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TRACER STUDIES ON ASCORBIC ACID FORMATION IN PLANTS

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Abstract—Tracer studies in which D-glucose-1-^{14}C, -2-^{14}C or -6-^{14}C; D-galactose-1-^{14}C; D-glucuronic acid-6-^{14}C; D-glucuronolactone-1-^{14}C or -6-^{14}C; D-galacturonic acid-1-^{14}C; D-galacturonate-U-^{14}C methyl ester; *myo*-inositol-2-^{14}C; or D-glucose-1-t or -6-t are administered to intact plant tissues, usually to the detached ripening strawberry or the detached parsley leaf, reveal the existence of two processes of L-ascorbic acid formation in higher plants. In one process, D-glucose and related sugars are utilized as their hexose phosphates. Oxidation proceeds at carbon 1 of the sugar to form the carboxyl group at carbon 1 of L-ascorbic acid. Except for partial equilibration of the hexose phosphate with triose and pentose phosphates, the six carbon chain is utilized without cleavage. Carbon 5 is epimerized from the D- to the L- configuration during the conversion. Carbon 6 remains reduced throughout the process. The other process of L-ascorbic acid formation proceeds from exogenously supplied D-glucuronolactone or D-galacturonate methyl ester. Here, the carboxyl group at carbon 6 of the uronic acid derivative is conserved, becoming carbon 1 of L-ascorbic acid. The aldehydic group of the uronic acid derivative is reduced to a primary alcohol corresponding to carbon 6 in the L-ascorbic acid. There is no cleavage of the carbon chain during the conversion. D-Glucuronic acid supplied exogenously or generated enzymatically within the plant tissue from *myo*-inositol is not utilized for L-ascorbic acid synthesis. The physiological significance of the two processes is discussed.

INTRODUCTION

DIRECT evidence for a precursor relationship of D-glucose to L-ascorbic acid was first obtained by Jackel, Mosbach, Burns and King[1] who observed that chloretone-treated albino rats, when administered uniformly-labeled D-glucose-^{14}C, excreted a uniformly labeled L-ascorbic acid in their urine. Supporting evidence obtained with D-glucose-1-^{14}C [2] and D-glucose-6-^{14}C[3] revealed that D-glucose-1-^{14}C was converted to urinary L-ascorbic acid labeled predominately in carbon 6, while D-glucose-6-^{14}C was converted to L-ascorbic acid labeled primarily in carbon 1. Subsequent studies uncovered the nature of this conversion as it occurred in animals.[4] Principal intermediates appear to be D-glucurono-γ-lactone and L-gulono-γ-lactone. Fig. 1 is a highly simplified scheme which illustrates the path of conversion.

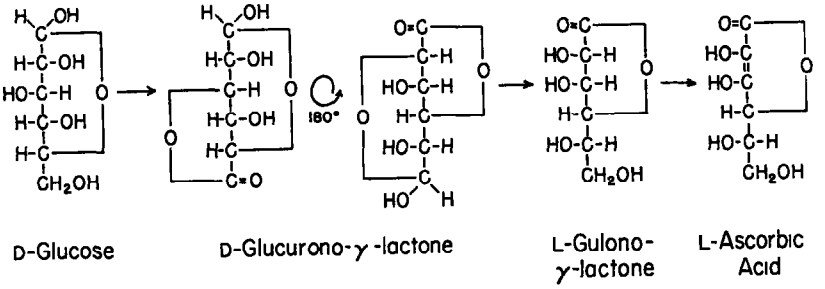

FIG. 1. CONVERSION OF D-GLUCOSE TO L-ASCORBIC ACID IN ANIMALS (C. G. KING AND CO-WORKERS).

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[1] S. S. JACKEL, E. H. MOSBACH, J. J. BURNS and C. G. KING, *J. Biol. Chem.* **186**, 569 (1950).
[2] H. H. HOROWITZ, A. P. DOERSCHUK and C. G. KING, *J. Biol. Chem.* **199**, 193 (1952).
[3] H. H. HOROWITZ and C. G. KING, *J. Biol. Chem.* **200**, 125 (1953).
[4] J. J. BURNS and G. ASHWELL in *The Enzymes*, (edited by P. D. BOYER, H. LARDY and K. MYRBACH), Academic Press, Inc., New York, 3B, 387 (1960).

Isherwood, Chen and Mapson[5] presented a comparative study of L-ascorbic acid biosynthesis in normal rats and in germinating cress seedlings. They found that of a number of sugar acid lactones studied, only D-glucurono-γ-lactone, L-gulono-γ-lactone and L-galactono-γ-lactone as well as the methyl ester of D-galacturonic acid would cause significant increases in the L-ascorbic acid content of cress seedlings or in the urine of normal rats. Of these four compounds, D-galacturonic acid methyl ester and L-galactono-γ-lactone were the best precursors in cress seedlings. Their findings led them to propose two quite similar pathways of L-ascorbic acid biosynthesis (Fig. 2).

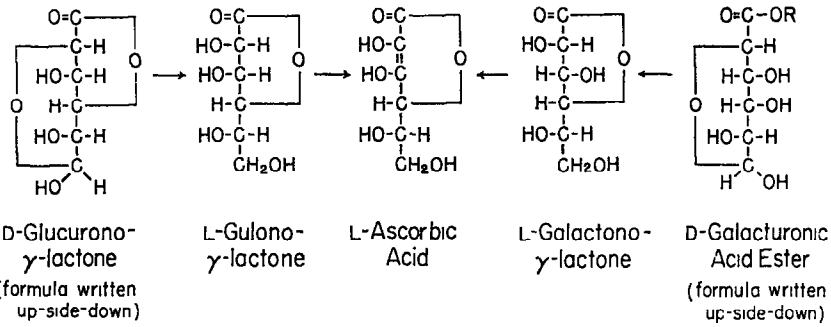

FIG. 2. CONVERSION OF URONIC AND ALDONIC ACID DERIVATIVES TO L-ASCORBIC ACID (ISHERWOOD, CHEN AND MAPSON).

Isherwood *et al.* also discovered that D-mannono-γ-lactone was converted in the rat to D-araboascorbic acid, whereas in cress seedlings D-altrono-γ-lactone was converted to the same product (Fig. 3).

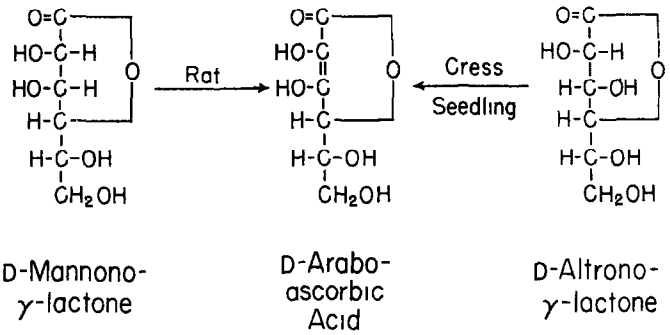

FIG. 3. FORMATION OF D-ARABOASCORBIC ACID FROM D-ALDONIC ACID LACTONES IN THE CRESS SEEDLING AND THE RAT (ISHERWOOD, CHEN AND MAPSON).

D-Altrono-γ-lactone and L-galactono-γ-lactone have the same configuration about carbons 2, 3 and 4. Since the cress seedling would convert D-altrono-γ-lactone to D-araboascorbic acid and L-galactono-γ-lactone to L-ascorbic acid, Isherwood *et al.* singled out the galacto- configuration as a structural requirement unique to L-ascorbic acid synthesis in plants. Additional support for this special role for the galacto- configuration was obtained from enzymatic studies.[6,7] These studies led Isherwood *et al.* to propose a scheme of L-ascorbic acid biosynthesis in plants that originated with D-galactose. This is outlined

[5] F. A. ISHERWOOD, Y. T. CHEN and L. W. MAPSON, *Biochem. J.* **56**, 1 (1954).
[6] L. W. MAPSON, F. A. ISHERWOOD and Y. T. CHEN, *Biochem. J.* **56**, 21 (1954).
[7] L. W. MAPSON and E. BRESLOW, *Biochem. J.* **68**, 395 (1958).

in Fig. 4, which resembles Fig. 1 but contains the galacto- rather than the gluco- configuration. It should be pointed out that no experimental evidence for participation of D-galactose as envisioned in this scheme was presented.

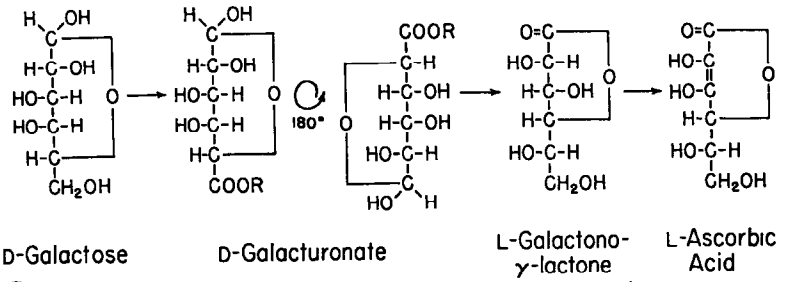

FIG. 4. CONVERSION OF D-GALACTOSE TO L-ASCORBIC ACID IN PLANTS (ISHERWOOD, CHEN AND MAPSON)

TRACER STUDIES WITH LABELED SUGARS

If the schemes of L-ascorbic acid biosynthesis outlined in Figs. 1, 2 and 4 actually represent the path of conversion from D-glucose or D-galactose in plants, one should be able to test these ideas with specifically labeled sugars in a manner similar to the experiments described by C. G. King and co-workers.

Conversion of D-*glucose to* L-*ascorbic acid in the strawberry*

A series of experiments was performed in which D-glucose-1-^{14}C, D-glucose-2-^{14}C and D-glucose-6-^{14}C were given to individual, detached, ripening strawberries (*Fragaria*), either through the severed stem or by injection directly into the berry.[8] After periods of metabolism ranging from 24 to 120 hr, each berry was macerated in 70% ethanol. After removal of the alcohol, L-ascorbic acid was recovered from the 70% ethanol-soluble portion of the berry by means of an ion exchange resin column (Dowex 1, formate). Ascorbic acid was eluted by means of a dilute formic acid gradient. To identify ascorbic acid-containing fractions, aliquots were titrated with 2,6-dichlorophenol indophenol, combined, diluted with a known quantity of L-ascorbic acid carrier, and recrystallized to constant specific activity. The internal ^{14}C distribution was determined by chemical degradation.[2,3] Results from a number of experiments are summarized in Table 1.

TABLE 1. DISTRIBUTION OF ^{14}C IN L-ASCORBIC ACID RECOVERED FROM RIPENING STRAWBERRIES GIVEN SPECIFICALLY LABELED D-GLUCOSE

Labeled glucose given and berry color	Metabolism (hr)	^{14}C distribution (per cent) Carbon 1	2	3	4	5	6
Glucose-1-^{14}C							
Green	42	55	19	4	1	2	19
White	40	67	1	10	7	1	14
Pink	24	66	1	14	3	1	15
Red	24	56	9	18	—2—		15
Red	120	62	6	9	—4—		19
Glucose-2-^{14}C							
White	23	0	73	0	12	10	5
White	46	0	69	6	11	12	2
Glucose-6-^{14}C							
Green	47	24	1	1	—2—		73

[8] F. A. LOEWUS, R. JANG and C. G. SEEGMILLER, *J. Biol. Chem.* **222**, 649 (1956).

In each instance, the carbon atom in L-ascorbic acid containing the most ^{14}C corresponded to the same numbered carbon atom in the original D-glucose. Thus, D-glucose-1-^{14}C, -2-^{14}C or -6-^{14}C labeled strawberries yielded L-ascorbic acid with 55 to 73 per cent of its ^{14}C in carbon 1, 2 or 6 respectively. Another 14 to 24 per cent of the ^{14}C redistributed into carbon 6 or 1 in L-ascorbic acid from berries labeled with D-glucose-1-^{14}C or -6-^{14}C. Such redistribution patterns have been routinely encountered in other studies on hexose products in plants given specifically labeled D-glucose.[9-12] Presumably, this is caused by partial equilibration with triose phosphate during passage of the labeled hexose through the hexose phosphate pool, but a fuller explanation of the observation is needed. Berries labeled with D-glucose-1-^{14}C or -2-^{14}C formed L-ascorbic acid in which a significant amount of ^{14}C appeared in carbons other than those characterizing triose phosphate metabolism. It would appear that this ^{14}C redistribution was due to pentose phosphate metabolism. Experiments with D-xylose-1-^{14}C and L-arabinose-1-^{14}C seem to bear out this view.[13] These results suggest that the carbon chain of D-glucose was utilized for L-ascorbic acid biosynthesis without complete rearrangement of the labeled carbon. Furthermore, the experiments indicate an oxidation of carbon 1 of D-glucose rather than carbon 6, as had been shown in animals and proposed for plants.

To clarify these early experiments, a new series* of three strawberries was labeled with D-glucose-6-^{14}C by means of the techniques described above. After 17, 41 and 65 hr, berries were macerated in 70% ethanol. L-Ascorbic acid and sucrose were recovered from the soluble fraction, and the sucrose was further hydrolyzed to D-glucose and D-fructose with invertase. The D-glucose moiety was diluted with carrier D-glucose and recrystallized to constant activity. Treatment of the insoluble residue with a fungal pectinase released considerable D-galacturonic acid into solution. This acid was recovered in crystalline form as its sodium calcium salt.[14] Finally, all three crystalline products were degraded to determine their internal ^{14}C distributions.

TABLE 2. DISTRIBUTION AND SPECIFIC ACTIVITY OF L-ASCORBIC ACID, SUCROSE-DERIVED D-GLUCOSE AND D-GALACTURONOSYL RESIDUES OF PECTIN OBTAINED FROM STRAWBERRIES GIVEN APPROXIMATELY 15 μc OF D-GLUCOSE-6-^{14}C BY INJECTION INTO THE BERRY PULP

Berry constituent	Metabolism (hr)	^{14}C distribution (per cent)		Specific activity (μc/mmole)
		Carbon 1	Carbon 6	
L-Ascorbic acid	17	19	78	0·84
	41	19	74	2·36
	65	20	77	1·13
D-Glucose (from sucrose)	17	14	85	17·7
	41	17	79	5·7
	65	16	76	1·6
D-Galacturonate (from pectin)	17	16	83	0·67
	41	19	78	1·59
	65	18	80	0·74

* F. A. LOEWUS and S. KELLY, unpublished observations.

[9] J. EDELMAN, V. GINSBURG and W. Z. HASSID, *J. Biol. Chem.* **213**, 843 (1955).
[10] A. C. NEISH, *Can. J. Biochem. Physiol.* **33**, 658 (1955).
[11] S. SHIBKO and J. EDELMAN, *Biochim. Biophys. Acta* **25**, 642 (1957).
[12] G. A. MACLACHLAN and H. K. PORTER, *Biochim. Biophys. Acta* **46**, 244 (1961).
[13] F. A. LOEWUS and R. JANG, *J. Biol. Chem.* **232**, 521 (1958).
[14] H. S. ISBELL and H. L. FRUSH, *J. Research Nat. Bur. Standards* **32**, 77 (1944).

In each instance, the product retained the greatest portion of ^{14}C in carbon 6 (Table 2). That portion of D-glucose-6-^{14}C utilized for L-ascorbic acid synthesis was oxidized at carbon 1, and the portion going to D-galacturonosyl units of pectin was oxidized at carbon 6. The labeling pattern of the D-glucose moiety of sucrose provided an internal check on the fate of the carbon chain of labeled D-glucose after its introduction into the strawberry. The ^{14}C in sucrose was equally divided between D-fructose and D-glucose. If the assumption is made that D-galacturonosyl residues of pectin are derived from the same biosynthetic pathway as the D-galacturonic acid intermediate proposed by the scheme in Fig. 4, one would expect most of the ^{14}C in L-ascorbic acid to appear in carbon 1. Clearly, this was not the case.

Even at 17 hr, almost maximum redistribution of ^{14}C from carbon 1 had taken place in all three products. Very little further movement of ^{14}C into other carbons took place up to 65 hr. The specific activity of both L-ascorbic acid and D-galacturonic acid continued to increase over 41 hr, but sucrose decreased beyond 17 hr. It would seem that both L-ascorbic acid and pectin are synthesized from a hexose precursor that can be formed from sucrose reserves in the berry without further carbon chain cleavage.

Conversion of D-*galactose to* L-*ascorbic acid in the strawberry*

Isherwood, Chen and Mapson singled out D-galactose as the principal precursor of L-ascorbic acid in plants (5). Their idea was tested by administering D-galactose-1-^{14}C to ripening strawberries in experiments similar to those just described for labeled D-glucose. The results of three separate experiments are presented in Table 3.[15,16] A greater percentage

TABLE 3. DISTRIBUTION OF ^{14}C INTO CARBONS 1 AND 6 ONLY OF L-ASCORBIC, SUCROSE-DERIVED D-GLUCOSE, AND D-GALACTURONOSYL RESIDUES OF PECTIN FROM STRAWBERRIES GIVEN D-GALACTOSE-1-^{14}C

Berry constituent	Experiment	^{14}C distribution (per cent)	
		Carbon 1	Carbon 6
L-Ascorbic acid	1	47	39
	2	65	23
	3	67	25
D-Glucose (from sucrose)	1	78	14
	2	78	14
	3	80	15
D-Galacturonate (from pectin)	1	67	21
	2	72	23
	3	68	25

of ^{14}C appeared in carbon 1 of L-ascorbic acid than carbon 6 in each experiment, but unlike the D-glucose-1-^{14}C labeled berries, appreciable redistribution into carbon 6 occurred. Pectin also reflected this increased redistribution, but the effect was less pronounced in the all-green berry. Again sucrose was used as an internal control to monitor the labeling pattern of hexose after incorporation. Both moieties of sucrose were equally labeled. The D-glucose moiety revealed far less redistribution than L-ascorbic acid or pectin. Apparently

[15] F. A. LOEWUS and R. JANG, *J. Biol. Chem.* **232**, 505 (1958).
[16] F. A. LOEWUS, R. JANG and C. G. SEEGMILLER, *J. Biol. Chem.* **232**, 533 (1958).

D-galactose is rapidly converted to hexose phosphate, then utilized in exactly the same way as D-glucose. Rapid conversion of D-galactose-1-^{14}C to sucrose in which about 72 per cent of the ^{14}C in D-glucose was in carbon 1 was observed in *Canna* leaf disks.[17] A similar finding was made on the incorporation of D-galactose-1-^{14}C into cell wall polysaccharides of wheat.[18]

Neither the D-glucose- nor the D-galactose-labeled sugar experiments can be explained using the schemes of L-ascorbic acid biosynthesis described by Isherwood *et al.* On the contrary, these tracer studies suggest that, in so far as the strawberry is a representative plant tissue, the conversion of these sugars to L-ascorbic acid follows a different route, one not involving the oxidation of carbon 6 of the parent sugar but rather, carbon 1. Thus it would appear that plants possess two processes of L-ascorbic acid formation.

Which of the two pathways of L-ascorbic acid biosynthesis is physiologically significant in intact plants? An adequate answer to this question required additional studies on the newly discovered conversion of D-glucose to L-ascorbic acid by way of a carbon 1 oxidation as well as an investigation of the process of uronic acid metabolism. To this end, experiments were performed which extended the strawberry observations to other plant and animal tissues. The exact isomeric nature of L-ascorbic acid formation in plants was re-examined. Studies of uronic acid metabolism were undertaken. Finally, the fate of terminally-bound hydrogen in the course of the D-glucose to L-ascorbic acid conversion was investigated with tritium isotope. A review of these studies is presented in the following sections.

Conversion of D-*glucose*-1-^{14}*C to* L-*ascorbic acid in other plant tissues*

Cress seedlings (Lepidum sativum). This tissue was used by Isherwood *et al.*[5] in their comparative study with the normal rat, therefore information on the distribution of ^{14}C in L-ascorbic acid from specifically labeled D-glucose would be highly relevant. Two-day-old etiolated seedlings, grown on filter paper disks from seeds which had been first washed in saturated calcium hypochlorite, were given a sterile solution of 0·1% D-glucose-1-^{14}C without disturbing the seedlings.[19] After two days in which the radioactive sugar was metabolized by the seedlings, the plant tissues, less their mucilaginous seed coats, were macerated, and the L-ascorbic acid was isolated, diluted with carrier, and degraded. Most of the ^{14}C in the recrystallized L-ascorbic acid remained in carbon 1 (Table 4), just as had been previously found in strawberries.

TABLE 4. DISTRIBUTION OF ^{14}C IN L-ASCORBIC ACID FROM CRESS SEEDLINGS GIVEN D-GLUCOSE-1-^{14}C

Carbon atom	^{14}C distribution (per cent)	Specific activity (cpm/mmole ascorbic acid)
1	73·5	2,330
1 + 2	77	2,450
3	2·5	80
4 + 5	3	100
6	17	540
Sum of fragments		3,170
Van Slyke–Folch combustion of whole molecule		3,240

[17] W. Z. HASSID, F. W. PUTMAN and V. GINSBURG, *Biochim. Biophys. Acta* **20**, 17 (1956).
[18] A. C. NEISH, *Can. J. Biochem. Physiol.* **36**, 187 (1958).
[19] F. A. LOEWUS and R. JANG, *Biochim. Biophys. Acta* **23**, 205 (1957).

Parsley leaves (Petroselinum). D-Glucose-1-^{14}C was administered to detached parsley leaves through the freshly severed petiole. The tracer was taken up within 30 min. The parsley leaves were then transferred to water and left to photosynthesize for 41 hr under ordinary room illumination. Experiment 1 was performed on relatively mature leaves; Experiment 2 involved younger leaves, fully emerged but still quite 'mossy' in appearance. At the end of that period, the leaves were frozen, pulverized, and macerated in 0·1% oxalic acid. L-Ascorbic acid was recovered, as well as sucrose-derived D-glucose, D-galacturonosyl units of pectin, and *myo*-inositol.

Table 5 lists the specific activity, per cent ^{14}C incorporation, and internal ^{14}C distribution pattern for each compound. About 80 per cent of the ^{14}C incorporated into

TABLE 5. DISTRIBUTION, AMOUNT INCORPORATED AND SPECIFIC ACTIVITY OF ^{14}C IN CONSTITUENTS FROM PARSLEY LEAVES GIVEN APPROXIMATELY 15 μc OF D-GLUCOSE-1-^{14}C

Constituent	Amount incorporated (per cent)	Specific activity (μc/mmole)	^{14}C distribution (per cent)		
			Carbon 1	Carbons 2–5	Carbon 6
Experiment 1					
L-Ascorbic acid	0·55	1·99	77	8	15
D-Glucose	6·97*	10·8	80	†	†
D-Galacturonate	2·1	0·69	78	6	16
myo-Inositol‡	0·2	2·32	14	16	70
Experiment 2					
L-Ascorbic acid	0·46	3·75	82	6	12
D-Glucose	3·69*	16·1	83	†	†
D-Galacturonate	3·8	2·81	79	5	16
myo-Inositol‡	0·39	6·6	12	6	82

* Total ^{14}C in sucrose before hydrolysis.

† Not determined.

‡ Inositol numbered according to Angyal and Anderson.[20]

L-ascorbic acid, D-galacturonic acid and D-glucose (sucrose) was located in carbon 1. *myo*-Inositol had a similar pattern, since carbons 1 through 6 or *myo*-inositol correspond to carbons 6 through 1 of D-glucose.[21] Therefore, even in the green leaf, as characterized by parsley, D-glucose is converted to L-ascorbic acid through the oxidation of carbon 1, whereas D-galacturonic acid is formed by an oxidation of carbon 6.

Mung bean or green gram (Phaseolus radiatus). Several years ago, Nath, Chitale and Belavady[22] found an appreciable increase in L-ascorbic acid in mung beans that had germinated in a solution containing the condensation product of D-glucose and ethyl acetoacetate, D-glucose-cycloacetoacetate ethyl ester. The identity of the L-ascorbic acid was established by paper chromatography[23] and animal assay.[24] Subsequently, Thangamani and Sarma[25] prepared the condensation product from uniformly labeled D-glucose-^{14}C and compared this product with the free D-glucose-^{14}C as an L-ascorbic acid precursor in mung bean seedlings. They found the condensation product nearly five times more effective as a ^{14}C precursor. Now Belkhode and Nath[26] have presented additional information

[20] S. J. ANGYAL and L. ANDERSON in *Advances in Carbohydrate Chemistry*, Interscience Pubs., Inc., New York, **14**, 135, (1959).
[21] F. A. LOEWUS and S. KELLY, *Biochem. Biophys. Research Commun.* **7**, 204 (1962).
[22] M. C. NATH, R. P. CHITALE and B. BELAVADY, *Nature* **170**, 545 (1952).
[23] M. C. NATH and M. L. BELKHODE, *Nature* **183**, 1258 (1959).
[24] M. C. NATH and M. L. BELKHODE, *Indian J. Physiol. Allied Sci.* **14**, 71 (1960).
[25] A. THANGAMANI and P. S. SARMA, *J. Sci. and Ind. Research (India)* **15C**, 157 (1956).
[26] M. L. BELKHODE and M. C. NATH, *J. Biol. Chem.* **237**, 1742 (1962).

concerning this conversion. They prepared the condensation product from D-glucose-1-^{14}C as well as from D-glucose-6-^{14}C. Freshly germinated seedlings were grown for 2 days in solutions of these labeled condensation products. Three experiments were performed in each instance. L-Ascorbic acid was recovered by ion exchange resin, diluted with L-ascorbic acid carrier, and crystallized from glacial acetic acid. Degradation[2,3] revealed that the L-ascorbic acid from plants labeled with the D-glucose-1-^{14}C ester contained 65–72 per cent of its ^{14}C in carbon 1 and another 14–18 per cent in carbon 6. When the ester was prepared from D-glucose-6-^{14}C, the resulting L-ascorbic acid had 11–17 per cent of its ^{14}C in carbon 1 and 64–67 per cent in carbon 6. These labeling patterns resemble others obtained from D-glucose-labeled strawberries or cress seedlings. The mechanism of the D-glucose-cycloacetoacetate ethyl ester conversion is not known, but it is readily apparent that the plant can utilize the sugar portion of this ester for ascorbic acid biosynthesis in much the same way that it uses D-glucose.

Conversion of D-*glucose-2-*^{14}*C to* L-*ascorbic acid in the rat*

Despite numerous studies of ascorbic acid biosynthesis performed with rats by King, Burns and others, certain technical considerations remained to be investigated. In the first place, it appeared desirable to conduct an experiment with D-glucose labeled at a carbon other than the terminal positions, 1 or 6. Also, the original findings would be strengthened considerably if the experimental time interval could be shortened and if the ascorbic acid were recovered directly from the liver, which is the presumed site of synthesis, rather than from the urine. Finally, if direct comparison could be made with another well-characterized hexose product of D-glucose metabolism, the L-ascorbic acid data would have more meaning.

In collaboration with H. H. Hiatt, three separate experiments were performed using non-drugged rats which were normally fed, fasted, and fasted plus exercised.[27] D-Glucose-2-^{14}C was injected intraperitoneally. After 3 hr the animals were sacrificed and L-ascorbic acid as well as glycogen recovered from their livers. Glycogen was first diluted with carrier glycogen, then hydrolyzed, diluted with carrier D-glucose, and crystallized. L-Ascorbic acid was recovered from a Dowex 1 formate exchange resin column by gradient elution with dilute formic acid. It was then diluted and recrystallized in exactly the same manner as had been employed in the strawberry experiments. The per cent conversion and ^{14}C distribution in each product are given in Table 6.

TABLE 6. DISTRIBUTION AND RECOVERY OF L-ASCORBIC ACID AND GLYCOGEN-DERIVED D-GLUCOSE FROM LIVERS OF RATS GIVEN D-GLUCOSE-2-^{14}C

Product	Experiment	Amount of ^{14}C incorporated (per cent)	^{14}C distribution by carbon atom (per cent)					
			1	2	3	4	5	6
Ascorbic acid	Normally fed	0·029	0	7	4	13	58	18
	Fasted	0·014	10	12	4	18	44	12
	Fasted+ exercised	0·002	16	17	7	8	40	12
D-Glucose (glycogen)	Normally fed	0·40	15	66	8	2	7	2
	Fasted	0·014	8	66	6	2	10	8
	Fasted+ exercised	0·004	6	71	11	2	3	7

[27] F. A. LOEWUS, S. KELLY and H. H. HIATT, *J. Biol. Chem.* **235**, 937 (1960).

In each experiment, D-glucose derived from glycogen was labeled primarily in carbon 2, and L-ascorbic acid was labeled primarily in carbon 5. Somewhat greater redistribution of ^{14}C into positions other than 2 or 5 occurred in the fasted and fasted-plus-exercised rats than in the normally fed rat, but the general observation held in all three experiments, to amply confirm the original findings of Horowitz, Doerschuk and King.[2] Apparently, stress does affect the L-ascorbic acid labeling pattern as well as the amount of ascorbic acid formed, but the exploratory studies presented here offer no explanation for these observations.

Identity of ascorbic acid formed from D-*glucose-6-*^{14}C *in the strawberry*

Isherwood and Mapson[28] have recently proposed that two different pathways of ascorbic acid biosynthesis arise from the D-glucose configuration. Their proposal stemmed from an attempt to explain the redistribution of ^{14}C in ascorbic acid recovered from strawberries tagged with specifically labeled D-glucose or D-galactose. They suggested that the product was really a mixture of two isomeric acids, L-ascorbic acid and D-araboascorbic acid, the former being formed by processes similar to those shown in Fig. 1 or 4, and the latter by oxidation of D-glucose at carbon 1, but without an epimerization about carbon 5 to form the L-configuration. Their proposal merits attention, but it should be pointed out in fairness to the early tracer studies that the ascorbic acid recovered from the strawberries was characterized reasonably well. It was diluted with L-ascorbic acid as carrier, then recrystallized until there was no further change in the specific activity. The recrystallized material was also converted to the *o*-dianil of dehydroascorbic acid with no loss of activity. Furthermore, the occurrence of labeling patterns similar to those reported in Tables 1, 2, 3, 4 and 5 has been reported in many other products of hexose metabolism in which the carbon chain is conserved.

In 1960, Takahashi, Mitsumoto and Kayamori[29] discovered D-araboascorbic acid in a number of *Penicillium* molds which had been grown on D-glucose, D-gluconate or sucrose. When D-glucose-1-^{14}C was employed as a tracer, D-araboascorbic acid was formed in which 80 per cent of the incorporated ^{14}C was in carbon 1. Further studies by Takahashi and Mitsumoto[30] led them to propose the scheme of D-araboascorbic acid biosynthesis outlined in Fig. 5. An aerodehydrogenase catalyzing a reaction similar to the initial reaction in Fig. 5, the oxidation of D-gluconic acid, has been described in citrus fruit vesicles.[31]

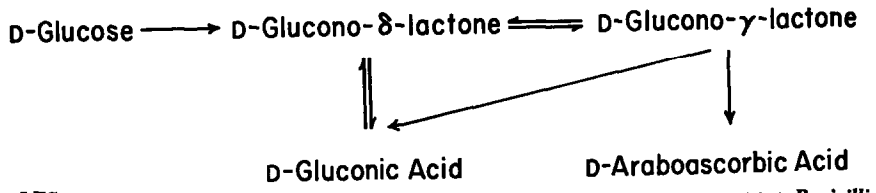

FIG. 5. SCHEME OF D-GLUCOSE OXIDATION AND D-ARABOASCORBIC ACID FORMATION *Penicillium* (TAKAHASHI AND MITSUMOTO).

The possible significance of D-araboascorbic acid biosynthesis in *Penicillium*, as it might relate to higher plants, made it imperative that the experiments in which labeled D-glucose was converted to L-ascorbic acid by the strawberry be carefully rechecked. The experiment

[28] F. A. ISHERWOOD and L. W. MAPSON, *Ann. N.Y. Acad. Sci.* **92**, 6 (1961).
[29] T. TAKAHASHI, M. MITSUMOTO and H. KAYAMORI, *Nature* **188**, 411 (1960).
[30] T. TAKAHASHI and M. MITSUMOTO, *Biochim. Biophys. Acta* **51**, 410 (1961).
[31] R. C. BEAN, G. G. PORTER and B. M. STEINBERG, *J. Biol. Chem.* **236**, 1235 (1961).

was performed as follows.[32] To a detached ripening strawberry was given 7 mg D-glucose-6-^{14}C containing 50 μc of activity. Within 3 hr, all the radioactivity had been imbibed through the cut stem. An additional 40 hr of metabolism was allowed while the berry took up distilled water through the stem. The berry was macerated at —40° in ethanol and its ascorbic acid recovered by the usual procedure from an anionic exchange resin column by elution with a dilute formic acid gradient. Ascorbic acid appeared as a discrete peak. Peak fractions were combined, mixed and then separated into two portions. To one was added 200 mg of authentic L-ascorbic acid while to the other portion was added 200 mg of D-araboascorbic acid. Each portion was then carried through three recrystallizations with glacial acetic acid. Table 7 summarizes the results. Only the L-ascorbic acid diluted portions retained its radioactivity throughout the recrystallizations.

TABLE 7. RETENTION OF ^{14}C ON RECRYSTALLIZATION OF CARRIER DILUTED ASCORBIC ACID FROM A STRAWBERRY GIVEN D-GLUCOSE-6-^{14}C

Number of crystallizations	Specific activity of ascorbic acid (mμc/mmole)	
	Diluted with L-ascorbic acid as carrier	Diluted with D-araboascorbic acid as carrier
1	74·7	8·2
2	68·7	2·0
3	66·5	1·0

As an added check, two derivatives of the reduced form of L-ascorbic acid were also prepared, the 2,3-diphenacyl derivative and the 5,6-isopropylidene derivative. Under experimental conditions employed, the latter forms only with L-ascorbic acid, not with D-araboascorbic acid. Both of these L-ascorbic acid derivatives retained radioactivity comparable to the specific activity of L-ascorbic acid itself.

The L-ascorbic acid diluted portion was degraded chemically. It contained 71 per cent of the incorporated ^{14}C in carbon 6 and 18 per cent in carbons 1+2, a pattern similar to those described in Table 2.

This experiment demonstrates that D-araboascorbic acid is not formed from D-glucose by the ripening strawberry. How generally this observation can be applied remains to be determined. It is still possible that some plants do form D-araboascorbic acid. Certainly, a re-investigation of the isomeric nature of ascorbic acid throughout the plant kingdom would be worthwhile.

TRACER STUDIES WITH LABELED URONIC ACIDS

Metabolism of D-*glucuronolactone by the ripening strawberry*

None of the data obtained from plant tissues given labeled sugars provided evidence of the participation of D-glucuronic acid, D-galacturonic acid, or their derivatives in L-ascorbic acid biosynthesis. Nevertheless, plants do utilize exogenously supplied uronic and aldonic acids and their lactones or esters. In particular, Isherwood *et al.*[5] found that D-glucurono-lactone, D-galacturonic acid methyl ester, L-gulonolactone and L-galactonolactone were effective in causing increases in L-ascorbic acid levels in intact cress seedlings. When enzyme preparations from pea seeds were studied, Mapson and Isherwood[33] found D-galacturonic acid methyl ester was reduced, presumably to L-galactonolactone, by an

[32] F. A. LOEWUS and S. KELLY, *Nature* **191**, 1059 (1961).
[33] L. W. MAPSON and F. A. ISHERWOOD, *Biochem. J.* **64**, 13 (1956).

NADP-dependent oxidoreductase. D-Glucuronolactone was also reduced, presumably to L-gulonolactone, but at a much slower rate. Only L-galactonolactone was oxidized by plant enzyme preparations to L-ascorbic acid.[6,7] The presence of free D-galacturonic acid, its methyl ester or L-galactonolactone in plants is problematical, therefore the significance of the enzymatic reactions is uncertain.

The overall conversion of D-glucose and D-galactose to D-galacturonosyl residues of pectin was first shown by Seegmiller *et al.*[34,35] Later, enzymatic steps in the oxidation of D-glucose to uridine diphosphate D-glucuronic acid (UDP D-glucuronic acid) were detailed[36,37] and the conversion of UDP D-glucuronic acid to UDP D-galacturonic acid,[38] as well as the phosphorylation of D-glucuronic acid[39] and D-galacturonic acid[40] in higher plants, was described. The final enzymatic steps leading to methyl esterification and pectin formation have not been found. Nucleotide intermediates, especially UDP D-glucuronic acid, probably play a major role in D-galacturonic acid synthesis, but whether this process ever provides free D-galacturonic acid or an ester-like derivative in the plant cell that can lead to L-ascorbic acid is not known.

In order to gain some insight into the differences between the biosynthetic pathway leading to pectin and the one leading to L-ascorbic acid, a rather detailed study of D-glucuronolactone metabolism was undertaken.[16,41,42] Other contributions to present knowledge of D-glucuronic and D-galacturonic acid metabolism have appeared.[42-50] The present discussion is limited to observations pertaining to L-ascorbic acid formation.

In the first exploratory experiments,[8,16] D-glucuronic acid-6-^{14}C was given to detached strawberries. Very little ^{14}C was found in the L-ascorbic acid, and even this radioactivity decreased with repeated recrystallization. The portion that still remained showed up, unexpectedly, upon chemical degradation, in carbon 3 which is the carbon atom obtained from the carboxyl position of the L-threonic acid fragment[2] after periodate oxidation. Any contaminating aldonic or uronic acid could contribute ^{14}C to this particular carbon dioxide fraction. Later studies revealed that this was exactly what happened; the L-ascorbic acid fraction had been contaminated by an adjacent aldonic acid peak which had been eluted from the ion exchange column just ahead of the ascorbic acid. The rather concentrated formic acid gradient used in these early studies did not permit complete resolution of the two peaks. The aldonic acid peak was subsequently shown to be composed primarily of L-gulonic acid.[51]

Among the early experiments was one (Experiment 12[8]) in which D-glucuronolactone-6-^{14}C was given to the strawberry. It gave an ascorbic acid fraction much more radioactive

[34] C. G. Seegmiller, B. Axelrod and R. M. McCready, *J. Biol. Chem.* **217**, 765 (1955).
[35] C. G. Seegmiller, R. Jang and W. Mann, Jr., *Arch. Biochem. Biophys.* **61**, 422 (1956).
[36] J. L. Strominger and L. W. Mapson, *Biochem. J.* **66**, 567 (1957).
[37] W. Z. Hassid, E. F. Neufeld and D. S. Feingold, *Proc. Natl. Acad. Sci. U.S.* **45**, 905 (1959).
[38] D. S. Feingold, E. F. Neufeld and W. Z. Hassid, *J. Biol. Chem.* **235**, 910 (1960).
[39] E. F. Neufeld, D. S. Feingold and W. Z. Hassid, *Arch. Biochem. Biophys.* **83**, 96 (1959).
[40] E. F. Neufeld, D. S. Feingold, S. M. Ilves, G. Kessler and W. Z. Hassid, *J. Biol. Chem.* **236**, 3102 (1961).
[41] F. A. Loewus, B. J. Finkle and R. Jang, *Biochim. Biophys. Acta* **30**, 629 (1958).
[42] B. J. Finkle, S. Kelly and F. A. Loewus, *Biochim. Biophys. Acta* **38**, 332 (1960).
[43] H. A. Altermatt and A. C. Neish, *Can. J. Biochem. Physiol.* **34**, 405 (1956).
[44] K. Sivarama Sastry and P. S. Sarma, *Nature* **179**, 44 (1957).
[45] E. A. M. Asselbergs, *Plant Physiol.* **32**, 326 (1957).
[46] M. Nakatani, *Bull. Agr. Chem. Soc. (Japan)* **22**, 261 (1958).
[47] A. C. Neish, *Can. J. Biochem. Physiol.* **36**, 187 (1958).
[48] W. G. Slater and H. Beevers, *Plant Physiol.* **33**, 146 (1958).
[49] L. Hough and J. B. Pridham, *Arch. Biochem. Biophys.* **59**, 17 (1955).
[50] G. Kessler, E. F. Neufeld, D. S. Feingold and W. Z. Hassid, *J. Biol. Chem.* **236**, 308 (1961).
[51] F. A. Loewus and S. Kelly, *Biochem. Biophys. Research Commun.* **1**, 143 (1959).

than the D-glucuronic acid-6-^{14}C experiments. This finding prompted a more carefully designed study aimed at retrieval of L-ascorbic acid free of the aldonic acid contaminant. The new series of experiments was designed also, to avoid another problem encountered in the exploratory work. Those experiments had revealed that a considerable portion of carbon 6 of D-glucuronic acid was lost as carbon dioxide.[8] Use of the lactone as well as comparison of D-glucuronolactone-1-^{14}C with D-glucuronolactone-6-^{14}C metabolism at appreciably higher activities made it possible to circumvent this as well as a number of other pitfalls previously encountered.

Once the method of L-ascorbic acid recovery had been modified by using a very dilute formic acid gradient for the elution of the acid from the ion exchange column, it was easy to show that both D-glucuronolactone-1-^{14}C and -6-^{14}C were effective precursors of L-ascorbic acid.[52] About 1 per cent of the lactone was converted to ascorbic acid in less than 2 days of metabolism within the berry. During the same time about 2·5 per cent was converted to D-galacturonosyl residues of pectin and about 5 per cent to L-gulonic acid. About 30 per cent of the ^{14}C from D-glucuronolactone-6-^{14}C appeared as carbon dioxide while only about 10 per cent of the ^{14}C from D-glucuronolactone-1-^{14}C was respired as carbon dioxide in the same time. Between 4 to 8 per cent of the ^{14}C from D-glucuronolactone-1-^{14}C was recovered as D-xylose-1-^{14}C. These results reveal the rather complex nature of D-glucuronolactone catabolism. Apparently several different processes contribute additively to the utilization of the lactone. The process involved in L-ascorbic acid formation requires the reduction of carbon 1 of the lactone. This is revealed, very clearly, in the labeling patterns of L-gulonic acid and L-ascorbic acid, as contrasted with the pattern of D-galacturonic acid from berries labeled with either D-glucuronolactone-1-^{14}C or -6-^{14}C (Table 8). The carboxyl function of the uronic acid is conserved, becoming the carboxyl function in L-ascorbic acid just as it does in animal tissues.

TABLE 8. DISTRIBUTION OF ^{14}C IN L-GULONIC ACID, L-ASCORBIC ACID AND D-GALACTURONATE FROM STRAWBERRIES GIVEN D-GLUCURONOLACTONE-1-^{14}C OR -6-^{14}C

Site of ^{14}C in glucuronolactone	Labeled constituent	^{14}C distribution (per cent)	
		Carbon 1	Carbon 6
-1-^{14}C	L-Gulonic acid	0	100
	L-Ascorbic acid	0	100
	D-Galacturonate	99	1
-6-^{14}C	L-Gulonic acid	100	*
	L-Ascorbic acid	95	2
	D-Galacturonate	*	99

* Not determined.

It is interesting to note that none of these labeling patterns reveal the characteristic redistribution of ^{14}C of hexose-labeled strawberries. Presumably, these effects are limited to substances that enter the hexose and triose phosphate pathways. In the case of D-glucuronolactone, these effects do not become appreciable until D-xylose is generated by the lactone metabolism. Even then, the effect can be detected only with D-glucuronolactone labeled in one or more of the first five carbons, which produces a labeled D-xylose that can re-enter normal sugar phosphate metabolism *via* the pentose phosphate pathway. This has been clearly demonstrated by the nature of the labeling pattern found in D-glucose derived from sucrose in berries labeled with D-glucuronolactone-1-^{14}C.[52]

[52] F. A. LOEWUS, *Ann. N.Y. Acad. Sci.* **92**, 57 (1961).

Isherwood and Mapson were inclined to discount the role of D-glucuronolactone and L-gulonolactone as precursors of L-ascorbic acid in plants. The tracer studies reported here show that D-glucuronolactone is readily utilized in this synthesis when supplied exogenously, but the nature of this conversion is not revealed. It is possible that L-gulonolactone is formed, then oxidized directly to L-ascorbic acid or L-gulonolactone may undergo an epimerization to L-galactonolactone, which is then oxidized by a system such as that already reported by Mapson *et al.*[6,7] Other possibilities also exist[52] although recent experiments of Isherwood and Mapson[28] would seem to make them less likely.

Metabolism of D-*galacturonic acid by the ripening strawberry*

D-Galacturonic acid recovered from the pectin of strawberries labeled with D-glucuronolactone-1-^{14}C contained nearly all of its ^{14}C in carbon 1 (Table 8). Enough of this label was recovered to perform a single experiment on the metabolism of this compound. Fortunately, all aspects of this study turned out successfully, and a reasonably complete assessment of the fate of D-galacturonic acid in the strawberry was possible.[53] D-Galacturonic acid-1-^{14}C was given as a partial salt (pH 3 to 4). It entered the stem of a ripening berry very slowly, but enough radioactivity was taken up to insure a survey of its metabolism. It resembled D-glucuronic acid in many respects. It formed an aldonic acid (L-galactonic acid), D-xylose, and a very small amount of L-ascorbic acid, and it was incorporated into pectin. An appreciable portion was oxidized to galactaric acid (mucic acid). A search for this particular acid was prompted by an earlier report of mucic acid synthesis from D-galacturonic acid in mung bean seedlings.[50]

The very small amount of ^{14}C associated with L-ascorbic acid made it imperative that this ^{14}C actually be demonstrated to be a part of the L-ascorbic acid molecule and not a radioactive impurity. To show this, ascorbic acid from the labeled strawberry was diluted with carrier L-ascorbic acid and then twice crystallized from glacial acetic acid. The crystalline product was oxidized to dehydroascorbic acid and then treated with *o*-phenylenediamine to form the di-*o*-anil derivative of dioxo-L-gulonic acid. This derivative readily crystallizes from water providing an added means of removing water-soluble radioactive impurities, if present. The dioxo-L-gulonic acid derivative was oxidized with periodate to yield carbons 1 through 4 as an insoluble reddish precipitate, carbon 5 as formic acid and carbon 6 as formaldehyde. The formic acid and formaldehyde were separated by procedures already described[34,35] and counted separately as carbon dioxide. Practically all of the ^{14}C present was recovered in the formaldehyde fraction (carbon 6). In Table 9, the total specific activity of L-ascorbic acid is compared with the specific activity of the

TABLE 9. COMPARISON OF THE SPECIFIC ACTIVITIES OF SEVERAL PRODUCTS OF D-GALACTURONATE-1-^{14}C METABOLISM IN THE STRAWBERRY WITH THE SPECIFIC ACTIVITIES OF THEIR MOST ACTIVE FRAGMENT

Product	Specific activity of product (dpm/mmole)	Fragment (carbon atom number)	Specific activity of fragment (dpm/mmole)
L-Ascorbic acid	790	6	750
L-Galactonic acid	2,260	6	2,240
L-Galactaric acid	13,700	1+6	13,400
D-Galacturonic acid (pectin)	38,900	1	35,600
D-Xylose	12,200	1	12,000

[53] F. A. LOEWUS and S. KELLY, *Arch. Biochem. Biophys.* **95**, 483 (1961).

carbon 6 fragment. This table also lists comparable results for the other products which were recovered and then degraded.

The D-galacturonic acid-1-^{14}C was incorporated into pectin without cleavage of the carbon chain or redistribution of the specific label (Table 9). It was reduced at carbon 1, as shown by the appearance of almost all the ^{14}C in carbon 6 of L-galactonic acid and L-ascorbic acid. It was also oxidized at carbon 1 to form galactaric acid. In this instance, periodate oxidation revealed the presence of all of the ^{14}C in the terminal carboxyl groups, but the two ends of the molecule could not be distinguished.

Surprisingly, the free pentose turned out to be D-xylose, not L-arabinose as one might postulate if direct decarboxylation had occurred. D-Xylose might have been formed from L-arabinose by epimerization or by a set of related conversions.[13] It might also have been formed in an oxidative decarboxylation of D-galacturonic acid followed by reduction of the five carbon fragment after rearrangement to the D-xylo- configuration. Additional studies are needed to clarify this interesting observation.

Metabolism of D-*galacturonic acid methyl ester by the strawberry*

As a companion experiment to the study of D-galacturonic acid metabolism, utilization of its methyl ester was also investigated.[53] This ester was prepared from uniformly labeled D-galacturonic acid, so the specific nature of labeling patterns in its products could not be determined. The methyl ester was an excellent precursor of L-ascorbic acid, many times more effective as a carbon source than the free acid. It was also incorporated into pectin, but only about one-third as well as the free acid was. It did not form galactaric acid or D-xylose. Although an appreciable quantity of radioactivity remained in the neutral (sugar containing) fraction of the ethanol soluble portion of the berry, none of the easily recognized sugars were labeled. The ^{14}C containing constituents of the neutral fraction chromatogrammed[15] well ahead of xylose with the unfortunate result that all except the slowest component were lost from the paper. This one substance was identified as an ester of L-galactonic acid (either the lactone or methyl ester). Methods of separation used precluded further characterization. A lactone or ester has been thought necessary to convert uronic acid to L-ascorbic acid.[5] Undoubtedly, where penetration through the cell wall is involved this is indeed the case. However, D-galacturonic acid introduced through the vascular system of the strawberry was converted to ascorbic acid. Whether this involved an enzymatic conversion in the vascular tissues prior to incorporation in the cells of the strawberry fruit is not known. The experiment will have to be repeated with D-galacturonic acid containing higher specific activity to clarify this aspect. In any case, the present studies indicate that D-galacturonic acid is relatively ineffective as an L-ascorbic acid precursor when supplied exogenously but its methyl ester and D-glucuronolactone are quite effective.

Recent studies by Jackson, Wood and Prosser[54] have shown that the oxidative step, L-galactono-γ-lactone to L-ascorbic acid, is present in many plants. In one instance they demonstrated that ascorbic acid increased from 1·5 to 12 per cent in parsley leaves placed in a 0.5% L-galactono-γ-lactone solution (calculated on a dry weight basis).

Metabolism of myo-*inositol in plants*

In 1957, Charalampous and Lyras[55] found an enzyme in rat kidney preparations that converted *myo*-inositol to glucuronic acid. Charalampous[56,57] reported that the reaction

[54] G. A. D. JACKSON, R. B. WOOD and M. V. PROSSER, *Nature* **191**, 282 (1961).
[55] F. C. CHARALAMPOUS and C. LYRAS, *J. Biol. Chem.* **228**, 1 (1957).
[56] F. C. CHARALAMPOUS, *J. Biol. Chem.* **234**, 220 (1959).
[57] F. C. CHARALAMPOUS, *J. Biol. Chem.* **235**, 1286 (1960).

involved an oxidative cleavage of the cyclitol between carbons 1 and 6,[20] to form D-glucuronic acid in which the carboxyl carbon was derived from carbon 1 of *myo*-inositol (Fig. 6).

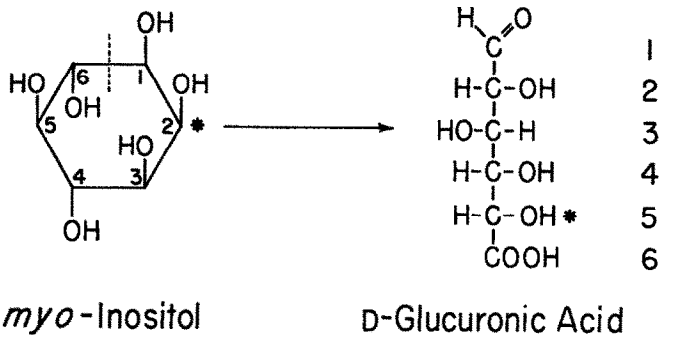

FIG. 6. OXIDATIVE CLEAVAGE OF *myo*-INOSITOL TO D-GLUCURONIC ACID.
The carbon–hydrogen bonds of *myo*-inositol are deleted in order to simplify the illustration. The dotted line between position 1 and 6 in *myo*-inositol indicates site of cleavage. The asterisk denotes the site of labeling in *myo*-inositol-2-^{14}C and its location in the product D-glucuronic acid.

Attempts to demonstrate the conversion of *myo*-inositol to L-ascorbic acid in animals were unsuccessful. Burns *et al.*[58] recovered labeled D-glucuronic acid and L-gulonic acid from the urine of rats injected intraperitoneally with *myo*-inositol containing tritium or ^{14}C but they were unable to detect labeled ascorbic acid.

Since the metabolism of *myo*-inositol has never been throughly explored in plants and since this substance might generate a uronic acid *in situ*, which could act as an ascorbic acid precursor, experiments were run on the metabolic fate of *myo*-inositol-2-t in ripening strawberry and parsley leaf and *myo*-inositol-2-^{14}C in ripening strawberry.[59] Results were roughly similar, regardless of the type of isotope employed, tritium or ^{14}C. Since, in the conversion of *myo*-inositol-2-t to D-glucuronic acid tritium appears in carbon 5 of the product, and since this carbon is eventually oxidized to the enediol configuration in the hypothetical L-ascorbic acid, one might expect loss of tritium during such a conversion. If the starting substance were *myo*-inositol-2-^{14}C, one would not be faced with these considerations. As it turned out, L-ascorbic acid from parsley or strawberry labeled with *myo*-inositol-2-t contained an insignificant amount of tritium and the presence or absence of a path of conversion from *myo*-inositol to L-ascorbic acid rested on results obtained with *myo*-inositol-2-^{14}C.

The experiment with *myo*-inositol-2-^{14}C was performed with a minimal quantity of radioisotope (0·7 μc). This label was administered to three ripening strawberries over a 2 hr period and the berries then were left to metabolize for an additional 68 hr. Labeled berries were macerated in 70% ethanol, centrifuged, and separated into washed 70% ethanol-insoluble and 70% ethanol-soluble fractions. About 53 per cent of the ^{14}C remained in the soluble fraction and washings. When run through the Dowex 1 formate exchange resin, 6 per cent of the ^{14}C remained bound. Less than 0·06 per cent of the total radioactivity was finally recovered in the eluted fractions identified as containing ascorbic acid. After a 17-fold dilution of the ascorbic acid and recrystallization, no detectable radioactivity was found in the final recrystallized L-ascorbic acid. The counting procedure was sufficiently

[58] J. J. BURNS, N. TROUSOF, C. EVANS, N. PAPADOPOULOS and B. W. AGRANOFF, *Biochim. Biophys. Acta* **33**, 215 (1959).
[59] F. A. LOEWUS, S. KELLY and E. F. NEUFELD, *Proc. Natl. Acad. Sci. U.S.* **48**, 421 (1962).

sensitive to detect less than 0·01 per cent conversion of *myo*-inositol-2-^{14}C to L-ascorbic acid, a value lower than the per cent conversion observed when D-glucose-^{14}C or D-glucuronolactone-^{14}C was used as the source of ^{14}C. This experiment should be repeated with a substantially higher quantity of ^{14}C but the results obtained in the present experiment appear to rule out *myo*-inositol as an important precursor of L-ascorbic acid.

Although *myo*-inositol-2-^{14}C did not form L-ascorbic acid, it did undergo an oxidative cleavage to D-glucuronic acid, L-gulonic acid, free D-xylose and the D-galacturonosyl, D-xylosyl, and L-arabinosyl residues of cell wall polysaccharides. This is the pattern of metabolism to be anticipated if cleavage occurred in the manner described by Charalampous.[56] Proof of cleavage between carbons 1 and 6 of *myo*-inositol was obtained[60] by reducing the D-galacturonic acid from pectin to L-galactonic acid with $NaBH_4$, converting the acid to its γ-lactone and then preparing the L-galactonamide. Both the L-galactonic acid and its amide were oxidized with periodate. Nearly all the ^{14}C was recovered in the formic acid fraction (carbons 2+3+4+5) of the L-galactonic acid, but virtually no ^{14}C was found in the formic acid fraction (carbons 3+4+5) of L-galactonamide. The ^{14}C was, by difference, in carbon 2 of the L-galactonic acid (carbon 5 of D-galacturonic acid).

The conversion of *myo*-inositol to pectin has been employed as a biochemical tool to trace the biosynthetic origin of *myo*-inositol. Since the strawberry readily metabolizes *myo*-inositol to D-galacturonosyl residues in pectin with retention of the carbon ring sequence, albeit reversed as concerns the convention of numbering, it follows that *myo*-inositol of any given labeling pattern can be given to the strawberry, and after a suitable period of metabolism, the D-galacturonosyl residues can be recovered, degraded chemically and assayed for radioactivity to determine the ^{14}C distribution related to the original *myo*-inositol.

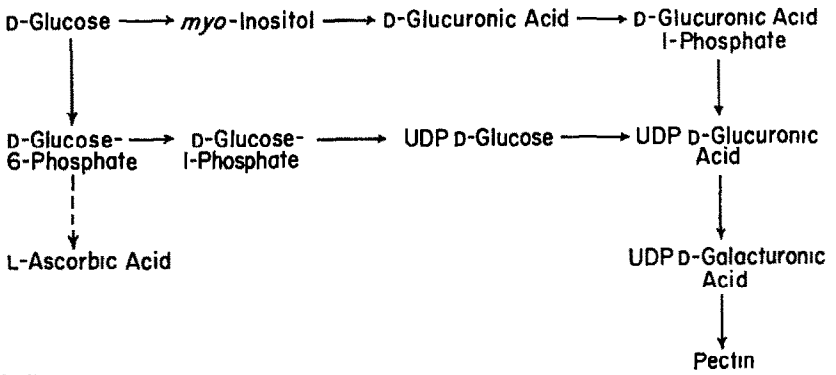

FIG. 7. COMPARISON OF ALTERNATE PATHWAYS OF URONIC ACID FORMATION IN PLANTS IN RELATION TO L-ASCORBIC ACID SYNTHESIS.

This has been done in the case where D-glucose-1-^{14}C had been converted to *myo*-inositol by the green parsley leaf. A portion of the labeled *myo*-inositol was converted to pectin in a strawberry and the resulting labeled pectin degraded.[21] Results of two such experiments were summarized in Table 5. The *myo*-inositol had 70–88 per cent of its ^{14}C in carbon 6 as judged by the distribution of label in the D-galacturonic acid recovered from the strawberries which metabolized this cyclitol. Thus, regardless of which pathway the plant actually uses for uronic acid synthesis, *via* UDP D-glucose with oxidation to UDP

[60] F.A. LOEWUS and S. KELLY, *Federation Proc.* **21**, 88 (1962).

D-glucoronic acid[36] or *via myo*-inositol with oxidative cleavage,[59] D-glucose-1-^{14}C will form uronic acid products labeled primarily in carbon 1. Neither pathway, apparently, is involved in L-ascorbic acid biosynthesis in plants, since D-glucose-1-^{14}C is converted to L-ascorbic acid by processes involving oxidation of carbon 1, not carbon 6.

Figure 7 outlines these processes of conversion from D-glucose to pectin on the one hand and to L-ascorbic acid on the other. The conversion of uronic acid esters to L-ascorbic acid in plants seems to be a process distinct from the pathway that utilizes D-glucose.

RECENT STUDIES ON THE CONVERSION OF D-GLUCOSE TO L-ASCORBIC ACID IN PLANTS

Specific information concerning the biosynthetic pathway from D-glucose to L-ascorbic acid must await a step by step elucidation of enzymatic processes, but there is information to be gained by studying the fate of hydrogen attached to the terminal carbons of D-glucose in this overall conversion.* Table 10 presents a comparison of tritium incorporation into

TABLE 10. SPECIFIC ACTIVITIES OF L-ASCORBIC ACID AND D-GALACTURONATE (PECTIN) RECOVERED FROM STRAWBERRIES GIVEN D-GLUCOSE-1-t OR -6-t

Site of tritium in glucose*	Activity of tritium given ($dpm \times 10^{-6}$)	Product†	Amount of product recovered (μmole)	Specific activity of product (dpm/μmole)	Ratio of specific activities $\frac{\text{product}}{\text{glucose}}$
Carbon 1	193	AA	45	3,700	0·0042
		G	130	1,900	0·0022
Carbon 6	249	AA	23	36,800	0·0291
		G	160	1,700	0·0014
Carbon 6	216	AA	27	15,700	0·0124
		G	180	1,800	0·0014
Carbon 6	218	AA	32	28,200	0·0223
		G	90	1,600	0·0013

* Specific activity: glucose-l-t, $0{\cdot}9 \times 10^6$ dpm/μmole; glucose-6-t, $1{\cdot}3 \times 10^6$ dpm/μmole

† L-Ascorbic acid (AA); D-Galacturonate (G)

L-ascorbic acid and D-galacturonic acid (pectin) from strawberries labeled with D-glucose-l-t or -6-t. These experiments were run under conditions quite similar to earlier ones in which the isotope was ^{14}C. As metabolism proceeded, some of the tritium appeared in the transpired water. By the time the experiment had been completed, several μc of tritium had exchanged into the water fraction of the berry. The possibility that secondary processes might possibly cause re-incorporation of tritium from tritiated water into carbohydrate constituents of the strawberry prompted experiments in which strawberries were given tritiated water containing approximately the same quantity of radioactivity as that present in the tritiated glucose studies. About 4 per cent of the label from this tritiated water entered non-exchangeable positions in the water-soluble non-volatile constituents in 40 hr. Secondary exchanges of this magnitude would have little effect, if any, on the data presented in Table 10.

L-Ascorbic acid from D-glucose-6-t labeled berries had higher specific activity than that from D-glucose-1-t. In order to compare the specific activities of D-glucose with those of L-ascorbic acid in each experiment, the ratio of specific activities of glucose to product was

* F. A. LOEWUS and S. KELLY, unpublished observations.

used (Table 10, last column), since the specific activities of the two types of labeled glucose were not identical. The ratio for L-ascorbic acid from D-glucose-6-t was 3–7 times as great as from D-glucose-1-t. This indicated preferential retention of hydrogen from carbon 6 as compared to carbon 1 of D-glucose. The fact that any tritium at all was retained from D-glucose-1-t is explained on the basis of the glycolytic processes which interconvert some carbon (with its tritium–carbon bond intact) between carbons 1 and 6. This process of redistribution was described in earlier sections and was amply illustrated in Tables 1, 2, 3, 4 and 5. It could cause the appearance at carbon 6 of as much as 40 per cent of the tritium bound to carbon 1 before the oxidation of carbon 1 removed the remaining tritium from that position. In a similar fashion, tritium at carbon 6 could be transferred into the carbon 1 position, there to be lost by oxidation prior to L-ascorbic acid formation. When this redistribution is taken into acount, and one assumes that the oxidation of carbon 1 takes place subsequent to this redistribution, then one would expect to find a ratio from D-glucose-6-t to L-ascorbic acid that is about four times the value obtained from D-glucose-1-t. This four-fold difference is bridged by the experimentally observed differences of 3–7.

The specific activities of pectin derived D-galacturonic acid were markedly lower than those of the L-ascorbic acid. Part of this was probably due to endogenous dilution of newly synthesized pectin by pectin already formed in the nearly ripe berry. Such dilutions have always been experimentally greater in the case of pectin than in the case of L-ascorbic acid. Comparison of the ratios from D-glucose-1-t and -6-t revealed the former to be slightly higher indicating preferential loss of hydrogen from carbon 6. Why the differences were not greater is not well understood. It might be related to the mechanism of D-galacturonic acid synthesis from D-glucose. Suppose, instead of oxidation of carbon 6 of UDP D-glucose, there was a cyclization of D-glucose followed by oxidative cleavage.[21] In this case, one might envision a process involving conservation of hydrogen (tritium) originally on carbon 6 of glucose by intramolecular transfer of this hydrogen to another carbon possibly carbon 1 of the resulting uronic acid, during the oxidative cleavage.

In the present results concerning synthesis of tritium-labeled L-ascorbic acid, it has been assumed that all tritium is bound to carbon 6. There are 4 non-exchangeable hydrogens in ascorbic acid, one each on carbons 4 and 5 and two on carbon 6. While there is little likelihood of transfer of tritium from either the 1 or 6 position of D-glucose to either carbons 4 or 5 of L-ascorbic acid, this possibility exists and must be considered. To establish the exact location of tritium in each labeled ascorbic acid isolated, these ascorbic acids were oxidized with sodium periodate. The formaldehyde corresponding to carbon 6 was recovered as its dimedon derivative. All of the tritium was found in this fraction.

DISCUSSION

Isotope studies involving administration of labeled substrates to intact tissues seldom provide specific information on the enzymatic processes involved, but they do give information on the fate of the label as well as related details on the nature of the physiological pathway and, quite often, insight into the reaction mechanisms. The present study has shown that the formation of L-ascorbic acid in higher plants proceeds by enzymatic reactions distinct from those leading to uronic acid synthesis. While the latter results from oxidation of carbon 6 of a hexose precursor, the former results from oxidation of carbon 1. This is diagrammed in Fig. 8.

At least two known enzymatic processes in higher plants cause the oxidation of a hexose at carbon 1, resulting in the corresponding hexonic acid or lactone. One is the familiar

direct oxidation pathway (Warburg–Dickens pathway). The other is a less familiar non-phosphorylated reaction that was found in citrus fruits.[31] Isotope studies support the idea of a process of L-ascorbic acid formation that is dependent upon hexose phosphate as an

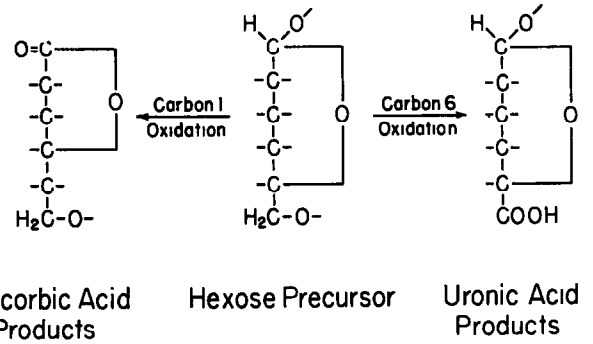

FIG. 8. OXIDATION PROCESSES ON TERMINAL CARBONS OF HEXOSE IN PLANTS.

ultimate precursor. Isherwood *et al.*[5] were inclined to favor a process that did not involve phosphate esters, yet their studies showed that D-glucono-γ-lactone was not effective in causing L-ascorbic acid increases in plants. Their observation might be interpreted as further support for participation of the direct oxidation pathway *via* D-glucose-6-phosphate rather than *via* free D-glucose.

The conversion of L-gulono-γ-lactone to L-ascorbic acid and the conversion of L-gulonate to carbon dioxide and L-xylulose in animal tissues[4] provide an interesting analogy to what might possibly be the path of L-ascorbic acid formation in plants. In animals, L-gulono-γ-lactone is probably oxidized to 2-oxo-L-gulono-γ-lactone which, upon rearranging, forms L-ascorbic acid.[61] L-Gulonate, on the other hand, is oxidized at carbon 3 to form 3-oxo-L-gulonate which undergoes decarboxylation to form L-xylulose.[62,63] Now, suppose D-glucose-6-phosphate is oxidized to D-gluconolactone-6-phosphate with subsequent hydrolysis of a portion of the lactone to D-gluconate-6-phosphate.[64] Given two separate processes of oxidation analogous to those shown to operate on L-gulonolactone and L-gulonate, one can visualize the oxidation of D-gluconolactone-6-phosphate to a substance of the same oxidation level as ascorbic acid, whereas D-gluconate-6-phosphate is oxidatively decarboxylated to D-ribulose-5-phosphate. This proposal provides no explanation for the conversion from a D- to an L-configuration, but it is conceivable that in the process of oxidation of the lactone, which is a δ-lactone, there is a rearrangement to a γ-lactone and that during this process epimerization occurs at carbon 5. The product of such a rearrangement would be more stable in its enolic form, L-ascorbic acid-6-phosphate. Conceivably, L-ascorbic acid accumulation in plants could be related to the irreversible hydrolysis of this phosphate ester.

The significance of plant processes that can utilize exogenously provided uronic acid esters or lactones for L-ascorbic acid synthesis is less apparent. There is now both enzymatic and isotopic evidence for these processes but comparable information supporting the view that they have physiological significance in intact plants is lacking. Maybe, as suggested

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in an earlier paper,[52] these reactions occur only when conditions of stress arise. Such conditions might include tissue wounding, pathological disturbances, senescence, and possibly certain host–parasite relations. Under these conditions one can visualize a series of degradative enzymatic reactions in which pectin and related cell wall polysaccharides are hydrolyzed to release free uronic acids and uronic acid esters for further catabolic reactions. These catabolic products, L-ascorbic acid, D-xylose, etc., then become avilable to the tissue for repair and resynthesis of plant cells. It is conceivable that L-ascorbic acid has a role in plant tissue repair similar to that already well documented in animal tissues.[65,66] The metabolism of D-xylose in plants has already been described.[13,67]

No attempt has been made, in this review of isotope studies on ascorbic acid synthesis in plants to detail the broad aspects of ascorbic acid formation, function or its fate in plants. For these details the reader is referred to recent reviews.[68-72]

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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