheximide added after the onset of greening affected Chl biosynthesis more rapidly than linoleate desaturation but, in both cases, the eventual decline in the activities suggests that protein turnover is occurring.

Damage of any plant tissue by abrasion, excision, or chopping may give rise to wounding responses that can dramatically affect the metabolism of both the tissue directly affected and the rest of the plant (12). When discs were excised from leaves of *Nicotiana tabacum*, the dark respiration rate increased rapidly, reaching a plateau within 15 min of excision (8). The capacity of cucumber cotyledons to convert various substrates into polyunsaturated fatty acids was drastically altered by tissue damage and fine chopping of the cotyledons completely removed this capacity. The intact plants were not only the least perturbed treatment but they were also the best at polyunsaturate formation. The enzymes responsible for polyunsaturate biosynthesis may be especially susceptible

to inactivation due to tissue damage and this may explain the relative lack of success in observing these activities *in vitro*.

The terminal desaturating mechanisms of greening cucumber cotyledons appear to be complex and subject to different levels of control. Light is the most important factor in both inducing and maintaining their activity and this was seen even in light-grown tissue subjected to a diurnal light/dark cycle. The desaturating activities respond dramatically to tissue damage and it is important for other investigators to be aware of this, especially in its implications for *in vitro* studies. Finally, while these findings hold for germinating cucumber cotyledons, we have found that other tissues, notably maize and spinach, behave differently and extrapolation of results from only one species is therefore inadvisable.

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