présence dans le plasma et son absence dans l'urine, permettent d'envisager qu'il est hydrolysé en partie dans les tissus par une arylsulfatase et que son constituant hormonal est désiodé.

3. Le rôle probable de ST_3 est celui d'une forme de réserve de T_3 accessible aux cellules après hydrolyse de la liaison ester.

BIBLIOGRAPHIE

[1] J. ROCHE, R. MICHEL, J. CLOSON ET O. MICHEL, *Biochim. Biophys. Acta*, 33 (1959) 461.
[2] M. DE WISSCHER ET C. BECKERS, *J. Physiol. (Paris)*, 49 (1957) 439.
[3] J. ROCHE, O. MICHEL, R. MICHEL ET J. TATA, *Biochim. Biophys. Acta*, 13 (1954) 471.
[4] J. ROBBINS AND J. E. RALL, *Recent Progress in Hormone Research*, 13 (1957) 161.
[5] J. ROCHE, R. MICHEL ET J. TATA, *Compt. rend. soc. biol.*, 147 (1953) 1574.
[6] O. CREPY, M. F. JAYLE ET F. MESLIN, *Compt. rend. soc. biol.*, 151 (1957) 322.
[7] A. TAUROG, *Brookhaven Symposia in Biol.*, 7 (1955) 111.

Biochim. Biophys. Acta, 38 (1960) 325–332

METABOLISM OF D-[1-^{14}C]- AND D-[6-^{14}C]GLUCURONOLACTONE BY THE RIPENING STRAWBERRY*

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SUMMARY

D-[1-^{14}C] and D-[6-^{14}C]glucuronolactone is metabolized by the detached ripening strawberry fruit to several six carbon acids including gulonic acid, L-ascorbic acid, D-galacturonic acid, and D-glucuronic acid. In addition, one of the acid intermediates is decarboxylated to yield free D-xylose from carbons 1 through 5 and CO_2 from carbon 6 of D-glucuronolactone. A portion of carbons 1 through 5 is also utilized in sucrose synthesis and in pentose residues of cell-wall polysaccharides. The ^{14}C distribution patterns and specific activities of the identified constituents suggest that two pathways of glucuronolactone utilization reside in the strawberry. The evidence for these pathways is discussed.

INTRODUCTION

Tracer studies of the over-all conversion of glucose*** to ascorbic acid by the detached, ripening, strawberry fruit[2,3] and by the etiolated, germinating, cress seedling[4] have revealed that the six carbon chain is conserved and that C-1 of glucose is oxidized forming the carboxyl (C-1) carbon of ascorbic acid. The utilization of glucose for

* A preliminary report of this work has been published[1].

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*** The prefixes D and L are omitted in all instances where the naturally-occurring isomer is mentioned and where there will be no question of configuration.

ascorbic acid formation in the strawberry appeared to follow normal processes of hexose phosphate metabolism[5–7].

We have, however, confirmed the existence of a path of conversion from glucuronolactone (GL) to ascorbic acid in the strawberry that corresponds to the stereochemical requirements of the scheme described by ISHERWOOD, CHEN AND MAPSON[8]. [1-^{14}C]GL was converted, in part, to ascorbic acid labeled almost exclusively in C-6[3]. This finding has prompted a more detailed study of the metabolism of GL.

EXPERIMENTAL

Materials and methods

[1-^{14}C]GL was synthesized chemically from [1-^{14}C]glucose[9]. [6-^{14}C]GL was prepared from [6-^{14}C]sodium glucuronate obtained from Dr. H. S. ISBELL, National Bureau of Standards, Washington, D. C. Both lactones were purified just prior to use by paper chromatography.

Field grown strawberries (*Fragaria*, variety Shasta) were harvested in the early white stage of ripening on July 21, 1958. The stems were cut under water and kept thus until placed in the radioactive solutions. After the radioactive material had been taken up by the berries (0.4 ml), water was added to keep the cut stems immersed until the respiration study was completed (41 h). At the end of this time, both berries were red and had gained 0.6 g. The respired CO_2 was trapped in *N* NaOH.

Separation of berry constituents and degradation procedures

The methods of separation and isolation of the carbohydrate constituents have been described previously[2,5,7]. A slight modification was introduced in the method by which ascorbic acid was eluted from the Dowex 1 (formate) exchange column in order to reduce the contamination of ascorbic acid with other labeled acidic products of GL metabolism[2,7]. The berry acids from the ethanol-soluble fraction that remained on the Dowex 1 (formate) (1 × 10 cm, 200–400 mesh, medium porosity) were eluted with a gradient of 0.02 → 0.06 *N* formic acid that consisted of a reservoir of 500 ml of 0.06 *N* formic acid which flowed through a mixing chamber containing 200 ml of 0.02 *N* formic acid onto the top of the resin column. After 500 ml of eluate had been collected in 5-ml fractions, 200 ml of 3 *N* formic acid was added to the reservoir and an additional 200 ml of eluate collected. Finally, 35 ml of 6 *N* formic acid was added directly to the top of the column and collection of fractions continued until the column was dry.

Galacturonic acid from the fungal pectinase hydrolysate of the 70 % ethanol-insoluble portion of the strawberries was also recovered using the anion exchange column and formic acid gradient just described.

All carbohydrate constituents scheduled for degradation were first recrystallized until there was no further change in radioactivity. The methods employed in degradation and counting have been described previously[2,5–7].

RESULTS

Distribution of ^{14}C among the berry fractions

A plot of the ^{14}C accumulation in respired CO_2 during the uptake and metabolism of labeled GL is shown in Fig. 1. ^{14}C from [6-^{14}C]GL appeared almost immediately

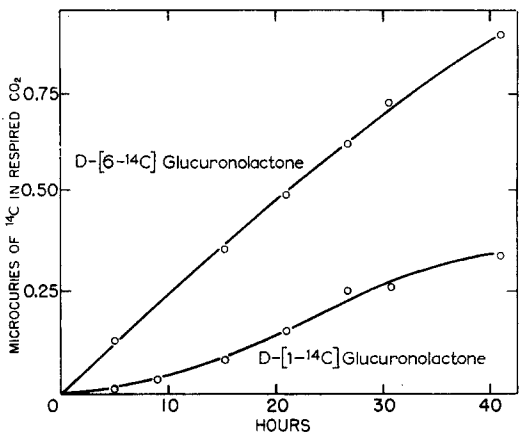

Fig. 1. A plot of ^{14}C accumulation in the respired CO_2 as a function of time. The ^{14}C values given for the [1-^{14}C]glucuronolactone have been reduced by a factor of 0.76 in order to permit direct comparison of respired activities between the two experiments.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN LABELED STRAWBERRIES

		D-glucuronolactone	
		[1-^{14}C]	*[6-^{14}C]*
		μC	*μC*
^{14}C	Administered	3.8 (1.46 mg)	2.9 (2.84 mg)
^{14}C	Recovered		
	Respired CO_2	0.44	0.87
	Berry, ethanol-soluble	0.90	0.91
	Stem and calyx, ethanol-soluble	0.82	0.51
	Pectinase solubilized	0.21	0.12
^{14}C	Unaccounted for*	1.43	0.5

* Seeds, unhydrolyzed cell wall material and related insolubles.

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN ETHANOL-SOLUBLE PORTION OF LABELED STRAWBERRIES

		D-glucuronolactone	
		[1-^{14}C]	*[6-^{14}C]*
		mμC	*mμC*
Activity added to Dowex 1		900	910
Activity of acidic pooled fractions*	A (0– 30 ml)	8	4
	B (90–215 ml)	150	170
	C (215–340 ml)	51	34
	D (340–450 ml)	14	31
	E (510–615 ml)	89	51
	F (640–700 ml)	32	17
Activity of neutral effluent		440	180
Activity of sucrose and xylose only		240	negligible
Activity lost during separations		120	420

* Pooled fractions corresponding to the various radioactive peaks eluted from the Dowex 1 (formate) resin with the dilute formic acid gradient. The successive volume increments are indicated in parentheses.

and continued to accumulate at a linear rate during the respiration. When the experiment ended, 30 % of the ^{14}C from [6-^{14}C]GL had been converted to CO_2. In contrast to this observation, [1-^{14}C]GL was metabolized to $^{14}CO_2$ by the strawberry only after a lag period. Less than 12 % of the administered ^{14}C appeared in the CO_2. [1-^{14}C]GL resembled [1-^{14}C]glucose and [1-^{14}C]xylose in this respect. The preferential decarboxylation of C-6 of GL has also been observed in corn coleoptiles by SLATER AND BEEVERS[10] and in rat kidney preparations by RABINOWITZ AND SALL[11].

A crude accounting of the ^{14}C distribution in the berries is presented in Table I. The ^{14}C remaining in the 70 % ethanol-insoluble portion of the berries after treatment with fungal pectinase has not been examined but the work of NEISH[12] has already demonstrated that considerable ^{14}C from [1-^{14}C]GL is incorporated into cell wall xylan and cellulose.

The distribution of activity among the various crude fractions of the ethanol-soluble portion of each berry after removal of the stem and calyx is given in Table II. About a third of this activity remained on the anion exchange column after it had been thoroughly washed with water. The neutral effluent which had previously been shown to consist primarily of free sugars (sucrose, glucose, fructose, xylose, and a trace of ribose[5]) also contained, in the present experiments, some labeled GL. Very little ^{14}C appeared in the free sugars from the [6-^{14}C]GL-labeled berry and the 180 mμC found in the neutral effluent was almost entirely due to GL. The neutral effluent from the alcohol solubles of the [1-^{14}C]GL-labeled berry contained, in addition to [^{14}C]GL, considerable activity in the free xylose and sucrose confirming our earlier report of the preferential labeling of these sugars[3].

Elution of the activity retained by the anion exchange resin resulted in several radioactive peaks. The fractions corresponding to these peaks were pooled as described in Table II. Elution patterns from [1-^{14}C]GL- and [6-^{14}C]GL-labeled berries were similar.

Pooled fractions "B" contained one major radioactive component and at least six minor components. The major component could be lactonized and did not react with aniline–trichloroacetic acid spray reagent (an aldose test). When chromatogrammed on paper in three different solvent systems, the lactone of the major component migrated identically with L-gulonolactone. Comparisons were made with D-mannonolactone, D-altronolactone, L-galactonolactone, L-idonolactone, and D-gluconolactone in ethyl acetate–acetic acid–water (3:1:3)[13], ethyl acetate–pyridine–water (8:2:1)[14] and 2,2-dimethyl-1-propanol–water–1-propanol–ethanol (4:2:1.3:0.5)[15]. In all of these systems, gulonolactone was the slowest moving aldonolactone tested.

Pooled fractions "C" included ascorbic acid which was recovered by dilution with unlabeled ascorbic acid followed by successive crystallizations from glacial acetic acid until the activity remained constant. Less than one-half of the activity in "C" could be ascribed to ascorbic acid.

Pooled fractions "D" were eluted in the fractions that characterized glucuronic acid in separate control runs. It gave an aniline–trichloroacetic acid spot test similar to glucuronic acid and probably represented the non-metabolized GL that was hydrolyzed to free acid by the berry. Pooled fractions "E" and "F" have not been investigated as yet.

Table II also records the portion of ^{14}C unaccounted for after anion exchange

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN PECTINASE-HYDROLYZED SOLUBLES OF LABELED STRAWBERRIES

	D-*glucuronolactone*	
	[1-^{14}C]	*[6-^{14}C]*
	mμC	*mμC*
Activity added to Dowex 1	194	114
Activity in galacturonic peak	73	80
Activity in other peaks (combined)	24	11
Activity of neutral effluent	94	2
Activity lost (unaccounted for)	3	21

resin treatment of the ethanol solubles. In the case of the berry labeled with [6-^{14}C]GL this loss represented almost one-half of the added activity. Presumably, this loss of activity was due to decarboxylation of unknown, labile, acidic components. Further work on the nature of this phenomenon is planned.

The distribution of radioactivity in the soluble pectinase hydrolysate of the 70 % ethanol insoluble portion of each berry is given in Table III. A major portion of the activity retained on the anion exchange column was located in galacturonic acid. A smaller radioactive peak of glucuronic acid followed the galacturonic acid in both experiments. Galacturonic acid was recovered from the pooled fractions as its sodium calcium salt[16].

A significant amount of ^{14}C appeared in the neutral effluent only when [1-^{14}C]GL was the source of label. Paper chromatography followed by radioautography[7] revealed just two radioactive components, arabinose and xylose. Neither glucose nor galactose residues were labeled although, quantitatively, these hexoses accounted for two-thirds of the sugar residues in the pectinase-hydrolysate.

Labeling patterns of ascorbic acid, galacturonic acid and sugar constituents

The activity and distribution of ^{14}C in the carbon chains of ascorbic acid and galacturonic acid residues are given in Table IV. Earlier attempts[2,7] to demonstrate

TABLE IV

RADIOACTIVITY AND SITE OF LABELING IN ASCORBIC ACID AND GALACTURONIC ACID FROM [1-^{14}C]- AND [6-^{14}C]GLUCURONOLACTONE LABELED STRAWBERRIES

	[1-^{14}C]glucuronolactone			*[6-^{14}C]glucuronolactone*	
	Ascorbic acid	*Galacturonic acid*		*Ascorbic acid*	*Galacturonic acid*
Recovered**, mg	6.1	31*	26.1	5.5	33.4
Dilution factor	21.6	1.4	5.8	27.7	4.7
Total activity, mμC	30	46	77	32	74
Specific activity, mμC/mg carbon	12	40	8	14	6
	Per cent of total activity				
Activity, carbon 1	—	99	99	95	—
Activity, carbon 6	99	—	1	2	99

* This column contains data on galacturonic acid recovered from a berry used in an earlier experiment[8].

** Based on assay before carrier dilution.

a conversion of ^{14}C from [6-^{14}C]GL to ascorbic acid were unsuccessful since the anion exchange procedure employed at that time could not separate ascorbic acid from the radioactive aldonic acid found in pooled fractions "B" in the present study.

The results in Table IV provide isotopic evidence for the over-all conversion of GL to ascorbic acid with inversion of the intact carbon chain as originally described by ISHERWOOD, CHEN AND MAPSON[8]. Our earlier demonstration of the conversion of [1-^{14}C]GL to [6-^{14}C]ascorbic acid[3] is confirmed in the present experiment and substantiated by the [6-^{14}C]GL to [1-^{14}C]ascorbic acid conversion. Both [1-^{14}C]GL and [6-^{14}C]GL were converted to galacturonic acid by an epimerization of C-4.

Very little ^{14}C from [6-^{14}C]GL was incorporated into the free sugars or the pectinase-solubilized cell wall polysaccharides. [1-^{14}C]GL was converted by the strawberry to free xylose and sucrose, and to arabinose and xylose residues in the

TABLE V

RADIOACTIVITY AND SITE OF LABELING IN SUCROSE-DERIVED GLUCOSE AND PENTOSES FROM [1-^{14}C]GLUCURONOLACTONE LABELED STRAWBERRIES

	Sucrose-derived glucose		*Free xylose*		*Pectinase-hydrolyzed*			
					Xylose		*Arabinose*	
	*		*		*		*	
Recovered**, mg	2.2	7.1	6	3.6	0.8	1.1	1.6	6.1
Dilution factor	46.4	15.2	61	57.3	175	93.7	89	17.9
Total activity, mμC	19	20	806	144	46	11	46	39
Specific activity, mμC/mg carbon	21	7	330	100	143	25	72	16
	Per cent of total activity							
Activity carbon 1	41.5	49	99	84	92	90	100	99
2	4	4.5						
3	13	15		11	8	8		
4	12	9.5						
5	5.5	2		5		2		
6	24	20						

* These columns contain data on sugars recovered from a berry used in an earlier experiment[3].
** Based on assay before carrier dilution.

cell-wall polysaccharides. Table V gives the labeling patterns from the present experiments as well as unreported data on the sugars of the previously reported [1-^{14}C]GL experiment[3]. Free xylose had the highest specific activity, 10 and 31 % of the administered label (1050 mμC/mg carbon). A portion of free xylose from the previous experiment was converted to 2:4, 3:5 dibenzilidene xylose dimethyl acetal, a reaction highly specific for xylose[17], with no change in radioactivity in the product. Another portion of this free xylose was degraded microbiologically with D-xylose adapted *Lactobacillus plantarum*. 99 % of the ^{14}C appeared in the acetic acid fragment (C-(1 + 2)) which chemical degradation revealed to be labeled only in the methyl carbon (C-1 of the original xylose). These data have been interpreted as evidence that the major free pentose product from [1-^{14}C]GL was D-xylose.

The glucose and fructose moieties of sucrose were equally labeled. Both their specific and total activities were considerably less than that of the free xylose. The activities and distribution patterns in the carbon chains of the glucose moieties are given in Table V. C-1 contained the most ^{14}C. C-3, C-4, and C-6 also contained

significant amounts of label. The appearance of ^{14}C in C-1 and C-3 is particularly significant since it suggests that a pentose labeled in C-1 was the precursor[18]. Redistribution from C-1 and C-3 into C-6 and C-4 respectively corresponds to the normal pattern of label redistribution among hexose phosphate-derived constituents of the strawberry[2,5,6] as well as those of other plants. A previous study of the metabolism of [1-^{14}C]xylose by the strawberry[6] revealed that sucrose was the primary free sugar product that became labeled. It appears reasonable to assume that in the conversion of [1-^{14}C]GL to ^{14}C-sucrose, a [1-^{14}C]pentose precursor was the principle intermediate.

Table V also records the activities and patterns of xylose and arabinose residues that were isolated from the pectinase hydrolysate of the 70% ethanol-insoluble portion of the [1-^{14}C]GL-labeled berries. Both pentose residues had lower specific activities than did free xylose. Essentially no redistribution of ^{14}C occurred in the arabinose, C-1 retained all the activity. In xylose residues, between 8 to 10% of the ^{14}C incorporated was in carbons other than C-1. These patterns differ from those obtained when [1-^{14}C]xylose was the source of label[6]. It is entirely possible that [1-^{14}C]xylose administered through the vascular tissues of the berry is utilized quite differently in polysaccharide synthesis from [1-^{14}C]pentose generated from [1-^{14}C]GL *in situ*.

DISCUSSION

The discovery that free D-xylose was a product and that it corresponded to the first five carbons, while C-6 of GL was lost as CO_2 implied a direct decarboxylation of GL. ALTERMATT AND NEISH[19] and NEISH[12] have already discussed this possibility in terms of uridine diphosphate derivatives in the course of their tracer studies of cellulose and xylan synthesis in wheat seedlings. HASSID *et al.*[20–22] have experimentally demonstrated the conversion of glucuronic acid to the uridine diphosphate derivatives of galacturonic acid, xylose and arabinose by plant extracts. Their findings explain the observed conversion of GL to cell wall polysaccharides in the strawberry. If the same path of conversion is responsible for free xylose formation, then a hydrolytic mechanism is also necessary although experimental evidence is still lacking. Alternatively, free xylose could be formed by non-phosphorylative reactions related to the production of gulonic acid and ascorbic acid during GL utilization by the berry. This was discussed in a previous paper[3].

The exact nature of the non-phosphorylative conversion of GL to gulonic acid and to ascorbic acid and, possibly, to xylose is not fully understood. ISHERWOOD, CHEN AND MAPSON[8] proposed a direct reduction of GL to L-gulonolactone which was then oxidized to an ascorbic acid precursor. Recent studies on the metabolism of GL and glucuronic acid by certain bacteria[23–25] have revealed that the first step is an isomerization to D-fructuronic acid followed by a reduction of this compound to D-mannonic acid[23]. Although the corresponding reaction has not yet been reported to be present in plants, it provides a reasonable explanation for the appearance of gulonic acid (the C-2 epimer of mannonic acid) which could be obtained from fructuronic acid by a stereospecific reduction. Assuming that fructuronic acid, and not gulonic acid, is the intermediate that is oxidized at C-4 (corresponding to C-3 of gulonic acid), then the product would be 3,5-diketogulonic acid. This diketo compound could be decarboxylated as a beta-keto acid to form a pentose directly, or it could conceivably enolize at the beta-keto function to form the 5-keto analog of ascorbic acid.

3,5-diketogulonic acid differs from 3,5-diketogluconic acid only in the configuration about C-2. Enolization of the C-3 keto function would destroy this difference, providing a common intermediate from GL *via* fructuronic acid and from glucose *via* 3-keto-6-phosphogluconic acid[3,7]. A stereospecific reduction of C-5 to L-ascorbic acid would represent the final step. Such a scheme would accommodate all of the tracer patterns obtained with labeled glucose or GL.

NOTE ADDED IN PROOF

Positive characterization of the major component of peak "B" as L-gulonic acid has recently been completed and reported elsewhere[26].

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REFERENCES

1 F. A. LOEWUS AND B. J. FINKLE, *Federation Proc.*, 18 (1959) 275.
2 F. A. LOEWUS, R. JANG AND C. G. SEEGMILLER, *J. Biol. Chem.*, 222 (1956) 649.
3 F. A. LOEWUS, B. J. FINKLE AND R. JANG, *Biochim. Biophys. Acta*, 30 (1958) 629.
4 F. A. LOEWUS AND R. JANG, *Biochim. Biophys. Acta*, 23 (1957) 205.
5 F. A. LOEWUS AND R. JANG, *J. Biol. Chem.*, 232 (1958) 505.
6 F. A. LOEWUS AND R. JANG, *J. Biol. Chem.*, 232 (1958) 521.
7 F. A. LOEWUS, R. JANG AND C. G. SEEGMILLER, *J. Biol. Chem.*, 232 (1958) 533.
8 F. A. ISHERWOOD, Y. T. CHEN AND L. W. MAPSON, *Biochem. J.*, 56 (1954) 1.
9 C. L. MEHLTRETTER, B. H. ALEXANDER, R. L. MELLIES AND C. E. RIST, *J. Am. Chem. Soc.*, 73 (1951) 2424.
10 W. G. SLATER AND H. BEEVERS, *Plant Physiol.*, 33 (1958) 146.
11 J. L. RABINOWITZ AND T. SALL, *Biochim. Biophys. Acta*, 23 (1957) 289.
12 A. C. NEISH, *Can. J. Biochem. and Physiol.*, 36 (1958) 187.
13 P. S. RAO, R. M. BERI AND RAO RAMACHANDRA, *Proc. Indian Acad. Sci. A.*, 34 (1951) 236.
14 K. T. WILLIAMS AND A. BEVENUE, *J. Assoc. Offic. Agr. Chemists*, 36 (1953) 969.
15 J. A. CIFONELLI, J. LUDOWIEG AND A. DORFMAN, *J. Biol. Chem.*, 233 (1958) 541.
16 H. S. ISBELL AND H. L. FRUSH, *J. Research Natl. Bur. Standards*, 32 (1944) 77.
17 L. J. BREDDY AND J. K. N. JONES, *J. Chem. Soc.*, (1945) 738.
18 M. GIBBS AND B. L. HORECKER, *J. Biol. Chem.*, 208 (1954) 813.
19 H. A. ALTERMATT AND A. C. NEISH, *Can. J. Biochem. and Physiol.*, 34 (1956) 405.
20 E. F. NEUFELD, D. S. FEINGOLD AND W. Z. HASSID, *J. Am. Chem. Soc.*, 80 (1958) 4430.
21 D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *Science*, 128 (1958) 1144.
22 D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *Federation Proc.*, 18 (1959) 224.
23 A. J. WAHBA, J. W. HICKMAN AND G. ASHWELL, *J. Am. Chem. Soc.*, 80 (1958) 2594.
24 W. J. PAYNE AND R. A. MCRORIE, *Biochim. Biophys. Acta*, 29 (1958) 466.
25 W. W. KILGORE AND M. P. STARR, *Biochim. Biophys. Acta*, 30 (1958) 652.
26 F. A. LOEWUS AND S. KELLY, *Biochem. Biophys. Research Commun.*, 1 (1959) 143.