Glucolipids of Zea mays and Pison sativum

Received for publication January 13, 1976 and in revised form February 23, 1976

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ABSTRACT

The glucolipids formed upon feeding (U-14C)glucose to embryos of Zea mays were partially characterized with respect to: (a) metabolic turnover, (b) acid lability, (c) phosphorus content, (d) chromatographic properties, and (e) hydrolysis products. The chloroform-methanol-soluble assimilated radioactivity was examined specifically for occurrence of a glycosylated prenol phosphate. With the extraction conditions used, no evidence was found for formation of a glucosylated prenol phosphate. Several, as yet unidentified, acid-labile glucolipids undergoing metabolic turnover were observed. Four diglycerides were characterized as hydrolysis products of a fraction that contained 14C-glucose and phosphorus, and was subject to metabolic turnover. Examination of the 1-butanol-soluble glucolipids from pea (Pison sativum) seedlings also demonstrated anionic glucolipids, evidence metabolic turnover but none with the properties of glycosylated prenol phosphate.

Demonstration of the importance of glycosylated phosphoprenols in microbial cell wall synthesis (14, 18, 19, 26) suggests that higher plants, where the bulk of the cell dry weight is exterior to the permeability barrier, should provide excellent experimental material for a study of glucolipid carrier lipids. The occurrence of prenols in plants is well established (16, 25), as is the participation of a lipid-soluble intermediate in cellulose biosynthesis (9). Data on the possible occurrence of a mannosyl prenol have been presented by Kauss (15) and Villemuez (24) and for a mannosyl and glucosyl prenol by Forsee and Elbein (12, 13), although in these cases chemical characterization was precluded by the small amount of material available. In this work we have surveyed the glucolipids formed by corn embryos (Zea mays) and by pea seedlings (Pison sativum). No evidence was found for incorporation of radioactive glucose into a glucosylated prenol phosphate. Evidence is presented for a number of glucosylated lipids, some of which, (a) turn over rapidly, (b) contain phosphorus, and (c) are acid-labile. A partial characterization of the diglyceride produced by hydrolysis of these fractions is presented.

MATERIALS AND METHODS

Experiments with Zea mays: Corn kernels (Stowell's Evergreen Hybrid sweet corn) were sterilized with 1% sodium hypochlorite solution for 20 min, then imbibed 15 hr in running tap water, and grown on wet filter paper for 1 day at 24 C. Embryos, including the scutellum, were pinched out of the kernels. Endosperm tissue, adhering to the scutellum, was removed. Radioactive lipids were prepared from about 100 embryos treated with 14C-glucose. A thin slice of the scutellum was removed and 10 μl of the 14C-glucose solution containing 0.1 μCi in 0.1 ml of glucose was placed on the cut scutellar surface and the embryos incubated in a Petri dish at 24 C for 1 hr. The tissue was macerated in CHC13-CH3OH (1:1, v/v), and the residue was extracted four times with the same solvent. Crude lipids were isolated (50 μg) using the scutellum exterior as a matrix. The lipids (about 6 g, 2.5 x 10^8 cpm) were applied to a silica gel column (60 g) and eluted successively with 500 ml of CHC13, 600 ml of acetone, 550 ml of CHC13-CH2OH, 1:1 (CHC13-CH2OH fraction), and 800 ml of CHC13-CH2OH-H2O, 1:1:0.3 (CHC13-CH2OH-H2O fraction). About 90% of the 14C-glucose incorporated into the lipid fraction is in glucosteroids or acylated glucosteroids (cf. 17). These were removed by the acetone elution. In some experiments, the CHC13-CH2OH fraction was chromatographed on a DEAE-cellulose column (2.3 x 17 cm) prepared in CHC13-CH2OH (2:1). The column was eluted with 600 ml of CHC13-CH2OH (2:1), 500 ml of CH2OH, and then 1 liter of a linear gradient of 0 to 0.08 M ammonium acetate in 99% CH2OH. The column effluent of the last solvent was collected in fractions of 11 ml each. The radioactive fractions, numbers 12 to 37, were pooled, and constituted the DEAE fraction.

Chasing Experiment. After incubating the embryos with 14C-glucose for 1 hr, unlabeled glucose, 5 μmol in 10 μl, was applied to each embryo and incubation continued for an additional 1 hr.

3P-labeling Experiment. About 150 embryos were incubated with 0.1 mM of 32P-phosphate in 30 ml of phosphate buffer (pH 6.5, 3 mM) for 3 hr at 24 C.

Experiments with Pison sativum. Pea seeds (var. Alaska) were grown for 3 days in the same way as corn. Ten g of the apical 3- to 5-mm tips of shoots and roots from 1.5-cm long seedlings were incubated in 30 ml of 14C-glucose solution, 50 μCl/50 μmol, for 1 hr at 24 C. In experiments designed to measure metabolic turnover, incubation for 1 hr in 14C-glucose was followed by incubation in unlabeled glucose solutions (5 mmol in 30 ml) for an additional 1 hr. Crude lipids were extracted with H2O-saturated 1-butanol and chromatographed on a DEAE-cellulose column (50 g) with the following gradients: 100 ml from 30 g of unlabeled prenol and added to the radioactive lipids. Elution was the same as described for experiments with corn embryos. The CHC13-CH2OH fraction was chromatographed on a DEAE-cellulose column (2.3 x 13 cm) and eluted with 400 ml of 3.7 mM and then 300 ml of 50 mm ammonium acetate in 99% CH2OH (designated as 7.5 mm-acetate fraction and 50 mm-acetate fraction, respectively). These fractions were treated with alkali to decylactate phospholipids according to the procedure of Scher et al. (18).

Thin Layer Chromatography. TLC was performed on pre

1 This work was supported, in part, by the National Science Foundation (GB-18353X and GB-40821X). Scintillation counting equipment was provided by the United States Atomic Energy Commission, Contract AT-(11-1)-1338, and mass spectrometric facilities by a National Institute of Health grant (PHS RR-00480) to Professor C. C. Sweeney. Journal Article 7089 from the Michigan Agriculture Experiment Station.

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3 Abbreviations: 14C-glucose: uniformly labeled glucose; M*: molecular ion; m/e: mass per electron; Si(CH3)3: trimethylsilyl.
coated silica gel plates (0.25 mm, Merck) with the following solvent systems: A, CHCl₃-CH₂OH-H₂O (65:25:4); B, benzene-CH₂OH (2:98); C, n-heptane-ethyl acetate (9:1). Visualization was accomplished with iodine vapor or Rhodamine 6G for lipids (22) and molybdenum reagent (10) for phospholipids. The bands and areas between the bands were scraped from the TLC plate and transferred to a scintillation vial for radioactivity determination with Bray's solution.

Gas-Liquid Chromatography. GLC analysis was with a Hewlett-Packard 402 chromatograph equipped with a flame ionization detector. The columns used are indicated in the legends. N₂ was the carrier gas with a flow rate of 60 ml/min. Combined GLC-MS analysis was conducted with an LKB-9000 instrument. Silylation was with trimethylsilylimidazole, 1 m in pyridine, for 1 hr at 50 °C.

Sources of Materials. DEAE-cellulose was from Sigma Chemical Co., U-¹⁴C-glucose from Biochemical Nuclear Corp., GLC-column packings from Applied Science-Anspec, seeds of Pisum sativum var. Alaska from Vaughan's Seed Co., silica gel TLC plates and silica gel for columns was a Merck product from Brinkmann, and Zea mays var. Stowell's Evergreen Hybrid from Ferry-Morse.

RESULTS AND DISCUSSION

Glucolipids from Zea. Polysoprenyl phosphate sugar has been shown to be an intermediate of sugar transfer from sugar nucleotides to acceptors such as cell wall polymers in bacteria (14, 18, 19, 26), yeast (20), and animal liver protein (6, 8). We undertook an examination of glucolipids in plant tissues to determine whether similar lipids, with similar functions, occurred in higher plants. In this work, ¹⁴C-labeled glucose was supplied to corn embryos and pea seedlings because glucose is a major endogenous sugar and glucans are abundant polymers. If polysoprenyl phosphate glucose was synthesized, it should be recovered in the CHCl₃-CH₂OH eluate of a silica gel column (14, 15) or in the ammonium acetate eluate of a DEAE column (cf. 18). If, however, polysoprenyl phosphate oligosaccharide is formed, as in animal liver, it should be recovered in the chloroform-methanol-water eluate of a silica gel fraction (7). With this information in mind the DEAE and CHCl₃-CH₂OH-H₂O fractions from corn germ were examined by TLC (Fig. 1), and the acid lability of the components was determined (Table 1). Bands 2, 4, 5, and 6 from the DEAE fraction and band 5-1 from the CHCl₃-CH₂OH-H₂O fraction were found to be incompletely hydrolyzed by 0.01 m HCl in 50% CH₂OH in 10 min at 100 C (Table 1). They were too stable to acid hydrolysis to be glucosyl phosphopolyprenols (18) and were not further examined. The radioactivity of band 5-2 from the CHCl₃-CH₂OH-H₂O fraction was 90% hydrolyzed by weak acid under the above conditions. Total phosphorus in fraction 5-2 was determined colorimetrically (1) and the glucose content by quantitative GLC. A small amount of phosphorus was detected in some preparations, the molar ratio of phosphate to glucose being 1:11, but in samples purified by repeated TLC no phosphorus was detected. The lipid moiety obtained from fraction 5-2 by acid hydrolysis was more polar than would be expected for prenol hydrolysis products (11, 14, 23) with an RF of 0.1 in solvent A and immobile in solvent C. The fraction was not subject to metabolic turnover since the radioactivity of 5-2 did not decrease after a 1-hr chase of ¹⁴C-glucose with unlabeled glucose and thus, apart from its acid lability, compound 5-2 differed from prenol phosphogluco-lose.

The CHCl₃-CH₂OH fraction was also characterized with respect to its TLC behavior and its metabolic turnover after a cold glucose chase (Fig. 2). Of the bands showing turnover, bands 6 and 10 had higher RF values, 0.5 and 0.8, respectively, than did prenol phosphoglucose with RF values of 0.15 and 0.3. Although no distinct band could be detected in regions 1 and 2, when these regions were eluted, concentrated, and chromatographed on TLC, four spots (RF in solvent A: 0.07, 0.14, 0.20 and 0.25) were detected, the two with lower RF being radioactive. These two fractions were partially purified by preparative TLC. About 95% of the radioactivity of both fractions was recovered in the H₂O phase after weak acid hydrolysis. As the amount of these fractions was too low for analysis of their phosphorus content, an experiment was conducted to determine whether ³²P was incorporated into the fractions. Incorporation of ³²P into the fractions of regions 1 and 2 was barely detectable. Since these data showed the labeled fractions of regions 1 and 2 to have a low RF value, a slow incorporation of ¹⁴C-glucose, and no incorporation of ³²P, we concluded that these fractions could not be prenol phosphoglucose.

The radioactivity in region 4 was high and subject to metabolic turnover. This region was eluted, concentrated, and examined by TLC. Following a single preparative TLC step, the fraction was found to be contaminated with bands 3 and 5. Since more material was being applied to the TLC plate, band 4 could now be detected with I₂ vapor or the phospholipid reagent (Fig. 3). Phosphorus was detected by the Ames' method (1) in a sample twice chromatographed by preparative TLC. About 70% of the

Table 1. Acid Lability of Radioactive Glucolipids from Zea mays

Hydrolysis was in 0.01 m HCl in 50% methanol at 100 C for 10 min. The hydrolysate mixture was extracted with chloroform and radioactivity remaining in the aqueous phase was used as a measure of the degree of hydrolysis to free glucose. Bands 2, 4, 5, and 6 were eluted from the DEAE fraction of Fig. 1. Bands 5-1 and 5-2 were eluted from sector 5 of Fig. 1 with 5-1 containing 35% of the radioactivity of band 5.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Hydrolysis</th>
</tr>
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<tbody>
<tr>
<td>DEAE-cellulose</td>
<td></td>
</tr>
<tr>
<td>Band 2</td>
<td>52</td>
</tr>
<tr>
<td>Band 4</td>
<td>14</td>
</tr>
<tr>
<td>Band 5</td>
<td>8</td>
</tr>
<tr>
<td>Band 6</td>
<td>23</td>
</tr>
<tr>
<td>Silica-gel, CHCl₃-CH₂OH-H₂O</td>
<td></td>
</tr>
<tr>
<td>Band 5-1</td>
<td>53</td>
</tr>
<tr>
<td>Band 5-2</td>
<td>92</td>
</tr>
</tbody>
</table>

Fig. 1. Polar and anionic lipids of Zea. Iodine vapor visualized substances and their radioactivity on a TLC (solvent A) of the DEAE and CHCl₃-CH₂OH-H₂O lipid fractions of Zea. The lipids were extracted from 1.5 and 0.5 g of corn embryos respectively for a and b.
The column of figures on the left shows the radioactivity of the indicated sector of the plate after a 1-hr incubation with $^{14}$C glucose, and the figures on the right show radioactivity as above, but after a 1-hr chase with unlabeled glucose. Chromatography was with solvent A.

radioactivity was recovered in $\text{H}_2\text{O}$ following hydrolysis with weak acid. The lipid moiety liberated by acid hydrolysis of band 4 (0.1 N HCl in 50% CH$_3$OH, 100 C, 8 min) was examined by TLC. Four spots were detected with $I_2$ vapors with $R_f$ in solvent B of: spot 1, 0.14; spot 2, 0.42; spot 3, 0.57; and spot 4, 0.91. GLC analysis of the Si(CH$_3$)$_3$ derivatives showed two discrete peaks, a and b, for each (Table II). Each peak was examined by combined GLC-MS.

Components 4-a and 4-b. From the GLC retention times (Table II), MS fragmentation patterns, and by comparison with authentic standards, 4-a and 4-b were identified as methyl palmitate ($M^+$ = 270) and methyl linoleate ($M^+$ = 294), respectively. Methyl esterification of fatty acids occurs during hydrolysis.

Components 2-a and 3-a. $M^+$: corresponding to palmitoyl linoleoyl diglyceride-Si(CH$_3$)$_3$ at m/e 664 is not observed (Table III), but M-CH$_3$ at 649 is seen as has previously been reported (3). The ion at m/e 574 is M-HOSi(CH$_3$)$_3$ while m/e 408 and 385 would be produced by loss of palmitic and linoleic acids, respectively. The ions m/e 395 and 371 correspond to loss of an acyloxy group together with methylene, M-CH$_2$ palmitate, and M-CH$_2$ linoleate, respectively (3). The large 395 and 371 in the spectra of 3a shows it to be a 1,3-diglyceride-Si(CH$_3$)$_3$ (3). The ion m/e 313 is CH$_2$(CH$_3$)$_4$COOSi(CH$_3$)$_3$ and m/e 129 is M-palmitic acid-linoleate. From the differences in fragmentation patterns (3) and the difference in polarity of 2-a and 3-a on TLC (22) it may be concluded that 2-a is a 1,2-diglyceride-Si(CH$_3$)$_3$ and 3-a is a 1,3-diglyceride-Si(CH$_3$)$_3$. Further, the intense ion at 385 in the spectrum of 2-a shows the linoleate to be at C-2 (3).

Components 2-b and 3-b. The molecular ion at m/e 690 is absent, but m/e 675 is M-CH$_3$ for oleoyl linolinoyl diglyceride-Si(CH$_3$)$_3$, and m/e 600 is M-HOSi(CH$_3$)$_3$. Loss of linoleate and oleate lead to m/e 411 and 409, respectively, and m/e 397 and 395 represent loss of an acyloxy group with methylene, M-CH$_2$ linoleate, and M-CH$_2$ oleate, respectively. The ion at m/e 129 is described above. Again, the relative mobilities of 2-b and 3-b and the m/e 397 and 395 in the spectrum of 3-b indicate it to be a 1,3-diglyceride-Si(CH$_3$)$_3$, whereas 2-b is a 1,2-diglyceride-Si(CH$_3$)$_3$ (3, 22). Since oleic acid was not detected as a product of acid hydrolysis, further characterization is required.

Components 1-a and 1-b. These were not identified. The highest m/e was at 313, but it is uncertain whether this signal is that of the molecular ion. Fragment ions differing by 14 mass units were obtained at m/e = 43, 57, 71, 85, and 99, and thus these components seem to be long chain hydrocarbons.

The water-soluble components of the acid hydrolysis were silylated and examined by GLC. Peaks for authentic $\alpha$ and $\beta$-glucose were observed at retention times of 4.1 and 6.6 min at a column temperature of 150 C and the silylated hydrolysis mix-

Table II. Chromatographic Analysis of Lipids

<table>
<thead>
<tr>
<th>Location on TLC</th>
<th>GLC.Columns</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(a) 1st peak</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td>^C</td>
</tr>
<tr>
<td>R$_f$ 0.14-Spot 1</td>
<td>30</td>
<td>4.65</td>
</tr>
<tr>
<td>R$_f$ 0.42-Spot 2</td>
<td>270</td>
<td>7.6</td>
</tr>
<tr>
<td>R$_f$ 0.57-Spot 3</td>
<td>270</td>
<td>8.1</td>
</tr>
<tr>
<td>R$_f$ 0.91-Spot 4</td>
<td>133</td>
<td>5.8</td>
</tr>
<tr>
<td>Methyl palmitate</td>
<td>133</td>
<td>5.8</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>133</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Table III. Mass Spectral Analysis of Lipids

Some characteristic ions in the 70 ev mass spectrum of the Si(CH$_3$)$_3$ derivatives of components 2a and 3a and 2b and 3b of Table II. The numbers represent relative amounts of fragment ions of the indicated mass when m/e = 73 is normalized to 100.
A plant not in acid-stable spot (RF stable to contained eluted, concentrated, solvent A). Weak acid lipids were shown which disclosed multiplicity of glucolipids of Mycoplasma (21). Some phospholipids were detected, but only RF spot = 2. In Fig. 2, RF spot = 1.5. No phospholipids were detected in the 50 mM-acetate fraction. The CHCl₃:CH₂OH:H₂O fraction (Fig. 4) contained a major radioactive phospholipid band (band 6), which showed some metabolic turnover, but was found to be stable to weak acid hydrolysis. When regions 1 and 2 were eluted, concentrated, and chromatographed, one phospholipid spot (RF = 0.25) was obtained. Its radioactivity was comparatively acid-stable since only 47% of its radioactivity was recovered in H₂O after weak acid hydrolysis. Thus, in the 1-butanol extracts of pea seedlings, some glucolipids containing phosphorus are found, but they are relatively acid-stable and do not have the properties of prenolipoglucolipase.

Examination of the glucolipids of corn and peas failed to disclose a compound with the chemical characteristics of a prenolipoglucolipase. Negative data are not conclusive, but the multiplicity of compounds exhibiting turnover and having the expected phosphorus content and acid lability suggests that these compounds could play a role analogous to that of the prenolipoglucolipase and would warrant detailed chemical characterization. Behrens and Cabib (5) and Ankel et al. (2), using other nonbacterial systems, were also unable to find evidence for a prenol intermediate of sugar transfer.

LITERATURE CITED