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Glycerolipid labelling kinetics in isolated intact chloroplasts

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Glycerolipid synthesis was studied in intact chloroplasts isolated from three different plant species. The sequential acylation of *sn*-glycerol 3-phosphate and lysophosphatidate (1-acyl-*sn*-glycerol 3-phosphate) was confirmed by monitoring the incorporation of oleate synthesized *in situ* into lysophosphatidate, phosphatidate and diacylglycerol. Lysophosphatidate was not only readily detected in these experiments, but was also present in the chloroplasts at the beginning of the time courses. The rate of glycerolipid synthesis depended primarily on *sn*-glycerol 3-phosphate supply, and given adequate *sn*-glycerol 3-phosphate, the proportion of newly synthesized fatty acids diverted into glycerolipids appeared to be determined by differing acyltransferase activities in the chloroplasts isolated from different plant species.

From studies utilizing a soluble *sn*-glycerol 3-phosphate acyltransferase purified from spinach (*Spinacia oleracea*) and pea (*Pisum sativum*) chloroplasts it may be deduced that the first committed step in chloroplast glycerolipid synthesis *in vivo* will be a transfer of oleate from oleoyl-ACP to the *sn*-1 position of *sn*-glycerol 3-phosphate (*sn*-G3P) (Frentzen *et al.*, 1983). A second acyltransferase is firmly bound to the chloroplast envelope (Joyard & Douce, 1977) and specifically transfers palmitate to the *sn*-2 position on 1-acyl-*sn*-glycerol 3-phosphate (Frentzen *et al.*, 1983). Reconstituted chloroplast systems will, when provided with acyl-CoA or acyl-ACP species as well as *sn*-G3P, synthesize 1-acyl-*sn*-glycerol 3-phosphate, phosphatidate (PA) and 1,2-diacylglycerol (DAG) (Joyard & Douce, 1977, 1979; Frentzen *et al.*, 1983).

Although lysophosphatidate (LPA) is readily detected in incubations utilizing purified enzymes or stromal preparations (Joyard & Douce, 1977; Bertrams & Heinz, 1981; Frentzen *et al.*, 1983), it has rarely (see, however, Mudd & Dezacks, 1981; Sparace & Mudd, 1982) been considered in studies on lipid metabolism by intact organelles (Murphy & Leech, 1978; McKee & Hawke, 1979; Roughan *et al.*, 1980; Drapier *et al.*, 1982). Since the synthesis of PA, DAG and even monoacylglycerol have been reported, it must be assumed that LPA was either present in inconsequential amounts, as might be the case in a concerted acylation of *sn*-G3P (Vick & Beevers, 1977), or was lost in the washing procedures used to purify lipid extracts (Gardiner *et al.*, 1984). Recent work (Heinz & Roughan, 1983; Gardiner et al., 1984) suggests that LPA could be a genuine intermediate in chloroplast glycerolipid synthesis. It is therefore important to investigate the role of LPA in chloroplast lipid metabolism and to show whether or not the reaction sequence

$$sn\text{-G3P} \rightarrow \text{LPA} \rightarrow \text{PA} \rightarrow \text{DAG}$$

can be validated by using intact organelles and acyl donors generated *in situ*.

Here we analyse in detail the initial stages of the incorporation of newly synthesized fatty acids into the glycerolipids of chloroplasts isolated from *Spinacia oleracea*, *Chenopodium quinoa* (quinoa) and *Pisum sativum*. These species represent plants in which the DAG moieties of diacylgalactosylglycerol, diacyldigalactosylglycerol and sulpholipid are derived to different extents from chloroplast glycerolipid synthesis, and from which isolated chloroplasts show different abilities to synthesize glycerolipids (Heinz & Roughan, 1983; Gardiner & Roughan, 1983; Gardiner *et al.*, 1984).

Abbreviations used: ACP, acyl-carrier protein; Chl, chlorophyll; DAG, diacylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; LPA, lysophosphatidate; PA, phosphatidate; *sn*-G3P, *sn*-glycerol 3-phosphate.

Experimental

Materials

Sodium [1-^{14}C]acetate (sp. radioactivity 61.6 Ci/mol) was obtained from Amersham International

and *sn*-G3P from Sigma, St. Louis, MO, U.S.A. Seeds were obtained from the same sources as described previously (Gardiner *et al.*, 1984).

Methods

Plants were grown as previously described (Gardiner *et al.*, 1984). Chloroplasts were isolated by the method of Nakatani & Barber (1977) modified by the addition of 0.25% (w/v) bovine serum albumin to the homogenizing buffer and 0.1% (w/v) bovine serum albumin to the wash buffer (Gardiner *et al.*, 1984), and any broken chloroplasts were removed by centrifugation through a Percoll cushion (Mills & Joy, 1980). Levels of CO_2-dependent O_2 evolution were at least 100 μmol/h per mg of Chl for *S. oleracea* chloroplasts and have previously been reported for the other species (Heinz & Roughan, 1983). The chloroplasts were incubated under illumination in a medium containing [1-^{14}C]acetate (sp. radioactivity 61.6 Ci/mol) and, where indicated, 0.2 mM-*sn*-G3P, as previously described (Gardiner *et al.*, 1984). Chl ranged from 9 to 54 μg per assay (Arnon, 1949). Methods of lipid recovery, purification and analysis were as described by Gardiner *et al.* (1984).

Time courses of incorporation of [1-^{14}C]acetate for chloroplasts isolated from three different plant species were performed in basal incubation medium (controls) and also in the presence of 0.2 mM-*sn*-G3P. The plants contained various amounts of galactosyl-2-hexadecatrienoyl-1-linolenoylglycerol (i.e. diacylgalactosylglycerol derived from chloroplast glycerolipid synthesis) in total diacylgalactosylglycerol (*S. oleracea*, 50%; *C. quinoa*, 8%; *P. sativum*, 0%) (Gardiner & Roughan, 1983). Chloroplasts from a single preparation were used for the two time courses with or without *sn*-G3P for each species.

Results

The incorporation of [1-^{14}C]acetate into the different acyl compounds synthesized by chloroplasts from *S. oleracea*, *C. quinoa* and *P. sativum* is shown in Fig. 1. The only instance where non-esterified fatty acid synthesis did not predominate over glycerolipid synthesis was that of *S. oleracea* chloroplasts provided with exogenous *sn*-G3P. In the absence of added *sn*-G3P, rates of glycerolipid synthesis in all cases declined markedly after 2–4 min, whereas rates of non-esterified fatty acid synthesis increased correspondingly (see also Gardiner *et al.*, 1984), thus maintaining a linear rate of total [1-^{14}C]acetate incorporation into lipids. As this decline in the rate of glycerolipid synthesis could be reversed in *S. oleracea* chloroplasts by adding *sn*-G3P at any time after beginning the incubation, it was probably caused by the depletion through glycerolipid synthesis of endogenous *sn*-G3P. The concentration of *sn*-G3P within *S. oleracea* chloroplasts freshly isolated from illuminated leaves appears to be quite low. A recent report (Sauer & Heise, 1983) suggested a concentration of 50 μM, assuming a chloroplast volume of 47 μl/mg of Chl (Wirtz *et al.*, 1980) and this would appear to be consistent with the marked effect of 50 μM exogenous *sn*-G3P on glycerolipid synthesis by isolated *S. oleracea* chloroplasts (Roughan *et al.*, 1979). From Table 1, it may be calculated that the 3 min initial linear period of glycerolipid synthesis by *S. oleracea* chloroplasts would decrease the endogenous *sn*-G3P concentration by 30 μM, i.e. from 50 μM to about 20 μM, which is below the value of 31 μM recorded for the K_m for *sn*-G3P of *sn*-glycerol 3-phosphate:oleoyl-ACP acyltransferase *in vitro* (Frentzen *et al.*, 1983).

In the presence of 0.2 mM exogenous *sn*-G3P, glycerolipid synthesis in chloroplasts isolated from all three species was maintained at a linear rate for the duration of the time course (Fig. 1). In agreement with the findings of previous studies employing longer incubation times (Heinz & Roughan, 1983; Gardiner & Roughan, 1983; Gardiner *et al.*, 1984), *S. oleracea* chloroplasts were at least twice as active in glycerolipid synthesis as were chloroplasts from *C. quinoa* and *P. sativum*, although rates of [1-^{14}C]acetate incorporation into total lipids were similar for all chloroplast preparations (Table 1). Exogenous *sn*-G3P stimulated initial rates of glycerolipid synthesis approx. 2-fold in *S. oleracea*, 3-fold in *C. quinoa* and 4-fold in *P. sativum* (Table 1). Only about 0.1%/min of the supplied *sn*-G3P was utilized for glycerolipid synthesis by *S. oleracea* chloroplasts. It may be assumed that glycerolipid synthesis in *S. oleracea* chloroplasts supplied with *sn*-G3P had reached a steady-state 3 min after beginning the incubation, since LPA and PA appeared to have become saturated with radioactivity, whereas DAG continued to accumulate it at a linear rate (Fig. 1). The equilibrium value of [1-^{14}C]acetate incorporated into LPA equates to a concentration of 14 μM during the linear accumulation of diacylglycerol by *S. oleracea* chloroplasts [assuming a chloroplast volume of 47 μl/mg of Chl (Wirtz *et al.*, 1980)]. This steady-state concentration of 14 μM-LPA in *S. oleracea* chloroplasts is to be compared with the steady-state concentrations of potential acylating agents of 1.6 μM-palmitoyl-ACP, 1.0 μM-stearoyl-ACP and 2.1 μM-oleoyl-ACP (Soll & Roughan, 1982).

Because steady-state glycerolipid synthesis was approached much later, less confidence can be attached to the results obtained with *C. quinoa*

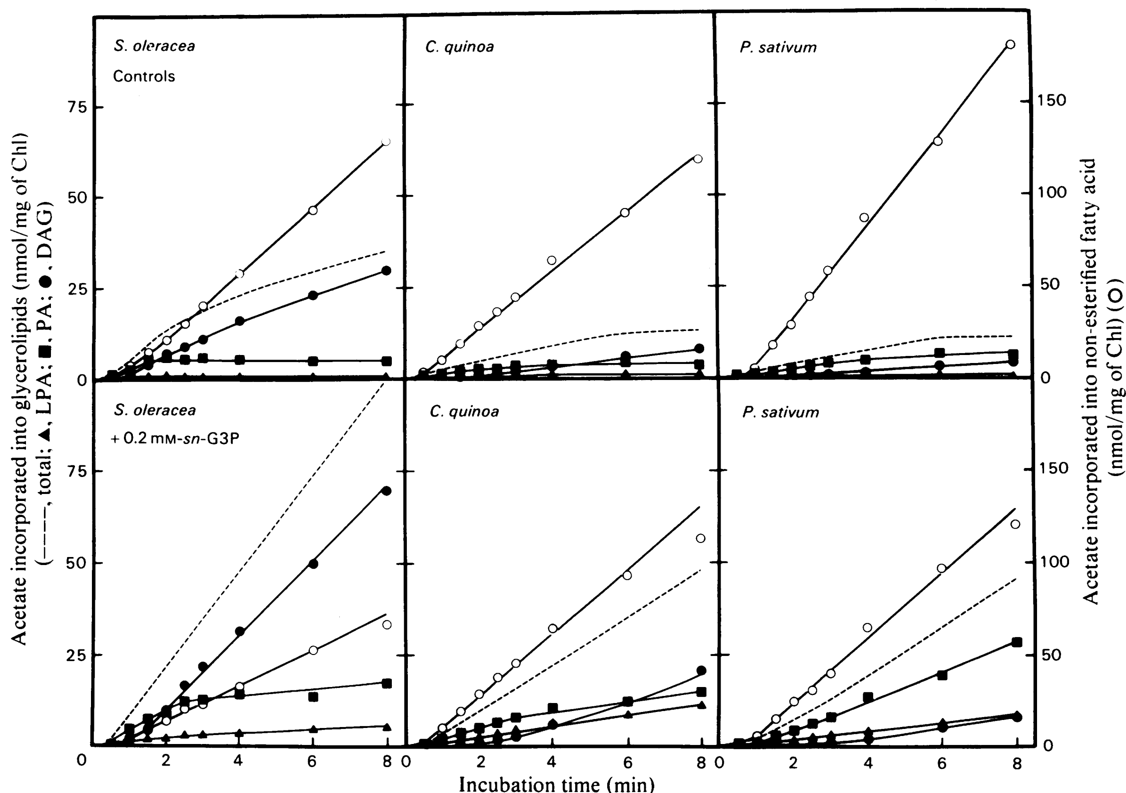


Fig. 1. *Time courses of [1-^{14}C]acetate incorporation by chloroplasts isolated from three different species*
Chloroplasts isolated from *S. oleracea*, *C. quinoa* and *P. sativum* were incubated in the light with shaking at 25°C in the basal incubation medium with [1-^{14}C]acetate (0.13mM and 61.6Ci/mol) (top panels) and also with the addition of 0.2mM-*sn*-G3P (bottom panels). The reactions were stopped at the times indicated and the lipids separated and radioactivity determined (Gardiner *et al.*, 1984). Total rates of acetate incorporation into lipids for controls and in the presence of 0.2mM-*sn*-G3P respectively were: *S. oleracea* 1470, 1470; *C. quinoa* 1210, 1530; *P. sativum* 1870, 1660nmol of acetate incorporated/h per mg of Chl. Fatty acids isolated from the aqueous methanol layer constituted less than 3% of total incorporation at 8min and were omitted from the Figure. Note that the glycerolipids are plotted on a ×2 expanded scale compared with that for non-esterified fatty acids. Key to symbols: ○, non-esterified fatty acids; ●, DAG; ▲, LPA; ■, PA; ----, sum of glycerolipids.

Table 1. *Initial rates of chloroplast glycerolipid synthesis with or without 0.2mM-sn-G3P*
Chloroplasts isolated from three different species were incubated in the light at 25°C with [1-^{14}C]acetate in either basal medium or with the addition of 0.2mM-*sn*-G3P. Reactions were stopped at intervals and lipids separated and radioactivity determined (Gardiner *et al.*, 1984). Initial rates of glycerolipid synthesis were derived from the linear portion of plots of total glycerolipids (lysophosphatidate plus phosphatidate plus diacylglycerol) labelled against time, and total rates for acetate incorporation from similar plots for radioactivity incorporated into total lipids.

		Rate of glycerolipid synthesis (pmol of glycerolipid/min per mg of Chl)		Total rate (nmol of acetate incorporated/h per mg of Chl)	
Plant	Expt.	Control	+*sn*-G3P	Control	+*sn*-G3P
S. oleracea	I	490	—	1090	—
	II	440	880	1470	1470
	III	500	920	1670	1670
C. quinoa	I	120	—	850	—
	II	130	380	1730	1340
	III	180	440	1210	1530
P. sativum	I	100	—	1580	—
	II	85	400	2180	2040
	III	120	440	1870	1660

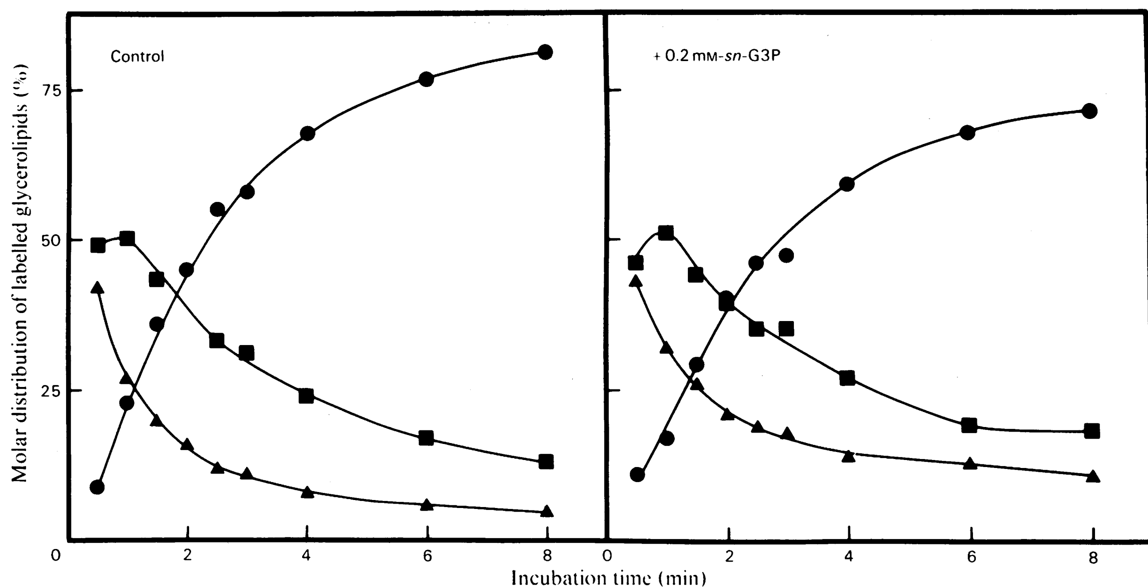
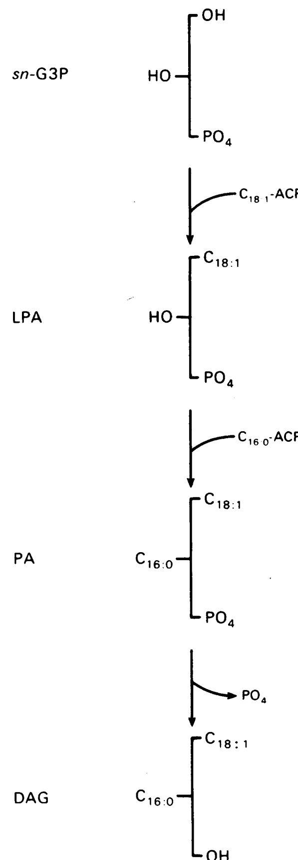


Fig. 2. *Molar ratios of LPA, PA and DAG labelled during initial stage of glycerolipid synthesis by S. oleracea chloroplasts*
The amount of individual glycerolipids synthesized from [1-^{14}C]acetate by *S. oleracea* chloroplasts was calculated from the data for incorporation given in Fig. 1 and expressed as the percentage of total glycerolipids synthesized *de novo* at each incubation time. Key to symbols: ▲, LPA; ■, PA; ●, DAG.

chloroplasts. Nevertheless, it can be seen in Fig. 1 that the steady-state concentration of phosphatidate will be approximately the same in chloroplasts isolated from *S, oleracea* or *C. quinoa*, but that of LPA in *C. quinoa* chloroplasts will be almost twice that in *S. oleracea* chloroplasts. In *P. sativum* chloroplasts, PA accumulated linearly throughout the time course and there was little indication that LPA was about to be saturated with radioactivity (Fig. 1). These results are consistent with the reported differences in phosphatidate phosphatase activities in chloroplasts from the different plant species (Gardiner & Roughan, 1983).

Glycerolipid labelling kinetics in the chloroplasts from different plants

Temporal changes in the molar ratios of the newly synthesized glycerolipids in *S. oleracea* chloroplasts did not suggest a precursor–product relationship between LPA and PA, but rather between LPA and DAG (Fig. 2). However, the putative sequence of initial reactions leading to nett glycerolipid synthesis in chloroplasts is as shown in Scheme 1 (Joyard & Douce, 1977; Frentzen *et al.*, 1983) so that the radioactivity in LPA should initially exceed that in PA and the curve for PA should extrapolate through zero at zero time. These anomalies might be explained if there were a significant pool of unlabelled LPA within the chloroplasts at the beginning of the incubation. Indeed, both the disproportionate incorporation of labelled palmitate into PA at the earliest sampling times



Scheme 1. *Probable reaction sequence for chloroplast glycerolipid synthesis*

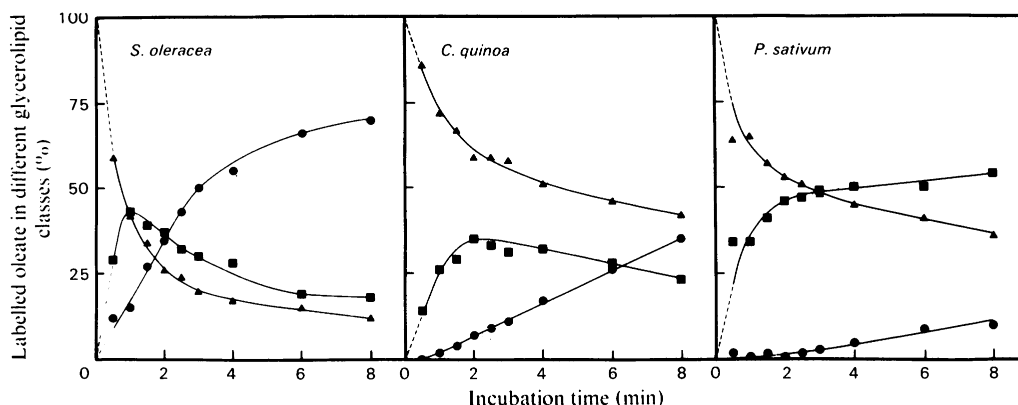


Fig. 3. *Molar ratios of different glycerolipids synthesized de novo as calculated by labelling of oleate from [1-^{14}C]acetate*
Portions of extracts from the experiment described in Fig. 1 (incubations containing 0.2mM-*sn*-G3P only) were analysed for content of labelled oleate in the individual glycerolipids (Gardiner *et al.*, 1984). Radioactivity in oleate in each glycerolipid was expressed as a percentage of total oleate label in glycerolipids for each incubation time. Key to symbols: ▲, LPA; ■, PA; ●, DAG.

(Fig. 4 below) and the slow turnover of LPA relative to PA in the dark when *S. oleracea* chloroplasts were alternated between light and dark (S. E. Gardiner, unpublished work) are consistent with this explanation. At earliest times both *sn*-G3P and unlabelled LPA would initially accept labelled acyl moieties, giving rise to an anomalously high level of label in PA. Since *sn*-G3P is acylated exclusively at the *sn*-1 position, and then predominantly with oleate, and since oleate is essentially excluded from the *sn*-2 position of PA and DAG synthesized by isolated chloroplasts and chloroplast preparations (Frentzen *et al.*, 1983; Heinz & Roughan, 1983; Roughan *et al.*, 1979), this labelling in PA (and DAG) would be expected to be disproportionately high in palmitate. Thus more realistic kinetic relationships might be established by considering only that radioactivity incorporated into the oleate of the glycerolipids, i.e. label incorporated only at the *sn*-1 position. The results obtained for *S. oleracea* chloroplasts by adopting this strategem show smooth changes in relative labelling with time, producing curves for LPA and PA that could be extrapolated at zero time through 100% and 0% respectively (Fig. 3). As this type of relationship was also obtained with *C. quinoa* and *P. sativum* chloroplasts only by ignoring palmitate incorporation into the glycerolipids, we suggest that unlabelled, but metabolically active, LPA was also present in those preparations. The results shown in Fig. 3 confirm the reaction sequence shown in Scheme 1 and suggest where the process might be rate-limited in chloroplasts from different species (discussed below).

Fatty acid compositions of labelled glycerolipids

Glycerolipids synthesized by isolated chloroplasts in the absence of added UDP-galactose have relatively simple fatty acid compositions with predominantly oleate at the *sn*-1 position and palmitate at the *sn*-2 position (Heinz & Roughan, 1983). However, Fig. 4 shows an apparent anomalously high incorporation of palmitate into the PA and DAG of all chloroplast types at the earliest times. The theoretical percentage of labelled saturated fatty acid in PA, calculated from the measured ratios in LPA and assuming subsequent esterification at *sn*-2 solely by labelled palmitate, was considerably lower than that actually measured. This lack of correspondence is not consistent with this radioactive LPA being the direct precursor of phosphatidate as indicated by the kinetics of oleate incorporation into the glycerolipids (Fig. 3), but may be explained (see also above) by the presence of unlabelled 1-acyl-*sn*-glycerol 3-phosphate at the beginning of the time course. Indeed, the curve for theoretical composition of phosphatidate converged with time upon the measured ratios to give reasonably close agreement by 8 min (Fig. 4). This, coupled with the tendency of the diacylglycerols to approach palmitate/oleate labelling ratios of unity (Fig. 4), suggests that the fatty-acyl-composition data is consistent with the kinetics of oleate incorporation when an unlabelled pool of LPA is taken into account, and that both lines of evidence do in fact support the pathway of glycerolipid synthesis shown above.

A gradual increase with time in the proportion of saturated fatty acid in LPA in all chloroplasts (Fig. 4) may have resulted from a preference for 1-oleoyl-*sn*-glycerol 3-phosphate over 1-palmitoyl-*sn*-glycerol 3-phosphate in the acyl-ACP:lysophosphatidate acyltransferase reaction. By contrast, there was little indication of discrimination in favour of a particular molecular species by the phosphatidate phosphatase of *S. oleracea* or *C.*

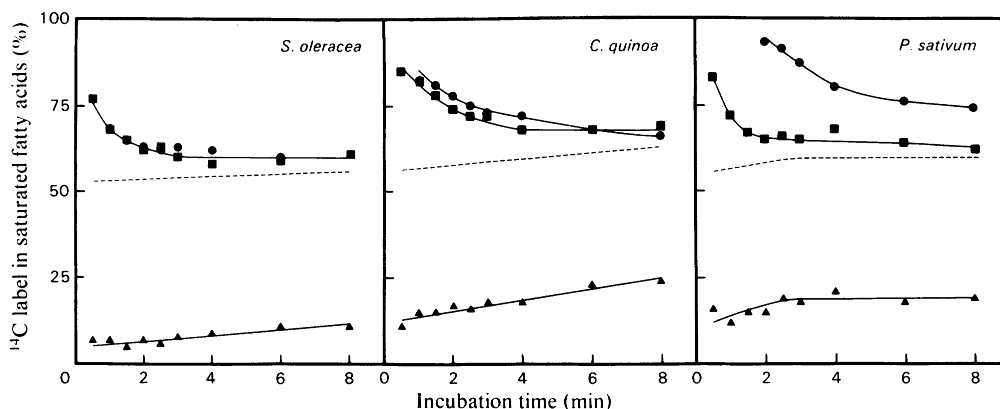


Fig. 4. *^{14}C label in saturated fatty acids as a percentage of total fatty acid ^{14}C in the different glycerolipids during time-course experiments with chloroplasts isolated from three different species and incubated with 0.2mM-sn-G3P*

The radioactive fatty acid composition of the different glycerolipids was determined as previously described (Gardiner *et al.*, 1984). The data for percentage of saturated fatty acids in LPA, PA and DAG were plotted directly, and that for LPA was also used to calculate the theoretical percentage composition for PA synthesized from that LPA, i.e.:

$$\text{Theoretical percentage of saturated fatty acid in PA} = \frac{\text{Percentage of saturated fatty acid measured in LPA} + 100}{2}$$

Key to symbols: ▲, LPA; ■, PA; ●, DAG; ----, calculated PA.

quinoa, since both PA and DAG were similarly labelled (Fig. 4). However, a large and more persistent difference in the fatty acyl labelling of DAG compared with PA in *P. sativum* chloroplasts would appear to suggest there was a preference for disaturated PA in the phosphatidate phosphatase reaction. Even this initial imbalance was probably corrected, however, as the oleate in PA increased with longer incubation times (Fig. 4 and also Heinz & Roughan, 1983).

Discussion

The sequence of reactions and the specificities of acylations leading to the net synthesis of glycerolipids as deduced from these studies with intact chloroplasts and acyl donors generated *in situ* agrees well with the proposals of Frentzen *et al.* (1983). LPA appears to be a free intermediate turning over only slowly in isolated chloroplasts in the dark. This would be expected if the main or sole acyl donors within chloroplasts were acyl-ACP species that are regenerated only in the light (Soll & Roughan, 1982). The LPA synthesized *in situ* contains rather more palmitate than would be expected from studies utilizing the purified acyl-ACP:*sn*-glycerol 3-phosphate acyltransferases from *P. sativum* and *S. oleracea* (Frentzen *et al.*, 1983). However, the enzyme from a number of chilling-sensitive plants may be even less specific *in vivo* for oleate compared with those within the isolated chloroplasts of the present study. Leaves of *Ipomoea batatas* (sweet potato) and *Colocasia antiquorum* (taro), for instance, produce, as possibly the sole product of chloroplast glycerolipid synthesis, phosphatidylglycerol that contains >60mol of palmitate/100mol at the *sn*-1 position (Murata, 1983).

In *S. oleracea* chloroplasts, LPA and PA turned over rapidly and were quickly reduced to minor constituents (Fig. 3); the acylation of *sn*-G3P was probably the rate-limiting step in the process. Compared with *S. oleracea*, *C. quinoa* chloroplasts have a much decreased phosphatidate phosphatase activity (Gardiner & Roughan, 1983), yet maintained LPA at a higher concentration than PA at steady state. This would seem to suggest a decrease in chloroplast acyl-ACP:lysophosphatidate acyltransferase activity in *C. quinoa* compared with *S. oleracea* and that this step could become rate-limiting. A similar conclusion could be drawn for *P. sativum* chloroplasts from the finding that LPA was maintained in a relatively high concentration in the presence (Fig. 3), but not in the absence (results not shown), of exogenous *sn*-G3P.

Thus the rate of glycerolipid synthesis in the light in each chloroplast type was primarily dependent on the concentration of *sn*-G3P, but was then limited by the activities of the *sn*-glycerol 3-phosphate acyltransferase in *S. oleracea* and the acyl-ACP: lysophosphatidate acyltransferase in *C. quinoa* and *P. sativum*. The products present when steady state is first achieved also reflect the large differences in the activities of phosphatidate phosphatase in each of the chloroplast preparations. However, the slow accumulation of DAG by *P. sativum* chloroplasts represents something of a dilemma since, unlike in *S. oleracea* and *C. quinoa*, in *P. sativum* leaves there is no significant synthesis of diacylgalactosylglycerol, diacyldigalactosylglycerol or sulpholipid containing a fatty acyl distribution that would be consistent with derivation from that DAG. It is possible that there is a non-enzymic breakdown of phosphatidate in *P. sativum* chloroplasts *in vitro*.

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