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Lipid Components of Leaves. V. Galactolipids, Cerebrosides, and Lecithin of Runner-Bean Leaves*

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Mono- and digalactosyl diglycerides have been isolated from runner-bean leaves and shown to have the structures 2,3-di-*O*-linolenoyl-1-*O*-β-D-galactopyranosyl-D-glycerol (I), and 2,3-di-*O*-linolenoyl-1-*O*-β-(6-*O*-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol (II), respectively. A cerebroside fraction has also been isolated and found to contain glucose as sole sugar constituent; α-hydroxy acids (chiefly α-hydroxypalmitic, α-hydroxy-C_{22}, and α-hydroxy-C_{24} acids) were the only fatty acids present, and the long-chain bases consisted chiefly of C_{18}-dehydrophytosphingosine with small amounts of C_{18}-phytosphingosine, C_{18}-dihydrosphingosine, and an isomer of C_{18}-sphingosine. The lecithin fraction was isolated in pure form and found to contain palmitic (27%), stearic (6%), oleic (4%), linoleic (38%), and linolenic (26%) acids; almost all the saturated acids were found to be in the α′ position.

The lipids of leaves are known to consist of more than a score of components. These include neutral lipids such as hydrocarbons, waxes, alcohols, quinones, sterols, sterol esters, and glycerides (Zill and Harmon, 1962; Weenink, 1962; Nichols, 1963; Lepage, 1964); phospholipids, such as lecithin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol and polyglycerol phosphatides (Benson and Maruo, 1958; Benson and Strickland, 1960; Wintermans, 1960; Kates, 1960; Wheeldon, 1960; Nichols, 1963); glycolipids such as mono- and digalactosyl diglycerides (Benson *et al.*, 1958, 1959a; Wintermans, 1960; Kates, 1960; Weenink, 1961; Zill and Harmon, 1962) and sterol glycosides (Nichols, 1963); and a sulfolipid (Benson *et al.*, 1959b; O'Brien and Benson, 1964).

Previous attempts in this laboratory to separate the lipids of runner-bean leaves by chromatography on silicic acid columns did not result in clear-cut resolutions of the components (Kates and Eberhardt, 1957). Four fractions were obtained, the main components of which were: fraction I, glycolipids (including phosphatidylglycerol and sulfolipid); fraction II, phosphatidylethanolamine and phosphatidylserine; fraction III, an amino acid–containing phosphatide; and fraction IV, lecithin. Subsequently, evidence for the presence of at least four glycolipids, designated as a, b, c, and d, was obtained by chromatography on silicic acid–impregnated paper (Kates, 1960). Zill and Harmon (1962) found evidence for four similar glycolipids in spinach leaves.

Three of these glycolipid components have now been isolated from fraction I and shown to be monogalactosyl diglyceride (glycolipid d), digalactosyl diglyceride (glycolipid a), and glucocerebrosides (glycolipid b); the lecithin in fraction IV has also been isolated and characterized. The isolation procedures and structure determinations are described here in detail.

MATERIALS AND METHODS

Extraction of Lipids.—Scarlet runner-bean (*Phaseolus multiflorus*) plants were grown in the greenhouse and harvested after 20–28 days of growth (Kates and Eberhardt, 1957). One kg of fresh primary leaves were extracted in 100-g batches with isopropanol followed by chloroform, as described previously (Kates and Eberhardt, 1957). The lipid material thus obtained weighed 9.38 g (9.5% of dry wt) and contained 82.2 mg of phosphorus; it was stored in chloroform solution at −10°.

Column Chromatography.—Total leaf lipids were chromatographed on columns of silicic acid–Celite (4:1, w/w) as described previously (Kates and Eberhardt, 1957). The glycolipid fraction obtained (fraction I, Table I) was rechromatographed on columns of silicic acid (Bio-Rad Laboratories, −325 mesh) made up in methanol and prewashed with 3 column volumes of chloroform; separation of glycolipid d from glycolipids a and b was thereby achieved. Columns of silicic acid (Bio-Rad Laboratories) prepared accord-

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ing to Hirsh and Ahrens (1958) were used for separation of glycolipids a and b. Purification of the lecithin fraction (fractions IV and V, Table I) was achieved by chromatography first on a column of alumina (Merck reagent grade) prepared in chloroform, and then on a column of silicic acid–Celite (3:1, w/w) prepared in chloroform-methanol (70:30, v/v), as described by Rhodes and Lea (1957). Elution of all columns was monitored by silicic acid–impregnated–paper chromatography (*vide infra*).

Paper Chromatography.—Lipids were chromatographed (single development) on silicic acid–impregnated Whatman 3MM paper in diisobutyl ketone–acetic acid–water (40:25:5, v/v) as described previously (Marinetti *et al.*, 1957; Kates, 1960). Because of the high concentrations of linolenic acid in the samples, the chromatograms were dried for not more than 10–15 minutes in the fume hood, and immediately stained with Rhodamine 6 G for total lipids, with ninhydrin for amino lipids, or with phosphomolybdate for choline-containing lipids (Marinetti, 1962). Vicinal glycol-containing lipids were detected as follows: The chromatogram was dipped through a 0.25% solution of sodium metaperiodate, left for 20 minutes, dipped through 1% sodium metabisulfite until the released iodine was decolorized, and finally passed through a solution of Schiff reagent (Marinetti *et al.*, 1957), diluted with an equal volume of 1% sodium metabisulfite.

Sugars, glycerol galactosides, and glycerol were chromatographed (descending) on Whatman No. 1 paper with pyridine–ethyl acetate–water (1:2.5:2.5, v/v, upper phase), and detected by spraying the chromatograms with a freshly prepared mixture of 2% sodium metaperiodate–1% potassium permanganate (2:1, v/v). The reactive compounds appeared first as yellow spots on a purple background; after 5–10 minutes the chromatograms were washed in water whereupon the spots appeared brown on a white background.

Analytical Procedures.—Phosphorus was determined by the method of Allen (1940); acyl ester groups were determined by the hydroxamic acid method (Snyder and Stephens, 1959), using methyl stearate as reference standard; choline as described previously (Kates, 1956); and total sugars were determined by the anthrone procedure (Morris, 1948). Glycerol was estimated by the periodate–chromotropic acid method of Lambert and Neish (1950); to correct for the presence of sugars, a standard solution of galactose was carried through the procedure. Periodate consumption and formaldehyde liberation by the galactosylglycerols was determined quantitatively according to Dyer (1956) and Lambert and Neish (1950), respectively. Iodine values were estimated by the method of Yasuda (1931–32).

Fatty acid methyl esters were analyzed by gas-liquid chromatography on 4 ft columns of 10% Apiezon L at 197° and 10% butanediol succinate polyester at 180°, using a Pye Argon chromatograph (James, 1960). Hydroxy acid methyl esters were analyzed on a 4 ft column of 10% butanediol succinate polyester at 200° and high flow rate (28 psi) essentially as described by O'Brien and Rouser (1964), and also on a short (53-cm) column of silicone (SE-52) at 200° (Stanacev and Kates, 1963). Long-chain aldehydes, as dimethylacetals, were analyzed on 10% Apiezon L at 197° and on 10% butanediol succinate polyester at 180° (Stanacev and Kates, 1963). The chain lengths of unsaturated acid and aldehyde components were checked by analysis of the samples after hydrogenation in methanol with platinum catalyst.

Hydrolytic Procedures.—All hydrolyses were carried out in a special extraction flask for separation of water-soluble and petroleum ether–soluble products, as described elsewhere (Kates, 1964). The lecithin and hydrolecithin samples were hydrolyzed in methanolic-HCl followed by NaOH, as described previously (Kates, 1964). The free fatty acids were weighed and converted to methyl esters by treatment with diazomethane in ethyl ether prior to analysis by gas-liquid chromatography.

The pure galactolipids and their saturated derivatives (10–15 mg) were methanolyzed in 4.5 ml of 2.5% methanolic-HCl (Kates, 1964), and the fatty acid methyl esters obtained were weighed and analyzed by gas-liquid chromatography. The water-soluble products were analyzed for total sugar, or hydrolyzed further in 2 N HCl at 100° for 4 hours and subjected to paper chromatography for identification of free sugars and glycerol.

To obtain the glycerol galactosides, the galactolipids (5–6 mg) were saponified in 5 ml of 0.3 N methanolic (90%)–sodium hydroxide under reflux for 1 hour; the mixture was shaken with Dowex-50 (H^+) to remove sodium ion, centrifuged free of resin, and extracted with petroleum ether to remove fatty acids. The neutral methanol-water phase was concentrated to dryness below 30°, and the residual galactosylglycerols were dissolved in water. Total sugar, periodate consumption, and formaldehyde liberation were estimated on suitable aliquots; glycerol was determined after hydrolysis in 1 N HCl at 100° for 1 hour. The glycerol galactosides and their acid hydrolysis products were also chromatographed on paper.

The cerebroside fraction was methanolyzed in 2 N methanolic–hydrochloric acid for 5 hours (Sweeley and Moscatelli, 1959), as described elsewhere (Kates, 1964). The fatty acid methyl esters were extracted with petroleum ether and weighed, and ester groups were determined; they were then analyzed by gas-liquid chromatography. The methanol-water phase was concentrated to a small volume, diluted with water, and made alkaline. The long-chain bases were extracted with ethyl ether, weighed, chromatographed on silicic acid–impregnated paper, and analyzed by the periodate oxidative procedure (*vide infra*). The aqueous phase was analyzed for total sugar, or hydrolyzed further in 2 N HCl at 100° for 4 hours for paper-chromatographic identification of the sugars present.

Long-Chain Base Composition.—The long-chain bases were analyzed by the periodate oxidative procedure of Sweeley and Moscatelli (1959), modified as follows (cf. Stanacev and Kates, 1963): The sample (1–5 mg) was dissolved in 4 ml of methanol in a special extraction flask (Kates, 1964), 0.5 ml of 0.5 M periodic acid in methanol (freshly prepared) was added, and the oxidation was allowed to proceed for 2 hours in the dark. Water (0.5 ml) was added, and the long-chain aldehydes were extracted with several 5-ml portions of petroleum ether (bp 30–60°). The petroleum ether extract was concentrated to dryness under nitrogen and the aldehydes were immediately converted to dimethylacetals by refluxing in 4.5 ml of 2.5% methanolic hydrogen chloride for 1.5 hours. To remove any methyl esters, 0.5 ml of 7 N aqueous sodium hydroxide was added, the mixture was refluxed for 1 hour, and the aldehyde dimethylacetals were extracted with low-boiling petroleum ether (Kates, 1964), and analyzed by gas-liquid chromatography.

To distinguish between phytosphingosine and sphingosine bases, the intact cerebroside fraction was also oxidized with periodic acid as described for the long-chain bases. In this case, the aldehyde dimethyl-

TABLE I
PRELIMINARY FRACTIONATION OF TOTAL LEAF LIPIDS ON SILICIC ACID–CELITE (4:1)[a]

Fraction	Solvent	Eluate Volume per Column (ml)	P in Fraction (% of total)	Weight of Fraction (% of total)	Main Constituents[b]
0	Chloroform	1100	0.9	26.6	Pigments, neutral lipids, PA (trace)
I	Chloroform-methanol (5:1)	200	45.1	53.5	PA, MGD(Gly-d), Di-PG, Gly-c, PE, PG, DGD (Gly-a), Gly-b, SL, PI
II	Chloroform-methanol (5:1)	350	9.4	4.9	PE, PG, PS, PI, SL
III	Chloroform-methanol (1:1)	175	4.9	3.9	PI, SL, unknown
IV	Chloroform-methanol (1:1)	450	28.6	7.5	PC
V	Methanol	250	0.74	1.4	PC
Recovery			89.6	97.8	

[a] Nine g of total lipids (containing 79.0 mg of lipid P) was dissolved in 60 ml of chloroform and distributed equally on four columns (3 cm diameter) each consisting of 75 g silicic acid–Celite (4:1). [b] *Abbreviations:* PA, phosphatidic acid; MGD (Gly-d), monogalactosyl diglyceride (glycolipid d); Di-PG, diphosphatidylglycerol; Gly-c, glycolipid c; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; DGD (Gly-a), digalactosyl diglyceride (glycolipid a); SL, sulfolipid; PI, phosphatidylinositol; PC, lecithin.

TABLE II
FATTY ACID COMPOSITION OF FRACTIONS OBTAINED AFTER SILICIC ACID–CELITE CHROMATOGRAPHY[a]

Fatty Acid	Composition (%)				
	Total Lipids	Fraction I	Fraction II	Fraction III	Fractions IV & V
14:0	0.3	Trace	1.1	1.7	0.8
16:0	13.2	10.8	31.6	29.8	24.8
16:1	1.2	1.8	1.6	2.5	Trace
16:2	Trace	Trace	1.4	1.9	Trace
18:0	2.2	1.5	3.2	3.4	5.6
18:1	2.0	1.3	2.0	4.4	4.0
18:2	9.2	5.9	10.8	14.4	31.0
18:3	72.0	78.5	48.5	41.8	33.0

[a] Analysis on butanediol succinate polyester at 179° (15 psi).

acetals obtained are derived only from the phytosphingosine-containing cerebrosides.

EXPERIMENTAL AND RESULTS

Preliminary Fractionation of Total Lipids

Chromatography of the total lipids of runner-bean leaves on silicic acid–Celite columns (Kates and Eberhardt, 1957) yielded the following main fractions (Table I, Fig. 1): fraction I, containing all the glycolipids together with most of the phosphatidylglycerol, sulfolipid, phosphatidylethanolamine and phosphatidylinositol; fraction II, containing mostly phosphatidylethanolamine and phosphatidylserine, and some phosphatidylglycerol; fraction III, containing phosphatidylinositol and an unknown acidic lipid; and fractions IV and V, consisting almost entirely of lecithin.

The fatty acid analyses (Table II) show a very high linolenic acid content in the total lipids and fraction I, almost twice that in the lecithin fraction (fraction IV). As discussed below, the high linolenic acid content is due to the occurrence of mono- and digalactosyldilinolenin as major components in fraction I.

Isolation and Purification of Glycolipids

The glycolipid-rich fraction (fraction I) was subjected to further chromatographic separation on silicic acid columns using the sequence of solvents given in Table III. The eluates were pooled to give a fraction rich in monogalactosyl diglyceride (fraction 1; see Fig. 2), and one rich in digalactosyl diglyceride and glycolipid b (fraction 4; see Fig. 3). Fractions rich in monogalactosyl diglyceride and glycolipid c (fraction 2), in phosphatidylglycerol (fraction 3), in phosphatidylethanolamine (fraction 5), and in phosphatidylserine (fraction 6) were also obtained (Table III), but these were not investigated further.

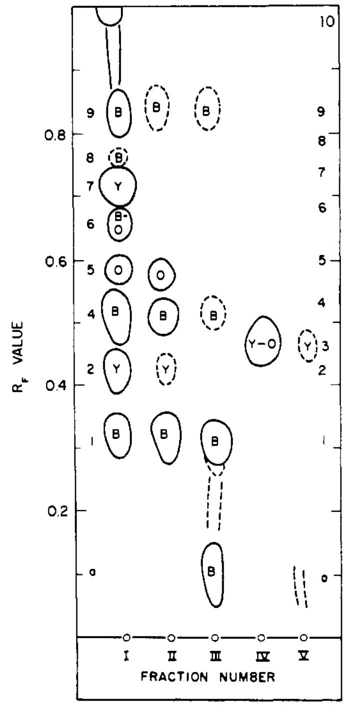


FIG. 1.—Chromatogram of total lipids of runner-bean leaves, stained with rhodamine 6 G (Marinetti *et al.*, 1957). Abbreviations: B, blue; Y, yellow; O, orange; dashed lines indicate faint spots. Identity of spots: a, unknown; 1, sulfolipid + PI; 2, DGD; 3, PC; 4, PG + PS + Glyc-b; 5, PE; 6, Di-PG + Glyc-c; 7, MGD; 8, unknown; 9, PA. (See Table I for abbreviation of component lipids.)

(*1*) *Monogalactosyl Diglyceride* (*Glycolipid d*).—The eluates containing glycolipid d (fraction 1; 1.79 g,

TABLE III
RECHROMATOGRAPHY OF GLYCOLIPID FRACTION (FRACTION I) ON SILICIC ACID[a]

Fraction	Eluent	Eluate Volume per Column (ml)	P in Fraction (% of total)	Weight of Fraction (% of total)	Main Constituents[b]
0	Chloroform Chloroform-methanol (98:2)	240 90	26.6	20.3	Pigments, unknown phosphatide, MGD
1	Chloroform-methanol (98:2)	90	24.6	37.9	MGD, PG
2	Chloroform-methanol (98:2)	168	9.6	6.8	MGD, Gly-c, PG
3	Chloroform-methanol (95:5)	132	13.5	5.6	PG, PE
4	Chloroform-methanol (95:5) Chloroform-methanol (9:1)	162 90	5.2 8.5	19.4 7.9	DGD, Gly-b, PE
5	Chloroform-methanol (9:1)	120	5.5	4.1	PE, DGD
6	Methanol	90	1.0	1.6	PE, PS
Recovery			94.5	103.6	

[a] Fraction I (4.72 g) (containing 34.9 mg P) was dissolved in 45 ml of chloroform and distributed equally on three columns of silicic acid (45 g/column). [b] Abbreviations as in Table I.

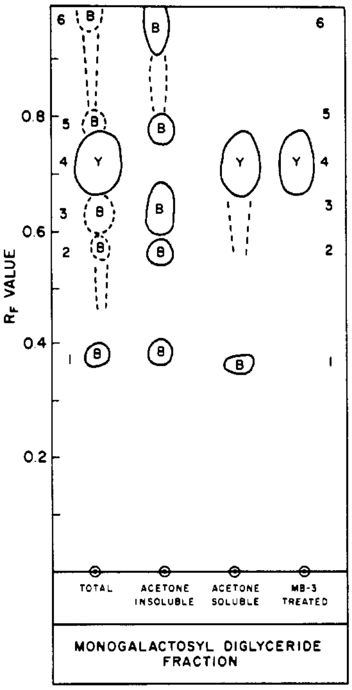


FIG. 2.—Chromatogram of monogalactosyl diglyceride–rich fraction (fraction 1, Table III) at various stages of purification. Identity of spots: 1, sulfolipid + PI; 2, PS; 3, PG; 4, MGD; 5, unknown; 6, PA. Abbreviations as in Table I.

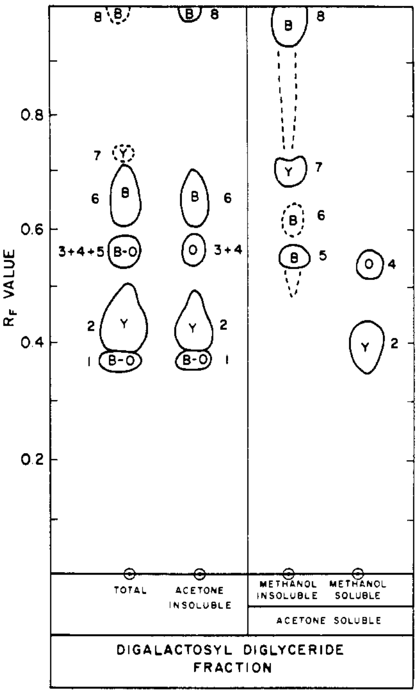


FIG. 3.—Chromatogram of digalactosyl diglyceride–rich fraction (fraction 4, Table III) at various stages of purification. Identity of spots: 1, PI + sulfolipid; 2, DGD; 3, PE; 4, Glyc-b; 5, PS; 6, PG; 7, Glyc-c; 8, PA. Abbreviations as in Table I.

containing 8.58 mg P) were concentrated *in vacuo* almost to dryness, the residue was dissolved in 3 ml of chloroform, and the solution was diluted with 10 volumes of acetone at 0°; the precipitate was removed by centrifugation at 0° and washed with cold acetone. The combined acetone supernatants were concentrated almost to dryness under nitrogen and the acetone precipitation procedure was repeated twice more. The acetone-insoluble material (Fig. 2) consisted of acidic phosphatides, chiefly phosphatidylglycerol, phosphatidic acid, phosphatidylserine, and phosphatidylinositol. The acetone-soluble material now consisted largely of glycolipid d but was still contaminated with a small amount of lipid P (2.44 mg), probably attributable to phosphatidylinositol (Fig. 2).

Further purification was achieved as follows: The acetone solution was concentrated *in vacuo* and the residue was dissolved in 10 ml of methanol-water (95:5, v/v). After centrifugation to remove some brown insoluble material, the solution was passed through a column of Amberlite MB-3 (20 g, 9 × 1.5 cm; prepared in 95% methanol), and the column was then eluted with 10 ml of methanol-water (95:5); this process was repeated twice more. The effluent was concentrated *in vacuo* (benzene was added to aid in removal of water) and the residue was immediately dissolved in chloroform.

Table IV
Analytical Data for Galactosyl Diglycerides

	Monogalactosyl Diglyceride			Digalactosyl Diglyceride		
		Hydrogenated			Hydrogenated	
Constituent	Unhydrogenated[a]	Found	Theory[b]	Unhydrogenated[a]	Found	Theory[c]
C (%)		68.84	68.6		65.10	64.7
H (%)		11.16	11.0		9.93	10.2
Galactose (%)		22.9	22.9		34.7	38.0
Glycerol (%)		12.6	11.7		9.3	9.7
Fatty acid methyl esters (%)		77.0	76.0		63.7	63.0
Mole ratio, galactose-glycerol-ester	1.00:1.00:1.97	1.00:1.07:2.04	1:1:2	2.00:1.11:2.04	2.00:1.05:2.10	2:1:2
Glycerol galactosides:						
HIO_4 consumed (moles/mole)	3.26	3.08	3.0	4.97	5.08	5.0
Formaldehyde liberated (moles/mole)	1.12	1.04	1.0	1.06	1.01	1.0

[a] Absolute analytical data could not be obtained for the unhydrogenated galactolipids because of their rapid autoxidation on drying *in vacuo.* [b] Calculated for monogalactosyldistearin, $C_{45}H_{86}O_{10}$ (786.6). [c] Calculated for digalactosyldistearin, $C_{51}H_{96}O_{15}$ (948.6).

The material now showed only one spot on paper, with R_F 0.71 (Fig. 2), and contained 1.46 g of monogalactosyl diglyceride (calculated from ester and sugar analysis); it was virtually free of phosphatides (0.04% P; calculated phosphatide contamination, 1%), but was still contaminated with brown pigments (mainly chlorophyll-degradation products). It contained galactose, glycerol, and fatty acid ester in the required mole ratio of 1:1:2 (Table IV), and its fatty acids consisted almost entirely (96%) of linolenic acid (Table V). Its infrared spectrum in carbon tetrachloride showed strong bands for OH (3400 cm^{-1}, broad), CH_2 (2940, 2860, 1460, and 710 cm^{-1}), ester C=O (1730 cm^{-1}) and C—O— (1165 cm^{-1}), alcohol C—O— (1070 cm^{-1}), and *cis* double bonds (3000, 1650, and 690 [shoulder] cm^{-1}).

Table V
Fatty Acid Composition of Galactosyl Diglycerides[a]

	Monogalactosyl Diglyceride		Digalactosyl Diglyceride	
Fatty Acid	Unhydrogenated	Hydrogenated	Unhydrogenated	Hydrogenated
16:0	2.3	1.0	4.5	2.3
16:1	Trace	0	Trace	0
18:0	Trace	96.2	1.0	97.7
18:1	Trace	0	Trace	0
18:2	2.2	2.8[b]	1.3	0
18:3	95.5	0	93.2	0

[a] Values are expressed as percentage of peak areas and are averages of analyses on Apiezon L at 198° and butanediol succinate polyester at 180°. [b] This component may be a conjugated diene produced during hydrogenation.

(2) *Digalactosyl Diglyceride* (*Glycolipid a*).—The eluates containing glycolipids a and b (fraction 4, Table III, 1.29 g; 4.77 mg lipid P) were brought almost to dryness *in vacuo,* the residue was dissolved in 1.5 ml of chloroform, and the phosphatides were precipitated with acetone, as described for glycolipid d. The acetone-insoluble material (Fig. 3) contained phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylinositol, together with some of the glycolipids a and b. The acetone-soluble material now had only traces of phosphatides (0.13 mg P) and contained glycolipids a and b with a trace of glycolipid c (Fig. 3). To remove the latter glycolipid and the remaining phosphatides, the acetone solution was brought to dryness under N_2, the residue was dissolved in 10 ml of methanol and kept at 0°, and the precipitate was removed by centrifugation. The methanol-soluble material now consisted only of glycolipids a and b (Fig. 3).

Separation of glycolipid a from glycolipid b was achieved as follows: The methanol-soluble material (*ca.* 350 mg) was dissolved in 5 ml of ethyl ether and applied to a column (16 mm diameter) of 10 g of silicic acid (Bio-Rad) prepared as described by Hirsch and Ahrens (1958). Elution with 200 ml ethyl ether removed only traces of material. Most of glycolipid b together with some glycolipid a was eluted with 37 ml of 5% methanol in ethyl ether, followed by 15 ml of 10% methanol in ether. The following 15 ml of 10% methanol in ether and 25 ml of 50% methanol in ether eluted the remaining glycolipid a with only a trace of glycolipid b. The 5% methanol-ether eluate containing glycolipids a and b was rechromatographed on a 10-g column of silicic acid, and the glycolipid b was removed by elution with 58 ml of 5% methanol in ether. The remaining glycolipid a was eluted with 100% methanol, and was combined with the glycolipid a eluates from the previous column; the combined eluates were concentrated to a small volume and dissolved in methanol.

This material (330 mg) now showed a single spot on silicic acid–impregnated paper (R_F, 0.40) which was strongly periodate-positive and fluoresced yellow with Rhodamine 6 G; it was entirely free of phosphatides but still contained some brown pigments and a trace of glycolipid b (*vide infra*). The ratio of fatty acid ester to glycerol to galactose was close to 2:1:2, as required for a digalactosyl diglyceride (Table IV), and its fatty acids consisted largely of linolenic acid (Table V). The infrared spectrum in carbon tetrachloride showed strong bands for OH, CH_2, ester, alcohol C—O—, and *cis* double bonds, as in that of monogalactosyl diglyceride.

Hydrogenation of Galactolipids: Mono- and Digalactosyldistearin.—Portions (130–150 mg) of the isolated mono- and digalactosyl diglycerides were hydrogenated in methanol solution (15 ml) with platinum oxide (37 mg) catalyst, at room temperature and atmospheric pressure. After hydrogen uptake was complete, the precipitated material was dissolved by

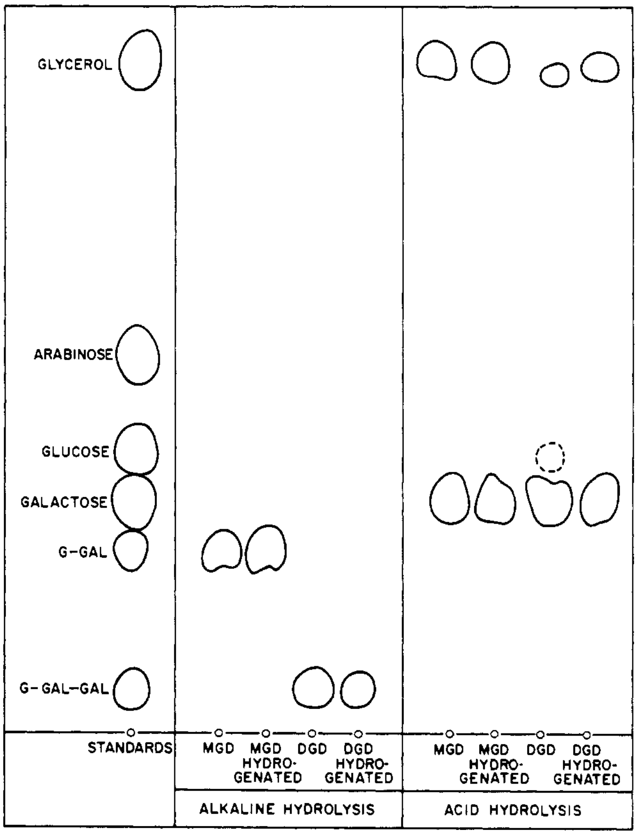


FIG. 4.—Chromatogram of water-soluble products obtained after alkaline or acid hydrolysis of purified mono- and digalactosyl diglycerides; solvent, pyridine–ethyl acetate–water (1:2.5:2.5, v/v, upper phase). Abbreviations as in Table I, and G-Gal = monogalactosylglycerol, G-Gal-Gal = digalactosylglycerol.

addition of warm chloroform, and the catalyst was removed by centrifugation and washed with chloroform. The combined supernatants were brought to dryness *in vacuo* and the residues were recrystallized several times from chloroform-methanol (1:9, v/v) for monogalactosyl derivative, or from hot methanol for digalactosyl derivative.

The saturated glycolipids were obtained as colorless crystalline powders (60–70 mg) having the following melting behavior: monogalactosyldistearin, transparent liquid crystal state, 122°, meniscus formation, 149°; digalactosyldistearin, transparent liquid crystal state, 135°, meniscus formation, 188–189°. Both saturated compounds were soluble in warm chloroform, warm benzene, hot ethyl acetate, or boiling methanol, and insoluble in cold methanol, acetone, ether, or ethyl acetate.

Their infrared spectra (KBr disks) showed the same main bands as the corresponding unsaturated compounds, except that the double-bond absorption bands (3000, 1650, and 690 cm^{-1}) were absent. The analytical data for the saturated galactolipids were very close to the values calculated for mono- and digalactosyldistearin, respectively (Table IV), and gas-liquid chromatographic analysis showed that their fatty acids consisted almost entirely (96 and 98%, respectively) of stearic acid (Table V). Their R_F values on silicic acid–impregnated paper were the same as the corresponding unsaturated galactolipids.

PROOF OF STRUCTURE AND CONFIGURATION OF GALACTOLIPIDS.—Saponification of mono- and digalactosyl diglycerides (unsaturated and saturated) yielded 2 moles of fatty acids and 1 mole of monogalactosylglycerol or digalactosylglycerol, respectively. The latter showed only one spot each on paper chromatograms, corresponding to those of authentic monogalactosylglycerol (R_{gal}, 0.80) and digalactosylglycerol, (R_{gal}, 0.20), respectively (Fig. 4); after acid hydrolysis, spots corresponding only to galactose and glycerol were detected on chromatograms (except for digalactosylglycerol, which also contained a trace of glucose from contaminating glycolipid b).

The molar ratio of periodate consumed to formaldehyde liberated was found to be close to the theoretical value of 3:1 for the monogalactosylglycerol, and 5:1 for the digalactosylglycerol (Table IV), proving that in both cases the point of attachment of the sugar portion was at carbon-1 of the glycerol.

For isolation of the galactosylglycerols, about 1 g of total runner-bean-leaf lipids was saponified in 0.5 N methanolic (90%) sodium hydroxide (see Methods), and the hydrolysate was extracted with petroleum ether and then passed repeatedly through a column of Amberlite-MB-3 (8 g; 12 mm diameter) to remove anionic and cationic substances (including chlorophyll-degradation products.) The neutral, yellow-colored effluent was concentrated to dryness and the residue was chromatographed on several sheets of Whatman 3MM paper with ethyl acetate–pyridine–water as solvent. Bands corresponding to monogalactosylglycerol and digalactosylglycerol were eluted with water, the eluates were concentrated to dryness, and the residues were crystallized from methanol-ether (monogalactosylglycerol) or from absolute ethanol (digalactosylglycerol), after seeding with authentic specimens (Carter *et al.*, 1956).

The crystalline monogalactosylglycerol obtained had mp (uncorr) 138–138.5° (reported mp uncorr, 139–140°, Carter *et al.*, 1956; corr, 140.5–141.5°, Wickberg, 1958a,b), and its infrared spectrum in KBr was identical in the region 700–1000 cm^{-1} with that reported for synthetic 1-*O*-β-D-galactopyranosyl-D-glycerol (Wickberg, 1958b). The crystalline digalactosylglycerol had mp (uncorr), 188–189° (reported mp, uncorr, 182–184°, Carter *et al.*, 1956; corr, 195–197°, Wickberg, 1958a), and its infrared spectrum in KBr was identical in the region 700–1000 cm^{-1} with that reported (Wickberg, 1958b) for 1-*O*-β-(6-*O*-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol isolated from wheat flour (Carter *et al.*, 1956) or from red algae (Wickberg, 1957, 1958a).

(*3*) *Cerebrosides* (*Glycolipid b*).—The glycolipid b-containing fraction obtained during purification of the digalactosyl diglyceride was virtually free from phosphatides, (0.1% P) but contained a small amount of digalactosyl diglyceride; it gave a negative Lieberman-Burchard reaction and thus was free from sterols. Separation of the contaminating digalactosyl diglyceride was achieved in two ways, as follows:

(a) The crude fraction was chromatographed on several sheets of silicic acid–impregnated paper, 20 spots (100–150 μg lipid per spot) being applied per sheet, using diisobutyl ketone–acetic acid–water (40:25:5, v/v) as solvent. The spots were located on guide strips by staining with the periodate-Schiff reagent and bands corresponding to glycolipid b were cut out from the unstained parts of the chromatograms and eluted with chloroform-methanol-water (75:25:5, v/v), followed by methanol. The eluates were combined and concentrated to dryness and the residual lipid was dissolved in chloroform.

(b) The crude fraction (*ca.* 7 mg) was saponified in 2 ml of methanol plus 0.2 ml of 2 N aqueous sodium hydroxide at 40° for 2 hours. The solution was then shaken for 15 minutes with Amberlite-MB-3 resin to remove sodium ion and free fatty acids. The resin was removed by centrifugation and washed twice with

TABLE VI
COMPOSITION OF FATTY ACIDS IN CEREBROSIDE FRACTION

Fatty Acid Methyl Ester	Retention Relative to Methyl Stearate at 200°[a]		Composition[b] (%)
	SE-52	Butanediol Succinate	
OH-16:0	0.78	1.87	29.9
OH-18:0	1.61	3.38	0.7
OH-19:0	2.22	4.50	Trace
OH-20:0	3.14	6.06	1.6
OH-21:0	4.68	8.23	Trace
OH-22:0	6.88	11.1	20.2
OH-23:0	9.66	15.0	4.6
OH-24:0	13.9	20.0	37.7
OH-25:0	19.6		2.0
OH-26:0	27.4		3.2

[a] Retention of authentic methyl esters relative to methyl stearate on SE-52 and butanediol succinate, respectively: α-OH-16:0, 0.79, 1.86; α-OH-18:0, 1.65, 3.40; α-OH-20:0, 3.30, 6.20; α-OH-26:0, 26.7, —. [b] Values are averages of results obtained with SE-52 and butanediol succinate columns.

TABLE VII
COMPOSITION OF LONG-CHAIN BASES IN CEREBROSIDE FRACTION

Aldehyde Dimethylacetal[a]	Retention Relative to 16:0 Acetal		Composition of Acetals (%)				Corresponding Long-Chain Base
			Long-Chain Bases		Intact Cerebrosides		
	Apiezon L, 198°	Butanediol Succinate, 180°	Unhydrogenated	Hydrogenated	Unhydrogenated	Hydrogenated	
15:1	0.57	0.78	64.0	0	62.1	0	C_{18}-Dehydrophytosphingosine
15:0	0.65	0.70	2.5	58.0	3.2	60.3	C_{18}-Phytosphingosine
16:1	0.88	1.14	2.1	0	+?	0	C_{18}-Sphingosine isomer
16:0	1.00	1.00	8.0	14.2	0	+?	C_{18}-Dihydrosphingosine
Unknown 1[b]	1.18	2.91	3.6	+?	+?	3.5	Unidentified
Unknown 2	1.98	5.00	19.3	25.0	32.0	36.0	Unidentified
Unknown 3[b]	2.76	5.67	1.4	2.9	0?	0?	Unidentified

[a] Several minor (<1%) fast-moving unidentified peaks were also present. [b] These components differ by two CH_2 groups in the chain.

1-ml portions of 90% methanol, and the combined methanolic supernatants were treated again with Amberlite-MB-3 to ensure removal of free fatty acids. The methanolic solution was brought to dryness *in vacuo*, and the residue was dissolved in 8 ml of methanol-chloroform (1:1, v/v) and 3.6 ml of water was added (see Bligh and Dyer, 1959). The mixture was centrifuged and the chloroform phase was withdrawn and concentrated to dryness in a stream of nitrogen; the residue (5.6 mg) was dissolved in chloroform.

The cerebroside fraction (glycolipid b) obtained by either procedure was free from ester groups and free fatty acids, and when chromatographed on silicic acid–impregnated paper it gave a single spot which had R_F, 0.59 (R_F of digalactosyl diglyceride, 0.48), fluoresced yellow with Rhodamine 6 G, was strongly positive with the periodate-Schiff reagent, and was ninhydrin negative.

STRUCTURE DETERMINATION.—Methanolysis of the cerebroside fraction (4.4 mg) yielded fatty acid methyl esters (1.5 mg), long-chain bases (1.6 mg), and sugar; molar ratio of fatty acids to sugar, 1.00:1.10. After hydrolysis in 2 N aqueous hydrochloric acid to cleave methyl glycosides, the sugar moiety was identified chromatographically as glucose.

Analysis of the fatty acid methyl esters by gas-liquid chromatography (Table VI) showed that they consisted entirely of hydroxy acids, predominantly the hydroxy derivatives of palmitic, C_{22}, and C_{24} acids. These are presumed to be α-hydroxy acids since their relative retentions were identical with those of authentic α-hydroxy acids; furthermore, according to O'Brien and Rouser (1964), the α isomers are well resolved from all other isomers. No linolenic or normal saturated acids were detected.

The long-chain base components, when chromatographed on silicic acid–impregnated paper, showed only one spot which gave a strongly positive reaction with the periodate-Schiff and the ninhydrin reagents, and fluoresced yellow when stained with Rhodamine 6 G; its R_F value was 0.48, identical with that of phytosphingosine prepared in the same way from yeast cerebrin (Stanacev and Kates, 1963) but lower than that of sphingosine (R_F, 0.52) obtained from beef-brain sphingomyelin or cerebroside (Kates, 1964). All samples showed small amounts of fast-moving material which was ninhydrin-negative and periodate-Schiff positive, and stained blue with Rhodamine 6 G.

The long-chain bases were found to contain more than 60% of a C_{18}-dehydrophytosphingosine and small amounts of C_{18}-phytosphingosine, C_{18}-dihydrosphingosine, and a C_{18}-sphingosine isomer (Table VII). Three unidentified phytosphingosine components, one of which was present to the extent of about 20% (unknown 2), were also detected (Table VII). The relative retentions of the acetals derived from these components suggest that they may be hydroxyphytosphingosines. However, it is also possible that they are products of incomplete periodate oxidation, or may be derived from the fast-moving ninhydrin-negative contaminants in the long-chain base preparation.

Isolation of Lecithin

The lecithin-rich fraction (fractions IV and V, Table I) analyzed as follows: P, 3.66%; N/P, molar ratio, 1.10; choline-N/P, molar ratio, 0.96; fatty acid/P, molar ratio, 2.17. It was contamined with about 10% of noncholine nitrogenous lipids and traces of green pigments, and was purified by chromatography first on a colum of alumina and then on a column of silicic acid (Table VIII).

The main fraction from the alumina column (fraction A2, Table VIII) now had choline-N/P and N/P atomic

TABLE VIII
CHROMATOGRAPHIC PURIFICATION OF LECITHIN FRACTION ON ALUMINA AND SILICIC ACID

Fraction	Solvent	Eluate Volume (ml)	Weight of Fraction (% of total)	P in Fraction (% of total)	Main Components
		Alumina Column[a]			
A 1	Chloroform	1100	1.5	0	Nonphosphatides
A 2	Chloroform-methanol (9:1)	1500	81.1	90.5	Lecithin + Lyso-PC
A 3	Chloroform-methanol (1:1)	800	2.9	4.1	Lyso-PC[b]
A 4	Ethanol-chloroform-water (5:2:2)	800	13.8	4.9	Aminophosphatides
	Recoveries		99.3	99.5	
		Silicic Acid–Celite Column[c]			
C 1	Chloroform-methanol (7:3)	200		0.6	
C 2	Chloroform-methanol (7:3)	640	84.5	79.8	Lecithin
C 3	Chloroform-methanol (7:3)	520	7.9	7.5	Lecithin + Lyso-PC
C 4	Methanol	250	7.6	9.9	Lyso PC[b]
	Recoveries		100.0	97.8	

[a] Crude lecithin (fractions IV & V; 843 mg, 30.9 mg P) applied to a column (3 × 17 cm) of 91 g of alumina prepared in chloroform. [b] These fractions had N/P and choline-N/P atomic ratios of 1.0 and fatty acid/P ratio of 0.91–0.97 as expected for lysolecithin. [c] Fraction A 2 (495 mg; 19.8 mg P) applied to a column (2.9 cm diameter) of 30 g silicic acid + 10 g Celite, prepared in chloroform-methanol (7:3, v/v).

ratios of 1.01, and a fatty acid/P mole ratio of 1.85. It was thus free from noncholine nitrogenous phosphatides, but contained about 10% of lysolecithin. Since the latter was not detected chromatographically in the lecithin-rich fractions (fractions IV and V, Fig. 1), it is presumed to have arisen by hydrolysis on the alumina column (see Renkonen, 1962). This contaminant was removed by chromatography on silicic acid (Table VIII). The eluates containing only lecithin (fraction C2) were concentrated to dryness *in vacuo*, and the residue was precipitated from ethyl ether (1 ml) by addition of 10 volumes of acetone at 0°, and stored in methanol solution at −10°.

The lecithin thus obtained (342 mg) was an almost colorless waxy material having the analytical data given in Table IX. When chromatographed on silicic acid–impregnated paper it gave a single spot (R_F, 0.50), which fluoresced yellow with Rhodamine 6 G and was strongly positive with the phosphomolybdate stain; no lysolecithin was detected.

TABLE IX
ANALYSIS OF PURIFIED LECITHIN AND HYDROLECITHIN

Constituent	Lecithin	Hydrogenated Lecithin	Theory[a]
P (%)	3.78	3.77	3.90
N (%)	1.69	1.73	1.76
N/P (atomic ratio)	0.99	1.01	1.00
Choline (%)	14.6	15.1	15.3
Choline-N/P (atomic ratio)	0.99	1.02	1.00
Fatty acids (%)	66.7	68.5	69.6
Neutral equivalent	278	276	277
Fatty acid/P (mole ratio)	1.97	2.04	2.00
Double bonds/mole lecithin	2.75	0	0

[a] Calculated for a 1:1 molar mixture of distearoyllecithin and palmitoylstearoyllecithin, $C_{43}H_{88}O_9NP$ (794).

Fatty acid analysis showed the presence of palmitic, linoleic, and linolenic acids as major components (Table X). Their distribution between the α′ and β positions was determined by hydrolysis with phospholipase A (cottonmouth venom) in ethyl ether (Hanahan, 1952; Long and Penny, 1957), and by analysis of the fatty acids liberated and those remaining in the lysolecithin formed (Table X). On the basis of the positional specificity established for phospholipase A (Tattrie, 1959; Hanahan *et al.*, 1960; De Haas and Van Deenen, 1961), the results show that almost all the saturated acids (palmitic and stearic) are present in the α′ position, and account for 63% of the total acids in this position, the remainder being unsaturated acids (oleic, linoleic, linolenic). In contrast the β position is occupied almost exclusively (97%) by the unsaturated acids.

TABLE X
COMPOSITION AND DISTRIBUTION OF FATTY ACIDS BETWEEN α′ AND β POSITIONS IN PURIFIED LECITHIN[a]

Fatty Acid Methyl Ester	Unhydrogenated Lecithin			Hydrogenated Lecithin
	Total	β Position	α′ Position	
16:0	26.6	2.3	51.0	24.9
16:1	Trace	Trace		0
17:0	0.2			0
18:0	6.0	0.4	11.6	75.1
18:1	3.7	4.6	2.8	0
18:2	37.7	55.3	20.3	0
18:3	25.7	37.1	14.4	0

[a] Fatty acids in the β position are those liberated by phospholipase A; those in the α′ position are the acids in the lysolecithin formed (Tattrie, 1959; Hanahan *et al.*, 1960; De Haas and Van Deenen, 1961). Gas-liquid chromatographic analysis done on butanediol succinate polyester at 178°; composition expressed as percentage of total peak area.

Hydrogenation of Lecithin

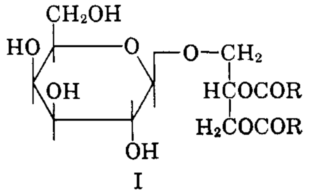
The purified lecithin (120 mg) was hydrogenated in methanol (35 ml) with platinum oxide catalyst (70 mg) at room temperature and atmospheric pressure. After hydrogen uptake had ceased (2 hours) the catalyst was removed by centrifugation and washed with chloroform, and the combined supernatants were concentrated to dryness. The residue was crystallized twice from 8 ml of chloroform-ether (1:3, v/v) by slow cooling to 0°; the crystalline hydrolecithin was filtered, washed with ether, and dried *in vacuo;* yield, 88 mg. The hydrolecithin contained stearic and palmitic acids in the ratio of 3:1 (Table X), and its analytical values were close to those of a 1:1 molar mixture of distearoyllecithin and palmitoylstearoylecithin (Table IX), the palmitic acid in the latter being almost entirely on the α'-position.

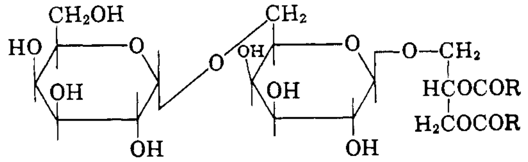
Discussion

Mono- and digalactosyl diglycerides were first discovered in wheat flour by Carter *et al.* (1956) and were subsequently shown to have the structures, 2,3-di-*O*-acyl-1-*O*-β-D-galactopyranosyl-D-glycerol (I), and 2,3-di-*O*-acyl-1-*O*-β-(6-*O*-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol (II), respectively (Carter *et al.* 1961a,b). Benson *et al.* (1958, 1959a) and Wintermans (1960) showed that these galactolipids constitute a major class of lipids in photosynthetic tissue. Galactolipids were subsequently isolated from red clover by Weenink (1961), and were found to have a high linolenic acid content and to account for at least 23% of the total lipids.

In the present study, the mono- and digalactosyl diglycerides isolated from runner-bean leaves accounted for 16.6% and 4%, respectively, of the total lipids. These values are based on the amounts of purified galactolipids isolated and are probably minimal, since small amounts of mono- and digalactosyl diglycerides were discarded during the purification procedures. The value of 20% for total galactolipids thus agrees fairly well with that reported by Weenink (1961) for red clover. The weight ratio of mono- to digalactosyl diglycerides found here was about 4:1, as compared to 2.7:1 estimated from Weenink's results. Lower galactolipid contents were reported for lipids of alfalfa leaves and *Chlorella* cells by O'Brien and Benson (1964), and the weight ratio of mono- to digalactosyl diglycerides was 1:2. These variations might be due to species difference and/or seasonal changes.

The present results show that the monogalactosyl diglyceride (glycolipid d) and the digalactosyl diglyceride (glycolipid a) in runner-bean leaves have the structures I and II, respectively, identical with those established for the corresponding compounds in wheat flour (Carter *et al.*, 1956, 1961b).


I


II

However, the runner-bean galactolipids and those from red clover (Weenink, 1961) contain linolenic acid almost exclusively (93–96%, Table V), whereas the wheat-flour galactolipids have palmitic, oleic, and linoleic acids and little or no linolenic acid. O'Brien and Benson (1964) also found high linolenic acid contents in the galactolipids of alfalfa leaves but those from *Chlorella* had a much lower proportion of linolenic acid (27%) and a high value (40%) for oleic acid.

The present studies have established the presence of glucocerebrosides in runner-bean leaves. This fraction accounts for less than 1% of the total lipids and is probably identical with the "glycolipid b" previously detected in runner-bean leaves (Kates, 1960). The cerebrosides contain glucose as the sole sugar and α-hydroxy acids as the only fatty acids; the long-chain base components contain chiefly a C_{18}-dehydrophytosphingosine with small amounts of C_{18}-phytosphingosine, C_{18}-dihydrosphingosine and an isomer of C_{18}-sphingosine. The same bases have been found in the cerebrosides of wheat-flour lipids (Carter *et al.*, 1961c), in which, however, the dehydrophytosphingosine was only a minor component. This new base has recently been shown to be a C_{18}-phytosphingosine with a double bond in the 8,9 position (Carter and Hendrickson, 1963), and it remains to be determined whether the corresponding base in runner-bean leaves has the same structure. The wheat-flour cerebrosides also contain α-hydroxystearic acid as major acid component, but this acid is only a trace component in the runner-bean-leaf cerebrosides.

The finding of glucose-containing cerebrosides in runner-bean leaves accounts, at least partly, for the glucose previously reported present in the lipids of these leaves (Kates and Eberhardt, 1957). Other glucose-containing lipids that may be present are sterol glucoside, recently found in lettuce leaves and believed to be identical with our glycolipid c (Nichols, 1963), and an acylated sterol glucoside found in potato tubers (Lepage, 1964). The previous finding (Kates, 1960) of glucose in glycolipids a and d, here identified as mono- and digalactosyl diglycerides, may now be explained as having been caused by streaking of the cerebroside (and sterol glucoside) spots on the chromatograms with resultant contamination of the galactolipid spots. The same explanation probably also accounts for the reported finding of glucose in the sulfolipid spot (Kates, 1960).

The lecithin component of runner-bean leaves was much more saturated than the galactolipids; saturated acids (palmitic and stearic) comprised about one-third of the acids present. The latter were almost entirely in the α' position whereas the unsaturated acids were present both in the β and α' positions (Table X). Although insufficient data are available for detailed calculation of the different isomeric lecithins present, it seems probable that α'-palmitoyl-β-linolenoyl-, α'-palmitoyl-β-linoleoyl-, dilinolenoyl-, and dilinoleoyl-lecithins are major constituents. Since diglycerides are presumed to be precursors of both lecithin and the galactolipids (Ferrari and Benson, 1961), there would appear to be a mechanism whereby dilinolenin is selectively galactosylated to form the galactolipids by the chloroplast enzymes (Neufeld and Hall, 1964). Alternatively, separate diglyceride pools may exist for biosynthesis of lecithin and galactolipids.

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Hydrolysis of Monogalactosyl and Digalactosyl Diglycerides by Specific Enzymes in Runner-Bean Leaves*

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Runner-bean leaves contain specific enzymes, associated both with the chloroplast and cell-sap-cytoplasm fractions, which catalyze the hydrolysis of monogalactosyldilinolenin and digalactosyldilinolenin to the corresponding galactosylglycerols and free linolenic acid. No evidence for the formation of "lyso" compounds was obtained, but these are presumed to be intermediates. The cell-sap cytoplasm also contains α- and β-galactosidases which catalyze hydrolysis of the galactosylglycerols to free galactose and glycerol. The galactolipid-hydrolyzing enzymes in the cell-sap cytoplasm, after 3-fold purification by ammonium sulfate fractionation, had the following properties: optimum *p*H, 7.0 for monogalactosyldilinolenin, 5.6 for digalactosyldilinolenin; apparent Michaelis-Menten constant, 7.8×10^{-3} M for monogalactosyldilinolenin, 1.5×10^{-3} M for digalactosyldilinolenin. This enzyme preparation was active only toward unsaturated galactolipids, and was free from lipase and phospholipase activities. Calcium ion had no effect and solvents such as ethyl ether were inhibitory rather than stimulating. Galactolipid-hydrolyzing activity has so far been demonstrated only in leaves of *Phaseolus* species and in commercial pancreatin.

Monogalactosyl and digalactosyl diglycerides occur widely throughout the plant kingdom (for pertinent references see Law, 1960; Zill and Cheniae, 1962; and Sastry and Kates, 1964). They constitute a major class of lipids in photosynthetic tissues (Benson *et al.*, 1959; Wintermans, 1960; Zill and Harmon, 1962) and have a high rate of turnover in algae (Ferrari and Benson, 1961) and in leaves (Kates, 1960). It might therefore be expected that specific enzymes should exist for the hydrolysis of this class of lipids. Enzymes catalyzing the stepwise hydrolysis of the fatty acid ester linkages in a related substrate, sulfoquinovosyl diglyceride, have recently been demonstrated in extracts of *Scenedesmus*, *Chlorella*, alfalfa leaves and roots, and other plant tissues, but activity towards galactosyl diglycerides was absent (Yagi and Benson, 1962).

During studies on the glycolipids of chloroplasts, it was observed that mono- and digalactosyl diglycerides

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