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Desaturation of linoleic acid from exogenous lipids by isolated chloroplasts

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When [^{14}C]diacylgalactosylglycerol was added to isolated pea or lettuce chloroplasts linolenate synthesis was seen. The desaturation of [^{14}C]linoleate in diacylgalactosylglycerol to [^{14}C]linolenate was stimulated by the addition of a soluble protein fraction containing lipid-exchange activity. Other [^{14}C]acyl lipids were ineffective, except that [^{14}C]phosphatidylcholine in the presence of UDP-galactose and *sn*-glycerol 3-phosphate could also supply [^{14}C]linoleate for desaturation. These results are consistent with a role for diacylgalactosylglycerol in linolenate synthesis, as indirectly suggested by labelling experiments.

Although α-linolenate is the world's most prevalent fatty acid, research on its biosynthesis has been hampered by the lack of active systems *in vitro* (Harwood, 1979*a*). Several aspects of its formation have been suggested by the use of whole-tissue preparations. These include the stereochemistry of hydrogen removal (Morris *et al.*, 1968), the overall pathway of formation (Murphy & Stumpf, 1979) and the possible involvement of diacylgalactosylglycerol (monogalactosyldiacylglycerol) in its synthesis (Heinz & Harwood, 1977; Siebertz & Heinz, 1977; Wharfe & Harwood, 1978; Joyard *et al.*, 1979). Experiments with a substituted pyridazinone, Sandoz 9785, gave results that were also consistent with a role for diacylgalactosylglycerol in the desaturation of linoleate to linolenate (Murphy *et al.*, 1979).

Recent experiments with isolated chloroplasts that had been incubated with [^{14}C]acetate gave results in keeping with the intimate involvement of diacylgalactosylglycerol in linoleate desaturation (Roughan *et al.*, 1979*b*). We have now tested the use of linoleate for desaturation directly by the use of exogenous lipids and isolated chloroplasts.

Experimental

Materials

Pea (*Pisum sativum* cv. Feltham First) seeds were grown in John Innes Seed Compost under normal daylight conditions and leaves were harvested after 3–4 weeks of growth. Lettuce (*Lactuca sativa*) was purchased from the local market. [1-^{14}C]Acetate (sp. radioactivity 60 Ci/mol) and [1-^{14}C]linoleic acid (sp. radioactivity 51 Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and UDP-galactose, ATP, NADPH, NADH and CoA from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Other reagents were of best available grades as detailed by Bolton & Harwood (1977).

Radiolabelled lipid substrates

[^{14}C]Acyl lipids prepared biosynthetically by incubating detached young pea leaves with [^{14}C]-acetate as described by Wharfe & Harwood (1978) or with [^{14}C]linoleate that was applied to the leaf surface in methoxyethanol. Leaves were incubated with [^{14}C]acetate for 6–8 h under an illumination of 105 (photosynthetically active wavelengths) $\mu E/m^2$ per s at 25°C. When incubated with [^{14}C]linoleate, leaves were allowed to metabolise the radiolabel for 16 h with low illumination ($<5\,\mu E/m^2$ per s). After incubation lipids were extracted (Wharfe & Harwood, 1978) and separated by t.l.c. by the method of Khan & Williams (1977). Glycosylacylglycerols were eluted from the plates by using acetone and phospholipids and also chloroform/methanol (2:1, v/v), evaporated to dryness and stored under N_2 in chloroform at −20°C.

Preparation of supernatant fractions

Supernatant preparations were made from pea or lettuce leaves by a modification of previous methods (Kader, 1975; Tanaka & Yamada, 1979). Samples of the postmitochondrial or the postmicrosomal supernatant were subjected to pH 5.1 treatment and 75% $(NH_4)_2SO_4$ precipitation as described by Kader (1975) and Tanaka & Yamada (1979) and the pellets were resuspended in 0.05 M-sodium phosphate buffer (pH 7.5) containing 0.01 M-2-mercaptoethanol and dialysed against the same buffer. Both

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the postmitochondrial and the postmicrosomal preparations gave similar activities and the latter source was generally used for the 'lipid-exchange protein fraction'.

Chloroplast incubations

Chloroplasts were isolated from 4-week-old pea leaves or from locally purchased lettuce by using a Waring blender and the method of Nakatani & Barber (1977). They were also further purified by using the Percoll technique (Mills & Joy, 1979). Intactness was estimated by phase-contrast microscopy and chlorophyll by the method of Bruinsma (1961). ^{14}C-labelled lipids were suspended in water by sonication in a Kerry 125 Pulsatron (Kerry Ultrasonics, Hitchin, Herts., U.K.) for 2h under N_2. Chloroplasts were usually incubated under the conditions described by Roughan *et al.* (1979*a*) and incubations were terminated by the addition of KOH (6%, w/v final concn.) or by the method of Garbus *et al.* (1963). Complex lipids were separated by t.l.c. (Khan & Williams, 1977) and quantified by g.l.c. after *trans*-methylation with H_2SO_4/methanol (2.5:97.5, v/v) and methyl pentadecanoate standard. Total ^{14}C-labelled fatty acids were extracted, methylated and quantified by radio-g.l.c. as previously described (Bolton & Harwood, 1977). The identity of ^{14}C-labelled fatty acids was ascribed by comparison with authentic markers on g.l.c. in 15% EGSS-X on 80–100 mesh Chromosorb W-AW, 15% DEGS on 80–100 mesh Gas Chrom. Q or 3% SE-30 on 80–100 mesh Supelcoport (Supelco, Bellefonte, PA 16823, U.S.A.). Hydrogenated ^{14}C-labelled fatty acids were also separated by g.l.c. to confirm their chain length and the methylated ^{14}C-labelled fatty acids were also separated by t.l.c. by using 5% $AgNO_3$/silica gel G plates and light petroleum (b.p. 60–80°C)/diethyl ether (9:1, v/v) as solvent.

Results and discussion

Several lines of evidence have pointed to the possible co-operation of different subcellular organelles in the conversion of photosynthetically fixed CO_2 into linolenate (Appelqvist & Stymne, 1979; Tremolieres *et al.*, 1979). These include time-course labelling experiments (Roughan, 1975; Williams *et al.*, 1976) as well as the correlation in specific radioactivities of oleate and linoleate in the major chloroplast lipids, the galactosylacylglycerols and the major extra-chloroplast lipid, phosphatidylcholine (Harwood, 1979*b*; Wharfe & Harwood, 1978). The latter result suggested the rapid transfer of oleate and of linoleate between the three lipids *in vivo*. Taken together with the likely connection of phosphatidylcholine with oleate desaturation (cf. Appelqvist & Stymne, 1979; Harwood, 1979*a*) an obvious experiment was to attempt the labelling of chloroplasts from exogenously supplied lipid. Two such attempts have been reported as unsuccessful (Heinz *et al.*, 1979; Tremolieres *et al.*, 1979).

Initially we used [^{14}C]phosphatidylcholine and incubated isolated chloroplasts with the lipid in the presence of a crude preparation of the plant phospholipid exchange protein (Kader, 1975). Although formation of [^{14}C]linolenate was seen, the amount of desaturation was extremely variable. Accordingly we attempted to maximize the conditions for fatty acid synthesis and, especially, polyenoate formation in the lettuce and pea chloroplast preparations. In full agreement with Roughan *et al.* (1979*a*) we have found that isolation of chloroplasts by the method of Nakatani & Barber (1977) followed by incubation under the conditions of Roughan *et al.* (1979*a*) gave the highest rates of fatty acid labelling/mg of chlorophyll. In an attempt to promote polyenoic acid synthesis we tested the addition of UDP-galactose and *sn*-glycerol 3-phos-

Table 1. *The effect of UDP-galactose and sn-glycerol 3-phosphate on the incorporation of radioactivity from [1-^{14}C]-acetate into lipid classes by chloroplasts*

Chloroplasts were isolated by the method of Nakatani & Barber (1977) and incubated in the assay system described by Roughan *et al.* (1979*a*). UDP-galactose and *sn*-glycerol 3-phosphate were added at 0.5mM and 1mM final concentrations respectively. Lipids were extracted by the method of Garbus *et al.* (1963), and separated and quantified as described in the Experimental section. Abbreviations used: n.m., not measured; MGDG, diacylgalactosylglycerol; PC, phosphatidylcholine; G-3-P, *sn*-glycerol 3-phosphate.

			Distribution of radioactivity (%)				
Chloroplast	Addition	Fatty acid synthesis (nmol of [^{14}C]acetate incorporated/h per mg of chlorophyll)	S-esters	Non-esterified fatty acid	MGDG	PC	Other lipids
Lettuce	None	33	n.m.	78	5	3	14
	+UDP-Gal+G-3-P	38	n.m.	75	9	3	13
Pea	None	148	12	77	4	5	2
	+UDP-Gal+G-3-P	151	17	61	14	4	4

phate, since these two compounds have been noted to increase the labelling of linolenate and/or diacylgalactosylglycerol from [^{14}C]acetate during chloroplast incubations (McKee & Hawke, 1978; Roughan *et al.*, 1979*b*; Tremolieres *et al.*, 1979). As the results in Table 1 show, addition of these two reagents resulted in a decrease in the total labelling of unesterified fatty acids and an increase in the labelling of diacylgalactosylglycerol. However, the change in the pattern of labelling was not as marked as that for spinach chloroplasts (McKee & Hawke, 1978; Roughan *et al.*, 1979*b*). In addition, although our data agreed with that for spinach in that UDP-galactose and *sn*-glycerol 3-phosphate primarily affected labelling patterns rather than total incorporation, there was very little labelling of linolenate (1–2% total ^{14}C-labelled fatty acids) from [^{14}C]acetate. In this regard it is pertinent to note that isolated lettuce chloroplasts synthesize 5–10% laurate, 20–30% myristate, 40–45% palmitate and 20–30% oleate from [^{14}C]acetate. Intact chloroplasts further purified by the Percoll technique had similar activity and characteristics to the crude chloroplast fraction.

Having established conditions where maximal fatty acid synthesis was likely to occur, we tested the addition of [^{14}C]acyl lipids to the isolated chloroplast system. The 'lipid-exchange protein fraction' was not extensively purified in case the crude preparation would also aid in the transfer of galactosylacylglycerols into the chloroplast. No change in the pattern of ^{14}C-labelled fatty acids was seen when [^{14}C]diacylgalabiosylglycerol, [^{14}C]-phosphatidylglycerol or [^{14}C]diacylsulphoquinovosylglycerol were added to lettuce chloroplasts and incubated in the presence or absence of the 'lipid-exchange protein fraction'. With [^{14}C]phosphatidylcholine little formation of [^{14}C]linolenate was seen but this was increased markedly by the addition of UDP-galactose and *sn*-glycerol 3-phosphate (Table 2). By far the most consistent and highest synthesis of [^{14}C]linolenate was seen when [^{14}C]diacylgalactosylglycerol was used. Stimulation of the desaturation was observed in the presence of the 'lipid-exchange protein fraction' (Table 2), though it is not clear at this stage whether this was due to true exchange or to an increase in the fusion of lipid micelles with the chloroplast envelope. Recovery of the counts added in fatty acids was complete, indicating that oxidation of fatty acids was not significant. Moreover, it also excluded the possibility that the enrichment in [^{14}C]linolenate in some of the experiments was due to the selective degradation of [^{14}C]linoleate. Results obtained from the separation of lipid classes at the end of the incubation indicated that, whereas diacylgalactosylglycerol was

Table 2. *Synthesis of* [^{14}C]*linolenate from exogenous phosphatidylcholine or diacylgalactosylglycerol by lettuce chloroplasts*

Lipids were labelled as described in the Experimental section from either [1-^{14}C]acetate or [1-^{14}C]linoleic acid Exogenous lipid (0.5–1 μg) and 70–180 μg of chloroplast chlorophyll/ml of incubation were used. Abbreviations used: n.d., not detected; tr., trace; G-3-P, *sn*-glycerol 3-phosphate; S.P., 'lipid-exchange protein fraction' (see the Experimental section). All tubes were incubated under the conditions of Roughan *et al.* (1979*a*) for 1 h. 'Lipid-exchange protein fraction' was added at 0.3 mg of protein/ml and UDP-gal and G-3-P were at 0.5 mM and 1 mM final concentrations respectively. Results from the five most recent (total 19) experiments, which are representative, are shown.

		Distribution of ^{14}C in fatty acids (%)			
Lipid substrate	Additions	$C_{16:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Diacylgalactosylglycerol	None (no-enzyme control)	n.d.	n.d.	59	41
	Chloroplasts + S.P.	n.d.	n.d.	54	46
Diacylgalactosylglycerol	None (no-enzyme control)	tr.	n.d.	61	39
	Chloroplasts	tr.	n.d.	54	46
	Chloroplasts + S.P.	n.d.	n.d.	46	54
	Sonicated chloroplasts + S.P.	tr.	n.d.	57	43
Diacylgalactosylglycerol	None (no-enzyme control)	25	48	27	tr.
	Chloroplasts	25	47	28	tr.
	Chloroplasts + S.P.	24	34	25	17
Phosphatidylcholine	None (no enzyme control)	24	76	tr.	n.d.
	Chloroplasts	26	72	tr.	2
	Chloroplasts + S.P.	28	66	1	5
Phosphatidylcholine	None (no-enzyme control)	n.d.	n.d.	100	n.d.
	Chloroplasts + S.P.	3	tr.	97	n.d.
	Chloroplasts + S.P. + UDP-gal + G-3-P	tr.	tr.	77	23

essentially the only complex lipid containing radioactivity, there was considerable label in unesterified fatty acids. This was probably due to acyl hydrolase activity, since the enzyme is soluble (Burns *et al.*, 1977) and therefore likely to be present in the 'lipid-exchange protein fraction' as well as in the chloroplast stroma (Anderson *et al.*, 1974). An intact organelle appeared to be necessary for effective linoleate desaturation since sonicated preparations, even in the presence of appropriate cofactors, were much less efficient. Since there were essentially no intact chloroplasts remaining at the end of a 60min incubation, the requirement for an intact organelle places a limit on the amount of desaturation of linoleate that can be achieved in the present system. Even so, in contrast with the experiments on oleate desaturation in *Chlorella* (Gurr *et al.*, 1969), the amount of linoleate desaturation achieved in the present experiments compares well with that reported for endogenously labelled diacylgalactosylglycerol (Roughan *et al.*, 1979*b*). In addition, the results represent direct evidence for the use of diacylgalactosylglycerol in linoleate desaturation, which was first suggested by Nichols, James and co-workers (Nichols *et al.*, 1967; Nichols, 1968). Unequivocal proof that diacylgalactosylglycerol is the actual substrate for the 15-ene desaturase must await further experiments, possibly with ether analogues as substrates (Heinz *et al.*, 1979). The system reported here should prove of great use in such experiments.

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