[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF NEBRASKA]

Structural Characterization of Products of Enzymatic Disproportionation of Lactose[1]

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Three oligosaccharides produced from lactose by an enzyme of *S. fragilis* have been shown to be disaccharides with the structures 3-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-glucose and 6-O-β-D-galactopyranosyl-D-galactose. Specific rotations for the oligosaccharides and melting points and X-ray diffraction data for the crystalline osazones are recorded. The lead tetraacetate oxidation procedure was used for establishing the position of the glycosidic linkage in the oligosaccharides.

In a previous communication[2] studies were reported pointing to the constitution of the galactosyl oligosaccharides produced enzymatically from lactose. The evidence at that time indicated that two of the oligosaccharides were disaccharides with the probable structure 6-O-β-D-galactopyranosyl-D-glucose and 6-O-β-D-galactopyranosyl-D-galactose. Further evidence for the proposed structures has now been obtained and is presented in this paper. In addition, methods for the preparation and structural characterization of a new galactosyl disaccharide, 3-O-β-D-galactopyranosyl-D-glucose, are described.

That enzyme preparations from yeasts and bacteria convert lactose into new oligosaccharides has been observed in several laboratories.[3-5] In a recent paper Roberts and Pettinati[6] have reported that eleven new oligosaccharides were present in a digest of lactose with an enzyme from *Saccharomyces fragilis*. In our experiments an enzyme preparation from *S. fragilis* also has been used. The oligosaccharides were isolated from the enzymatic digest by column[7,8] and paper chromatography.[9]

In Table I R_f values for the pure compounds and for the compounds present in the original digest are recorded. According to the relationship of paper chromatogram mobility and carbohydrate structure developed by French and Wild,[10] the R_f values of compounds I, II and III are typical of disaccharides, while the R_f values of compounds IV and V are typical of trisaccharides. On the basis of the chromatographic evidence, oligosaccharides 7, 5, 4, 3, 2 observed by Roberts and Pettinati[6] should prove to be identical with compounds I, II, III, IV and V, respectively.

Compound I is a reducing compound composed of glucose and galactose. The specific rotation of the amorphous but chromatographically pure compound is +28°. Prolonged acid hydrolysis converted the compound quantitatively to glucose and galactose in equal molar concentrations, while partial acid hydrolysis yielded glucose, galactose and unhydrolyzed compound. Since galactose was the reducing component in an acid hydrolysate of the aldonic acid of compound I, the glucose is the monosaccharide moiety with the reducing group of the original disaccharide. Lead tetraacetate oxidation of the compound as outlined by Perlin[11] required one mole of lead tetraacetate per mole of compound. In the hydrolysate of the oxidized product, arabinose and galactose were identified by paper chromatographic methods and specific colorimetric tests. This lead tetraacetate oxidation pattern is characteristic of 1,3-linked disaccharides.[11] The low specific rotation and the stereospecificity of enzymatic transglycosylations[9,12,13] strongly suggest that the linkage in the compound is the β-configuration. The phenylosazone derivative of compound I was prepared in crystalline form and melting point and X-ray diffraction data are recorded in the Experimental section. On the basis of the evidence presented, the structure for the compound appears to be 3-O-β-D-galactopyranosyl-D-glucose.

TABLE I

APPARENT R_f VALUES OF OLIGOSACCHARIDES

Compound	Individually	Digest
Glucose	0.88	0.87
Galactose	.82	.80
Compound I	.67	.65
Lactose	.52	.51
Compound II	.44	.42
Compound III	.34	.34
Compound IV	.23	.23
Compound V	.12	.13

Compound II moved on paper at a slower rate than lactose. Acid hydrolysis of the compound yielded glucose and galactose in equal molar concentrations, while acid hydrolysis of the aldonic acid yielded galactose as the reducing product. Chromatographically pure compound II exhibited a specific rotation of +25°. This value is in agreement with the values reported for the specific rotation of allolactose (6-O-β-D-galactopyranosyl-D-glucose).[14,15] In the lead tetraacetate oxidation procedure the compound consumed 3 moles of lead tetraacetate per mole. Examination of the acid hydrolysate of the oxidized product showed that

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galactose was the monosaccharide unit resistant to oxidation. These oxidation data are consistent with a 1,6-linked disaccharide. The melting point of the crystalline phenylosazone of the compound is in agreement with the value reported for the osazone of allolactose (6-O-β-D-galactopyranosyl-D-glucose),[15] and the X-ray diffraction data on the phenylosazone are recorded in the Experimental section. All the structural evidence substantiates the previously proposed structure, 6-O-β-D-galactopyranosyl-D-glucose, for compound II.

Compound III was the slowest moving of the galactosyl disaccharides thus far examined. Several lines of evidence indicate that compound III is probably galactobiose (6-O-β-D-galactopyranosyl-D-galactose.)[16-18] The rotation of compound III is +31° and is in close agreement with the literature value of +34°[17,18]; acid hydrolysis of compound III converts it quantitatively to galactose; the behavior of the compound on lead tetraacetate oxidation is characteristic of a 1,6-linked disaccharide; the crystalline phenylosazone of compound III melted with decomposition at 188–190°, a value recently reported by Weinland[18] for the phenylosazone of galactobiose. These observations are consistent with the structure 6-O-β-D-galactopyranosyl-D-galactose suggested for compound III.

Experimental

Isolation of the Oligosaccharides.—To a solution of 20 g. of C.P. lactose in 100 ml. of water was added 100 ml. of 4% enzyme solution.[19] The mixture was covered with toluene and allowed to stand at room temperature for 4 hours. The water layer was separated, heated at 100° for 5 minutes and concentrated by vacuum distillation to 50 ml. The carbohydrates in the solution were adsorbed on Celite and charcoal mixture[20] (1:1) and then eluted by washing with ethanol–water mixtures containing increasing amounts of alcohol; 500-ml. eluates of 0, 2.5, 5, 7.5, 10, 12.5 and 15% alcohol were collected and were concentrated by vacuum distillation to about 10 ml. Qualitative chromatograms showed that fractions with 0 and 2.5% alcohol were enriched in monosaccharide components; fractions with 5, 7.5 and 10% alcohol were enriched with the disaccharide components (compounds I, II, III and lactose); and fractions with 12.5 and 15% alcohol with the trisaccharides (compounds IV and V). The compounds in the 5, 7.5 and 10% eluates were separated on paper chromatograms as previously described.[9] The solution of compound I contained a small amount of sucrose present originally in the enzyme preparation. Since sucrose possesses an R_f value similar to compound I, it is difficult to eliminate by chromatography. Hydrolysis of the solution in 0.1 *N* hydrochloric acid for 1 hour at 80° converted the sucrose to glucose and fructose which were separated from compound I by paper chromatography. Compounds II and III possess very similar R_f values and required further purification by chromatography on paper. Finally the compounds were taken to dryness in a vacuum oven; yields 0.2 g. of compound I, 0.7 g. of compound II and 0.5 g. of compound III; specific rotations +28° for compound I, +25° for compound II and +31° for compound III.

Acid Hydrolysis of the Oligosaccharides.—From 5 to 10 micromoles of pure oligosaccharides in 0.2 ml. of water were mixed with 0.2 ml. of 0.2 *N* hydrochloric acid and heated at 100° for 6 hours in a tightly stoppered tube. Aliquots of 0.1 ml. of the cooled hydrolysates were placed on chromatograms and developed as previously described.[21] Marker strips were used for locating the glucose and galactose on the paper and the monosaccharides were extracted from the unsprayed portions of the chromatogram with 2 ml. of water. Aliquots of the solution were used for the quantitative determination of glucose and galactose by the diphenylamine procedure.[21] The results were as follows: 5.1 micromoles of compound I yielded 5.0 micromoles of glucose and 4.9 micromoles of galactose; 7.0 micromoles of compound II yielded 6.9 micromoles of glucose and 6.4 micromoles of galactose; and 9.5 micromoles of compound III yielded 18.6 micromoles of galactose.

Preparation and Acid Hydrolysis of Aldonic Acids of the Oligosaccharides.—A sample of 5 mg. of the oligosaccharide was dissolved in 0.2 ml. of 0.1 *N* sodium hydroxide and shaken with a small amount of crystalline iodine for 2 hours. The oxidized mixture was acidified by addition of 0.2 ml. of 0.5 *N* hydrochloric acid and heated at 100° for 3 hours in a sealed tube. Paper chromatographic examination of the hydrolysate of compound I showed that a reducing monosaccharide with an R_f value identical to that of galactose was produced. Galactose was similarly identified in the hydrolysates of the aldonic acids of compounds II and III. These results establish glucose as the reducing moiety in compounds I and II and substantiate the disaccharide structure for all three oligosaccharides.

Preparation of the Osazones.—A mixture of 0.1 g. of oligosaccharide, 0.2 g. of phenylhydrazine hydrochloride and 0.4 g. of sodium acetate in 2.0 ml. of water was heated in a boiling water-bath for 0.5 hour. The osazones of compounds I, II and III that precipitated from the solution were collected and dried on a filter, and finally crystallized from 1 ml. of ethyl alcohol; melting points for the osazone of compound I, 176°; for the osazone of compound II, 186–188°; literature[15] value 188–189°; and for the osazone of compound III 188–190°; literature values 207°[17] and 188–189°.[18] The X-ray diffraction data[22] on the osazones were as follows: osazone of compound I, 8.83[23]–50,[24] 8.10–40, 6.60–20, 5.60–50, 5.24–50, 4.92–40, 4.74–100, 4.45–70, 4.26–40, 4.09–40, 3.89–40, 3.72–80, 3.49–90, 3.23–60, 3.07–10, 2.93–10, 2.73–20, 2.52–10; osazone of compound II (allolactose), 6.31–40, 5.56–40, 5.30–40, 4.87–60, 4.61–60, 4.30–80, 4.11–80, 3.79–100, 3.54–20, 3.32–40, 3.15–100, 2.96–30, 2.81–30, 2.62–70, 2.46–20; for the osazone of compound III (galactobiose), 8.57–20, 7.18–30, 5.63–30, 4.69–100, 4.35–80, 3.98–80, 3.58–60, 3.32–10, 3.10–5.

Anal. Calcd. for $C_{24}H_{32}N_4O_8$: C, 55.38; H, 6.20; N, 10.77. Found: Osazone of comp. I: C, 55.02; H, 6.53; N, 10.48. Osazone of comp. II: C, 54.46; H, 6.17; N, 10.64. Osazone of comp. III: C, 54.75; H, 6.26; N, 10.71.

Lead Tetraacetate Oxidation.—To 2 mg. of oligosaccharide in 0.02 ml. of water was added 0.8 ml. of lead tetraacetate reagent (10 mg. of lead tetraacetate in glacial acetic acid) and the reaction was allowed to proceed at 27° for 2

TABLE II

LEAD TETRAACETATE OXIDATION OF OLIGOSACCHARIDES

Compound	Total moles of lead tetraacetate consumed per mole of compound 15 min.	50 min.	Consumed by reducing moiety[a] 15 min.	50 min.	Expected value[b]
Compound I	2.2	2.8	1.1	1.1	1
Lactose	3.0	3.8	1.9	2.1	2
Compound II	3.6	4.6	2.5	2.9	3
Compound III	3.7	4.7	2.6	3.0	3
Melibiose	4.0	4.9	2.9	3.2	3

[a] Determined by subtraction of values of 1.1 for the 15 min. and 1.7 for the 50 min. from the total consumption. The 1.1 and 1.7 were the moles of lead tetraacetate consumed per mole of methyl α-D-galactoside or *p*-nitrophenyl β-D-galactoside. [b] Calculated for a 1,3-, 1,4- and 1,6-linkage in the compounds.

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(19) The enzyme preparation of *S. fragilis* was kindly provided by Dr. W. M. Connors, National Dairy Research Laboratories, Long Island, N. Y.

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(21) J. H. Pazur, *J. Biol. Chem.*, **205**, 75 (1953).

(22) The X-ray patterns were obtained at the Instrumentation Laboratory of the University of Nebraska.

(23) Interplanar spacings, Å., CuK_α radiations.

(24) Relative intensities on basis of 100 for the strongest line.

hours. The oxidation was stopped by addition of 0.4 ml. of oxalic acid solution (10% in glacial acetic). The reaction mixture was filtered and the filtrate was concentrated by vacuum distillation to approximately 0.1 ml. Next 0.1 ml. of 0.1 *N* hydrochloric acid was added and hydrolysis was effected at 100° for 3 hours. Paper chromatograms of the hydrolysate and reference materials were prepared and sprayed with aniline oxalate reagent[25] for locating pentoses and with copper sulfate and molybdic acid reagents[9] for locating the reducing compounds. The apparent R_f values (2 ascents of solvent) of the carbohydrates in the hydrolysate of compound I and reference compounds were: arabinose, 0.74; pentose in hydrolysate, 0.74; glucose, 0.68; galactose, 0.61; other reducing product in the hydrolysate 0.61. From the oxidized and hydrolyzed compounds II and III, reducing products with R_f values of 0.61 and 0.62 were produced; R_f value of galactose under the same conditions was 0.61.

Other samples of the oligosaccharide (0.4 to 0.8 mg.) were oxidized with lead tetraacetate in the Warburg apparatus as outlined by Perlin.[11] Lead tetraacetate consumption was determined by an iodometric procedure and formic acid production was measured manometrically by its conversion to carbon dioxide. Values for the oligosaccharides at 15 to 50 minute reaction periods are recorded in Table II. These values have been corrected for the contribution of the galactosyl moiety to the reaction by determining the lead tetraacetate consumption for methyl α-D-galactoside and *p*-nitrophenyl β-D-galactoside.

LINCOLN, NEBRASKA

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[CONTRIBUTION FROM THE CANCER RESEARCH AND CANCER CONTROL UNIT OF THE DEPARTMENT OF SURGERY, AND THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, TUFTS UNIVERSITY SCHOOL OF MEDICINE]

The Presence of Maltose, Maltotriose and Maltotetraose in Liver[1]

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In the course of a search for hepatic glucosiduronic acids, material was isolated from fresh rat liver which had the physical and chemical properties of a mixture of oligosaccharides. This mixture was separated into three major components and a number (5–8) of minor constituents by a combination of column and paper chromatographic techniques. The three major components were positively identified as maltose, maltotriose and maltotetraose and the remaining components have been described as members of an ascending homologous series of glucosyl oligosaccharides. The product of acid hydrolysis of the original mixture was isolated and identified as glucose.

Originally, we were conducting a search for physiologically produced glucosiduronic acids in liver as an extension of studies on glucuronylation of steroids[2,3] and on enzyme-catalyzed glucuronyl transfer.[4,5] In this connection, it was found that glucosiduronic acids, *e.g.*, 8-hydroxyquinoline glucosiduronic acid, could be adsorbed from aqueous solution on charcoal and also could be readily eluted from this material by hot alcohol. When this charcoal treatment was applied to zinc hydroxide filtrates[4] of liver homogenate, a creamy-white solid ("X") was obtained whose amount exceeded by far the weight expected from analytical values[6] for glucosiduronic acid.

This was the point of departure of the present investigation which has now culminated in the isolation and identification of the main components of "X" as a homologous series of glucosyl oligosaccharides.

Experimental

Isolation of Liver Oligosaccharides.—The liver of a freshly-killed rat was cooled immediately in ice. An aqueous homogenate (*i.e.*, 1 g. per 10 ml. of ice-cold H_2O) was prepared by means of a chilled glass homogenizer (one-half minute). This mixture was then deproteinized by adding to each 10 ml. of homogenate first 1 ml. of $ZnSO_4$ (10%) and then dropwise 1 ml. of NaOH (0.5 *N*) solution with intermittent agitation. After filtration, the protein-free filtrate was treated with charcoal[7] (1.5 g. per gram of original liver tissue) for at least 30 minutes with intermittent stirring. The charcoal was removed by filtration and was washed repeatedly with water (250–500 ml., total volume). Elution was done with at least 100 ml. of hot ethyl alcohol. Evaporation of the solvent yielded a dry creamy-white solid ("X"). Since "X" was completely soluble in alcohol and it dialyzed readily through a cellophane membrane, it was clearly not glycogen. Its aqueous solutions exhibited reducing power (which increased substantially upon acid hydrolysis) and instability to alkali; optical rotation was $[\alpha]^{24}D$ +134.3° (H_2O).[8] Purified "X" acetate contained (%) C, 49.17; H, 5.59; O, 45.25; acetyl, 46.7; $[\alpha]^{24}D$ +116.5° ($CHCl_3$); mol. wt. (Rast), 779. Tests for nitrogen and phosphorus were negative.

Separation of these oligosaccharides was effected by means of both column and paper chromatography. Thus, 150 mg. of "X" was placed on a 2 × 18 cm. charcoal:Celite column prewashed with the elution mixture and water. First, 250 ml. of H_2O was passed through and discarded. Then, the material was eluted with an ethyl acetate–alcohol–water (3:1:1) mixture and 5-ml. fractions were collected. These were analyzed for hexose.[9] As indicated in Fig. 1, the plot of sugar *versus* tube number indicated three peaks. The contents of the tube corresponding to each peak was examined by descending paper chromatography (Whatman no. 1, solvent system 4 (Table I), ammoniacal silver nitrate[10]). The fastest moving spot (A) was the first to be eluted from the column. "B" and "C" travelled on paper, also in order of their elution from charcoal. Upon prolonged paper chromatography of "X" it was possible to observe 9 spots (Fig. 2) and the $\log_{10}(1/Rf - 1)$ of these spots was plotted as a function of hexose units per molecule (Fig. 3).

(1) These data were presented on December 8, 1956 at the Ninth Annual Meeting of Scientific Advisors, Harvard Club, Boston, and at the Annual Meeting of the American Society for Biological Chemists, April 19, 1957, Chicago, Illinois (W. H. Fishman and H.-G. Sie, *Federation Proc.*).

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(7) Activated charcoal (Howe and French, Inc., Boston) was previously washed with copious amounts of hot alcohol and water. No oligosaccharides were found in the alcohol eluate of the charcoal before it was employed in this study.

(8) We wish to thank Dr. R. J. Jeanloz of the Massachusetts General Hospital for extending the hospitality of his laboratory to us for measurements of optical rotation. His interest in this work is appreciated.

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