Biochimica et Biophysica Acta, 526 (1978) 387—397

BBA 68557

URIDINE DIPHOSPHATE GLUCOSE:FATTY ACID GLUCOSYL TRANSFERASE ACTIVITY OF RAPE (*BRASSICA NAPUS* L.) ANTHER TISSUE

G.R. CHANDRA, N. MANDAVA, J.D. WARTHEN, Jr.

USDA, ARS, Agricultural Research Center, Beltsville, Md. 20705 (U.S.A.)

(Received March 3rd, 1978)

Summary

Particulate fractions from mature rape anther tissue catalysed the incorporation of glucose from UDPglucose into endogenous lipids. The UDPglucose:fatty acid glucosyl transferase was tightly bound to particulates that could be suspended in 0.1% Triton X-100. The apparent K_m for UDPglucose was $3.5 \cdot 10^{-6}$ M. In Tris buffer the enzyme has a pH optimum of 8.0 and a temperature optimum of 30°C. In the presence of NaF, the transferase activity was proportional to protein concentration, time, and other variables of the reaction mixture. ADPglucose, GDPglucose, and CDPglucose did not compete with the activity of the UDPglucose-dependent glucosyl transferase. Gas chromatographic and high performance liquid chromatographic analyses indicated that the endogenous fatty acids glucosylated in vitro had carbon chain lengths of C_{16} to C_{20}. This is the first report on the biosynthesis of non-phosphorylated glucosyl esters of fatty acids in higher plants.

Introduction

Several plant enzyme systems that catalyze the formation of sugar-linked lipids have been reported [1]. In mung bean seedlings the incorporation of mannose from GDPmannose into endogenous lipids is mediated by a particulate-bound transferase [2,3]. A similar particulate-bound enzyme from cotton fibre catalyzes the incorporation of mannose from GDPMan and glucose from UDPGlu into endogenous membrane-bound lipids [4—6]. In both these plant systems, the donor of the carbohydrate moiety is a nucleoside diphosphate sugar, but the nature of the acceptor lipids is unknown. Little is known of the

Use of a company or product name does not imply approval or recommendation by the Department of the product to the exclusion of others which may also be suitable.

biosynthesis of glycolipids, though some progress has been made in identifying the intermediates in the formation of glucophospholipids. For example, as in mammalian systems [7], glycophospholipids in the mung bean and cotton fibre systems are derived from glycosyl phosphoryl polyisoprenols [8,9]. Also, cell-free preparations from *Tetrahymena pyroformis* catalyze the incorporation of glucose from UDPG into lipids with properties similar to those of dolichyl phosphate sugar derivatives [10].

Our interest in the biosynthesis of glycolipids was generated by the discovery [11] that lipids with phytohormone activity could be fractionated from rape pollen tissue. Bulk lipid preparations from rape pollen yielded a complex containing many long chain (C_{16}—C_{24}) nonphosphorylated glycolipids [12]; and one component of the complex was a glucosyl ester of linoleic acid. We, therefore, undertook to characterize UDPGlu:fatty acid (UDPGlu:FA) transferase activity of rape anther tissue and identify the enzymatically formed glucolipids.

Materials and Methods

Rape plants (*Brassica napus* L.) were grown in the greenhouse. Mature floral buds were harvested 2—3 days before opening; and the anther tissue was excised and then stored at −18° C.

Based on many preliminary experiments, the following procedure was adopted for the routine preparation of the particulate-bound glucosyl transferase from rape anther tissue. The frozen tissue was thawed and refrozen twice before extraction. 1 g frozen tissue was mixed with 1 g acid-washed sand and homogenized with 5 ml grind buffer (50 mM Tris/HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, and 0.1 mM dithiothreitol). The homogenate was diluted to 25 ml with grind buffer and centrifuged at 12 000 × *g* for 10—15 min. The supernatant was further centrifuged at 105 000 × *g* for 1 h and the pellet (bulk particulates) was frozen in solid CO_2 and homogenized with 1 ml 0.1% Triton X-100. The particulate suspension was centrifuged at 500 × *g* for 5 min, and the turbid supernatant was used as source of the glucosyl transferase enzyme.

Protein was determined according to a simplified Lowry's procedure [13]. All assays were performed in duplicate or triplicate and values were averaged.

Activity of the UDPGlu:fatty acid glucosyl transferase was assayed in 250 µl reaction mixture: 25 µmol Tris/HCl (pH 8.0), 10 µmol KCl, 10 µmol $MgCl_2$, 0.5 µmol dithiothreitol, 0.1 µCi UDP-[^{14}C]glucose (SA 310 Ci/mole, Amersham), 50 µmol NaF and enzyme solution corresponding to 100 µg protein. The mixture was incubated at 30° C for 30 min, and 10 µmol non-radioactive UDP-Glu was then added to terminate incorporation of radioactivity into the product.

The reaction mixture was freeze-dried and the residue was extracted, 4 times, with 2.5 ml $CHCl_3/CH_3OH$ (2 : 1, v/v). The combined extract was evaporated to dryness under a N_2 stream and the residue was dissolved in 100 µl CH_3OH. This solution was streaked onto Silica gel G plates (0.25 mm thick, Analtech), which were then developed with benzene/CH_3OH/acetic acid (45 : 8 : 4, v/v). The R_F values of glucolipids so chromatographed range from 0.40 to 0.45 [11]. In our chromatograms, free UDPGlu and glycopeptides were among the compounds that remained at the origin. Silica gel, from the zone

corresponding to the glucolipids was scraped from the plates and extracted with methanol for high-performance liquid chromatography analysis.

Distribution of radioactivity on thin layer chromatograms or eluates from HPLC was determined by liquid scintillation spectroscopy. Methanolic eluates of narrow bands of thin layer, as well as eluates from high-performance liquid chromatography, were analyzed in 10 ml toluene containing 40 mg PPO and 0.5 mg POPOP.

High-performance liquid chromatography, gas chromatography, and mass spectra analysis of glucolipids

HPLC was carried out at ambient temperatures on a Waters Associates ALC-100 liquid chromatograph equipped with M-6000 pumps, a U6K injector, a Model 660 programmer, a multi-wavelength ultraviolet detector (Schoeffel Instrument Corp. SF 770), and a 200-fraction collector (Buchler Fractomette). The labeled glucolipids from TLC were purified by high-performance liquid chromatography on a Waters Associates Porasil B C_{18} column (122 cm × 0.2 cm internal diameter, particle size 37—74 μm). Gradient elution (program 7) from 60 to 90% CH_3OH/H_2O over 30 min and then isocratic elution with 100% CH_3OH for 20 min were used at 2 ml/min to give 50 2-ml fractions.

The labeled glucolipids from the Porasil column were further purified on a Waters Associates μ Bondapak C_{18} column (30 cm × 0.4 cm internal diameter, particle size 10 μm). The column was eluted isocratically with methanol/water (9 : 1, v/v) at 1 ml/min, and 40 2-ml fractions were collected.

For identification, non-radioactive glucolipids were prepared and purified by the above methods. Then they were converted to their methyl esters by treatment with either methanolic HCl or BF_3/CH_3OH.

The esters were separated by a Hewlett Packard 7620A research chromatograph equipped with flame ionization detector. The column was of stainless steel (6′ × 1/6″) packed with 3% Silar 5CP on 80/100 mesh gas chrom Q. N_2 at 60 ml/min was the carrier gas and oven temp was 171°C. An LKB-9000 mass spectrometer equipped with a gas chromatograph was used for mass spectral analysis at 70 eV. The methyl esters were introduced into the mass spectrometer via a glass GC column packed with 0.75% SE-30 (178°C) and the separation was monitored by a total ion current monitor.

Results

Cellular distribution and preparation on UDPGlu:FA glucosyl transferase enzyme

To determine the cellular distribution of the glucosyl transferase, we isolated particulate fractions from tissue homogenates in buffered 0.25 M sucrose by differential centrifugation. The pelleted fractions were suspended in 0.1% Triton X-100, and transferase activity was assayed. Most of the glucosyl transferase activity was associated with the particulate fractions pelleted at 26 000 and 59 000 × *g* (Table I). The amounts of glucose incorporated into lipids by these two fractions were comparable. Microscopic examination showed that the fractions pelleted at 6700 and 26 000 × *g* were contaminated with intact pollen grains, cell wall fragments, starch grains, and other aggregated structures;

TABLE I

INCORPORATION OF [^{14}C]GLUCOSE FROM UDP-[^{14}C]GLUCOSE INTO ENDOGENOUS LIPIDS CATALYZED BY PARTICULATE FRACTIONS FROM RAPE ANTHER TISSUE

Enzyme preparation corresponding to 100 μg protein was incubated in the reaction mixture stated in the text.

Centrifugal force for fractionating particulates (1000 × *g*)	[^{14}C]glucose (pmol)	
	30 min	60 min
6.7	0.47	0.43
26.0	20.20	29.48
59.0	23.32	27.42
105.0	1.78	4.32

whereas, the other two fractions had little contamination and were considerably enriched with vesicular structures. When the particulate fractions suspended in Triton X-100 were centrifuged at high speed (105 000 × *g*), over 95% of the glucosyl transferase activity was recovered with the translucent vesicular materials pelleted. No measurable transferase activity could be demonstrated either in those supernatants or in high-speed supernatants derived from whole cell homogenates. It is possible that the fractions soluble in the detergent lack acceptor lipids. From many such experiments we concluded that the bulk of the UDPG-FA glucosyl transferase was particulate bound, presumably associated with vesicular structures. Thus, we used the procedure described in Materials and Methods for routine preparation of the glucosyl transferase. The enzyme preparation, in 0.1% Triton X-100, was also used as the source of endogenous acceptor lipids. High speed centrifugation of this suspension yielded a pellet devoid of all intact organelles and consisting essentially of transluscent, vesicular materials. Typically, the enzyme preparation (100 μg protein) incorporated 25—30 pmol glucose into endogenous lipids in 30 min at 30° C. Active enzyme preparations were also made from fresh pollen grains separated from anther tissue, as well as from pollen grains collected by bees. With respect to the transferase activity per unit weight of protein, the fresh pollen preparation was comparable to the anther preparation and 10 times as active as the preparation from the bee-collected pollen.

Kinetic properties of UDPGlu:FA glucosyl transferase

At 30° C, the enzyme preparation catalyzed at a linear rate the incorporation of [^{14}C]glucose from UDPGlu into endogenous lipids (Fig. 1). The rate of incorporation was proportional to the amount of protein in the reaction mixture, and was also dependent upon incubation temperature. The incorporation rate increased linearly between 0 and 30° C and leveled off at 40° C. The enzyme was inactivated when the preparation was heated at 95° C for 5 min.

NaF (a general inhibitor of oxidase activity) markedly promoted glucolipid formation. The incorporation of [^{14}C]glucose into glucolipids was 60—75% greater in assay mixtures with 50 μmol NaF than in those without NaF. Concentrations of NaF higher than 50 μmol had no adverse effect on the reaction. As compared to NaF, NaN_3 at 50 μmol per assay promoted glucolipid forma-

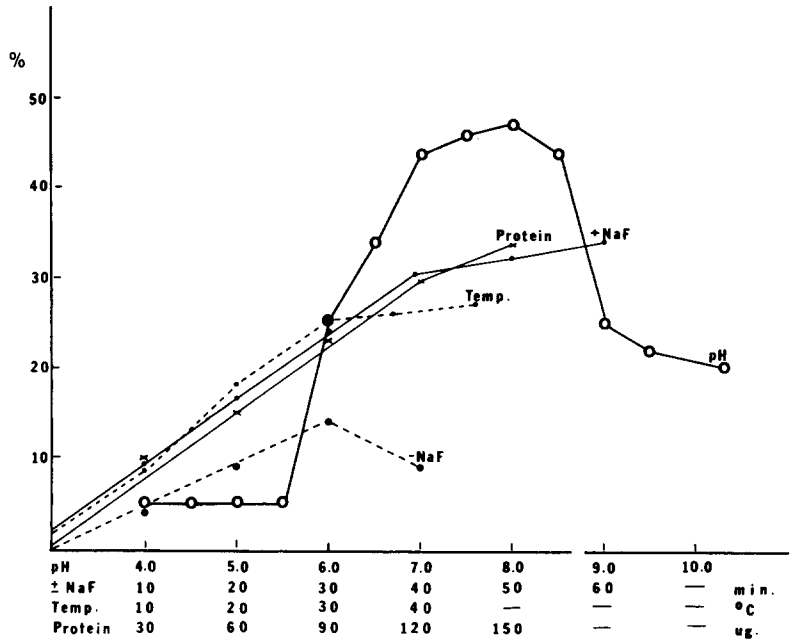

Fig. 1. Properties of UDPGlu:fatty acid glucosyltransferase. Unless indicated otherwise, the percent incorporation of [^{14}C]glucose from 0.1 μCi UDP-[^{14}C]Glu into endogenous lipids was determined in assays, containing 100 μg protein and 50 μmol NaF, incubated for 30 min at 30°C. The pH optimum was determined by incubation for 60 min at 30°C.

tion by only 40%. These results suggest that the endogenous acceptor lipids are susceptible to general oxidative degradation. The incorporation of [^{14}C]glucose into lipids also depended on the pH of the medium. Below pH 6.0, the enzyme was relatively inactive. Glucosyl transferase activity was high between pH 7.0 and 8.5 and appeared to be maximum at pH 8.0 (Fig. 1).

Table II shows the specificity of UDPGlu as a glucose donor for the formation of glucolipids. Incorporation of [^{14}C]glucose was not affected by the addition of 10 μmol GDPGlu and ADPGlu to the assay mixture but was decreased about 25% by 10 μmol CDPGlu. Because the inhibition by CDPGlu was low, we conclude that ADPGlu, CDPGlu, and GDPGlu did not compete with the UDPGlu in the UDPGlu-dependent formation of glucolipids. As expected, addition of 10 μmol non-radioactive UDPGlu completely diluted the specific activity of UDP-[^{14}C]glucose and, consequently, that of the glucolipids. Increasing the concentration of UDP-[^{14}C]glucose in the reaction mixture increased the amounts of [^{14}C]glucolipids formed. Since $7.5 \cdot 10^{-6}$ M was the lowest concentration of UDP-[^{14}C]glucose for maximum incorporation into lipids, the apparent K_m for UDPGlu was $3.75 \cdot 10^{-6}$ M.

Results of the kinetic and other properties of the glucosyl transferase provide little information on the nature of the endogenous acceptor lipids. The chemical identification of the glucolipids (see next section) prompted us to test the effects of a number of known fatty acids on the in vitro formation of glucolipids. However, none of the acids promoted glucolipid formation (Table III). In fact, all the C_{18} unsaturated fatty acids were relatively inhibitory. On

TABLE II

EFFECTS OF DIFFERENT NUCLEOSIDE DIPHOSPHATE GLUCOSES AND UDPG CONCENTRATIONS ON THE INCORPORATION OF [^{14}C]GLUCOSE FROM UDP-[^{14}C]GLUCOSE INTO ENDOGENOUS LIPIDS

Reaction mixture, as stated in the text, was incubated with different nucleoside diphosphate glucoses at 30°C for 5 min prior to the addition of UDP-[^{14}C]glucose. Results are expressed as pmol [^{14}C]glucose incorporated per 100 μg protein, 30°C for 30 min.

Nucleoside diphosphate glucose (10 μmol)	UDP-[^{14}C]glucose (μmol)	[^{14}C]glucolipids (pmol glucose)
—	1.0	23.7
CDPGlu	1.0	17.6
GDPGlc	1.0	22.8
ADPGlu	1.0	23.5
UDPGlu	1.0	0.3
—	1.5	33.0
—	3.0	44.5
—	4.5	54.0
—	7.5	99.0
—	10.0	99.5

the other hand, methyl linoleate was not inhibitory. We centrifuged the enzyme preparation, extracted the lipids from the pellet with acetone or methanol and reconstituted the enzyme preparation. However, we could never reconstitute a preparation with enzyme activity. Because of 15 such unsuccessful attempts, we think that the free fatty acids are probably not the immediate acceptor molecules for the formation of glucolipids. The glycosylation of free fatty acids may involve a labile intermediate and/or stereospecific lipid protein interactions.

TABLE III

EFFECTS OF FATTY ACIDS ON THE INCORPORATION OF [^{14}C]GLUCOSE FROM UDP-[^{14}C]-GLUCOSE INTO ENDOGENOUS LIPIDS

Results are expressed as pmol [^{14}C]glucose incorporated per 100 μg protein in 60 min incubation at 30°C. Fatty acids were obtained from P.L. Biochemicals, Inc.

Fatty acids (10 μmol/assay)	[^{14}C]glucolipids (pmol glucose/100 μg protein)
α-Hydroxylauric acid	40.8
α-Hydroxymyristic acid	43.5
α-Hydroxypalmitic acid	42.8
Stearic acid	40.5
α-Hydroxystearic acid	43.7
Linoleic acid	34.8
Linoelaidic acid	32.9
Linolenic acid	21.5
γ-Linolenic acid	22.5
Homo-γ-linolenic acid	25.1
Control	45.3
(Methyl linoleate)	(49.3)

Characterization of the enzymatically formed glucolipids

The glucolipids were stable to base and sensitive to acid hydrolysis. In 0.5 M HCl at 100°C, 97% of the glucolipids were hydrolyzed within 5 min. The mobility of the glucolipids on TLC depended upon the pH of the solvent system. The R_F values were 0.43 for pH 3.1, 0.81 for neutral pH and zero for a basic pH (Fig. 2). Iodine staining and distribution of ^{14}C-radioactivity on the thin layers indicated that TLC at pH 3.1 separated the glucolipids from 15 other components that were present in the extract (Fig. 2). The glucolipid fraction obtained by TLC was further purified by high-performance liquid chromatography on a Porasil B, C_{18} column. The glucolipids were separated from phospholipids, steroids, and other compounds, and were collected in tubes Nos. 34—40 (Fig. 3). TLC of the contents of these tubes and analysis of the distribution of radioactivity on the plates showed only the presence of glucolipids. The bulk glucolipids were then resolved, by reversed-phase chromatography on the Bondapak column, into three groups according to polarity. Radioactivity analysis indicated that the three peaks in Fig. 3 corresponded, respectively, to 29%, 62%, and 9% [^{14}C]glucolipids.

Gas chromatographic analyses showed that the glucolipids purified by HPLC on the reversed phase columns, where derivatives of 8 fatty acids. 6 of the acids were identified by carbon chain length and number of double bonds as 16 : 0, 18 : 0, 18 : 1, 18 : 2, 18 : 3, and 20 : 0 (Fig. 4). The identities of these acids

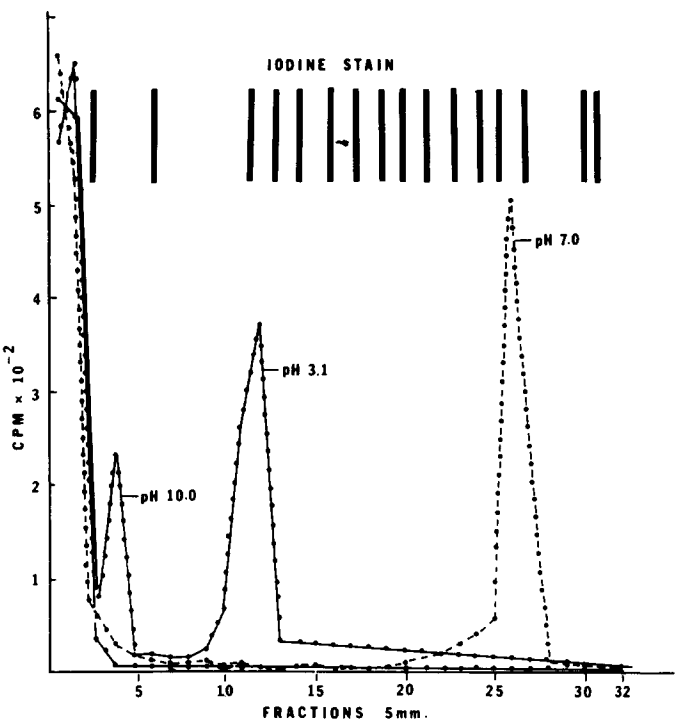

Fig. 2. TLC separation of [^{14}C]glucolipids. Solvents 45 ml benzene, 8 ml methanol, and 4 ml acetic acid (pH 3.1), 4 ml ammonium hydroxide (pH 10.0), or 4 ml distilled water (pH 7.0). Iodine stain of plates developed in pH 3.1 solvent.

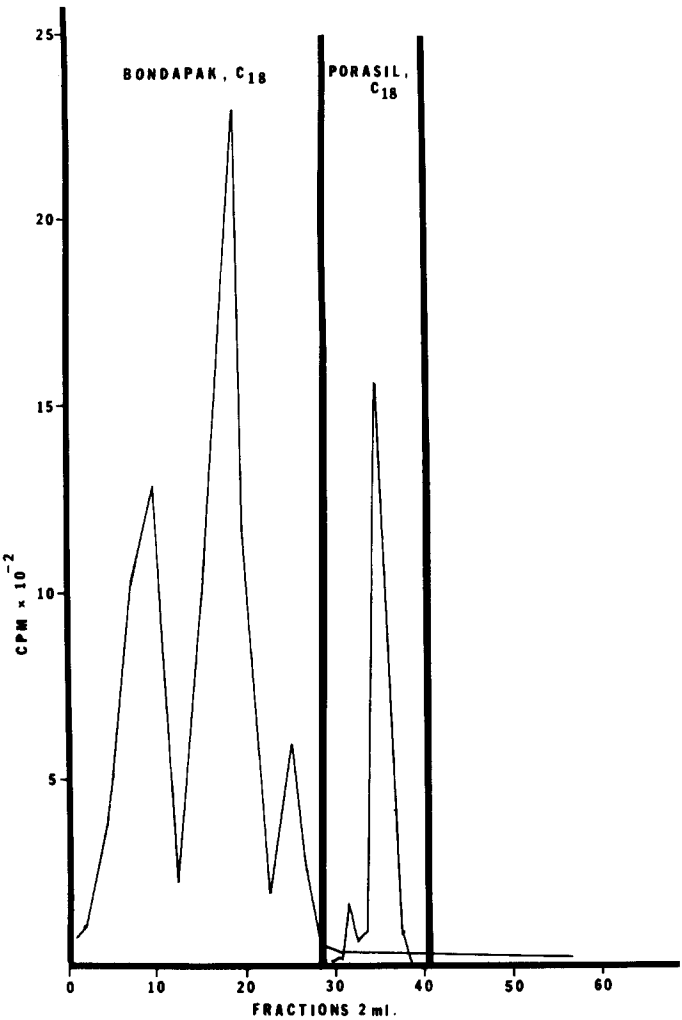

Fig. 3. HPLC profile of [^{14}C]glucolipids on μ Bondapak C_{18} and Porasil B C_{18} columns, respectively.

were established with internal standards and by gas-liquid chromatography-mass spectrometry data. The gas-liquid chromatography retention times of the other two acids indicated 17 : 0 and 17 : 1 fatty acids. However, the identifications are tentative. Although the fatty acids for gas-liquid chromatography analysis were derived from HPLC purified glucolipids (Fig. 3, Porasil C_{18}) it is not certain that all of the 8 fatty acids (Fig. 4) are glucosylated.

Discussion

Particulate-bound enzyme systems from cotton fibre, mung bean seedlings [2,6] and other plant tissue [1] are reported to catalyze the incorporation of sugar from nucleoside diphosphate sugar into endogenous acceptor lipids. Like the particulates from the cotton fibre, those from rape anther and pollen tissue catalyzed the incorporation of glucose from UDPGlu into endogenous lipids.

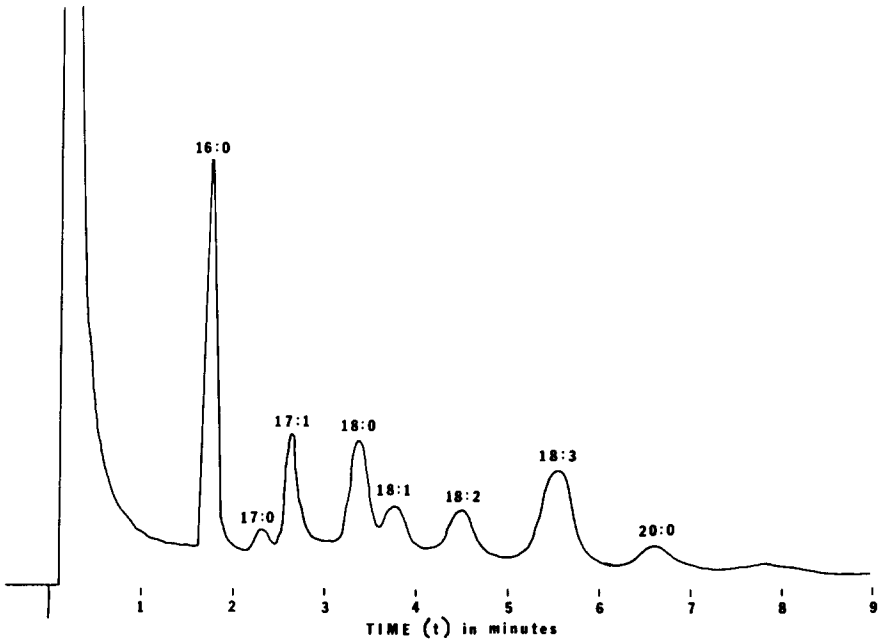

Fig. 4. GC profile of glucolipids. The glucolipids were purified by HPLC on Porasil B C_{18} and Bondapak columns (Fig. 3).

Most of the activity of the UDPGlu:fatty acid glucosyl transferase was associated with particles that could be sedimented by a centrifugal force of 26 000 or 59 000 × *g*. Further, when suspensions of both these types of particulates in 0.1% Triton X-100 were centrifuged at high speed, enzyme activity was recovered with the pellets, which consisted of vesicular structures. Clearly, therefore, the glucosyl transferase and the acceptor lipids were tightly bound to vesicular or membranous structures. The glucose transferase of rape anther tissue was specific for UDPGlu because the addition of excess ADPGlu, GDPGlu, or CDPGlu had little or no effect on the incorporation of [^{14}C]glucose from UDPGlu into lipids. At the optimum pH, 8.0, and temperature, 30°C, the apparent K_m for UDPGlu was $3.75 \cdot 10^{-6}$ M. For the sake of comparison, characteristics of GDPMan:fatty acid transferase from cotton fibre are: $K_m = 6.7 \cdot 10^{-7}$ M, pH optimum = 7.5. In preliminary experiments we had observed that the rate of formation of glucolipids in different preparations was not consistent with temperature changes. Since the glucolipids were relatively stable in the assay, we reasoned that the endogenous acceptor lipids were labile. Membrane fractions are known to be rich in general oxidases and phosphatases. In the presence of NaF, a general inhibitor of oxidase activity, the rate of formation of glucolipids was proportional to enzyme concentration and other variables of the assay. A similar beneficial effect of NaF on the glycosyl transferase activity of rat liver plasma membrane has been reported [14].

Although some progress has been made in identifying the intermediates for the formation of glycophospholipids in plant systems [8,9], little is known about the lipid precursors of glycolipids. In our study, we were unable to reconstitute an active enzyme preparation from its lipid and non-lipid compo-

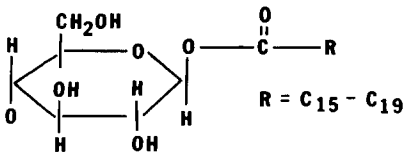

Fig. 5. Proposed structure of enzymatically synthesized glucosyl esters of fatty acids.

nents. Like the sugar transferases of cotton [6], the glucosyl transferase of rape anther tissue was inactivated by extraction with acetone or methanol. Addition of known fatty acids, differing in carbon chain length and unsaturation, had no promotive effect on the glucosyl transferase activity. Glucolipids are only one of the several glycolipids that are present in the membrane structure. Bulk phospholipids purified from cotton bolls contain arabinose, mannose, galactose, glucose, and a few other unidentified sugars linked to lipids [6]. Are all sugars attached to the same lipid or are different sugars attached to different lipids of the membrane structure? We have attempted to answer this question by a critical analysis of the enzymatically synthesized glucolipids. As determined by gas-liquid chromatography analysis, the distribution of fatty acids among the glucolipids was C_{16}, 28.8%; C_{17}, 14.2%; C_{18}, 51.5%; and C_{20}, 5.4%. Based on the elution profiles of standards, the order in which the labeled glucolipids were eluted from the Bondapak column was, in terms of their acid moieties, 18 : 3 first, followed by 18 : 2, 18 : 1, 16 : 0, 18 : 0 and 20 : 0. Fatty acid methyl esters analysis showed that the first group of lipids eluted from the μ Bondapak C-18 column (Fig. 3) contained 16 : 0, 18 : 1, 18 : 2, and 18 : 3 acid moieties; whereas, the second group mainly contained 18 : 0 acids. Thus, GC and HPLC data (Figs. 3 and 4) strongly indicate that the UDPGlu:fatty acid glucosyl transferse in rape anther tissue catalyzes the formation of glucosyl esters of fatty acids with carbon chain length C_{16} to C_{20} (Fig. 5).

The fact that purified preparations of the glucolipids were sensitive to acid hydrolysis and stable to base hydrolysis suggests that glucose was linked to the fatty acids via ester linkages. The extent to which unsaturation in the fatty acid molecule alters its acceptor properties remains to be determined in plants. The observation that free fatty acids did not promote the formation of glucolipids raises important questions concerning intermediates and stereospecificity of the lipid:protein interactions in the biosynthesis of glucosylated esters of fatty acids. Glucolipids were separated from glucophospholipids by high-performance liquid chromatography. Indeed, TLC of the glucolipids purified by the reverse-phase columns showed the complete absence of phospholipids. To our knowledge this is the first report on the biosynthesis of glucosyl esters of fatty acids in higher plants.

Acknowledgements

The authors are research chemists, G.R.C. of the Seed Research Laboratory, Agricultural Marketing, Research Institute, N.M. of the Plant Hormone and Growth Regulators Laboratory, Plant Physiology Institute, and J.D.W., Jr. of the Biologically Active Natural Products Laboratory, Agricultural Environmental Quality Institute.

References

1 Hassid, W.Z. (1972) in Biochemistry of the Glycosidic Linkage (Piral, R. and Pontis, H.G., eds.), pp. 315—335, Academic Press, New York
2 Kauss, H. (1969) FEBS Lett. 5, 81—84
3 Villemez, C.L. and Clark, A.F. (1969) Biochem. Biophys. Res. Commun. 36, 57—60
4 Villemez, C.L., Vodak, B. and Albersheim, P. (1968) Phytochemistry 7, 1561—1564
5 Forsee, W.T. and Elbein, A.D. (1972) Biochem. Biophys. Res. Commun. 49, 930—939
6 Forsee, W.T. and Elbein, A.D. (1973) J. Biol. Chem. 248, 2858—2867
7 Lucas, J.J. and Waechter, C.A. (1976) Mol. Cell. Biochem. 11, 67—78
8 Forsee, W.T. and Elbein, A.D. (1975) J. Biol. Chem. 250, 9283—9293
9 Forsee, W.T., Valkovich, G. and Elbein, A.D. (1976) Arch. Biochem. Biophys. 174, 469—479
10 Keenan, R.W., Kruczek, M. and Fusinato, L. (1975) Arch. Biochem. Biophys. 167, 697—705
11 Mitchell, J.W., Mandava, N., Worley, J.F., Plimmer, J.R. and Smith, M.V. (1970) Nature 225, 1065—1066
12 Mandava, N. and Mitchell, J.W. (1972) Chem. Ind. C. 2, 930—931
13 Schacterle, G.R. and Pollack, R.L. (1973) Anal. Biochem. 51, 654—655
14 Merritt, W.D., Morre, D.J., Franke, W.W. and Keenan, T.W. (1977) Biochim. Biophys. Acta 497, 820—824