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DESATURATION OF OLEATE-[^{14}C] IN LEAVES OF BARLEY

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; leaves; desaturation; oleate-[^{14}C]; linoleate; linolenate; phosphatidylcholine; monogalactosyl diacylglycerol.

Abstract—Etiolated barley leaves when exposed to light desaturate oleate-[^{14}C] to linoleate. The production of substantial amounts of radioactive linolenate was found only in very young, tightly rolled leaves. In oleate-[^{14}C] pulse experiments, radioactive linolenate first appeared in phosphatidylcholine (PC) and only after a lag period did it begin to accumulate in monogalactosyldiacylglycerol (MGDG). The results indicate that in young, immature barley leaves linolenate is synthesized from oleate on the parent lipid, PC, and is then transferred to MGDG.

INTRODUCTION

The plastid lipids of higher plants contain relatively large quantities of C_{18} polyunsaturated fatty acids. Earlier work [1] with higher plants and algae indicated that linoleic and linolenic acids were synthesized by the successive desaturation of oleic acid. More recently Tremolières and Mazliak [2], using pea leaves, found that as much as 20 and 50% of the radioactivity of oleate-[^{14}C] resided in linolenate and linoleate, respectively, after a 6-hr incubation period. In these experiments the substrate was added as 'microdroplets' to the whole leaf. On the other hand, Stumpf (see [3] for review) has demonstrated the synthesis of linolenate by the elongation of 16:3 which in turn may arise from the successive elongation of 12:3 acids. The significance of such a pathway still awaits appraisal although it is of interest that the elongation of 16:3 and the desaturation of oleate are both involved in the synthesis of linolenate in rape leaves [4].

There is growing support which implicates phosphatidylcholine (PC) as the major site for the synthesis of linoleate. Roughan [5, 6] found that $^{14}CO_2$ and acetate-[^{14}C] were rapidly incorporated into the unsaturated acids of PC in pumpkin leaves, and Willemot and Verret [7] have demonstrated the synthesis of 18:2/18:3 rich PC with choline-[^{14}C] in alfalfa leaves. However, the parent lipid substrate on which linolenate can be synthesized still remains obscure with suggestions that it is PC [8] or monogalactosyldiacylglycerol (MGDG) [9, 10].

This paper describes the desaturation of oleate-[^{14}C] in pulse experiments with greening barley leaves in an attempt to relate desaturation activity with leaf growth and maturity, and to elucidate the major substrate for linoleate and linolenate production.

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RESULTS

In preliminary experiments it proved difficult to demonstrate the synthesis of linolenic acid from oleate-[^{14}C] in etiolated or fully green barley leaves. In such material, however, adequate conversion of oleate to linoleate occurred. It was apparent that the maturity of the experimental material was extremely critical in determining whether the full desaturation of oleate to linolenate took place. Fig. 1 shows the relative radioactivity of linoleic and linolenic acid synthesized from oleate-[^{14}C] in leaf segments from progressively older dark-grown barley leaves which had been transferred to the light for 10 hr. Maximum amounts of linoleate-[^{14}C] were produced in the very young leaves (5 days dark growth) which had just emerged from the leaf sheath and which were still tightly rolled before transfer to the light. In the segments from these young leaves as much as 20% of the total radioactivity recovered in lipid was present in linolenic acid. The formation of linolenate from oleate-[^{14}C] decreased rapidly in older leaves where 'unrolling' was more advanced. Barley leaves at an early stage of growth and still tightly 'rolled', therefore, provide excellent material for an investigation of the sites and substrates for the desaturation of oleic acid.

Leaf sections from 5-day-old leaves were illuminated for 10 hr and then given a short 'pulse' of oleate-[^{14}C]. After thorough washing, the leaf segments were transferred to buffer and samples removed at regular intervals. During the incubation in buffer, the leaf samples were kept under continuous illumination. The incorporation of radioactivity from oleate-[^{14}C] was determined in the lipid groups, the individual complex lipids and the C_{18} unsaturated fatty acids of the total lipid. The results (Fig. 2) show that the radioactivity in the free fatty acids (FFA) rapidly declined during the first 2 hr of the incubation period. At all incubation times the radioactivity in the FFA

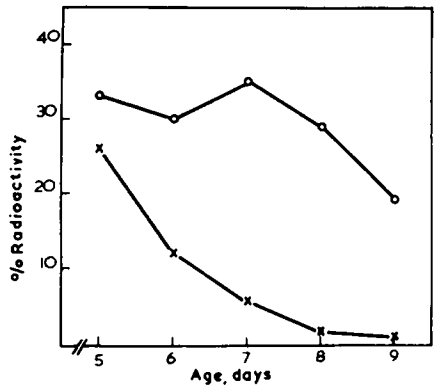

Fig. 1. Effect of leaf age on the relative incorporation of oleate-[^{14}C] into linoleate and linolenate. Seedlings were grown in the dark for 5–9 days and then exposed to light for 10 hr. Leaf sections (1.5 cm long) were prepared from the leaves 1 cm back from the leaf tip. Leaf sections were infiltrated under vacuum with oleate-[^{14}C] (NH_4^+, 8 μCi) and incubated in K-Pi buffer at 25° in the light. After 10 hr in buffer, samples were removed and the radioactivity present in the mono-, di- and trienoic fatty acids determined. The results are expressed as a percentage of the total activity in the unsaturated acids of the total lipid. (O——O) linoleate, (×——×) linolenate.

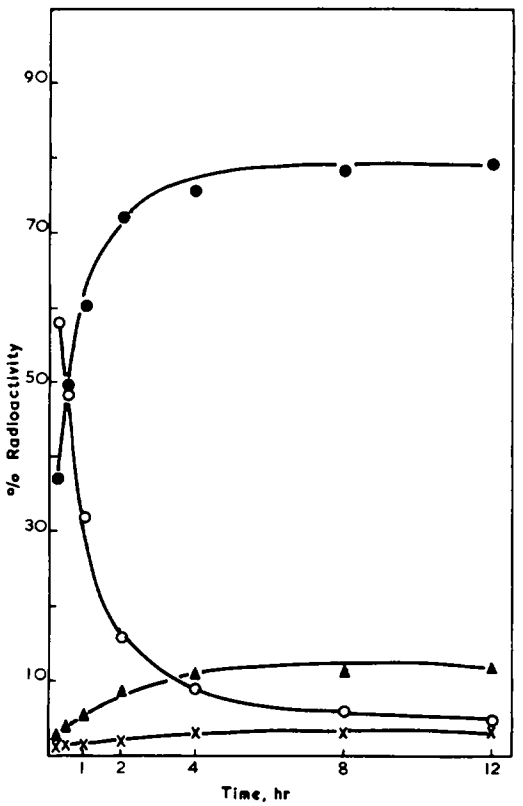

Fig. 2. Relative incorporation of radioactivity in the lipid fractions of leaf segments 'pulse labelled' with oleate-[^{14}C]. Leaf sections (1.5 cm long) were prepared from the primary leaves of barley seedlings which had been grown in the dark for 5 days and then given a 10 hr light treatment. The leaf sections were infiltrated under vacuum with oleate-[^{14}C] (NH_4^+ salt, 8 μCi) and incubated in K-Pi buffer at 25° in the light. Samples for analysis were removed at regular intervals. The results are expressed as a percentage of the activity in the total lipid against time after the ^{14}C-pulse. (●——●) polar lipid, (O——O) free fatty acid, (▲——▲) triacylglycerols, (×——×) diacylglycerols.

was essentially present in oleate with no detectable activity in free linoleic or linolenic acid. During the rapid depletion in free oleate-[^{14}C], there was an increase in the radioactivity recovered in the 'total' polar lipid fraction. The rate of incorporation of label into the triacylglycerols (TG), whilst slower than the rate of incorporation in the 'polar' lipid, also increased up to the 4 hr incubation time. The radioactivity recovered in the diacylglycerols (DG), although relatively small, steadily increased throughout the experiment.

The 'polar lipids' were analysed and the relative incorporation of radioactivity determined in PC, MGDG, digalactosyldiacylglycerol (DGDG), and phosphatidylethanolamine (PE) plus phosphatidylglycerol (PG) (Fig. 3). The rapid increase in the incorporation of oleate-[^{14}C] into the total polar lipid fraction, which was observed during the initial 2 hr incubation period, was largely reflected in the relative increase in the radioactivity in PC. The radioactivity in PC after 2 hr declined throughout the remaining incubation period. Whilst the incorporation of label into PE+PG was relatively low, it increased throughout the first 4 hr of incubation. The rate of incorporation of radioactivity into MGDG also increased up to 5–6 hr incubation time, after which the rate decreased.

To obtain information on the substrates and synthesis of the polyunsaturated C_{18} acids, the radioactivity in oleic, linoleic and linolenic acids was determined in the fatty acids of the total lipid fraction and separately in PC, MGDG, PE+PG and TG. The relative label present in the acids of the 'total lipid' is given in Fig. 4. The contribution made by oleate rapidly decreased during the initial 4 hr incubation and then slowed quite considerably. The radioactivity in linoleate increased to a maximum after 4 hr incubation and then decreased throughout the remainder of the experiment. The relative rate of incorporation into the linolenate of the total lipid fraction displayed different kinetics to the other acids and was more sigmoidal in shape.

The contribution in radioactivity made by each unsaturated C_{18} fatty acid in each parent lipid is given in Fig. 5. During the first 1 hr incubation, a rapid net increase in radioactivity was found in the oleate of PC after which a steady decline occurred. The linoleate of PC was labelled during the early periods of incubation and the relative activity increased at a steady rate for *ca* 4 hr after which it slowed and then decreased. The relative activity in PC–linolenate increased, after a short lag, to reach a maximum at 4 hr and then declined.

The relative activity in the oleate of TG, although much lower than that found in PC, increased to a maximum after 4 hr incubation and then declined. The oleate in PE and PG increased slowly throughout the incubation period. The incorporation of activity in the linoleate and linolenate of PE+PG exhibited a substantial lag before significant amounts of label were detected. On the other hand, the relative activity in the linoleate of TG increased linearly for 4 hr and

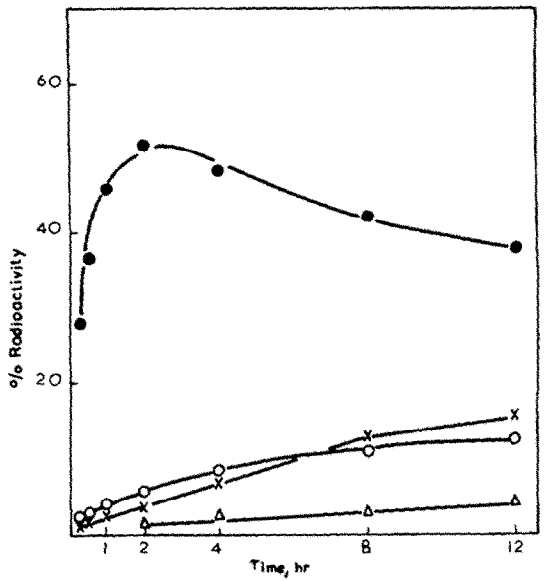

Fig. 3. Relative incorporation of radioactivity in the polar lipids in leaf segments 'pulse-labelled' with oleate-[^{14}C]. The 'polar lipids' were separated by TLC and assayed for radioactivity. The results are expressed as a percentage of the activity in the total lipid against time after the ^{14}C-pulse. (●——●) phosphatidylcholine, (○——○) phosphatidylethanolamine + phosphatidylglycerol, (×——×) monogalactosyl diacylglycerol, (Δ——Δ) digalactosyl diacylglycerol.

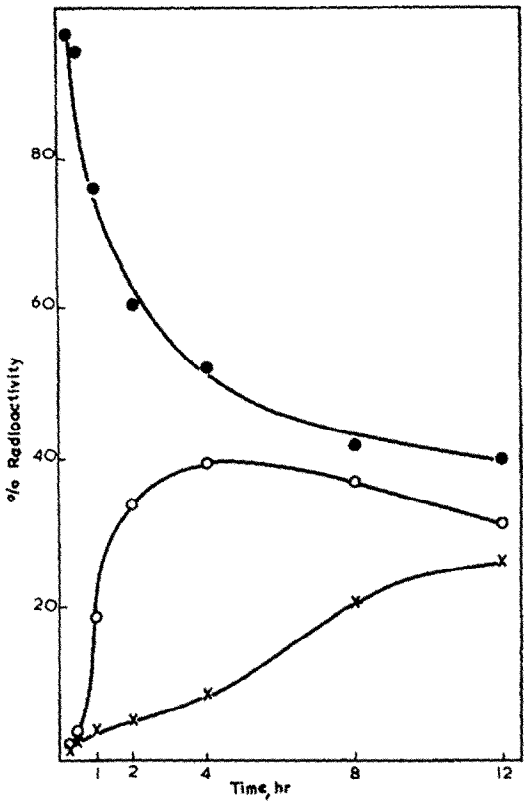

Fig. 4. Relative incorporation of radioactivity in linoleate and linolenate in leaf segments 'pulse-labelled' with oleate-[^{14}C]. Fatty acid methyl esters were prepared from the total lipid extract and purified by $AgNO_3$-TLC. The results are expressed as a percentage of the total activity in the mono-, di- and trienoic fatty acids against time after the ^{14}C-pulse. (●——●) oleate, (○——○) linoleate, (×——×) linolenate.

even at the end of the experiment it was still increasing. The linolenate of TG was not labelled until at least 2 hr incubation and then the incorporation of radioactivity increased at a linear rate.

The activity in the oleate of MGDG was relatively low; however, it increased during the first 2 hr of incubation and then remained fairly constant for the rest of the experimental period. A slight increase in the relative activity in linoleate of MGDG occurred during the initial 2 hr incubation period after which a substantial rate of incorporation was observed. Little or no label was found in the linolenate of MGDG during the first 1 hr of incubation. After this lag the relative rate of incorporation of radioactivity into the linolenate of MGDG increased in a linear fashion.

The initial lag in the appearance of radioactivity in the linolenate of MGDG compared to that of PC was further confirmed in a series of short term experiments. The results (Fig. 6) show that measurable radioactivity was present in the linolenate of PC at very early incubation times and this gradually increased for *ca* 2.5 hr after which a decline was always observed. Radioactivity in the linolenate of MGDG, on the other hand, was not found until after at least 1 hr incubation and this was then followed by a linear rate of incorporation.

In these experiments we used the leaves from 5-day-old dark-grown barley seedlings which had been transferred to the light for 10 hr before samples were taken. Very little, if any, linolenate synthesis from oleate-[^{14}C] was observed in leaves from dark-grown seedlings of a similar age without the light treatment. To investigate the effect of illumination on the formation of linolenate from oleate, 5-day-old dark-grown seedlings were transferred to the light and leaf segments prepared at regular intervals. The segments were 'pulse-labelled' with oleate-[^{14}C] and incubated in K-Pi buffer in complete darkness. Samples were removed after 6 hr and the radioactivity present in the unsaturated fatty acids determined. Chlorophyll, produced in the leaves, was also measured during the light treatment. The results (Fig. 7) show an increase in the relative incorporation of radioactivity in linoleate even in the leaves of seedlings given a short light treatment. The production of labelled linolenate was low at the onset of illumination. However, after 30 min light a substantial increase was found in the relative incorporation of oleate-[^{14}C] into linolenate. The chlorophyll content of the leaves began to increase after 2 hr in the light.

DISCUSSION

The leaves of dark-grown barley seedlings exposed to light synthesize radioactive linoleate from oleate-[^{14}C]. Labelled linolenate was produced only in relatively young and immature leaves. The age of the leaf and its state of development appears to play an extremely critical role in its ability to desaturate oleate to linolenate. Adequate synthesis of linolenate from

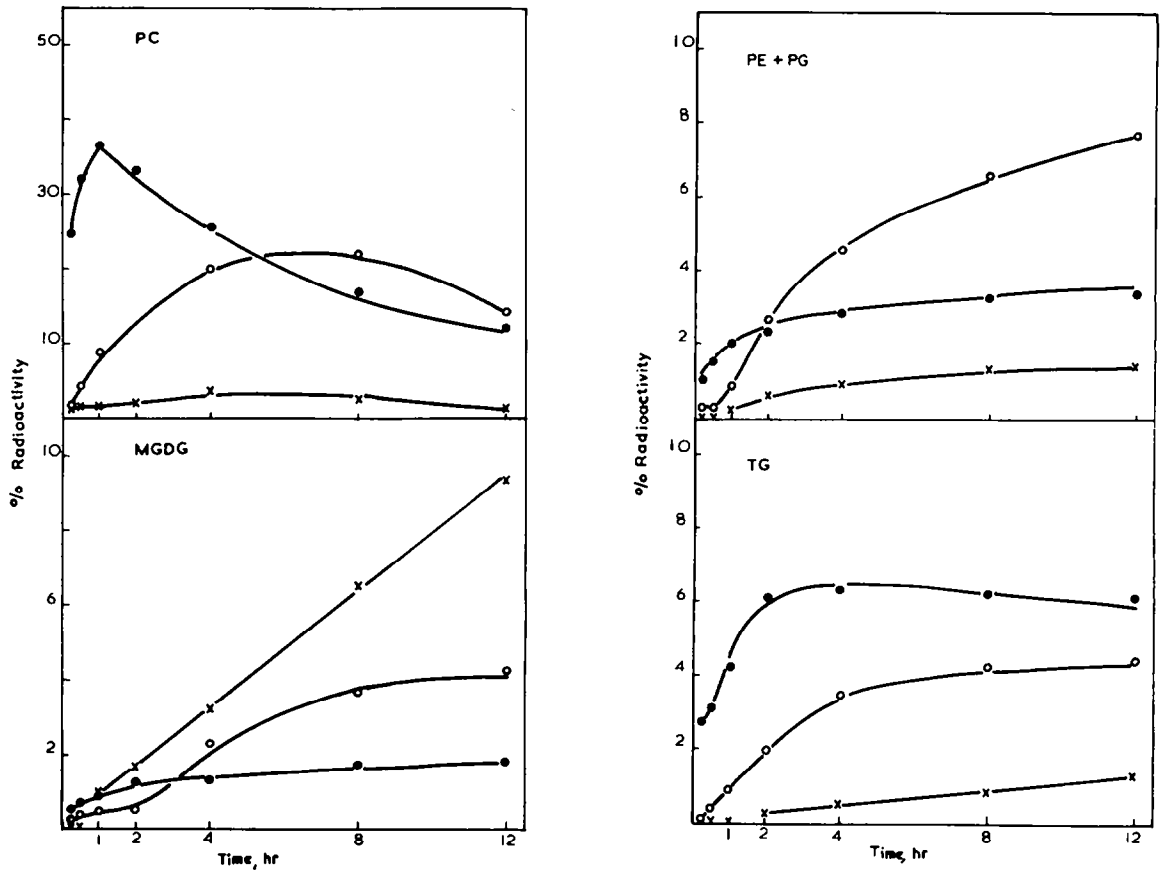

Fig. 5. Relative incorporation of radioactivity in the unsaturated fatty acids of parent lipids in leaf segments 'pulse-labelled' with oleate-[^{14}C]. The purified lipids were transmethylated and the fatty acid methyl esters separated by $AgNO_3$-TLC. The results for each acid are expressed as a percentage of the activity in the total lipid fraction against time after the ^{14}C-pulse. (●——●) oleate, (○——○) linoleate, (×——×) linolenate.

oleate via linoleate was demonstrated only in leaves tightly rolled and 'just' bursting from the leaf sheath prior to the light treatment. As much as a 20% or more conversion of oleate-[^{14}C] to linolenate was found in such young leaves. Little or no linolenate synthesis was evident in fully expanded leaves although substantial quantities of radioactive linoleate were always found. Appelqvist *et al.* [11] also noted that linoleate was always highly labelled in greening leaves of barley fed acetate-[^{14}C] although as much as 16% of the total fatty acid radioactivity resided in linolenate in leaves illuminated for 12 hr before incubation in isotope.

It is pertinent that Kannangara and Stumpf [12] found that only plastids from immature leaves converted acetate-[^{14}C] into linoleate and linolenate. The immature plastids were isolated from 'young pale green unexpanded leaves' [12] in which cell enlargement had probably just started. The tissue of these leaves would still be undergoing cell division. However, Kannangara and Stumpf suggested that some 'factor' necessary for the unsaturation reactions was lost during the preparation of the organelles from mature tissue. In a survey on the desaturation of oleate and linoleate in a number of plant species, Cherif *et al.* [13] observed that the 'age' and 'physiological state of the plant organs' were extremely important. Certainly in the work with leaves of barley full desaturation was observed only in tissue newly derived from a basal meristem and in which cell expansion was not complete. However, it should be noted that the leaves of dark-grown barley seedlings older than 5 days still produce substantial chlorophyll and viable chloroplasts on exposure to light [14] yet their apparent ability to synthesize linolenate by the desaturation of oleate is small. The contribution made by the elongation of 16:3 to 18:3 in such tissue would be of great interest.

Radioactive oleate was rapidly transferred to PC and desaturated to linoleate. This was in accord with other reports in which labelled acetate [5], CO_2[6] and choline [7] were fed to leaf tissue. Radioactive linolenate accumulated in PC soon after giving the leaf a 'pulse' of oleate-[^{14}C] and this was before its detection in any other parent lipid. A short, but definite, lag period was always observed before the appearance of linolenate-[^{14}C] in MGDG. The radioactivity in the linolenate of PC increased for a short period after the pulse of oleate-[^{14}C] and then decreased during the rapid accumulation of linolenate-[^{14}C] in MGDG. We feel that this is consistent with the view that in leaves, particularly young, immature leaves, oleic acid is sequentially desaturated to linolenic acid in PC and the linolenate is then rapidly transferred to other lipids,

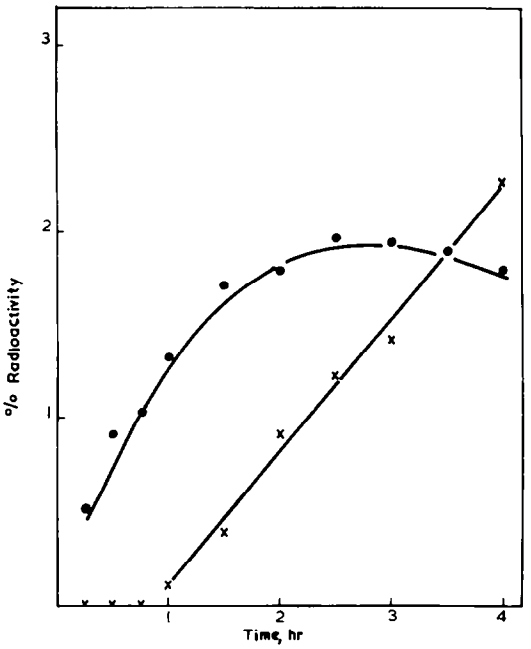

Fig. 6. Relative incorporation of radioactivity in the linolenate of phosphatidylcholine and monogalactosyl diacylglycerol in leaf segments 'pulse-labelled' with oleate-[^{14}C]. Leaf sections (1.5 cm long) were prepared from the primary leaves of barley seedlings which had been grown in the dark for 5 days and then given a 10 hr light treatment. The leaf sections were infiltrated under vacuum with oleate-[^{14}C] (NH_4^+ salt, 8μCi) and incubated in K-Pi buffer at 25° in the light. Samples for analysis were removed at regular intervals over a 4 hr incubation period. The results are expressed as a percentage of the activity in the total lipid against time after the ^{14}C-pulse. (●——●) phosphatidylcholine–linolenate, (×——×) monogalactosyldiacylglycerol–linolenate.

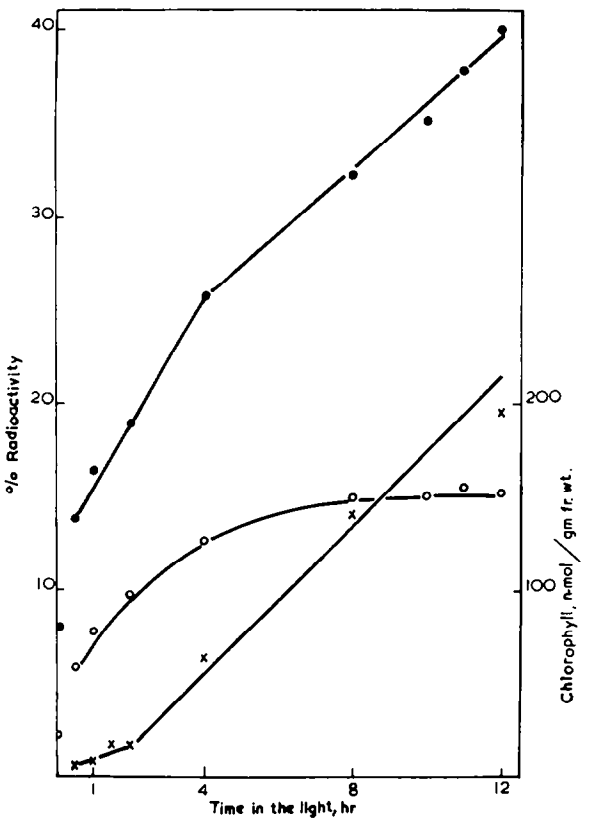

Fig. 7. Effect of 'greening' on the relative incorporation of radioactivity in linoleate and linolenate in leaf segments 'pulse-labelled' with oleate-[^{14}C]. Dark-grown 5-day-old barley seedlings were placed in the light. At regular intervals leaf sections (1.5 cm long) were prepared from the primary leaves. The segments were infiltrated, under vacuum, with oleate-[^{14}C] (NH_4^+, 8 μCi) and incubated in K-Pi buffer at 25° in the dark. After a 6 hr incubation in buffer, samples were removed and the radioactivity present in the mono-, di- and trienoic fatty acids of the total lipid determined. The results are expressed as a percentage of the total activity in the unsaturated fatty acids. The chlorophyll content of the leaves was also measured during the light treatment (×——×). (●——●) linoleate, (○——○) linolenate.

particularly MGDG. In studies on the incorporation of $^{14}CO_2$ into the lipids of *Vicia* leaves, Williams *et al.* [15] consider that the desaturation of oleate and linoleate occurred in PC and that some final desaturation may also occur in MGDG [16]. Harwood [9, 10], on the other hand, found in acetate-[^{14}C] feeding experiments that radioactive linolenate accumulated in MGDG with relatively little in PC and concluded that the linolenate was synthesized mainly in MGDG. We also found relatively small amounts of radioactivity in the linolenate of PC in oleate-[^{14}C] pulse experiments. However, the kinetics of incorporation indicate that the full desaturation of oleate to linolenate occurs in PC after which the linolenate is rapidly removed and transferred to MGDG.

It is relevant at this stage to consider the site of oleate and linoleate desaturation in plant cells. The microsome may be the prime location for the formation of linoleate from oleate [17, 18], whereas the plastid [2] may carry out the further desaturation of linoleate. If there exists a strict compartmentation of the 'desaturases' then a number of possible explanations of the results obtained with young leaves of barley are plausible. The linoleate in PC, which is formed in the microsome from PC–oleate may be transferred to the pastid where it is further desaturated to PC–linolenate with the linolenate finally accumulating in MGDG. It is also of interest that linoleate is formed from PC–oleate in microsomes of developing safflower seeds [19] and recently evidence for the full sequence of desaturation in PC has been obtained with homogenates from developing soya seeds [20]. It is possible, therefore, that in leaves containing young cells and relatively immature organelles, the linolenate can be synthesized in the microsome and this is then transferred to the plastid either as a PC species of via DG.

A light treatment of only short duration stimulated the desaturation of oleate to linolenate in the leaves of young dark-grown barley seedlings. The formation of linolenate-[^{14}C] preceded the light-induced synthesis of chlorophyll. The small quantity of chlorophyll present in the leaves up to *ca* 2 hr illumination can be accounted for by the endogenous protochlorophyllide in the dark-grown leaf at the start of the light treatment. The finding that only a relatively short period of

illumination is necessary to stimulate the synthesis of linolenate-[^{14}C] in leaves of dark-grown seedlings would indicate that active photosynthesis is not directly involved.

DCMU had no effect on the desaturaion of oleate in dark-grown leaves given a light treatment (A.K. Stobart, unpublished). It is of interest that the plastids of dark-grown barley leaves contain substantial reductant in the form of NADPH and that this is rapidly oxidized upon illumination [21]. If the natural reductant for linolenate synthesis is NADPH then its utlilzation in illuminated plastids from dark-grown leaves may account for some of our observations.

EXPERIMENTAL

Barley seeds (*Hordeum vulgare* cv Mona) were obtained from the Swedish Seed Association, Svalof. Oleic acid-[1-^{14}C] (58 Ci/mol) was purchased from the Radiochemical Centre, Amersham, U.K.

The seeds were soaked 18 hr in H_2O and sown in trays of moist Perlite. The seedlings were grown in complete darkness at 20°. At the desired age the seedlings were given a light treatment and their ability to desaturate oleate-[^{14}C] determined. Sections (1.5 cm long) were cut from the leaves in a region 1 cm back from the leaf tip. Groups of 25 segments were loaded onto pins and briefly stored on ice (*ca* 15 min). As many as 12 'loaded' pins were placed in a 100 ml conical flask and covered with 12 ml K-Pi buffer (0.1 M, pH 7.5) containing $CaSO_4$ (final concn 10μM). Ammonium oleate-[^{14}C] (8 μCi) was added and the material infiltrated *in vacuo* ×5 with a 1 min interval between each. After thorough washing in K-Pi buffer, the tissue was transferred to 12 ml fresh buffer and incubated at 25° with constant shaking (150 strokes/min). At regular intervals the 'loaded' pins were removed and the tissue immediately extracted by the method of ref. [22]. All samples were stored in $CHCl_3$ at −15° under N_2.

Polar lipids and neutral lipids were separated by TLC on Si gel with $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (10:4:2:2:1) or hexane–Et_2O–HOAc (70:30:1), respectively. Lipids were located by briefly exposing the plates to I_2 vapour. In the system used to separate polar lipids, the PE overlapped slightly with PG. Me esters of fatty acids were separated by $AgNO_3$–Si gel TLC (1:9). The plates were developed twice in haxane–Et_2O–HOAc (85:15:1) and the esters located by spraying with dichlorofluorescein (0.05% in EtOH) and viewing under UV light.

Areas of gel corresponding to lipid were moistened with H_2O and removed from the plates. Individual lipids and lipid groups were assayed for radioactivity by liquid scintillation in PCS and xylene. Me esters of the mono-, di- and trienoic fatty acids were counted in PPO and POPOP in toluene (4 g PPO + 0.2 g POPOP/l. toluene). All counts were corrected for quenching. (In other studies [9, 10] Me esters of fatty acids after separation by $AgNO_3$–TLC were assayed for radioactivity in PCS. However, in the present work it was found that PCS reacted with Ag^+-impregnated gel to give complete quenching within *ca* 15 min.)

Me esters of fatty acids of parent lipids and total lipid were prepared with BF_3 in MeOH after saponification [23].

Chlorophyll was estimated in 80% Me_2CO [24].

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