CONCENTRATIONS OF PHOSPHATIDES AND GLYCOLIPIDS IN LEAVES AND CHLOROPLASTS

J. F. G. M. WINTERMANS

Laboratory of Plant Physiological Research, Agricultural University, Wageningen (Netherlands) *

(Received March 5th, 1960)

SUMMARY

Deacylated leaf and chloroplast lipids were separated by twodimensional chromatography. Quantitative determinations were made of the 4 major phosphate diesters, and of the 3 major carbohydrate–glycerol compounds. From the comparison of whole leaves and chloroplasts, and similarly from the comparison of green and yellow leaves, it was concluded that the green chloroplasts are particularly enriched in phosphatidylglycerol, and in mono- and digalactosyl lipids, the latter compounds occurring in concentrations approaching or exceeding 10^{-2} *M*. Lecithin, cephalin, phosphatidylinositol, and the plant sulfolipid were not specifically linked to the photosynthetic apparatus.

INTRODUCTION

After the recognition of phospholipids and glycolipids as important constituents of the photosynthetic apparatus[1–3], it became of interest to further establish the quantities involved by applying techniques other than those using radioactive tracers or neutron activation. This was achieved by a slight modification of the technique used by BENSON *et al.*[3], and the results confirm the particularly close association of phosphatidyl glycerol and of the galactolipids with the photosynthetic apparatus.

MATERIALS AND METHODS

Leaves and chloroplasts from spinach, *Spinacia oleracea* and sugarbeet, *Beta vulgaris* were used. Spinach chloroplasts were isolated in 0.4 *M* sucrose, 0.01 *M* NaCl, 0.05 *M* Tris pH 7.8 (see ref. 4); beet chloroplasts were isolated in 0.35 *M* NaCl, 0.001 *M* $MgSO_4$, 0.067 *M* Tris pH 7.4 (see ref. 5).

Green and yellow leaves of two other species were compared: elder, *Sambucus nigra*, and bean, *Phaseolus vulgaris*. From the first species normal green plants were available and an *aurea* form, in which the chlorophyll is abnormally sensitive to light.

Abbreviations: GP, glycerophosphate; GPC, glycerophosphoryl choline; GPE, glycerophosphoryl ethanolamine; GPG, glycerophosphoryl glycerol; GPI, glycerophosphoryl inositol; GPS, glycerophosphoryl serine; GPGPG, 1,3-diglycerophosphoryl glycerol; G-gal, monogalactosyl glycerol; G-gal-gal, digalactosyl glycerol; G-gal-SO_3, glyceryl galactoside sulfonic acid.

* 201st communication; 74th communication on photosynthesis.

In this variety especially the outer leaves, which are exposed to full sunlight are very poor in chlorophyll, survival probably depending on photosynthesis by the less exposed shade leaves. Microscopic examination shows that the yellow leaves have reduced palissade parenchyma, and few chloroplasts. It can be assumed that the photosynthetic apparatus in these leaves is damaged by excess of light.

In the experiments with bean, seedlings were used which had been grown in red light at 3 different daylengths, viz. 0.5, 2 and 8 h daily. The shortest illumination induced very little chlorophyll formation, but allowed the plants to reach normal shape; especially stem length and leaf size were normal, in contrast to fully dark grown plants. The plants grown at the short daylengths can be assumed to be poor in chlorophyll owing to lack of light.

Leaves separated from petioles, or packed chloroplasts were killed in boiling 95 % ethanol and further extracted with boiling ethanol–toluene mixtures, until colourless. An aliquot from the combined supernates was diluted with ethanol for the estimation of chlorophyll, using the extinction coefficients given by HEIERLE[6]. The bulk of the extract was concentrated *in vacuo*, lipids were taken up in chloroform, and thoroughly washed with water. The lipid phase was dried, and taken up in methanol–toluene. An aliquot was used for the determination of total lipid P, the remainder was hydrolysed by the addition of an equal volume of methanol–0.2 *N* KOH, and incubation at 37° for 15 min. Water and chloroform were added to the hydrolyzate, and the supernatant, containing the water soluble deacylated lipids was neutralized with Amberlite IR-120, H-form. Since pigments and fatty acids still are present in the supernate at this stage, the latter is again thoroughly washed with chloroform. Emulsions can be broken by centrifugation, or by the addition of ethanol. In the latter case, practically colourless solutions can be obtained, but some P stays in the chloroform phase. This removal of fatty material was desirable, since, in its presence, a very low limit is set upon the amounts of substance that can be separated by paper chromatography.

The water phase is dried *in vacuo*, taken up in 50 % ethanol, and applied to the origin of paper chromatograms. Whatman No. 4 paper was used throughout, and

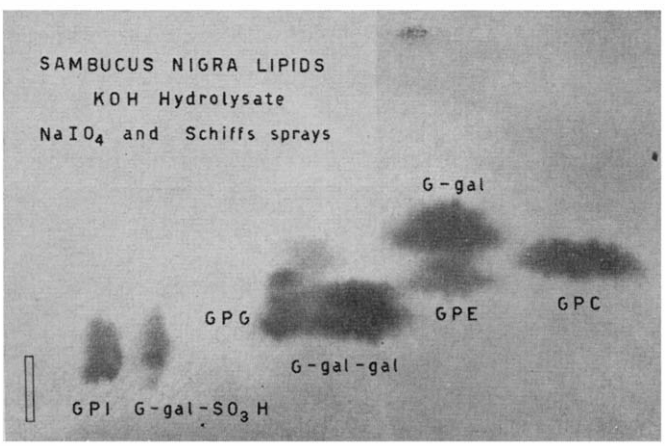

Fig. 1. Paper chromatogram of deacylated lipids of *Sambucus nigra cv. aurea*. Development with phenol–water, left to right, and with butanol–propionic acid–water upwards.

solvents used were phenol–water (3:1), and butanol–propionic acid–water (14:7:10, v/v). Up to 150 μg P, equivalent to about 1.5 g of leaf, or about 0.7 ml of packed chloroplasts can be conveniently separated in this way.

Phosphates were detected with molybdate reagent[7], followed by 10 min heating at 70° and u.v. irradiation. Traces of phenol are particularly interfering, and 16–24 h drying after the phenol run is therefore recommended.

The deacylation products of the galactolipids, as well as those of the phosphatides can also be detected by periodate-Schiff reagent[8]. A typical chromatogram obtained in this way, is shown in Fig. 1. For the identification of the P containing spots, see ref. 1; for the identification of the carbohydrate containing spots, see ref. 3. For quantitative P determinations, the spots or corresponding areas of duplicate chromatograms were eluted. Total P was determined after destruction of the eluate with sulfuric and nitric acids. Recovery usually was from 60–90 % of the amount applied to the origin. The galactose containing spots were always eluted with water from the duplicate chromatogram. 1-ml aliquots were taken for total carbohydrate determination using the anthrone method[9].

EXPERIMENTAL RESULTS AND DISCUSSION

The averaged results of these observations are given in Table I. From the ratio of chlorophyll concentrations in leaf and chloroplasts an estimate of the volume percent of chloroplasts in the leaves§ can be obtained. Hence, the amounts of chloroplast phosphatides and glycolipids per unit volume of leaves can be calculated, and com-

TABLE I

PLANT PHOSPHOLIPIDS, GLYCOLIPIDS, AND CHLOROPHYLL

Concentrations in (moles/l) × 10^4

Source	*P-lipids*						*Chlorophyll*	*Glycolipids*		
	GPI	*GPG*	*GPE*	*GPC*	*GP*	*Total*		*G-gal-SO₃H*	*G-gal-gal*	*G-gal*
Spinach, leaves	2	6	4	11	0.5	23	11			
Spinach, chloroplasts	6	24	3	19	1	53	105			
Beet, leaves	5	9	11	22	0	47	14	9	13	24
Beet, chloroplasts	6	23	9	24	7*	69	72	15	68	121
Elder, green	3	17	5	19	0	44	28	5	26	56
Elder, yellow	4	7	5	18	0	33	2.5	4	11	17
Bean seedlings						Unknowns				
0.5 h light daily	3.5	5.5	6	15	5	35	1	2	6	10
2 h light daily	3.5	7	6.5	11.5	5	33.5	6	4	9	13
8 h light daily	3.5	9.5	6	13	3	35	27	2	14	39

* Since GP was observed in the chloroplasts, but not in the whole leaves, it must be an artifact, arising during the preparation of the chloroplasts. See for chloroplast lecithinase C: KATES[21]. In the data of Table II, a correction has been made for this partial hydrolysis. This does not, however, substantially influence the results and the conclusions.

§ Leaf volume is estimated from fresh weight, assuming an average specific weight = 1.

TABLE II

PHOSPHOLIPIDS AND GLYCOLIPIDS:
AMOUNTS PER UNIT VOLUME OF FRESH LEAVES, EXPRESSED AS (MOLES/l) × 10^4

	GPI	*GPG*	*GPE*	*GPC*	*Total P*	*G-gal-SO_3H*	*G-gal-gal*	*G-gal*
Spinach, total leaf:	2.45	5.2	2.8	11.05	21.5			
(1) chloroplasts (10% of leaf volume)	0.75	2.6	0.1	2.2	5.65			
(2) cytoplasm (by difference)	1.7	2.6	2.7	8.85	15.85			
Percent in chloroplasts:	30	50	3	20	26			
Beet, total leaf:	4.8	8.7	10.8	22.5	47	9	13	24
(1) chloroplasts (20% of leaf volume)	1.3	5.2	2.1	5.2	14	3	13.5	24
(2) cytoplasm (by difference)	3.5	3.5	8.7	17.3	33	6	0	0
Percent in chloroplasts:	27	60	20	23	30	33	100	100

pared with total cellular phosphatides, and glycolipids. The actual concentrations in cytoplasm cannot be estimated directly, without reliable data on the volume of the vacuoles.

The averaged results from those experiments in which the leaf- and chloroplast lipids were analysed from a single batch of material are given in Table II. The distribution of phosphatide-P among the 4 major fractions, in chloroplasts and cytoplasm, is visible in Table III.

TABLE III

PHOSPHATIDE COMPOSITION OF CHLOROPLASTS AND CYTOPLASM

Source	% *phosphatidyl ester*			
	GPI	*GPG*	*GPE*	*GPC*
Spinach: chloroplasts	13	46	2	39
cytoplasm	11	16	17	56
Beet: chloroplasts	9	38	15	38
cytoplasm	11	11	26	52

Phosphatides

The high percentage of GPG-lipid in the chloroplasts is again evident from comparison of the concentrations in leaf *vs.* chloroplasts in the two species *Spinacia* and *Beta*.

The data of Table II show that the chloroplasts are not particularly rich in phosphatides. For spinach leaves MENKE AND JAKOB[10] reported that the chloroplasts contain about 35 % of total leaf protein, and about 18 % of the dry weight. Taking into consideration that the cell walls constitute an important fraction of the dry weight —26.5 % for spinach leaves[10]—, we calculate that the chloroplasts contain about 25 %

of the cell contents and we must conclude that the ratios of phosphatide : protein or phosphatide : dry weight of cell contents, for chloroplasts and other cell contents are not significantly different. (On the other hand, it is well proved that the chloroplasts are particularly rich in lipoid matter (see ref. 11 p. 372).) The composition of the phosphatides is quite different, however, in chloroplasts and other cell contents, as appears from Table III. Chloroplasts are particularly rich in GPG-lipid, as was concluded already from earlier experiments[3]. The remainder of the cell has a much higher percentage of the classical, N-containing phosphatides, lecithin and cephalin.

In the experiments with green *vs.* yellow leaves, the same trend is observed, both in the damaged leaves of *Sambucus* and in the immature leaves of *Phaseolus*. In *Sambucus*, the total phosphatide-P is significantly lower in the yellow leaves; this difference is entirely due to the lack of phosphatidyl glycerol. With the beans of different degrees of greening, the only important difference is also in the GPG-lipid.

Some unknown compounds have a certain importance in *Phaseolus*. Judging from the R_F values, among the observed spots may be GPS and GPGPG, the deacylation products of, respectively, phosphatidyl serine, and the recently discovered diphosphatidyl glycerol[12]. Unknown spots, similar to those seen in the *Phaseolus* experiments have also been observed with *Xanthium* leaves and with *Lemna*.

From theoretical considerations regarding the structure of the chloroplasts, importance has been attached to the molar ratio of chlorophyll : phosphatides; see for a discussion ref. 11, p. 391 and p. 1742. From data by GODNEV *et al.*[13], a molar ratio can be calculated: 5.0 for rye, and 2.2 for lettuce. From the present experiments the ratio for spinach chloroplasts is calculated: 2.0, and for beet chloroplasts: 1.05. Incidentally, the latter value closely approaches the "theoretical" value, postulated by HUBERT's theory[14] (see also ref. 11).

Glycolipids and sulfolipid

Since analyses of glycolipids and sulfolipid are still scarce, it seems worthwile to give some data regarding quantities involved.

The method of determination used, has been reported to be equally sensitive for mono-, di- and polysaccharides[9,15]. Hence, it can be assumed that acid-labile linkages do not interfere, and the measured extinctions have been evaluated by comparison with a galactose standard. However, the carbohydrate-sulfonate linkage in the sulfolipid is acid-stable[16], and the results regarding the G-gal-SO_3 would be more accurate, if an authentic sample had been available. Indeed, the time course of colour development with the anthrone reagent was somewhat slower in this case, than with the galactosyl-glycerols and the galactose standard. By comparison of the relative size and intensity of the spots (see Fig. 1), however, the distribution of sugar over the three compounds investigated seems to agree reasonably with the chemical data of Table I.

For the galactolipids, the concentrations measured indicate that they are major lipids of the green leaves. Moreover, the results with beet leaves and chloroplasts (Table II) show that the galactolipids are found specifically in the chloroplasts, where they are found in concentrations up to, and even exceeding 10^{-2} *M*, in agreement with earlier estimates[3]. In the experiments with *Sambucus* and *Phaseolus*, the concentrations in the green leaves were significantly higher than in the yellow ones. The fact that there are still important amounts of galactolipids, even when chlorophyll concen-

trations are very low, does not prove that these lipids occur in the cytoplasm, since there may be a more or less developed plastid system. This seems supported by the recent observation that one short illumination permitted development of the plastids of dark-grown *Phaseolus vulgaris* to near-normal size, without concomitant formation of chlorophyll[17].

The sulfolipid, in our experiments, seemed less specifically connected with the photosynthetic apparatus. In the experiments with green *vs.* yellow leaves, the concentrations of this compound were not significantly different at different chlorophyll concentrations. In the beet experiments, it appeared that the compound occurred both in the cytoplasm and in the chloroplasts.

In connection with this publication, it is interesting to draw attention to a series of investigations on plant phosphatides by WINTERSTEIN, HIESTAND and coworkers, now over 50 years ago[18,19]. From various plant materials, they isolated lipid fractions which were submitted to acid hydrolysis. Apart from P and N in varying percentages and ratios, important amounts of reducing sugars were found. Maximum reducing power was only liberated after prolonged hydrolysis, from which it was concluded that chemical binding, rather than adsorption or coprecipitation had to be assumed. Galactose was isolated from the hydrolyzate in measurable amounts, and also glucose and pentose were demonstrated. It seems very probable that the galactolipids were involved. The highest % of sugar was found in lipids from wheat flour—the same source from which the galactolipids were isolated by CARTER *et al.*[20].

ACKNOWLEDGEMENTS

The author owes many thanks to Professor A. A. BENSON for his continued interest and illuminating advice. Similarly to Professor E. C. WASSINK for valuable suggestions and stimulating criticism. Finally to Mr. D. PETERS, whose cooperation is herewith sincerely appreciated.

REFERENCES

1 A. A. BENSON AND B. MARUO, *Biochim. Biophys. Acta*, 27 (1958) 189.
2 A. A. BENSON, R. WISER, R. A. FERRARI AND J. A. MILLER, *J. Am. Chem. Soc.*, 80 (1958) 4740.
3 A. A. BENSON, J. F. G. M. WINTERMANS AND R. WISER, *Plant Physiol.*, 34 (1959) 315.
4 A. T. JAGENDORF AND M. AVRON, *J. Biol. Chem.*, 231 (1958) 277.
5 R. HILL AND D. A. WALKER, *Plant Physiol.*, 34 (1959) 240.
6 E. HEIERLE, *Ber. schweiz. botan. Ges.*, 44 (1935) 17.
7 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
8 J. G. BUCHANAN, G. A. DEKKER AND A. G. LANG, *J. Chem. Soc.*, (1950) 3162.
9 E. W. YEMM AND A. J. WILLIS, *Biol. J.*, 57 (1934) 508.
10 W. MENKE AND E. JAKOB, *Z. physiol. Chem. Hoppe Seyler's*, 272 (1942) 227.
11 E. RABINOWITCH, *Photosynthesis* I; II, 2, Interscience Publ.
12 A. A. BENSON AND E. H. STRICKLAND, *Biochim. Biophys. Acta*, 41 (1960) 328.
13 T. N. GODNEV, A. A. SHLIK AND N. K. TRETJAK, *Doklady Akad. Nauk SSSR*, 87 (1952) 493.
14 B. HUBERT, *Rec. trav. botan. néerl.*, 32 (1935) 323.
15 R. M. MCGREADY, J. GUGGOLS, V. SILVIERA AND H. S. OWENS, *Anal. Chem.*, 22 (1950) 1156.
16 A. A. BENSON, H. DANIEL AND R. WISER, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1582.
17 J. MEGO AND A. T. JAGENDORF, *Plant Physiol.*, 34 (1959) suppl. xix.
18 E. WINTERSTEIN AND O. HIESTAND, *Z. physiol. Chem. Hoppe Seyler's*, 54 (1908) 288.
19 E. WINTERSTEIN AND K. SMOLENSKI, *Z. physiol. Chem. Hoppe Seyler's*, 58 (1909) 506.
20 H. E. CARTER, R. H. MCCLUER AND E. C. SLIFER, *J. Am. Chem. Soc.*, 78 (1956) 3735.
21 M. KATES, *Can. J. Biochem. and Physiol.*, 34 (1956) 967.