[CONTRIBUTION FROM THE RESEARCH LABORATORY OF THE GREAT WESTERN SUGAR COMPANY]

Isolation and Identification of O-α-D-Galactopyranosyl-*myo*-inositol and of *myo*-Inositol from Juice of the Sugar Beet (*Beta Vulgaris*)

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By application of chromatographic procedures, two previously unrecognized natural carbohydrate constituents of sugar beet juice have been isolated. One has been identified as *myo*-inositol. Hydrolysis of the second, $C_{12}H_{22}O_{11}.2H_2O$, with α-galactosidase yielded only two products, identified as *myo*-inositol and α-D-galactose, establishing the identity of the compound as O-α-D-galactopyranosyl-*myo*-inositol. This new compound, which we have named "galactinol," can be a source of serious error in analysis of beet sugar processing liquors because of its high dextrorotation and large drop in rotation accompanying hydrolysis with melibiase.

Introduction

During the course of an investigation designed to develop a method for the determination of raffinose in mother beets by means of paper chromatography, it was observed that the raw beet juice contained two carbohydrates which did not react to the common aldose and ketose color tests, in paper chromatography, but did react to ammoniacal silver nitrate.

The course of these products during sugar making operations was followed by paper chromatography, and it was found that they pass through the carbonation process into the thin juice and concentrate in the molasses. They are largely precipitated in the Steffens[1] process along with sucrose and raffinose, and reach a comparatively high concentration in "discard"[2] molasses. Little, if any, of these compounds is precipitated along with sucrose in the barium saccharate process for recovery of sucrose from "discard" molasses, and they are therefore found in relatively high concentration in the barium saccharate filtrate. This filtrate also contains some sucrose and raffinose, together with various organic and inorganic components of beet juice.

After isolation in crystalline form, the compounds were identified as an α-galactoside of *myo*-inositol, a previously unrecognized compound, hereinafter called "galactinol," and as *myo*-inositol.

The finding that inositol is a natural component of beet juice was unexpected since Lippmann[3] reported in 1901 that, after an extensive investigation of the waste liquor from the strontia process for desugarizing beet molasses, he was unable to find inositol, but he did isolate a small amount of a tetrahydroxycyclohexane which he named "betit." The isolation and identification of galactinol was also unexpected. As recently as 1950, Sumner and Myrback[4] stated that raffinose is presumably the only naturally appearing substrate for α-galactosidase. Climate may be an important factor in determining the absolute quantities of the two compounds present in sugar beet juices. Lippmann found no inositol in juice from European beets. Tests made in this laboratory on Steffens saccharate sirup and on beet molasses from California showed very little inositol and only a trace of galactinol in each. However, all beet sugar factory processing liquors from the Rocky Mountain area, tested to date, show significant quantities of both components. Individual mother beets have been found which contain more of galactinol than of raffinose.

Interest of the beet sugar industry in the two compounds is derived from two sources. First, since both are precipitated by lime in the Steffens process, improved factory operations would result if their quantities in beet juice could be reduced by suitable beet breeding. Second, galactinol possesses an unusually high dextrorotation, and it is precipitated in varying degrees from its solutions by basic lead acetate, which is employed for clarification of the processing liquors prior to polarization procedures. It therefore causes variable unknown errors in analysis.

The two compounds, inositol and galactinol, can be isolated by application of a modification of the Whistler and Durso[5] technique, in carbon column chromatography, to deionized beet molasses solution, wherein inositol is leached from the column with water, and galactinol is eluted with 2% ethanol.[6]

Preferably, for a variety of reasons, galactinol and inositol are concentrated by chemical means, prior to employment of the carbon column for their isolation.

Experimental

Preparation of Concentrate of Galactinol and Inositol.—Filtrate from the barium saccharate process for recovery of sucrose from "discard" molasses, was carbonated to remove BaO, and then treated by standard laboratory technique in the Steffens process, for preparation of saccharate sirup. This sirup contained essentially all of the sucrose and raffinose, and a high percentage of the galactinol and inositol originally present in the barium saccharate filtrate. To 1 kg. of dry substance in this sirup, at *ca.* 30% dry substance, were added *ca.* 2 l. of basic lead acetate solution of 55 Bx. and 6.6 *p*H. After standing overnight, the mixture was filtered, washed with H_2O, and the precipitate was decomposed with H_2S. The filtrate from the PbS was boiled to remove excess H_2S, and then passed, at *ca.* 7.5% dry substance through a column of anion exchanger (Duolite

(1) Steffens process: the process for recovery of sucrose from beet molasses wherein suitably diluted molasses is treated in the cold with powdered calcium oxide to precipitate sucrose and other carbohydrate matter, and, after filtration the precipitated matter is released by carbonation. The sucrose containing sirup thereby obtained is called "saccharate sirup."

(2) "Discard" molasses is the final molasses obtained in the factories employing the Steffens process, when further recovery of sucrose by that process becomes uneconomic because of excessive accumulation of non-sucrose matter precipitable by calcium oxide.

(3) E. O. von Lippmann, *Ber.*, **34**, 1159 (1901).

(4) James B. Sumner and Karl Myrback, "The Enzymes," Vol. 1, Part 1, Academic Press, Inc., New York, N. Y., 1950, p. 263.

(5) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

(6) All references to ethanol, mentioned herein, refer actually to Formula No. 30 specially denatured alcohol.

A-2) until the *p*H of the effluent dropped to 5.0 *p*H. A 1 liter column of ion exchanger yielded 2.2 liters of deacidified effluent containing 100 g. of dry matter.

Carbon Column Chromatography of Galactinol–Inositol Concentrate. Control Tests. A. Reagents.—*Ammoniacal silver nitrate:* Dissolve 5.0 g. of $AgNO_3$ crystals in 50 ml. of distilled H_2O. Add concd. NH_4OH until precipitate just redissolves. Prepare fresh daily. This reagent is referred to hereinafter as A.S.N. *α-Naphthol reagent:* Dissolve 1.0 g. of α-naphthol in 100 ml. of ethanol. Add to this solution 10 ml. of sirupy H_3PO_4. **B. Spot Tests.**—*Sugars, galactinol and inositol:* Place a drop of solution to be tested on filter paper. Dry. Spray with A.S.N. Dry. Heat at *ca.* 120° for *ca.* 1 min. A positive reaction is a brown to black spot. *Ketose-containing sugars:* Spot filter paper with test solution, dry, spray with α-naphthol reagent, dry and heat at 90° for 5 min. A positive reaction is a bluish color. **C. Color test for Galactinol and Sugars, but not Inositol:** *Modified Dische[7] test:* Mix 1 ml. of test soln. and 1 ml. of α-naphthol reagent in a 1″ × 6″ test-tube. Add 2 ml. of concd. H_2SO_4 and mix. When boiling ceases, add 6 ml. of concd. H_2SO_4. Purplish color denotes positive reaction.

Carbon Column Operation and Control.—A small scale test was run on the deacidified galactinol concentrate using the original Whistler and Durso[5] technique, in carbon column chromatography. The three fractions of effluent, water fraction, 5% ethanol fraction, and 10% ethanol fraction, were tested by paper chromatography. The 5 and 10% ethanol fractions were tested before and after hydrolysis with invertase and invertase containing melibiase.

The water fraction showed a single spot, reacting only to A.S.N., and unaffected by enzyme treatment. R_g values, compared to sucrose as 1.0, were *ca.* 0.4 in both pyridine and collidine solvents. This was inositol.

The 5% ethanol fraction showed one spot only (sucrose) to α-naphthol and P.D.A.[8] It showed 3 spots to A.S.N. They were sucrose, inositol and galactinol. R_g values of galactinol were: *ca.* 0.2 in both pyridine and collidine solvents. Hydrolysis with invertase had no effect on either inositol or galactinol. Hydrolysis with invertase containing melibiase resulted in disappearance of the galactinol spot, increased the intensity of the inositol spot, and caused the appearance of a spot which reacted to both P.D.A. and A.S.N. at R_g *ca.* 1.2 (galactose).

The 10% ethanol fraction showed essentially only raffinose–R_g *ca.* 0.4 in pyridine solvent and *ca.* 0.6 in collidine solvent. Hydrolysis with enzymes produced only the changes anticipated in the presence of raffinose.

A large column was used for isolation and recovery of inositol and galactinol. The Whistler and Durso[5] technique was modified by employment of 2% ethanol to obtain a fraction containing galactinol free from sucrose. The carbon column contained *ca.* 13 liters of an equal part, by weight, mixture of Darco G-60 and Johns–Manville Celite 535 held in a 36″ section of 6″ Pyrex pipe, with an 18.5 cm. buchner funnel, held in place with Woods metal, as a base. The carbon mixture was washed with *ca.* 20 liters of H_2O and then charged with a volume of the previously described deacidified galactinol concentrate containing 180 g. of dry matter. The charged column was washed with *ca.* 50 liters of distilled water which flowed at *ca.* 5 liters per hour. The water effluent was concentrated for inositol recovery (Fraction No. 1). This solution gave blank modified Dische test and positive A.S.N. spot test, showing absence of invert sugar and galactinol and presence of inositol. The water leach was followed with 2% ethanol in distilled water. Up to this point, the effluent had shown a blank modified Dische test. Soon after water had been displaced by 2% ethanol, a positive modified Dische test, and a slight positive rotation were noted. During the following 14 liters of effluent, taken in 2 to 3 liter increments, the rotation rose to 0.42°V. in a 400 mm. tube. This fraction, which contained both inositol and galactinol, was discarded. The volumes and rotations of succeeding increments, saved for galactinol recovery, are given in Table I.

Up to this point, the effluent had given a negative α-naphthol spot test, and positive modified Dische test, showing presence of galactinol and absence of sucrose.

(7) C. A. Browne and F. W. Zerban, "Sugar Analysis," 3rd edition, John Wiley & Sons, Inc., New York, N. Y., 1941, p. 737.

(8) See below, under Color Reagents for Paper Chromatography.

TABLE I

ROTATION OF GALACTINOL FRACTIONS

Volume, l.	°V. in 400 mm. tube	Volume, l.	°V. in 400 mm. tube
3.5	4.60	3.0	1.45
1.9	6.50	3.8	0.90
2.8	5.00	1.4	.60
3.4	3.70	3.5	Final .42
3.6	2.50		
		26.9	

Collection of effluent for galactinol recovery was discontinued here because previous experience had shown that sucrose would appear before all galactinol was eluted.

Based on mean rotation, the fraction retained for galactinol recovery contained *ca.* 49 g. of galactinol. This fraction was concentrated for recovery of crystalline galactinol (Fraction No. 2).

Identification of Galactinol and Inositol. Reagents. A. Enzymes.—*Invertase free from melibiase:* A solution of 1.5 g. of Wallerstein scales in 100 ml. distilled water. This solution showed an invertase activity in excess of the standard.[9] *Invertase containing melibiase:* A solution of 5.0 g. of Wallerstein scales in 100 ml. of distilled water. This solution showed a melibiase activity in excess of the standard.[10] **B. Solvents for Paper Chromatography.**—*Pyridine solvent:* A single phase solution prepared by mixing: 50 vol. *n*-butanol, 30 vol. pyridine, 30 vol. H_2O and 4.5 vol. benzene. *Collidine solvent:* Mix 1 vol. of redistilled 2,4,6-collidine with 10 vol. of same saturated with H_2O at room temp. **C. Color Reagents for Paper Chromatography.**—*Ammoniacal silver nitrate* (A.S.N.)*:* For sugars, galactinol and inositol, and *α-naphthol*, for ketose containing sugars, as above described. *Phenylendiamine:* For aldose-containing sugars. Dissolve 0.2 g. of phenylenediamine hydrochloride, and 2 g. of oxalic acid in 100 ml. of ethanol. This indicator is referred to hereinafter as P.D.A.

Galactinol.—Fraction No. 2, from the carbon column, containing an estimated 49 g. of galactinol, after concentration *in vacuo*, was treated by the Whistler and Durso[11] butanol–methanol technique. Two crops of crystals were taken, totalling 43.6 g. These crystals were dissolved in hot water and recrystallized from ethanol; yield 34 g.

Identification of the Galactose Moiety of Galactinol. Hydrolysis.—A solution of galactinol in water was unaffected by treatment with invertase, Clerget acid, or emulsin which contained no α-galactosidase, as shown by failure to hydrolyze raffinose. Crystalline galactinol (2.005 g.) was dissolved in water in a 100-ml. flask, made to volume at 20° and read in a 400-mm. tube in a saccharimeter; observed reading: +31.45°V., equivalent to 225.3°V. for 26 g. of anhydride read in a 200 mm. tube. To a 50-ml. aliquot of this solution, in a 100-ml. flask, was added 5.0 ml. of solution of invertase containing melibiase. After 48 hr., at room temperature, the solution was made to volume at 20° and read in a 400-mm. tube at 20.0°; observed reading, corrected for enzyme: +4.37°V.; theoretical for galactose at $[\alpha]^{20}D$ 79.7°[12]; +4.42°V.

Reducing sugar in the hydrolyzed solution was determined by titration of 10.0 ml. of mixed Quisumbing and Thomas[13] reagents using the Lane and Eynon[14] technique. Standard tables do not exist for boiling procedures at the elevation of Denver, Colo. A table of factors for the above procedure was established. After correcting for the blank, the hydrolyzed solution showed reducing power equivalent to 240.9 mg. galactose per 100 ml.; theoretical 238.6 mg./100 ml.

Preparation of the Osazone.—Crystalline galactinol (2.0 g.) was hydrolyzed as above. Inositol was precipitated from the hydrolyzed solution with ammoniacal lead acetate solution. The precipitate was filtered and washed with

(9) A.O.A.C., Methods of Analysis, 7th Edition, Sec. 29.21, p. 500.

(10) A.O.A.C., Methods of Analysis, 7th Edition, Sec. 29.24, p. 503.

(11) R. L. Whistler and D. F. Durso, THIS JOURNAL, **73**, 4189 (1951).

(12) C. A. Browne, "Handbook of Sugar Analysis," 1st edition John Wiley and Sons, New York, 1912, p. 177.

(13) A.O.A.C., Methods of Analysis, 7th Edition, Sec. 29.46, p. 511.,

(14) A.O.A.C., Methods of Analysis, 7th Edition, Sec. 29.33–29.34, p. 506.

dilute ammoniacal lead acetate solution. Filtrate and washings were freed from lead with H_2S and concentrated to 16 ml. Phenylhydrazine hydrochloride (1.6 g.) and sodium acetate (2.4 g.) were added and the solution immersed in a boiling water-bath. Crystalline galactose osazone appeared in 17 min. The crystals were separated and recrystallized from 50% ethanol; m.p. 186° (uncor.), mixed m.p., with authentic specimen of galactose phenylosazone 185° (uncor.).

Paper Chromatography.—The hydrolyzed galactinol solution was run in both pyridine and collidine solvents. Reactions to both solvents were similar. α-Naphthol reagent showed no ketoses present. P.D.A. reagent showed a single aldose present, in the galactose location. A.S.N. reagent showed two spots, the second being inositol.

Identification of the Inositol Moiety of Galactinol.—The lead precipitate containing inositol, described above, was freed from lead with H_2S. The filtrate from the lead sulfide was concentrated, and inositol was crystallized from ethanol solution; m.p. 220–222° (uncor.), mixed m.p. with authentic specimen of inositol 220–222° (uncor.); microbiological analysis, 100% inositol.[15] Scherer's[16] test for cyclitols applied to the crystals gave a positive reaction.

Paper Chromatography.—A solution of the crystals run in both pyridine and collidine solvents gave single spots, at the inositol area, which reacted only to ammoniacal silver nitrate.

Anal. Calcd. for $C_6H_{12}O_6$: C, 39.98; H, 6.72. Found: C, 39.85; H, 6.67.[17]

Galactinol Dihydrate.—Galactinol was proved to be a compound containing α-D-galactose and *myo*-inositol. The molecular weight was determined by the freezing point depression of a solution containing 1.890 g. of crystalline galactinol in 10.0 g. of H_2O. The freezing point was depressed 0.906°, corresponding to mol. wt. 388, in the disaccharide range.

Crystalline galactinol (0.2997 g.) heated *in vacuo* at 90° for 16 hr. lost 0.0287 g. equivalent to 9.57% H_2O; theoretical for $C_{12}H_{22}O_{11}\cdot 2H_2O$: 9.52%.

Anal. Calcd. for $C_{12}H_{22}O_{11}\cdot 2H_2O$: C, 38.07; H, 6.93; O, 54.99. Found: C, 38.19; H, 6.99; O (by analysis), 54.85.[17]

These data proved galactinol to be a molecule of 378 mol. wt., containing D-galactose and *myo*-inositol, and crystallizing as a dihydrate. Hydrolysis with melibiase indicates the α-D-galactopyranosyl configuration. Therefore, modern terminology assigns the descriptive name O-α-D-galactopyranosyl-*myo*-inositol.

Some physical properties of galactinol which have been determined are given in Table II.

TABLE II

PHYSICAL PROPERTIES OF GALACTINOL DIHYDRATE

Melting point, open tube, heated slowly:	220–222° (uncor.)
Closed tube:	113–114° (uncor.)
$[\alpha]^{20}D$, estimated by saccharimeter, 2% soln. in H_2O	*ca.* +135.6°
Solubility in H_2O at 20°, by refractometer:	satd. soln. shows 49.9% as anhydride
Mutarotation:	none

myo-**Inositol.**—Fraction No. 1, from the carbon column was concentrated *in vacuo* and treated with ethanol for crystallization. No extensive tests for identification of the crystals were made, it being considered sufficient to accept the evidence of various qualitative tests. The crystals gave the same reactions in paper chromatography as given by the *myo*-inositol recovered from hydrolyzed galactinol, and by authentic inositol. Scherer's test[16] gave a positive reaction for cyclitol; m.p. 219° (uncor.), mixed m.p. with authentic specimen 219° (uncor.).

DENVER, COLORADO

(15) Analysis made by the Laboratory of Vitamin Technology of Chicago, Illinois, by the yeast growth method of L. Atkin, A. S. Schultz, W. L. Williams and C. N. Frey, *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

(16) C. A. Browne, "Handbook of Sugar Analysis," ref. 12, p. 758.

(17) Analysis by Huffman Microanalytical Laboratories of Denver, Colorado.

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Synthesis of Phenyl β-D-Glucopyruronoside[1]

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Phenyl β-D-glucopyruronoside was synthesized by the catalytic oxidation of phenyl β-D-glucopyranoside in the presence of platinum black. Its identity to the natural product was shown by its physical properties and its hydrolysis by β-D-glucuronidase. A previously reported synthesis by Neuberg and Neimann is discussed.

Phenyl β-D-glucopyruronoside (I) was first isolated from urine by Külz[2] in 1890, and although it has since continued to be of biological interest as a detoxification product of phenol,[3] only recently has it been properly characterized (m.p. 161–162°, $[\alpha]^{23}D$ −90.5°).[4] Its synthesis was claimed by Neuberg and Neimann[5] from phenol and acetobromoglucurone in the presence of alkali (m.p. 150–151°, $[\alpha]^{17}D$ −83.3°, solvent not mentioned). The product was compared with I isolated from the urine of a sheep (m.p. 148–150°),[6] but no mixed melting point with the synthetic product was given. Both Goebel and Babers[7] and we were unable to repeat the Neuberg and Neimann synthesis.

In the course of developing a synthesis for 2-naphthyl β-D-glucopyruronoside[8] from 2-naphthyl β-D-glucopyranoside by the catalytic oxidation first described by Fernandez-Garcia, *et al.*,[9] we were able to prepare I by the same procedure from phenyl β-D-glucopyranoside. Marsh, however, reported[10] that he was unable to prepare I by this

(1) This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, Federal Security Agency and by an Institutional Grant to Harvard University from the American Cancer Society.

(2) E. Külz, *Z. Biol.*, **27**, 248 (1890).

(3) N. E. Artz and E. M. Osman, "Biochemistry of Glucuronic Acid," Academic Press, Inc., New York, N. Y., 1950, p. 39.

(4) G. A. Garton, D. Robinson and R. T. Williams, *Biochem. J.*, **45**, 65 (1949).

(5) C. Neuberg and W. Neimann, *Z. physiol. Chem.*, **44**, 114 (1905).

(6) E. Salkowski and C. Neuberg, *Biochem. Z.*, **2**, 307 (1906).

(7) W. F. Goebel and F. H. Babers, *J. Biol. Chem.*, **100**, 743 (1933).

(8) K.-C. Tsou and A. M. Seligman, THIS JOURNAL, **74**, 5605 (1952); presented before the Organic Division at the 122nd National Meeting of the American Chemical Society, September 14, 1952.

(9) R. Fernandez-Garcia, L. Amores, H. Blay, E. Santiago, H. Soltero-Diaz and A. A. Colon, *El. Crisol.*, **4**, 40 (1950).

(10) C. A. Marsh, *J. Chem. Soc.*, 1578 (1952).