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THE GLYCEROLIPID COMPOSITION OF LEAVES

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Abstract—Leaf lipids were separated by a combination of DEAE-cellulose column and thin-layer chromatography. Estimations of the separated glycerolipids were performed using the most recent techniques for phospho- and glycolipid analyses which obviate the necessity to elute the lipid from the adsorbent. Twenty photosynthetic tissues ranging from the unicellular green alga *Mesotaenium caldariorum* to the leaves of higher plants were examined as was parsnip root cortex.

INTRODUCTION

FEW QUANTITATIVE analyses of the glycerolipid components of plant tissues or subcellular fractions of plants have been reported in the literature. In 1966, James and Nichols[1] noted the occurrence of particular lipids in a variety of photosynthetic organisms but no quantitative figures for relative percentage compositions were given. Mudd,[2] in a recent review on plant lipid metabolism, commented on the paucity of such quantitative data. In fact, only three investigations designed to provide reliable figures for the concentrations of the total glycerolipids in leaves, algae or subcellular fractions are available.[3–5]

This paper reports the results of quantitative analyses of the major lipids in photosynthetic tissues of a variety of plants. Recently developed methods were used for their separation and estimation.

RESULTS AND DISCUSSION

The results of the analyses are presented in terms of both fresh weight (Table 1) and chlorophyll content (Table 2) of the tissue; the plants are listed in phylogenetic sequence according to Hutchinson.[6]

These results extend the range of species for which quantitative glycerolipid values are available, from four (*Chlorella*,[4] beet, bean and elder leaves[3]) to twenty-four and give the first results for root tissue. Although there are considerable species differences in the amounts of the individual lipids present, the average values (in μmole/g) agree reasonably well with those values calculated from the results of Wintermans[3] (Table 3). Variability between species was less pronounced when the amounts of individual components were calculated relative to chlorophyll (Table 2) instead of fresh weight of tissue (Table 1). The excessively high values for *Camellia* are due to the very low content of chlorophyll in young leaves.

[1] A. T. JAMES and B. W. NICHOLS, *Nature* **210**, 372 (1966).
[2] J. B. MUDD, *Ann. Rev. Plant Physiol.* **18**, 229 (1967).
[3] J. F. M. G. WINTERMANS, *Biochim. Biophys. Acta* **44**, 49 (1960).
[4] R. A. FERRARI and A. A. BENSON, *Arch. Biochem.* **93**, 185 (1961).
[5] C. F. ALLEN, P. GOOD, H. F. DAVIS, P. CHISUM and S. D. FOWLER, *J. Am. Oil. Chem. Soc.* **43**, 223 (1966).
[6] J. HUTCHINSON, *The Genera of Flowering Plants*, Vol. 1, p. 9, Oxford University Press, London (1964).

TABLE 1. GLYCEROLIPID COMPOSITION OF PLANT TISSUES I

Plant	μMoles of lipid per g fresh weight of leaf							
	MGD	DGD	SL	PC	PG	PE	PI	DPG
Mesotaenium caldariorum	10·00	5·50	1·30	1·10	1·67	0·80	0·80	Tr.
Marchantia berteroana	1·07	0·66	0·19	0·18	0·16	0·09	0·07	0·16
Moss (see text)	2·68	1·50	0·48	1·26	0·40	0·40	0·15	0·05
Blechnum fluviatile	5·60	2·30	0·38	0·80	0·60	0·40	0·13	0·35
Ginkgo biloba	4·70	2·80	0·30	1·80	0·85	0·55	0·25	0·27
Pinus radiata	2·80	1·95	0·52	0·81	0·55	0·28	0·37	0·29
Rose (*Rosa cv*)	5·60	4·60	0·54	1·50	0·60	0·55	0·27	0·20
Rowan (*Sorbus aucuparia*)	10·20	7·16	0·46	2·20	0·97	1·70	0·37	0·30
White clover (*Trifolium repens*)	8·60	5·20	0·76	1·41	1·13	0·87	0·25	0·28
Lucerne (*Medicago sativa*)	8·60	5·20	1·72	0·75	0·67	0·50	0·28	0·58
Poplar (*Populus italica*)	4·95	3·80	0·62	1·80	1·10	0·80	0·50	0·35
Camellia japonica	3·10	3·10	0·23	2·00	0·65	0·90	0·20	0·90
Squash (*Cucurbita pepo*)	4·10	2·70	0·30	1·60	0·91	1·00	0·14	0·09
Tomato (*Solanum lycopersicum*)	5·08	2·46	0·31	1·10	0·43	0·45	0·10	0·10
Lettuce (*Lactuca sativa*)	0·68	0·68	0·03	0·31	0·10	0·21	0·06	0·13
Xanthium orientale	6·10	5·90	0·62	0·50	0·25	0·15	0·05	0·04
Cocksfoot (*Dactylis glomerata*)	8·00	5·10	0·62	1·10	1·10	0·80	0·10	0·28
Perennial ryegrass (*Lolium perenne*)	5·10	3·95	0·95	1·35	0·75	0·55	0·20	0·20
Paspalum dilatatum	6·00	3·60	0·62	0·38	0·48	0·18	0·10	0·10
Maize (*Zea mays*)	3·10	2·30	0·35	0·45	0·48	0·24	0·12	0·24
Range	0·68–10·20	0·68–7·16	0·03–1·72	0·18–1·00	0·10–1·67	0·09–1·70	0·05–0·80	<0·01–0·90
Parsnip root (*Pastinaca sativa*)	0·17	0·34	Tr.	0·33	0·07	0·18	0·11	0·19

These results are the means of duplicate and in some cases triplicate and quadruplicate analyses of a single extract. For PE and PI, duplicates that agreed to within 10 per cent were considered acceptable but for the other components only those duplicates that agreed to within 5 per cent were taken.

Key: PC, PE, PI, PG = phosphatidyl-choline, -ethanolamine, -inositol and -glycerol, respectively; MGD, DGD = mono- and di-galactosyl glycerides; SL = sulpholipid; DPG = diphosphatidyl glycerol.

TABLE 2. GLYCEROLIPID COMPOSITION OF PLANT TISSUES II

Plant	μMoles of lipid per mg of total chlorophyll							
	MGD	DGD	SL	PC	PG	PE	PI	DPG
Mesotaenium	6·25	3·42	0·81	0·69	1·04	0·50	0·50	Tr.
Marchantia	3·25	1·90	0·53	0·50	0·45	0·25	0·19	0·45
Moss	2·60	1·46	0·47	1·22	0·39	0·39	0·14	0·05
Blechnum	3·08	1·26	0·21	0·44	0·33	0·22	0·07	0·19
Ginkgo	2·90	1·70	0·19	1·12	0·53	0·34	0·16	0·17
Pinus	5·10	3·50	0·95	1·47	1·00	0·51	0·67	0·53
Rose	3·97	3·26	0·38	1·06	0·43	0·39	0·19	0·14
Rowan	3·95	2·82	0·18	0·86	0·38	0·67	0·15	0·11
White clover	3·98	2·40	0·35	0·66	0·52	0·40	0·12	0·13
Lucerne	4·30	2·60	0·86	0·38	0·33	0·25	0·14	0·28
Poplar	4·20	3·30	0·53	1·55	0·94	0·68	0·43	0·30
Camellia	6·74	6·74	0·50	4·44	1·95	1·41	0·44	1·95
Squash	4·10	2·70	0·30	1·60	0·90	1·00	0·14	0·05
Tomato	5·08	2·46	0·31	1·10	0·43	0·45	0·10	0·10
Lettuce	2·76	2·76	0·12	1·48	0·40	0·96	0·24	0·52
Xanthium	3·80	3·70	0·39	0·31	0·16	0·09	0·03	0·03
Cocksfoot	3·30	2·10	0·24	0·46	0·46	0·33	0·02	0·11
Perennial ryegrass	3·08	2·35	0·58	0·81	0·44	0·32	0·12	0·10
Paspalum	4·46	2·70	0·46	0·28	0·36	0·13	0·08	0·13
Maize	2·70	2·05	0·31	0·40	0·43	0·22	0·11	0·22
Range	2·60–6·74	1·26–6·74	0·12–0·95	0·28–4·44	0·16–1·95	0·09–1·41	0·02–0·44	<0·01–1·95

Variations between species might be less if the chloroplast lipids were considered separately. The molar ratios of these lipids (monogalactosyl diglyceride, digalactosyl diglyceride,

TABLE 3. COMPARISON OF RESULTS WITH THOSE OF WINTERMANS[3]

	μMole lipid per g fresh weight of leaf							
	MGD	DGD	SL	PC	PG	PE	PI	DPG
Present study*	5·30	3·57	0·57	1·12	0·69	0·57	0·22	0·25
Wintermans†	3·97	1·79	0·53	1·80	1·18	0·73	0·38	—

* Averages of twenty species.
† Averages of three species: elder, beet and bean (8 hr seedlings).

TABLE 4. MOLAR RATIOS OF "CHLOROPLAST" LIPIDS (PG = 1·0)

	MGD	DGD	SL	PG
Mesotaenium	6·00	3·30	0·78	1·0
Marchantia	6·70	4·12	1·19	1·0
Moss	6·70	3·75	1·20	1·0
Blechnum	9·25	3·83	0·63	1·0
Ginkgo	5·54	3·29	0·35	1·0
Pinus	5·10	3·54	0·94	1·0
Rose	9·35	7·65	0·90	1·0
Rowan	9·50	7·00	0·45	1·0
White clover	7·60	4·60	0·67	1·0
Lucerne	12·90	7·80	2·58	1·0
Poplar	4·50	3·16	0·56	1·0
Camellia	4·77	4·77	0·35	1·0
Squash	4·50	2·96	0·33	1·0
Tomato	11·80	5·72	0·72	1·0
Lettuce	6·80	6·80	0·30	1·0
Xanthium	24·10	23·60	3·28	1·0
Cocksfoot	7·28	4·64	0·56	1·0
Perennial ryegrass	6·80	5·28	1·28	1·0
Paspalum	12·50	7·50	1·29	1·0
Maize	6·35	4·80	0·73	1·0
Means ± Standard deviation	8·40 ± 4·23	5·90 ± 4·67	0·95 ± 0·80	
Xanthium omitted from analysis				
Means ± Standard deviation	7·05 ± 4·14	4·97 ± 2·85	0·83 ± 0·57	

phosphatidyl glycerol and sulpholipid) were recalculated based on phosphatidyl glycerol ≡ 1·0. The results of this recalculation (Table 4) show that although these components may be the only lipids of chloroplasts[1] (but see Ref. 5), these organelles show quite different molar ratios of the four compounds. The large standard deviations are due mainly to the results

for *Xanthium* which had a very low level of phosphatidyl glycerol, but even when *Xanthium* was omitted from the analysis the standard deviation was still relatively high (e.g. MGD, 7·05 ± 4·14; DGD, 4·97 ± 2·85; and SL, 0·83 ± 0·57). The ratio of monogalactosyl diglyceride/digalactosyl diglyceride varied from 2·4 in *Blechnum* to 1·0 in *Camellia* and lettuce but digalactosyl diglyceride did not exceed monogalactosyl diglyceride in concentration in any leaf sample.

Since these leaves were performing essentially the same functions under very similar conditions, the large, between-species variations in the concentrations of glycerolipids relative to chlorophyll were unexpected. This is particularly true of the lipids considered to be confined to chloroplasts. Even when *Camellia* is omitted from consideration (because of the relatively low chlorophyll content of the sample taken), only the galactolipids show a reasonably constant distribution varying by approximately 50 per cent from the average over the range of species examined. Both of the remaining chloroplast glycerolipids, SL and PG, showed variations around the mean value of more than 100 per cent. "Extrachloroplast" lipids also showed high variations but this might be expected with chlorophyll content as the basis for comparison since variable amounts of non-photosynthetic tissue (i.e. vascular tissue) would be encountered in different leaves. The most variable of the glycerolipids was DPG, which was detectable in *Mesotaenium* (cultured under continuous light) only by radioisotope techniques and yet was a major phospholipid (by weight) of *Camellia* leaves. It is not impossible that these variations portent seasonal or diurnal variations in the concentrations of these components in leaves, especially since analyses of red clover leaves (Roughan, unpublished results) have suggested sulpholipid levels in the early morning may be reduced three-fold compared with later in the day.

Glycerolipids other than those listed in Table 1 may have been present in some of the extracts and were either not detected by the light iodine-staining technique or appeared in only trace amounts. Typical of such constituents would be phosphatidyl serine which has been detected in sweet clover leaves.[7] *Lyso*phosphatidyl choline was found in significant amounts only in *Paspalum* and maize (0·14 and 0·17 μmole/g respectively) and two unknown lipids, together with triglyceride, were found in high concentrations in *Mesotaenium*. From their charring reactions[8] compared with known glyco- and phospholipids, the unknown lipids were estimated at 2·4 and 0·9 μmole/g and, from its glycerol content,[9] the triglyceride was estimated at 2·3 μmole/g. The structures of the unknown substances are being investigated.

The combination of DEAE-cellulose column and thin-layer chromatography provides a convenient procedure for the separation of the major glycerolipids of plants. This, together with recent methods developed for the quantitative estimation of phospholipids[10] and glycolipids[11] which obviate the necessity to separate these compounds from thin-layer adsorbent, gives a reproducible and rapid analytical procedure for the quantitative fractionation and estimation of plant lipids.

Methods used by other investigators have presented difficulties either in poor recoveries or complexities of the analytical procedure. Wintermans'[3] separation of deacylated lipids by paper chromatography followed by spot elution gave poor recoveries of the phospholipids (60–90 per cent). Paper elution can also complicate glycolipid estimations through the

[7] A. A. Benson, J. F. M. G. Wintermans and R. Wiser, *Plant Physiol.* **34**, 315 (1959).
[8] J. B. Marsh and D. B. Weinstein, *J. Lipid Res.* **7**, 574 (1966).
[9] O. Renkonen, *Biochim. Biophys. Acta* **50**, 367 (1962).
[10] G. Rouser, A. N. Siakatos and S. Fleischer, *Lipids* **1**, 85 (1966).
[11] P. G. Roughan and R. D. Batt, *Anal. Biochem.* **22**, 74 (1968).

presence of cellulose fibres.[12] For determinations in *Chlorella*, Ferrari and Benson[4] grew the organism in the presence of $^{14}CO_2$ to give uniformly labelled lipids which were deacylated and separated by paper chromatography. Phosphoryl compounds were estimated by neutron activation and the concentrations of glycosyl compounds were calculated from ^{14}C contents compared with the values in known phosphoryl compounds. Although accurate, this procedure is unnecessarily complicated for routine estimations of leaf lipids. In addition, reservations have been reported by Marinetti[13] concerning the use of deacylation techniques for lipid analyses. For the estimation of glycerolipids in spinach lamellae, Allen *et al.*[5] calculated molar concentrations from the weights of fractions recovered from DEAE-cellulose columns. Although this method could be expected to be satisfactory for the major components (e.g. monogalactosyl diglyceride and digalactosyl diglyceride), other fractions of only 3 or 4 mg weight would be difficult to estimate accurately, especially in the absence of special precautions to avoid the hygroscopic absorption of water. The elution of ammonium acetate with the acidic lipid fraction would present an additional problem with this gravimetric procedure which could probably not be used for whole leaf lipid.

"Chloroplast" glycerolipids have been detected in potato tuber[14] and their presence may be inferred in other roots (e.g. carrot) which also have the ability to become green in the light. Parsnip root, however, does not develop chloroplasts and yet has relatively high concentrations of these lipids. It seems likely that these lipids are localized in the proplastids of root tissue although these organelles do not contain the prolamellar bodies typical of chloroplast precursors in etiolated leaves. Instead, root proplastids are apparently involved with the synthesis of storage starch and, in potato tuber and mature carrot root tissue, can be recognized only as a thin membrane surrounding large starch grains.[15] A similar situation is likely to exist in mature parsnip root cells. It has been suggested that the galactolipids of chloroplasts may be involved in sugar transport across the chloroplast membrane[16] and these lipids could play a similar role in the starch-storing plastids of root tissue.

EXPERIMENTAL

The youngest, fully expanded leaves from a number of plants were sampled in the mid-afternoon in the spring and early summer of 1967–1968. In the cases of ryegrass and cocksfoot, flag leaves were taken from plants in which the inflorescence was just emerging from its sheath. No attempt was made to dissect out the vascular tissue but as little petiole as possible was included in the samples. With compound leaves only leaflets were taken. Pine needles, 5–6 cm in length, were gathered from the base of new shoots of about 18 cm in length. The liverwort *Marchantia* was grown in a mist house under natural daylength. As much of the rhyzoid material as could be was removed from the thalli before weighing and extracting. The green alga *Mesotaenium caldariorum* was grown in liquid culture at 18° under continuous illumination from fluorescent tubes. This organism was harvested in the middle of the log phase of growth and the results given in terms of fresh weight are based on the assumption that 1 ml of packed cells weighed 1 g. The moss was a mixture of equal amounts of *Furoria* and *Leptobryum pyriforme*. Parsnip root was washed free of soil before samples were cut from the cortex for weighing and extracting.

Samples were extracted into chloroform/methanol within 5 min of harvest and extracts were washed against dilute NaCl.[11] An aliquot of the washed lipid extract in chloroform was separated into neutral plus dipolar lipids (fraction A) and acidic lipids (fraction B) by DEAE-cellulose column (3 × 1 cm i.d.) chromatography[11] and individual lipid species isolated by TLC. Phospholipids were determined in the presence of adsorbent by the method of Rouser *et al.*[10] and glycolipids were measured, also in the presence of adsorbent,

[12] J. F. M. G. WINTERMANS, *Coll. Int. Centre Nat. Recherche Sci.* (*Paris*) **119**, 381 (1962).
[13] G. V. MARINETTI, *J. Lipid Res.* **3**, 1 (1962).
[14] M. LAPAGE, *J. Chromatog.* **13**, 99 (1964).
[15] S. TOYAMA, *Sci. Rep. Tokyo Kyoiku Diagaku* **12**, 245 (1967).
[16] A. A. BENSON, in *Proceedings of the Fifth Congress of Biochemistry*, Vol. 6, p. 340, Pergamon Press, Oxford (1963).

by the method of Roughan and Batt.[11] In preliminary experiments it was found that some leaves (e.g. lettuce) had a relatively low ratio of lipid to fresh weight and, as chlorophyll content was probably a more reliable guide to the total lipid content of a leaf, the amount of an extract required for DEAE-cellulose chromatography was judged more on the basis of chlorophyll than of fresh weight as had previously been done.[11] For instance, an amount of extract containing 5 mg of chlorophyll (equivalent to 2·5 g of white clover leaflets, 5 g of squash leaves or 20 g of lettuce leaves) was within the capacity of the 3 × 1 cm (i.d.) DEAE-cellulose column and an amount of fraction A containing 100 to 150 μg of chlorophyll was as much as could be applied as a 2 cm streak[17] to a thin layer of Silica Gel HR (Merck.) without overloading the chromatogram. Silica Gel G (Merck.) was capable of taking a greater load but gave higher blanks in the glycolipid analysis. This quantity of fraction A (i.e. equivalent to 100–150 μg of chlorophyll) normally contained monogalactosyl diglyceride and digalactosyl diglyceride in concentrations that were just within the upper limit of the phenol/H_2SO_4 assay. However, it was necessary to take aliquots of this size so that sufficient phosphatidylethanolamine and phosphatidylcholine could be isolated for the simultaneous analysis of phospho- and glycolipids. For some leaves (*Xanthium*, *Paspalum*) the levels of phosphatidylethanolamine and phosphatidylcholine relative to the galactolipids were so low that a simultaneous analysis could be performed only by reverting to an "indirect" method for galactolipid analysis. In these cases, amounts of fraction A equivalent to 150–200 mg of fresh leaf were streaked across 4 cm of a thin layer. After chromatography, the galactolipid zones were scraped into straight-sided centrifuge tubes, as for the "direct" method,[11] 2–4 ml of 2 N H_2SO_4 added and the loosely stoppered tubes placed in a boiling bath for 60 min. During this period the contents of the tubes were gently mixed three or four times. After cooling and centrifuging, an aliquot of the clear hydrolysate (0·6 ml) was added to 5% phenol (0·4 ml), followed by concentrated H_2SO_4 (4 ml). Adsorbent blanks and standards were taken through the whole procedure and absorbances were measured at 480 nm. In general, amounts of the acidic lipid fraction (B), in which phosphatidyl inositol was the lower limiting factor, equivalent to four times the quantity of fraction A (in terms of fresh weight of leaf) were required for the accurate analyses of the lipids in this fraction.

The solvent used for TLC was chloroform/methanol/acetic acid/water (85:15:10:3),[18] in which phosphatidic acid, if present, would be expected to co-chromatograph with diphosphatidylglycerol. In many cases, therefore, and in all those samples which gave a relatively high value for diphosphatidylglycerol, a second thin-layer separation was carried out using chloroform/methanol/ammonia (65:25:2). With this solvent mixture, phosphatidic acid remained at or near the origin[18] while diphosphatidylglycerol was still the most mobile of the phospholipids in this fraction. In no case where this check was performed was phosphatidic acid detected.

Total chlorophyll was estimated in methanol solutions of the total lipids, using the spectrophotometric factors of Comar and Zscheile.[19]

[17] P. G. Roughan and C. G. Tunnicliffe, *J. Lipid Res.* **8**, 511 (1967).
[18] B. W. Nichols in *New Biochemical Separations* (Edited by A. T. James and L. J. Morris) p. 321, Van Nostrand., London, (1964).
[19] C. L. Comar and P. F. Zscheile, *Plant Physiol.* **17**, 198 (1942).