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METABOLISM OF DULCITOL IN *EUONYMUS JAPONICA*

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Abstract—Dulcitol-1(6)-^{14}C was administered to leaves of *E. japonica* and samples were taken for time periods ranging from 0·5 to 24 hr. For each time period the absolute activity of the glucose, galactose and dulcitol pools was determined. Such studies demonstrated that dulcitol is converted to glucose and galactose. The initial product was glucose, some of which was converted to galactose, glacturonic acid and glucuronic acid. Fractionation of a leaf sample into its pectin, lignin, hemicellulose and α-cellulose components, with subsequent hydrolysis, showed that the dulcitol pool is used in the synthesis of structural carbohydrates. The activity of these fractions was shown to reside in dulcitol, glucose, galactose, galacturonic acid and glucuronic acid residues.

INTRODUCTION

MOST POLYOLS are formed by the reduction of an aldose and the first step in the metabolism of these compounds is the re-oxidation to the ketose form.[1-6] The ketose, tagatose (*lyxo*-hexulose), which corresponds to dulcitol (galactitol), however, has been shown to be absent in *E. japonica*.[7] The metabolism of dulcitol must therefore proceed via a different route. The present investigation was undertaken to determine the metabolic fate of dulcitol and to determine the role of the large dulcitol pool in *E. japonica* (2·7–11·6% dry wt).

RESULTS

Tables 1 and 2 list the activity of the glucose, galactose and dulcitol pools following infiltration of dulcitol-1(6)-^{14}C, in terms of percentage of the total activity incorporated into the leaf samples and as specific activity (μCi/mM). Since the amount of labelled dulcitol incorporated into the leaf samples varied, the specific activities are expressed on the basis of 100 000 dpm in the methanolic extract.

The results in Tables 1 and 2 do not indicate whether glucose or galactose was the initial product of the dulcitol substrate. Leaf samples, vacuum infiltrated with labelled dulcitol with subsequent stoppage of the ensuing reactions after 1–5 min, yielded glucose

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Table 1. Specific activity of carbhydrate pools and the ratio of the unit activity present in the residue/to the total activity following dulcitol81(6)-^{14}C infiltration

Extract (hr)	Specific activity of carbohydrates μCi/mM* Galactose	Dulcitol	Glucose	Unit activity: Residue total / Tissue total†
Run No. I				
0·5	0·885	0·159	0·150	0·0244
1	0·443	0·324	0·117	0·0483
2	0·386	0·130	0·012	0·0263
4	1·061	0·378	0·374	0·0479
8	0·952	0·139	0·082	0·0375
24	0·297	0·186	0·064	0·0947
Light				
24	0·346	0·135	0·044	0·0388
Dark				
Run No. II				
0·5	0·939	0·228	0·184	0·0467
1	1·267	0·227	0·314	0·0412
2	0·695	0·166	0·103	0·0336
4	1·388	0·197	0·145	0·0296
8	0·680	0·206	0·195	0·0488
24	0·309	0·229	0·047	0·0870
Light				
24	0·381	0·238	0·114	0·0741
Dark				

* Based on standardized 100 000 dpm in methanolic extract.
† Tissue total = dpm methanolic extract + dpm residue.

Table 2. Percentage activity present in the carbohydrate pools following dulcitol (16)-^{14}C infiltration

Extract (hr)	Dulcitol %	Galactose %	Glucose %	Total activity in tissue (dpm × 10^{-3})
Run No. I				
0·5	51·99	11·79	6·87	290
1	78·61	4·34	6·25	188
2	45·03	2·98	3·24	407
4	69·22	10·30	13·72	134
8	51·86	9·46	4·80	224
24 Light	66·29	5·99	5·17	303
24 Dark	41·43	4·34	3·14	448
Run No II				
0·5	51·72	8·02	8·43	146
1	71·79	12·37	13·19	134
2	51·09	12·94	7·89	203
4	54·04	10·87	6·45	217
8	61·0	6·40	8·40	236
24 Light	58·24	6·50	4·09	310
24 Dark	64·89	6·52	8·76	242

and galactose pools with approximately equal activities. The results in Table 3 give the activity of the extracted glucose, galactose and dulcitol for very short time periods (15 sec–1·5 min) after a solution of labelled dulcitol had been injected into the midrib vein of the leaves. Since the amount of dulcitol injected into the leaf samples varied, a direct comparison of the glucose or galactose activities for the different time periods cannot be made. The

TABLE 3. ^{14}C ACTIVITIES OF GLUCOSE, GALACTOSE AND DULCITOL FOLLOWING DULCITOL-1(6)-^{14}C INJECTION*

Time	(dpm) Activity: Galactose	Dulcitol	Glucose
Run No. I			
15 sec	35·7	152·8	42·6
45 sec	30·1	83·4	33·5
1·5 min	30·7	88·1	42·2
45 sec (10% glucose also added)	51·6	97·9	80·5
45 sec (10% galactose also added)	34·0	70·4	30·4
1·5 min (10% galactose)	38·2	79·1	39·1
Run No. II			
15 sec	1·3	35·9	10·3
45 sec	11·5	54·7	34·13
1·5 min	8·3	64·5	25·1
45 sec (10% glucose also added)	24·0	58·1	15·1
1·5 min	10·5	40·0	29·1
45 sec (10% galactose also added)	11·4	19·3	40·1
1·5 min (10% galactose)	10·2	30·0	7·7
Run No. III			
15 sec	4·5	33·9	14·6
45 sec	8·4	36·2	12·2
1·5 min	8·2	50·9	23·1
45 sec (10% glucose also added)	12·7	60·5	32·7
1·5 min (10% glucose)	18·0	63·7	22·6
45 sec (10% galactose also added)	9·2	36·6	12·3
1·5 min (10% galactose)	9·9	22·5	12·4

* Activities expressed were collected by preparative GLC from a 30 μl injection of a silylated solution.

results in Table 4 have therefore been extrapolated so that the dulcitol activity was equal to 100 dpm. The results in Table 5 express the activity residing in the glucose, galactose and dulcitol pools as a fraction of the total activity in the leaf sample.

To confirm the identity of the first product formed from the metabolism of dulcitol, some leaf samples were injected with 10 μl of a 10% glucose or galactose solution along with dulcitol-1(6)-^{14}C. It was hoped that the exogenous, cold monosaccharides would alter the metabolism of dulcitol sufficiently to add further evidence to the data obtained from a strict time sequence.

The data in Table 1 also showed that, following infiltration of a leaf sample with dulcitol-

TABLE 4. ADJUSTED ^{14}C ACTIVITIES* OF GALACTOSE AND GLUCOSE (dpm)

Experimental series No.	Time	^{14}C activity (dpm) Galactose	Glucose
I	15 sec	23·6	28·1
II	15 sec	3·6	28·8
III	15 sec	13·5	43·3
I	45 sec	36·1	40·2
II	45 sec	21·0	62·0
III	45 sec	23·3	33·8
I	45 sec (10% glc)	52·0	82·0
II	45 sec (10% glc)	41·2	26·0
III	45 sec (10% glc)	21·1	47·7
I	45 sec (10% gal)	42·8	38·3
II	45 sec (10% gal)	—	—
III	45 sec (10% gal)	25·2	33·6
I	1·5 min	34·9	47·9
II	1·5 min	12·7	38·5
III	1·5 min	16·2	45·4
I	1·5 min (10% glc)	58·1	92·5
II	1·5 min (10% glc)	26·4	72·6
III	1·5 min (10% glc)	28·2	35·5
I	1·5 min (10% gal)	48·3	49·7
II	1·5 min (10% gal)	34·0	25·7
III	1·5 min (10% gal)	44·0	55·3

* Adjusted activity of galactose and glucose in terms of 100 dpm for dulcitol pool.

1(6)-^{14}C, the activity of the nonextractable residue increased steadily with time. To determine into what structural polysaccharide the radioactive monosaccharides were being incorporated and the identity of these carbohydrates, a fractionation, hydrolysis and separatory GLC procedure was employed. The results of this study are given in Tables 6 and 7. From the results in Tables 1–7, a pathway for the metabolism of dulcitol was advanced (Fig. 1).

DISCUSSION

After infiltration of leaves of *E. japonica* with dulcitol-1(6)-^{14}C labelled glucose and galactose are found in the methanolic extract. The activities of these compounds Tables 1 and 2) however, show that no definite pattern arises during the time sequence study of the metabolism of dulcitol. This implies that the rate of dulcitol metabolism is dependent on the internal conditions of the individual leaf samples. Indeed, if a comparison of the size of the dulcitol pool with the rate of incorporation of activity from dulcitol-1(6)-^{14}C into the nonextractable residue is made (Table 8) one can see that the smaller the size of the polyol pool the greater the incorporation. The time sequence study of dulcitol metabolism (Table 1), however, shows that the rate of incorporation of dulcitol into the nonextractable residue generally increases with time.

Since these results did not indicate which monosaccharide was the initial product of the dulcitol, an ultrashort time sequence study was performed (Tables 3–5). The purpose of introducing cold glucose and galactose into the leaves of *E. japonica* along with dulcitol-1(6)-^{14}C was to alter the rate of dulcitol metabolism. For example, it was possible that

TABLE 5. ACTIVITIES OF GLUCOSE, GALACTOSE AND DULCITOL POOLS AS A FRACTION OF THE TOTAL SOLUBLE ACTIVITY

Time	Fraction of activity		
	Dulcitol/total	Glucose/total	Galactose/total
Run No. I			
15 sec	0·534	0·149	0·125
45 sec	0·399	0·160	0·144
45 sec (10% glc)	0·347	0·285	0·183
45 sec (10% gal)	0·402	0·166	0·162
1·5 min	0·525	0·251	0·183
1·5 min (10% glc)	0·286	0·265	0·166
1·5 min (10% gal)	0·408	0·202	0·197
Run No. II			
15 sec	0·347	0·099	0·012
45 sec	0·366	0·227	0·077
45 sec (10% glc)	0·390	0·101	0·160
45 sec (10% gal)	0·228	0·474	0·135
1·5 min	0·241	0·093	0·030
1·5 min (10% glc)	0·405	0·294	0·107
1·5 min (10% gal)	0·199	0·051	0·067
Run No. III			
15 sec	0·366	0·157	0·049
45 sec	0·317	0·106	0·073
45 sec (10% glc)	0·387	0·208	0·045
45 sec (10% gal)	0·302	0·101	0·076
1·5 min	0·274	0·125	0·045
1·5 min (10% glc)	0·250	0·089	0·095
1·5 min (10% gal)	0·370	0·205	0·162

added galactose would inhibit the metabolism of dulcitol if galactose was the initial product. It was also reasoned that the cold intermediates injected along with labelled dulcitol should also alter the rates of formation of glucose and galactose from dulcitol.

While the results are not consistent, certain initial conclusions are postulated:

(1) Comparison of the activity of the glucose and galactose pools in extracts of samples in which the reactions had been stopped after 15 sec, 45 sec and 1·5 min yields values of glucose which are consistently larger than those of galactose. These results suggest that the reverse pathway is probably dulcitol–glucose–galactose.

TABLE 6. DISTRIBUTION OF ACTIVITY IN STRUCTURAL COMPONENTS OF LEAVES OF *E. japonica* FOLLOWING DULCITOL INFILTRATION

Fraction	Total activity (dpm)
Pectin	2284
Lignin	4444
Hemicellulose	5724
α-Cellulose	177

TABLE 7. DISTRIBUTION OF IDENTIFIABLE ACTIVITY IN THE HYDROLYSATES OF *E. japonica* FRACTIONS

	Sample	Activity (dpm)	
	Pectin hydrolysate		
	60 μl TMS solution	77·7	
60 μl injection	Glucose	24·4	Total activity = 66·1
	Galactose	10·6	
	Galacturonic Acid	10·2	
	Glucuronic Acid	4·7	
	Dulcitol	16·2	
	Hemicellulose hydrolysate		
	50 μl TMS solution	147·0	
50 μl injection	Glucose	24·0	Total activity 102·2
	Galactose	32·1	
	Galacturonic Acid	9·6	
	Glucuronic Acid	10·7	
	Dulcitol	25·8	
	Lignin Hydrolysate		
	Glucose	41·4	
	Galactose	25·1	
	Galacturonic Acid	34·7	
	Glucuronic Acid	7·5	
	Dulcitol	24·5	

(2) If the reactions in leaves injected with dulcitol-1(6)-^{14}C and labelled dulcitol plus unlabelled glucose were stopped after the same period of time, a comparison of the glucose, galactose and dulcitol activity in these samples showed the following trends. First, the injection of glucose into leaves along with dulcitol-1(6)-^{14}C generally interferes with the metabolism of dulcitol. Second, the activity of glucose, except with 45 sec, and galactose increases. Comparison of the relative increase when the activity of the dulcitol sample has

TABLE 8. COMPARISON OF THE SIZE OF THE DULCITOL POOL WITH THE RATE OF INCOPORATION OF ACTIVITY FROM DULCITOL-(16)-^{14}C INTO THE NONEXTRACTABLE RESIDUE

Sample (hr)	Size dulcitol pool (mg)	Activity Residue total / Tissue total
0·5 No. 1	27·45	0·204
0·5 No. 2	19·50	0·046
1 No. 1	20·85	0·048
1 No. 2	27·00	0·041
2 No. 1	28·92	0·026
2 No. 2	25·98	0·033
4 No. 1	15·75	0·047
4 No. 2	23·10	0·029
8 No. 1	31·65	0·037
8 No. 2	25·50	0·048
24 Light No. 1	32·10	0·094
24 Light No. 2	22·80	0·087
24 Dark No. 1	26·00	0·038
24 Dark No. 2	24·10	0·074

been adjusted to 100 dpm yields an increase in the glucose activity of 100%, while the galactose activity increases to somewhat less than twice its original value.

(3) If the reactions in leaves injected with dulcitol-1(6)-^{14}C and labelled dulcitol plus unlabelled galactose were stopped after the same period of time, a comparison of the glucose, galactose and dulcitol activity in these samples showed that the injection of galactose into leaves along with dulcitol-1(6)-^{14}C increases the rate of dulcitol metabolism. Also, the activity of glucose decreases slightly or remains approximately the same while the activity of galactose increases.

A pathway was then proposed consistent with the foregoing results:

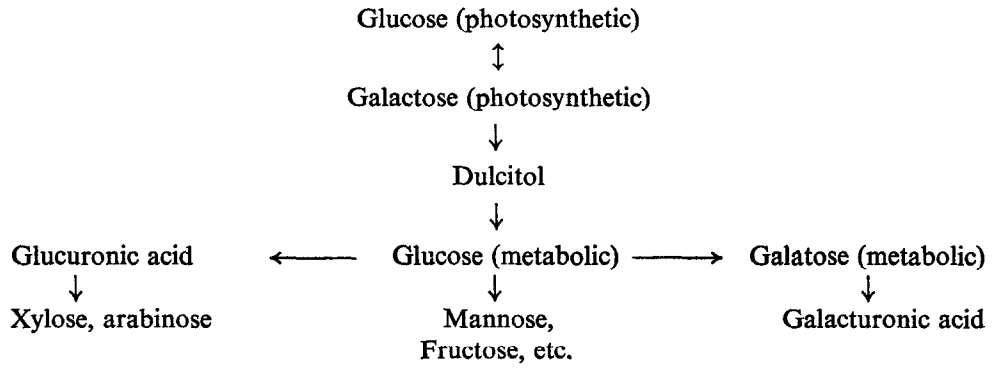

FIG. 1.

The order in which the pathway has been set up is justified by the following points.

(1) Reactions which were stopped after very short periods of time consistently contain a glucose pool with greater activity than the galactose pool from the same leaf sample.

(2) The rate of dulcitol metabolism in leaves injected with labelled dulcitol and cold glucose is lower (except in one 45-sec run) than the metabolic rate in leaves injected with only dulcitol-1(6)-^{14}C if the reaction were stopped after equal periods of time. Thus a large buildup of the metabolic glucose pool would tend to decrease the metabolism of the dulcitol pool.

The increase in activity of the glucose pool is consonant with the proposed scheme since with a larger glucose pool any labelled glucose formed has a smaller probability of being further metabolized. The initial rate of dulcitol metabolism in plants fed dulcitol plus glucose would probably be unaffected by the glucose injection whereas the exogenous glucose would inhibit the metabolism of labelled glucose from the very start. No reason can be found for the increase in galactose activity; however, the proportional increase in glucose activity is greater than the increase in activity of the galactose pool.

(3) The rate of dulcitol metabolism in leaves injected with dulcitol-1(6)-^{14}C and unlabelled galactose is higher than the metabolic rate in leaves injected with only labelled dulcitol if the reactions were stopped after equal periods of time. Galactose infused into the leaves of *E. japonica* would enter both the photosynthetic and metabolic galactose pools. However, since the first galactose pool is metabolically adjacent to the dulcitol pool whereas the second galactose pool is farther removed, the influx of dulcitol into the dulcitol pool from reduced exogenous galactose may increase the metabolism of dulcitol (i.e. a push–pull mechanism). This is also congruent with the results of $^{14}CO_2$ fixation which showed a

constant biosynthesis of dulcitol under photosynthetic conditions although the size of the dulcitol pool remains fairly constant.

Feedings of labelled dulcitol and unlabelled galactose also display a galactose pool with an increased activity, whereas the activity of the glucose pool remains relatively static. The increased galactose activity may be the result of the decreased probability of further metabolism of the galactose molecules formed from labelled dulcitol via glucose in the presence of a large concentration of unlabelled exogenous galactose. The constancy of the activity of the glucose pool would seem logical, however, since metabolic glucose is the precursor for many carbohydrates other than galactose. Although as might be expected that the large concentration of exogenous galactose infused into the leaves should inhibit the formation of a galactose pool. There is considerable leeway in terms of intermediates between the glucose and galactose pools.

(4) The inclusion of glucuronic acid and galacturonic acid as intermediates in the pathway came about after proof had been obtained regarding their presence in the leaves of *E. japonica* following the administration of labelled dulcitol to the leaves. The methodology used in this procedure is explained below.

(5) After infiltration with labelled dulcitol, compounds in the extract of the leaves with retention times very similar to arabinose and xylose were collected. These compounds were found to be active although the percentage activity of these substances of the total activity present was less than the percentage activity found in the glucose pool.

The results in Tables 6 and 7 list the activity in the fractions obtained from the fractionation of 64·1 mg of dried leaf tissue which had been previously infiltrated with dulcitol-1(6)-^{14}C. Also listed are the identifiable activities in the hydrolysed fractions and separated by GLC.

The activity initially attributed to lignins were subsequently found to be the result of carbohydrates associated with the lignin fraction. This was demonstrated by hydrolysing the lignin fraction and extracting the lignin molecules with ether. The ether solution contained compounds which strongly absorbed UV light (251, 257 and 264 nm) but which were found to be nonradioactive. Carbohydrates were extracted from the lignin hydrolysate with pyridine. These were separated by GLC.

Previous work[8] had shown that the concentration of dulcitol in *E. japonica* had decreased only a maximum of 1·29% from an average value of 9·59% (dry wt basis) after 24–96 hr of respiratory conditions. This result plus the absence of tagatose in *E. japonica* rules out the possibility of dulcitol being utilized for the interconversion of sugars, the storage of reducing power, or the storage of carbohydrates. The extremely high concentration of this polyol also would seem to rule out the role of coenzyme regulation. The relatively high activity of the carbohydrates associated with the lignin fraction, the pectic substances and hemicelluloses as compared to the α-cellulose fraction may indicate a form of differentiating the synthesis of these substances.

EXPERIMENTAL

Procedure for time sequence 0·5–24 hr. Dulcitol-1(6)-^{14}C (50 μCi, 25 mCi/mM, Calbiochem) was incorporated into the leaf samples by dissolving the dulcitol in 150 ml H_2O and vaccum infiltrating the leaves at 10 mm Hg. The infiltration procedure was carried out in a 5 l. desiccator. After infiltration the leaves were bathed in distilled water and placed in Petri dishes with the petioles immersed in water.

Source of illumination. Steady illumination was provided in an apparatus 61 cm square which contained 7, 46-cm Cool White and 9, 46-cm Gro-Lux fluorescent light tubes.

[8] R. HOPE, B.S.P. Thesis, College of Pharmacy, University of Saskatchewan.

Procedure for time sequence 15 sec–1·5 min. A solution of dulcitol-1(6)-^{14}C was injected into the midrib vein of individual leaves. Leaf samples which were injected with labelled dulcitol plus cold glucose or galactose were injected with 10 μl of a 10% solution of the unlabelled monosaccharide.

Extraction procedure. Leaf samples were placed in a 25 × 80 mm cellulose extraction thimble, frozen with liquid N_2 and comminuted with an aluminum rod. The extraction thimble was then placed in a Soxhlet and extracted with methanol for 10 hr. Following extraction the methanol solution was evaporated to a few ml and the final vol. made upto 50 ml with H_2O. This solution was defatted with two 25 ml portions of ether. An aliquot portion of the water solution was then evaporated, redissolved in 1 ml pyridine and silylated with hexamethyldisilazane and trimethylchlorosilane.

Fractionation procedure. Fractionation of the leaf tissue which had previously been infiltrated with dulcitol-1(6)-^{14}C and allowed to metabolize the polyol for 36 hr, was accomplished using the procedure of Thornber and Northcote.[9] However, a Soxhlet was used under the following conditions: (1) water extraction at 74° for 24 hr; (2) ethanol–benzene (1:2) extraction at 61° for 12 hr; (3) methanol extraction at 55° for 24 hr.

The final methanol extraction was added to ensure complete extraction. Also, since the temp. specified by the authors for extraction with water and ethanol–benzene could not be reached in a Soxhlet the times of extraction with these solvents was increased. No activity could be detected in a final water extraction, indicating that the extraction procedure used had been effective.

Hydrolysis and extraction. The extracted fractions were hydrolysed, using a 10% HCl solution, by refluxing for 48 hr. The pectic fraction was refluxed for 1 week. Following hydrolysis the solutions were neutralized with NH_4OH and the water evaporated. The carbohydrates were extracted from the residue with three 25-ml portions of pyridine. The carbohydrates were silylated using the procedure described previously.

Procedure for GLC. See Ref. 10.

Procedure for liquid scintillation counting. See Ref. 10. The activity of the nonextractable residue was determined by grinding the residue in a stainless steel mortar, screening through a No. 100 sieve and counting an aliquot portion (40–60 mg) as a suspension in CAB-O-SIL.[11] This value also had to be multiplied by a factor, determined previously, to compensate for the quenching which occurs when the sample was counted as a suspension.

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Key Word Index—Euonymus japonica; celastraceae; dulcitol; carbohydrate; metabolism.