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Effect of BASF 13-338, a Substituted Pyridazinone, on Lipid Metabolism in Leaf Tissue of Spinach, Pea, Linseed, and Wheat[1]

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CLAUDE WILLEMOT[2], C. ROGER SLACK, JOHN BROWSE, AND P. GRATTAN ROUGHAN
Plant Physiology Division, D.S.I.R., Palmerston North, New Zealand

ABSTRACT

A substituted pyridazinone (BASF 13-338) inhibited photosynthesis in spinach (*Spinacia oleracea*, Hybrid 102 Arthur Yates Ltd.) leaf discs and reduced the incorporation of [1-^{14}C]acetate into trienoic acids of diacylgalactosylglycerol while causing radioactivity to accumulate in diacylgalactosylglycerol dienoic acids. Although BASF 13-338 inhibited photosynthesis in isolated spinach chloroplasts, it did not prevent dienoate desaturation. In discs, the labeling of fatty acids was affected by the inhibitor only in diacylgalactosylglycerol. Very little radioactivity was incorporated into trienes of phosphatidylcholine and the proportion of the label recovered in the fatty acids of phosphatidylcholine was not changed by BASF 13-338. The herbicides caused an increase in the proportion of the lipid ^{14}C incorporated into diacylgalactosylglycerol and a decrease in labeling of phosphatidylcholine, whereas the proportion of ^{14}C recovered in other lipids remained unchanged. Similar results were obtained with pea (*Pisum sativum* cv. Victory Freeze), linseed (*Linum usitatissimum* cv. Punjab), and wheat (Triticum aestivum cv. Karamu). With these species, a greater proportion of the label was incorporated into phosphatidylcholine and less into diacylgalactosylglycerol than with spinach. The data indicate that trienoate synthesis uses diacylgalactosylglycerol as substrate. BASF 13-338 appears to act at that step, and seems to cause in spinach a shift in polyenoate synthesis from the pathway involving microsomal phosphatidylcholine to the pathway operating inside the chloroplast.

In a number of plant tissues, 4-chloro-5-dimethylamino-2-phenyl-3(2H)-pyrizadinone (BASF 13-338[3], Sandoz 9785) has been shown to cause a decrease in linolenic acid content and a corresponding increase in linoleic acid with little change in other fatty acids (3, 7, 11, 22, 25) by preventing linoleic acid desaturation on diacylgalactosylglycerol (5, 11).

In spinach leaves, which contain hexadecatrienoic acid esterified in DGG,[3] two pathways leading to trienoate DGG fatty acids have been proposed (18). One pathway (eucaryotic) involves microsomal PC as substrate for desaturation of oleate to linoleate (21), and glyceride intermediates with a 18 carbon fatty acid at the 2-position. The other (procaryotic) occurs completely within the chloroplast and involves glyceride intermediates with a 16 carbon fatty acid at the 2-position. Linoleate and linolenate synthesis by isolated spinach chloroplasts has been recently demonstrated (17).

It was of interest to test the effect of BASF 13-338 on fatty acid biosynthesis in this system. The inhibitor might act only on one of the two proposed pathways leading toward trienoate synthesis. Desaturation of fatty acids might be inhibited preferentially at one of the two positions where desaturation is thought to occur in glycerides. The use of BASF 13-338 might permit identification of the actual substrate of linoleic acid desaturase by causing the immediate precursor of linolenic acid to accumulate. Additionally, the effect of the substituted pyridazinone on the fatty acid composition of the various lipid classes might give some information on the relationship existing between them.

MATERIALS AND METHODS

Materials. Expanding leaves were taken from plants of spinach (*Spinacia oleracea*, Hybrid 102 Arthur Yates Ltd., Auckland, New Zealand), grown in aerated solution culture (20), and from plants of pea (*Pisum sativum* cv. Victory Freeze), linseed (*Linum usitatissimum* cv. Punjab) and wheat (*Triticum aestivum* cv. Karamu), grown in a pumice/vermiculite support and irrigated with half-strength Hoagland nutrient solution. Sodium [1-^{14}C]acetate at 58.1 Ci/mol was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, U. K.; Hepes, Mes, and all other biochemicals were from Sigma.

Methods. Chloroplast isolation, measurement of HCO_3^--dependent O_2 evolution, incorporation of [1-^{14}C]acetate into lipids of isolated chloroplasts, and TLC separation of the lipid extracts were as described previously (14–17). The standard 2-ml assay medium for measuring O_2 evolution using an O_2 electrode contained 0.33 M sorbitol, 25 mM Hepes/NaOH (pH 7.9), 10 mM $NaHCO_3$, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4, catalase (2,000 units/ml) and washed chloroplasts equivalent to approximately 25 μg Chl/ml. For [1-^{14}C]acetate incorporation into DGG, 0.25 ml of the same buffer, without catalase, was supplemented with 0.2 mM [1-^{14}C]acetate (8 mCi/mmol), 0.25 mM *sn*-glycerol 3-P, 130 μM Triton X-100, and 0.1 mM UDP-galactose and contained chloroplasts equivalent to 180 to 250 μg Chl/ml.

Discs 2 mm in diameter were punched from expanding lamina of spinach leaves, washed in distilled H_2O and vacuum infiltrated in a buffer containing 0.33 M sorbitol, 25 mM Mes/NaOH (pH 5), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.5 mM KH_2PO_4. The randomized discs were divided into groups of 50 which were incubated at 25°C with illumination in 1 ml of the same buffer to which [1-^{14}C]acetate was added. Leaves of pea, linseed, and wheat were sliced transversely into 2 mm strips using a razor blade, and 0.1 g fresh weight of the small segments were incubated in the same way as the spinach leaf discs. All experiments were repeated, and all treatments were duplicated. Incubations were stopped by adding 5 ml of hexane:isopropanol (3:2, v/v) (4) to the chloroplast suspensions or to the leaf tissue recovered from incubation media. Leaf tissue was comminuted in the organic solvent using a Ten-

[1] Contribution No. 190, Ste-Foy Research Station.

[2] Present address: Agriculture Canada, Research Branch, 2560 Blvd. Hochelaga, Ste-Foy, Quebec G1V 2J3 Canada. To whom reprint requests should be sent.

[3] Abbreviations: BASF 13-338, 4-chloro-5-dimethylamino-2-phenyl-3(2H)-pyridazinone; DGG, diacylgalactosylglycerol; PC, phosphatidylcholine.

Broeck homogenizer. Extracts were washed with 2 ml 6.5% (w/v) Na_2SO_4, evaporated to dryness under a stream of N_2 and the lipid residues were redissolved in 0.5 or 1 ml of chloroform.

Molecular species of DGG were separated on 5% (w/v) $AgNO_3$ in silica gel G using chloroform:methanol:acetone:acetic acid (80:15:5:1, by volume) as developing solvent. Fatty acid methyl esters were prepared from isolated lipids using $NaOCH_3$ (20) and were analyzed either by radiogaschromatography or by a combination of $AgNO_3$ TLC, reverse-phase TLC and autoradiography.

Initially, BASF 13-338 was added to incubation media in 25% (v/v) ethanol solution (final concentration, 0.125%, v/v, for the O_2 evolution assay, and 1%, v/v, for the [1-^{14}C]acetate incorporation). Ethanol was observed to affect fatty acid synthesis and to modify the effect of the herbicide so that, in later experiments, the compound was added to tubes or flasks in acetone solution, and the acetone evaporated off before adding incubation media. BASF 13-338, 2×10^{-4} M, was completely solubilized under these conditions.

RESULTS

Inhibition by BASF 13-338 of Photosynthesis and Linolenate Synthesis in Isolated Chloroplasts. When added to reaction media in aqueous ethanol, BASF 13-338 caused an immediate inhibition of HCO_3^--dependent O_2 evolution by isolated spinach chloroplasts. In three separate experiments, O_2 evolution was measured at 233 ± 81 μmol O_2 per h per mg Chl for the control and at 57 ± 7 μmol (24% of control) in the presence of 10 μM BASF 13-338 and 20 ± 7 μmol (9% of control) in the presence of 100 μM of the inhibitor. However, even at a concentration of 80 μM the herbicide had no effect either on [1-^{14}C]acetate incorporation into total lipids and DGG or on the high rates of linolenate synthesis by these isolated chloroplasts (Table I). Linolenate contained 40% of the DGG acyl radiocarbon after 60 min of unilluminated incubation. The presence of Triton X-100 in incubation media was found to alleviate to some extent the effect of the herbicide on O_2-evolution by isolated chloroplasts, but even when the detergent was omitted from incubations incorporating [1-^{14}C]acetate into polyunsaturated fatty acids, we could never detect any inhibition of linolenate synthesis by BASF 13-338. This lack of inhibition of the desaturation of linoleate was confirmed in the absence of Triton X-100 and ethanol and at higher concentrations (0.16 and 0.32 mM) of the inhibitor. At such concentrations BASF 13-338 is expected to partition progressively into the chloroplast membrane lipids.

Effect of BASF 13-338 on Photosynthesis by Spinach Leaf Discs. Since lipid metabolism by isolated chloroplasts appeared to be unaffected by the herbicide, we tested its effect on small (2 mm diameter) discs from spinach leaves similar to those used for chloroplast isolation. Within 3 min after addition of BASF 13-338, O_2 evolution by the discs was 60 to 70% inhibited by low concentrations (40 μM), and abolished at high concentrations (400 μM), of the herbicide.

Table I. *Effect of BASF 13-338 on Linolenic Acid Synthesis by Isolated Spinach Chloroplasts*

Chloroplasts equivalent to 48 μg Chl were incubated with [1-^{14}C]acetate for 20 min in the light followed by 60 min in the dark (17). BASF 13-338 was added to the media in 25% ethanol, v/v (final concentration of ethanol in control and treated sample: 1%, v/v).

	Time in Dark	[1-^{14}C]Acetate	DGG Label in Trienes
	min	*nmol/mg Chl into DGG*	*%*
Control	0	174.5[a] ± 39.0	9.0 ± 2.5
BASF 13-338, 80 μM		212.2 ± 66.7	8.7 ± 2.5
Control	30	224.7 ± 64.3	25.6 ± 5.5
BASF 13-338, 80 μM		230.0 ± 60.1	24.6 ± 6.5
Control	60	237.6 ± 81.6	35.0 ± 8.4
BASF 13-338, 80 μM		236.9 ± 72.5	34.0 ± 7.2

[a] Mean of six samples: duplicates from three experiments ± SD.

Table II. *Effect of Increasing Concentrations of BASF 13-338 on the Incorporation of [1-^{14}C]Acetate into DGG and PC of Spinach Leaf Discs*

Discs (67 μg Chl) were incubated for 2 h in buffer containing 0.05 mM [1-^{14}C]acetate (0.4 μCi), and the concentrations of inhibitor indicated.

Lipid	Inhibitor Concn.	[1-^{14}C]Acetate Incorporated (% of Total Lipid ^{14}C)	Total Fatty Acid ^{14}C			
			0[b]	1	2	3
	mM	*nmol*	*%*			
DGG	0	110[a] (35.5)	35	15	21	30
	0.05	114 (40.8)	36	14	23	28
	0.1	116 (43.7)	39	15	25	21
	0.2	125 (47.0)	41	15	28	17
PC	0	55 (18.0)	26	64	7	3
	0.05	56 (19.6)	23	68	7	3
	0.1	49 (18.0)	23	68	7	3
	0.2	40 (15.4)	24	66	7	3

[a] Average of duplicate samples.
[b] Number of double bonds/molecule.

Table III. *Effect of BASF 13-338 on the Incorporation of [1-^{14}C]Acetate into Different Lipids of Spinach Leaf Discs*

Discs (34 and 61 μg Chl) were incubated with [1-^{14}C]acetate (0.2 mM, 1.5 μCi) for 2 h. Lipids were separated by 2-dimensional TLC. Means of nine samples from two experiments ± SD.

Lipid Class	Control	0.2 mM BASF 13-338
Phosphatidylinositol	1.0[a] ± 0.2	0.8 ± 0.3
Phosphatidylcholine[b]	19.9 ± 2.3	14.5 ± 1.1
Diacylsulphoquinovosylglycerol	2.5 ± 0.7	2.8 ± 0.9
Diacyldigalactosylglycerol	6.1 ± 1.4	4.4 ± 0.9
Phosphatidylglycerol	16.0 ± 2.0	14.1 ± 3.0
Phosphatidylethanolamine	2.6 ± 0.3	2.2 ± 0.5
Phosphatidic acid	2.3 ± 0.2	1.9 ± 0.3
Diacylgalactosylglycerol[b]	42.2 ± 2.4	48.8 ± 3.7
Neutral lipids	7.4 ± 2.0	10.1 ± 3.0
Total lipid ^{14}C, dpm × 10^{-3}	1,018 ± 110	620 ± 90
nmol Acetate/mg Chl incorporated into lipids	933.8 ± 100.2	477.6 ± 83.8

[a] Percent of total lipid radioactivity.
[b] Differences between control and treated samples were very significant by the *t* test ($p < 0.01$).

Effect of BASF 13-338 on Lipid Metabolism by Spinach Leaf Discs. Increasing concentrations of BASF 13-338 reduced the total incorporation of [1-^{14}C]acetate into the lipids of leaf discs. Incorporation into DGG, when expressed as percentage of total lipid ^{14}C, increased in all experiments while incorporation into PC was depressed (Table II). Within DGG there was a progressive decrease in trienoate labeling and a corresponding increase in the labeling of dienoate and saturated fatty acids. It was further demonstrated by a combination of $AgNO_3$ TLC and reverse-phase TLC that only those molecular species of DGG containing 16 carbon acyl chains at position 2 incorporated [1-^{14}C]acetate. The relative increase in DGG labeling and the inhibition of trienoate labeling was manifested within 15 min of exposure to the inhibitor. PC contained only small amounts of ^{14}C-labeled di- and trienoic fatty acids, and the distribution of the label among the fatty acids of this lipid was unaltered by the herbicide.

The effect of BASF 13-338 on the incorporation of [1-^{14}C] acetate into the glycerolipids of spinach leaf discs is shown in Table III. Total [1-^{14}C]acetate incorporation into lipids was reduced by 39% in the presence of the herbicide. As noted above, the proportion of the lipid radioactivity incorporated into DGG increased, while that incorporated into PC decreased. When tested by the *t* test, the differences between labeling in control and treated discs where shown to be highly significant ($p < 0.01$) for DGG and PC. Labeling of the other lipids was but little affected. When the fatty acid methyl esters derived from these lipids were separated by $AgNO_3$ TLC, only the labeling of the fatty acids of DGG, and to a lesser extent of diacyldigalactosylglycerol, was shown to be affected by BASF 13-338. Little change was observed in the distribution of the label among the fatty acids of PC, phosphatidylglycerol and the other, less labeled lipids.

In the hope of observing a transfer of radiocarbon from PC to DGG (20), and of measuring (18:3–18:3) DGG synthesis from diglycerides released from (18:2–18:2) PC, leaf discs were incubated with high specific radioactivity [1-^{14}C]acetate for 30 min and then transferred to media containing unlabeled acetate, with and without 200 μM BASF 13-338. However, during the course of the 120 min chase there was apparently no transfer of ^{14}C from PC to the galactolipid and there was no significant desaturation of oleate to linoleate within the phospholipid (Fig. 1). Instead, the redistribution of the label among the fatty acids of DGG during the chase was consistent with a sequential desaturation of oleate to linolenate and a slower rate of palmitate desaturation within DGG.

Incorporation of [1-^{14}C]Acetate into Whole Spinach Leaves in the Presence of BASF 13-338. When spinach leaves were provided via the cut petiole with [1-^{14}C]acetate in aqueous solution with and without the herbicide, there were no differences either in incorporation rates of the precursor or in the lipids and fatty acids synthesized.

Inhibition of Linolenate Synthesis in Pea, Linseed, and Wheat by BASF 13-338. Since the spinach leaf discs incorporated [1-^{14}C] acetate directly into DGG and not through the mediation of PC (20), the effect of the herbicide on [1-^{14}C]acetate incorporation into the lipids of leaf segments from wheat, pea and linseed, species which do not accumulate $C_{16:3}$ in galactolipids, was examined. All three species incorporated the precursor primarily into PC and only to a lesser extent into DGG. Incorporation of [1-^{14}C]acetate into total lipids in the presence of BASF 13-338 was inhibited by 31% in pea, 18% in linseed and 50% in wheat and reflected a general decrease in labeling of all lipids examined. The proportion of the lipid radioactivity incorporated into DGG and PC decreased in the three species (Table IV). Accumulation of labeled linolenate within DGG was inhibited by the herbicide while label increased in linoleate. Only little label was incorporated into linolenate of PC and the distribution of radiocarbon among the fatty acids of PC was largely unaffected. Pea leaves exhibited the most active linoleate desaturation which was the most affected by the herbicide. When young seedlings were treated with BASF 13-338 via the roots, desaturation of linoleic acid was most inhibited in wheat, only slightly in linseed, and was not affected in pea. Linoleate desaturation was also not inhibited in treated spinach seedlings.

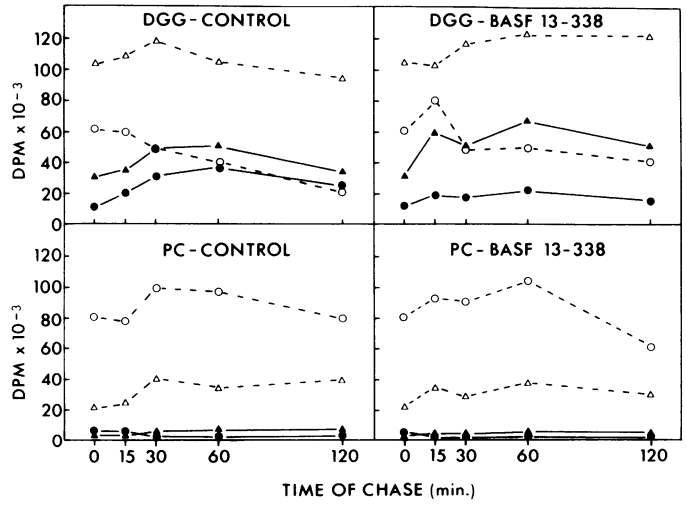

FIG. 1. Redistribution of ^{14}C among fatty acids of DGG and PC of spinach leaf discs during a pulse chase with and without BASF 13-338. Discs (73 μg Chl) were incubated with [1-^{14}C]acetate (0.2 mM, 2 μCi) for 30 min, then transferred to media containing unlabeled acetate (25 mM) with and without BASF 13-338 (0.2 mM). △, palmitate, ○, oleate, ▲, linoleate, ●, linolenate.

DISCUSSION

The present results (Table II) confirm that BASF 13-338 acts mainly on the desaturation of diene to triene fatty acids (3, 5, 7, 11, 22, 25). The effect was shown to occur almost exclusively in the labeling of DGG. Incorporation of radioactivity into linoleate and linolenate of PC was not affected. These observations are strong evidence that DGG is a major site of diene to triene desaturation, as suggested for leaves (6, 9, 19, 24) and as demonstrated with isolated spinach chloroplasts (18), and that the herbicide acts at this step (5, 11). The only glycerolipid showing a similar response to BASF 13-338 was diacyldigalactosylglycerol (5, 7, 11, 22). The fact that BASF 13-338 may inhibit to some extent all desaturation steps within DGG may have been overlooked because species were used which desaturated only linoleic acid on DGG.

The significant decrease in the percentage of the label incorporated into PC in spinach leaf discs in the presence of BASF 13-338 and the concomitant increase of that in DGG (Table III) suggests that the inhibitor caused a shift in spinach leaf tissue from the eucaryotic pathway for synthesis of DGG species with 18 carbon fatty acids in position 2, which involves microsomal PC, to the procaryotic pathway for synthesis of DGG species with 16 carbon fatty acids in position 2, which is localized inside the chloroplast. Further indication for an increased participation of this pathway was the relative increase in labeling of saturated fatty acids in DGG in the presence of BASF 13-338 (Table II).

The high level of radioactivity of [1-^{14}C]acetate incorporated into PC of pea, linseed and wheat leaf segments, as compared with that in DGG (Table IV), reflects the predominance of the eucaryotic pathway in these species. The relatively low percentage of the DGG fatty acid radioactivity recovered in saturated fatty acids did not increase in the presence of BASF 13-338, in contrast with the spinach leaf discs.

Inhibition of desaturation of linoleic acid by BASF 13-338 within 15 min makes it unlikely that changes in fatty acid composition would be the consequence of impaired lamellar assembly (1). The lack of inhibition of linoleic acid desaturation in isolated chloroplasts by 80 μM BASF 13-338 while 100 μM of the herbicide caused almost immediate interruption of photosynthesis suggests that these effects of the substituted pyridazinone are independent. Active desaturation of linoleate in the dark (Table I) is further evidence that inhibition of the desaturase is not a secondary effect of the interruption of photosynthesis as suggested by Porter and Bartels (12), but a direct inhibition of the desaturase by the herbicide (8). The immediate effect of the herbicide on photosynthesis by isolated chloroplasts shows that BASF 13-338 entered the organelles and reached the site of action (8), in disagreement with Ridley and Ridley (13). The inhibition of linoleic acid desaturation in discs, but not in isolated chloroplasts, suggests that for this action the herbicide has to be modified in the cytoplasm

Table IV. *Effect of BASF 13-338 on the Incorporation of [1-^{14}C]Acetate into the Fatty Acids of DGG and PC in Leaf Segments of Pea, Wheat, and Linseed*

Leaf slices were incubated with [1-^{14}C]acetate (0.2 mM, 1.5 μCi) for 2 h. Means of four samples in two experiments ± SD.

Lipid Class	Plant Species	0.2 mM BASF 13-338	Total Lipid ^{14}C	^{14}C in Class			
				Sat	Mono	Di	Tri
				%			
DGG	Pea	−	13.0 ± 1.1	7.0 ± 1.0	12.9 ± 0.6	33.2 ± 1.5	47.0 ± 0.2
		+	10.8 ± 0.8	6.3 ± 1.3	11.1 ± 0.4	68.8 ± 2.3	13.9 ± 1.4
	Wheat	−	8.3 ± 0.9	34.0 ± 5.7	32.0 ± 3.0	21.6 ± 4.0	12.4 ± 4.6
		+	7.5 ± 0.2	38.6 ± 6.7	26.6 ± 1.4	29.5 ± 3.4	5.4 ± 3.3
	Linseed	−	21.6 ± 3.2	21.6 ± 3.2	26.5 ± 3.4	28.8 ± 1.3	23.1 ± 2.0
		+	9.1 ± 0.6	21.3 ± 4.5	28.0 ± 4.5	39.4 ± 4.2	11.4 ± 4.3
PC	Pea	−	29.7 ± 0.8	16.1 ± 0.7	26.3 ± 1.8	55.1 ± 1.1	2.8 ± 0.3
		+	27.8 ± 0.5	13.8 ± 1.0	25.2 ± 0.6	59.0 ± 1.3	2.0 ± 0.5
	Wheat	−	29.8 ± 0.9	9.2 ± 0.5	58.9 ± 2.5	29.0 ± 2.7	3.0 ± 0.3
		+	23.1 ± 1.0	9.6 ± 0.8	51.1 ± 2.5	36.4 ± 1.5	2.9 ± 0.5
	Linseed	−	27.4 ± 0.4	26.8 ± 0.4	34.9 ± 1.4	36.8 ± 1.0	1.4 ± 0.2
		+	21.6 ± 0.4	23.2 ± 0.7	39.5 ± 1.9	36.1 ± 1.6	1.2 ± 0.5

before entering the organelle.

The experiments with leaf segments of pea, linseed and wheat showed that linoleate desaturase was sensitive to the herbicide in all three species. The tolerance of linoleate desaturase in seedlings of linseed, pea and spinach for BASF 13-338, also observed by Murphy *et al.* (11), as well as in spinach leaves fed via the cut petiole, might be explained by either reduced translocation of the herbicide to the site of action (23) or by metabolism of the herbicide to less toxic products (10).

The 2 mm discs incorporated twice as much radioactivity into DGG as into PC (Table II). This was the opposite of the situation in attached spinach leaves fed by petiole uptake (18). In fact the leaf discs behaved rather like isolated chloroplasts (17), which may be related to the accumulation of photosynthetic intermediates, *e.g.* glycerol 3-P. This behavior of the discs was further illustrated by the results of the chase experiment showing desaturation of palmitate and oleate within DGG and no transfer of ^{14}C from PC to DGG, and by the fact that only those molecular species of DGG containing 16 carbon acyl chains at position 2 incorporated [1-^{14}C]acetate.

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