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RADIOLABELLING STUDIES OF FATTY ACIDS IN *PISUM SATIVUM* AND *VICIA FABA* LEAVES AT DIFFERENT TEMPERATURES

JOHN L. HARWOOD

Department of Biochemistry, University College, P.O. Box 78, Cardiff, CF1 1XL, U.K.

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Abstract—Leaves of pea and broad bean plants were incubated with acetate-[^{14}C] at temperatures varying from 7 to 34°. No significant difference was observed in the distribution of radioactivity between phosphatidylcholine and the galactosylglycerides in pea with different temperatures. However, increasing temperatures increased the labelling of phosphatidylcholine in broad bean leaves, at the expense of polar lipids other than the galactosylglycerides. The incubation temperature had no significant effect on the pattern of labelling of the fatty acids of the major leaf lipids. A correlation was seen in the specific radioactivity of oleate and linoleate in phosphatidylcholine and, especially, in the galactosylglycerides. The data emphasise the rapid equilibration of oleate and linoleate (which probably occurs by transacylation) between the two galactosylglycerides and phosphatidylcholine in leaf tissues.

INTRODUCTION

Higher plant leaves contain large amounts of acyl lipids, much of which is associated with the thylakoid membranes. These lipids are remarkably rich in the polyunsaturated fatty acids, linoleic and α-linolenic, which may account for up to 95% of the acyl moieties of certain lipids [1]. In spite of the huge quantities of lipid involved, the details of polyunsaturated fatty acid synthesis in plants are not only unclear but also, at present, a subject of some controversy [2].

Palmitoyl-acyl carrier protein is produced by fatty acid synthetase, elongated by palmitate elongase and the resultant stearoyl-acyl carrier protein is desaturated by a Δ9-desaturase to form oleate. These 3 steps in leaves are probably mainly localized in the chloroplast [3]. Two types of substrate have been suggested for oleate (and linoleate) desaturation. Firstly, oleoyl-CoA has been used as a substrate in two storage tissues [4, 5]. From their studies with oleoyl-CoA in leaves, however, Slack *et al.* [6] suggested that the acyl moiety was transferred to phosphatidylcholine which was the true substrate. Indeed, some years before, the high rates of labelling of the fatty acid moieties of a number of complex lipids in photosynthetic tissues had led to the suggestion that intact lipids could act as substrates for desaturation. These proposals included diacylgalactosylglycerol which was implicated in green and blue-green algae [7] and phosphatidylcholine which was suggested to be involved (possibly as the actual substrate) in linoleate synthesis in *Chlorella* [8]. Direct evidence for phosphatidylcholine as a substrate for oleate desaturation has been obtained in rat liver [9] and in various micro-organisms [10–12]. This has supported the hypothesis that this lipid is the substrate for oleate and linoleate desaturation in higher plant leaves [13–16]. The same lipid has also been proposed as a donor for linolenate of the chloroplastic galactolipids [14, 16], possibly via a phospholipase reaction [17]. In addition to a sequential desaturation of fatty acids at the C_{18} level to α-linolenate [18, 19], a second pathway has been proposed which involves desaturation at the C_{12} level to a trienoic acid which is then elongated [20].

Environmental temperature has been found to cause significant changes in the endogenous fatty acid composition of plant tissues, including leaves. Part of the reason for these changes is undoubtedly the correlation of membrane fluidity with enzyme activity [21]. The mechanism(s) by which plants are able to adapt to temperature variation are, at present, unclear. The solubility of oxygen [22, 23], and induction [24] or modification [25] of desaturase activity have all been proposed.

We have used the incubation of leaf preparations at different temperatures to obtain some information concerning the above proposals. In addition, large differences in temperature were used in an effort to change the strikingly similar specific radioactivities which were observed for oleic and linoleic acids in phosphatidylcholine and the galactolipids of several plant leaves [26]. This similarity has important implications in the proposed routes for fatty acid desaturation. Two plants were chosen for the experiments, *Pisum sativum* and *Vicia faba*, both of which exhibit high rates of fatty acid synthesis but markedly different esterification of the newly synthesized moieties [26, 27]. In addition, the specific preparations used in the present experiments were physiologically different (see Experimental) from those used previously [26, 27].

RESULTS AND DISCUSSION

As a preliminary to the examination of fatty acid labelling, I determined the distribution of radiolabel amongst different acyl lipid fractions (Table 1). In common with other studies using mature or expanded leaves [13, 16], the fatty acids of phosphatidylcholine were highly labelled in pea leaves. Interestingly, raising the

Table 1. Distribution of radioactivity in complex lipids of pea and broad bean leaves incubated at different temperatures with acetate-[^{14}C]

	Lipid	Distribution (% total)		
		7°	24°	34°
Pea	NL	—	11.2 ± 2.4	9.5 ± 2.2
	MGDG	—	3.2 ± 0.2	3.3 ± 0.2
	DGDG	—	4.1 ± 0.5	3.7 ± 0.2
	PC	—	81.5 ± 2.5	83.5 ± 1.1
Broad bean	NL	2.9 ± 0.6	2.8 ± 0.6	2.6 ± 0.1
	MGDG	38.5 ± 1.0	33.9 ± 3.2	36.8 ± 1.4
	DGDG	6.7 ± 2.1	7.1 ± 1.5	7.2 ± 0.4
	PC	12.4 ± 1.1	20.2 ± 3.4	27.6 ± 3.6
	PL	39.7 ± 1.9	36.6 ± 5.8	25.5 ± 4.8

Results are expressed as means ± s.d. (n = 4, pea; n = 3, broad bean). MDGD = Diacylgalactosylglycerol, DGDG = diacylgalabiosylglycerol, NL = other neutral lipids, PC = phosphatidylcholine, PL = other polar lipids (mainly phosphatidylglycerol, phosphatidylethanolamine, and sulpholipid). Incubations were carried out for 1–4 hr, with no difference in distribution of counts observed between different incubation times.

temperature of incubation from 24 to 34° did not alter the relative distribution of counts between the galactosylglycerides and phosphatidylcholine. Since the broad bean tissue was younger, more counts were observed in the diacylgalactosylglycerol fraction, again in keeping with previous results [15, 26] including broad bean [27]. Because the previous data with developing pea, barley and wheat leaves had indicated such a close relationship between the fatty acids of galactosylglycerides and phosphatidylcholine [26], we included other polar lipids in our study of broad bean (Table 1). With increasing incubation temperature, the fatty acids of these polar lipids were less well-labelled, while those of phosphatidylcholine were more heavily labelled. These data are interesting since they may indicate an adaptive alteration in the rate of synthesis of certain complex lipids as a result of environmental temperature. In a similar fashion, changes in the growth temperature of alfalfa did not alter the fatty acid compositions of individual lipids—variations were induced by increases in the relatively more saturated lipids [28]. In broad bean as with pea (Table 1), the proportion of total label in the galactosylglycerides was not changed with incubation temperatures.

We next studied the fatty acid moieties of the acyl lipid fractions (Tables 2 and 3). Phosphatidylcholine was highly labelled in its oleoyl moiety, particularly in the case of pea leaves. Broad bean leaves contained a higher proportion of radioactivity in palmitic acid and the 'other polar' lipids also contained a substantial percentage of label in palmitate. The galactosylglycerides of pea contained relatively more radiolabel in linoleic acid when compared with phosphatidylcholine but in the broad bean preparations the distribution of counts were rather similar. The general features of labelling seen in Tables 2 and 3 are broadly similar to previous data from higher plant leaves [13–16, 26, 27, 29]. However, it is important to note that, presumably because of the different physiological state of the tissues used, α-linolenate was only poorly labelled in the present experiments (cf. [27]). In addition, the labelling patterns were not significantly affected in either tissue by the temperature

Table 2. Distribution of radioactivity in the fatty acids of phospholipids of pea and broad bean leaves incubated at different temperatures

Plant	Lipid	Incubation temp. (°)	[^{14}C]-Fatty acids (% total) 16:0	18:0	18:1	18:2	Others
Pea	PC	24	8.5 ± 1.7	1.1 ± 0.3	72.7 ± 3.0	17.3 ± 4.3	0.4 ± 0.1
	PC	34	10.6 ± 1.9	1.5 ± 1.2	64.2 ± 1.5	23.6 ± 3.1	0.1 ± tr
Broad bean	PC	7	32.5 ± 1.9	1.7 ± 1.1	52.5 ± 3.5	18.2 ± 0.6	0.1 ± tr
	PC	24	34.4 ± 3.4	1.7 ± 0.2	46.5 ± 1.5	18.0 ± 2.6	tr
	PC	34	29.5 ± 0.5	1.5 ± 0.6	52.6 ± 0.7	16.3 ± 3.2	0.1 ± tr
	PL	7	21.5 ± 0.7	1.5 ± 0.8	57.7 ± 0.8	20.2 ± 1.2	0.1 ± tr
	PL	24	21.3 ± 1.1	1.5 ± 0.5	49.5 ± 1.0	27.8 ± 6.5	tr
	PL	34	20.9 ± 0.4	1.9 ± 0.4	53.3 ± 1.5	21.6 ± 0.4	2.3 ± 0.8

Results are expressed as means ± s.d. (n = 4, pea; n = 3, broad bean). For abbreviations, see Table 1.

Table 3. Distribution of radioactivity in the fatty acids of galactolipids of pea and broad bean leaves incubated at different temperatures

Plant	Lipid	Incubation temp. (°)	[^{14}C]-Fatty acids (% total) 16:0	18:0	18:1	18:2	Others
Pea	MGDG	24	3.0 ± 1.2	tr	54.3 ± 6.4	35.6 ± 5.9	7.0 ± 2.0
	DGDG	24	16.4 ± 4.1	tr	55.2 ± 5.2	28.4 ± 3.1	tr
	MGDG	34	3.7 ± 0.7	tr	43.2 ± 3.8	40.9 ± 4.9	12.2 ± 2.9
	DGDG	34	18.9 ± 3.2	tr	47.0 ± 2.3	34.1 ± 4.0	tr
Broad bean	MGDG	7	19.4 ± 0.4	4.5 ± 2.1	58.0 ± 2.3	18.1 ± 0.5	tr
	DGDG	7	17.6 ± 1.5	1.8 ± 0.7	59.3 ± 3.1	19.8 ± 1.4	2.4 ± 0.8
	MGDG	24	22.9 ± 2.9	tr	54.0 ± 0.6	21.6 ± 1.4	1.5 ± 0.5
	DGDG	24	22.1 ± 2.5	0.7 ± 0.2	53.6 ± 1.2	23.6 ± 1.6	tr
	MGDG	34	24.8 ± 7.2	2.8 ± 1.9	53.0 ± 5.5	18.9 ± 3.3	0.5 ± 0.1
	DGDG	34	21.5 ± 2.9	2.3 ± 0.2	57.6 ± 2.5	17.8 ± 3.2	0.8 ± 0.2

Results are expressed as means ± s.d. (n = 4, pea; n = 3, broad bean). For abbreviations, see Table 1.

Table 4. Relative specific radioactivities of fatty acids in the major acyl lipids of pea and broad bean leaves

	Lipid	Fatty acid (relative specific activity) 16:0	18:1	18:2
Pea (n = 10)	PC	0.36 ± 0.14	2.83 ± 0.61	0.76 ± 0.27
	MGDG	0.22 ± 0.12	3.03 ± 0.87	0.74 ± 0.32
	DGDG	0.16 ± 0.13	3.00 ± 0.64	0.69 ± 0.24
Broad bean	PC	1.14 ± 0.77	6.54 ± 2.33	0.56 ± 0.32
(n = 9)	MGDG	0.95 ± 0.38	4.94 ± 0.57	0.48 ± 0.13
	DGDG	0.54 ± 0.31	5.85 ± 2.75	0.50 ± 0.28
	PL	0.97 ± 0.47	7.61 ± 6.06	0.96 ± 0.62

Results were calculated from the specific radioactivities (dpm/mg fatty acid) for individual experiments divided in each case by the uptake (dpm) of precursor acetate-[1-^{14}C]. Means ± s.d. are shown. For abbreviations, see Table 1.

of incubation. This indicates that the amount of fatty acid desaturation in pea and broad bean leaves is probably changed only by the slow process of altering the net levels of the relevant enzymes and not by the availability of oxygen for dehydrogenation [22, 23]. The importance of genetic regulations of desaturases has been noted in other studies with plant tissues [30, 31] although Trémolières *et al.* [25] concluded that both effects may occur in maturing rapeseed.

Together with previous data (e.g. [26]) we have information regarding the specific radioactivities of fatty acids for leaf lipids labelled under several different conditions. In order to see if the striking correlations previously noted [26] are also held in the present work, we analysed the results statistically (Table 4). Because the uptake of radioactive precursor varies between individual leaves, the specific radioactivities of fatty acids were divided by the precursor uptake so that different experiments could be compared. It will be readily seen that the relative specific radioactivities of oleic acid are similar for phosphatidylcholine and the two galactosylglycerides in both pea and broad bean leaves. The same is also true for the linoleate of these 3 lipids. The correlation for palmitic acid was less good, with diacylgalabiosylglycerol apparently less well-labelled than diacylgalactosylglycerol and phosphatidylcholine. The correlation for the two unsaturated fatty acids between the galactosylglycerides was particularly striking. These results clearly show, especially when taken in conjunction with previous data [26], that newly synthesized oleate and linoleate equilibrate rapidly between the major leaf lipids (phosphatidylcholine and galactosylglycerides).

Roughan and Slack [6, 14, 15, 17, 32] have recently published results with plant tissue which they interpret to indicate that oleate is desaturated probably while esterified to phosphatidylcholine. The latter molecule is then a donor of linoleate to other lipids possibly by means of phospholipase and phosphatidate phosphatase enzymes. Although during the short incubation periods which we have used there is no significant net transfer of label from phosphatidylcholine to diacylgalactosylglycerol, the rather similar relative specific radioactivities for oleate or for linoleate in these two lipids (Table 4) would be expected from the above theory. In fact, if anything, the relative specific radioactivities in phosphatidylcholine are slightly higher as might be predicted for a precursor–product interrelationship. However, it is difficult to explain the remarkably good correlation in relative specific radioactivities of oleate and linoleate between the galactosylglycerides by the phosphatidylcholine-donor theory. Diacylglycerol eventually destined for diacylgalabiosylglycerol synthesis either must pass entirely [33] or partially [34] through diacylgalactosylglycerol. By either route, newly synthesized (and radioactive) diacylglycerol moieties enter a large non-radioactive pool in diacylgalabiosylglycerol. (The same arguments also apply to a lesser extent to diacylgalactosylglycerol.) When comparing specific radioactivities over the first 4 hr of labelling, we never noticed any marked change in the ratios between phosphatidylcholine and the galactosylglycerides (see also [26]). I therefore consider [26] that there is free interchange of oleate and linoleate between phosphatidylcholine and the galactosylglycerides. This exchange is most probably due to transacylation reactions. It should be noted that the exchange must involve all molecules of these 3 lipids in order to easily explain why the specific radioactivities are similar.

The exchange of oleate and linoleate as suggested above does not necessarily favour either oleoyl-phosphatidylcholine or oleoyl-CoA as the substrate for oleate desaturation. My results cannot be used to support either theory since both interpretations are equally valid. In situations where there is net transfer of double-labelled diacylglycerol from phosphatidylcholine to diacylgalactosylglycerol [17] or where oleoyl-phosphatidylcholine has, apparently, been used as substrate for desaturation [6, 35], the results tend to favour the oxygen–ester theory originally proposed by Gurr *et al.* [8]. However, in the presence of the rapid acyl-exchange indicated by the present data, it is impossible to unequivocally exclude CoA-esters as the true substrates for the desaturase [36]. This could only be done in an *in vitro* system where acyl transfer was absent.

In summary, therefore, it is important to consider the rapid acyl exchange occurring *in vivo* between leaf lipids when trying to obtain evidence for particular substrates in oleate desaturation. Although phosphatidylcholine has, apparently, been excluded as a substrate for linoleate desaturation [26, 27, 37, 38] it remains a likely candidate for oleate desaturation. However, in view of the clear demonstration that both thioesters and complex lipids can be used for fatty acid elongation [39, 40], there seems no reason why both types of substrates could not be used for the same desaturation step [cf. 12, 35]. Clarification of the relative contribution of phosphatidylcholine and/or oleoyl CoA to linoleate formation must await purification of the Δ^{12}-desaturase.

EXPERIMENTAL

Pea seeds (*Pisum sativum* cv Kelvedon Wonder) were obtained from Thompson and Morgan Ltd., Ipswich, and broad bean seeds (*Vicia faba* cv Beagle) from the Scottish Horticultural Research Institute, Invergowrie, Scotland. Seeds were grown in John Innes Seed Compost under normal daylight conditions (14 hr daylight, 15–19°). Expanded pea leaves were used from plants *ca* 20 days old. Broad bean plants were pruned after 10 days and the first pair of expanding leaves which subsequently developed were used. Both prepns, therefore, contrasted with pea and broad bean leaves previously used [26, 27].

Incorporation studies. Leaves were detached and incubated with Na acetate-[1-^{14}C] (Radiochemical Centre, Amersham, 60 mCi/mmol) as previously [26]. Incubations were for 0.5 to 4 hr under constant temp. of 7, 24 or 34°. Total lipids were extracted from leaves [26] and total radioactivity in the non-lipid and lipid portions determined. In addition, aliquots were taken from the incubation soln as an additional check for total uptake of radioisotope.

Lipid analysis. Total lipids were separated by TLC on Si gel G using Me_2CO–C_6H_6–H_2O (90:31:8) and visualized with 0.001% aq. Rhodamine 6G. Radioactivity in the separated lipids was detected with a spark chamber scanner and fatty acids transmethylated with 0.5 M NaOMe in MeOH. Fatty acid Me esters were separated and quantitated as previously [26].

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