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SORBITOL-1-PHOSPHATE AND SORBITOL-6-PHOSPHATE IN APRICOT LEAVES

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Abstract—Sorbitol-1-phosphate and sorbitol-6-phosphate were isolated from *Prunus armeniaca* leaves that had been labelled with ^{14}C by photosynthesis in $^{14}CO_2$. Each hexitol phosphate was present at *ca* 7 μmol/kg fr. wt in the tissue and formed *ca* 4% of the hexose monophosphate fraction. ^{14}C-specific activity measurements suggest that each hexitol monophosphate is formed from a hexose monophosphate, and that one or other could be an intermediate in photosynthesis of sorbitol from CO_2.

INTRODUCTION

Although sorbitol is the major carbohydrate present in leaves of the woody Rosaceae, its synthetic pathway in higher plants has not been established. Bieleski and Redgwell [1] suggested that in apricot, *Prunus armeniaca* photosynthesis from CO_2 follows the normal pathway from 3-P-glyceric acid to Fru-6-P, which is then reduced to sorbitol-6-phosphate (Sor-6-P), and subsequently dephosphorylated. In this paper, we report the isolation of Sor-1-P and Sor-6-P from apricot leaves, and some specific activity data which support the hypothesis.

RESULTS AND DISCUSSION

Phosphate esters in plant tissues are present in low concentrations, 0.5–100 μmol/kg fr. wt [2]. Our preliminary results suggested that a sorbitol phosphate was present in apricot leaves at *ca* 1 μmol/kg fr. wt [1]. Hence, in order to be able to use sufficiently sensitive detection procedures, we labelled apricot leaf tissue with ^{14}C, using photosynthesis from $^{14}CO_2$ under conditions giving maximum incorporation of ^{14}C into the hexose monophosphate fraction [1]. (Hexitol monophosphates would be expected to have properties very like those of the hexose monophosphates, and to be recovered in the hexose monophosphate fraction). Subsequent extraction and purification procedures were those used in our earlier studies on plant phosphate esters [1, 3–8]. We extracted 1050 g fr. wt leaf tissue in all: 50 g was radioactively labelled and was extracted as one portion; the remainder was unlabelled and was extracted as ten 100 g portions in order to avoid problems of bulking-up. The initial killing procedure halts phosphatase action [4], extracts phosphate esters and removes some interfering material in a $CHCl_3$ phase [3]. The 11 extracts so obtained were combined prior to the next step, linear gradient elution on QAE Sephadex [7]. The composition of each fraction was monitored by TLE [5, 6], and the column procedure was found to separate all sugars, all amino acids, most of the organic acids and most other phosphate esters from the hexose monophosphates (Table 1). The sugar-containing fractions were kept for later study. Fractions containing hexose monophosphates were bulked and further purified by preparative paper electrophoresis (PE): this step removed all remaining organic acids and phosphate esters other than the hexitol- and hexose-monophosphates; although it was necessary to repeat the step a second time in order to remove all *Pi*. The resulting hexose monophosphate fraction (total wt 86 mg, $(NH_4^+)_2$ salts) was finally fractionated by prep-PC, chromatograms were autoradiographed, and the radioactive band with the same R_f as authentic sor-6-P was cut out and eluted with H_2O. The PC procedures removed Glu-6-P, Fru-6-P, Man-6-P, and at least one other ^{14}C-containing compound. Material from the 'Sor-6-P' band was finally purified by partition chromatography on Sephadex LH 20 to remove contaminants from the PC procedures.

Table 1. Purification of hexitol monophosphate from 1.05 kg fr. wt apricot leaves

Purification step	Compounds removed by step	Dry wt after step
$MeOH/CHCl_3$ extract	Cellulose, starch, lipid, pigment, phospholipid	78.0 g
Ion exchange (QAE)	Sugars, amino acid, most organic acid, most phosphate esters	0.93 g
Prep-PE	Organic acid, *Pi*	86 mg
Prep-PC	Separation of individual hexose and hexitol monophosphates	
LH 20 Sephadex	Solvent and paper residues	7.8 mg

The resulting fraction (7.8 mg, $(NH_4^+)_2$ salt) appeared to contain a single compound that was chromatographically and electrophoretically indistinguishable from Sor-6-P in several TLE and TLC systems. The material was stable to acid hydrolysis (2 hr at 100° in 6 M HCl), conditions that extensively hydrolyse Glu-6-P, Fru-6-P, Glu-1-P, etc. This is known characteristic of hexitol monophosphates [9]. When the fraction was hydrolysed by action of acid phosphatase (EC 3.1.3.2), a single

^{14}C-containing product was obtained: it was chromatographically and electrophoretically indistinguishable from sorbitol, even by GLC (as the TMSi derivative) and by a TLE system known to separate other hexitols from sorbitol [10]. Thus the fraction appeared to contain a sorbitol phosphate.

When the TMSi derivative of the unhydrolysed fraction was subjected to GLC [11], two peaks were obtained, some ^{14}C being present in each. The faster-travelling compound A had a R_t and MS identical to that of TMSi-Sor-6-P. The slower-travelling compound B also gave a MS identical to that of TMSi-Sor-6-P, despite its longer R_t. TMSi-Sor-1-P was prepared, and found to have characteristics identical to those of B. In contrast the R_t of TMSi-mannitol-1-P (=TMSi-mannitol-6-P) differed from those of A and B.

The evidence shows that two compounds are present in the hexitol monophosphate fraction. The acid stability and MS characteristics show that the compounds must be either hexitol-1- or -6-phosphates, but there is only one hexitol released by reaction with phosphatase, that being sorbitol. We therefore conclude that both Sor-1-P and Sor-6-P are present in apricot leaves.

The amounts of the main sugars in the sugar fraction, and of total hexose monophosphate in the hexose monophosphate fraction, were obtained by quantitative GLC using TMSi derivatives of the free sugars (phosphate esters being hydrolysed by phosphatase prior to their assay), and their sp. act. were calculated (Table 2). Amounts of fructose and Fru-6-P were insufficient for measurement of their sp. act. and other minor components of the hexose monophosphate fraction were not studied. The total hexose monophosphate plus hexitol monophosphate content was about 160 nmol/g fr. wt, with hexitol monophosphate, about 14 nmol/g fr. wt, comprising 9% of the total. Separation of Sor-1-P and Sor-6-P was incomplete, even by GLC of the TMSi derivatives, but it was possible to estimate the proportion of one to the other. Thus Sor-1-P and Sor-6-P were present in relative amounts of 0.9:1, with relative radioactivities of 0.35:1, and therefore relative sp. act. of 0.4:1. Both hexitol phosphates had sp. act. considerably higher than that of sorbitol, but lower than those of the two hexose monophosphates (Table 2), indicating that they were synthesized from a hexose phosphate by reduction rather than from sorbitol by phosphorylation. The results are not inconsistent with there being a photosynthetic pathway in apricot leaves from 3-P-glyceric acid to Fru-6-P to Sor-6-P to sorbitol [1]. The occurrence at the same time of Sor-1-P is an unexplained anomaly. Is it derived from Glu-1-P, or Fru-1-P, or from Fru-1,6-P_2 by way of a sorbitol diphosphate, or by the action of a mutase? And what is its role? Resolution of these questions will probably require a study of the enzyme systems involved.

Table 2. Amounts, radioactivities and specific activities of sugars and phosphate esters in apricot leaves after 10 min photosynthesis with $^{14}CO_2$. Values for Sor-1-P and Sor-6-P are estimated from the value for hexitol monophosphate after measuring the proportionate amount and radioactivity in each ester by prep-GLC.

Compound	Tissue content μmol/g fr. wt	Radioactivity nCi/g fr. wt	Sp. act. nCi/μmol C
Sorbitol	70.7	558	1.32
Sucrose	15.3	74	0.40
Glucose	6.0	130	3.61
Hexitol monophosphate	0.0135	0.68	8.4
Sorbitol-1-phosphate	0.0064	0.18	4.7
Sorbitol-6-phosphate	0.0072	0.50	11.6
Mannose-6-phosphate	0.0173	6.15	59.2
Glucose-6-phosphate	0.089	39.5	74.0

EXPERIMENTAL

^{14}C-labelling. Freshly-picked apricot leaves, 50 g fr. wt, carried out photosynthesis in a 1 l. container in 2 mCi $^{14}CO_2$, 2 klx light, 25°, 10 min [1]. Tissue extracted as for main tissue sample.

Tissue extraction. 10 freshly-picked samples 100 g fr. wt apricot leaves were each frozen in 400 ml MeOH–$CHCl_3$–H_2O–HCO_2H (12:5:2:1) at −25°, kept 18 hr at −25° to inactivate phosphatase [4], then extracted [3]. Each $CHCl_3$ phase was discarded and each MeOH phase was kept, the tissue residues being combined and reextracted with 2 l. 2% HCO_2H in aq. 20% MeOH [3]. All aq. MeOH phases, including that containing ^{14}C, were combined and evapd to near-dryness on a rotary evaporator (bath temp. <40°), diluted to 100 ml with H_2O and frozen and thawed in order to produce a flocculent white ppt. that was discarded after centrifuging at 0°. The supernatant was again evapd to near-dryness and remaining H_2O and formic acid were removed in a desiccator (solid P_2O_5 and KOH, 10^{-3} τ, 48 hr). After being weighed, the extract was dissolved in 500 ml H_2O, dispensed into 10 × 50 ml aliquots and stored frozen. Major organic contaminants at this stage were sugars and oligosaccharides

Analytical TLC and TLE and detection of compounds. For monitoring the steps in purification, TLC and TLE used solvents and conditions described elsewhere [5, 6]. For separation of hexitols, TLE (MN 300 cellulose, 6% basic PbAc, 1000 V, 20 mA, 50 min) adapted an existing PE procedure [10]. Radioactive compounds were detected by autoradiography, phosphate esters by molybdate [3], amino acids by ninhydrin [6] and sugars by *p*-anisidine: KIO_4 [13]. All radioactivity measurements were by scintillation spectrometer, in Triton-X scintillant, with quench correction by channels ratio method.

Column chromatography. Ion exchange chromatography used QAE Sephadex A-25 and linear gradient elution with 0.05–0.5 M NH_4HCO_3 as for isolation of phaseolotoxin [7]. Partition chromatography used Sephadex LH20 swollen in MeOH–$CHCl_3$–0.25 M aq. NH_3 (3:1·1) (MCA) in a 55 × 4 cm column, prewashed with MCA (40 ml/hr, 18 hr) and eluted with MCA (30 ml/hr) [7]. Fractions were monitored by TLC and TLE.

Preparative and analytical PC and PE. Extract was applied as a band origin to 46 × 57 cm sheets of chromatography paper. PE used pH 7.9, 0.15 M NH_4HCO_3 buffer on Whatman 120 paper in a Savant flatbed apparatus; 900 V, 300 mA, 2 hr. Prep-PC employed 4 solvents: (i) Whatman 1 paper, *t*BuOH–H_2O–picric acid (aq. satd)–H_3BO_3 (40:10:2:1 v/v/wt/wt) (picric solvent) [14] (ii) Whatman 3 MM paper, *n*-BuOH–HOAc–H_2O–C_6H_5N (11:3·9·9) (BAWP) [15]. (iii) Whatman 3 MM paper, *n*-BuOH–H_2O–HOAc (5·4:1) upper phase (BAc solvent). (iv) Whatman 1 paper, MEK–HOAc–satd aq H_3BO_3 (9:1·1) (boric solvent) [1].

Purification of hexose monophosphates and hexitol monophosphate. In turn, each portion of tissue extract (50 ml ≡ 105 g fr. wt tissue) was diluted to 1.2 l. with 0.05 M NH_4HCO_3, filtered and fractionated by ion exchange column chromatography: 40 ml fractions were collected, hexose monophosphates being

in fractions 8–10 (0.18–0.23 M NH_4HCO_3). All fractions containing hexose monophosphate were combined, evapd weighed and rechromatographed as a single portion on one column as above. The major contaminant in this crude hexose monophosphate fraction was P_i (>90% dry wt).

The crude hexose monophosphate fraction was divided into 4 parts, each being subjected to prep-PE. Bands containing hexose monophosphate were eluted, but overloading caused P_i removal to be incomplete, so prep-PE was repeated, yielding a clean hexose monophosphate fraction. Individual hexose monophosphates and hexitol monophosphate were then separated by a succession of prep-PC steps. (i) The clean fraction was divided into 16 parts, each being chromatographed in picric solvent and yielding 'Glu-6-P', 'Fru-6-P' and 'crude Sor-6-P' fractions. (ii) The crude Sor-6-P fraction was divided into 8 parts, each being chromatographed in BAWP and yielding 'Sor-6-P' and 'Fru-1-P' fractions. (iii) 'Glu-6-P', 'Fru-6-P', 'Fru-1-P' and 'Sor-6-P' fractions were each rechromatographed in BAWP. The 'Glu-6-P' fraction yielded a second component, 'Man-6-P'. Finally the Sor-6-P fraction was subjected to partition column chromatography on Sephadex LH20. After the void vol. had passed, 5 ml fractions were monitored, and fractions 3–7 were combined then dried down to give a pure hexitol monophosphate fraction. This was shown to be essentially free from ^{14}C-hexose monophosphate by its resistance to acid hydrolysis (6 M HCl, 100°, 2 hr) [9]. Stock solns of each fraction (1.5–5 mg/ml) and of standards (10 mg/ml) were made up and stored frozen.

Preparation of hexitol monophosphate standards. Hexitol monophosphates were synthesized by borohydride reduction [16] of the appropriate sugar phosphates: Sor-6-P from Glu-6-P; mannitol-1-P from Man-6-P; and a mixture of Sor-1-P plus mannitol-1-P (ratio *ca* 1:2) from fructose-1-phosphate. Other standards (including Sor-6-P) were purchased.

Purification of sugars. A fraction containing sugars was isolated from the tissue extract during ion exchange chromatography 0.4% of the total was further fractionated by prep-PC. The portion was divided into 3 parts, each being chromatographed in BAc solvent to give 'Glu', 'Suc' and 'Fru' fractions. Insufficient fructose was present in the 'Fru' fraction for further study, but 'Suc' and 'Glu' fractions were submitted to prep-PC in boric solvent, the 'Suc' fraction yielding sucrose and the 'Glu' fraction being further resolved to yield glucose and sorbitol. Borate was removed by volatilization with MeOH [16]. Stock solns of each fraction (2–5 mg/ml) and of standards (10 mg/ml) were made up and stored frozen.

GLC and GC–MS of TMSi-phosphate esters. TMSi derivatives of the phosphate esters were prepared [11]: 50 µl hexitol monophosphate soln or 10 µl standard soln in a Reactivial was dried over P_2O_5, 50 µl Trisil reagent (Pierce Chem. Co.) was added, the vial was shaken for 10 min and then heated (100°, 1 hr). Analytical GLC (FID) used a 1.5 m × 2.28 mm i.d. stainless steel column packed with 3% SE 30 on 100–120 mesh Varaport 30 (180° for sugars, 210° for phosphate esters). Prep-GLC (FID, splitter and liq N_2 trap) used a 2 m × 2.28 mm i.d. stainless steel column packed with 5% Silicone OV 11 on H.P Chromasorb W at 210°. The latter procedure gave longer R_ts but better resolution. GC–MS used the analytical GLC procedure in conjunction with an AEI MS 30 at 20 eV. Some fragments obtained from Sor-6-P were *m/e* 751 (M^+-Me), 663 (M^+-CH_2OTMSi), 116 ($CH_2{=}CHO^+TMSi$), 101 ($CH_2{=}CHO^+SiMe_2$), with the most abundant ion being *m/e* 387 ($(TMSiO)_4P^+$) [17].

Hydrolysis and quantitation of phosphate esters. For qualitative study of the hexitol monophosphate fraction, 50 µl stock soln in a Reactivial was dried then hydrolysed with phosphatase (wheatgerm acid phosphatase, 1 mg in 100 µl, 0.05 M acetate buffer, pH 5, 37°, 24 hr, toluene as bacteriocide) [1]. Products were separated by PC in boric solvent, eluted and measured for radioactivity. Alternatively, the products were submitted to analytical TLE. In each case, sorbitol was the only ^{14}C-containing material detected. For quantitative estimation of hexitol monophosphate, Glu-6-P and Man-6-P, the same procedure was used but with less phosphatase (0.05 mg in 50 µl buffer). Products were separated by PC in boric solvent, eluted, and then identified and quantitated by GLC of TMSi derivatives [1, 18].

Quantitation of sugars. Identity of each sugar was confirmed and amount determined by quantitative GLC of TMSi derivatives [18].

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