PREPARATION OF ^{14}C-LABELED PECTIC ACID AND D-GALACTURONIC-^{14}C ACID

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ABSTRACT

^{14}C-Labeled D-galacturonan [poly(D-galacturonic acid)] was isolated from the alga *Chara globate* grown in the presence of $^{14}CO_2$. The polysaccharide provides a convenient source for the preparation of uniformly ^{14}C-labeled oligo(D-galacturonic acids) and D-galacturonic acid.

INTRODUCTION

The synthesis of small amounts of ^{14}C-labeled pectin has been achieved in several investigations[1–6] designed to study the pathways of biosynthesis of the polysaccharide. In these experiments, plant tissue was incubated with ^{14}C-labeled monosaccharides, the ^{14}C-labeled pectin was subsequently isolated, and D-galacturonic-^{14}C acid was obtained from its hydrolyzate. The ^{14}C-labeled products obtained by these procedures were of low specific activities, and the use of relatively expensive ^{14}C-labeled substrates was involved; thus, the methods could not be recommended for the production of pectin-^{14}C and D-galacturonic-^{14}C acid in large quantities.

A need for ^{14}C-labeled substrates for a study of the pattern of action of D-galacturonan hydrolases[7] prompted us to develop a procedure for the preparation of these ^{14}C-labeled compounds. The method employed was based on Anderson and King's observation[8,9] that pectic acid having a zero (or only a very low) methoxyl content is a structural component of the sweet-water algae of the *Characea* family, plants that can readily be grown in the laboratory.

The present study describes the isolation of pectic-^{14}C acid and D-galacturonic-^{14}C acid from *Chara globate* cells grown with carbonate-^{14}C. Some 3 to 5% of the total carbon-14 incorporated into the cellular matter could be accounted for as D-galacturonic acid. The specific activity of the hexuronic acid obtained lay between 0.5 and 1.0 mCi·mmol^{-1} (or 0.08 to 0.16 μCi per μatom of carbon). As, in the present procedure, carbonic-^{12}C acid ions were employed to prevent the medium from becoming too alkaline, significant dilution of the applied $^{14}CO_2$ occurred. It is, therefore, conceivable that, under physiological conditions allowing growth of algae with a proper control of pH but in a way that would not result in a significant increase of the salt concentration of the medium, a higher specific activity for the final ^{14}C-

labeled product could be achieved. The difficulty in developing such conditions lies in the fact that growth of the *Chara* strain employed is inhibited by the relatively low salt concentrations (10 mM) tried as effective buffering agents. In addition, were carbonate-^{14}C having a specific activity higher than that employed in the present study commercially available, it could be useful in obtaining highly radioactive, ^{14}C-labeled products.

MATERIALS AND METHODS

Organism. — A strain of *Chara globate* was isolated from an uncovered rain-water reservoir (Mamilla Pool) in Jerusalem.

Chemicals and enzymes. — Pectic acid, D-galacturonic acid, and, unless otherwise stated, most of the other biochemical compounds used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo., U. S. A.).

Pectic acid hydrolyase complex was isolated from a culture medium of *Aspergillus niger*[7]. Two "pectinase" preparations purchased from Sigma Chemical Co. and Nutritional Biochemical Co. (Cleveland, Ohio) were also employed. A purified endopolygalacturonase was isolated from cultures of[10] *Saccharomyces fragilis ATCC 8589*. Sodium hydrogen carbonate-^{14}C was obtained from the Radiochemical Centre (Amersham, Bucks., England).

Chromatographic procedures. — Oligo(D-galacturonic acids) up to nonamers were separated effectively by descending chromatography on Whatman No. 3 filter paper, with 50:25:42 (v/v) ethyl acetate–glacial acetic acid–water as the developing solvent. Alkaline silver nitrate reagent[11] was employed for detection of spots. Paper-chromatographic examination for the presence of neutral sugars was performed with 5:1:4 (v/v) butanol–ethanol–water as the developing solvent, and an *o*-aminobiphenyl spray reagent[12] for color development.

Thin-layer chromatographic separation of oligo(D-galacturonic acids) up to the octamer was conducted on plates of $CaSO_4$-free Kieselgel G (Camag AG, Muttenz, Switzerland) with 2:3:1 (v/v) butanol–formic acid–water as the solvent[13]. Spots were revealed by spraying the plates with a 5% solution of sulfuric acid in propanol, followed by heating for 10 min at 110°.

Colorimetric analysis. — Total sugar was estimated by the phenol–sulfuric acid method[14]. Hexuronic acids were determined with the carbazole–sulfuric acid reagent, with or without borate, to enable a specific evaluation of D-galacturonic acid residues[15,16]. Reducing D-galacturonic acid residues were determined with the copper acetate reagent[17].

Enzymic determination. — D-Galacturonic acid was determined spectrophotometrically by Nagel and Hasegawa's method[18]. The D-galacturonate isomerase and NADH–D-tagaturonate dehydrogenase used for this purpose were isolated from *Bacillus polymyxa* cells which had been grown on a pectin hydrolyzate[18]. The standard assay system (1.0 ml) contained: 0.1 M phosphate buffer, pH 7.0; NADH, 0.2 mM; an excess of the enzymic complex; and 0.01 to 0.18 mM D-galacturonate.

The decrease in optical absorbance at 340 nm was measured in cuvets having a 1.0-cm light-path by using a Gilford Model 2000 automatic, recording spectrophotometer.

Measurement of radioactivity. — Radioactivity was measured in a Packard Tri-CARB scintillation spectrometer by using Permablend II in toluene (Packard Instruments Co., Inc., Downers Grove, Illinois) as the scintillator solution, with 80% counting efficiency for carbon-14.

Determination of the extent of polysaccharide hydrolysis. — The degree of hydrolysis of the α-D-(1→4)-glycosidic linkages in poly(D-galacturonic acid) by hydrolytic enzymes was monitored by measuring the amount of free reducing ends cuprimetrically[17]; t.l.c. was employed to determine the distribution of products having low molecular weight.

A rapid fractionation procedure to measure the degree of hydrolysis of poly-(D-galacturonic acid) and the amount of free D-galacturonic acid liberated was performed as follows. A sample of an enzymic reaction mixture (containing 1 to 4 μmoles of reducing hexuronic acid) was applied to a column (0.9 × 3 cm) of Dowex-1 X-2 (formate) ion-exchange resin made in a standard, 2.5-ml, disposable, polyethylene syringe. A series of such columns can be used for rapid, simultaneous assays of many samples. After application of the sample, the column was washed successively with water (~20 ml), 0.2M formic acid (20 ml), 0.5M formic acid (10 ml), and 4M formic acid (5.0 ml). Each eluant was collected separately, and samples were assayed colorimetrically for reducing sugar and for hexuronic acid, and counted for radioactivity. A sample taken for the enzymic determination of free D-galacturonic acid must first be neutralized.

EXPERIMENTAL

Growth. — The medium employed was prepared as follows. A suspension of garden soil (100 g) in water (500 ml) was boiled for 30 min, and filtered through Whatman No. 1 filter paper. The filtrate was diluted 1:20 in sterile, aqueous solution containing sodium carbonate (20 p.p.m.) and calcium carbonate (50 p.p.m.). This culture medium (batches of 500 ml) was used for growing the algae in 600-ml, blood-plasma bottles equipped with screw-cap covers and self-sealing, neoprene plugs, All connections to these culture bottles, for the purpose of adding or withdrawing material, were made with hypodermic needles.

Each bottle containing the liquid medium was swirled on a rotary shaker while being flushed with 19:1 air–carbon dioxide at 20 lb. in.$^{-2}$ for ~3 h before incubation. A suspension (10 ml) of water-washed, algal "starter" (~0.5 g fresh weight) was added, and this was followed by the injection of $NaH^{14}CO_3$ (13.5 mg in 3.0 ml of water) (6.72 mCi or 4.8 mCi/matom of carbon). The bottle was swirled manually while flushing with the air–carbon dioxide mixture was continued for an additional hour. All connections were then severed by withdrawing the needles, and the tightly sealed bottle was incubated at 22°, without shaking, between two 40-W, standard, fluorescent tubes, at a distance of 30 cm from each lamp.

After three weeks, the culture bottle, while still being illuminated, was connected for 48 h to a wash-bottle containing 300 ml of a 0.05M barium hydroxide solution. The algae were then collected by filtration on gauze, and washed with three 300-ml portions of cold water. The yield of algae was 10–15 g of fresh-packed material (0.9–1.4 g, dry weight) per bottle; it was stored in a deep freezer until used.

The initial pH of the medium was 7.5, and, by the time growth had terminated, it had increased to 8.5. When pre-equilibration with the air–carbon dioxide mixture was insufficient, the pH of the medium rose rapidly to 9, or higher, and growth of the algae was poor. Introduction of such salts as phosphates, for the purpose of buffering the medium, was not beneficial, as growth was severely retarded by relatively low ionic strength (5 mM phosphate buffer was inhibitory).

Isolation of pectic-^{14}*C acid.* — The procedure employed was essentially that described by Anderson and King[8,9], with slight modifications as follows. Washed algae (equivalent to 1.0 g, dry weight) were blended twice with 4:1 (v/v) acetone–water (100 ml), and collected by filtration on a Büchner funnel. The dry powder was then blended with two 100-ml portions of 4:1 (v/v) chloroform–methanol, and the precipitate was collected by filtration, washed with ether, and dried. The resulting powder was extracted overnight with water (400 ml) in a Soxhlet extractor, and the extract discarded. The solid residue was extracted by three successive, 30-min treatments by boiling with 100-ml portions of 0.5% oxalic acid plus 0.5% ammonium oxalate solution (adjusted to pH 4.5 with ammonia). The oxalate extracts were combined, and 0.01M hydrogen chloride in ethanol (2 vols.) was added. The precipitate was collected by centrifugation at 5,000 *g* for 30 min, and washed successively with 0.01M hydrochloric acid, 60% ethanol, and absolute ethanol.

The precipitate was suspended in water (20 ml), and 1M sodium hydroxide was

TABLE I

FRACTIONATION[a] OF ^{14}C-LABELED *Chara* EXTRACTS

Fraction	*Total d.p.m.* $\times 10^{-8}$	*Carbohydrate*			*Specific activity (d.p.m./μmole of hexose* $\times 10^5$*)*
		Total hexose (μmoles)	*"Anhydrogalacturonic" acid (μmoles)*	(%)	
Organic phase	30	<5	0		
Hot-water extract	19.2	651	100	15	29.4[b]
Oxalate extract	4.2	430	233	54	9.77
First acid-alcohol precipitate	2.4	190	119	62	12.63
Precipitate after acid hydrolysis	2.0	145	110	76	13.80
Insoluble residue[c]	89				

[a]Procedures of extraction and fractionation are described in the text. Values given are for a sample of washed algae equivalent to 1.0 g of dry weight. [b]This fraction contained sugars of low molecular weight, water-soluble polysaccharides, and other ^{14}C-labeled metabolites that were not assayed colorimetrically as carbohydrate. [c]This fraction consists mostly of structural polysaccharides[8] and some denatured protein.

slowly added, with constant stirring, until complete dissolution occurred (pH 5.0). This fraction, designated "oxalate extract" (see Table I), could be further enriched in its content of D-galacturonic acid by several procedures[9]. In the present study, another cycle of precipitation from acid solution in the cold, sometimes with prior boiling for 1 h in 0.1M hydrochloric acid to remove some neutral sugar components, was employed. Fractions of pectic acid thus obtained contained 62–75% of D-galacturonic acid residues (see Table I) and some neutral sugars, in particular, arabinose, xylose, galactose, and rhamnose, as revealed by chromatographic analysis of acid hydrolyzates. Small amounts of glucose detected were usually contributed by a glycogen contaminant, which could be partially removed by treatment with *alpha*-amylase or by acid hydrolysis as already described[8]. The properties of the pectic-^{14}C acid isolated here correspond fairly well with the composition of the pectic acid isolated from land-plant tissues in general[19–23].

Partial, enzymic hydrolysis of pectic-^{14}C acid. — Samples of pectic acid obtained from the oxalate fraction by repeated precipitations (see Table I) were partially hydrolyzed by *Saccharomyces fragilis* polygalacturonase to yield a series of oligo(D-galacturonic-^{14}C acids) of degree of polymerization (d.p.) 1 to 8 in the following way. Pectic-^{14}C acid (8.4×10^7 d.p.m.; 61 μmoles of "anhydro-D-galacturonic" acid having a specific activity of 1.38×10^6 d.p.m./μmole) was dissolved in 5 ml of 0.05M acetate buffer (pH 5.0) containing 9 units of *Saccharomyces fragilis* endopolygalacturonase[10]. Incubation was conducted at 30° until the average d.p. reached a value of ~3 (assayed by t.l.c. analysis of the oligosaccharides-^{14}C in the reaction mixture, as well as by an assay of reduced equivalents appearing in a parallel, similar system using nonradioactive pectic acid). The reaction system was boiled for 5 min to stop enzymic activity, treated with about 2 g of Dowex 50 (H^+) ion-exchange resin, and filtered. The filtrate was put on a column (1 × 20 cm) of Dowex-1 X-2 (formate) (200–400 mesh) in 0.1M formic acid. Elution was performed with a linear gradient (0.1–5.0M) of formic acid, and fractions were collected.

In a similar experiment, quantitative paper chromatography on Whatman No. 3 MM filter paper was employed, and the oligosaccharides-^{14}C were eluted from paper strips with hot water. The results are given in Table II.

The oligosaccharides obtained were found to be useful in the study of the pattern of action of pectic acid hydrolyases[7]. The products of the enzymic hydrolysis of *Chara* pectic acid had the same chromatographic mobility as those of oligo(D-galacturonic acids) obtained by partial, enzymic hydrolysis of apple and citrus pectin (see Table II). Separation of the individual components was achieved by quantitative paper chromatography and subsequent elution of the carbon-14 zones, by chromatography on a column[24] of Dowex-1 X-2 (formate) ion-exchange resin with a linear gradient of 0.1 to 5.0 M formic acid, or by gel filtration on columns of Sephadex G-25 (Pharmacia, Uppsala) with 1 mM hydrochloric acid as the eluant[7].

Isolation of D-galacturonic-^{14}C acid. — *Method a.* Pectic-^{14}C acid (30 μmoles of "anhydro-D-galacturonic" acid residues; 1.88×10^6 d.p.m./μmole) obtained from the "oxalate extract" fraction (see Table I) in 10 ml of 0.05M acetate buffer, pH 5.0,

TABLE II

PARTIAL ENZYMIC HYDROLYSIS OF PECTIC-^{14}C ACID

Degree of polymerization	D-*Galacturonic acid* (*μmoles*)	α-(*1→4*)-D-*Galactosyluronic linkages*[a]	*Total d.p.m.* × 10^6	*Specific activity* (*d.p.m.* × 10^6/*μmole*)	*Paper chromatography* (R_{GalA})	*T.l.c.* (R_F)	K_{av}[b]	*Specific turbidity*[c]	$[\alpha]_D^{20}$ (*degrees*)[d]
1	19.3		26.3	1.36	1.00	0.49	0.54	0	+52
2	9.3	1.1	13.0	1.39	0.76	0.37	0.37	0.012	+151
3	7.1	2.1	9.2	1.30	0.61	0.29	0.32	0.018	+188
4	4.6	3.1	5.9	1.29	0.47	0.26	0.26	0.029	+205
5	2.1	4.2	2.4	1.15	0.35	0.22	0.20	0.072	+231
6	2.7	4.9	3.6	1.33	0.25	0.21	0.14	0.125	+247
7	3.9	6.1	5.0	1.28	0.17	0.12	0.08	0.180	+257
>8[e]	6.0	not determined	14.0		0.10	0.08	0.05	0.240	+265

[a]Indicates the number of reducing D-galactosyluronic residues appearing after exhaustive hydrolysis by *Aspergillus niger* pectin hydrolase preparation[7]. [b]Partition coefficient for gel filtration on Sephadex G-25 (Ref. 30). [c]Specific turbidity value, at 400 nm, per μmole of D-galacturonic acid, determined in the cetylpyridinium bromide reaction[31]. [d]These values (*c* 1, in water) were obtained for products isolated from an enzymic hydrolyzate of a large batch of nonradioactive, *Chara* pectic acid ($[\alpha]_D^{20}$ +245°), and of orange poly(D-galacturonic acid). The oligosaccharides obtained were also used as standards for chromatographic analyses. [e]This fraction did not move appreciably in paper chromatography. It contained significant amounts of neutral sugars-^{14}C which could be qualitatively seen in chromatograms of acid hydrolyzates, as well as in hydrolyzates obtained by use of crude, commercial, "pectinase" preparations. The latter were found to possess significant amylase, cellulase, and hemicellulase activities.

was mixed with nonradioactive poly(D-galacturonic acid) (170 mg; 875 μmoles of "anhydro-D-galacturonic" acid) to yield a specific activity of 6.2×10^4 d.p.m./μmole of D-galacturonic acid. Pectinase (20 mg; Sigma Chemical Co.) was added, and the mixture was incubated for 48 h at room temperature. The solution, which reached a level of 90% hydrolysis as determined by the copper acetate assay[17], was passed through a small column of Dowex-50 (H^+) ion-exchange resin. The acidic effluent was treated with Darco G-60 activated carbon (~100 mg) and the suspension was filtered through a Millipore PH membrane (Millipore Corporation, Bedford, Mass.). The filtrate was concentrated to 1.0 ml under diminished pressure at 30°, and the solution was put on a column (0.9 × 10 cm) of Dowex-1 X-2 (formate) ion-exchange resin. After the column had been washed with 20 ml of 0.1M formic acid, 50 ml of 1.2M formic acid was passed through, to elute the D-galacturonic-^{14}C acid, the effluent was evaporated to dryness under diminished pressure at 40°, and the residue was washed with diethyl ether. The dry material (805 μmoles of hexuronic acid, as determined by the carbazole test) was dissolved in water (10 ml), 1 ml of 0.5M sodium hydrogen carbonate and 200 mg of calcium carbonate were added as the solution was carefully warmed to 80°, and the suspension was rapidly filtered through a Millipore filter. After concentration to 3.0 ml, the turbid solution was kept for several days at 4°. Crystals of $CaNa(^{14}C_6H_9O_7)_3 \cdot 6\,H_2O$ (Ref. 25) separated, and were filtered off, washed with ice-cold water, and dried in a vacuum desiccator. The yield of this double salt of D-galacturonic-^{14}C acid was 409 mg.

A sample of the salt was converted into the free acid by dissolving it in warm water and adding an equivalent amount of oxalic acid, or by treatment with Dowex 50 (H^+) ion-exchange resin. The D-galacturonic-^{14}C acid obtained had a specific activity of 6.1×10^4 d.p.m./μmole. It formed a (2,5-dichlorophenyl)hydrazone[26], m.p. 178°, and gave crystalline galactaric acid on oxidation with bromine water, similar to that formed by authentic D-galacturonic acid. The product served as a substrate in the specific enzymic test for D-galacturonic acid[18], and exhibited the c.d. and o.r.d. spectra in the far-ultraviolet region expected for this hexuronic acid[28,29].

Method b. For the preparation of D-galacturonic-^{14}C acid of higher specific activity, another batch of pectic-^{14}C acid was enzymically hydrolyzed as already described, but without dilution with "carrier" poly(D-galacturonic-^{12}C acid). The D-galacturonic-^{14}C acid was obtained in the formic acid eluate from the Dowex-1 (formate) column as already described. Analyses indicated the presence of chromatographically pure D-galacturonic-^{14}C acid (21 μmoles) having a specific activity of 1.85×10^6 d.p.m./μmole. A crystalline preparation of the monohydrate of the acid[27] could also be isolated, although in low yield, from hot, aqueous methanol solutions.

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