Metabolism of \(\beta\)-Glucuronic Acid and \(\beta\)-Galacturonic Acid by Phaseolus aureus Seedlings*

GIAN KESSLER, ELIZABETH F. NEUFFELD, DAVID S. FEINGOLD, AND W. Z. HASSID

From the Department of Biochemistry, University of California, Berkeley, California

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\(\beta\)-Glucuronic acid and its lactone are known to be converted to polysaccharides by plant tissues. Ripeening strawberries have been shown to incorporate the intact carbon skeleton of \(\beta\)-glucuronic acid into pectin (1). It has also been demonstrated that wheat plants and corn coleoptiles incorporate \(\beta\)-glucuronic acid and its lactone into the pentosyl moieties of pentosan with loss of carbon 6 (2,3).

In the present investigation, results of feeding uniformly \(^{\text{14}}\)C-labeled \(\beta\)-glucuronic acid and \(\beta\)-galacturonic acid to Phaseolus aureus seedlings are presented. It is shown that \(\beta\)-galacturonic acid as well as \(\beta\)-glucuronic acid is readily incorporated into pectin and hemicellulose. Furthermore, evidence is presented for a novel aspect of uronic acid metabolism in plants involving the oxidation of hexuronic acids to hexaric acids.

**EXPERIMENTAL PROCEDURE**

*General Analytical Methods*—Paper electrophoresis was carried out in five different buffer solutions: 0.2 M ammonium formate buffer pH 3.7 (I), 0.2 M ammonium acetate buffer pH 5.8 (II), 0.1 M ammonium carbonate buffer pH 9.0 (III), 0.05 M sodium carbonate buffer pH 9.0 (IV), or 0.05 M sodium tetraphosphate buffer pH 9.2 (V). The electrophoretic mobility of individual compounds is given relative to the mobility of picric acid (M	extsubscript{pa}).

Paper chromatography was performed in the following solvents expressed as volume per volume: n-butanol-acetic acid-water, 52:13:35 (4) (VI), ethyl acetate-acetic acid-water, 3:1:1:3 (5) (VII), ethyl acetate-pyridine-water, 8:2:1 (6) (VIII), 2,2-dimethyl propanol-water-n-propanol-ethanol, 4:2:1:3:0.5 (7) (IX), isopropanol-pyridine-water-acetic acid, 8:8:4:1 (8) (X), ethyl acetate-5% aqueous boric acid-pyridine, 12:5:4 (XI), n-propanol-water-ethyl acetate, 7:2:1 (9) (XII), and water-saturated phenol (10) (XIII).

After the separation of the compound by paper chromatography and paper electrophoresis, sugars, uronic acids, aldonic acids, and aldaric acids were detected by the periodate-benzidine reaction and paper electrophoresis, sugars, uronic acids, aldonic acids, and aldaric acids were detected by the periodate-benzidine reaction and paper electrophoresis, sugars, uronic acids, aldonic acids, and aldaric acids were detected by the periodate-benzidine reaction and paper electrophoresis, sugars, uronic acids, aldonic acids, and aldaric acids were detected by the periodate-benzidine reaction and paper electrophoresis, sugars, uronic acids, aldonic acids, and aldaric acids were detected by the periodate-benzidine reaction and paper electrophoresis, sugars, uronic acids, aldonic acids, and aldaric acids were detected by the periodate-benzidine reaction and paper electrophoresis.

Preparation of Labeled Uronic Acids.—UDP-\(\beta\)-glucuronic acid labeled with \(^{\text{14}}\)C in the \(\beta\)-glucuronic acid moiety was prepared by enzymatic oxidation of radioactive UDP-\(\beta\)-glucose and purified as described by Feingold et al. (13). UDP-\(\beta\)-galacturonic acid was prepared from UDP-\(\beta\)-glucuronic acid using a particular enzyme preparation from radish root (13). Upon fractionation of the reaction mixture, UDP \(\beta\)-galacturonic acid was recovered in a yield corresponding to approximately 15% of the initial UDP-\(\beta\)-glucuronic acid activity. \(\beta\)-Glucuronic acid and \(\beta\)-galacturonic acid, uniformly labeled with \(^{\text{14}}\)C, were obtained from the corresponding sugar nucleotides by hydrolysis with a mixture of nucleotide pyrophosphatase (14) and seminal phosphomonoesterase. The liberated uronic acids were separated from the reaction mixture by paper electrophoresis at pH 5.8 (II) and further purified by paper electrophoresis at pH 9.0 (III).

**Germination of Mung Bean Seeds and Feeding of Labeled Substrates**—Mung bean (Phaseolus aureus) seeds were obtained from a local grocer. The seeds were immersed for 5 minutes in a solution containing 1 g of HgCl\textsubscript{2} and 2.5 ml of concentrated HCl in 500 ml of water, and washed thoroughly with sterile water. All subsequent operations were performed under aseptic conditions. The disinfected seeds were germinated in water with aeration at room temperature for 20 hours, and then the cotyledons were carefully cut from the seedlings and discarded. The remaining portions of the seedlings were used for the extraction of endogenous hexaric acids and for experiments with labeled substrates.

The substrate solutions were sterilized before use by passage through a Millipore filter. Thirty seedlings were placed in 2

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1 C. E. Ballou, personal communication.
ml of an aqueous solution (pH 6.5) containing either 0.5 μmole of ammonium d-glucuronate (18 μc), or 0.17 μmole of ammonium d-galacturonate (6 μc), for periods of time varying from 90 minutes to 6 hours. At the end of the incubation period, the external solution was removed with a pipet, and the seedlings were washed with 5 portions of 0.5 ml of water. The washed samples were transferred into 5 ml of boiling 95% ethanol, and the suspension was boiled for 1 minute. Subsequently the seedlings were extracted by suspension in 1-ml portions of hot 80% ethanol and centrifugation until the supernatant liquid was free from radioactivity. Before analysis, the external solution was concentrated to a small volume. Similarly, the combined alcohol extracts and the residual insoluble material were concentrated to dryness.

Fractionation and Analysis of Labeled Metabolic Products

Products Present in External Solution—Examination of the solution in which the mung bean seedlings had been incubated showed the presence of newly formed metabolic products. Presumably, the removal of the cotyledon exposes the vascular system of the seedlings and permits an interchange of metabolites between the outside solution and the interior of the plants.

Examination of the external solution by paper electrophoresis at pH 3.7 (I) showed that when d-glucuronate was made available to the seedlings, two major metabolic products (Compounds A and C) were present in addition to residual d-glucuronate and its lactone. Furthermore, a number of other compounds (neutral and anionic) were detected on the paper after prolonged autoradiography (72 hours). However, since the activity of these individual components did not exceed 5 × 10⁻⁴ μc, their identification was not attempted. When d-galacturonate was used as substrate, only one major new metabolic product appeared on the electrophoretic pattern (Compound B). Several minor compounds, each containing less than 5 × 10⁻³ μc of total activity, were not further investigated.

1. Compound A—Examination of the electrophoretic mobility of Compound A showed that at pH 3.6 it was slightly greater than that of d-glucuronic acid (Mₚ₋₃-d-glucuronic acid, 0.89; Compound A, 1.06). At pH 5.8, however, the mobility of the new metabolite was twice that of d-glucuronic acid (Mₚ₋₃-d-glucuronic acid, 0.98; Compound A, 2.00). This suggested that Compound A has at least one more ionizable group than uronic acid. Treatment of Compound A with seminal phosphomonoesterase failed to cause a change in electrophoretic mobility. This showed that Compound A is not a phosphate ester.

Further information concerning the structure of Compound A was obtained by examination of the products of periodate oxidation. When a sample of Compound A was oxidized with unlabeled d-gluaric (d-glucosaccharic) acid and treated with sodium periodate under very mild conditions (0°C, pH 7.5, 5 to 30 minutes, ratio of periodate to glucurate = 8), both labeled and unlabeled formate, glyoxalate, and an unidentified intermediate were shown to be present in the reaction mixture by paper electrophoresis at pH 9.0 (IV). Under more vigorous conditions (25°C, pH 7.5, 3 hours, ratio of periodate to glucurate = 8), formate and CO₂ were the only products that could be detected. This is in agreement with the known behavior of glyoxalate upon vigorous periodate oxidation (15). The presence of radioactive CO₂ was shown by oxidizing Compound A in a Conway vessel containing alkali in the center well, and precipitating the CO₂ as BaCO₃. The formation of glyoxalate from Compound A indicates the presence of a glyceric acid structure in the molecule.

Since no radioactive formaldehyde could be detected among the products of vigorous periodate oxidation, the parent compound did not contain a primary hydroxyl group. This was shown as follows. Compound A was oxidized with periodate for 1 hour at 25°C at pH 7.5, and ethylene glycol was added to destroy the unreacted periodate and to form unlabeled formaldehyde in the reaction mixture. After the iodate was removed as the insoluble lead salt, the formaldehyde was precipitated as the 2,4-dinitrophenylhydrazone by adding an excess of dinitrophenylhydrazine (0.001 M, dissolved in 2 N HCl). The precipitate was thoroughly washed with water, dissolved in chloroform, and plated for counting. No radioactivity above background could be detected. C¹⁴-labeled sorbitol and erythritol yielded the theoretical amount of radioactive formaldehyde when subjected to the same treatment. These experiments eliminate not only the presence of formaldehyde among the oxidation products of Compound A, but also of any carbonyl compound which would yield an insoluble 2,4-dinitrophenylhydrazone.

The results of periodate oxidation show the presence in Compound A of carboxyl and secondary hydroxyl groups only, indicating an aldonic acid structure.

Compound A could not be distinguished from the hexaric acids, d-glucaric acid or galactaric acid, by electrophoresis (I, II) or chromatography (IX). Since all the theoretically possible isomers of hexaric acid can be differentiated by reduction and subsequent separation of the resulting hexitols by a combination of paper chromatography and electrophoresis, Compound A was transformed to the corresponding alcohol.

An aliquot (1 × 10⁻³ μc) was diluted with 0.2 μmole of unlabeled d-glucaric acid and mixed with 100 μl of 5% methanolic HCl. The solution was then scaled in a capillary tube and heated at 120°C for 1 hour. This treatment resulted in a conversion of the acid to its dimethyl ester and other reaction products, possibly methyl ester monolactone and dilactone. After removal of the methanolic HCl by evaporation, the mixture was reduced by treatment with an excess of aqueous sodium borohydride for 80 minutes. The exoexorhodirhydride was destroyed by acidification to pH 3 with Dowex 50-H⁺ resin, which also adsorbed the sodium ions from the reaction mixture. The solution was then decanted from the resin and concentrated to dryness. Boric acid was removed as the methyl ester by repeated addition of 0.1-ml aliquots of dry methanol and evaporation to dryness. The radioactive hexitol obtained by reduction of the unknown hexaric acid was identical with glucitol upon electrophoresis in 0.05 M sodium borate (V). Of the known hexitols, only iditol and glucitol do not separate well electrophoretically. The radioactive sugar alcohol was therefore chromatographed on paper in a solvent which separates iditol and glucitol (XI). The chromatographic analysis confirmed that the labeled hexitol was identical with glucitol. Compound A was thus shown to be glucaric acid.

2. Compound B—Compound B was identified as galactaric (mucic) acid by a characterization procedure identical to that described for Compound A. Reduction of the sugar acid yielded a polyhydroxy alcohol, which was shown to be galactitol (dulcitol).

3. Compound C—The electrophoretic mobility of Compound C in formate buffer (I) was slightly lower than that of d-galactaric acid (Mₚ₋₃-d-galactaric acid, 0.74; Compound C, 0.67)
and was found to be identical with that of \( \text{d-glucuronic acid} \). However, since a number of uronic and aldonic acids occupy a similar position on the electrophoretogram, data for the additional characterization of the compound were necessary.

Since uronic acids are readily oxidized to aldaric acids by hypophosphite solution, whereas aldonic acids are unaffected, the two types of acids can be differentiated by their behavior toward this reagent. The following experiment was therefore performed. An aliquot of Compound C (2 \( \times 10^{-8} \) \( \mu \)c) was diluted with 1 \( \mu \) mole of unlabeled \( \text{d-glucuronic acid} \) and subjected to oxidation by treating with hypophosphite solution according to the method of Auerbach and Bodlander (16, 17). When the sample subjected to the oxidant was analyzed by electrophoresis (II), there was no change in electrophoretic mobility, showing that no aldaric acid was produced, and hence that Compound C is not a uronic acid.

The presence of a primary hydroxyl group in Compound C was demonstrated by periodate oxidation (25*, pH 7.5, 3 hours, ratio of periodate to gluconate = 8) and determination of the liberated formaldehyde. An aliquot containing 2527 counts yielded 427 counts of formaldehyde, which corresponds to 15.1% of the initial activity. The theoretical formaldehyde recovery for a hexonic acid was 16.7%. The aldonic acid was converted to a lactone (18), which was found to be chromatographically identical with glucuronolactone in three different solvents (VII, VIII, IX). Furthermore, reduction of Compound C by methods previously described yielded glucitol.

Additional evidence that Compound C is glucuronic acid was obtained by a modified Ruff degradation (19). An aliquot (1 \( \times 10^{-8} \) \( \mu \)c) was diluted with 40 \( \mu \)l of 1% unlabeled calcium \( \text{d-glucuronate} \) solution and mixed with 12 \( \mu \)l of 1.4 m barium acetate and an equal volume of 0.42 m ferric sulfate. A small precipitate was removed by centrifugation, and 25 \( \mu \)l of 50% hydrogen peroxide were added to the supernatant. The reaction mixture was warmed in a water bath until gas evolution commenced. It was then kept at 50°C for 1 hour to ensure complete oxidation. A second 25-\( \mu \)l portion of hydrogen peroxide was subsequently added, and the reaction was terminated upon appearance of a purple precipitate. The solids were removed by centrifugation, and the supernatant liquid was passed through a mixed resin bed (Dowex 30-H+, Dowex 1-OH-, 1:1 by volume). The de-ionized solution was concentrated to near dryness and analyzed by two-dimensional chromatography (XIII, VI). The only labeled product present in the concentrate (10% yield) was a compound chromatographically identical with arabinose.

Products in Ethanolic Extracts—Examination of the ethanolic extract fractions showed very little radioactivity (1 \( \times 10^{-2} \) \( \mu \)c). Two-dimensional chromatography (XIII, VI) of the extract revealed the presence of xylose, arabinose, and several unidentified spots.

Insoluble Material—Inubation of mung bean seedlings with radioactive \( \text{d-glucuronate} \) or \( \text{d-galacturonate} \) resulted in the incorporation of label into insoluble constituents of the plant material. These were separated into pectin, hemicellulose, and alkali-insoluble residue.

Pectin—The residues of the culinum-extracted seedlings were ground with a little sand in 0.5 ml of water and the pectin degraded as described by Albersheim and Bonner (20). Purified yeast polygalacturonase in sodium acetate at pH 4.5 (final buffer concentration, 0.1 M) was used for enzymatic hydrolysis. The hydrolysates mixtures were filtered; the filtrates were concentrated to near dryness under reduced pressure and then passed through a Dowex 50 column. The effluents were reduced to small volumes, adjusted to pH 9 with dilute ammonia, and adsorbed on a Dowex 1 column. The column was washed with 4 bed volumes of water and eluted with 0.5 M formic acid. The eluates were collected in 2-ml fractions and evaporated to dryness under vacuum. Electrophoretic analysis of the fractions at pH 5.8 (II) showed the presence of labeled galacturonic acid and two compounds which were electrophoretically identical with digalacturonic and trigalacturonic acid. Hydrolysis of each of these compounds with 1 N HCl at 100°C for 2 hours produced galacturonic acid as the sole product.

Hemicellulose—After the pectinase treatment, the hemicellulose was extracted from the residual plant material with sodium hydroxide, according to the method of Edelman et al. (21). The solubilized hemicellulose fractions were acidified to pH 4.5 with concentrated HCl and dialyzed for 24 hours against running tap water. After evaporation to a small volume under reduced pressure, the fractions were lyophilized.

Aliquots of the lyophilized fractions were suspended in 200 \( \mu \)l of 1 N HCl and hydrolyzed at 100°C for 1 hour. Analysis of the partial hydrolysates by two-dimensional paper chromatography (XIII, VI) and paper electrophoresis (I) showed that they contain labeled arabinose (1 part), xylose (2 parts), glucuronic and galacturonic acid (5 parts), and unhydrolyzed material (35 parts). These figures are representative of the alkali-soluble fraction of all three incubation experiments.

Alkali-insoluble Residue—The plant material which resisted alkali extraction was washed exhaustively with water and quantitatively analyzed for incorporated label. For this purpose, portions of the alkali-insoluble residue were ground to a homogeneous suspension with 0.1 ml of water, and the finely divided material was transferred to a filter paper disk, dried, and counted.

Relative Quantities of Labeled Metabolic Products—Table I represents the relative amounts of the various labeled products synthesized by the mung bean seedlings from \( \text{C}^{14} \)-labeled \( \text{d-glucuronate} \) or \( \text{d-galacturonate} \). In Experiment 1, 18 \( \mu \)c of ammonium \( \text{d-glucuronate} \) were supplied to the seedlings for 90
yielded two compounds which migrated as galactitol and glucitol in borate buffer (V) and in borate buffer (V), thus demonstrating the presence of galactaric and glucaric acid in the seedling extract. The precise ratio of the two compounds could not be determined because of the small quantity of material available. However, it was estimated that glucaric and galactaric acids were extracted in comparable amounts from the plant material.

**DISCUSSION**

The demonstration that mung bean seedlings are capable of incorporating D-glucuronate into pectin and hemicellulose is in agreement with the results of physiological experiments obtained by other workers (1-3) who showed that higher plants can utilize exogenous D-glucuronic acid for the synthesis of cell wall polysaccharides. Finkle et al. (1) presented evidence that D-glucuronic acid labeled in the C-6 position is incorporated into the pectin fraction of ripening strawberries, with but little randomization of label. Experiments with wheat plants (3) and corn coleoptiles (2) further indicated that specifically labeled D-glucuronic or its lactone are transformed into pentosyl moieties by loss of C-6. As shown in this work, substitution of D-glucuronic acid for D-glucuronate also leads to the formation of pectin and other cell wall polysaccharides in mung bean seedlings.

Present concepts suggest that when D-glucuronic acid is introduced into the plant, it must enter a sugar nucleotide pool before conversion to polysaccharide. Enzymes that would account for these reactions were shown to be present in mung bean seedlings (22). A number of polysaccharides and other complex saccharides have also been demonstrated to be formed from sugar nucleotides by transglycosylation in animals, microorganisms, and plants (22). It is therefore reasonable to assume that pectin and hemicellulose in plants are formed from D-glucuronic acid by the following pathway (Fig. 1).

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\begin{align*}
\text{D-Glucuronic acid} & \rightarrow \text{D-glucuronic acid-1-phosphate} \\
& \rightarrow \text{UDP-D-glucuronic acid} \\
& \rightarrow \text{UDP-D-galacturonic acid} \\
& \rightarrow \text{UDP-D-galacturonic acid-1-phosphate} \\
& \rightarrow \text{D-galacturonic acid} \\
& \rightarrow \text{D-galacturonic acid} \\
\end{align*}
\]

Fig. 1. Suggested pathway for the formation of pectin and hemicellulose from D-glucuronic acid and D-galacturonic acid in mung beans.

The aldaric acid peak was shown to consist of glucaric and galactaric acids as follows. The fraction was dissolved in 0.5 ml of 0.5 M formic acid, followed by 2 M formic acid. Aldaric acids were located by spotting an aliquot of each fraction on paper and testing by the periodate-benzidine method (8). They emerged from the chromatogram as a single peak shortly after the concentration of the eluant was increased to 2 M. Final purification of the aldaric acid peak was achieved by paper electrophoresis at pH 5.8 (II) and pH 9.0 (III). A yield of 5 mg was obtained.

The aldaric acid peak was shown to consist of glucaric and galactaric acids as follows. The fraction was dissolved in 0.5 ml of 5% methanolic HCl and esterified as previously described. Upon cooling of the reaction mixture, a small quantity of colorless needles crystallized from the solution. After separation from the mother liquor, the crystalline material was purified by recrystallization from dry methanol. These crystals were identified as dimethylgalactarate by its melting point and mixed melting point with an authentic specimen (184-186°, uncorrected). This compound had the same mobility as that of authentic dimethylgalactarate when subjected to circular electrophoresis at pH 5.8 (II) and pH 9.0 (III). A yield of 5 mg was obtained.

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elastica (24), and galactaric acid has been isolated from various fruits (25, 26). Their existence in plants suggests that D-glucuronic acid and D-galacturonic acid may be utilized by the plant for energy production.

A pathway of uronic acid catabolism in bacteria has been described by Kilgore and Starr (27). These authors obtained cell-free extracts from D-glucuronate- and D-galacturonate-grown cells of Pseudomonas syringae which catalyze the DPN-linked oxidation of the two uronic acids. In addition, Blumenthal (28) showed that a strain of Escherichia coli can convert D-glucarate or galactarate to pyruvate.

Mung bean seedlings also convert D-glucuronic acid to gluconic acid. Such a conversion has not been previously shown, although formation of the aldonic acid, D-gulonic acid, from D-glucuronolactone by ripening strawberries was reported by Loewus and Kelly (18). This reaction is considered to involve reduction of the carbonyl group of D-glucuronolactone to form L-gulonate. On the other hand, the mechanism of the formation of gluconate from D-glucuronate is not understood.

Uronic acids are seldom found in the free state in plants. During seed germination, uronic acids could be produced by hydrolysis of uronate-containing polysaccharides stored in the endosperm or other seed tissues. The free D-glucuronic acid or D-galacturonic acid could then either enter the sugar nucleotide pathway and be used for synthesis of cell wall material in the developing seedling, or be catabolized with the production of energy.

SUMMARY

A study of the utilization of D-glucuronic acid and D-galacturonic acid by mung bean (Phaseolus aureus) seedlings showed that these uronic acids are readily incorporated into pectin and hemicellulose by the seedlings.

Mung bean seedlings are capable of oxidizing D-glucuronic acid and D-galacturonic acid to the corresponding aldaric acids, gluconic acid and galactaric acid.

Gluconic acid is also produced from D-glucuronic acid by the seedlings.

Gluconic acid and galactaric acid were shown to occur in mung seedlings as natural products.

REFERENCES

Metabolism of d-Glucuronic Acid and d-Galacturonic Acid by *Phaseolus aureus* Seedlings

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