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

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Received April 15, 1964

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

* Issued as N.R.C. No. 8127. A preliminary account of this work (Sastry and Kates, 1963b) was presented at the 145th Meeting of the American Chemical Society, New York, September 8–13, 1963.

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were completely absent in water homogenates and chloroplast preparations of runner-bean leaves, although these galactolipids are present in high concentration in the intact leaves (Sastry and Kates, 1963a, 1964). These observations suggested that runner-bean leaves contain galactolipid-hydrolyzing enzymes which begin to act as soon as the leaf cells are broken. Subsequently, direct evidence for the presence of such enzymes was obtained, and this communication describes their partial purification, properties, and mode of action.

Materials and Methods

Plant Material.—Scarlet runner-bean (*Phaseolus multiflorus*) plants were grown in the greenhouse in soil containing vermiculite. The tops of the plants were removed after 14 days of growth to prevent formation of pinnate leaves. Primary leaves, harvested between the third and fourth week of growth, were used for the enzyme preparations. The leaves were rinsed in tap water, blotted, wrapped in Saran wrap, and kept at 5° for 1 hour before use.

Kidney-bean (*Phaseolus vulgaris*) and mung-bean (*Phaseolus aureus*) plants were also grown in the greenhouse. Primary leaves were harvested after 4–5 weeks of growth. Sugar-beet, squash, and soybean plants were also grown in the greenhouse.

Cabbage, spinach, and carrots were obtained from the local market.

Substrates.—Monogalactosyldilinolenin, digalctosyldilinolenin, and lecithin were isolated from runner-bean leaves as described previously (Sastry and Kates, 1963a, 1964); these were stored in chloroform solution at 0°. Mono- and digalactosyldistearin were prepared by catalytic hydrogenation of the corresponding dilinolenin derivatives as described elsewhere (Sastry and Kates, 1964). The α,β-diglyceride derived from the leaf lecithin was obtained after enzymatic hydrolysis with *Clostridium perfringens* toxin (phospholipase C) by the procedure of Hanahan and Vercamer (1954). The fatty acids of the lecithin and of the diglyceride consisted of palmitic (27%), stearic (6%), oleic (4%), linoleic (38%), and linolenic (25%) acids (Sastry and Kates, 1964). Triolein was a product of the Hormel Institute, Minneapolis, Minn.

Preparation of Leaf-Cell Fractions.—(A) Whole chloroplasts.—Fresh runner-bean leaves (76 g) were ground in a mortar with sea sand at 0° with an equal weight of cold 0.35 M sodium chloride solution. The homogenate was filtered through cheesecloth and the juice was centrifuged lightly for 1–2 minutes to remove sand and debris, and then at 1000 × *g* for 7 minutes at 0° to sediment unbroken chloroplasts. The sediment was resuspended in 76 ml of 0.35 M sodium chloride solution, recentrifuged at 1000 *g* for 7 minutes at 0°, resuspended in 10 ml of 0.35 M sodium chloride solution, and finally centrifuged for 1 minute at 800 × *g* to remove any whole cells. The suspension thus obtained contained 35.6 mg dry wt of whole chloroplasts per ml.

(B) Broken chloroplasts.—These were prepared from the once-washed whole chloroplasts by suspension in distilled water (20 ml for one-half the above preparation) at 0°, and after 15 minutes, centrifuging at 1000 *g* for 1 minute to remove whole cells and whole chloroplasts, and then at 15,000 × *g* for 20 minutes to sediment the broken chloroplasts. The latter were then suspended in distilled water to a concentration of 18.6 mg dry wt/ml.

Whole and broken chloroplast fractions from spinach leaves were also prepared as described, except that the grinding medium consisted of a solution of 0.35 M sodium chloride, 0.01 M sodium ascorbate, 0.001 M EDTA disodium salt, and 0.01 M potassium phosphate buffer, *p*H 7.4. These procedures are essentially those of Arnon *et al.* (1956) and Stumpf and James (1962).

Extraction of Lipids.—Whole leaves were extracted with hot isopropanol as described elsewhere (Kates and Eberhardt, 1957; Sastry and Kates, 1964). Leaf homogenates and whole or broken chloroplast suspensions were extracted by either of the following procedures:

(1) Two ml of the suspension was stirred with 16 ml of isopropanol at 60–70° for 3–4 minutes. The mixture was centrifuged, the green supernatant was decanted, and the sediment was extracted, in succession, with 16 ml hot isopropanol, 16 ml of isopropanol-chloroform (1:1), and 16 ml of chloroform, with centrifugation between changes of solvent. The combined extracts were concentrated to dryness *in vacuo*, the residue was dissolved in 20 ml of chloroform, and the solution was washed twice with distilled water. The chloroform solution was concentrated *in vacuo* and the residue was finally dissolved in chloroform (10 ml).

(2) To 1.6 ml of suspension was added 4 ml of methanol and 2 ml of chloroform, and the mixture was shaken for a few minutes and left at room temperature for several hours with frequent shaking. Two ml of chloroform and 2 ml of water were added, and the mixture was shaken and centrifuged. The chloroform phase was withdrawn and concentrated to dryness in a stream of nitrogen, and the residue was dissolved in chloroform (10 ml). Procedure (2) is based on that of Bligh and Dyer (1959).

Enzyme Preparation.—Fresh runner-bean leaves (70 g) were blended with an equal weight of distilled water for 2 minutes in an Omni-mixer cooled on ice. The crude homogenate was filtered through cheesecloth and centrifuged at 15,000 × *g* for 20 minutes at 0° to remove the chloroplast fraction. The greenish-yellow supernatant (cell-sap cytoplasm, 84 ml) was further fractionated by either of the following procedures:

(A) By dialysis.—The cell-sap cytoplasm (40 ml) was dialyzed against distilled water for 48 hours. The precipitate formed was centrifuged and suspended in 10 ml of distilled water; the supernatant was retained for further testing (see Results). The precipitated enzyme preparation (designated as enzyme preparation I) was used in unbuffered systems for chromatographic studies of the water-soluble products of hydrolysis.

(B) By ammonium sulfate precipitation.—The cell-sap cytoplasm (44 ml) was centrifuged further at 104,000 × *g* for 60 minutes at 0° in a Spinco preparative centrifuge to remove microsomal particles plus some chloroplast fragments. The resulting clear yellow supernatant was dialyzed against 0.05 M phosphate buffer (*p*H 7.0) for 12 hours. Solid ammonium sulfate was added to the dialysate, and the precipitates formed at 0–35%, 35–55%, and 55–75% saturation were centrifuged at 15,000 × *g*, dissolved in 0.05 M phosphate buffer (*p*H 7.0), dialyzed overnight against the same buffer, and diluted with buffer to half the original volume of the supernatant. Protein content and enzyme activity were determined for each ammonium sulfate fraction (see Results, Table II). The fraction precipitating up to 70% saturation with ammonium sulfate was used routinely in subsequent experiments, and is designated as enzyme preparation II. It was finally dissolved in buffer to a volume such that 1 ml

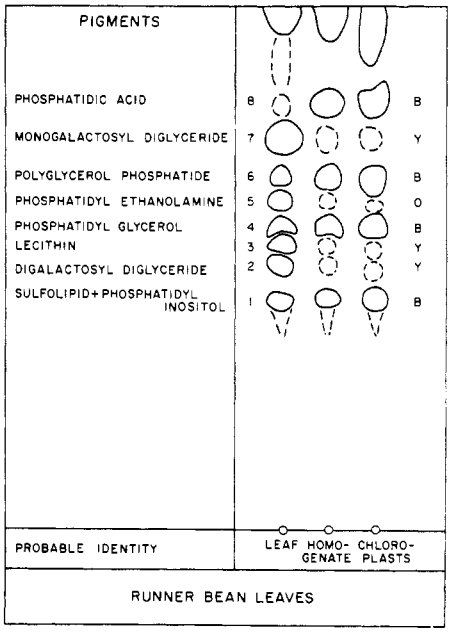

FIG. 1.—Tracing of chromatogram of lipids from intact runner-bean leaves, leaf homogenate, and chloroplast fraction (whole or broken), on silicic acid–impregnated paper (Marinetti, 1962); chromatogram stained with Rhodamine 6G and viewed under ultraviolet light (abbreviations: B, blue, Y, yellow, O, orange; dashed lines indicate faint spots). Tentative identity of spots as indicated in the figure.

was equivalent to 4 g of leaves; protein concentration, 15–16 mg/ml.

Enzyme Preparations from Other Sources.—Enzymes from the other plant sources were prepared by blending the tissue with distilled water, and centrifuging at 15,000 × *g* for 20 minutes and at 104,000 × *g* for 60 minutes, as described for runner bean. The clear yellow supernatants thus obtained were used without further purification.

The pancreatin preparation was made by grinding 2 g of lyophilized pancreatin extract (Parke-Davis) with 10 ml of 1% sodium chloride solution at 5° for 5 minutes, and centrifuging at 1500 rpm for 20 minutes. The supernatant was used without further purification.

Phospholipase B was prepared from *Penicillium notatum* as described by Dawson (1958). Lyophilized snake venom (*Agkistrodon piscivorus piscivorus*) was obtained from the Ross Allen Reptile Institute.

Analytical Methods.—Phosphorus was determined by the method of Allen (1940), acyl ester by the procedure of Snyder and Stephens (1959), and protein by the method of Lowry *et al.* (1951). Total sugar was estimated by the anthrone procedure (Morris, 1948), and (free) reducing sugar by the method of Nelson (1944), adapted to a micro scale. Free fatty acids liberated enzymatically were determined quantitatively by gas-liquid chromatography, using methyl stearate as internal reference standard (see Kates *et al.*, 1964).

Paper Chromatography.—Lipids were chromatographed on silicic acid–impregnated paper (Whatman 3MM), using the solvent system diisobutyl ketone–acetic acid–water (40:25:5, v/v) (Marinetti, 1962). The lipids were detected by staining the chromatograms with Rhodamine 6G and viewing under ultraviolet light (360 mμ); glycolipids were detected by staining with the periodate-Schiff reagent, as described elsewhere (Sastry and Kates, 1964). The lipid components were identified as described previously (Kates, 1960).

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). These were detected by spraying the chromatograms with a freshly prepared mixture (2:1, v/v) of 2% sodium periodate–1% potassium permanganate (Sastry and Kates, 1964).

Enzyme Assay.—An aliquot of the chloroform solution of monogalactosyldilinolenin or digalactosyldilinolenin, containing about 1 μmole (2 μeq acyl ester) of substrate was taken just to dryness in a stream of nitrogen, and suspended in 0.05 ml of methanol followed by 0.1 ml of 0.5 M phosphate buffer (*p*H 7.0 for monogalactosyldilinolenin or 5.6 for digalactosyldilinolenin). An aliquot of the enzyme preparation (e.g., 0.2 ml of purified enzyme, equivalent to 0.8 g of leaves), and water to 0.8 ml were added, and the mixture was incubated at 30° with shaking for 30 or 60 minutes as required. To stop the reaction, 2 ml of methanol was added and the sample was kept in a hot-water bath (80°) for 30 seconds. Lipids in the enzyme digest were then extracted by the procedure of Bligh and Dyer (1959). One ml of chloroform was added, the mixture was shaken, and after 20–30 minutes a further 1 ml of chloroform plus 1 ml of water were added; the mixture was shaken and centrifuged, and the chloroform phase (2.0 ml) was carefully withdrawn. A suitable aliquot (0.5–1.0 ml) of the chloroform phase was taken to dryness in a stream of nitrogen and the acyl ester content was determined. Control tubes containing either substrate and buffer or enzyme and buffer were also run simultaneously. Enzyme activity is expressed as μeq reduction in acyl ester per hour (or % hydrolysis per hour), after correction for the endogenous ester in the incubated enzyme preparation.

RESULTS

Lipid Components in Intact Leaves, Homogenate, or Chloroplasts.—Lipids extracted with isopropanol from intact runner bean leaves, total leaf homogenate, and chloroplasts (whole or boken) showed some striking differences in composition (Fig. 1). In contrast to the whole leaves, which contained large amounts of mono- and digalactosyldilinolenin (spots 7 and 2), both the leaf homogenate and the chloroplast preparations were almost completely devoid of these lipids, and also of lecithin (spot 3) and phosphatidylethanolamine (spot 5). The disappearance of the latter two components is attributable to the action of phosphatidase D (Kates, 1956), since both the homogenate and chloroplast preparations contained a large amount of phosphatidic acid (spot 8). The amounts of phosphatidylinositol + sulfolipid (spot 1), phosphatidylglycerol (spot 4), and diphosphatidylglycerol (spot 6) appeared to be unaffected, but no quantitative data are available on this point. The same results were obtained when the extractions were carried out by the procedure of Bligh and Dyer (1959). In contrast, chromatograms of the lipids extracted with isopropanol from intact spinach leaves, homogenate, or chloroplasts showed little or no change in concentration of mono- or digalactosyldilinolenin. It therefore appeared that runner-bean leaves possess enzyme(s) that rapidly hydrolyze the galactolipids and that this activity is manifested as soon as the leaf cells are broken.

Distribution of Enzyme Activity.—Incubation of mono- or digalactosyldilinolenin with the total water homogenate of runner-bean leaves at *p*H 7 for 1 hour resulted in pronounced decreases in the acyl ester

TABLE I
DISTRIBUTION OF GALACTOLIPID-HYDROLYZING ENZYME(S) IN RUNNER-BEAN-LEAF CELL FRACTIONS[a]

Fraction	Enzyme Activity (reduction in acyl ester/60 min)			
	Monogalactosyldilinolenin		Digalactosyldilinolenin	
	(μeq)	(%)	(μeq)	(%)
Homogenate	2.71	60.6	4.56	100
Chloroplasts (broken)	1.59	35.5	4.56	100
Cell-sap-cytoplasm	0.68	15.0	2.81	61.7
"Microsome" particles	0.07	1.4	0.59	13.0
Supernatant	0.34	7.5	2.30	50.6

[a] Reaction mixtures contained 2.24 μmoles (4.48 μeq) of monogalactosyldilinolenin or 2.28 μmoles (4.56 μeq) of digalactosyldilinolenin, cell fraction equivalent to 1.4 g fresh leaf, 0.2 ml of 0.5 M Tris buffer (*p*H 7.0), and water to 1.6 ml. Incubation at 30° for 60 minutes as described in text.

content of the substrates (Table I). This activity was associated mostly with the broken-chloroplast fraction and to a lesser extent with the cell-sap cytoplasm fraction. The activity of the latter fraction remained with the supernatant after centrifugation at 104,000 × *g* and relatively little was associated with the sedimented microsome fraction (Table I).

Since the enzymes in the cell-sap cytoplasm thus appeared to be in soluble form, further studies were carried out with this fraction rather than with the chloroplast fraction.

Partial Purification of Enzymes.—(a) BY DIALYSIS (ENZYME PREPARATION I).—When the cell-sap cytoplasm was dialyzed against distilled water, a precipitate formed which was centrifuged and resuspended in distilled water (see Methods). On testing equivalent amounts of the precipitate of the supernatant for galactolipase activity all the activity toward monogalactosyldilinolenin was found in the precipitate, while that toward digalactosyldilinolenin was distributed between the precipitate and the supernatant in the ratio 7:3, respectively. A partial separation of the two enzymes appeared to have been achieved but this was not further investigated. The precipitated enzyme preparation I was used in unbuffered systems when chromatography of the water-soluble products was desired.

(b) BY AMMONIUM SULFATE FRACTIONATION (ENZYME PREPARATION II). The results of ammonium sulfate fractionation of the cell-sap cytoplasm (after sedimentation of the microsomal particles at 104,000 × *g*), are shown in Table II. The fraction obtained between 35% and 55% saturation showed a 2.6- and a 2.9-fold increase in specific activity toward monogalactosyldilinolenin and digalactosyldilinolenin, respectively. No significant further increase in specific activity in the fraction obtained from 55 to 75% saturation was observed. Attempts to purify the enzymes further were not carried out. In subsequent experiments, the fraction precipitated between 0 and 70% saturation was used (enzyme preparation II).

Properties of the Enzymes.—(A) *p*H DEPENDENCE.—The effect of *p*H on the rate of hydrolysis of monogalactosyldilinolenin and digalactosyldilinolenin is shown in Figure 2. With monogalactosyldilinolenin as substrate, the optimum was at *p*H 7.0, whereas for digalactosyldilinolenin the optimum was at 5.6 (in both acetate and phosphate buffers). At their respective *p*H optima, activity was greater toward digalactosyldilinolenin than toward monogalactosyldilinolenin. Unbuffered runner-bean-leaf supernatants have a *p*H of about 5.6, which is optimum for digalactosyldilinolenin. This factor and inadequate buffering with the Tris buffer (*p*H 7.0) used would account for the higher activity toward digalactosyldilinolenin than toward monogalactosyldilinolenin observed with the various cell fractions (see Table I).

TABLE II
AMMONIUM SULFATE FRACTIONATION OF GALACTOLIPID-HYDROLYZING ENZYMES

Ammonium Sulfate Fraction	Protein		Relative Specific Activity[a]	
	(mg/fraction)	(%)	Monogalactosyldilinolenin	Digalactosyldilinolenin
Initial enzyme[b]	145.5	100.0	1.00	1.00
Precipitate at 0–35% saturation	68.8	47.2	1.48	1.16
35–55%	37.8	26.0	2.60	2.86
55–75%	27.0	18.5	2.65	
Supernatant after 75% saturation	22.1	15.2		

[a] Reaction mixtures contained 1.05 μmoles (2.10 μeq ester) of monogalactosyldilinolenin or 0.90 μmole (1.80 μeq ester) of digalactosyldilinolenin, equivalent amounts enzyme fractions, and 0.1 ml 0.5 M Tris buffer (*p*H 7.0); final volume 0.8 ml; incubation at 30° for 30 minutes. Specific activities are given relative to those of the initial enzyme (0.28 μeq ester reduction/30 min/mg protein for monogalactosyldilinolenin; 0.18 μeq/30 min/mg protein for digalactosyldilinolenin). [b] Cell-sap cytoplasm after centrifugation at 104,000 × *g* for 1 hour and dialysis against 0.05 M Tris buffer (*p*H 7).

(b) DEPENDENCE ON ENZYME CONCENTRATION.—Under optimum conditions of *p*H the rates of hydrolysis were linear functions of enzyme concentration, up to a concentration of 6 mg protein/ml for monogalactosyldilinolenin and 4 mg protein/ml for digalactosyldilinolenin (Fig. 3). Higher enzyme concentrations were inhibitory for monogalactosyldilinolenin hydrolysis, but not for digalactosyldilinolenin hydrolysis.

(c) DEPENDENCE ON SUBSTRATE CONCENTRATION.—The effect of substrate concentration on the rate of hydrolysis of mono- and digalactosyldilinolenin at their respective optimum *p*H is shown in Figure 4. When plotted according to Lineweaver and Burk (1934), the data indicated a linear relation between $1/v$ and $1/[S]$ up to substrate concentrations of 2.7×10^{-3} M for monogalactosyldilinolenin and 1.9×10^{-3} M for digalactosyldilinolenin; higher substrate concentrations were inhibitory. The apparent Michaelis-Menten constants (K_m) calculated from the linear portions of the curves were 7.8×10^{-3} M and 1.5×10^{-3} M, for mono- and digalactosyldilinolenin, respectively, indicating that digalactosyldilinolenin has a greater affinity for the enzyme(s) than has monogalactosyldilinolenin.

(d) SUBSTRATE SPECIFICITY.—The partially purified enzyme preparation II was tested for activity toward the various substrates shown in Table III.

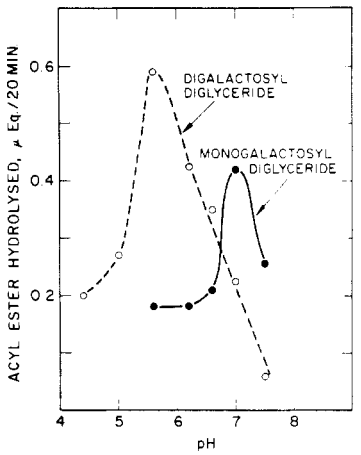

FIG. 2.—Dependence of hydrolysis rate on *p*H. Reaction mixtures contained 1.01 μmoles monogalactosyldilinolenin or 1.00 μmoles digalactosyldilinolenin, 0.1 ml of 0.5 M buffer (acetate buffer, *p*H 4.4–5.6; phosphate buffer, *p*H 5.6–7.5), 0.2 ml of enzyme (preparation II, 3.28 mg protein), and 0.5 ml of water; incubation at 30° for 20 minutes.

TABLE III
SUBSTRATE SPECIFICITY[a]

Substrate	(μmoles)	*p*H	Enzyme Activity (% reduction in acyl ester/ 20 min)
Monogalactosyldilinolenin	1.09	7.0	42
Monogalactosyldistearin	1.27	7.0	0
Digalactosyldilinolenin	1.03	5.6	54
Digalactosyldistearin	0.71	5.6	0
Runner-bean leaf-lecithin	1.28	7.0	3
α,β-Diglyceride derived from leaf lecithin	1.54	7.0	3
Triolein	0.61	7.0	0

[a] Reaction mixtures contained the indicated amount of substrate, 0.2 ml of enzyme preparation II, 0.1 ml of 0.5 M phosphate buffer, and water to 0.8 ml total volume. Incubation at 30° for 20 minutes.

No significant hydrolysis occurred with the saturated mono- or digalactosyl diglycerides, whereas the natural unsaturated galactolipids were rapidly hydrolyzed. It should be noted, however, that the saturated substrates did not form micellar dispersions whereas their unsaturated counterparts were readily dispersible. Lack of accessibility of substrate for the enzyme might thus be a rate-limiting factor.

The enzyme preparation showed little or no activity toward leaf lecithin, the α,β-diglyceride derived from it, or triolein (Table III). The preparation therefore appears to be free from phospholipase A and B and lipase activities. Furthermore, when the lecithin recovered after incubation with the enzyme was subjected to chromatography on silicic acid–impregnated paper only a single spot corresponding to intact lecithin was found, and neither phosphatidic acid nor diglyceride could be detected. The enzyme preparation II is thus also free from phospholipase D and C activities.

(e) EFFECT OF SOLVENTS AND CALCIUM ION ON ACTIVITY.—Since ethyl ether is known to stimulate plant–phosphatidase D activity (Kates, 1957), the effect of this solvent on the rate of hydrolysis of mono- and digalactosyldilinolenin was tested (Table IV). Ethyl ether did not stimulate the activity and actually produced a marked decrease in the rate of hydrolysis of both mono- and digalactosyldilinolenin. Addition

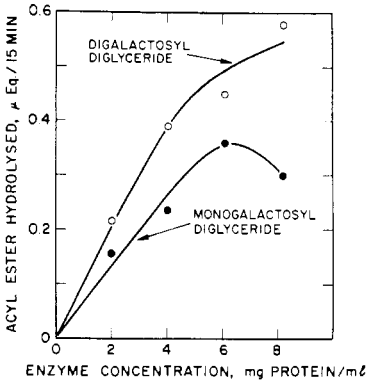

FIG. 3.—Dependence of hydrolysis rate on enzyme concentration. Reaction mixtures contained 1.01 μmoles of monogalactosyldilinolenin or 1.00 μmole digalactosyldilinolenin, 0.1 ml of 0.5 M phosphate buffer (*p*H 7.0 for monogalactosyldilinolenin, 5.6 for digalactosyldilinolenin), 0.1–0.4 ml of enzyme preparation II (16.4 mg protein per ml), and water to 0.8 ml total volume; incubation at 30° for 15 minutes.

TABLE IV
EFFECT OF SOLVENTS AND CALCIUM ION ON RATE OF HYDROLYSIS OF MONO- AND DIGALACTOSYL DIGLYCERIDES[a]

Substrate	Additions	Hydrolysis (%/ 30 min)
Monogalactosyldilinolenin (1.09 μmoles)	None	42
	Ethyl ether (0.2 ml)	12
	$CaCl_2$ (5 μmole)	44
Digalactosyldilinolenin (1.03 μmoles)	None	54
	Ethyl ether (0.2 ml)	<1
	$CaCl_2$ (5 μmole)	54
Monogalactosyldistearin (1.32 μmoles)	None	0
	Methanol (0.2 ml)	0
	Methanol (0.2 ml) + ethyl ether (0.2 ml)	5
	Methanol (0.2 ml) + chloroform (0.1 ml)	0
Digalactosyldistearin (0.75 μmole)	None	0
	Methanol (0.2 ml)	0
	Methanol (0.2 ml) + ethyl ether (0.2 ml)	0
	Methanol (0.2 ml) + chloroform (0.1 ml)	0

[a] Reaction mixtures contained indicated amount of substrate, 0.1 ml of 0.5 M phosphate buffer (*p*H 7.0 for monogalactosyldilinolenin; 5.6 for digalactosyldilinolenin), 0.2 ml of enzyme II, and 0.5 ml of water (total volume, 0.8 ml). Additions as indicated; incubation at 30° for 30 minutes.

of Ca^{2+} in a final concentration of 0.0062 M did not affect the rate of hydrolysis significantly.

Since the presence of methanol had been found to enhance the ethyl ether stimulation of phosphatidase D activity (Kates, 1957), the effect of methanol together with ethyl ether (or chloroform) was tested using the saturated galactolipids as substrates. No significant hydrolysis of these substrates in the presence or absence of the additional solvents was observed (Table IV).

Kinetics and Mode of Action.—Incubation of mono- and digalactosyldilinolenin with enzyme preparation I (unbuffered) for 60 minutes resulted in the changes shown in Table V. With each substrate the reduction in acyl ester was accompanied by a release of fatty acids (linolenic acid) and water-soluble carbohydrate. The molar ratios of ester hydrolyzed to fatty acid liberated to total water-soluble carbohydrate were

TABLE V

PRODUCTS AND STOICHIOMETRY OF ENZYMATIC HYDROLYSIS OF MONO- AND DIGALACTOSYL DIGLYCERIDES[a]

Products Formed	Monogalactosyl-dilinolenin	Digalactosyl-dilinolenin
Acyl ester reduction (moles/mole substrate)	0.43	0.79
Linolenic acid liberated (moles/mole substrate)	0.42	0.75
Total water-soluble sugar (moles/mole substrate)	0.27	0.84
Mole ratio (ester–fatty acid–total sugar)	1.02:1.00:0.64	1.05:1.00:1.12

[a] Reaction mixtures contained 2.24 μmoles monogalactosyldilinolenin or 2.28 μmoles digalactosyldilinolenin, and 0.8 ml of enzyme preparation I (7.4 mg protein/ml); incubation at 25° for 60 minutes, without buffer. Values are corrected for endogenous ester or sugar in the enzyme preparation by means of a zero-time control.

close to the expected ratios of 1:1:0.5 for monogalactosyldilinolenin and 1:1:1 for digalactosyldilinolenin. Chromatography of the chloroform-soluble products (Fig. 5) showed spots corresponding to unreacted substrate and free fatty acid, but none corresponding to galactosyl monoglycerides (lyso compounds). Chromatograms of the water-soluble products from monogalactosyldilinolenin showed a major spot corresponding to monogalactosylglycerol and faint spots corresponding to free galactose and glycerol; that from digalactosyldilinolenin showed a major spot for digalactosylglycerol and three minor ones corresponding to monogalactosylglycerol, free galactose, and free glycerol (Fig. 5).

These results were confirmed by a study of the kinetics of the enzymatic hydrolysis of mono- and digalactosyldilinolenin (Fig. 6). With monogalactosyldilinolenin, the rate of hydrolysis of the fatty acid ester groups on a molar basis was about twice that of the release of water-soluble sugar during the period studied; free galactose was essentially absent in the early stage of hydrolysis, but was released slowly after 40 minutes. With digalactosyldilinolenin, the rate of ester hydrolysis and water-soluble sugar release were identical throughout the course of the hydrolysis. Again, free galactose was essentially absent in the early stage of hydrolysis but was slowly released after 10–20 minutes.

Chromatography of the chloroform-soluble reaction products on silicic acid–impregnated paper again showed only the presence of unreacted substrate and fatty acids; "lyso" intermediates (mono- or digalactosyl monoglyceride) could not be detected even in the early stages of hydrolysis of either substrate.

These results show that the enzymes catalyze the hydrolysis of the two fatty acid ester groups in mono- and digalactosyldilinolenin, with the formation of the corresponding glycerol galactosides. The appearance of free galactose after considerable lag periods suggests the presence of galactosidases which would cleave monogalactosyl- and digalactosylglycerol.

Galactolipid-hydrolyzing Enzymes in Various Plant and Animal Sources.—Leaf supernatant fractions from various plants were tested for hydrolyzing activity towards mono- and digalactosylidilinolenin (Table VI). The three species of *Phaseolus* showed considerable activity whereas the other leaf tissues tested (and also carrot root) showed little or no activity.

Of the enzyme preparations from sources other than plants, both snake venom (phospholipase A) and extract of *P. notatum* (phospholipase B) were devoid of galactolipase activity. Pancreatic extract, however, showed considerable activity toward monogalactosyldilinolenin but little or none toward digalactosyl-

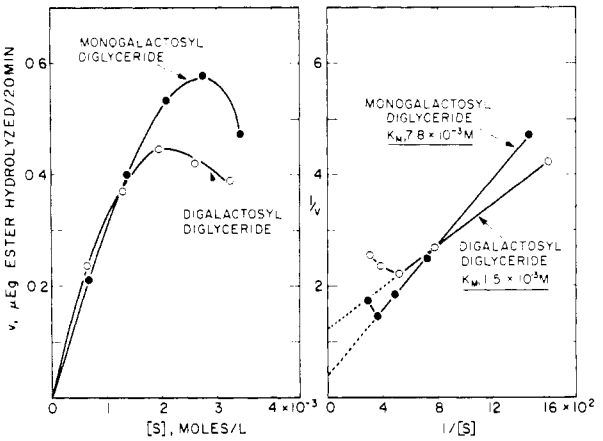

FIG. 4.—Dependence of hydrolysis rate on substrate concentration. Reaction mixtures contained 0.54–2.71 μmoles monogalactosyldilinolenin, or 0.51 to 2.57 μmoles digalactosyldilinolenin, 0.1 ml of 0.5 M phosphate buffer (*p*H 7.0 for monogalactosyl diglyceride; 5.6 for digalactosyl diglyceride), 0.2 ml of enzyme preparation II, and 0.5 ml of water (total volume, 0.8 ml); incubation at 30° for 20 minutes.

TABLE VI

GALACTOLIPID-HYDROLYZING ENZYME ACTIVITY IN VARIOUS PLANT AND ANIMAL EXTRACTS

Enzyme Source	Incubation Time (min)	Enzyme activity[a] (% reduction in acyl ester): Monogalactosyl-dilinolenin	Enzyme activity[a] (% reduction in acyl ester): Digalactosyl-dilinolenin
Runner-bean leaves (*Phaseolus multiflorus*)	30	25	21
Mung-bean leaves (*Phaseolus aureus*)	60	36	21
Kidney-bean leaves (*Phaseolus vulgaris*)	60	21	0
Soybean leaves	30	0	0
Spinach leaves	30	1	3
Cabbage leaves	30	0	0
Sugar-beet leaves	30	0	0
Squash leaves	30	0	0
Carrot root	30	0	1
Phospholipase B of *P. notatum*	60	0	0
Phospholipase A (venom of *Agkistrodon piscivorus*)	60	0	2
Pancreatin	30	17	0
Pancreatin	60	42	1

[a] Reaction mixtures contained 0.73 μmole monogalactosyldilinolenin or 1.0 μmole of digalactosyldilinolenin, 0.1 ml of 0.5 M phosphate buffer (*p*H 7 for monogalactosyldilinolenin, 5.6 for digalactosyldilinolenin) and 0.7 ml of plant extract or pancreatin. With the *P. notatum* enzyme, 0.2 ml of enzyme + 0.5 ml of water + 0.1 ml of 0.5 M acetate buffer, *p*H 4.4, was used. With snake venom, 25 μl of a solution containing 1.0 mg lyophilized venom per ml of 0.005 M $CaCl_2$ solution + water to 0.8 ml total volume was used; buffer was omitted and the mixture was shaken with 1 ml of ethyl ether. Incubation at 25° for times indicated.

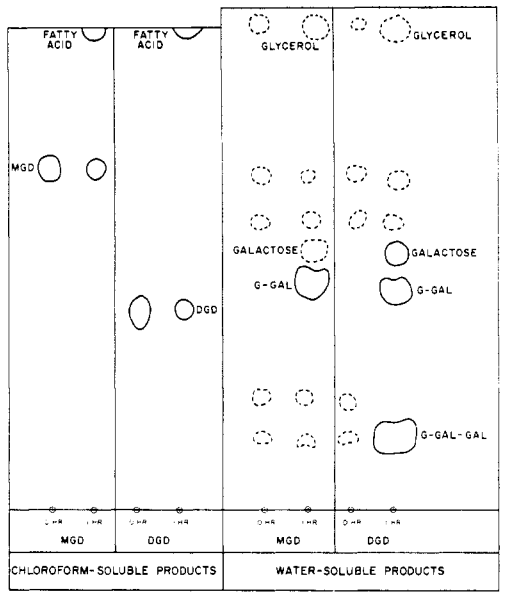

FIG. 5.—Chromatograms of chloroform-soluble and water-soluble enzymatic-hydrolysis products of monogalactosyl diglyceride and digalactosyl diglyceride; reaction mixtures as in Table V. Abbreviations: G-Gal, galactosylglycerol; G-Gal-Gal, digalactosylglycerol; MGD, monogalactosyl diglyceride; DGD, digalactosyl diglyceride. Dashed lines indicate faint spots. The faint spots found for the zero-time controls arise from the enzyme preparation.

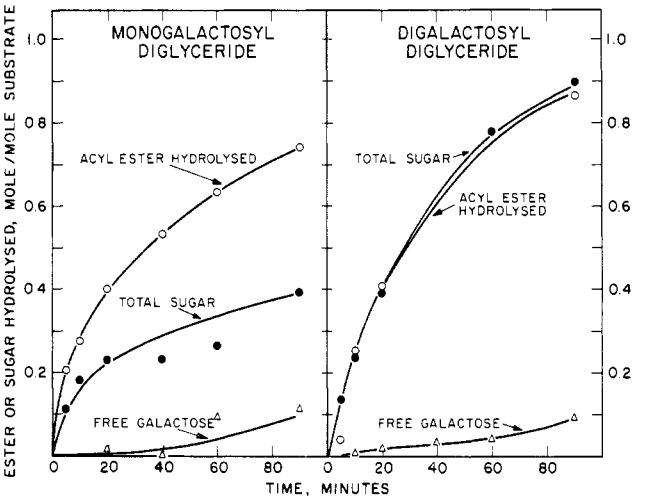

FIG. 6.—Enzymatic hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride as a function of time. Reaction mixtures contained 1.65 μmoles of monogalactosyldilinolenin or 1.55 μmoles of digalactosyldilinolenin, 0.15 ml of 0.5 M phosphate buffer (*p*H 7.0 for monogalactosyl diglyceride; 5.6 for digalactosyl diglyceride), 0.3 ml of enzyme preparation II, and 0.75 ml of water (total volume, 1.2 ml); incubation at 30° for various times up to 90 minutes.

dilinolenin. Chromatography of the chloroform-soluble products of the pancreatin digest of monogalactosyldilinolenin on silicic acid–impregnated paper showed the presence of unreacted monogalactosyldilinolenin and a considerable amount of neutral lipid, probably mono- and diglycerides and free fatty acids. The enzymes in pancreatin thus appear to differ in their mode of action from the runner-bean enzymes.

DISCUSSION

The foregoing results establish the presence in runner-bean leaves of enzymes catalyzing the hydrolysis of monogalactosyl- and digalactosyldilinolenin. These enzymes are associated with the chloroplasts and are also present in soluble form in the cell-sap cytoplasm. On the basis of the stoichiometric (Table V) and chromatographic (Fig. 5) data, the following reactions are presumed to be catalyzed by these enzymes:

monogalactosyldilinolenin → [galactosyl monoglyceride] + linolenic acid
→ galactosylglycerol + linolenic acid (1)

digalactosyldilinolenin → [digalactosyl monoglyceride] + linolenic acid
→ digalactosylglycerol + linolenic acid (2)

Further hydrolysis of the galactosylglycerols is catalyzed by α- and β-galactosidases, also present in the cell-sap cytoplasm, as follows:

Digalactosylglycerol
$\xrightarrow{\alpha\text{-galactosidase}}$ monogalactosylglycerol
$\xrightarrow{\beta\text{-galactosidase}}$ glycerol + galactose (3)

Thus runner-bean leaves possess the enzymes necessary for complete breakdown of galactolipids to fatty acids, glycerol, and galactose, and this breakdown is manifested immediately upon breaking the leaf cells. Presumably these reactions may also occur in the intact cells, but the breakdown products are then reconverted to galactolipids by the biosynthetic systems in chloroplasts (Ferrari and Benson, 1961; Neufeld and Hall, 1964).

It should be mentioned that no direct evidence for the intermediate formation of "lyso" compounds (galactosyl monoglycerides) in the above sequence of reactions could be obtained. Presumably the rates of deacylation of the "lyso" compounds are much greater than those of the parent galactolipids, thus preventing the accumulation and detection of the lyso compounds. This explanation is supported by the fact that water-soluble sugar released was always in stoichiometric proportion to the ester hydrolyzed even in the very early stages (Fig. 6). If appreciable amounts of lyso compounds accumulated, the ratio of ester hydrolyzed to water-soluble sugar released would be greater than theoretical.

The question arises whether reactions (1) and (2) are catalyzed by one enzyme or by separate enzymes, each specific for mono- or digalactosyldilinolenin. Although the present data are insufficient to settle this question unequivocally, the following observations are pertinent: (a) Considerable decreases in the activity of enzyme preparation II toward digalactosyldilinolenin but not toward monodigalactosyldilinolenin occurred on storage at 4° for several days. (b) During preparation of enzyme preparation I, the supernatant obtained after removal of the precipitated enzyme was active only toward digalactosyldilinolenin but not monogalactosyldilinolenin, whereas the precipitate and original starting material were active toward both substrates. (c) The optimum *p*H and Michaelis-Menten constants are different with the two substrates. These observations suggest that two separate enzyme systems may be involved, and that attempts to separate them would be feasible.

The enzyme preparation appears to have a specificity with regard to the degree of unsaturation of the fatty acids in the substrates, since no hydrolysis of the fully saturated galactolipids could be detected (Table III).

In the absence of stimulating solvents, plant phospholipase D also appeared to require unsaturated substrates; however, in the presence of ethyl ether or methanol-ether mixtures, saturated substrates were readily hydrolyzed (Kates, 1956, 1957). Unfortunately, ethyl ether could not be used as stimulating solvent for the galactolipid-hydrolyzing enzymes because of its inhibitory effect (Table IV). However, by analogy with the plant phospholipase D, it seems reasonable to suppose that the apparent requirement for unsaturated substrates is due to inaccessibility of the saturated substrates to the galactolipid-hydrolyzing enzymes, and that activity could probably be demonstrated if the saturated substrates were put into true micellar dispersion.

Significant galactolipid-hydrolyzing activity was found in the cell-sap cytoplasm of the leaves of the three species of *Phaseolus* tested, but was absent in similar preparations from soybean, a member of a closely related family (Table VI). Galactolipid-hydrolyzing activity is also absent in extracts of *Scenedesmus* (Yagi and Benson, 1962). These enzymes thus appear to be confined to the *Phaseolus* family. However, it is still possible that galactolipases are actually present in the other plant sources (perhaps associated with plastids) but are prevented from acting on their substrates by the presence of inhibitors. Thus inhibitors of phospholipase D have been demonstrated in the cell-sap cytoplasm of spinach leaves (Kates, 1954) and cabbage leaves (Tookey and Balls, 1956) and account for the low activity in these preparations; the washed chloroplast fractions are, however, fully active. A more extensive and detailed survey, in which chloroplast as well as cytoplasm fractions are tested, is therefore necessary to establish whether or not galactolipid-hydrolyzing enzymes are widely distributed in the plant kingdom.

Since herbivores consume large quantities of galactolipids and sulfolipids, which are present in grasses (Weenink, 1961, 1963), it is reasonable to expect that they are able to digest these substances. This has now been established by the present finding that beef pancreatin shows galactolipid-hydrolyzing activity (Table VI), and by the previous finding of Yagi and Benson (1962) that pancreatin has sulfolipase activity. It should be noted, however, that the mode of hydrolysis of galactolipids by pancreatin appears to be different from that found for the leaf enzymes.

From the present results (Fig. 1) it is clear that runner-bean chloroplasts isolated by conventional procedures are deficient in galactolipids, lecithin, and phosphatidylethanolamine, as a result of enzyme degradation during the isolation procedure. The previously reported losses of lipids during isolation of Black Valentine bean chloroplasts (Mego and Jagendorf, 1961) may have been caused by galactolipase and phospholipase action, and the fact that higher lipid contents are obtained with chloroplasts isolated in the presence of formaldehyde offers a method for circumventing this enzymatic degradation.

Although enzymatic degradation of galactolipids and phosphatides does not appear to be extensive during the preparation of spinach chloroplasts, the previously reported lipid analyses of chloroplasts from spinach and other leaves (Benson *et al.*, 1959; Wintermans, 1960; Zill and Harmon, 1962) may have to be reevaluated in the light of the present findings.

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