SYNTHESIS OF HAMAMELOSE-DIPHOSPHATE BY ISOLATED SPINACH CHLOROPLASTS

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1. Introduction

The branched-chain hexose hamamelose (2-C-(hydroxymethyl)-D-ribose) occurs in most higher plants. Sellmair [1] and Van Scherpenberg et al. [2] found this sugar in 75 percent of 560 species of higher plants (belonging to 110 families). However, in most cases the amounts of hamamelose in the leaves were so small that detection was only possible by the isotope method of Van Scherpenberg et al. [2].

Experiments on the biosynthesis of hamamelose with whole leaves demonstrated that this sugar is formed only in the light and that its synthesis is most likely located in the chloroplasts [3, 4].

The present paper shows the formation of hamamelose diphosphate by isolated spinach chloroplasts.

2. Methods

2.1. *Preparation of chloroplasts*

Spinach leaves were homogenized with an Ultraturrax for 5 sec in the medium described by Cockburn et al. [5]. After filtration through cheesecloth the suspension was centrifuged for 1 min at 2000 *g*. The pellet was suspended in the buffer B of Jensen and Bassham [6]. Centrifugation at 30 *g* (1 min) removed whole cells and greater particles. From the supernatant the chloroplasts were spun down at 500 *g* (1 min) and resuspended in the same buffer. Microscopic observation of the suspension indicated almost no broken chloroplasts. All steps were performed at 0°.

2.2. ^{14}C-*Fixation by chloroplasts*

For CO_2-fixation the chloroplasts were incubated in 1 ml of the buffer C used by Jensen and Bassham [6]. The concentrations of $NaH^{14}CO_3$ (4.6 mCi/mmole) and of chlorophyll were adjusted to those given by the same authors. The experiments were run in conical glass tubes; to avoid sedimentation of the chloroplasts, nitrogen was bubbled through the suspension (2 bubbles/sec). The tubes were held in a water bath at 20°. The chloroplasts were illuminated with 110,000 lux. The reactions were stopped by adding an equal volume of boiling methanol. The chloroplast extracts were obtained by boiling the methanol-containing suspension for 3 min and removal of the membranes by centrifugation.

2.3. *Measurement of* ^{14}C-*fixation*

Aliquots of the extract and the membranes were treated with conc. acetic acid (final concn. 50%) at at 100° for 3 min to release $^{14}CO_2$. The remaining radioactivity was measured in a scintillation counter (Beckman 250 LS) using the dioxane cocktail [7]. The unacidified part of the extract was dried in high vacuo.

2.4. *Analysis of the chloroplast extract*

An aliquot of the extract was separated by 2-dimensional paper chromatography using solvents I and II (see table 2). The remaining extract was separated by paper electrophoresis on Whatman no. 3 in ammonium-formate buffer (pH 3.7). Radioautograms were prepared with Agfa X-ray paper. The distribution of the radiocarbon on the chromatograms and pherograms was investigated with a methane flow counter (Friesecke and Hoepfner, model 407).

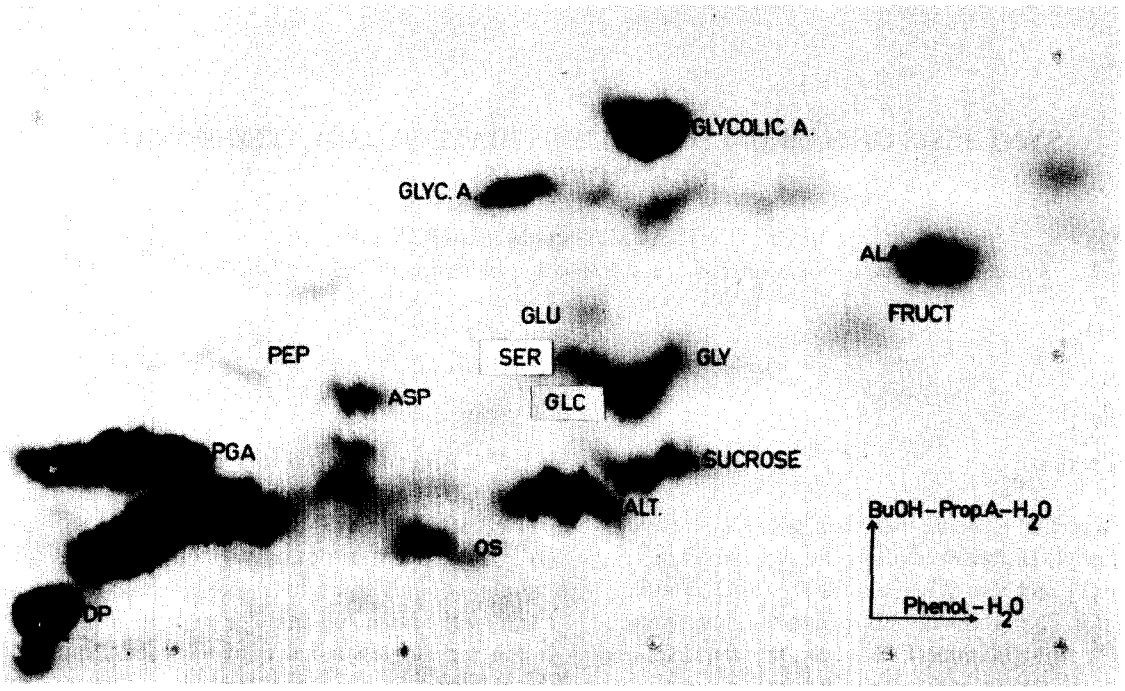

Fig. 1. Chromatogram of ^{14}C-labeled products of 15 min photosynthesis by isolated spinach chloroplasts. *Abbreviations:* ALA = alanine, ASP = aspartic acid, GLU = glutamic acid, GLY = glycine, SER = serine, GLYC.A. = glyceric acid, PEP = phosphoenolpyruvate, PGA = 3-phosphoglyceric acid, MP = sugar monophosphate, DP = sugar diphosphate, FRUCT = fructose, GLC = glucose, MALT = maltose, OS = oligosaccharide.

3. Results

^{14}C-Fixation by the chloroplasts was linear between 6 and 20 min. The fixation rates (μM CO_2/mg chlorophyll/hr) were 10 to 30 in winter and 60 to 100 in summer. CO_2 fixation by whole spinach leaves was 130 to 190 (in summer); the method used in the experiments with whole leaves was that described by Van Scherpenberg et al. [2].

A chromatogram of the soluble products of ^{14}C-fixation is shown in fig. 1 and the distribution of the radiocarbon in the extract is given in table 1. Labeled free hamamelose was detectable neither in the extract nor after dephosphorylation of the sugar monophosphates. However, when the sugar-diphosphates were dephosphorylated, a radioactive substance, which ran on the chromatogram like hamamelose, was found.

Table 1
Percentage distribution of ^{14}C in the chloroplast extract after 15 min photosynthesis in labeled bicarbonate.

Compound	% ^{14}C of the chloroplast extract
3-Phosphoglyceric acid	46.30
Sugar-monophosphates	29.40
Ribulose-diphosphate	0.37
Hamamelose-diphosphate	0.90
Fructose-diphosphate	3.94
Sedoheptulose-diphosphate	1.56
Glucose-diphosphate	0.78
Sucrose	1.38
Maltose	2.36
Hexoses	1.30
Glyceric acid	0.73
Glycolic acid	3.32
Amino acids	3.50
not identified	4.16

Table 2

$R_{glucose}$-values of hamamelose, hamamelitol, xylose, ribose and fructose.

Compound	$R_{glucose}$-value in solvent							
	I	II	III	IV	V	VI	VII	VIII
Hamamelose	1.51	1.50	1.27	1.18	1.53	1.27	1.37	1.30
Hamamelitol	1.55	1.54	1.20	1.20	1.50	1.17	1.45	1.35
Xylose	1.25	1.35	1.30	1.26	1.55	1.23	1.37	1.35
Ribose	1.56	1.67	1.50	1.53	1.68	1.45	1.51	1.50
Fructose	1.37	1.28	1.21	1.21	1.33	1.06	1.31	1.13

Solvents:
I: 88% (w/w) phenol:water: acetic acid:1 M Na_2EDTA = 480:160:10:1
II: butanol–water (solution 1):propionic acid–water (sol. 2) = 1:1
soln. 1: butanol:water = 750:50
soln. 2: propionic acid:water = 352:448
III: butanol:pyridine:water:acetic acid = 60:40:30:3
IV: butanol:ethylacetate:acetic acid:water = 4:3:2.5:4
V: butanol:acetic acid:water = 4:1:5 (upper phase)
VI: ethylacetate:pyridine:water = 140:70:30
VII: isopropanol:water:acetic acid = 75:15:10
VIII: butanol:ethanol:water = 5:1:4
The chromatograms were run on Whatman paper 1; for cochromatography the chromatograms were sprayed with alkaline silver nitrate [11] after preparation of the radioautograms.

Table 3

$R_{picrate}$-values of hamamelose, hamamelitol, xylose, ribose, ribulose and glucose on paper electrophoresis.

Compound	$R_{picrate}$-value in buffer		
	I	II	III
Hamamelose	2.00	0.81	0.92
Hamamelitol	1.87	0.83	1.23
Xylose	1.85	0.38	0.95
Ribose	1.03	0.77	0.93
Ribulose	1.76	0.84	1.42
Glucose	0.00	0.24	1.51

Buffer system conditions:
I: 0.1 M sodium-molybdate, pH 5.0, 20 V/cm, 8–10°, 6 hr
II: 0.05 M sodium-germanate, pH 10.7, 15 V/cm, 8–10°, 8 hr
III: 0.1 M sodium-borate, pH 9.2, 20 V/cm, 8–10°, 6 hr

All pherograms were run on Whatman paper 1. The pherograms were sprayed with periodate/bendizine. The germanate- and borate-complexes were destroyed by spraying the pherograms with conc. acetic acid prior to the reaction with periodate.

For further identification larger amounts of this sugar were prepared by the sixfold incubation mixture described in the methods.

3.1. *Identification of hamamelose*

Paper chromatography with 8 solvents (see table 2) and paper electrophoresis in 3 different complexing buffers (see table 3) showed no difference between the radioactive sugar and authentic hamamelose, which was prepared from hamamelitannine (for procedure see 3).

Furthermore radiogaschromatography was performed with a mixture of the radioactive sugar and authentic hamamelose (see fig. 2). The radiogaschromatogram showed agreement between the radioactive sugar and the authentic hamamelose*.

The identity of the two sugars was further confirmed by crystallization to constant specific radioactivity. For this purpose the radioactive sugar was mixed with 25 mg of authentic hamamelose and crystallized according to the method of Glick (unpublished). An aliquot of the crystals was dissolved in water and the radioactivity was measured in the scintillation counter

* Gaschromatography of the trimethylsilylated hamamelose from hamamelitannine and of synthetic D-hamamelose [8] showed full agreement. We are grateful to Dr. Williams for generous gift of synthetic hamamelose.

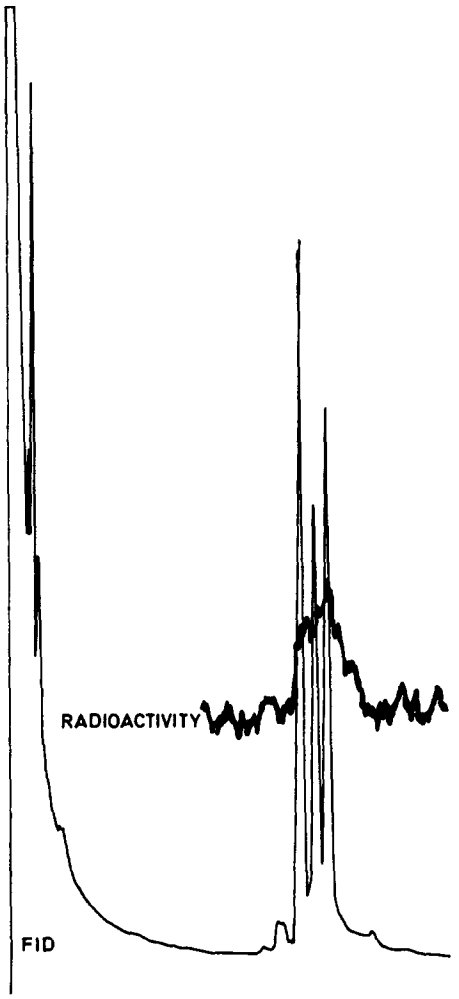

Fig. 2. Radiogaschromatogram of the trimethylsilylated mixture of ^{14}C-hamamelose (1300 dpm) from the chloroplast sugar-diphosphates and of authentic hamamelose (signal from the FID—— and from the flow scintillation counter▬). The gaschromatograms were performed with a Packard radiogaschromatograph (model 7461/564). Column: 4% SE 54 on Gaschrom Q (60–80 mesh). Carrier: N_2. Temperature program: start 120° (10 min) followed by a temperature increasing rate of 3°/min. The sugars were trimethylsilylated using *N*-methyl-*N*-trimethyl-silytrifluoracetamine as solvent and reagent. Retention times (min) for trimethylsilylated hamamelose and glucose (for comparison):

Compound	Glucose		Hamamelose		
Signals from the FID	28.4	31.8	25.4	26.9	27.9
Signals from the flow-scintillation counter	28.6	32.4	26.2	27.2	28.0

using the dioxane cocktail. Already the first two crystallizations provided the same specific activity.

Finally the radioactive sugar was mixed with 100 μg of non labeled hamamelose, dried in vacuo and reduced by addition of 100 μl of water and 1 mg $NaBH_4$. After 12 hr a pherogram of the reaction mixture was performed using buffer 1 (table 3).

The pherogram showed 2 labeled compounds moving like hamamelitol and hamamelose, respectively. When the carrier was located on the pherogram with alkaline silver-nitrate hamamelitol and hamamelose also appeared. Thus although the reduction seemed to be incomplete, the production of radioactive hamamelitol confirmed the identification of hamamelose.

3.2. *Evidence for the occurrence of the diphosphate-ester of hamamelose*

a) ^{14}C-Hamamelose is released from the sugar-diphosphates which move on paper chromatography as a single spot (see fig. 1).

b) The original hamamelose-containing substance moved on the pherogram at pH 3.7 as a double charged substance and as far as fructose-diphosphate which served as reference substance. Dephosphorylation of the radioactive spot yielded ^{14}C-labeled sugars, which are known to occur as diphosphates, namely fructose, sedoheptulose, ribulose, glucose (and hamamelose). In addition to these glyceric acid was found, which is derived from 3-phosphoglyceric acid (see fig. 3).

c) Hamamelose was released from the original compound by a highly pure alkaline phosphat (Boehringer Mannheim).

4. Discussion

The data presented show that spinach chloroplasts produce hamamelose-diphosphate in significant amounts (about 1% of the ^{14}C assimilated during 15 min photosynthesis). This finding confirms the conclusions drawn from in vivo experiments with leaves of Primula clusiana [3].

Up to now, the role of hamamelose-diphosphate in plant metabolism is not known. Some years ago, Kandler found a compound related to hamamelose-diphosphate in whole Chlorella cells after photoassimilation of $^{14}CO_2$, namely hamamelonic acid-diphosphate [9]. In these experiments the incu-

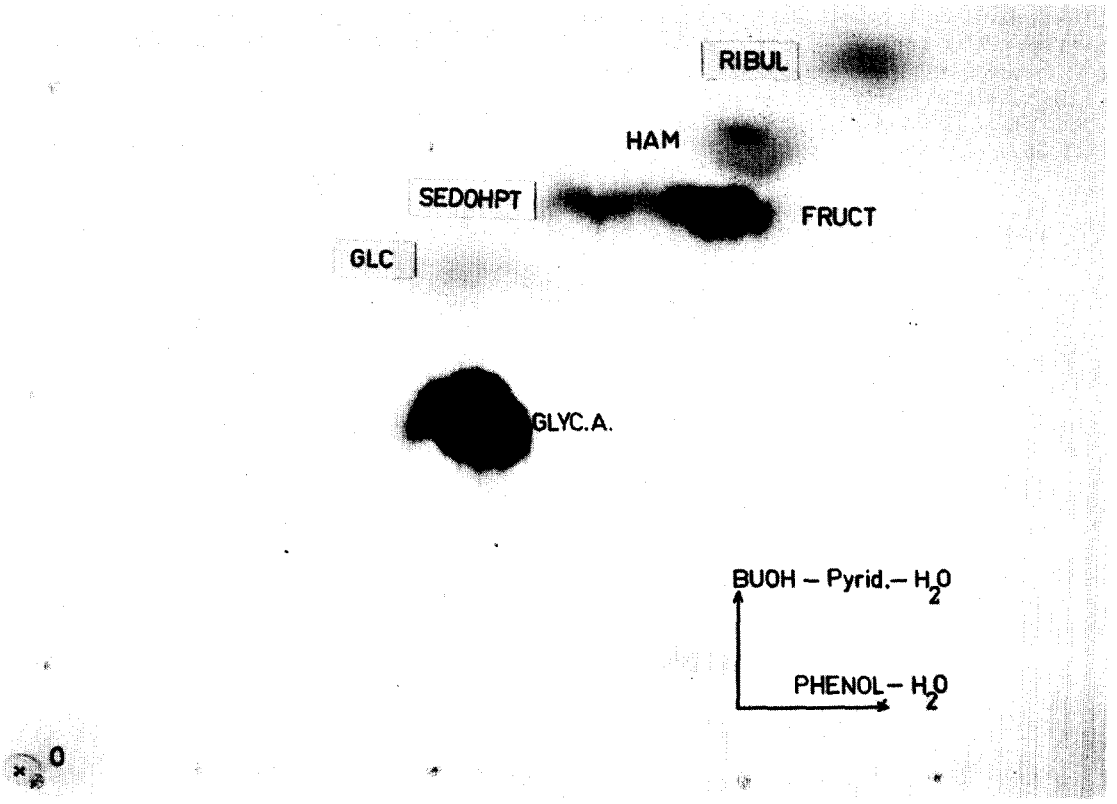

Fig. 3. Chromatogram of the ^{14}C-labeled compounds released by the action of alkaline phosphatase on substances moving as far as fructose-diphosphate on paper electrophoresis with the ammonium-formate buffer. *Abbreviations*: RIBUL = ribulose, HAM = hamamelose, FRUCT = fructose, SEDOHPT = sedoheptulose, GLC = glucose, GLYC. A. = glyceric acid, O = origin.

bation was terminated by addition of high concentrations of cyanide. Later on Kandler also showed that hamamelonic acid-diphosphate was an artefact arising by cyanohydrin synthesis of cyanide and ribulose-diphosphate [10]. In our experiments there is no reason to suppose that hamamelose-diphosphate might be such an artefact. Although hamamelose-diphosphate was not detectable in the extract of whole spinach leaves after photosynthesis in $^{14}CO_2$, labeled hamamelose-monophosphate and free hamamelose (both less than 0.1% of the radiocarbon of the extract) were found.

Thus we conclude that hamamelose-diphosphate is produced in the photosynthetic carbohydrate cycle, transfered to the cytoplasm and dephosphorylated by a phosphatase which is not present in the chloroplasts but in the cytoplasm.

Acknowledgements

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