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ISOLATION AND CHARACTERIZATION OF D-Arabino-HEXOSULOSE-CONTAINING OLIGOSACCHARIDES DERIVED FROM LACTOSE BY BORATE ION-EXCHANGE CHROMATOGRAPHY

A DISSERTATION
SUBMITTED TO THE GRADUATE FACULTY
in partial fulfillment of the requirements for the
degree of
DOCTOR OF PHILOSOPHY

by

BETTY JANE NEUKOM WHITE

Oklahoma City, Oklahoma

1972

ISOLATION AND CHARACTERIZATION OF D-Arabino-HEXOSULOSE-CONTAINING OLIGOSACCHARIDES DERIVED FROM LACTOSE BY BORATE ION-EXCHANGE CHROMATOGRAPHY

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Roger

ACKNOWLEDGMENTS

I wish to express my sincere appreciation and thanks to Dr. Raul Carubelli not only for his invaluable guidance, advice, and help during the course of this investigation, but also for his quiet patience and personal understanding throughout.

I would like to thank the Biochemistry Section of the Oklahoma Medical Research Foundation for the use of research facilities to conduct these experiments and to personnel who were so gracious with their help.

Special thanks go to Mr. Melvin Miller who provided assistance especially in the early stages of this research.

Appreciation is extended to Dr. Martin Griffin who helped both in the design and performance of the HeLa cell studies.

I am grateful to Dr. Paul B. McCay for his help in the use of gas chromatography as a tool in this study and also as a member of the Reading Committee. Thanks are extended to other members of this committee--Dr. Albert Chandler, Dr. Wallace Friedberg and Dr. Allan Stanley.

And, finally, I would like to thank Dr. John A. Schilling for his unfailing support and encouragement to undertake and finish this endeavor.

This study was supported in part by Contract No. DA-49-193-MD-2811 from the U.S. Army Medical Research and Development Command and by U.S. Public Health Service grant NS 09176 from the National Institutes of Health.

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CHAPTER I

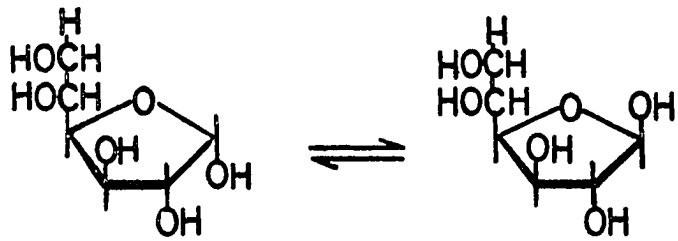
INTRODUCTION

The fractionation of neutral sugars by ion-exchange column chromatography is complicated by the fact that these compounds are very weak electrolytes and, therefore, have very little tendency to react with ion-exchangers.

Borate-Sugar Complexes

One solution to this problem, involving the non-charged nature of sugars, was provided by the use of the Böeseken complexes. This Dutch chemist conducted a thorough study of the complexes formed by sugars with the borate ion (from boric acid or its salts). These sugar-borate complexes are negatively charged due to the unpaired electron on the boron atom. It was known as far back as 1874 that the conductivity of boric acid solutions was influenced by polyalcohols. Böeseken began his study of this phenomenon in 1911 whereby he systematically examined the conductivity of solutions of polyhydroxy compounds both in water and in 0.5 M boric acid. From these studies he concluded (1) that the formation of sugar-borate complexes depends particularly on the favorable position of adjacent hydroxyl groups. In studies of five membered ring systems, which have a planar configuration, he found that adjacent hydroxyls

in cis position are favorable for the formation of borate complexes. It was later discovered that mutarotation of sugars had a profound effect on the formation of borate complexes. When his investigations began, the furanose structure of sugars was the assumed structure for monosaccharides having 4 or more carbon atoms. The furanose structures for α-and β-D-glucose under mutarotating equilibrium conditions, written now according to Haworth structures, would be as shown:



I II

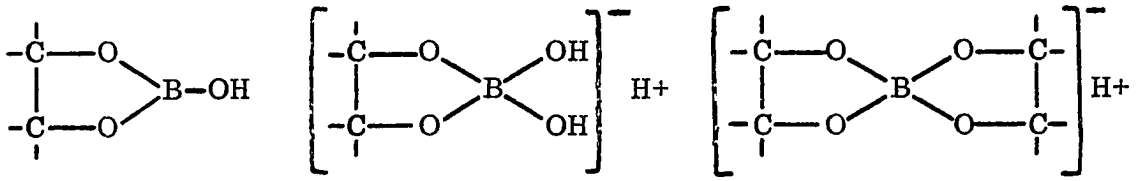
From measurements of conductivity and optical rotation, it was found that α-D-glucofuranose (I) enhanced conductivity of boric acid while β-D-glucofuranose (II) did not. He attributed this phenomenon to the cis and trans configurations of the hydroxyl groups on C_1 and C_2 of the α- and β-D-glucose respectively. This simple interpretation, however, is not fully acceptable now since it does not take into account our present knowledge of sugar mutarotation. According to the hypothesis of Isbell et al. (2) during mutarotation the ring opens for a moment and a pseudo-acyclic intermediate is formed which has a conformation very similar to the parent sugar. This intermediate passes through transition states via other intermediates to get to pyranoses, furanoses and other products as follows:

$$\alpha\text{-pyranose} \rightleftharpoons \mu\alpha p \rightleftharpoons \mu\beta p \rightleftharpoons \beta\text{-pyranose}$$

$$\alpha\text{-furanose} \rightleftharpoons \mu\alpha f \rightleftharpoons \mu\beta f \rightleftharpoons \beta\text{-furanose}$$

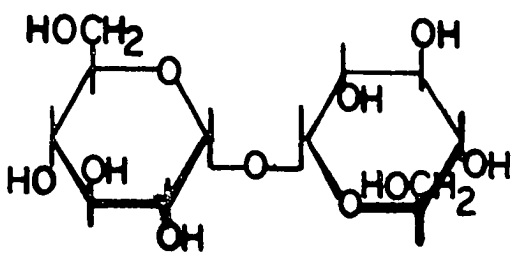
equilibrium with other acyclic and ring modifications

It is obvious that mutarotation with furanose-pyranose interconversions involving various intermediates is very important in the formation of sugar-borate complexes. Böeseken further emphasized that the furanose isomer was the most favorable configuration for complex formation. Three types of borate-sugar complexes have been proposed (3) for the interaction between polyols and boric acid as a result of the measurement of changes in optical rotation, conductivity, acidity, freezing point and solubility.



I II III

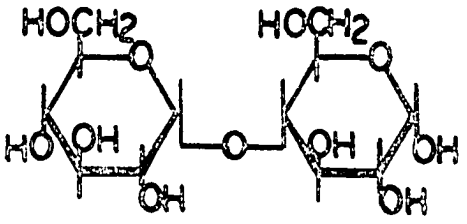
The exact nature of these sugar-borate complexes in aqueous solutions still remains somewhat uncertain but, in specific instances, probable structural forms can be predicted on the basis of the present theory. Evidence shows that some sugars can form all three types of complexes, however, an equilibrium of these types is formed which is dependent not only on pH, but also on the ratio of sugar to borate and absolute concentration of the sugar itself. Type II complexes are said to predominate in dilute solutions of sugars containing large quantities of borate. Some disaccharides form borate-diol complexes of type I which are only slightly ionized. An example is the non-reducing sugar, trehalose, which has no adjacent <u>cis</u>-hydroxyl.



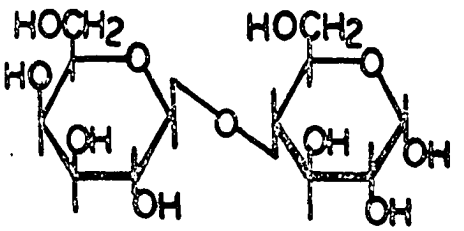
trehalose

Lactose and maltose are reducing sugars which have the possibility of adjacent <u>cis</u> hydroxyl groups by mutarotation, but in lactose there is an additional pair

of adjacent cis hydroxyl groups on the galactosyl moiety. Maltose and lactose form borate-diol compounds represented by structures II or III.



maltose



lactose

Borate Ion-Exchange Chromatography

Variation in Operating Conditions

The general application of borate-sugar complexes in the fractionation of sugar mixtures has proved to be of great value in carbohydrate chemistry. In recent years the chromatographic separation of sugars has even achieved the sophistication, precision, and automation that characterizes amino acid analysis, but not enough attention has been paid to defining the conditions under which a given separation can be made. There has been considerable variation in experimental conditions as the borate ion-exchange methodology evolved and the implications of these variable conditions need to be considered in detail because they affect not only the efficiency of the separation but also the stability of the sugars themselves.

The separation of sugars by borate ion-exchange chromatography was introduced by Khym and Zill in 1952 (4). They used the strong base anion exchanger, Dowex-1, in the borate form. Sugars were dissolved in dilute $K_2B_4O_7$ solutions (0.005 M) and eluted with stepwise increases in $K_2B_4O_7$ concentration. A relatively large volume of eluent was required to elute the sugars which, at the flow rates utilized, took several days to complete. The recoveries of sugars from the column were reported to be essentially quantitative. They later (5) extended their work to include, in addition to hexoses, the separation of related

compounds (hexitols, heptoses, etc.). The operating temperature was not mentioned specifically and hence it was assumed to be room temperature while the pH of the eluting solutions varied from 8 to 9. As expected, it was found that the greater the number of charges, the more tightly the sugar complex was bound to the resin, requiring proportionately greater concentrations of $K_2B_4O_7$ for elution. The more highly ionized disaccharides, maltose and lactose, showed greater affinity for the exchanger than the weakly ionized trehalose. However the affinity of disaccharides such as lactose and maltose for the ion-exchange resin was much less than that observed with monosaccharides which could pass into furanose forms. One exception was the disaccharide melibiose, which has a (1⟶6) linkage and can pass into a furanose isomer. This disaccharide was so tightly bound by the exchanger that it was eluted after glucose, the most tightly bound and last sugar to be eluted. These comparisons show that as the various sugar-borate complexes move down the ion-exchange columns, equilibria are established not only with the exchanger but also with the various forms of the sugar present under the existing operating conditions.

In 1966 Green (6) reported an improved, automated method. By increasing the temperature to 50°C the resolution was enhanced and the operating time was reduced from 60 hours to 12 hours. The resin was also improved by reducing the particle size and increasing the uniformity in shape.

In 1967 Kesler (7) compared 3 resin types — Dowex 1-X8, Ag 1-X8 (a more purified and sized Dowex 1-X8), and the Technicon resin which was quite uniform in particle size. Better resolution was achieved with more pure and uniformly sized resins. The operating temperature reported was 53°C, but the operating time was reduced to 6 hours. The pH varied from 7-10 and the borate concentration was 0.2-0.6 M. A similar method was reported in the same year by Ohms et al. (8) using a custom made, strongly basic anion-exchange resin of highly uniform spheres with a nominal 8% divinyl benzene cross-linking. This group separated 12 neutral sugars in 7.5 hours using a pH of 8.9 and a temperature of 50°C.

In 1969, and again in 1971, the group of Lee et al. (9, 10) reported another automated method of sugar separation and quantitation involving borate ion-exchange chromatography with Chromobeads S, a low-pressure type from Technicon, Inc. This method was based on Kesler's system but was faster and more suitable for routine analysis for neutral sugars especially those from glycoproteins. In this method the elution time for glucose was 5 hours. They claim the low alkalinity (pH 7-10) and short time of exposure of sugars to alkaline buffers explain the good recoveries stated to be 91-100%.

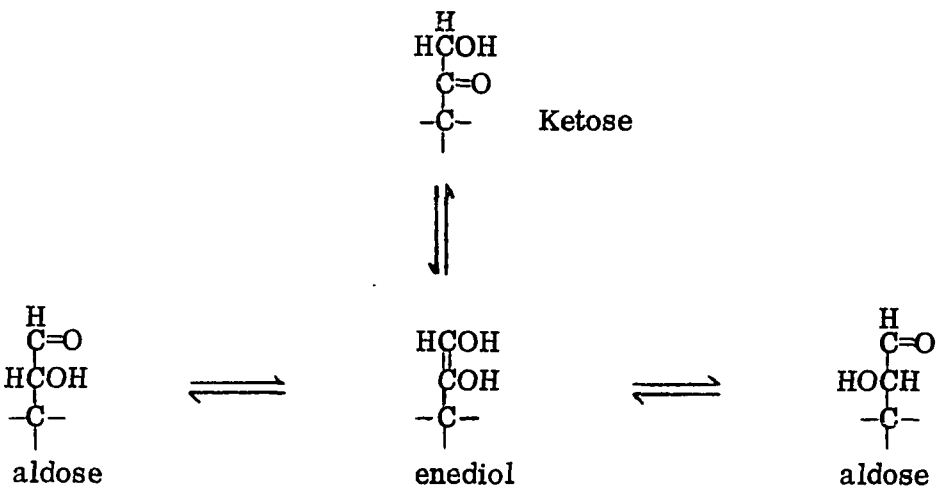
Perhaps the first method to take into account the possible detrimental effects of combined use of alkaline conditions and elevated temperatures was that of Walborg in 1965 (11). His method employed a boric acid-glycerol buffer of pH 6.8 with an operating temperature of 50°C. His was the first paper to mention recoveries which ranged from 91 to 100%. The original technique employed Dowex 2-X8 from Fluka AG, Buchs SG, Switzerland, but a more recent and improved method in 1970 (12) used Aminex A-14, a 4% cross-linked resin, of uniform spherical size. The flow rate was increased from 3 to 160 ml per hour.

Operating Conditions vs. Stability of Neutral Sugars

The foregoing has demonstrated that during the operating of borate ion-exchange columns neutral sugars can be subjected to a variety of conditions. There have been variations in type, purity, sizing and shape of resins; in concentration and pH of buffers (mostly alkaline); in temperature (mostly elevated above room temperature); and in length of time of exposure to such conditions. All these conditions can affect the sugars being analyzed in several ways.

Although reducing sugars exhibit moderate stability to acids at room temperature, they are profoundly affected by alkalies even under mild conditions. The changes under alkaline conditions usually take place in one or more of the following ways (13): isomerizations at the reducing end, fragmentation, internal oxidations and reduction, and even polymerization under certain conditions.

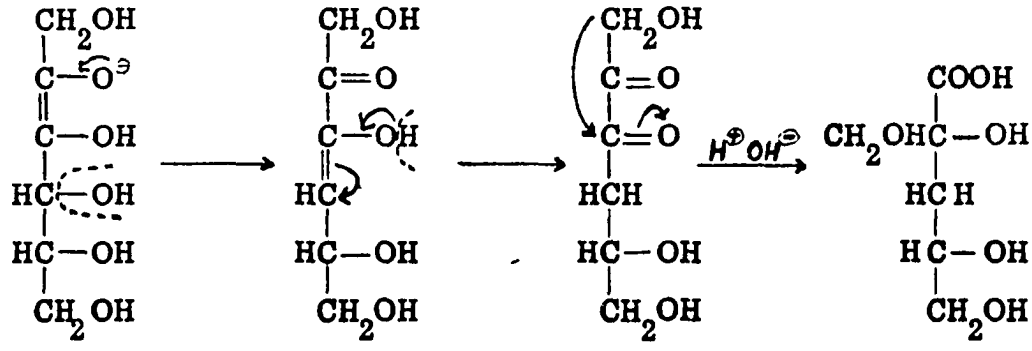
Isomerizations. The simplest isomerization reaction of reducing sugars is the classical Lobry de Bruyn-Alberda van Ekenstein transformation (14). For example, when glucose is subjected to alkaline conditions at room temperature, fructose and mannose can be separated from the products of the reaction. The mechanism of this reaction involves the base catalyzed removal of a proton which leads to the formation of an intermediate enediol compound.



The asymmetry of carbon atom 2 is thus destroyed, and the epimeric aldoses as well as the corresponding ketose will be in equilibrium as shown above. This illustrates how the treatment of aldoses with alkali can be utilized for the preparation of ketoses and at the same time it shows how the operating conditions of borate columns may affect the product yield of the ion-exchange fractionation of neutral sugars. The operation of borate columns under the conditions described above in automated procedures would tend to favor the alteration of sugars by the Lobry de Bruyn-Alberda van Ekenstein transformations. The isomerization of lactose to lactulose (conversion of the terminal glucose into fructose) was reported by Carubelli (15) using Dowex 1-X8 borate columns operated at room temperature. Kesler (7) has also described the appearance ("infrequently") of unknown peaks which he thought were transformed products of one of the original sugars, however, recoveries were not reported in his paper.

Oxidations. In addition to isomerizations, sugars undergo oxidation in the presence of alkali. This is a complex phenomenon and the products depend largely upon the presence or absence of an oxidant, the concentration of alkali, the temperature, and the type of cation present in the alkali used.

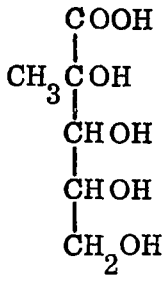
In the absence of oxidant. Internal oxido-reductions and migrations of groups occur with the formation of a group of saccharinic acids (isomeric with the initial sugars) and the corresponding lactones (saccharins). The formation, structure and properties of these compounds have been extensively reviewed (16, 17). According to the Nef-Isbell theory (16) the saccharinic acids are thought to be formed from reducing sugars by the following successive steps: the formation and ionization of an enediol, the β-elimination of a hydroxyl or alkoxyl group, rearrangement to an α-dicarbonyl intermediate, and a benzilic acid type rearrangement to the saccharinic acid.



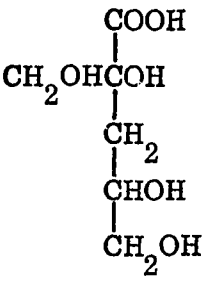
2,3-enediol

D-glucoisosaccharinic acid

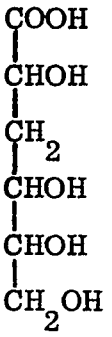
The nature of the saccharinic acid product is determined by the initial sugar and the position of the ensuing dehydration or leaving group. There are three types of six-carbon saccharinic acids, D-glucosaccharinic, D-glucometasaccharinic and D-glucoisosaccharinic acids, that are formed from the sugars D-glucose, D-mannose, D-fructose and their derivatives.



Saccharinic Acid



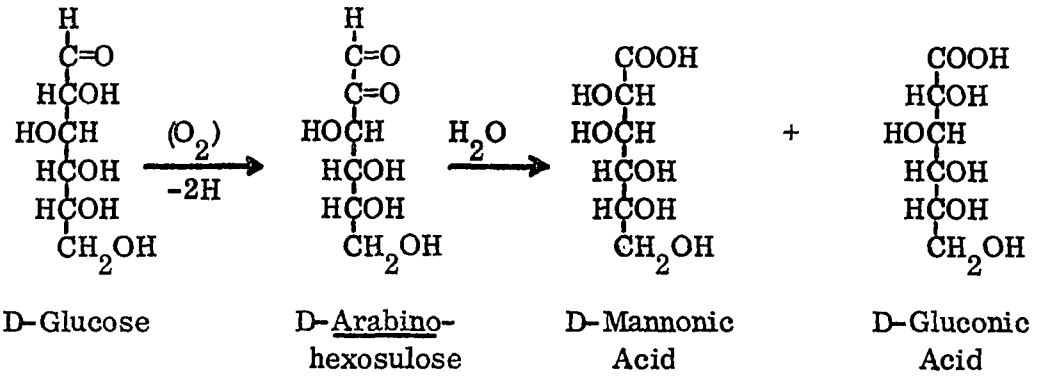
Isosaccharinic Acid



Metasaccharinic Acid

There are two possible isomers (α and β) of each type of saccharinic acid due to the formation of the new asymmetric center. It is well established that 3-deoxy-D-erythro-hexosulose (3-deoxy-D-glucosone) is the dicarbonyl intermediate formed during the production of α - and β -D-glucometasaccharinic acids under alkaline conditions in an inert atmosphere (18-20).

In the presence of an oxidant. Under alkaline conditions sugars sometimes undergo oxidation reactions which give rise to aldonic acids, instead of saccharinic acids (21). The path to aldonic acids may result in an oxidative carbon-carbon bond cleavage yielding an aldonic acid containing one carbon atom less than the starting sugar. The later case is usually observed when the alkali employed is monovalent (22), e.g., sodium or potassium hydroxide which favor fragmentation of the carbon skeleton. When alkali containing divalent cations (e.g. calcium or barium) is used stereospecific rearrangements occur. In a study of the influence of oxygen on glucose in a monovalent alkaline medium (23) it was found that 50% of the acid product formed was arabinonic acid. The next most prominent acid found was mannonic acid. Small amounts of gluconic, erythronic and ribonic acids were also found. The mannonic and gluconic acids were thought to be formed from D-arabino-hexosulose, a common precursor. The latter sugar has been postulated as the primary oxidation product (and dicarbonyl intermediate) when glucose is oxidized with polysulfide in strongly alkaline solution (24).



D-Glucose | D-Arabino-hexosulose | D-Mannonic Acid | D-Gluconic Acid

Polymerization. Nef (21) showed that a reducing sugar, in an alkaline solution, may yield more than 100 products. He found that under certain conditions polymerization takes place with the formation of resins and polysaccharides of unknown composition.

New Products Derived From Lactose During Borate Ion-Exchange Chromatography

In addition to the isomerization of lactose into lactulose, previously reported (15), the formation of other galactose-containing compounds from lactose has been investigated. The results show that borate ion-exchange chromatography under certain conditions leads to the oxidation of the glucose moiety on the reducing end of lactose or to the oxidation of fructose moiety on the reducing end of lactulose to yield the dicarbonyl compound D-arabino-hexosulose. Galactose was still attached to the latter sugar by a β-galactosidase-susceptible glycosidic bond. Isolation and characterization of a D-arabino-hexosulose-containing disaccharide (lactosone) and a tetrasaccharide (a lactosone dimer) indicate that both oxidation of lactose and polymerization of the resulting lactosone have taken place during borate ion-exchange chromatography.

The main objectives of this investigation have been:

1. To isolate, characterize, and identify sugars derived from the borate ion-exchange chromatography of lactose.

2. To determine how changes in experimental conditions may alter the yield of sugars from such columns.

3. To study the mechanism involved in the production of new products obtained by this method.

4. To determine the biological significance or application of the sugars obtained by this method.

CHAPTER II

MATERIALS AND METHODS

Materials

Chemicals and Solvents

All chemicals and solvents used in this investigation were of reagent grade. The potassium bromide used for infrared studies was of infrared quality. Reference standard carbohydrates as trimethylsilyl (TMS) derivatives: D(+)-galactose-TMS, α-D-glucose-TMS and β-D-glucose-TMS were 10% solutions in hexane. These were used for gas-liquid chromatography and were obtained from the Sigma Chemical Company of St. Louis, Missouri. One ml ampoules of the silylation reagent which contained hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS) and pyridine in a ratio of 3:1:9 were purchased from Supelco, Inc. of Bellefonte, Pennsylvania.

Instruments and Equipment

Electrophoresis apparatus. A Model E-800-2 RSCO (Research Specialties Co., Berkeley, California) was used. The instrument is of the horizontal type and was operated with water cooling.

Fraction collectors. Automatic collection of fractions of small volume (2 to 10 ml) was made with a MISCO Fraction Collector Model 6500 from Microchemical Specialties Co., Berkeley, California. For larger

effluent volumes (up to 150 ml) the fraction collector was utilized without the reel, and a glass tube connected by tygon tubing to the column was attached to the collector center post. Effluent samples were collected in 250 ml Erlenmeyer flasks or beakers properly spaced in a circle beneath the rotating glass delivery tube.

Gas Chromatograph. The instrument used was a Perkin-Elmer Model 881 with a hydrogen flame ionization detector, attached to a Leeds-Northrup Vapor Fractometer Recorder.

Lyophilization apparatus. Aqueous solutions of carbohydrates were freeze-dried by lyophilization using a Virtis Unitrap Freeze Dry Apparatus, Model 10-100 obtained from the Virtis Company of Gardiner, New York.

Melting-Point apparatus. Melting points were measured on small samples placed between glass cover-slips and visualized through the Nalge Apparatus No. 4015 from the Nalge Co. of Rochester, New York. Samples were also placed in small capillary tubes for use in the Mel-Temp Apparatus from Laboratory Devices of Cambridge, Massachusetts.

Rotary evaporators. Evaporation of samples was carried out with a Rinco Rotary Evaporator from the Rinco Company of Greenville, Illinois, the Buchi Evaporator R of the Buchi Company of Switzerland, or a Calab Model C Evaporator from the Calab Company of Oakland, California. Accessory equipment employed included a 2 liter glass condenser flask, a glass "cold finger" dry-ice condenser, and a stainless steel dry-ice condenser from the Virtis Company.

Spectrophotometers. Spectrophotometric measurements were made using a Beckman DU Spectrophotometer from the Beckman Instruments Company of South Pasadena, California. For variable absorption studies and for recording spectra both in the visible and in the ultraviolet ranges, the Cary 14 recording spectrophotometer from the Cary Instrument Corp. of Monrovia, California was used. Infrared absorption spectra were recorded

using a double-beam Model 21 Recording Infrared Spectrophotometer from the Perkin-Elmer Corporation, Norwalk, Connecticut.

Chromatographic Materials

Column chromatography. Dowex 50 W-X8-H^+, (20-50 mesh) was purchased from J. T. Baker of Phillipsburg, New Jersey. The exchange capacity was 2 meq per ml of wet volume. Dowex 50 is a strongly acidic cation exchange resin composed of sulfonic acid exchange groups which are attached to a styrenedivinyl benzene polymer lattice. The improved W designates that the polymer lattice is not darkened by sulfonation. This commercial grade resin was used on larger volumes and in early stages of purification of sugars while the highly purified analytical grade, AG5OW-X12-H^+ (200-400 mesh), purchased from Biorad Laboratories, Richmond, California was used on smaller volumes in the final stages of purification of sugars. Dowex 1-X8-Cl^- (50-100 mesh), a strongly basic anion exchange resin composed of quaternary ammonium exchange groups attached to a styrene-divinyl-benzene polymer lattice, was purchased originally from J. T. Baker of Phillipsburg, New Jersey and later, when it was no longer available from this supplier, it was purchased from Biorad Laboratories of Richmond, California. The exchange capacity was 2.3 meq per ml of wet volume resin bed. This commercial form of the resin was employed in borate form in early studies for the separation of individual sugars from mixtures and it was from columns of this resin that the new sugars observed in this study were eluted. AG1-X2-Cl^- (200-400 mesh), the analytical grade resin, obtained from Biorad Laboratories, was converted to formate form and utilized for removal of anions ($SO_4^=$) from H_2SO_4 hydrolysates of sugars and for removal of acidic sugars during purification procedures. The exchange capacity was 0.8 meq per ml of wet volume resin bed.

For carbon column chromatography, Darco G-60, activated carbon was obtained from Fisher Scientific Company. This was combined with an

equal proportion of Celite 535 Filter-aid, a Johns-Manville product.

Separation of oligosaccharides according to molecular size was performed on columns of Sephadex G-15, purchased from Pharmacia Fine Chemicals, Inc. of Piscataway, New Jersey. Avicel, a microcrystalline cellulose from FMC Corporation, American Viscose Division, Newark, Delaware, was utilized for cellulose column chromatography.

Paper chromatography. Whatman chromatographic papers made by W. & R. Balston, Ltd., England, were utilized. A thick paper, Whatman No. 3 MM, was used for preparative paper chromatography and for paper electrophoresis. Whatman No. 1 paper was used for the chromatographic identification of sugars.

Thin-layer chromatography. Glass plates, 20 x 20 cm., pre-coated with a uniform thickness (250μ) of Silica Gel G ("Mannograms") were purchased from Swartz/Mann Division of Becton, Dickinson and Company from Van Nuys, California.

Gas-liquid chromatography. A coiled pyrex glass column, 6 ft in length with inner diameter of 2 mm from Supelco, Inc. of Bellefonte, Pennsylvania was utilized. The column packing was the silicone 3% SE-30 on 100/120 mesh silane-treated Gas Chrom Q which was prepared and pretested by Applied Science Laboratories of State College, Pennsylvania. For injection of samples a Hamilton Syringe No. 7001N of 1 μl capacity was utilized.

Enzymes

Beta-galactosidase was purchased from Nutritional Biochemicals Company of Cleveland, Ohio.

Galactose oxidase, in a specially prepared kit ("Galactostat"), was purchased from Worthington Chemical Company of Freehold, New Jersey.

Methods

Analytical Methods

Anthrone method for hexoses (25). Concentrated H_2SO_4 reacts with hexoses to yield furfural derivatives which, in the presence of anthrone, give a green chromogen whose absorption peak is in the 620 mμ region. This reaction was used to quantitate hexoses and to monitor column effluents containing hexoses.

Anthrone reagent. This was prepared as a 0.2% w/v solution in concentrated sulfuric acid at least 4 hours prior to use, and it was stored at 4°C for no longer than 9 days.

Procedure. Suitable aliquots containing hexose amounts ranging from 10-50 μg in 1 ml of water were pipetted into pyrex ground-glass stopper type tubes. The tubes were chilled in ice-water for 5 minutes prior to the addition of 2 ml of ice-cold anthrone reagent. The tube contents were mixed and placed in a boiling water bath for 10 minutes and then cooled in an ice-bath for 5 minutes. The tubes were kept at room temperature for 5 minutes and the optical densities were read at 620 mμ. A water blank and standards of galactose were run concurrently.

Park-Johnson ferricyanide reducing sugar method (26). This method is based on the reduction of ferricyanide in alkaline solution followed by the formation of Prussian blue (ferric ferrocyanide) upon the addition of ferric ions. The use of the detergent Duponol keeps the Prussian blue in suspension to allow spectrophotometric analysis.

Reagent. The carbonate-cyanide solution contained 5.3 g of sodium carbonate and 0.65 g of potassium cyanide dissolved in 1 l of water. The ferricyanide solution consisted of 0.5 g of potassium ferricyanide dissolved in 1 l of water and stored in a brown bottle. For the ferric iron solution 1.5 g of ferric ammonium sulfate and 1.0 g of Duponol were dissolved in 1 l of 0.05 N sulfuric acid.

Procedure. A 1 ml sample was pipetted into a glass-stoppered tube. To this was added 1 ml of the carbonate-cyanide solution plus 1 ml of ferricyanide solution. The contents were mixed and either allowed to stand at room temperature (strongly reducing compounds) for 15 minutes or placed in a boiling water bath (sugars) for 15 minutes. Five ml of ferric iron solution were then added, the tubes were carefully mixed, and allowed to stand at room temperature for an additional 15 minutes. The optical density of the samples was read at 690 mμ using a water-reagent blank. Standards of glycolaldehyde in concentrations of 50-150 μg were run concurrently.

Steinhoff copper acetate reducing-sugar method (27). This method utilizes the Nelson-Somogyi arsenomolybdic reagent (28). The increase in reducing power as monosaccharides are released from disaccharides was measured with this reagent in progressive acid hydrolysis studies and in the assay of lactase (β-galactosidase) activity. In this modified method (29) the reducing power was indicated by the development of the arsenomolybdate chromogen by the action of the reduced copper formed.

Reagents. The copper reagent was prepared by dissolving 50 g of crystallized sodium acetate in 100 ml of 7% copper sulfate. The arsenomolybdic reagent was prepared by mixing 25 g of ammonium molybdate dissolved in 450 ml of water with 21 ml of concentrated sulfuric acid, adding 25 ml of a solution containing 3 g of $NaHAsO_2 \cdot 7H_2O$. After mixing, the solution was incubated for 1 to 2 days at 37°C. The reagent was diluted with one volume of 1.5 N sulfuric acid and then stored in a glass-stoppered brown bottle.

Procedure. For the reaction, 0.5 ml of each sample was pipetted into glass-stoppered tubes. To this was added 1 ml of the copper reagent, 0.2 ml of n-butanol and the contents mixed. (The layer of n-butanol over the samples prevents re-oxidation of cuprous ions during the reaction.) The tubes were immersed in a water bath at 80°C and then the stoppers were inserted. After 20 minutes the tubes were cooled to room temperature and

1 ml of the arsenomolybdic reagent added. This was followed by the addition of 0.8 ml of 5 N sulfuric acid and 1 ml of water. After mixing, the optical density of the samples was read at 520 mμ. Water-reagent blank and galactose standards of 100-300 μg were run concurrently.

Galactose oxidase assay for galactose. A coupled enzyme system of galactose oxidase and peroxidase in kit form, called the "Galactostat" reagent, was used for quantitative assay of galactose.

Reagents. The galactostat vial contained 20 units of galactose oxidase (Worthington), 2.5 mg peroxidase (Worthington) and 0.5 millimoles of phosphate buffer, pH 7.0. The chromogen vial contained 2.5 mg o-tolidine. The contents of the chromogen vial were dissolved in 0.5 ml of methanol and this and several rinsings were transferred to a 50 ml volumetric flask containing 10 ml of water. To the galactostat vial was added 3 ml of water and this was transferred with rinsings to the chromogen solution and the volume was brought to 50 ml with distilled water. The galactostat reagent was stored in a dark bottle protected from light.

Procedure. According to the Sempere modification (30), the sample, water blank, and standard galactose solution (ranging from 25-100 μg) in a volume of 1.5 ml were incubated for 10 minutes at 37°C prior to addition of 1.5 ml of the galactostat reagent. After a 1 hour incubation, the reaction was terminated by the addition of 0.2 ml of 0.5 M EDTA. The absorbance was read at 425 mμ. The color is stable for several hours.

Periodate consumption. Periodic acid is a powerful oxidant which, as observed by Malaprade in 1928 (31), cleaves 1, 2-glycols. This provided a convenient means to determine the number of vicinal OH groups and to study the structure of carbohydrates. There are a number of methods to determine the amount of periodate consumed by carbohydrates. One method is a direct determination of periodate consumed by using ultraviolet spectrometry according to the method of Aspinall and Ferrier (32) which, in turn, is a modification of the Dixon and Lipkin method (33). The basis of the method is

the ultraviolet absorption of the periodate ion at 222.5 mμ. The decrease in absorption at this wave length, due to the oxidation of vicinal OH groups in carbohydrates, is proportional to the amount of periodate consumed. In the modified method (32) a correction is made for the absorption due to the iodate ion produced during the reaction. The method can be carried out on very small samples (2-3 mg in some cases) depending upon the periodate consumption. The quantities of carbohydrate required were calculated from the formula, M x P = 70-100, where M is the micromoles of carbohydrate and P is the number of molecular proportions of periodate reduced. A 6-9 mg quantity of a monosaccharide consuming 2 molecular proportions of periodate would be suitable whereas 12 to 18 mg of a substance would be needed if the consumption were 1 molecular proportion of periodate.

Samples were dissolved in 10 ml of 0.015 M sodium metaperiodate and stored in the dark at room temperature. At intervals, aliquots of 0.2 ml were withdrawn and diluted 250 times (50 ml volumetric flask). The uv spectrum in the range of 200 to 300 mμ was recorded for each sample using the Cary 14 spectrophotometer. A correction for the absorption due to iodate produced in the reaction was made by previously measuring the absorbancy of mixtures of iodate and periodate ions in various concentrations and plotting a curve of concentration vs absorbancy at 222 mμ. A sodium metaperiodate solution of 0.015 M diluted 250 times gave an optical density of ca. 0.1. A conversion factor obtained from this plot was used to calculate the micromoles of periodate consumed by samples during periodate oxidation.

Gas-liquid chromatography. The separation and characterization of carbohydrates by gas chromatography of trimethylsilyl (TMS) derivatives was carried out according to the method of Sweeley et al.(34).

Procedure. For preparation of the TMS derivatives, 10 mg samples were accurately weighed into screw-cap tubes; or, in some cases, a suitable aliquot of a solution of known concentration was evaporated to dryness in a screw-cap tube. The silylation reagent (from 1 ml ampoule)

was added, and the mixture was shaken vigorously for about 30 seconds and then allowed to stand for 5 minutes at room temperature prior to chromatography. During this time, a white precipitate of ammonium chloride appeared but this interferred in no way with subsequent gas chromatography. To prevent possible contamination in the column, however, the samples were centrifuged for 10 minutes and the supernatants were transferred to other screw-cap tubes.

Aliquots of 0.1 to 1.0 μl of the TMS derivatives were injected into the gas chromatograph. The carrier gas (nitrogen) flow rate obtained by a pressure of 30-35 psi produced sharp separation of peaks. The column was either maintained isothermally at 150°C or it was programmed from 120°-180°C at an increase of 2°C per minute. For separation of aldonic acids produced in the alkaline degradation studies of glucosone, the column was maintained at 140°C. For separation of disaccharides the column temperature was set at 210°C.

Chromatographic Methods

Paper Chromatography. Paper chromatography was used to identify sugars, to follow the course of hydrolysis or of alkaline degradation and for the preparative isolation of monoses.

Analytical techniques. Whatman No. 1 filter paper was used and the chromatograms were developed by the descending technique. Standard samples, 5 μl of 0.5% solutions, were applied as small spots containing approximately 25 μg of each sugar which could be visualized by most reagents. Some sugars had to be applied in greater amounts. For preparative chromatography, Whatman No. 3 MM was used. The papers were pre-washed in 5% acetic and air dried. The samples were applied in a narrow band with the aid of a Pasteur pipette, on a line 8 cm from the end of the paper. The chromatogram was developed by descending technique with the following solvent system; ethyl acetate-glacial acetic acid-water (3:3:1, v/v). After development,

the paper was air dried and 2 guide strips were cut from each edge. These were dipped in the aniline-diphenylamine-phosphoric acid (reagent A) and the color developed in a chromatography oven at 100° for 5 minutes. The sugar bands were located with the aid of these guide-strips and the corresponding zone of the paper was removed by cutting. For elution of material from single sheets of paper, the zone was eluted in a closed elution chamber. When as many as 6 sheets were processed, the zone from each paper was cut into long strips which were rolled and placed in the bottom of a 2 liter beaker. Elution in a volume of 300 ml of distilled water was repeated 3 times or until all the sugar was eluted. The eluates were then concentrated by rotary evaporation in pre-weighed evaporating flasks.

Thin-layer chromatography (tlc). Thin-layer chromatography is an effective tool for characterizing and separating complex sugar mixtures. The separation achieved is very rapid (less than an hour in some cases) and highly sensitive due to the minute quantities of sample required. By charring of the plate with sulfuric acid-dichromate, this method can be used to detect carbohydrates not detectable by any other means.

Silica gel G (with an intert binder) "Mannograms", hermetically packed in plastic to protect from moisture, were used without prior activation. Aqueous solutions of samples and standards were applied 2 cm from the edge of the plate and were spaced 1 to 1.5 cm apart. The plates were developed by ascending technique in rectangular tanks (25 x 30 x 10 cm) lined with 3 MM filter paper and containing 100 ml of the solvent system. After development, the plates were air-dried and sprayed with one or a combination of spray reagents.

Solvent systems (v/v).

Solvent 1, ethyl acetate-glacial acetic acid-water (3:3:1).

Solvent 2, n-butanol-pyridine-water (5:3:2).

Solvent 3, n-butanol-glacial acetic acid-water (4:1:1).

Solvent 4, n-butanol-glacial acetic acid-water (4:1:5, upper phase).

Solvent 5, phenol-water (4:1).

Solvent 6, n-butanol-95% ethanol-water (4:1:1).

Solvent 7, ethyl acetate-95% ethanol-water (6:3:1).

Solvent 8, toluene-ethyl acetate (1:1).

Solvent 9, water-saturated n-butanol.

Solvent 10, water-saturated ethyl acetate.

Solvent 11, benzene-95% ethanol-water-glacial acetic acid (200:47:15:1).

Solvents 1 to 7 were used for paper chromatography while solvents 6 to 11 were used for tlc.

Sugar detection reagents. For the detection of sugars, paper chromatograms were dipped in, while thin-layer plates were sprayed with, the following reagents.

Reagent A, Aniline-diphenylamine (35). One gram of diphenylamine hydrochloride was dissolved in 100 ml of acetone. To this was added 1 ml of aniline and 9 ml of 85% phosphoric acid, in that order. The chromatogram was heated at 100°C for 5 minutes. This reagent was used to detect reducing sugars which gave specific colors.

Reagent B, Aniline hydrogen phthalate (36). This reagent consisted of 1.66 g of o-phthalic acid and 0.91 ml of aniline dissolved in 48 ml of n-butanol, 48 ml of ethyl ether and 4 ml of water. The reagent is stable in the refrigerator for several weeks. The chromatogram was heated at 105°C for several minutes after treatment with this reagent.

Reagent C, 2-Aminodiphenyl-oxalate (37). This reagent contained 1.69 g of 2-aminodiphenyl, 0.9 g of anhydrous oxalic acid, 5 ml of glycerol, 10 ml of water and 84 ml of acetone. For development of colors the chromatograms were heated for 5 to 10 minutes at 100°C.

Reagent D, Naphtholresorcinol (35). One ml of 85% phosphoric acid was added to 9 ml of water and this solution was added to 50 ml of 0.2% naphtholresorcinol in acetone. Colors were developed in 8 minutes in an

oven at 95°C containing a large beaker of water; colors fade quickly.

Reagent E, p-Anisidine hydrochloride (38). The chromatogram was dipped in a solution of p-anisidine hydrochloride (3%) in water-saturated n-butanol and heated at 100°C for 2 to 10 minutes.

Reagent F, 3,5-Dinitrosalicylic Acid (39). This reagent consisted of 2 solutions. The first was a 0.5% solution of 3, 5-dinitrosalicylic acid in acetone. The second was a 4% solution of sodium hydroxide in 95% ethanol. The chromatogram was dipped in the first solution and allowed to dry, and then in the second solution and dried again. The chromatogram was then heated in an oven at 110°C for 5 minutes to develop the brown spots.

Reagent G, Anisaldehyde (40). This reagent was prepared by mixing ethanol-anisaldehyde-concentrated H_2SO_4 in 36:2:2 proportions by volume. This was used only for thin-layer plates which were heated for 4 minutes at 140° for development of color.

Reagent H, Silver nitrate-alcoholic sodium hydroxide (41). A saturated $AgNO_3$ solution (0.5 ml) was diluted to 100 ml with acetone and water was added until complete solution was obtained. The 0.5N NaOH was made by diluting 5.2 ml 50% NaOH to 200 ml with 95% ethanol, and 6N NH_4OH, was prepared by diluting concentrated NH_4OH (40 ml) to 100 ml with water. The chromatogram was dipped in the silver nitrate solution, air-dried at room temperature for 5 minutes and then immersed in the alcoholic NaOH until spots developed sufficiently. The excess background was removed by dipping in the ammonium hydroxide solution briefly. The paper was washed for at least one hour in running tap water and then air dried.

Reagent I, Periodate-benzidine (37). A 0.1 M stock solution of periodic acid, which was stable for months at 0°C, was prepared by dissolving 228 mg of H_5IO_6 in 10 ml of distilled water. Just prior to use, 1 ml of stock solution was diluted with 19 ml of acetone yielding 0.005 M periodic acid. The chromatogram was dipped in this solution, and after air drying, it was dipped in a 0.01 M benzidine solution. The latter reagent, made by

dissolving 184 mg of benzidine in a mixture of 0.6 ml of glacial acetic acid, 4.4 ml of distilled water and 95 ml of acetone, detects unreacted periodic acid on the chromatograms. The sugars appear as white spots in the dark-blue background of unreacted periodate.

Reagent J, Sulfuric acid-dichromate. The reagent was prepared by carefully adding 38 ml of concentrated sulfuric acid to a solution containing 0.5 g of sodium dichromate in 62 ml of water. After lightly spraying, the tlc plate was heated for 5 to 10 minutes at 100°C in an oven to char the carbohydrate substances. (Fructose and deoxyribose do not char).

Ion-exchange column chromatography. Dowex 1-X8-(borate) column chromatography has been used in this laboratory for a number of years to separate neutral sugars during studies on the biosynthesis of lactose and neuramin lactose by rat mammary gland preparations (42, 43). It was found that under certain conditions, sugars other than those applied to the column could be found in the eluates. For example, if the Dowex 1-X8 (borate) columns were utilized at 25°C instead of 4°C, part of the applied lactose was found transformed to lactulose and was eluted with 0.015 M potassium tetraborate (15). After elution of the lactulose peak, 0.1 M $NaHCO_3$ was found to elute a third peak of sugars when lactose was chromatographed on Dowex 1-X8 (borate) columns operated at 37°C. A method for preparing large quantities of this sugar material was developed. This procedure, which involves the use of three different ion-exchange resins, i.e. Dowex 1-X8-(borate), Dowex 50W-X8 (H^+), and Ag 1-X2 (formate), will be described in the following sections.

Dowex 1-X8-Cl^- (50-100 mesh). This resin was converted to the borate form by a modification of the method of Khym and Zill (4). The resin was first washed batchwise with 4N HCl, then poured into a column (4 x 60 cm) and washed with additional 4N HCl. The conversion to borate form required 15 column volumes of 0.1 M sodium tetraborate (borax). Columns of 1 x 18 cm dimensions were used generally in this step of the pro-

cedure. For large scale experiments batteries of up to 12 small columns were used at one time to produce the desired sugars. This was found to be necessary since a different compound was produced if the same amount of resin was combined in a single large column. Temperature was also a critical factor controlled in these experiments. The use of both 25°C and 37°C operating temperatures was explored since sugar products eluted at these temperatures differed (as described in "results"). A 0.005 M solution of borax was passed through the column for several hours in order to equilibrate the resin with this solution prior to the introduction of the sugar. Lactose solutions (0.5 g per 100 ml of 0.005 M borax) were prepared several hours in advance of their application to each column and were applied at a rate of 50 ml per hour. Four liters of 0.005 M borax solution were passed through the column at a rate of 75-100 ml per hour (ca. 48 hours) in order to elute non-transformed lactose. The same volume of 0.015 M borax was passed through the column in another 48 hour period to remove lactulose formed during this process. Elution of the third peak of sugars was usually completed after a volume of 200 ml of 0.1 M of $NaHCO_3$ had passed through the column. Effluent samples were monitored for sugar content with the anthrone reagent. Effluent peak fractions containing each sugar were pooled with the corresponding fractions of other columns and processed as described below.

Dowex 50W-X8-H^+ (20-50 mesh). To regenerate this resin it was first washed batchwise with 2 volumes of 2 N H_2SO_4 and then poured into a column and the process repeated. Prior to use, the excess acid was removed from the resin by washing it with water until a neutral pH was obtained; otherwise, the resin was stored under slightly acid conditions. The resin was used to remove sodium ions from the 0.1 M $NaHCO_3$ eluate of the Dowex 1-X8 (borate) columns described above. The pooled eluate from 12 columns (ca. 2-3 liters) was added to one-half its volume of Dowex 50 (H^+) resin and the mixture allowed to stand at room temperature for one hour with

occasional shaking to facilitate evolution of the CO_2. The resin was removed by filtration and the filtrates were stored at -20°C. When ready for processing, the solution was thawed and concentrated to a volume of 200 ml by rotary evaporation under vacuum. The concentrate was passed through a Dowex 50W-X8(H^+) column (3 x 40 cm) at 4°C to remove remaining traces of Na^+. The eluate from this column was evaporated to dryness to yield a white material containing a mixture of sugars and boric acid. By rotary evaporation with methanol, boric acid was removed in the form of methyl borate as suggested by Khym and Zill (5). The final sugar material appeared as a clear syrup when concentrated, and it was stored frozen in a more dilute form (1 mg per ml by anthrone assay with galactose as standard). The pH of this sugar solution was about 3.0.

Ag 1-X2-Cl^- (200-400 mesh). This resin was placed in a column and converted to the hydroxyl form by passing 9 volumes of 1 N NaOH through it. The resin was washed with water until neutrality was reached and then the column was converted to formate with 2 volumes of 1 N formic acid and rinsed again with distilled water. This ion-exchange resin was used to remove sugar acids from the pH 3.0 sugar mixture described above. The acidic sugar mixture (approximately 300 mg by anthrone) was dissolved in 30 ml of water and passed through the column (2.6 x 30 cm) which was then washed with 300 ml of water. Formic acid in the pooled effluent and washings was removed by repeated rotary evaporation under reduced pressure at 40°C. The pH of the sugar mixture after this treatment was about 6.0.

Darco-Celite column chromatography. Adsorption of sugars to charcoal and desorption by water and alcohol solutions of increasing concentration was first studied by Tiselius (44). In our work, the Darco-Celite column method of Whistler and Durso (45) was used with modifications. Twenty-five g of Darco G-60 was mixed dry with 25 g of Celite 535 and the mixture was slurried with 200 ml of water. This thick slurry was poured into a glass column containing a loose plug of glass wool covered with a circle

of filter paper. After the column settled to final dimensions of 2 x 18 cm, it was washed overnight with approximately 100 ml of 6 N HCl. To remove all traces of chloride required washing the column with water for 7 to 10 days. A solution of the neutralized sugar mixture from the Ag 1-X2 formate column, 200 mg (anthrone analysis with galactose standard) in 5 ml of water, was applied to the column. Elution of the column at a rate of 75 ml per hour required that the reservoir be at a height of 8 feet above the column outlet. Elution began with water followed by stepwise increases in ethanol concentration of 1, 2, 4, 10, and 50%. The effluent was collected in fractions of 150 ml each. The sugar content was monitored on 1 ml aliquots of each fraction by the anthrone reagent.

Cellulose column chromatography. The method for separation of carbohydrates on cellulose was reported first by Hough, Jones, and Wadman (46). A modification of this method, described by Kato for separation of hexosuloses from hexoses (47), was employed for the isolation and purification of D-arabino-hexosulose encountered in this investigation. One liter of dry micro-crystalline cellulose (Avicel) was mixed with 1 liter of 1 N NaOH. This mixture was neutralized with 1 liter of 1 N HCl and filtered on a large Buchner funnel. The cellulose was washed with water repeatedly until residual inorganic ions were removed. The cellulose was then mixed in a beaker with 2 liters of 95% ethanol and filtered again. In the final washing of the cellulose on the Buchner funnel, absolute ethanol was used. The cellulose was dried for several days in a desiccator under reduced pressure. The pulverized powder was packed into a glass column (4.6 x 60 cm) with a circle of filter paper covering the sintered glass at the bottom. The powder was tamped gently while the column was packed to a height of 42 cm. A large sugar sample (12 g of syrup) was mixed with an equal weight of cellulose with the aid of 50% ethanol and the mixture dried under vacuum in a desiccator. The dried powder was packed on top of the prepared cellulose column and an additional 8 g of cellulose was packed on top of this. The column was

eluted with solvent system 1 (ethyl acetate-acetic acid-water, 3:3:1, v/v) from a 2 liter reservoir placed at a height of 8 ft above the column outlet. Fractions of 75 ml were collected each hour and monitored by paper chromatography using solvent system 1 (ethyl acetate-acetic acid-water, 3:3:1, v/v) or by thin-layer chromatography in solvent system 7 (ethyl acetate-ethanol-water, 6:3:1, v/v).

Gel Filtration

One hundred grams of Sephadex G-15 gel was allowed to swell overnight in 0.05 M NaCl and then the slurry was packed in a column (2.2 x 94 cm). The column was washed with 0.1 M acetic acid and then with degassed distilled water until the effluent solution was chloride free and of neutral pH. The column was kept flowing continually until needed. The column was standardized with a mixture of sugars containing 5 mg each of stachyose (tetrasaccharide), raffinose (trisaccharide), lactose (disaccharide) and galactose monosaccharide in 0.5 ml of distilled water containing 2.5 mg of Blue Dextran 2000. The standard mixture or the sugar sample (20 mg in 0.7 ml) was loaded carefully onto the column with a Pasteur pipette. For elution with distilled water, the column flow rate was adjusted to 6 ml per hour with the use of a Mariotte flask containing 2 liters of degassed water. Fractions of 3 ml were automatically collected in tubes and monitored by anthrone assay.

Paper Ionophoresis

This method was used to characterize and identify sugars. Whatman No. 3 MM paper of dimensions 18 x 52 cm was used. Aqueous sugar samples were applied one inch apart in amounts of 0.5 to 5 μmoles along a line 15 cm from the negative electrode end. The paper was dipped in 0.05 M borax of pH 9.2, blotted, and placed with its ends in the electrode compartments containing the same solution. A voltage of 375 was applied for a period of 5 hours after which the paper was removed and

allowed to dry at room temperature overnight. The paper was then treated with reagent A (aniline-diphenylamine).

Functional Group Analysis

Various tests were carried out on the unidentified oligosaccharides and on their constituent monosaccharides in order to gain some insight as to the presence or absence of specific functional groups.

Alkene function. The method employed was based on the addition of bromine to the double bond (48). A standard bromine-bromide solution, 0.01 N in bromine, was prepared immediately before use by making a 1:40 dilution of Br_2 in a 14.44% w/v solution of NaBr in methanol. A 5 mg sugar sample was dissolved in 2 ml of 50% methanol in a glass-stoppered test tube and 2 ml of the 0.01 N bromine solution was then added. A blank containing 2 ml of 50% methanol was run concurrently with each sugar sample tested. The tubes were kept at room temperature, in the dark, for periods of 3 or 6 hours. At the end of this time, 1 ml of freshly prepared 2% KI and 1 ml of 6 N H_2SO_4 were added. The remaining free bromine liberated I_2 which was titrated with standard 0.005 N sodium thiosulfate using 1% starch as the indicator.

Lactone. Since lactones are internal esters, some methods for determination of esters may be applied to the detection of the lactone function. The hydroxamic acid test of Abdel-Akher and Smith (49) for paper chromatograms was used. Paper chromatograms containing the unknown sugars and suitable standards were developed in solvent system 6 (n-butanol-ethanol-water, v/v) and air dried. Just prior to use, a 1 N solution of hydroxylamine hydrochloride in methanol (3.48 g in 50 ml) was mixed with an equal volume of 1.1 N solution of potassium hydroxide in methanol (6.17 g in 100 ml). This solution was sprayed lightly onto the chromatogram, and after 10 minutes the chromatogram was sprayed with a freshly prepared solution of 2% ferric chloride in 1% hydrochloric acid. A blue or mauve color is quickly formed in

areas containing esters or lactones.

A qualitative hydroxamic acid test described by Davidson (50) was also used. A drop of sample, standard solution of D-glucurono- δ -lactone (ca. 5 µg) or water as a blank were placed in test tubes. To each was added 1 ml of freshly mixed hydroxylamine-potassium hydroxide in methanol described above. The tubes were heated in a boiling water bath for 5 minutes, acidified with 0.5 ml of 2 N HCl, and a drop of 10% ferric chloride was added. An orange color is given by esters and/or lactones.

Anhydro-sugars. For the determination of anhydro-sugars, the colorimetric procedure for 3-6-anhydro-galactose with the resorcinol reagent was used (51). The stock reagent contained 130 mg of resorcinol in 100 ml of absolute ethanol. Ten ml of the stock was added to 100 ml of 12 N HCl just before use. A 2-ml sample containing 5-40 µg of 3, 6-anhydro-galactose (an equivalent amount of agar was utilized)or 30-200 µg of unknown sugar was pipetted into a test tube placed in an ice bath and 10 ml of the resorcinol reagent was added. After mixing, the tube was heated for 10 minutes at 80°C, cooled for 15 minutes in an ice bath and read at 550 mµ within 15 minutes against a water blank treated in the same manner.

Reducing power. In addition to the analytical methods of Nelson-Somogyi (29) and Park-Johnson (26), the ferricyanide reduction method employed by Borsook et al. (52) for strongly reducing fructose-amino acids was also used. This employs the ferric sulfate reagent of Folin and Malmros (53) which contains gum ghatti instead of Duponol as in the Park-Johnson method. Twenty g of gum ghatti, wrapped in cheese-cloth, is suspended over-night just below the surface of one liter of water in a measuring cylinder. After straining this solution, 5 g of anhydrous ferric sulfate in 75 ml of 85% phosphoric acid and 100 ml of water was added . About 15 ml of 1% potassium permanganate was added dropwise to destroy any reducing materials present in the gum ghatti. The slight turbidity disappears if the solution is kept at 37°C for a few days. For the reaction 0.1 ml of the sugar

solution was mixed with 1 ml of 0.1% potassium ferricyanide in water and the reaction is allowed to proceed at room temperature for 5 minutes. One ml of the ferric sulfate reagent is added and after another 5 minutes 5 ml of water is added. The blue color which appears can be read at 690 mμ. This method can detect as little as 2 μg of reducing compound.

The test for reducing power with Fehling's reagent was also applied (54). The reagent consists of 2 solutions. Solution I has 3.46 g of $CuSO_4 \cdot 5H_2O$ in 50 ml of water containing a few drops of 10% sulfuric acid. Solution II has 6 g of NaOH and 17.3 g of sodium potassium tartrate in 50 ml of water. Immediately before use, a small amount of reagent was prepared by mixing equal proportions of each solution. Then 0.4 ml of the reagent was added to a drop of sugar solution (5 mg). Glycolaldehyde and ascorbic acid were used as positive test compounds. These react strongly at room temperature giving a red precipitate to cuprous oxide.

Reductone. According to Weygand et al. (55) the customary reagents for reductones are Tillman's reagent (2:6-dichlorophenolindophenol in acid solution) and methylene blue in alkaline solution.

Tillman's reagent (56). The dye (0.05%) was prepared by dissolving 10 mg of 2:6-dichlorophenolindophenol in 20 ml of water, acidifying the solution with 0.03 N HCl until a red color is obtained, and extracting the dye into 20 ml of xylene. The water layer was discarded. One ml of sample was added to 1.4 ml of water, 1.4 ml of 0.03 N HCl or 1.4 ml of 0.03 N NaOH. After mixing, 0.1 ml of the dye solution was added. Ascorbic acid at concentrations as low as 0.06 mg/ml reduces (decolorize) the red color of the dye under acid conditions.

Methylene blue. The dye was made up in 0.001% aqueous solution. The samples were tested under the same conditions as above. The sample (0.1 ml) was added to 1.4 ml of water, 1.4 ml of acid or 1.4 ml of base. One ml of the dye was added to this. Ascorbic acid (0.06 mg) decolorized the dye in 7-10 minutes under acid conditions but not in water

or alkali.

Enolic group. A 1% solution of ferric chloride was used to test for the presence of enol-type compounds (57). A drop of phenol and resorcinol standards (0.1% solutions) gave a blue color with a drop of the reagent.

Dicarbonyl group. One of Feigl's spot tests (58) for aliphatic 1, 2-dioxo groups can be performed by reacting one drop of the compound to be tested with one drop of a solution of hydroxylamine hydrochloride (1 g) and sodium acetate (1 g) in 2 ml of water. The mixture is warmed for 2 minutes at 100°C in a test tube, applied to a piece of filter paper and treated with one drop of 5% nickel acetate solution. A yellow-red color appears at once or after exposure to ammonia. Diacetyl (0.5 μg) reacted positively with this reagent.

Another Feigl spot test for dicarbonyls involves a condensation reaction with thiophene (59). A 1 mg sample of the compound to be tested is dissolved in 3 drops of concentrated H_2SO_4. The mixture is spotted on filter paper and a drop of 0.3% thiophene in benzene is added. Ninhydrin gives a positive blue color with this reagent.

Dicarbonyl compounds, react with phenylhydrazine reagents under mild conditions. A reagent solution was prepared (60) by heating 5 mg of 2:4-dinitrophenylhydrazine placed in a test tube in a boiling water bath with 0.84 ml of concentrated HCl and 1 ml of water. This reagent, 0.5 ml, was added to 10 mg of the sugar in a test tube. Reducing sugars such as glucose require heat for the reaction to take place, but dicarbonyl compounds form red precipitates at room temperature.

Methods of Synthesis of Reference Compounds

3-Deoxy-D-erythro-hexosulose. The preparation of this compound was done by the Maillard reaction employing amino acids as described

by Otsuka and Egyud (61). D-Glucose (90 mg) and D, L-leucine (66 mg) were added to 2 ml of 0.025 M phosphate buffer, pH 7.0. The suspension (the amino acid was not very soluble) was stirred for 3 hours at 37°C. Arsenous trioxide (237 mg) was then added and the stirring continued for another 16 hours. The mixture was filtered, and after several water rinses the pooled filtrate was concentrated to about 5 ml and deionized on coupled columns (1 x 12 cm each) of Ag50W-X8(H^+) and AgI-X2(formate). Formic acid in the water effluent was volatilized during rotary evaporation in vacuo. For subsequent studies the product was diluted with distilled water to a volume of 10 ml.

In the second method (47) a primary amine, n-butylamine (17 ml), was added to D-glucose (30 g) in 20 ml of methanol. This mixture was stirred for 30 minutes at 65° C. The yellow solution was cooled quickly to 25°C, glacial acetic acid (10 ml) was added and the mixture stirred again for 30 minutes at 65°C. The blackened solution was cooled again to 25°C and diluted with 300 ml of distilled water. Darco G-60 (40 g) was added and the mixture was stirred for 1 hour, after which the Darco was removed by filtration yielding a straw-colored liquid. This solution was passed through a column (4 x 60 cm) of Ag 50W-X8(H^+) resin which was then washed with water. Rotary evaporation of the effluent solution, under reduced pressure at 25°C, yielded a thick, light-yellow syrup weighing about 6 g. The syrup was dissolved in a minimum volume (ca. 50 ml) of 95% ethanol and stored at -20°C.

D-Arabino-hexosulose. This compound can be prepared by a variety of methods (62). Perhaps the better known methods are those involving the oxidation of reducing hexoses and the osazone cleavage method. The N-glycoside degradation method of Kato (47), described above, can also be utilized since he later (63) isolated this compound from the final mixture of products.

Oxidation methods. The oxidation of glucose by cupric acetate

was carried out by the method of Evans et al. (64). Nine g of glucose was added to 250 ml of aqueous cupric acetate (25 g), and the mixture was stirred at 25°C in a stoppered flask for a period of 4 days. Aliquots were taken at 24 hour intervals for paper chromatographic examination of the products. The solution was then filtered to remove cuprous oxide and the residual copper ions were removed by passage through Dowex 50W-X8-H^+ (20-50 mesh) in a column of 4 x 60 cm dimensions. The water eluate (ca. 1 liter) was collected in a 2 liter evaporation flask, concentrated to a syrup by rotary evaporation, and stored in 50% ethanol at -20°C until purified further by preparative paper or column chromatographic methods.

The same procedure was followed using fructose on a smaller scale, using 0.9 g with 25 ml of cupric acetate (2.5 g). The reaction was carried out at 25°C and concluded at 24 hours by filtering off the red precipitate of cuprous oxide. The filtrate was passed through a column (1.2 x 25 cm) of Dowex 50W-X8-H^+ (20-50 mesh), as described above for glucose. The effluent products were examined by paper chromatography.

The preparation of D-arabino-hexosulose from fructose, using selenium dioxide as the oxidant, was described by Dixon and Harrison (65). In our modification, fructose (500 mg) and selenous acid (150 mg) were added to 2 ml of water in a reaction flask and the mixture was refluxed for 1 hour. The solution was cooled and a red precipitate filtered off. The filtrate was passed through a small Dowex 50 column (1.2 x 25 cm) to remove residual selenium ions, as described above for the cupric acetate oxidation of fructose. The products of the fructose and selenium dioxide oxidation were examined by paper chromatography.

Osazone-cleavage methods. For this method the regeneration of D-arabino-hexosulose from fructosephenylosazone was utilized (66, 67). The latter compound was prepared from fructose and phenylhydrazine reagent as described in the following section under "derivatives". The recrystallized

phenylhydrazine derivative of fructose (200 mg) was suspended in a mixture of water (0.3 ml), 95% ethanol (2.2 ml), and 1.9 M HCl (0.95 ml) in a 20 x 150 mm test tube. The suspension dissolved when heated to 40°C and then 2 M sodium nitrite (0.7 ml) was added dropwise with shaking. A dark-brown color from the evolution of nitrous acid was noted. A glass marble was placed on the top of the tube and heating was continued for 1 hour at 40°C. Following this treatment, the ethanol was evaporated off at 25°C under diminished pressure using the rotary evaporator. The precipitated material which appeared was removed by centrifugation and the supernatant was extracted with chloroform. The reaction products remaining in the aqueous phase were examined by paper and thin-layer chromatography and by paper ionophoresis.

Methods for Preparation of Derivatives

Phenylosazones. One of the most characteristic derivatives formed by carbohydrates are the osazones formed by their reaction with phenylhydrazine. The phenylhydrazine reagent (68) was made fresh each time by adding 10 ml of water to a mixture of 1 g of phenylhydrazine hydrochloride and 1.5 g of sodium acetate. After the mixture was dissolved, it was filtered directly into a 25-ml flask which contained 0.5 g of the carbohydrate. For the formation of this derivative, most carbohydrates require heating with the phenylhydrazine reagent while dicarbonyl compounds react with phenylhydrazine at room temperature. For sugars requiring heat for this reaction a small amount (1.25 ml) of a saturated solution of sodium bisulfite was added to prevent tarry products, and the flask was covered with a glass marble, was placed in a boiling water bath for 20 minutes. After the appearance of the yellow phenylosazone precipitate, the solution was cooled and allowed to stand at 4°C for 1 hour. In the case of lactose, the phenylosazone was soluble during the heating period and the

yellow precipitate did not appear until the cooling period. The precipitate was collected on filter paper using a Buchner funnel; and it was washed successively with water, 0.1 M acetic acid, water, 50% ethanol, and finally with 95% ethanol. The material was dried under vacuum in a desiccator. For recrystallization, 250 mg of the derivative was dissolved in 20 ml of absolute ethanol in a small beaker heated on a hot plate and under continuous stirring with a glass rod. Heating was continued to concentrate the solution by evaporation until crystallization started. Crystallization was allowed to proceed at 4°C for 24 hours. The crystals were filtered on a Buchner funnel and dried in a desiccator under vacuum.

2:4-Dinitrophenylosazones. The 2:4-dinitrophenylosazones of aldehydes and dicarbonyl-containing carbohydrates are formed at room temperature (69). Most carbohydrates, however, require heating for this reaction to take place. The reagent was prepared just prior to use, by adding 2 ml of concentrated sulfuric acid to 0.4 g of 2:4-dinitrophenylhydrazine (DNPH) followed by dropwise addition of 3 ml of water with stirring. After the solution was complete, 10 ml of 95% ethanol was added to the solution while still warm. A solution of the carbohydrate, prepared by dissolving 0.5 g of the sugar in 20 ml of 50% ethanol, was mixed with the freshly prepared DNPH reagent and the mixture was allowed to stand at room temperature or heated on a boiling water bath for aldoses until an orange-red precipitate appeared. After cooling the precipitate at 4°C for one hour, filtration was carried out on a Buchner funnel, and the precipitate was washed extensively with water to insure the removal of traces of sulfuric acid. The precipitate was then washed with 50% ethanol, 95% ethanol, and finally dried on the filter paper overnight in a desiccator under vacuum. Recrystallization was performed by suspending the crushed precipitate in about 30 ml of 95% ethanol and heating on a hot plate until solution took place. It was usually necessary to add some 5 to 15 ml of ethyl acetate to achieve complete solubilization. The solution was filtered while still hot and allowed to stand at 4°C overnight or until crystallization

was complete.

Reduction with Sodium Borohydride

The sugars were subjected to reduction with sodium borohydride using a 1:5 w/w proportion of sugar to borohydride in a boric acid-borax buffer of pH 7.6. In initial experiments, the reduction was carried out at 25°C for 2 hours and then continued at 4°C for another 2 hours. Some sugars, however, require longer treatment with borohydride for the reaction to proceed to completion (70). Best results were obtained when the temperature remained at 4°C and the reaction time was prolonged to 16 hours. At the end of this treatment, the solution was acidified with acetic acid to pH 6 and passed through a column of Dowex 50W-H^+. The eluate was concentrated to a syrup by rotary evaporation and the boric acid removed as volatile methyl borate as described previously.

CHAPTER III

RESULTS

Temperature plays an important role in the sugar transformations observed during borate ion-exchange chromatography of neutral sugars. When lactose was complexed with borate and chromatographed on Dowex 1-X8 (borate) columns at 25°C, a partial isomerization to the ketosugar lactulose was observed (15). In an attempt to increase the yield of lactulose, the column temperature was increased to 37°C. However, since the sugar recoveries from these columns were poor after borate elution, a solution of 0.1 M sodium bicarbonate was passed through the column, resulting in the elution of a third sugar-containing peak (Figure 1). This investigation is concerned with the sugar products derived from lactose found in this third peak.

Isolation and Characterization of Neutral Oligosaccharides Derived from Lactose

Prior to the examination of the various sugars present in the third peak mentioned above, it was necessary to remove the sodium bicarbonate and sodium tetraborate. The details of purification procedures for the sugar mixture are given in the section on methods. Briefly, this involved the removal of sodium ions with Dowex 50W-X8(H^+) and concomitant decomposition of $NaHCO_3$ and evolution of CO_2. Boric acid was then removed as volatile methyl borate by the addition of methanol and repeated evaporation under vacuum. The solution of the residual sugar material was quite acid (pH ca. 3.0). This low pH was probably due to sugar acids, which were removed on a column of Ag 1-X2 (formate). After rotary evaporation, the aqueous solution of the sugar mixture exhibited a

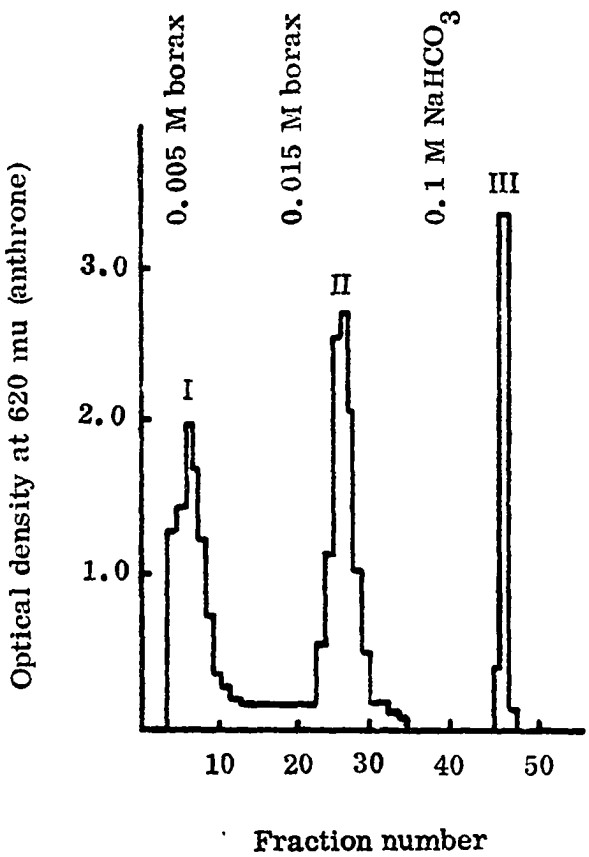


Figure 1. Elution pattern of a 500 mg sample of lactose subjected to ion-exchange chromatography on a column (1 x 18 cm) of Dowex 1-X8- (borate form, 50-100 mesh) at 37°C.

Lactose (peak I) was eluted with 0.005 M sodium tetraborate (borax), lactulose (peak II) was eluted with 0.015 M sodium tetraborate, and unidentified sugars (peak III) were eluted by 0.1 M $NaHCO_3$. Fraction volumes were ca. 75 ml each.

pH of approximately 6.0.

Oligosaccharides and Their Monosaccharide Composition

Oligosaccharide I. Two types of oligosaccharides were found in the sugar mixture eluted by the 0.1 M $NaHCO_3$ solution. One of them (oligosaccharide I) was visualized as a slow-moving, elongated purple spot (Figure 2) on paper chromatograms developed with solvent system 1 and treated with reagent A. In comparison to disaccharides (lactose and lactulose) the material appeared to migrate chromatographically as an oligosaccharide of disaccharide size or possibly larger.

Component monosaccharides. Identification of the monosaccharide units of this oligosaccharide posed some problems due to the extreme alkali lability of one component. This alkaline instability was detected because of discrepancies observed in the results of acid hydrolysis. When the carbohydrate material was hydrolyzed in sulfuric acid, 0.01 N and 0.5 N at 100°C for 2 hours, sulfate ions were removed by precipitation with barium hydroxide. Paper chromatography of the mild acid hydrolysate (0.01 N) showed two spots--a brown spot and a second spot which stained gray and migrated as galactose (Figure 3). Only the galactose spot was found in the 0.5 N acid hydrolysate. Acid hydrolysis with 1 N hydrochloric acid (100°C for 1 hour and for 6 hours), on the other hand, gave different results; paper chromatography showed the presence of another monosaccharide spot in addition to galactose (Figure 4). This new spot, like the undegraded oligosaccharide I, stained purple and was quite elongated. The difference in chromatographic pattern given by the HCl and H_2SO_4 hydrolysates was found to be due to the use of barium hydroxide to remove sulfate ions. While HCl was removed from hydrolysates by repeated evaporation in vacuo, saturated barium hydroxide was used to precipitate sulfate ions from H_2SO_4 hydrolysates. In a separate experiment (Figure 5) it was found that direct addition of $Ba(OH)_2$ at room temperature altered the oligosaccharide such that it lost its purple staining ability. Also the presence on the chromatogram

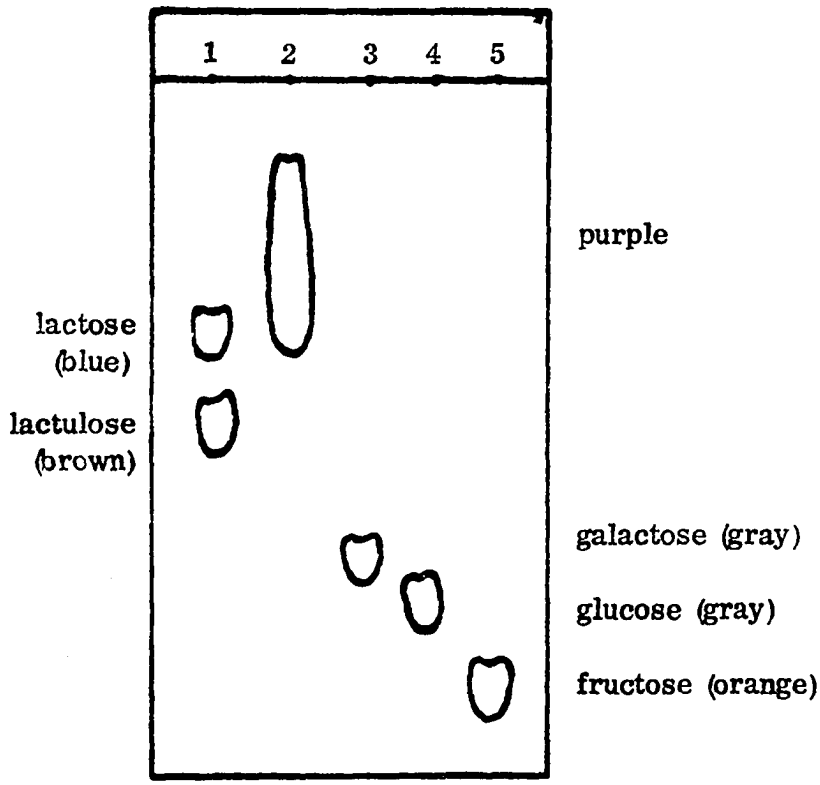


Figure 2. Paper chromatography of sugars eluted in peak III (with 0.1 M $NaHCO_3$) from borate ion-exchange column chromatography of lactose at 37°C.

(1) = standard mixture of lactose and lactulose

(3) = standard galactose

(4) = standard glucose

(5) = standard fructose

(2) = unidentified sugars from peak III of column

Chromatogram was developed with solvent system 1 and stained with reagent A.

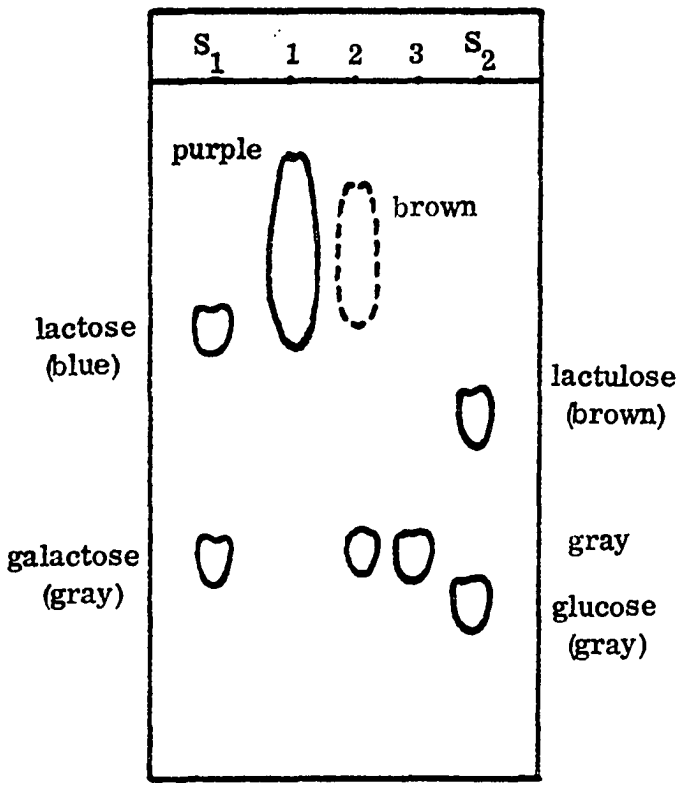


Figure 3. Paper chromatography of sulfuric acid hydrolysates of sugars eluted in peak III (with 0.1 M $NaHCO_3$) from borate ion-exchange column chromatography of lactose at 37°C.

(S_1) = standard mixture of lactose and galactose

(S_2) = standard mixture of lactulose and glucose

(1) = unidentified sugars from peak III of column before hydrolysis

(2) = (1) hydrolyzed in 0.01 N H_2SO_4 at 100°C for 2 hours

(3) = (1) hydrolyzed in 0.5 N H_2SO_4 at 100°C for 2 hours

Sulfate was precipitated from hydrolysates with $Ba(OH)_2$. Excess Ba^{2+} was removed as $BaCO_3$ by adding powdered Dry Ice. Chromatogram was developed with solvent system 1 and stained with reagent A.

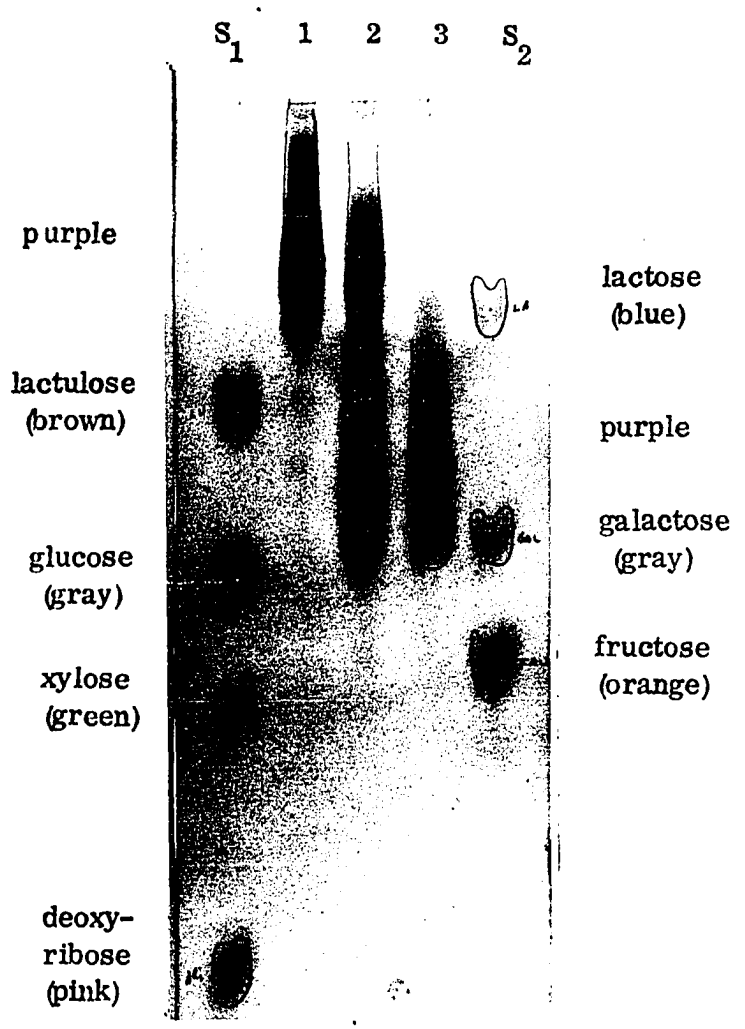


Figure 4. Paper chromatography of hydrochloric acid hydrolysates of sugars eluted in peak III (with 0.1 M $NaHCO_3$) from borate ion-exchange column chromatography of lactose at 37°C.

(S_1) = standard mixture of lactulose, glucose, xylose, and deoxyribose

(S_2) = standard mixture of lactose, galactose, and fructose

(1) = unidentified sugars from peak III of column before hydrolysis

(2) = (1) hydrolyzed in 1 N HCl at 100°C for 1 hour

(3) = (1) hydrolyzed in 1 N HCl at 100°C for 6 hours

HCl was removed from hydrolysates by repeated evaporation in vacuo. Chromatogram was developed with solvent 1 and stained with reagent A.

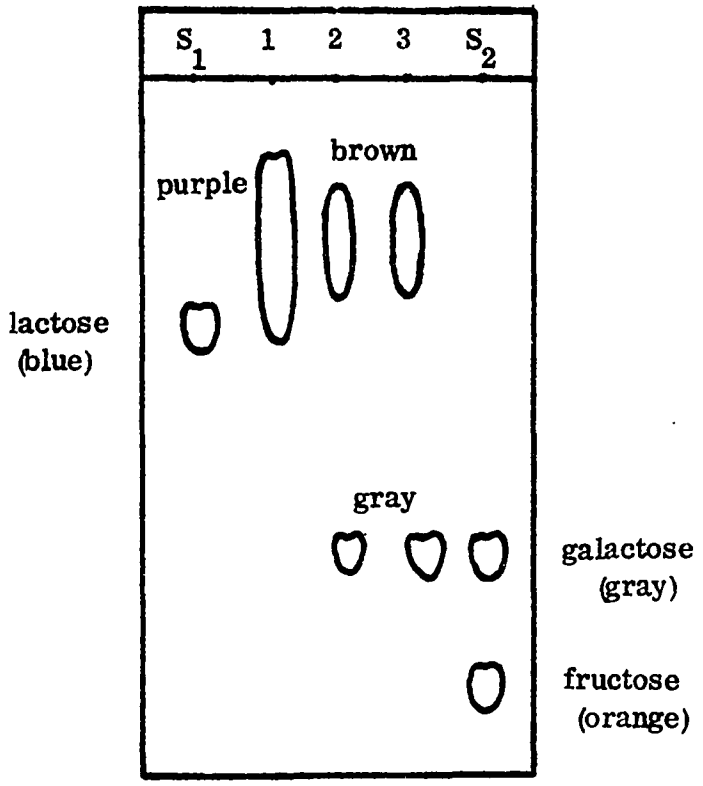


Figure 5. Paper chromatography of sugars derived from lactose which were treated either with 0.01 N H_2SO_4 or water at 25°C and then with sat. $Ba(OH)_2$.

(S_1) = standard lactose

(S_2) = standard mixture of galactose and fructose

(1) = unidentified sugars from peak III of column before treatment

(2) = (1) in 0.01 N H_2SO_4 for 10 minutes, sat. $Ba(OH)_2$ added to remove sulfate, and Dry Ice added to remove excess Ba^{2+}

(3) = (1) in water at 25°C for 10 minutes, sat. $Ba(OH)_2$ added, and Dry Ice added to remove Ba^{2+}

Chromatogram was developed with solvent system 1 and stained with reagent A.

(Figure 5) of what appeared to be galactose in the samples (in acid or water at 25°C) treated with $Ba(OH)_2$ suggested that release of galactose by beta-elimination might be taking place.

Oligosaccharide II. The presence of another oligosaccharide, staining brown with reagent A (aniline-diphenylamine) was established during the acid hydrolysis studies. Oligosaccharide II was not detected in Figure 2 because it was masked by the purple-staining oligosaccharide I. Both oligosaccharides had the same mobility with solvent system 1. However, oligosaccharide II can be seen unmasked in Figures 3 and 5 due to the experimental conditions described which resulted in decomposition of the purple-staining compound. Compared to oligosaccharide I, oligosaccharide II was alkali stable. These two oligosaccharides were resolved by paper chromatography of the undegraded material with other solvent systems. The best separation of these oligosaccharides was achieved by a solvent system containing butanol-pyridine-water (5:3:2, v/v) as shown in Figure 6.

Component monosaccharides. Paper chromatography of the HCl hydrolysate of oligosaccharide II, in Figure 6, revealed the presence of only one monosaccharide spot--galactose. No other reducing monosaccharide could be detected by paper chromatographic methods.

Studies of Molecular Size of Oligosaccharides I and II

Darco-Celite columns. Darco-Celite column chromatography was quite effective for the fractionation of the oligosaccharide mixture into classes according to molecular size. The sugar mixture, after elution from the column with distilled water and ethanol solutions of increasing concentrations (1%, 2%, 4%, 10% and 50%), was found to contain monosaccharides and oligosaccharides of several sizes (Figure 7). A peak of anthrone positive material was eluted with each solution; the largest quantity of sugar came out in the 10% ethanol fraction. Paper chromatography of a concentrate of each peak (Figure 8) demonstrated that monosaccharides--galactose, fructose, and the purple-staining

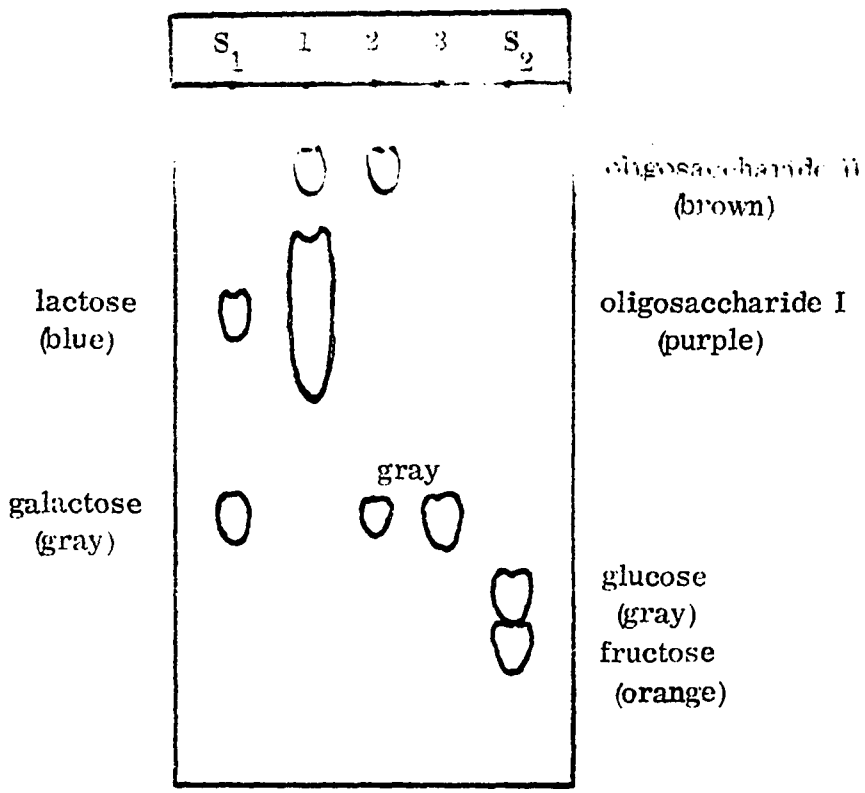


Figure 6. Chromatographic separation of sugars derived from lactose showing the presence of oligosaccharide I and oligosaccharide II eluted from the column in peak III (with 0.1 M $NaHCO_3$) followed by treatment of this oligosaccharide mixture with $Ba(OH)_2$ and 1 N HCl at 100°C respectively.

(S_1) = standard mixture of lactose and galactose

(S_2) = standard mixture of glucose and fructose

(1) = mixture of oligosaccharide I and oligosaccharide II

(2) = (1) in 0.01 N H_2SO_4 at 25°C for 10 minutes, sat. $Ba(OH)_2$ and Dry Ice added to remove sulfate and excess Ba^{2+} respectively

(3) = (2) followed by hydrolysis in 1 N HCl at 100°C for 2 hours and removal of HCl by evaporation in vacuo

Chromatogram was developed with solvent system 2 and stained with reagent A. Note disappearance of oligosaccharide I after $Ba(OH)_2$ treatment.

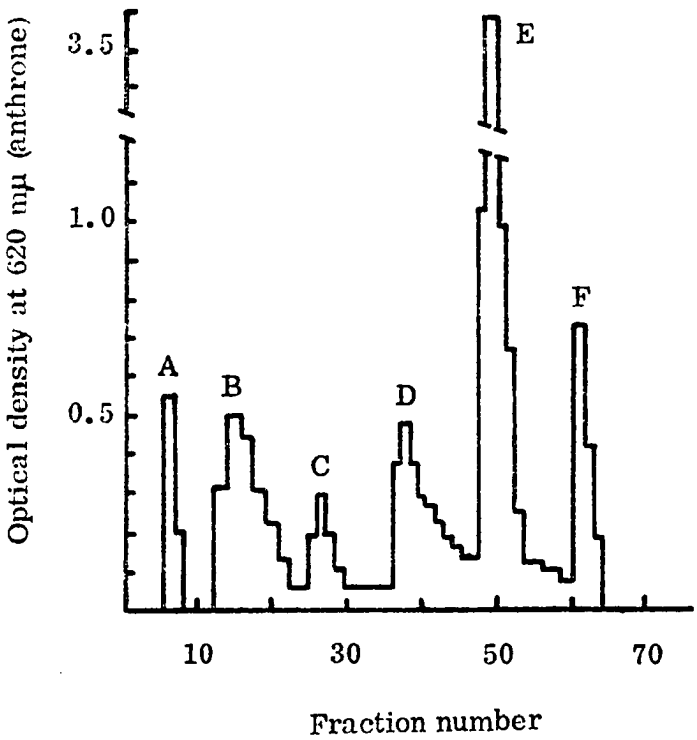


Figure 7. Darco 60-Celite column fractionation of sugars derived from lactose found in peak III eluted from a Dowex 1-X8 (borate) column with 0.1 M $NaHCO_3$.

A 200 mg sample (anthrone) was applied to the column (2 x 18 cm). Fractions were eluted with (A) = water, (B) = 1% ethanol, (C) = 2% ethanol, (D) = 4% ethanol, (E) = 10% ethanol, and (F) = 50% ethanol. Fraction volumes were 100-125 ml each.

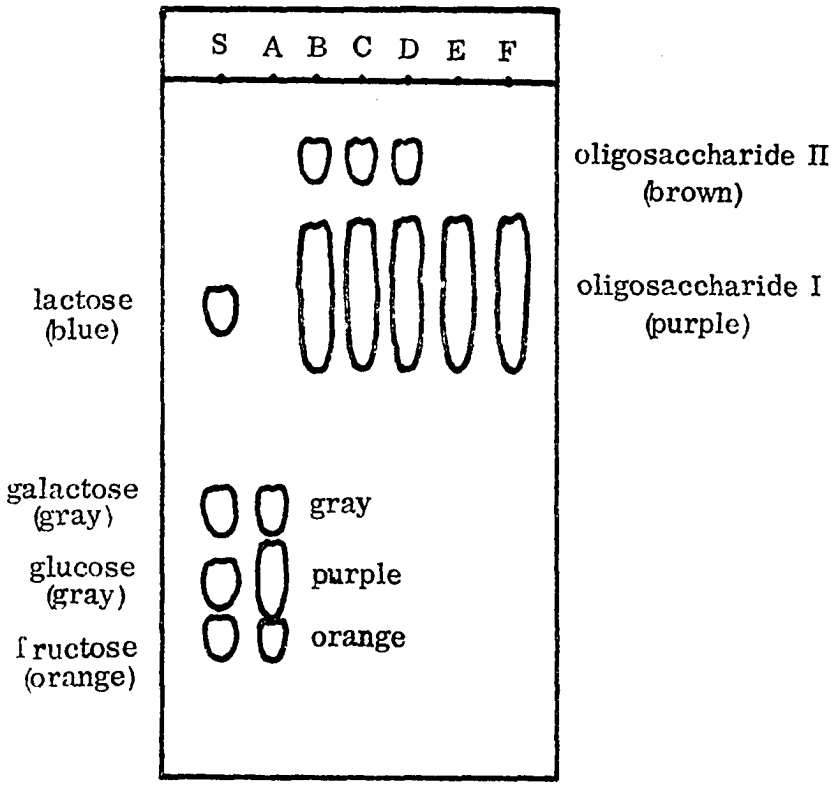


Figure 8. Paper chromatography of fractions (obtained by chromatography on a Darco 60-Celite column) of sugars in peak III derived from lactose.

Standards (S) = mixture of lactose, galactose, glucose, and fructose

Darco 60-Celite column fractions eluted with:

(A) = water

(B) = 1% ethanol

(C) = 2% ethanol

(D) = 4% ethanol

(E) = 10% ethanol

(F) = 50% ethanol

Chromatogram was developed with solvent system 2 and stained with reagent A. Note appearance of oligosaccharide II in fractions B, C, and D (staining brown).

monosaccharide component--were all found in the water fraction. Oligosaccharide I (purple) appeared in all alcohol fractions while oligosaccharide II (brown) appeared only in the 1, 2 and 4% fractions. According to Whistler and Durso (45), a solution of 5% ethanol is usually sufficient to displace disaccharides while ethanol solutions of concentrations greater than 5% are usually necessary to displace sugars larger than disaccharide size. It was concluded that oligosaccharide II was probably a disaccharide while oligosaccharide I might be a mixture of a disaccharide and larger oligosaccharides.

When a large Dowex 1-X8 (borate) column (3.5 x 35 cm) was used to chromatograph a large batch (20 g) of lactose, a longer elution time (3 weeks) was required, and the purple-staining product was not found in the effluent solution. Instead, only the brown-staining compound (oligosaccharide II) was obtained in this experiment. The relative proportion of oligosaccharides I and II found in the $NaHCO_3$ eluate appears to be affected by the time of contact between resin and sugar complexes as well as by the temperature of the column. For the preparation of large amounts of oligosaccharide I, which was selected for study in this dissertation, multiple small columns (instead of one large column) were utilized, and the process carried out at 25°C instead of 37°C. Under these conditions there was no formation of oligosaccharide II. As shown in Figure 9 a typical chromatogram of the Darco-Celite fractions reveals the absence of oligosaccharide II when the borate ion-exchange chromatography was done in small columns run at 25°C.

Sephadex G-15 columns. Gel filtration on Sephadex G-15 was employed to fractionate the purple-staining oligosaccharide mixture I according to molecular size. In an initial experiment, aliquots containing approximately 10 mg of sugar fractions B, E, and F from the Darco-Celite column chromatography were concentrated to 0.5 ml by rotary evaporation. Each sample was applied individually in separate experiments to the top of the column (2.2 x 90 cm) which was then eluted with degassed distilled water. The elution profile of each fraction is shown in Figure 10. Peak B (Darco-Celite 1% ethanol

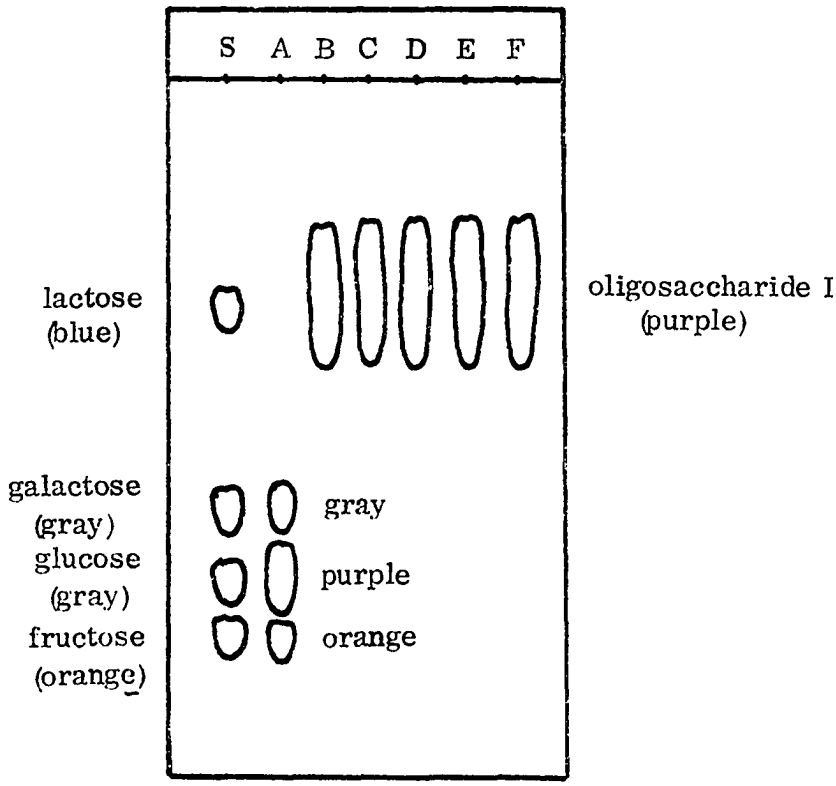


Figure 9. Paper chromatography of Darco 60-Celite column fractions from oligosaccharide I mixture derived from lactose on a borate ion-exchange column operated at 25°C instead of 37°C.

Standards (S) = mixture of lactose, galactose, glucose, and fructose

Darco 60-Celite column fractions eluted with:

(A) = water

(B) = 1% ethanol

(C) = 2% ethanol

(D) = 4% ethanol

(E) = 10% ethanol

(F) = 50% ethanol

The chromatogram was developed with solvent system 2 and stained with reagent A. Note that only oligosaccharide I appeared in fractions B through F; oligosaccharide II, which stains brown, was not produced at this temperature (25°C).

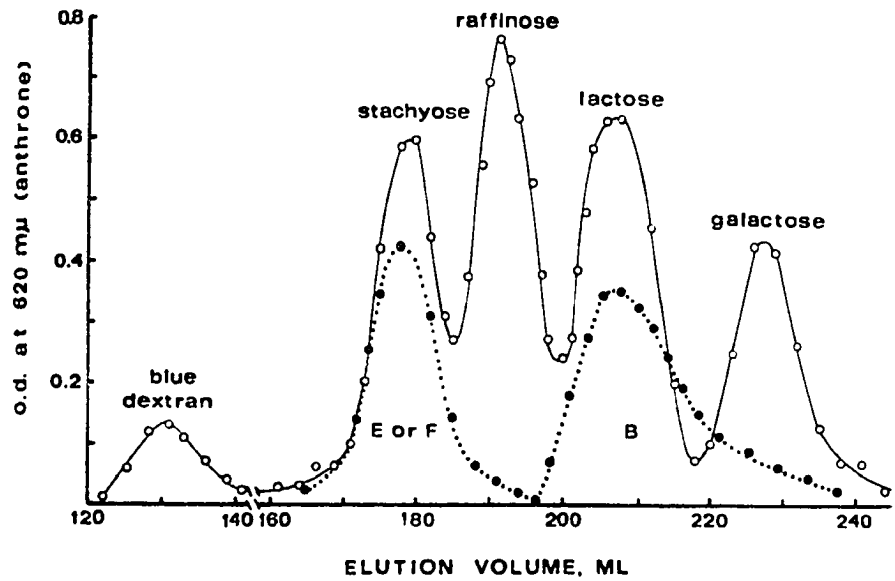


Figure 10. Elution patterns of Darco-Celite column sugar fractions subjected to gel filtration on a column (2.2 x 90 cm) of sephadex G-15 eluted with degassed distilled water. The solid line represents the pattern obtained with a mixture of standard sugars which contained 5 mg each of: galactose (monosaccharide), lactose (disaccharide), raffinose (trisaccharide), and stachyose (tetrasaccharide). The void volume is shown by the position of elution of Blue Dextran.

When applied separately to this column, Darco-Celite fractions E (10% ethanol) or F (50% ethanol) were eluted in a volume typical for a tetrasaccharide whereas Darco-Celite fraction B (1% ethanol) was eluted in a volume typical for a disaccharide.

fraction) was eluted in a volume typical for a disaccharide (lactose) while peaks E and F, the 10% and 50% ethanol fractions, respectively, were eluted in a volume typical for a tetrasaccharide (stachyose). Elution of the column with 0.1 M acetic acid yielded the same elution profile indicating lack of ionic interactions between the gel and the sugars.

To determine the molecular weight distribution of the sugars present in the 0.1 M $NaHCO_3$ eluate of the Dowex 1-X8 (borate) column, the entire sugar mixture was chromatographed directly on the Sephadex G-15 column, omitting the Darco-Celite column step. The elution pattern (Figure 11) shows that 58% of the applied sugar mixture was of tetrasaccharide size, 24% was of disaccharide size while the remaining 18% was of monosaccharide size. There was no indication of the presence of trisaccharide material. The amount of sugar eluted in each peak is expressed as galactose equivalents since the column was monitored by the anthrone reaction using galactose as the standard. Therefore, since the color intensity varies from sugar to sugar, these figures might not reflect the true percentage composition of the mixture. Since the results of the Sephadex G-15 fractionation of the sugars indicated only mono-, di-, and tetrasaccharides in the peak III from the Dowex column, the elution scheme of the Darco-Celite column was simplified so that, after removal of monosaccharides with water, the disaccharides were eluted with 4% ethanol while the bulk of the tetrasaccharide was eluted with 10% ethanol. A small amount of tetrasaccharide which remained on the column could be eluted with 50% ethanol. A typical elution pattern is shown in Figure 12.

Gas-liquid chromatography (glc). Qualitative analyses were carried out by glc techniques employing the procedure of Sweeley et al. (34). The trimethylsilyl (TMS) ether derivative of the purple-staining disaccharide isolated from oligosaccharide I mixture was chromatographed on a glass column packed with 3% SE-30 and maintained at 210°C under isothermal conditions as suggested (34) for sugars of this size. The major peak in the glc pattern of this sugar (Figure 13) had a disaccharide retention time of 17.4 minutes; two small additional peaks of unknown identity having retention values of 23.6 and 25.6 minutes

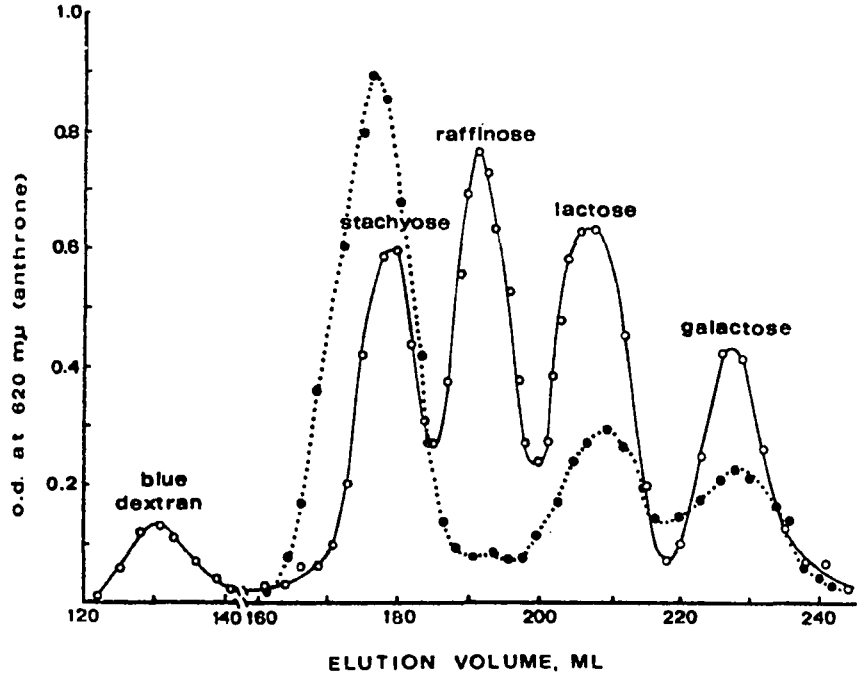


Figure 11. Fractionation on Sephadex G-15 of the mixture of sugars present in peak III from Dowex 1-X8 (borate) column. The mixture (dotted line) contained 21 mg of sugars (anthrone), and it was fractionated under the conditions described in Figure 10. The solid line represents the pattern obtained with a mixture of standard sugars which contained 5 mg each of: galactose (monosaccharide), lactose (disaccharide), raffinose (trisaccharide), and stachyose (tetrasaccharide). The void volume is shown by the position of elution of Blue Dextran.

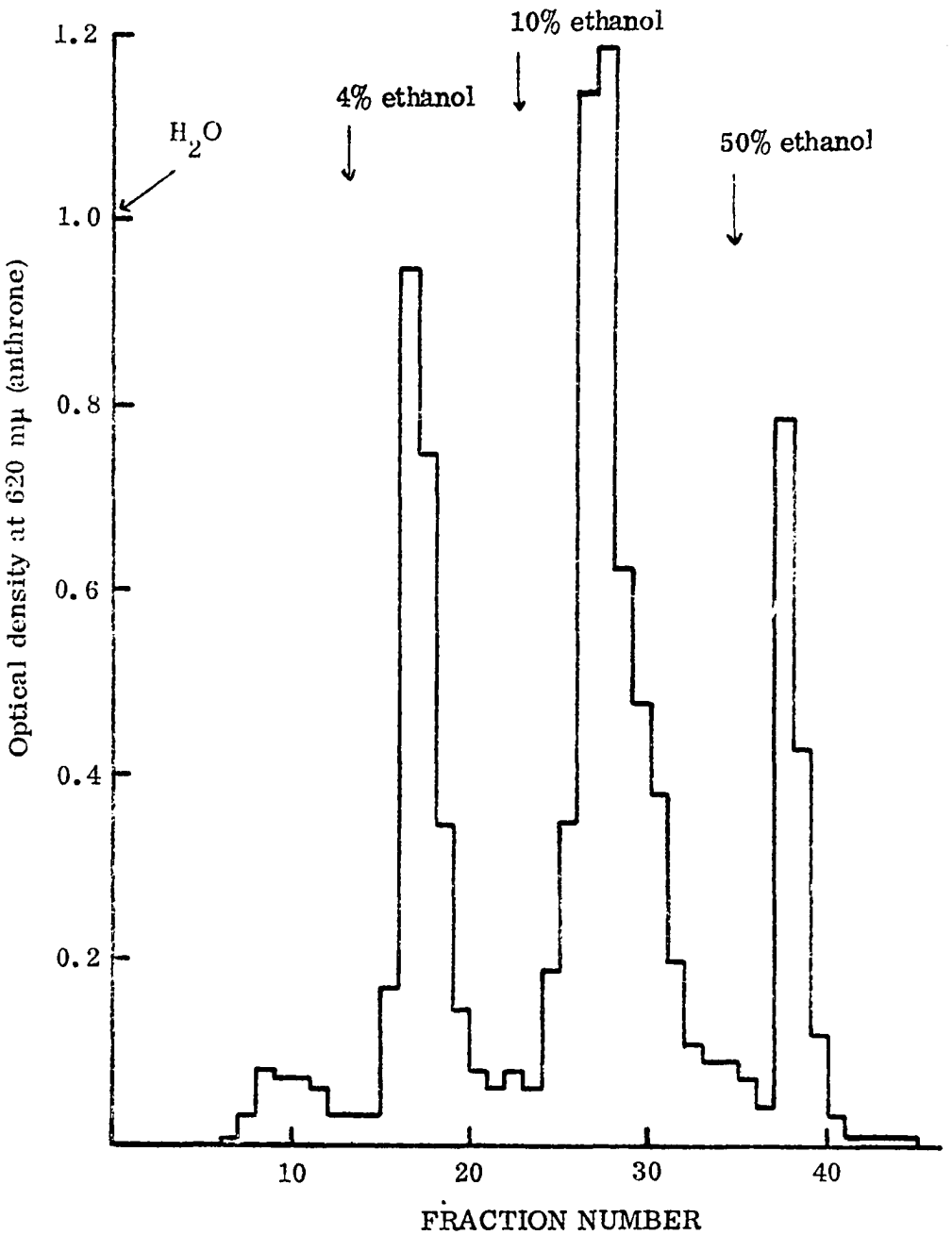


Figure 12. Elution profile of oligosaccharide I mixture on Darco 60-Celite, 1:1, column (3.2 x 20 cm). The mixture contained 200 mg (anthrone) of sugar from peak III of a Dowex 1-X8 (borate) column. The fraction volumes were approximately 100 ml each.

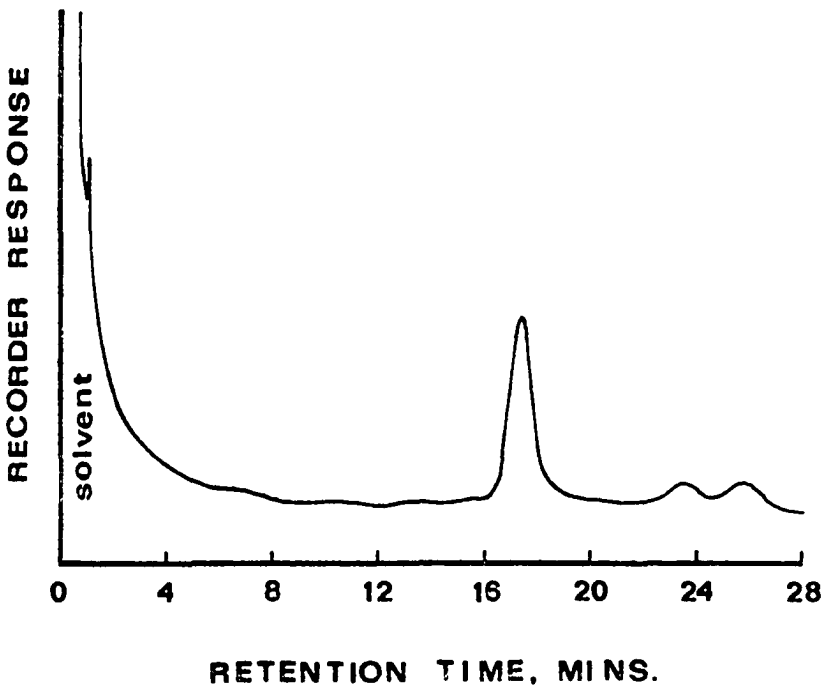


Figure 13. Gas-liquid chromatography of O-trimethylsilyl ether (TMS) derivative of disaccharide isolated from oligosaccharide I mixture. The column (3% SE-30) was maintained isothermally at 210°C. The T_R value of TMS-lactose under identical conditions was 17.8 minutes.

respectively were also detected. The retention time of a lactose reference standard (a single peak) was 17.8 minutes.

Paper ionophoresis. Paper ionophoresis of the oligosaccharide I mixture produced a somewhat elongated spot with migration similar to that of lactulose, $M_{glu} = 0.73$. When the disaccharide and tetrasaccharide were examined individually, they were found to have $M_{glu} = 0.75$ and $M_{glu} = 0.62$ respectively (Figure 14 and Table 1). However, a mixture of these compounds could not be resolved by this method due to the overlapping of spots which resulted in a single elongated spot. The ionophoretic migration of the isolated unknown monosaccharide component of the oligosaccharide I mixture was similar to that of glucose.

Chromatographic Characteristics of Oligosaccharides and Monosaccharide Components

Paper chromatography. The unidentified oligosaccharide I mixture was subjected to paper chromatography in a number of solvent systems. In all instances, the chromatograms were characterized by elongated spots (see Figures 2-6) suggesting the existence of the sugars in an equilibrium mixture of several forms. The results of the chromatographic studies with various solvent systems are shown in Table 2. Mobilities are expressed relative to that of standard glucose, i.e. R_{glu}, because the solvent front was always allowed to run off the paper. The best separation of glucose from the monosaccharide component was achieved with solvent system 1 containing ethyl acetate, acetic acid, and water. Solvent systems devoid of ethyl acetate and containing large proportions of phenol or butanol tended to increase the rate of migration of the monosaccharide relative to that of glucose. None of the solvent systems examined would allow the separation of the disaccharide from the tetrasaccharide due to the extreme elongation of spots on chromatograms.

Thin-layer chromatography (tlc). Carbohydrates being strongly hydrophilic require polar solvent systems for paper chromatography. Polar solvent systems, however, have relatively slow migration rates in tlc systems.

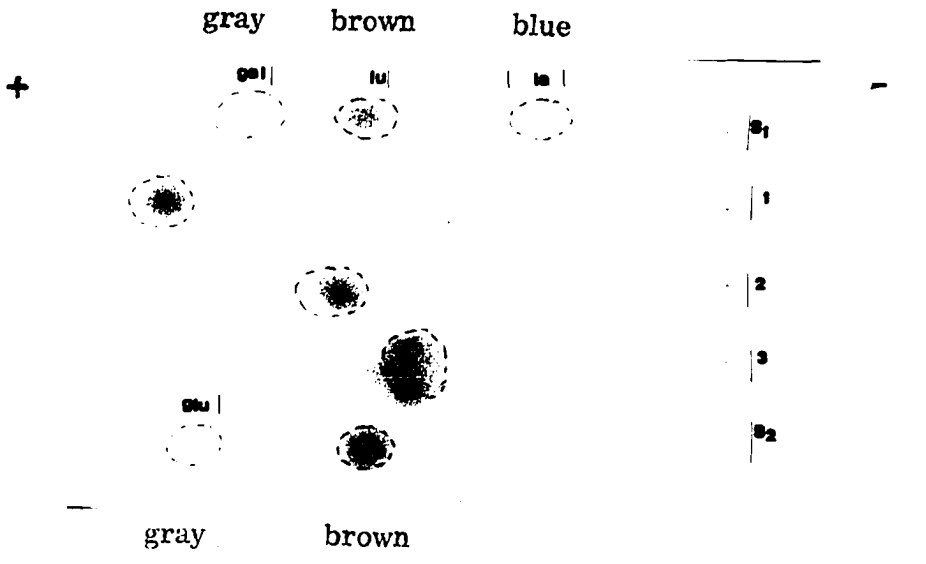


Figure 14. Ionophoretic migrations of sugars isolated from oligosaccharide I mixture derived from lactose by borate ion-exchange chromatography.

(S_1) = standard mixture of lactose (la), lactulose (lu), and galactose (gal)

(S_2) = standard mixture of lactulose and glucose (glu)

(1, 2, 3) = monosaccharide, disaccharide, and tetrasaccharide respectively, isolated from oligosaccharide I mixture and all staining purple

Ionophoresis performed in 0.05 M borax, pH 9.2, for 5 hours at 375 volts. After drying the paper was stained with reagent A.

TABLE I

IONOPHORETIC MIGRATION OF SUGARS ISOLATED FROM OLIGOSACCHARIDE I MIXTURE DERIVED FROM LACTOSE BY BORATE ION-EXCHANGE CHROMATOGRAPHY

Sugar	Mglucose[a]
Reference Standards:	
glucose	1.00
galactose	0.91
fructose	0.90
lactose	0.44
lactulose	0.71
Unknown Sugars:	
monosaccharide	1.06
disaccharide	0.75
tetrasaccharide	0.62

[a]The Mglucose values were calculated from uncorrected migration distances observed with 0.05 M borax, at 375 volts for 5 hours.

TABLE 2

PAPER CHROMATOGRAPHIC MOBILITIES OF SUGARS ISOLATED FROM OLIGOSACCHARIDE I MIXTURE DERIVED FROM LACTOSE BY BORATE ION-EXCHANGE CHROMATOGRAPHY

Solvent Number	Composition	(v/v)	Rglucose	
			Monosaccharide	Oligosaccharide I Mixture
1	Ea:A:W[a]	(3:3:1)	0.55	0.33
7	Ea:Et:W	(6:3:1)	0.73	N.D.[b]
5	Ph:W	(4:1)	0.77	0.86
2	Bu:Py:W	(5:3:2)	1.00	0.80
6	Bu:Et:W	(4:1:1)	1.12	N.D.
4	Bu:A:W	(4:1:5)	1.13	0.58
3	Bu:A:W	(4:1:1)	1.20	0.55

[a]Abbreviations are: Ea = ethyl acetate, A = glacial acetic acid, W = Water, Et = 95% ethanol, Ph = phenol, Bu = n-butanol, Py = pyridine.

[b]N.D. = not determined.

Hence, most solvent systems used for paper chromatography were of no use in tlc. The solvent systems examined in this study are listed in Table 3. The most effective solvent system was number 7 which contained ethyl acetate, ethanol, and water. Separation of the unknown monosaccharide (R_{glu} = 0.73) from glucose was achieved on plates developed for 90 minutes. Water-saturated butanol was not useful for paper chromatography but was found to be effective for tlc. The R_{glu} value for the unidentified monosaccharide in this system was 1.15. Two other solvent systems investigated were water-saturated ethyl acetate (71) and a more volatile system of benzene, ethanol, water, and acetic acid (72). Both of these solvent systems were ineffective for separation of the oligosaccharide mixtures and monosaccharide components.

The fact that the unidentified monosaccharide portion of the oligosaccharides exhibited relative mobilities so similar to glucose and galactose in nearly all systems suggested that this sugar was still of hexose size, i.e. that it had not undergone carbon-carbon scission during the borate ion-exchange treatment.

Gas-liquid chromatography (glc). This method can be used effectively not only to classify the sugar as to molecular size, but it can also give some information as to the nature of the sugar. Qualitative analysis of the monosaccharide was carried out by glc techniques employing trimethylsilyl (TMS) ether derivatives on a 3% SE-30 column. It was found that paper chromatographically purified preparations of the monosaccharide gave multiple peaks under these conditions. An aliquot (0.1 ml) of the monosaccharide solution (10 mg in 0.2 ml pyridine) was converted to the TMS derivative after 5 minutes (Figure 15A) and another aliquot after 17 hours (Figure 15B) in pyridine at 25°C. Seven peaks appeared in the glc pattern of the sugar which had been in pyridine for 5 minutes. Peak 6 was found to decrease considerably when the sugar had been in pyridine for 17 hours, prior to conversion to the TMS derivative.

Reaction with Selective Carbohydrate Detection Reagents

The reactions observed with a series of selective carbohydrate

TABLE 3

THIN-LAYER CHROMATOGRAPHIC MOBILITIES OF SUGARS ISOLATED FROM OLIGOSACCHARIDE I MIXTURE DERIVED FROM LACTOSE BY BORATE ION-EXCHANGE CHROMATOGRAPHY

Solvent Number	Composition	(v/v)	Rglucose	
			Monosaccharide	Oligosaccharide I Mixture
7	Ea:Et:W[a]	(6:3:1)	0.73	0.25
9	Bu:W	(water sat.)	1.15	0.35
10	Ea:W	(water sat.)	0	0
11	Bz:Et:W:A	(200:47:15:1)	0	0

Thin-layers of Silica gel G were utilized, and the chromatograms were developed for 90 minutes.

[a]Abbreviations are: Ea = ethyl acetate, Et = 95% ethanol, W = water, Bu = n-butanol, B_z = benzene, A = glacial acetic acid, sat. = saturated.

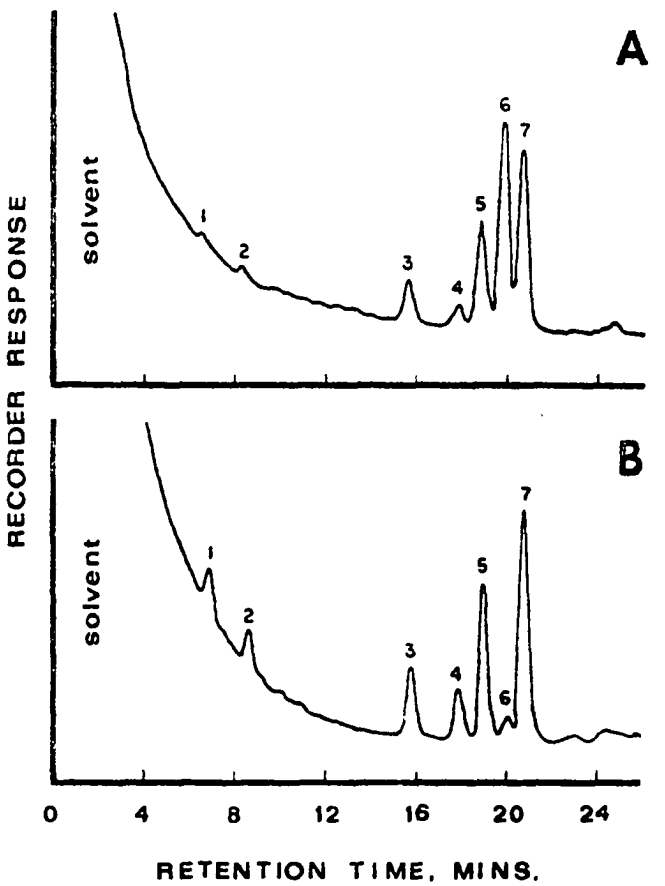


Figure 15. Gas-liquid chromatography of O-trimethylsilyl ether (TMS) derivative of monosaccharide isolated from hydrolysates of oligosaccharide I mixture derived from lactose. The solution of the sugar in pyridine was kept at 25°C for 5 minutes (A) and at 17 hours (B) prior to O-trimethylsilylation. The temperature of the column was programmed to start at 120°C and increase by 2°/minute. The T_R value of TMS-D-glucitol was 22.9 minutes under the programmed conditions.

detection reagents provided useful information concerning the structure and/or identity of the carbohydrates studied by paper chromatography, thin-layer chromatography, and paper ionophoresis. A summary of the results obtained with the various tests applied is shown in Table 4.

Amine reagents. The unknown sugars reacted readily with amine containing reagents (A, B and C). The reaction with aniline-diphenylamine (reagent A) is very specific yielding a purple color with both the monosaccharide and oligosaccharide I mixture (disaccharide and tetrasaccharide). On a dry weight basis, however, the same amount of disaccharide gave a much darker purple spot than the corresponding amount of tetrasaccharide. For detection purposes, the dry weight amount of sugar required for visualization on 3 MM paper, as in Figure 14, was 3 mg for the tetrasaccharide, 0.5 mg for the disaccharide and 1 mg for the monosaccharide.

Phenolic reagents. Phenols are also useful to detect ketoses. The naphtholresorcinol reagent (D) reacted, although rather weakly, with the unknown monosaccharide.

Furfural reagents. These reagents employ strong acid to produce furfural derivatives of the sugars which then combine with organic compounds to yield chromogens. The unknown monosaccharide did not react with p-anisidine (E) or with 3,5-dinitrosalicylic acid (F). The oligosaccharides, however, gave colors (green and brown respectively) with the latter two reagents which are due entirely to the galactose moiety of the molecule.

Anisaldehyde. This reagent gives a variety of colors with reducing sugars, is useful for the detection of α, β-unsaturated sugars, and detects furaldehyde-type degradation products of sugars. The unidentified monosaccharide and oligosaccharides I gave a brown color with this reagent (G).

Oxidizing reagents. The alkaline silver nitrate test (H) is perhaps the most sensitive test for carbohydrate derivatives and gives a permanent record of the chromatogram. Its main drawback is that it lacks specificity, reacting even with non-carbohydrate compounds. The unknown saccharides gave

TABLE 4

REACTION OF SUGARS ISOLATED FROM OLIGOSACCHARIDE I MIXTURE WITH SELECTIVE DETECTION REAGENTS

Reagent[a]	Contents	Monosaccharide[b]	Oligosaccharide I Mixture[c]
A	aniline-diphenylamine	purple	purple
B	aniline-phthalate	yellow	yellow
C	o-amino-biphenyl	yellow	tan
D	naphtholresorcinol	blue-green	tan
E	p-anisidine	none	green
F	3,5-dinitrosalicylic acid	none	brown
G	anisaldehyde	brown	brown
H	silver nitrate	+	+
I	periodate-benzidine	+	+

[a] Reagents were sprayed on TLC plates. Paper chromatograms and pherograms were dipped in the reagents.

[b] Monosaccharide-component of oligosaccharide I mixture subsequently identified as D-arabino-hexosulose.

[c] Mixture contained both disaccharide and tetrasaccharide sugars.

a positive reaction which was rather weak when compared to standard aldoses.

Glycol reagent. The periodate-benzidine reagent (I) is useful for polyols in general. A positive reaction was recorded indicating the presence of vicinal OH groups in the sugars under investigation.

From these results it appeared that the unidentified monosaccharide component of oligosaccharide I mixture was a ketose due to the yellow color with reagents B and C, listed in Table 4. In addition the monosaccharide did not react with furfural-dependent carbohydrate reagents (E and F).

Progressive Hydrolysis Studies of Oligosaccharide I Mixture

Acid hydrolysis. The release of galactose from acid degraded oligosaccharides was studied as a function of time (Figure 16). Changes in amounts of galactose, hexose, and reducing sugars were assayed by galactose oxidase, anthrone, and the Nelson-Somogyi methods respectively. Samples of the disaccharide and tetrasaccharide were analyzed by these methods before and during hydrolysis in 0.5 N sulfuric acid at 100°C for varying periods of time. The results of the galactose oxidase reaction which accounted for 23% of the initial dry weight before hydrolysis, increased to 50% after hydrolysis. The anthrone reaction (galactose standard) accounted for 48% of the total dry weight both before and after hydrolysis, while the reducing power of the hydrolysate accounted for about 50% of the initial dry weight (galactose standard). These results could be accounted for by the galactose portion of the tetrasaccharide. On the basis of this evidence, the unknown monosaccharide portion of the oligosaccharide appeared to be a non-reducing compound which did not react with the anthrone reagent.

Enzyme hydrolysis. The tetrasaccharide was treated with β-galactosidase from E. coli. Paper chromatography (solvent systems 1 and 2) of the enzyme hydrolysate of the tetrasaccharide revealed the appearance of both galactose and the purple-staining monosaccharide component found previously in acid hydrolysates (Figure 17). The galactose portion of the original lactose was apparently still intact and connected by a β glycosidic linkage to an unidentified

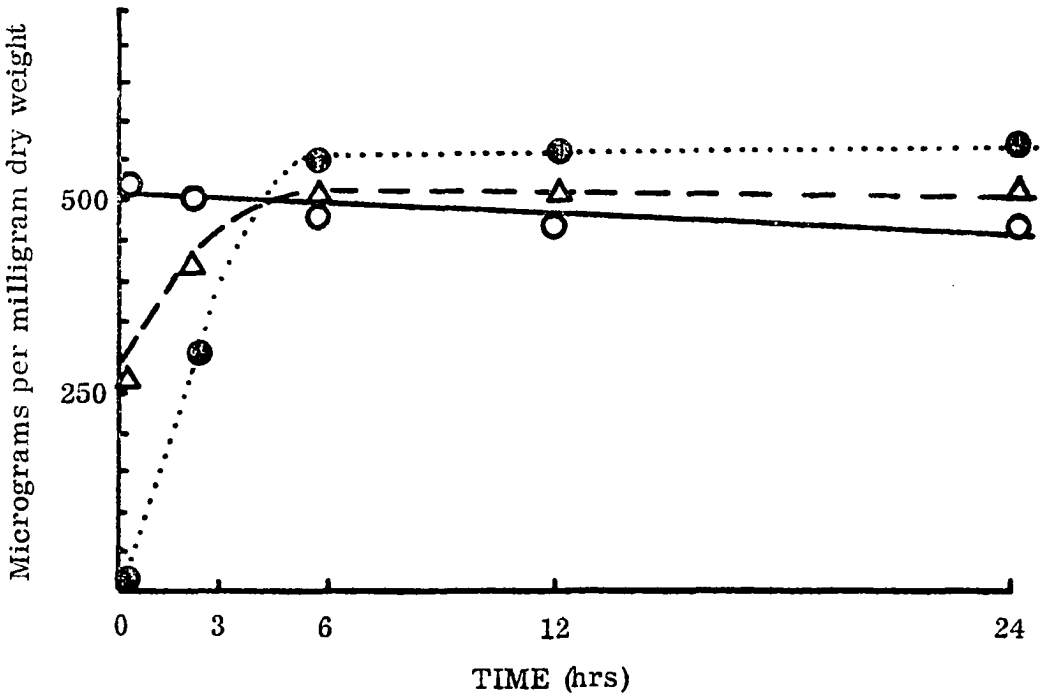


Figure 16. Release of monosaccharides by progressive acid hydrolysis of the tetrasaccharide isolated from oligosaccharide I mixture (derived from lactose by borate ion-exchange chromatography). The hydrolysis was conducted in 0.5 N sulfuric acid at 100°C for varying periods of time. The hydrolysates were analyzed for galactose by the Galactostat method (△), for hexose by the anthrone method (○), and for reducing power by the Nelson-Somogyi method (●). The disaccharide produced the same results.

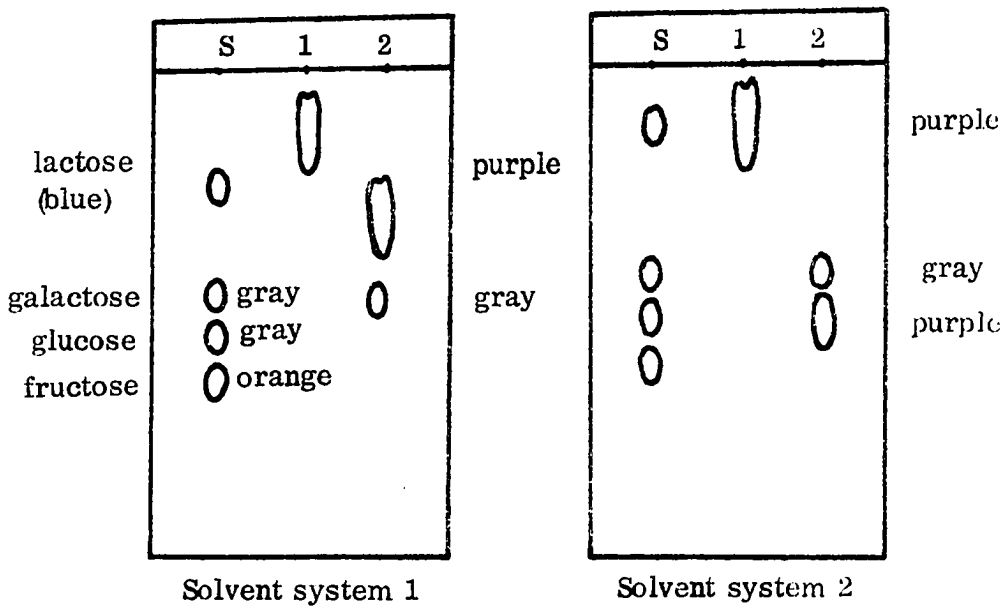


Figure 17. Paper chromatography of products released by β-galactosidase digestion of tetrasaccharide isolated from oligosaccharide I mixture (derived from lactose by borate ion-exchange chromatography).

(S) = standard mixture of lactose, galactose, glucose, and fructose

(1) = tetrasaccharide

(2) = tetrasaccharide hydrolyzed with β-galactosidase for 24 hours at 37°C

Chromatograms were developed with solvent systems 1 or 2, as shown, and stained with reagent A.

monosaccharide which did not react with the Nelson-Somogyi nor with the anthrone reagent.

Functional Group Analysis

Qualitative analyses were carried out on both the undegraded oligosaccharides and the unidentified monosaccharide component to provide some insight as to the presence or absence of specific functional groups.

Unsaturation. Both the tetrasaccharide and monosaccharide were treated with bromine water for 3 hours and 16 hours as described under "Methods." Since these compounds consumed less bromine than either galactose or lactose, the test for unsaturation was considered negative.

Lactone. A specific hydroxamic acid test for sugar lactones was applied to a paper chromatogram of the unknown saccharides. Standard D-glucurono-lactone, run on the same chromatogram, gave a typical purple color, but a negative test was obtained with all of the unknown saccharides. As a check on the paper chromatographic method, a qualitative hydroxamic acid test in solution was also performed again with negative results.

Anhydro-sugars. The resorcinol color reaction, used for the quantitative determination of 3,6-anhydrogalactose, was performed on the unknown monosaccharide and tetrasaccharide with negative results.

Reductone. Enediol compounds with unusual reducing power, such as ascorbic acid, are classified as reductones (73) by their reaction with dichlorophenolindophenol (Tillman's reagent). The unknown sugars (a disaccharide-tetrasaccharide mixture and the monosaccharide alone) were tested under neutral, acid (0.03 N HCl), and alkaline (0.04 N NaOH) conditions with negative results.

Another test for reductones with methylene blue under the same conditions was also tried, but negative results were obtained.

Enolic group. The unknown sugars were tested for enol content with 1% ferric chloride reagent, but no color indicative of an enol was obtained. However, since many enol compounds do not give a positive test with this reagent,

a negative result could not be considered conclusive.

Reducing power. The negative results obtained with functional group tests, combined with the failure of the unknown sugars to show any reducing power with the Somogyi-Nelson reagent, narrowed the possibilities for the unknown monosaccharide. However, the extreme lability of the oligosaccharide in alkali at room temperature casted some doubt on the interpretation of the results obtained with the Nelson-Somogyi test which is run in alkali at 80°C, and this pointed out the need to re-examine the reducing power using milder conditions. When the ferricyanide reduction method by Borsook et al. (52) for detection of fructose-amino acids was tried on the unknown saccharides they were found to exhibit strong reducing power at room temperature. In this method the sugar is treated with 0.1% potassium ferricyanide in 0.1 N sodium hydroxide for 5 minutes at room temperature followed by addition of the acidic ferric sulfate reagent. Reduction of ferricyanide to ferrocyanide is revealed by the development of the Prussian Blue color. Similar results were obtained with the improved ferricyanide method of Park-Johnson which was subsequently adopted for quantitative studies. In addition it was found that these saccharides reduced Fehling's cupric reagent at room temperature; a similar reaction was observed with solutions of ascorbic acid. Both compounds gave a red precipitate of cuprous oxide in 5 minutes at 25°C. The strong reducing power of the unknown sugars suggested the presence of more than one carbonyl group in their structure.

Dicarbonyl function. A spot test for the presence of aliphatic 1,2-dioxo groups, based on the formation of nickel dioxime, was negative when applied to the unidentified oligosaccharides while an intense pinkish color was observed with diacetyl.

Another spot test for dicarbonyl function, the condensation reaction with thiophene, gave a negative test with the oligosaccharides while ninhydrin gave a dark blue color.

Compounds having dicarbonyl function react readily with phenylhydrazine reagents even at room temperature. Both the oligosaccharide sugars

and the unidentified monosaccharide component gave a red-orange precipitate with the 2,4-dinitrophenylhydrazine reagent within 5 minutes at room temperature. Precipitates with glucose or galactose appeared only after 10 minutes in a boiling water bath. A study of these 2,4-dinitrophenylhydrazine derivatives was conducted by thin-layer chromatography according to the method of Anet (74). Samples of derivatives of the unknown sugars along with the corresponding derivative of glycolaldehyde, were dissolved in acetone (0.1%, w/w). Approximately 5 µl of each sample was applied to the plate and development was carried out in solvent system 8 for a period of 30 minutes. The derivatives could be visualized during the development as yellow spots on the plate. After drying, the plate was treated with a spray reagent consisting of 2% sodium hydroxide in 90% ethanol. The spots corresponding to the unknown compounds turned a blue or purple color while the glycolaldehyde spot gave a brown color. According to Anet, the blue or purple color is specific for 1,2-bis derivatives while the brown color is specific for mono-derivatives.

Identification of Saccharides Derived from Lactose

Synthesis of Reference Compounds

Monosaccharides. The most likely candidates for monosaccharide reference compounds were aldosuloses, known to give elongated spots on chromatograms (75). The dicarbonyl compounds are formed as by-products during both acid and alkaline degradation of sugars and from the Maillard reaction of reducing sugars with amines. Among these compounds, 3-deoxy-D-erythro-hexosulose (3-deoxy-D-glucosone) has been widely investigated. Its preparation was attempted first by a somewhat simplified sugar-amine reaction. As described under "Methods", glucose was reacted with leucine to form a fructosyl-amino acid which was then treated with arsenous trioxide. A small amount of the product, 3-deoxy-D-erythro-hexosulose, was demonstrated in the final reaction mixture. A chromatogram of the final products treated with reagent A (aniline-diphenylamine) showed a blue-staining spot of 3-deoxy-D-erythro-hexosulose and a considerable amount of unreacted glucose; the typical elongated

purple spot characteristic of the unidentified monosaccharide was absent. In another method, glucose is reacted with n-butylamine, to form the N-glycoside (47) which degrades to 3-deoxy-D-erythro-hexosulose upon treatment with acetic acid. Also formed are the epimeric aldose, mannose, and some furfural derivatives. Thin-layer plates were prepared to visualize the reaction products (Figure 18). Seven products were visible and their R_{glu} values and staining characteristics are noted in Table 5. The major products on the plate were labelled b and e. From their migration and staining properties products b, c, and d were identified as glucose, fructose, and mannose respectively. Products a, e, and f all gave elongated spots, but product a stained purple while products e and f gave blue and gray colors respectively with aniline-diphenylamine. The mobility and staining property of product e was found to be identical to that obtained with an authentic sample of 3-deoxy-D-erythro-hexosulose generously donated by Dr. Roger Rowell. The marked difference between the R_{glu} of 2.77 for 3-deoxy-D-erythro-hexosulose shown in Table 5 and the value of 1.3 reported by Rowell et al. (22) is due to a difference in the thin-layer support[1]. These workers did not mention the presence in the reaction mixture of the slower migrating compound which stained purple with reagent A. However, it was revealed by personal communication that "two very minor spots at R_{glu} = 0.9 and 1.9" were found. Perusal of the original publication of Kato (47) revealed a footnote which referred to the fact that the fractions were "contaminated by a considerable amount of an osazone-forming substance" which they thought might be glucosone. A literature search produced a subsequent paper by Kato (63) where the identification of D-glucosone among the products of this reaction was confirmed. Synthesis of D-glucosone (D-arabino-hexosulose) was then carried out on a larger scale by Kato's method, (63) and the compound was separated and purified by preparative paper chromatography in solvent system 1 which provides the best

[1] Dr. Rowell stated in a private communication that tlc plates coated with silica gel H were used in his studies of 3-deoxy-D-erythro-hexosulose. The tlc plates used in this investigation, obtained from Mann Laboratories, were precoated with silica gel G.

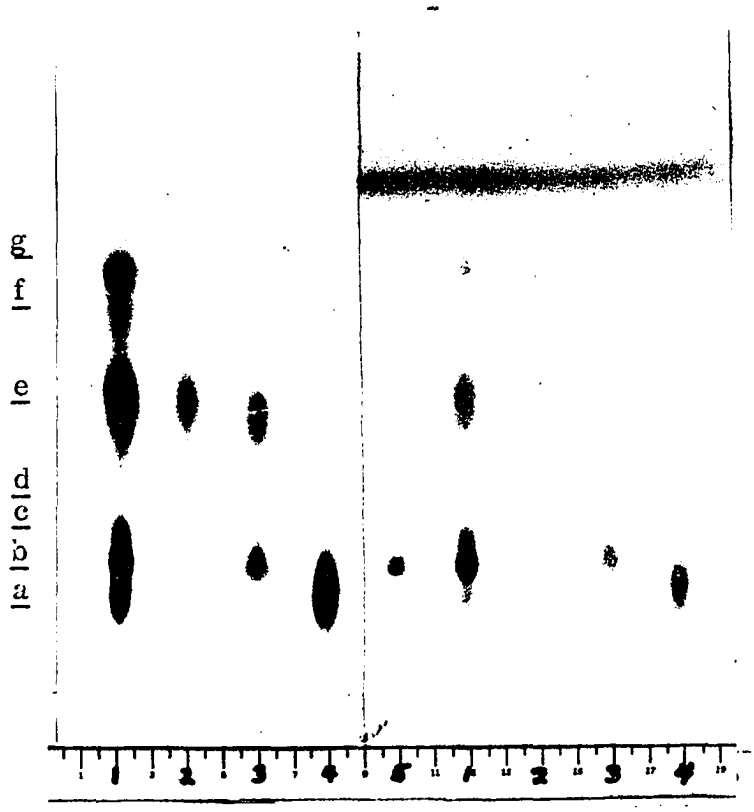


Figure 18. Silica gel G thin-layer chromatography of reference standards and products of the reaction between D-glucose and n-butylamine in the synthesis of 3-deoxy-D-erythro-hexosulose.

(1) = reaction product mixture showing 7 products lettered a to g

(2) = authentic 3-deoxy-D-erythro-hexosulose

(3) = impure 3-deoxy-D-erythro-hexosulose (containing glucose)

(4) = monosaccharide (from oligosaccharide I mixture derived from lactose)

(5) = standard of glucose

Plate was developed with solvent system 7 and sprayed on the left side with reagent A and on the right side with reagent H.

TABLE 5

CHROMATOGRAPHIC ANALYSIS OF PRODUCTS FOUND IN THE REACTION MIXTURE OF D-GLUCOSE AND n-BUTYLAMINE DURING SYSTHESIS OF 3-DEOXY-D-erythro-HEXOSULOSE

Spot	Rglucose[a]	Color[a]	Identification
a	0.73	purple	unknown[b]
b	1.00	blue-gray	glucose
c	1.23	orange-pink	fructose
d	1.41	blue-gray	mannose
e	2.77	blue-gray	3-deoxy-D-erythro-hexosulose
f	3.56	gray	unknown
g	4.27	blue-gray	unknown

[a]Data obtained from thin-layer plate shown in Figure 23.

[b]Mobility and staining of this unknown is identical to unidentified monosaccharide produced during borate ion-exchange chromatography of lactose.

separation from glucose. A sample of this purified substance was found to co-chromatograph with the unknown monosaccharide on paper with solvent system 1, 2 and 7, on tlc plates with solvent systems 7 and 9, and by borate paper ionophoresis.

A more specific method for the preparation of aldosuloses is by direct oxidation of hexoses with copper acetate or selenium dioxide (see "Methods"). In the first experiment, D-arabino-hexosulose was prepared by treating glucose or fructose with an aqueous saturated solution of copper acetate at 50°C. In order to cut down on unwanted by-products, the reaction with glucose was allowed to proceed at room temperature (25°) for varying periods of time. Aliquots were taken at 24 hour intervals over a period of 4 days, and the products were checked by paper chromatography and paper ionophoresis. Optimal amounts of the main product, an elongated spot staining purple with reagent A appeared at 24 hours. This compound was found to co-chromatograph in all solvent systems mentioned above with D-arabino-hexosulose, and with the unknown monosaccharide component of oligosaccharide I derived from lactose. A drawback of the copper acetate in the original method was the presence of large amounts of copper salts at the end of the reaction; however, the sugar was easily recovered from the mixture after removal of the copper with Dowex 50(H^+) ion-exchange resin. The production of D-arabino-hexosulose from fructose was demonstrated in two separate experiments. The first using the copper acetate method was carried out at 25°C for 24 hours. This was followed by an experiment using fructose and selenium dioxide by the method of Dixon and Harrison (65). Fructose was heated in an aqueous solution of selenous acid for 1 hour under reflux. Paper chromatography of the products obtained in these three experiments can be seen in Figure 19.

The third method of preparation of aldosuloses employs phenylhydrazine sugar derivatives. Fructose was converted to fructosephenylosazone (same compound as glucosephenylosazone) by treatment with phenylhydrazine reagent. Liberation of the dicarbonyl compound, D-arabino-hexosulose, was

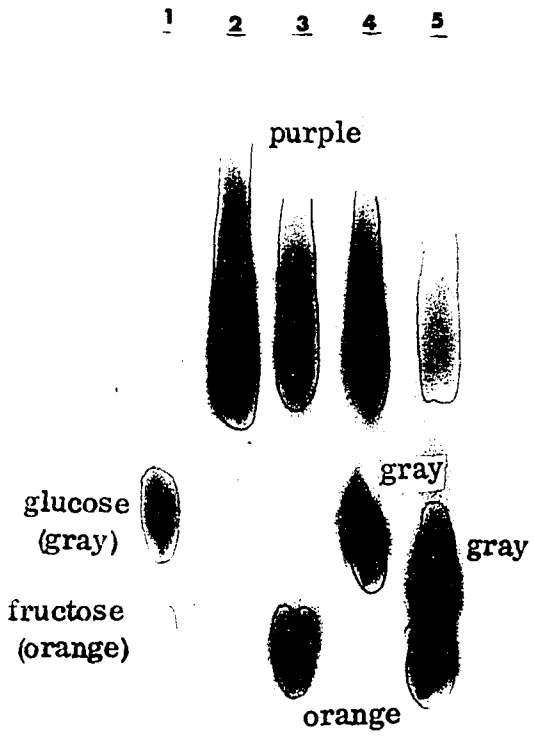


Figure 19. Paper chromatography of reference compound, D-arabino-hexosulose, prepared from direct oxidation of glucose or fructose.

(1) = standard mixture of glucose and fructose

(2) = Monosaccharide from oligosaccharide I mixture derived from lactose

(3) = D-arabino-hexosulose from the reaction of fructose with copper acetate

(4) = D-arabino-hexosulose from the reaction of glucose with copper acetate

(5) = D-arabino-hexosulose from the reaction of fructose with selenium dioxide

Chromatogram was developed with solvent system 1 and stained with reagent A.

achieved by the action of nitrous acid at 40°C. The reaction products were examined chromatographically on paper and on thin-layer plates and by paper ionophoresis. The major product, D-arabino-hexosulose, stained purple with reagent A and co-chromatographed with the monosaccharide component of oligosaccharide I mixture (derived from lactose) in solvent systems 1, 2, 7 and 9 and by borate paper ionophoresis.

Figure 20 is a photographic reproduction of a paper chromatogram showing the identity in mobility, shape, and color reaction of the spots of the monosaccharide under study and of reference samples of D-arabino-hexosulose prepared by three methods: the reaction of glucose with n-butylamine; the reaction of glucose with a saturated solution of copper acetate; and the reaction of fructosephenylosazone with nitrous acid. Figure 21 depicts a typical borate ionophoresis study of the same compounds examined in Figure 20.

Disaccharide reference compounds. The methods used for the preparation of D-arabino-hexosulose and 3-deoxy-D-erythro-hexosulose were tested using lactose as the starting material. The procedure involving treatment with leucine and arsenous trioxide was unsuccessful. The method based on the reaction of n-butylamine gave low yields. However, most of the unreacted lactose was crystallized out at 4°C leaving a final product which was identified as a mixture of lactose and lactosone.

Preparation of lactosone by the oxidation of lactose with copper acetate was not successful. Since the preparation of lactosone from lactosephenylosazone had been described by Fischer in 1888 (76), we followed his procedure except for the decomposition of lactosephenylosazone to liberate lactosone. This was performed by a nitrous acid modification proposed by Ohle (66) for the preparation of D-arabino-hexosulose (D-glucosone). Figure 22 shows the paper chromatographic migration of the disaccharide isolated from oligosaccharide I mixture and of lactosone prepared both by the butylamine and by the osazone methods; the chromatogram was developed with solvent system 1. The ionophoretic migrations of these compounds are shown in Figure 23.

Since the chromatographic and electrophoretic evidence shown

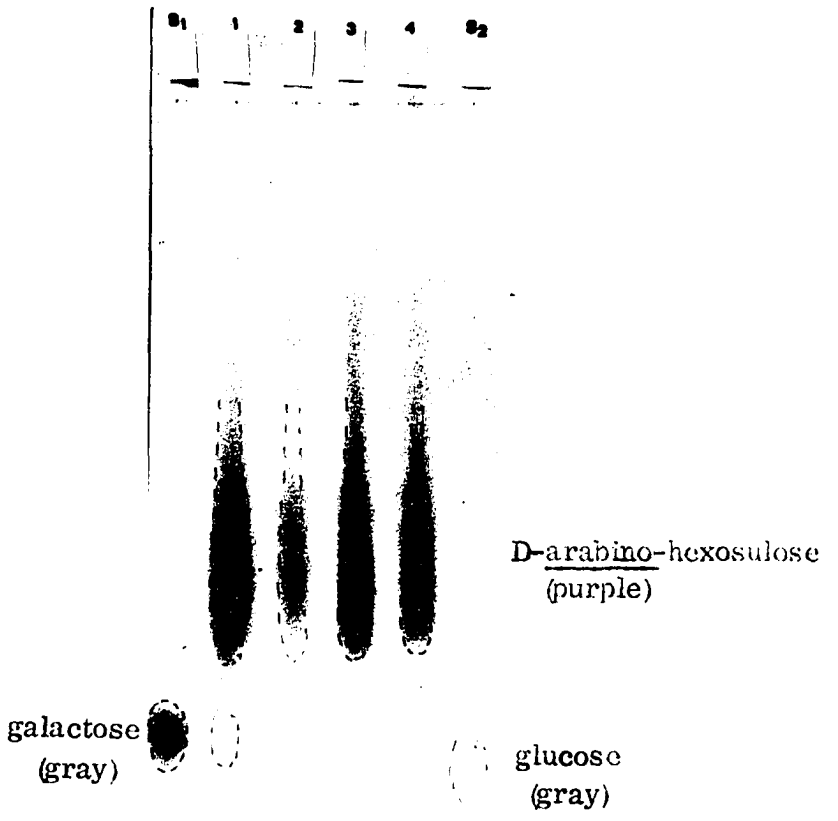


Figure 20. Paper chromatography of the monosaccharide isolated from hydrolysates of oligosaccharide I mixture (derived from lactose) and reference compound, D-arabino-hexosulose, prepared from glucose and fructose by three different methods.

(S_1) = standard galactose

(S_2) = standard glucose

(1) = D-arabino-hexosulose from oligosaccharide I mixture (contaminated with galactose)

(2) = D-arabino-hexosulose from the reaction of glucose with n-butylamine

(3) = D-arabino-hexosulose from the reaction of glucose with copper acetate

(4) = D-arabino-hexosulose from the reaction of fructosephenylosazone with nitrous acid

Chromatogram was developed with solvent system 1 and stained with reagent A.

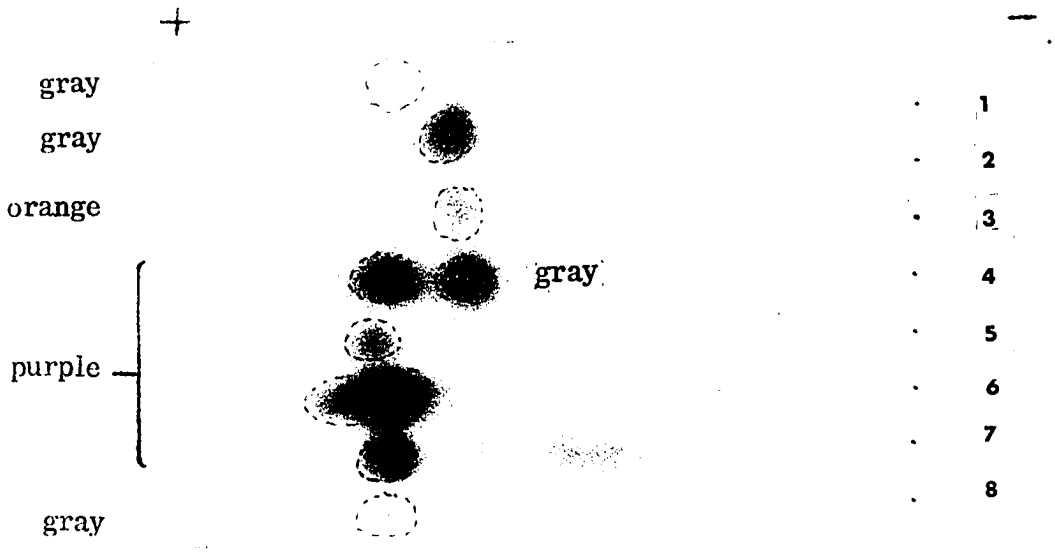


Figure 21. Ionophoretic migration of monosaccharide isolated from oligosaccharide I mixture (derived from lactose) and reference compound, D-arabino-hexosulose, prepared from glucose and fructose by three different methods.

(1, 8) = standard glucose

(2) = standard galactose

(3) = standard fructose

(4) = D-arabino-hexosulose from oligosaccharide I mixture (contaminated with galactose)

(5) = D-arabino-hexosulose from the reaction of glucose with n-butylamine

(6) = D-arabino-hexosulose from the reaction of glucose and copper acetate

(7) = D-arabino-hexosulose from the reaction of fructosephenylosazone with nitrous acid

Ionophoresis was performed in 0.05 M borax, pH 9.2, for 5 hours at 375 volts. After drying, the paper was stained with reagent A.

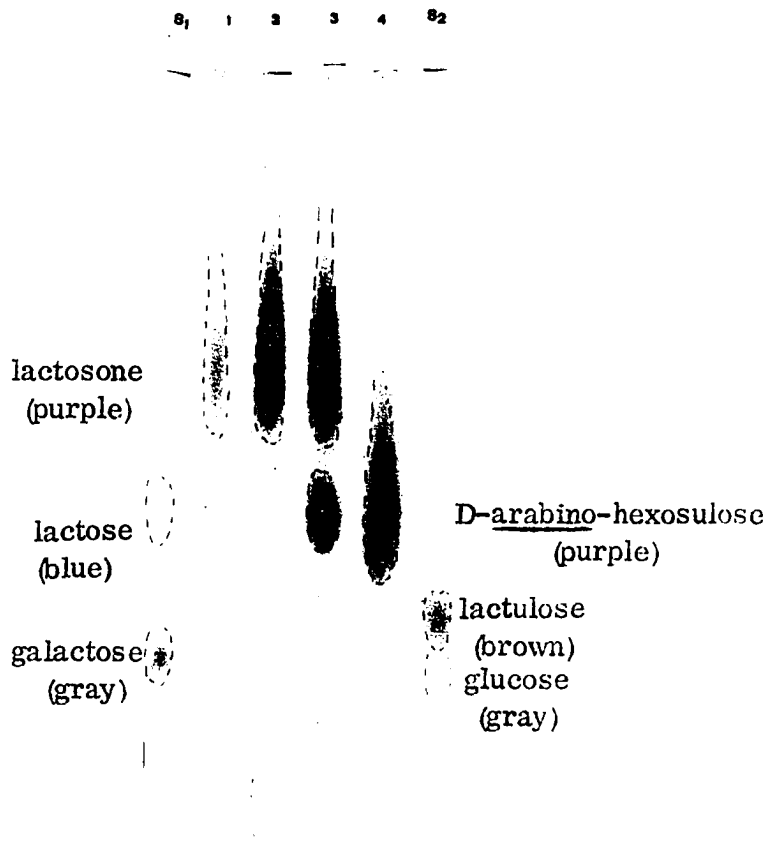


Figure 22. Paper chromatography of the disaccharide component from oligosaccharide I mixture (derived from lactose by borate ion-exchange chromatography) and reference compound, lactosone, prepared from lactose by two different methods.

(S_1) = standard mixture of lactose and galactose

(S_2) = standard mixture of lactulose and glucose

(1) = disaccharide component from oligosaccharide I mixture

(2) = lactosone from the reaction of lactosephenylosazone with nitrous acid

(3) = lactosone from the reaction of lactose with n-butylamine

(4) = monosaccharide, D-arabino-hexosulose, isolated from hydrolysate of oligosaccharide I mixture

Chromatogram was developed with solvent system 1 and stained with reagent A.

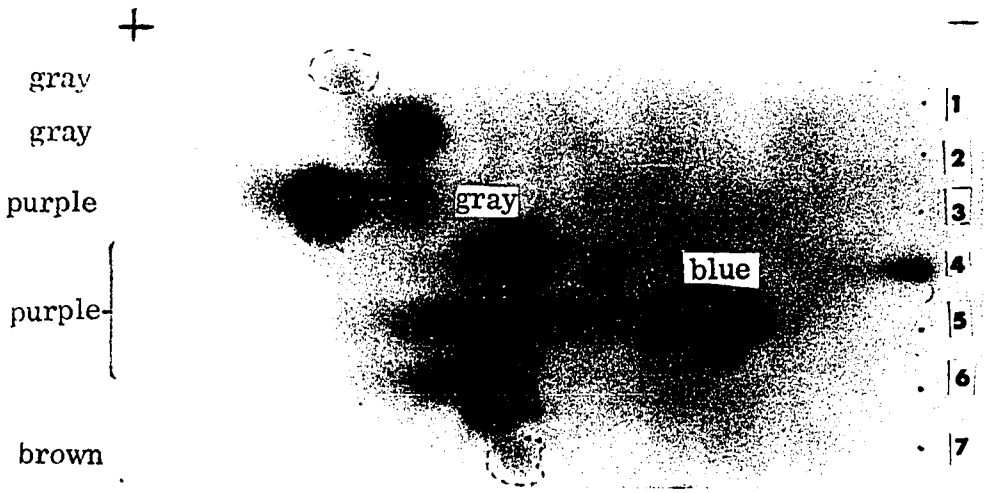


Figure 23. Ionophoretic migration of the disaccharide component from oligosaccharide I mixture (derived from lactose by borate ion-exchange chromatography) and reference compound, lactosone, prepared by two different methods.

(1) = standard glucose

(2) = standard galactose

(3) = monosaccharide, D-arabino-hexosulose, isolated from hydrolysate of oligosaccharide I mixture (contaminated with galactose)

(4) = lactosone from the reaction of lactosephenylosazone with nitrous acid

(5) = lactosone from the reaction of lactose with n-butylamine (contaminated with lactose)

(6) = disaccharide component from oligosaccharide I mixture

(7) = standard lactulose

Ionophoresis was performed in 0.05 M borax, pH 9.2, for 5 hours at 375 volts. After drying the paper was stained with reagent A.

above suggested an identity between D-arabino-hexosulose and the monosaccharide component and between lactosone and the disaccharide component of oligosaccharide I mixture respectively, a more detailed chemical characterization of these compounds was undertaken. These studies required the isolation and purification of larger quantities of the monosaccharide component from the oligosaccharide I mixture, and also preparation of highly purified specimens of D-arabino-hexosulose to be used for comparative purposes. The amount of reference lactosone, derived from synthetic methods described above, was small and therefore, it was utilized only for chromatographic purposes.

Isolation and Purification of D-Arabino-hexosulose

The osuloses (or osones) exist as amorphous or syrupy materials and, consequently, they are difficult to isolate and purify. The monosaccharide identified in this study could be obtained either by acid or enzymic hydrolysis of the disaccharide, tetrasaccharide or oligosaccharide mixture. In the acid hydrolysis method an oligosaccharide sample of 200 mg dry weight (approximately 100 mg of anthrone positive material) was hydrolyzed in 5 ml of 2 N H_2SO_4 at 100°C for 3 hours. The hydrolysate was cooled and deionized by passing it through coupled columns (1 x 18 cm) of Ag 50W-X8-H^+ and Ag 1-X2-$HCOO^-$. The columns were eluted with water until a negative anthrone test was obtained. The hydrolysate was concentrated by evaporation under vacuum, and it was then subjected to preparative paper chromatography with solvent system 1 until the final product was chromatographically homogeneous. Solutions of the isolated monosaccharide (10 mg of syrup per ml of water) were stored frozen.

D-Arabino-hexosulose formed as a by-product in the reaction between glucose and n-butylamine contained considerable quantities of 3-deoxy-D-erythro-hexosulose in addition to glucose. Since D-arabino-hexosulose is more stable to acid than its related deoxy form, the mixtures were freed of the deoxy sugar by utilizing the acid hydrolysis step described in the previous paragraph.

For purification of the D-arabino-hexosulose prepared from the

reaction of glucose with copper acetate on a larger scale, cellulose column chromatography was used. As much as 12 g of syrup obtained in this reaction was chromatographed on columns of microcrystalline cellulose developed with solvent system 1, in order to separate D-arabino-hexosulose from glucose and other contaminating sugars. The column eluate was monitored by paper chromatography with solvent system 1 or by thin-layer chromatography using solvent system 7. A typical tlc pattern can be seen in Figure 24. Glucose was completely eluted in one column volume (about 750 ml). Some overlap appeared in fractions 13 to 18 but the hexosulose appeared to be free of glucose by fraction 20. Fractions 20 to 50, containing only hexosulose, were pooled and concentrated to a syrup by rotary evaporation at 30°C. Water was added and the evaporation repeated several times until all traces of acetic acid were removed. The final syrup (1 g) was diluted to 20 ml with 50% ethanol and the solution stored at -20°C.

Degradation Studies of Oligosaccharide I Mixture and Component D-Arabino-hexosulose

During the course of purification it was found that, although D-arabino-hexosulose was fairly stable under acid conditions, repeated concentration of its solutions even by lyophilization caused changes, and in some cases, decomposition of this product. This appears to be typical of dicarbonyl compounds of this type (67). A slower-moving, purple-staining compound (with reagent A) which is probably a polymer was detected a number of times, especially on tlc plates (see for example spot 4 on Figure 18).

D-Arabino-hexosulose was found to exhibit some absorption in the ultraviolet range under certain conditions. The uv spectra of D-arabino-hexosulose isolated from the oligosaccharide I mixture was compared to that of reference standards prepared from the reactions of glucose both with butylamine and with cupric acetate. Only the uv spectra of D-arabino-hexosulose isolated from oligosaccharide I mixture is shown in Figure 25 since the uv spectra of these

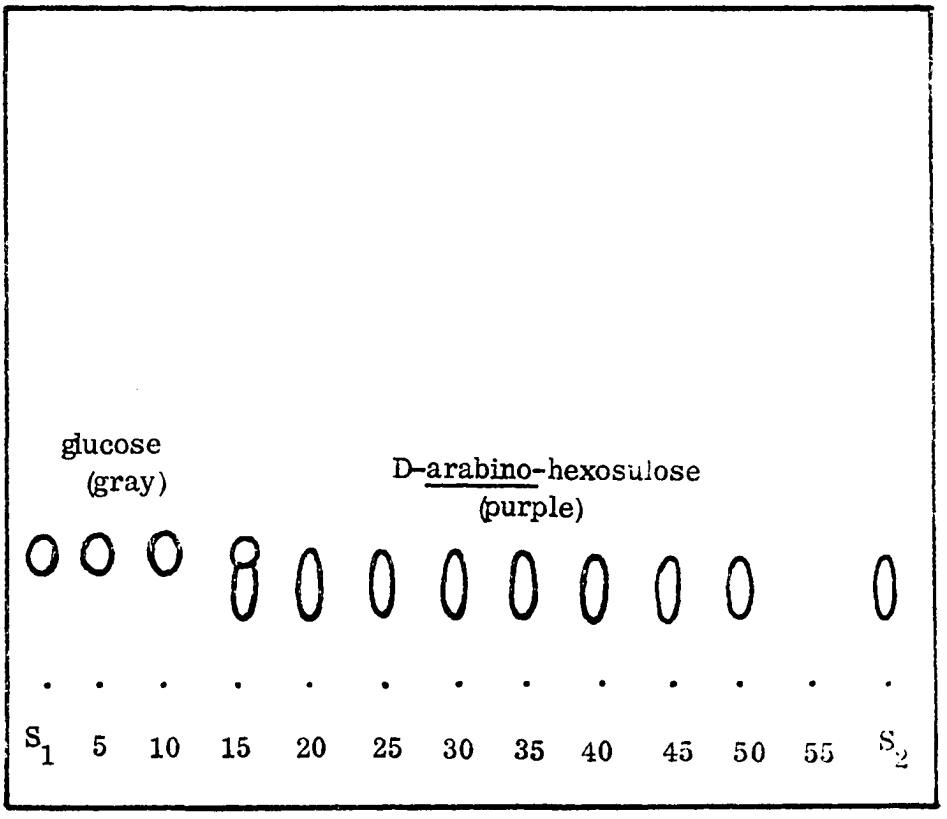


Figure 24. Thin-layer chromatography of cellulose column fractions obtained during the purification of D-arabino-hexosulose prepared by reacting glucose with copper acetate. Sugar products, visualized on the tlc plate, were glucose and D-arabino-hexosulose which exhibited the same migration as standard glucose (S_1) and chromatographically homogeneous D-arabino-hexosulose (S_2) prepared previously from the reaction of glucose with n-butylamine.

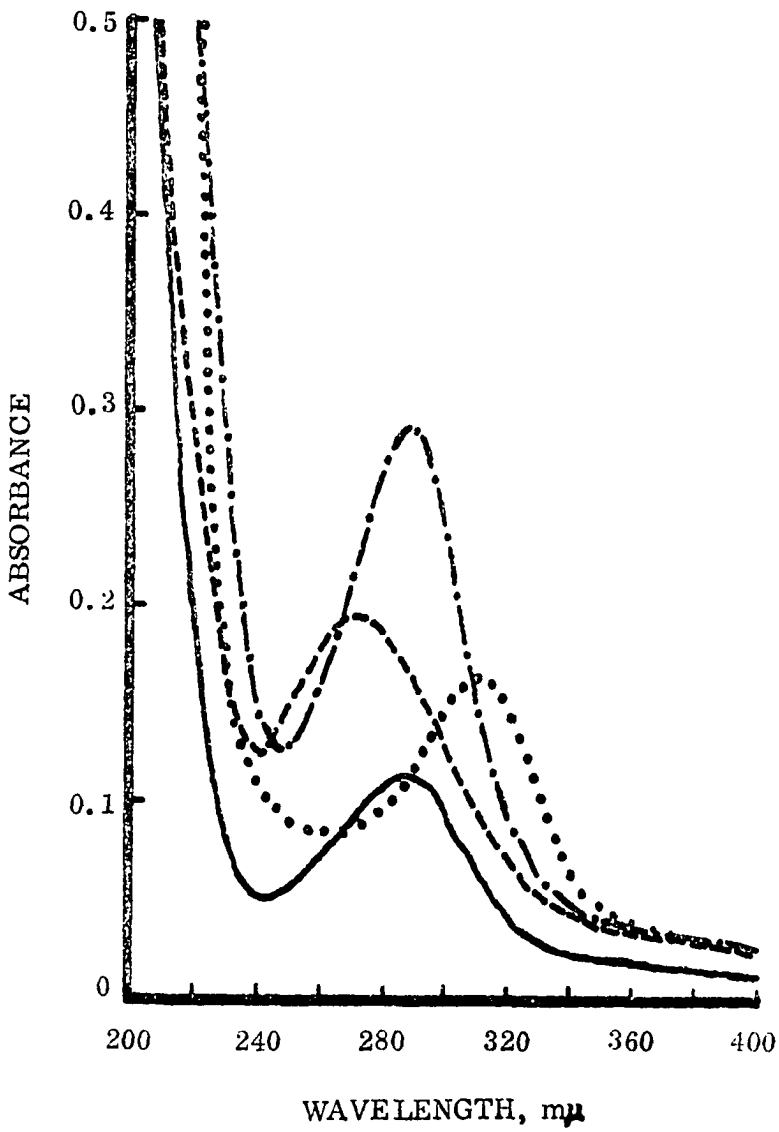


Figure 25. Ultraviolet absorption spectra of solutions of the monosaccharide component (D-arabino-hexosulose) isolated from oligosaccharide I mixture. Absorption spectra were recorded on 0.1% solutions of the sugar in distilled water (———), 0.1 N HCl (- - - -), and methanol (—·—), and on a 0.01% solution of the sugar in 0.1 N NaOH (·····).

preparations were qualitatively similar. The λ max which appeared at 315 mμ for each sample (0.1 mg per ml dissolved in 0.1 N NaOH) was transient and disappeared after 30 minutes. The uv absorptions of the three glucosone preparations in aqueous, methanol, or 0.1 N HCl solutions varied somewhat in intensity requiring higher concentrations (1 mg per ml) than the alkaline solutions. The reference standard prepared from glucose and cupric acetate reactions produced very little uv absorption in the 200-400 mμ range ($E^{1\%}_{1cm}$ values were 0.3 or less). On the other hand, D-arabino-hexosulose prepared from the glucose and butylamine reaction and that isolated from oligosaccharide I mixture both exhibited λ max of 275 mμ in 0.1 N HCl and λ max of 285-290 mμ in aqueous and methanol solutions, but the magnitude of the $E^{1\%}_{1cm}$ value was only 3 or less.

The uv absorption spectra of the disaccharide (Figure 26) and the tetrasaccharide (Figure 27) in aqueous, methanol, and acidic solutions were very weak and featureless above 200 mμ which is typical of most sugars. Under alkaline conditions (pH 13), however, in addition to the transient absorption peak with max of 315 mμ, another peak appeared at 260 mμ. The disaccharide gave the highest value for $E^{1\%}_{1cm}$ at both 315 mμ and 260 mμ with values ten times those of the tetrasaccharide.

Acid degradation. The stability of D-arabino-hexosulose from oligosaccharide I mixture and reference standards was studied under acid conditions. The experimental protocol used by Anet (77) for the study of 3-deoxy-D-erythro-hexosulose was employed for these studies, and the latter sugar was run concurrently for comparative purposes. Solutions of each compound, 2 mmol/ml, in 0.03 N acetic acid and in 2 N hydrochloric acid were heated at 100°C. Aliquots were withdrawn at various intervals and their ultraviolet spectra were recorded. The absorption spectra in 0.03 N acetic acid solutions (recorded at 0, 15, and 60 minutes) are shown in Figure 28. The absorption peak at 284 mμ was much stronger for the deoxy-hexosulose indicating that D-arabino-hexosulose had greater stability under acid conditions. The appearance of a breakdown product, thought to be 5-hydroxymethyl-2-furaldehyde because of its

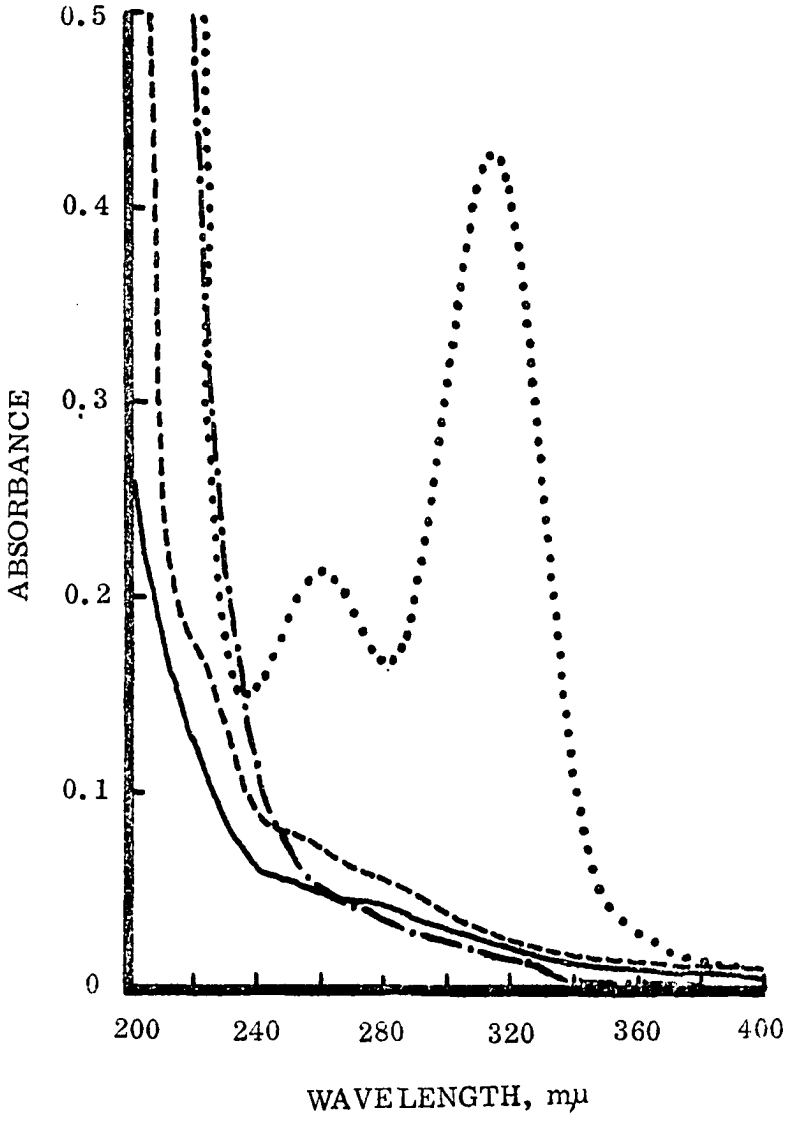


Figure 26. Ultraviolet absorption spectra of solutions of the disaccharide (lactosone) component isolated from the oligosaccharide I mixture. Absorption spectra were recorded on 0.1% solutions of the sugar in distilled water (———), 0.01 N HCl (- - - - -), and methanol (—·—), and on a 0.01% solution of the sugar in 0.1 N NaOH (· · · · · ·).

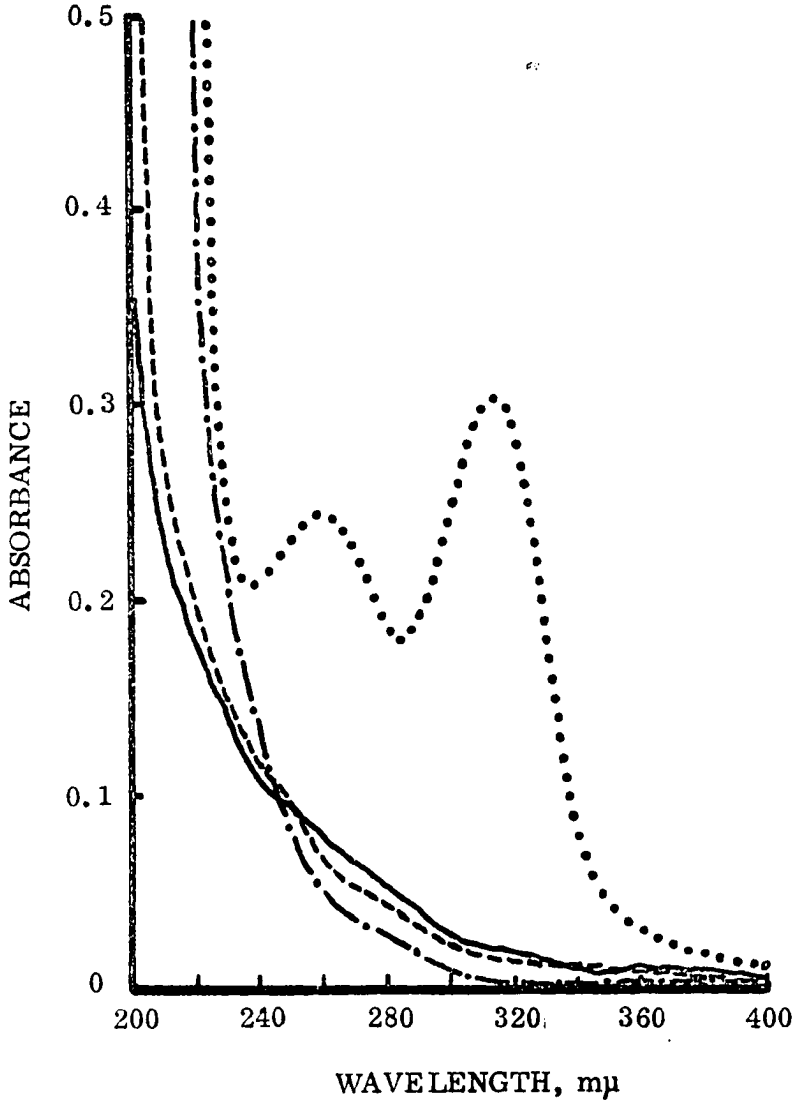


Figure 27. Ultraviolet absorption spectra of solutions of the tetrasaccharide (lactosone dimer) component of the oligosaccharide I mixture. Absorption spectra were recorded on 0.1% solutions of the sugar in distilled water (———), 0.1 N HCl (– – – –), methanol (—•—), and 0.1 N NaOH (• • • • •).

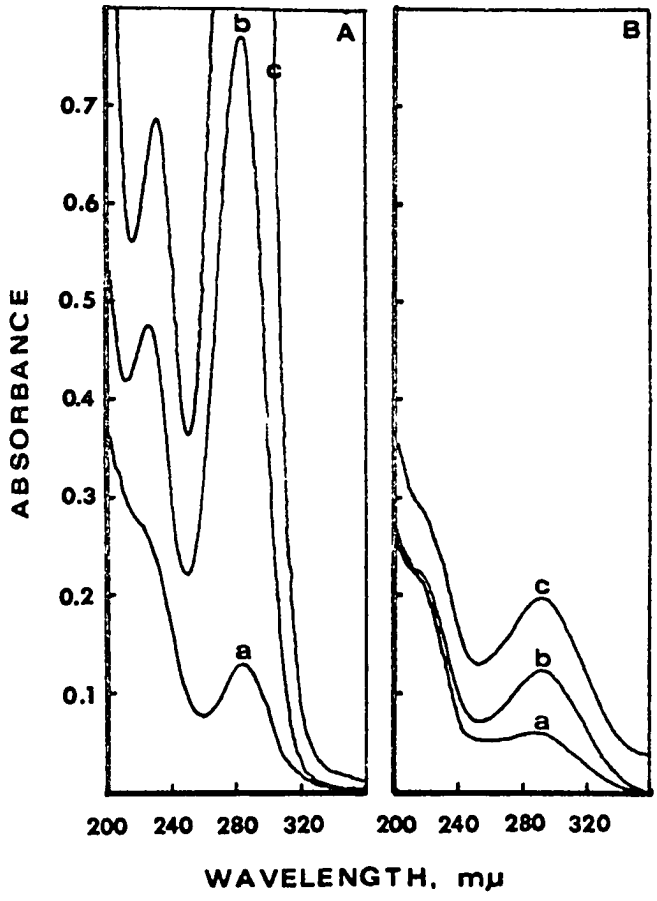


Figure 28. Ultraviolet absorption spectra of solutions of 3-deoxy-D-erythro-hexosulose (A) and D-arabino-hexosulose (B) in 0.03 N acetic acid. The latter sugar was isolated from oligosaccharide I mixture. Absorptions were recorded on 2 mmol per ml solutions of the sugars after heating at 100°C for: (a) 15 min, (b) 30 min, and (c) 60 min.

strong absorption at 284 mμ (78), occurred rapidly when the deoxy-hexosulose was heated in 2 N hydrochloric acid. The strong 284 mμ absorption observed at 15 minutes (Figure 29) disappeared rapidly with time. D-arabino-hexosulose, on the other hand, had a weaker ultraviolet absorption; the highest absorption for the major peak (at 265-270 mμ) was observed after 5 hours in 2 N HCl at 100°C. By 23 hours, two peaks were recorded at 290 mμ and at 230 mμ respectively. These results show that the acid stability of D-arabino-hexosulose was much greater than that of the deoxy-hexosulose; furthermore, it is apparent that the main acid degradation product of D-arabino-hexosulose was not 5-hydroxymethyl-2-furaldehyde. The different behavior of these compounds in 2 N HCl at 100°C becomes quite striking when the absorbancy at 284 mμ is plotted as a function of time (Figure 30). The course of the reaction in 2 N HCl at 100°C was also followed by tlc (Figure 31). The spot of D-arabino-hexosulose staining purple with reagent A was still visible after 1 hour of acid treatment, but it was absent after 23 hours. The 3-deoxy-D-erythro-hexosulose was easily degraded by acid and no trace of this compound was detected after 15 minutes of acid treatment. The yellow-green-staining spot of 5-hydroxymethyl-2-furaldehyde produced by acid degradation of deoxy-hexosulose appeared at 15 minutes, but it was absent after 1 hour.

Alkaline degradation. The experimental protocol and techniques employed by Lindberg and Theander (79) in their studies on the effect of various alkalies on D-arabino-hexosulose were applied to the monosaccharide component isolated from oligosaccharide I mixture. The compound, in 10 mg aliquots each, was treated with 5 ml of 0.04 M sodium hydroxide at 50°C for 2 hours, with 14 ml of 0.04 M sodium hydroxide at 100°C for 5 hours, and with 14 ml of 0.02 M calcium hydroxide at 100°C for 5 hours. Cations were removed on columns (1 x 10 cm) of Ag 50W-X8(H^+), and the water eluate was concentrated by rotary evaporation. Aliquots were then examined by glc. The results of these experiments show very good agreement with the published data and further establish the identity of our compound as being D-arabino-hexosulose. Gas chromatography

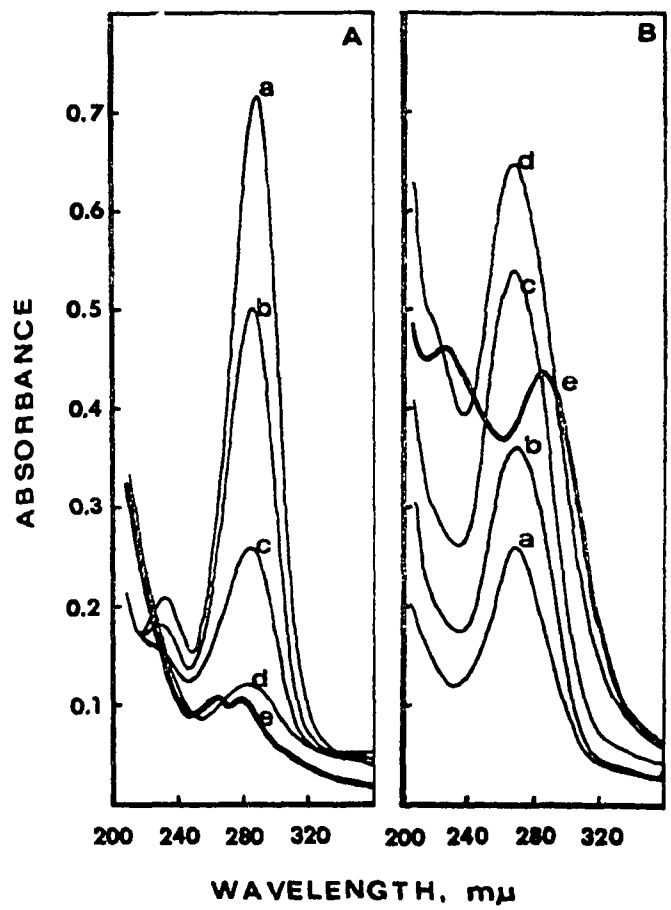


Figure 29. Ultraviolet absorption spectra of solutions of 3-deoxy-D-erythro-hexosulose (A) and D-arabino-hexosulose (B) in 2 N HCl. The latter sugar was isolated from oligosaccharide I mixture. After heating at 100°C for (a) 15 min, (b) 30 min, (c) 1 h, (d) 5 h, and (e) 23 h, the solutions (2mmol per ml) were diluted (A= 1:10, B = 1:1) with 2 N HCl and the ultraviolet spectra were recorded.

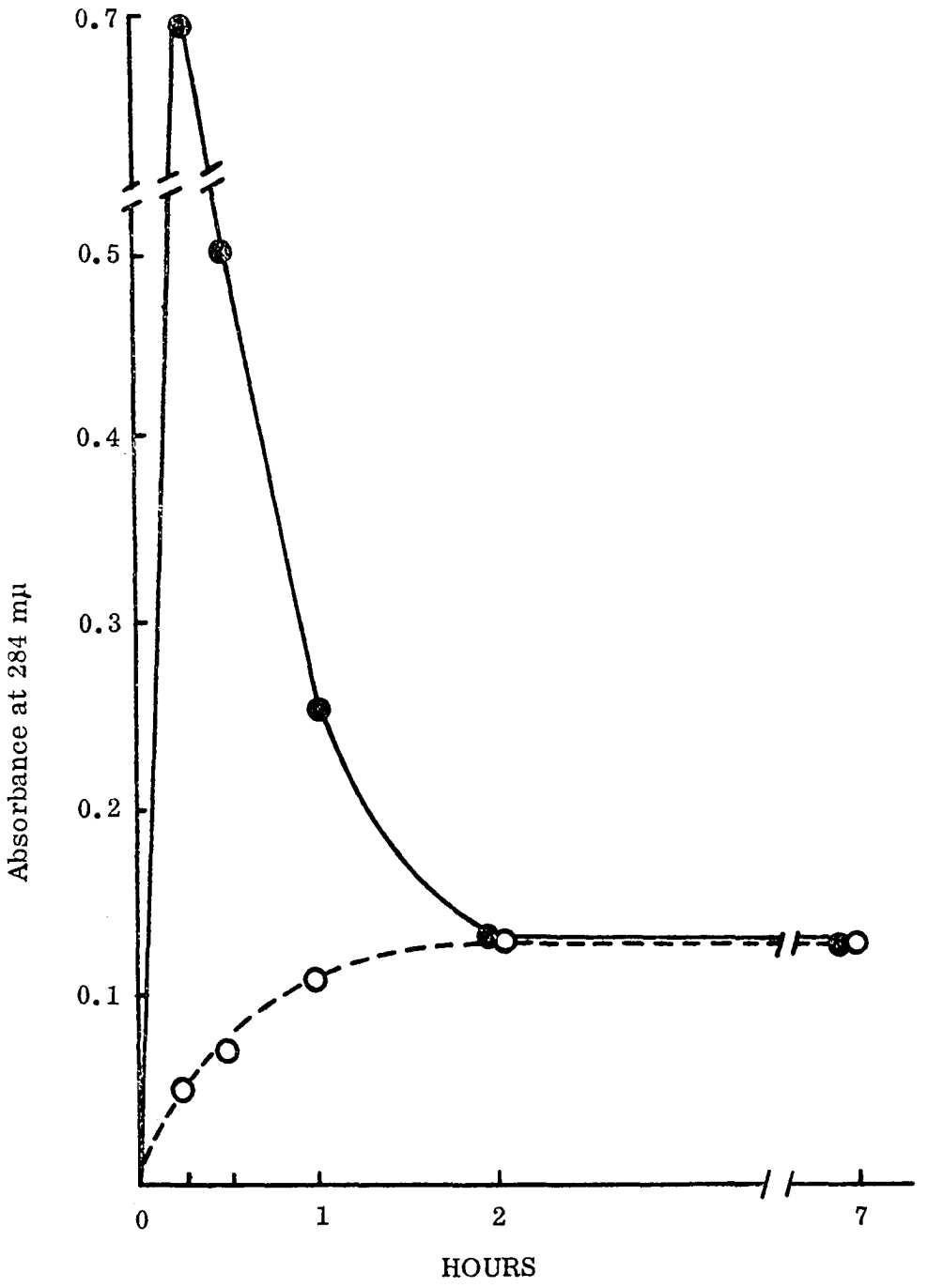


Figure 30. Comparison of the absorbancies at 284 mμ observed in solutions (2 mmol per ml) of D-arabino-hexosulose (-O-) isolated from oligosaccharide I mixture and 3-deoxy-D-erythro-hexosulose (—●—) subjected to treatment with 2 N HCl at 100°C for various lengths of time.

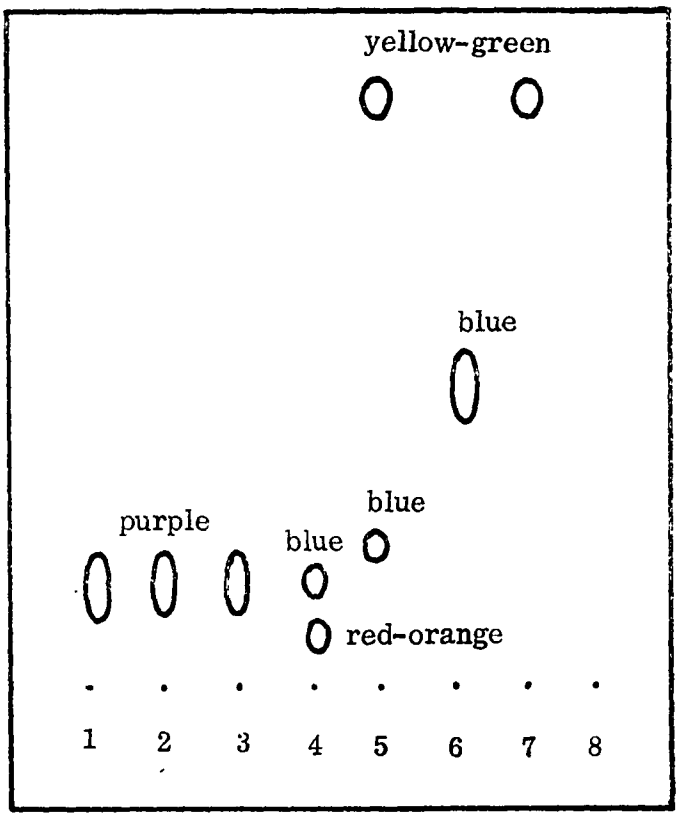


Figure 31. Thin-layer chromatography of D-arabino-hexosulose and 3-deoxy-D-erythro-hexosulose after heating at 100°C in 2 N HCl for various lengths of time.

(1) = D-arabino-hexosulose isolated from oligosaccharide I mixture

(2,3,4) = (1) after heating in acid for 15 min, 1 h, and 23 h, respectively

(5) = standard mixture of glucose and 5-hydroxy-methyl-2-furaldehyde

(6) = 3-deoxy-D-erythro-hexosulose

(7,8) = (6) after heating in acid for 15 min and 1 h, respectively

Plate was developed with solvent system 7 and stained with reagent A.

of the trimethylsilyl (TMS) ethers of the products obtained by sodium hydroxide treatment (Figures 32 and 33) showed that the main sugar acid (in lactone form) was arabinonic acid. While some erythronic, ribonic, gluconic and mannonic acids were present after 2 hours at 50°C, they were found in very low amounts in the material treated for 5 hours at 100°C. In the calcium hydroxide treated sample (Figure 34), mannonic acid was the main product. Lesser amounts of arabinonic, as well as small amounts of erythronic, ribonic, and gluconic acid were also present.

The behavior of lactosone, isolated from oligosaccharide I mixture, was also investigated under mild alkaline conditions. A comparison of the effects of 0.04 M sodium hydroxide and 0.02 M calcium hydroxide on this sugar at 37°C for 3 hours can be seen on the chromatogram shown in Figure 35. The characteristic purple-staining spot of the disaccharide disappeared after treatment with calcium hydroxide, but some of it was still visible in the sodium hydroxide treated sample. Considerable quantities of galactose were found on the chromatogram probably as a result of beta-elimination. The appearance of a keto sugar (probably fructose) was also found in the calcium hydroxide treated sample. If the alkaline treatment was conducted at 100°C for 5 hours, and the products were then subjected to paper chromatography, no sugar spots were detected with reagent A. No conversion of oligosaccharide I (disaccharide) to the oligosaccharide II was detected as a result of any of these alkaline treatments.

Borohydride Reduction Studies

Reduction of the tetrasaccharide component was not very informative since this treatment neither decreased the anthrone values nor changed the paper chromatographic mobility of the sugar. The only change observed after this treatment was that this sugar failed to give the purple aniline-diphenylamine color and gave instead a faint tan color. The disaccharide, on the other hand, was reduced to a sugar having the mobility and brown staining characteristics of lactulose (see Figure 36). This indicated that the monosaccharide on the

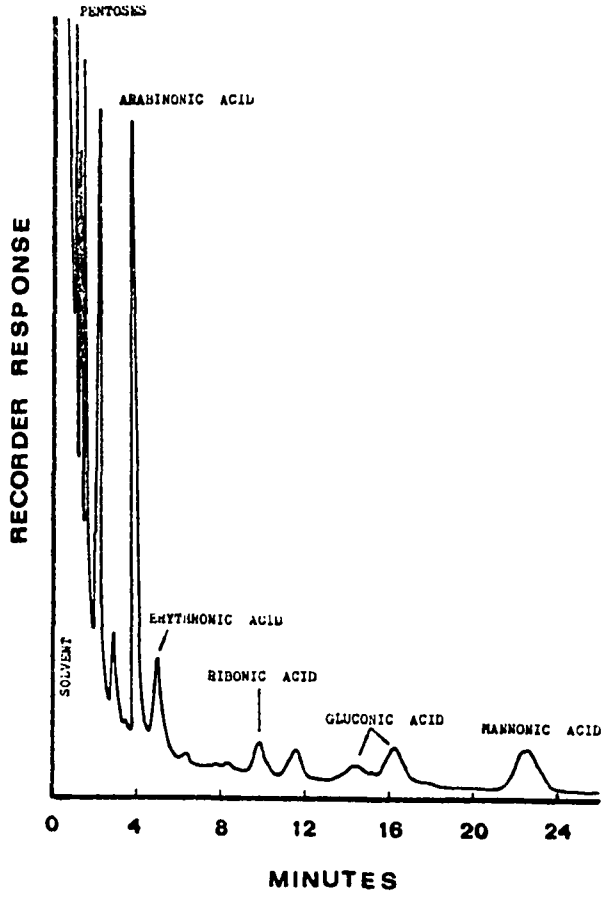


Figure 32. Gas-liquid chromatography of TMS derivatives of lactones produced following the treatment of D-arabino-hexosulose (isolated from oligosaccharide I mixture) with 0.04 M NaOH at 50°C for 2 hours. The column (3% SE-30) was maintained isothermally at 140°C. The T_R value of α-D-glucose-TMS, under identical conditions, was 17.6 minutes.

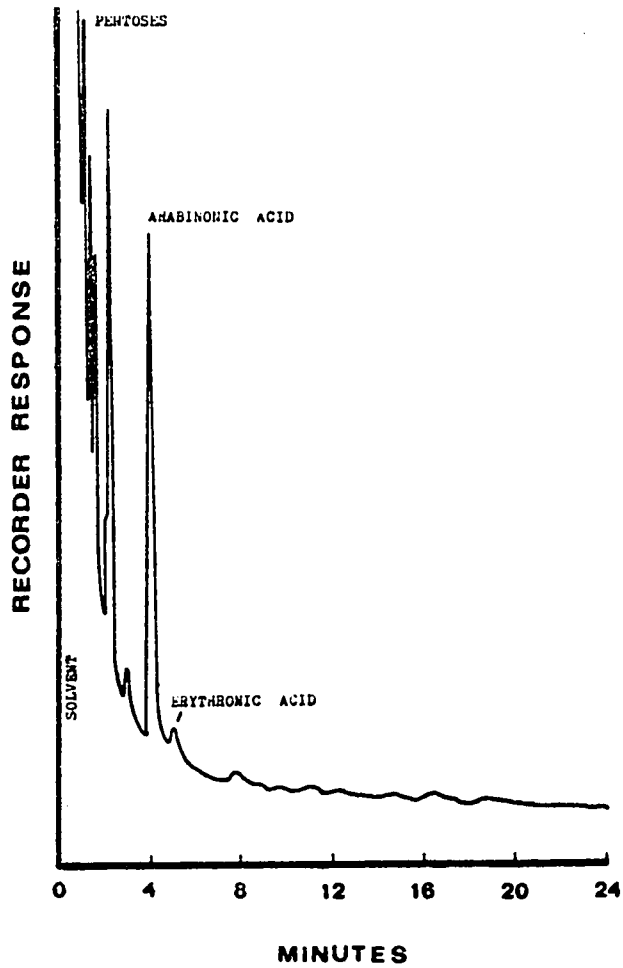


Figure 33. Gas-liquid chromatography of TMS derivatives of lactones produced following the treatment of D-arabino-hexosulose (isolated from oligosaccharide I mixture) with 0.04 M NaOH at 100°C for 5 hours. The column (3% SE-30) was maintained isothermally at 140°C. The T_R value of α - D-glucose-TMS, under identical conditions, was 17.6 minutes.

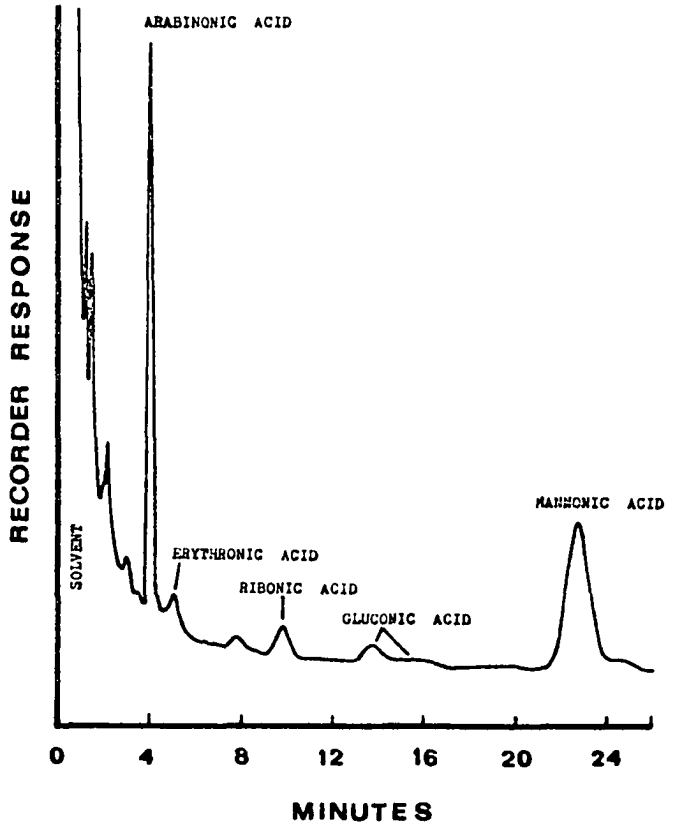


Figure 34. Gas-liquid chromatography of TMS derivatives of lactones produced following the treatment of D-arabino-hexosulose (isolated from oligosaccharide I mixture) with 0.02 M $Ca(OH)_2$ at 100°C for 5 hours. The column (3% SE-30) was maintained isothermally at 140°C. The T_R value of α-D-glucose-TMS, under identical conditions, was 17.6 minutes.

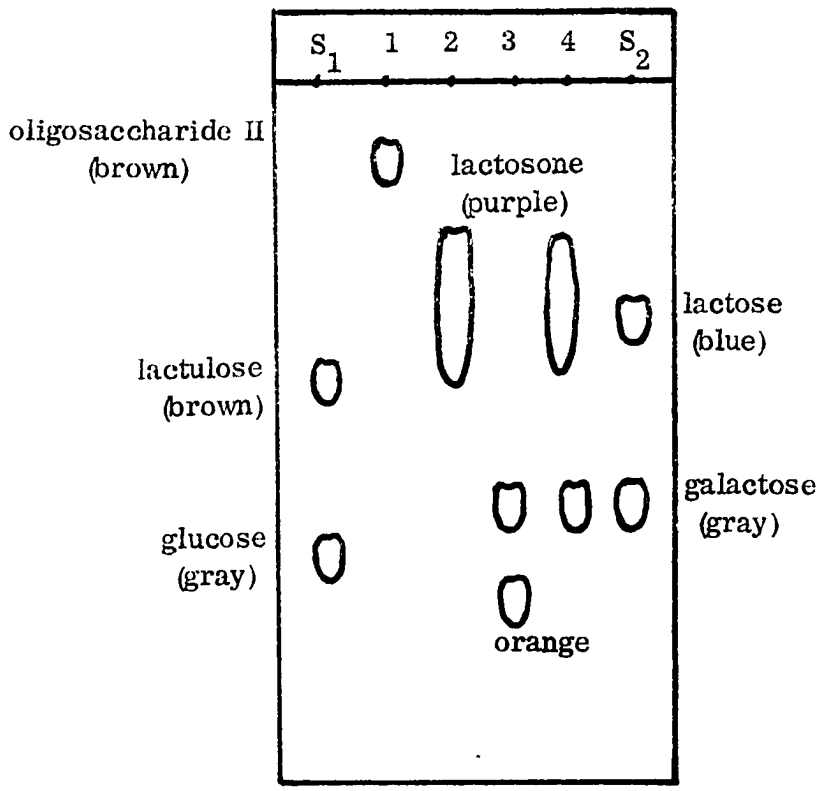


Figure 35. Paper chromatography of alkali-treated disaccharide, lactosone, isolated from oligosaccharide I mixture.

(S_1) = standard mixture of lactulose and glucose

(S_2) = standard mixture of lactose and galactose

(1) = unidentified sugar (oligosaccharide II) from borate ion-exchange column at 37°C

(2) = lactosone isolated from oligosaccharide I mixture

(3) = (2) treated with 0.02 M $Ca(OH)_2$ at 37°C for 3 hours

(4) = (2) treated with 0.04 M NaOH at 37°C for 3 hours

Chromatogram was developed with solvent system 2 and stained with reagent A.

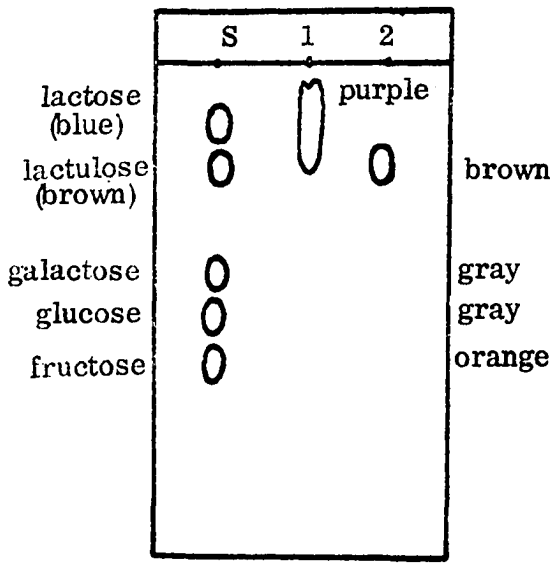


Figure 36. Paper chromatography of sodium borohydride reduced disaccharide, lactosone, isolated from oligosaccharide I mixture derived from lactose by borate ion-exchange chromatography (peak III).

(S) = standard mixture of lactose, lactulose, galactose, glucose, and fructose

(1) = lactosone isolated from oligosaccharide I mixture

(2) = (1) reduced with $NaBH_4$ in a 0.1 M solution of borate buffer, pH 7.7

Chromatogram was developed with solvent system 1 and stained with reagent A.

reducing end of the disaccharide (lactosone) could be reduced to fructose. Independent and direct proof of this transformation was obtained by reducing a purified sample of the monosaccharide component (isolated from the acid hydrolysate of the disaccharide) to fructose as shown in Figure 37. Reference standards of D-arabino-hexosulose were also reduced to fructose by this procedure.

Preparation of Crystalline Derivatives of D-Arabino-hexosulose

Since glycosuloses are difficult to identify and determine, the hydrazine derivatives are particularly useful for this purpose.

Phenylosazones. Fischer (76) first reported that D-glucosone (D-arabino-hexosulose) forms D-glucosonephenylosazone (the same compound as D-glucosephenylosazone) on reaction with phenylhydrazine acetate at room temperature. This compound was prepared from the phenylhydrazine reagent at room temperature as described in "Methods". Under these mild conditions the formation of osazones from aldoses would be precluded. The yellow compound was recrystallized several times from absolute ethanol yielding bright yellow needles having a melting point of 203°C (dec.) as recorded in Table 6. Elemental analysis of the derivative prepared from D-arabino-hexosulose isolated from oligosaccharide I mixture was conducted by Schwarzkopf Microanalytical Laboratory of Woodside, N.Y. and gave the following results:

	$C_{18}H_{22}N_4O_4$
Calculated:	C 60.33%, H 6.15%, N 15.64%
Found:	C 60.03%, H 6.36%, N 15.87%

The osazones of three related hexoses--glucose, mannose, and fructose--are identical to that of D-arabino-hexosulose because their differences are confined to carbon 1 and carbon 2 which are involved in the osazone formation. Infrared spectra of the phenylosazones prepared from D-arabino-hexosulose, glucose, fructose, and galactose are compared in Figure 38. The infrared spectrum of the D-arabino-hexosulose derivative is identical to the spectra

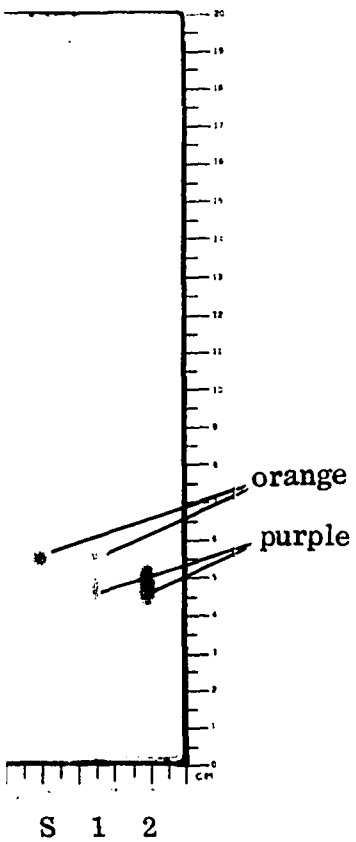


Figure 37. Thin-layer chromatography of sodium borohydride reduced D-arabino-hexosulose isolated from oligosaccharide I mixture.

(S) = standard fructose

(1) = D-arabino-hexosulose reduced, in part, to fructose by treatment with $NaBH_4$ in a 0.1 M solution of borate buffer, pH 7.7

(2) = D-arabino-hexosulose, aliquot before reduction

Plate was develped with solvent system 7 and stained with reagent A.

TABLE 6

MELTING POINTS OF CRYSTALLINE DERIVATIVES OF SUGARS ISOLATED FROM OLIGOSACCHARIDE I MIXTURE

Sugar Derivatives	Observed M. P. (°C)[a]	Literature M. P. (0°C)	ref.
D-Arabino-hexosulose-phenylosazone[b]	203 (dec.)	206-208	(80)
D-Arabino-hexosulose-phenylosazone[c]	203 (dec.)		
D-Arabino-hexosulose-2,4-dinitrophenylosazone[b]	249-250 (dec.)	254 (dec.) 252	(63) (81)
D-Arabino-hexosulose-2,4-dinitrophenylosazone[c]	241-242 (dec.)		

[a] Melting points were not corrected.

[b] Hexosulose sugar was isolated from oligosaccharide I mixture.

[c] Hexosulose sugar was prepared in the reaction between glucose and cupric acetate.

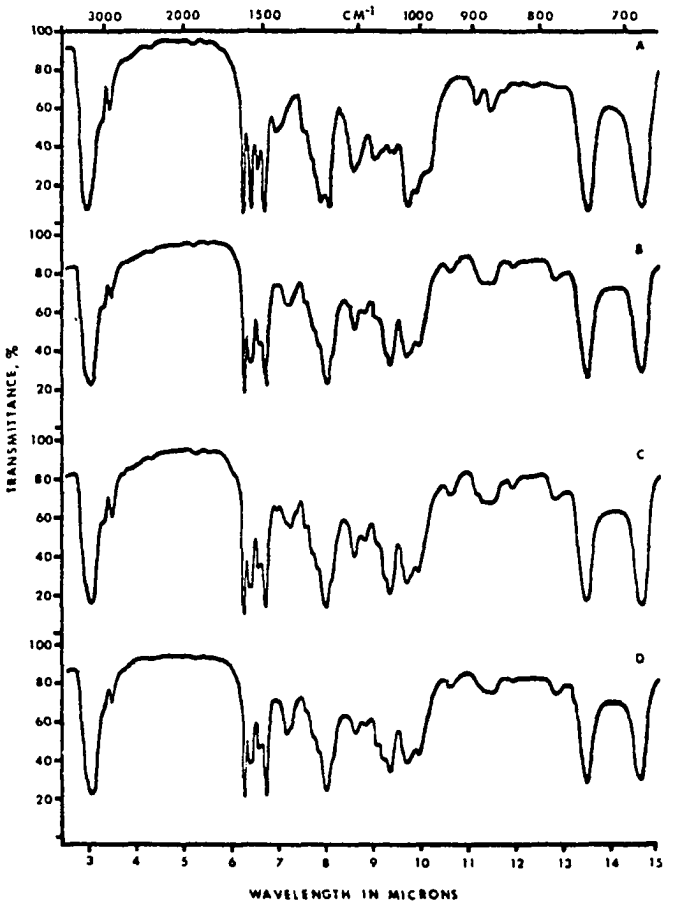


Figure 38. Infrared spectra of crystalline phenylosazone derivatives of D-galactose (A), D-fructose (B), D-glucose (C), and D-arabino-hexosulose (D). The latter sugar was isolated from oligosaccharide I mixture. All samples were examined in KBr pellets.

of glucose and fructose derivatives whereas that of the galactose derivative is different.

2,4-Dinitrophenylosazones. The 2,4-dinitrophenylhydrazine reagent described in "Methods" was used to prepare this characteristic red-orange derivative of D-arabino-hexosulose. As shown in Table 6, the melting point of 249-250°C (dec.) of the derivative prepared from the D-arabino-hexosulose component of oligosaccharide I mixture was in better agreement with the literature values of 252°C and 254°C than was that of the reference compound synthesized in the reaction of glucose with cupric acetate (241-242°C dec.). These derivatives are difficult to redissolve even in hot ethanol-ethyl acetate solutions for recrystallization purposes, and the difference observed in the melting point of these two preparations may be due to retention of impurities formed during the preparation of this sugar by the cupric acetate method. Confirmation of the identity of the derivative prepared from D-arabino-hexosulose isolated from the oligosaccharide I mixture was provided by its elemental analysis as reported by Schwarzkopf Microanalytical Laboratory:

	$C_{18}H_{18}N_8O_{12}$
Calculated:	C 40.15%, H 3.35%, N 20.82%
Found:	C 40.09%, H 3.59%, N 20.93%

A comparison of the infrared spectra of the 2,4-dinitrophenylosazone derivatives of fructose, glucose, and D-arabino-hexosulose isolated from oligosaccharide I mixture and from the reaction of glucose with copper acetate is shown in Figure 39.

Additional Observations

Melting points. Although crystallized forms of the oligosaccharides could not be obtained, highly purified preparations of the disaccharide and tetrasaccharide were obtained by chromatography on Darco-Celite columns. The dry, lyophilized preparations were white, amorphous hygroscopic powders. The disaccharide exhibited a melting point of 130-133°C while the tetrasaccharide had

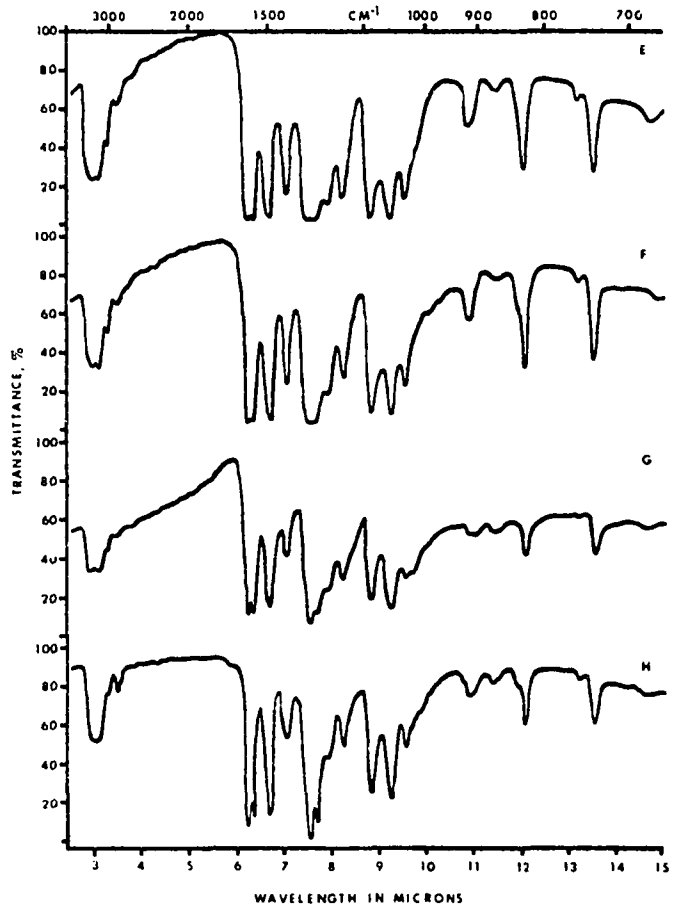


Figure 39. Infrared spectra of crystalline 2,4-dinitrophenylosazone derivatives of D-fructose (E), D-glucose (F), D-arabino-hexosulose from the reaction of glucose with copper acetate (G), and D-arabino-hexosulose isolated from oligosaccharide I mixture (H). All samples were examined in KBr pellets.

a melting point of 141-143°C.

Fractions containing monosaccharides, eluted with water from the Darco-Celite column, were concentrated and fractionated by preparative paper chromatography as described in "Methods". The purple-staining D-arabino-hexosulose, separated from galactose in this manner, was eluted with distilled water from non-stained areas of the paper. Lyophilization of the sugar solution, after partial concentration by rotary evaporation, yielded a bubbly, yellow, very hygroscopic material. Concentration of the monosaccharide solution by rotary evaporation alone gave a yellow syrup. Treatment of the syrup with 10 volumes of absolute ethanol resulted in precipitation of a white substance which could be separated by centrifugation. The white powder was amorphous and decomposed at temperatures above 126°C failing to give a sharp melting point. The powder turned yellow upon prolonged exposure to atmospheric moisture.

Elemental analysis. A nitrogen determination (Kjeldahl) performed on the isolated monosaccharide (D-arabino-hexosulose) yielded negative results.

Lyophilized samples of the di-and tetrasaccharides were sent to Schwarzkopf Microanalytical Laboratory, Woodside, N.Y., for elemental analyses. The values found were:

Disaccharide: C 37.96%, H 6.48%, O 55.56%.

Tetrasaccharide: C 38.67%, H 6.47%, O 54.86%.

The data were calculated in terms of atomic ratios (82) and a comparison of these data with some theoretically derived formulas is shown in Table 7 for the disaccharide and in Table 8 for the tetrasaccharide. From these considerations it would appear that the elemental analysis data for the lyophilized disaccharide are compatible with the calculated values for lactosone dihydrate while the analysis for the tetrasaccharide corresponds to a lactosone dimer retaining 4 or 5 molecules of water; some minor contamination with other sugars might also have occurred.

Infrared absorption spectra. Mixtures containing 200 mg of potassium bromide and 1 mg samples of lyophilized di- or tetrasaccharide were pressed into pellets. Figure 40 shows the infrared absorption spectra along with the

TABLE 7

COMPARISON BETWEEN ELEMENTAL ANALYSIS DATA AND CALCULATED VALUES FOR LACTOSE-DERIVED DISACCHARIDE

Elemental Analysis Data			
Disaccharide[a]:			
% Composition, found[b]	C 37.96%, H 6.48%, O 55.56%		
Atomic ratio, calculated	1.00	2.03	1.10
Molecular formula, calculated	$C_{12}H_{24}O_{13}$		

Calculated Values for Lactose-derived Disaccharide	
Disaccharide:	
1. Two aldohexoses, hydrated,	
Molecular formula	$C_{12}H_{22}O_{11} \cdot H_2O$
% Composition	C 38.09%, H 6.94%, O 54.97%
2. Aldohexose-aldohexosulose, hydrated	
Molecular formula (monohydrate)	$C_{12}H_{20}O_{11} \cdot H_2O$
% Composition	C 40.22%, H 6.20%, O 53.58%
Molecular formula (dihydrate)	$C_{12}H_{20}O_{11} \cdot 2H_2O$
% Composition	C 38.29%, H 6.44%, O 55.27%

[a] Lyophilized preparation isolated from oligosaccharide I mixture obtained during borate ion-exchange chromatography of lactose.

[b] Values were reported for C and H whereas O value was obtained by difference.

TABLE 8

COMPARISON BETWEEN ELEMENTAL ANALYSIS DATA AND CALCULATED VALUES FOR LACTOSE-DERIVED TETRASACCHARIDE

Elemental Analysis Data			
Tetrasaccharide[a]:			
% Composition, found[b]	C 38.67%, H 6.47%, O 54.86%		
Atomic ratio, calculated	1.00	1.99	1.06
Molecular formula, calculated	$C_{24}H_{48}O_{25}$		

Calculated Values for Lactose-derived Tetrasaccharide	
Tetrasaccharide:	
1. Four aldohexoses, hydrated	
Molecular formula	$C_{24}H_{42}O_{21} \cdot 4H_2O$
% Composition	C 39.01%, H 6.84%, O 54.15%
2. Two aldohexoses-two aldohexosuloses, hydrated	
Molecular formula (tetrahydrate)	$C_{24}H_{38}O_{21} \cdot 4H_2O$
% Composition	C 39.23%, H 6.32% O 54.44%
Molecular formula (pentahydrate)	$C_{24}H_{38}O_{21} \cdot 5H_2O$
% Composition	C 38.29%, H 6.44%, O 55.27%

[a] Lyophilized preparation isolated from oligosaccharide I mixture obtained during borate ion-exchange chromatography of lactose.

[b] Values were reported for C and H whereas O value was obtained by difference.

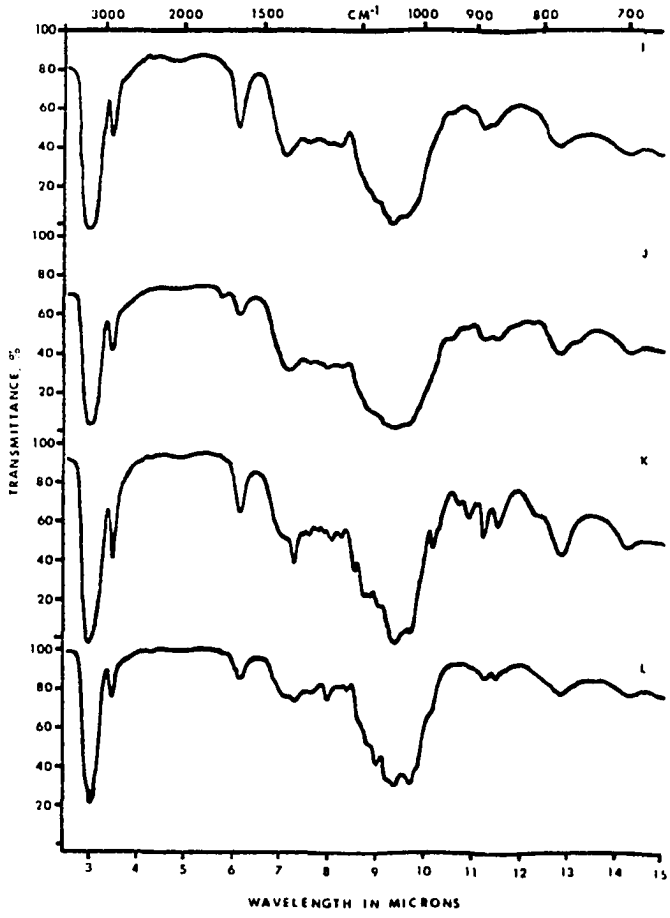


Figure 40. Infrared spectra of lyophilized preparations of the tetrasaccharide or lactosone dimer (I) and disaccharide or lactosone (J) which were isolated from oligosaccharide I mixture, lactose (K), and lactulose (L). All sugars were examined in KBr pellets.

spectra obtained from similarily prepared pellets of standards of lactose and lactulose. The infrared spectrum of an ethanol powder of the unknown monosaccharide was obtained by the same procedure, and it is shown in Figure 41 together with spectra of standards of D-glucose, D-fructose, and 3-deoxy-D-erythro-hexosulose.

Periodate oxidation studies. Oxidations were carried out at room temperature, in the dark, in 0.015 M sodium periodate solution. Sorbitol, methyl α-D-glucoside, D-glucose, lactose, maltose, sucrose, and stachyose were used as reference compounds. The results are shown in Table 9. The consumption of periodate by the monosaccharide reached a value of 2.6 moles/mole after 48 hours. This value compares very well with the value of 3 obtained during a five-day oxidation of D-arabino-hexosulose with lead tetraacetate (81). The periodate consumption by the disaccharide is close to the value of 4 moles calculated for galactosyl β 1 → 4 glucosone. The tetrasaccharide consumed considerably more periodate than anticipated (cf. standard stachyose).

Summarized Procedure for Preparation of D-Arabino-hexosulose-Containing Oligosaccharides by Borate Ion-exchange Chromatography of Lactose

A summary of the final procedure adopted for the preparation and purification of oligosaccharides I derived from lactose is outlined in Figure 42. The total amount of lactose, applied to a series of 12 columns (1 x 18 cm each), was 6.0 g (i. e., 0.5 g per column). As mentioned previously, attempts to use proportionally larger columns to handle the entire 6 g of lactose, under identical experimental conditions, were unsuccessful because the purple-staining oligosaccharide I fraction was absent from the 0.1 M $NaHCO_3$ eluted peak III which contained instead only the brown-staining oligosaccharide II. Using the small columns, the yield of anthrone positive sugars eluted by 0.1 M $NaHCO_3$ was ca. 8% (see Table 10). However, this reflects only 50% of the real yield because only the galactose moiety of the oligosaccharide material was anthrone positive. The real yield, based on dry weight, might approach 16%.

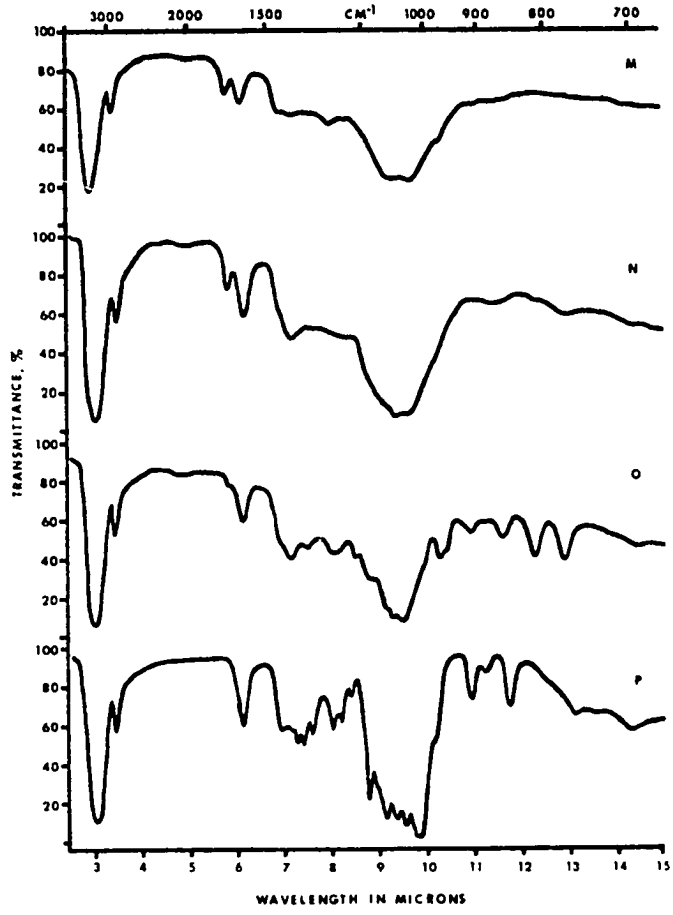


Figure 41. Infrared spectra of authentic crystalline 3-deoxy-D-erythro-hexosulose (M), ethanol powder of D-arabino-hexosulose isolated from oligosaccharide I mixture (N), lyophilized powder of D-fructose (O), and lyophilized powder of D-glucose (P). All sugars were examined in KBr pellets.

TABLE 9

PERIODATE OXIDATION STUDIES OF SUGARS ISOLATED FROM OLIGOSACCHARIDE I MIXTURE

Sugar	Moles of periodate consumed per mole of sugar			
	observed		reported	
	24 hr	48 hr	final[a]	ref.
Unknowns:				
Monosaccharide	2.3	2.6	3[b]	(81)
Disaccharide	3.6	3.5	-	
Tetrasaccharide	8.8	9.4	-	
Reference Standards:				
Methyl-α-D-glucose	1.9	2.0	2	(83, 84)
D-Glucose	4.3	4.6	5	(84)
Lactose	4.3	4.8	5	(48)
Stachyose	6.0	6.0	7	(84)

[a]This value represents the approximate final consumption reported.

[b]This value represents moles of lead tetraacetate consumed per mole of D-arabino-hexosulose in a progressive oxidation taking 5 days.

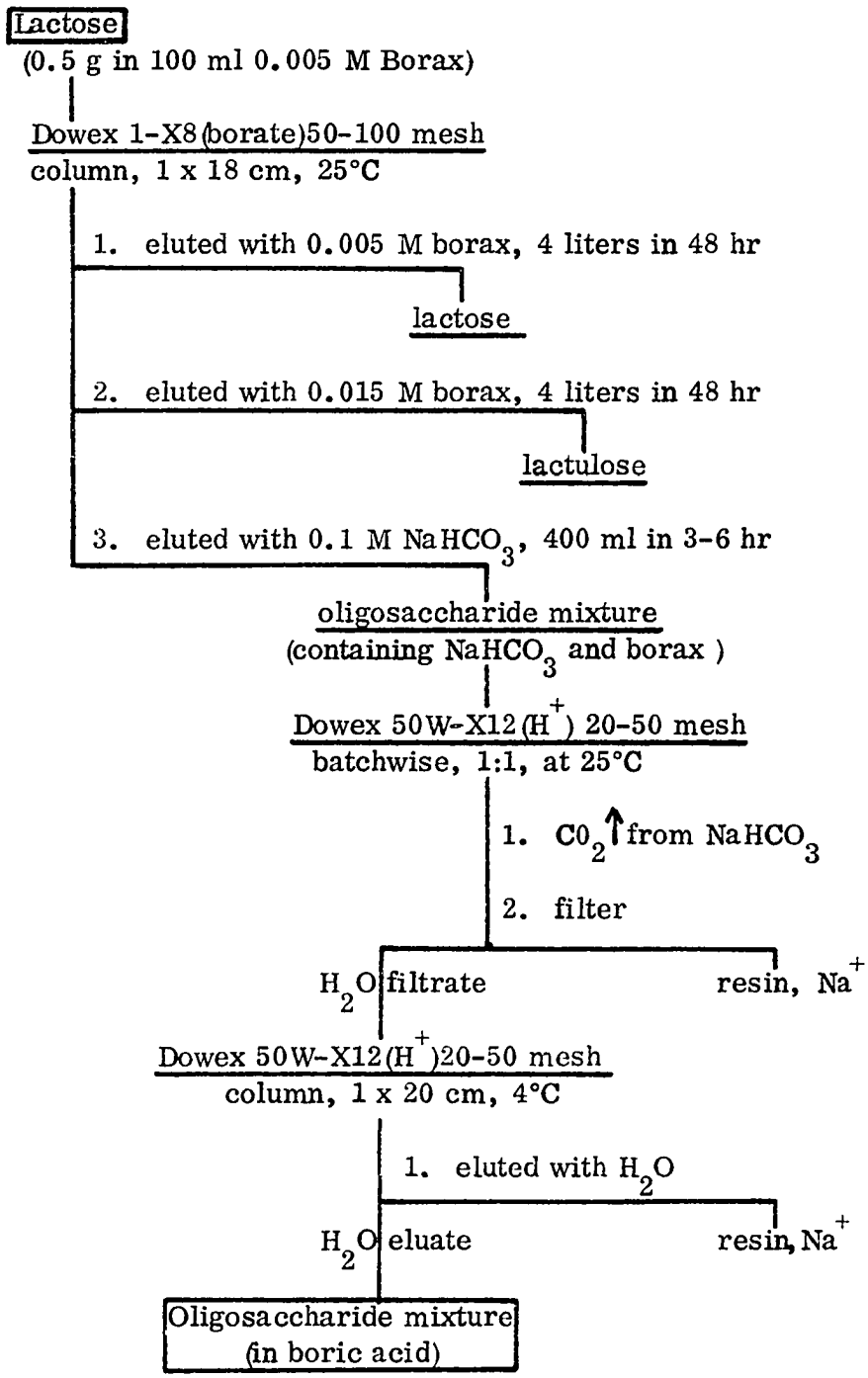


Figure 42. Procedure for the preparation and fractionation of oligosaccharides derived from lactose during borate ion-exchange chromatography.

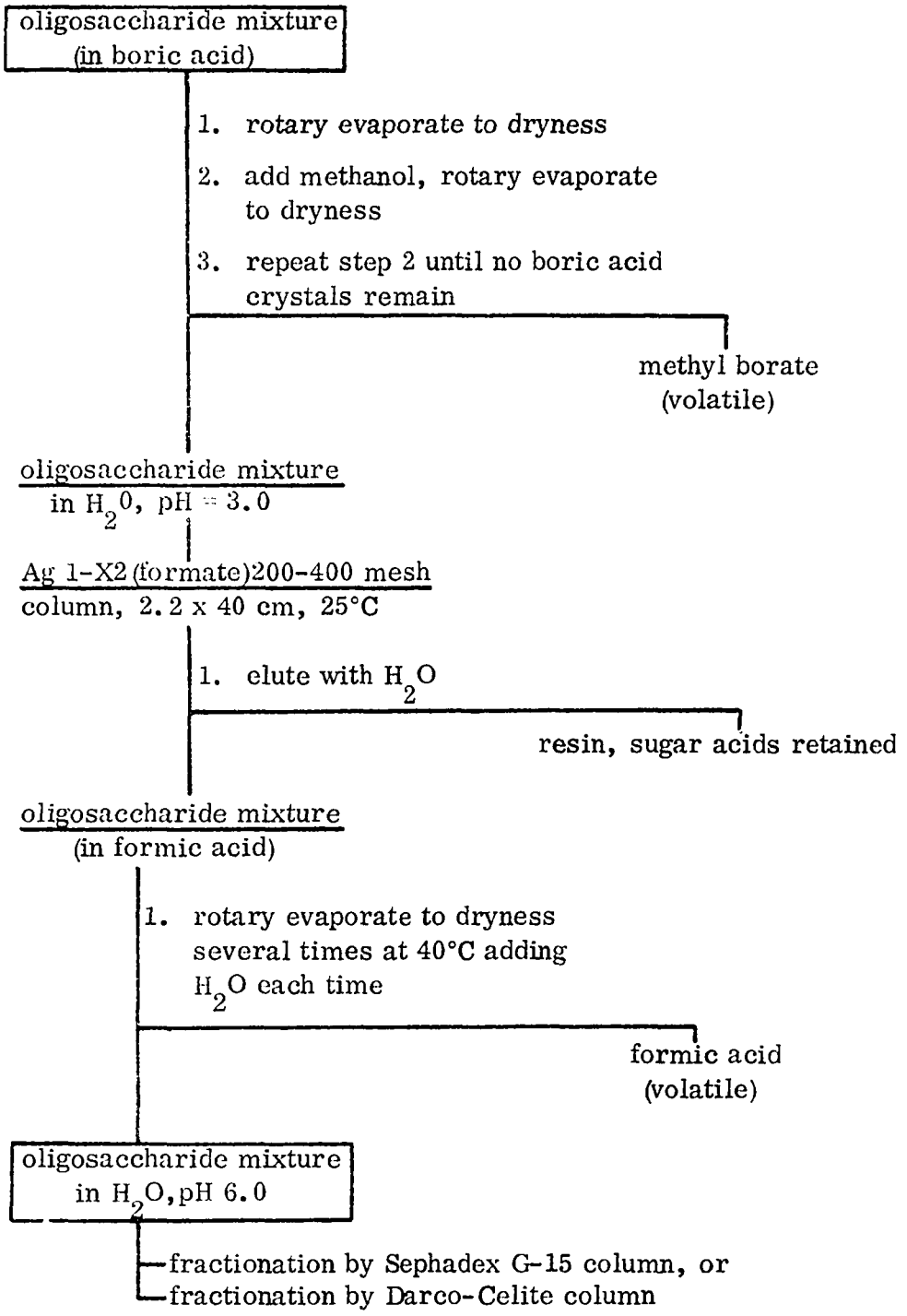


Figure 42 (continued). Procedure for the preparation and fractionation of oligosaccharides derived from lactose by borate ion-exchange chromatography.

TABLE 10

YIELD OF MIXED OLIGOSACCHARIDES (PEAK III) PREPARED BY DOWEX 1-X8(BORATE) ION-EXCHANGE CHROMATOGRAPHY OF LACTOSE

Experiment[a] No	Resin Batch	Column Temperature °C	Yield[b] per column mg	Yield[c] %
1	I	25	41	8.3
2	I	25	42	8.4
3	I	25	48	9.7
4	I	25	24	4.8
5	II	25	5	1.0
6	II	25	7	1.4
7	II	37	35	7.0
8	II	37	17	3.4

[a]Experiments 1 to 4 were done using Dowex 1-X8-Cl^- (50-100 mesh) supplied by J. T. Baker Co. (Batch I). Experiments 5 to 8 were done with Batch II of the same resin purchased from Biorad Laboratories, Inc.

[b]Yield of Peak III in mg was based on anthrone reaction of the O.1M sodium bicarbonate effluent; galactose was used as the standard.

[c]Yield in percent was calculated from anthrone data (not corrected for anthrone negative moiety) and was based on initial dry weight of lactose applied to the column (0.5 g per column).

About 20 % of the anthrone positive sugars of this fraction was retained on the Ag 1-X2 (formate) column during the removal of acidic substances. The final yield of neutral sugars, based on the anthrone test with galactose as standard, was found to vary from 300 to 600 mg which represented 5 to 10 % of the initial lactose (6 g). A summary of the yields obtained in 8 different experiments is given in Table 10. The anion exchange resin Dowex 1-X8 (Cl^-) of 50 to 100 mesh, utilized in early experiments, was supplied by the J. T. Baker Co. Since this resin is no longer available from this company, the product, i.e. Dowex 1-X8 (Cl^-) of 50 to 100 mesh, was purchased from Biorad Laboratories, Inc. As can be seen from Table 10, considerable difference in the yields was obtained with these two batches of the same resin supplied by these two companies. The yield with the new batch of resin was between 1/5 and 1/10 of that obtained with the previous resin when the experiments were conducted at the 25°C temperature. The yield with the new batch of resin could be increased if the working temperature was raised to 37°C.

Biological Studies with D-Arabino-hexosulose

The toxic effect of D-arabino-hexosulose *in vivo* was first described by Hynd (85) who administered the compound to a number of animal species. The effect of the sugar was said to be similar to that of a large dose of insulin. The margin between the dose of the compound which produced symptoms and a lethal dose was a small one, i. e. in mice 2.4 mg per g of body weight was toxic but non-lethal while 2.6 mg per g of body weight was lethal. The amount of substance which was required to produce toxic symptoms was remarkably constant in all species, being in the neighborhood of 1 to 2 mg per g of body weight. The effect of this aldosulose on the carbohydrate metabolism of various cell systems *in vitro* has been tested repeatedly and appears to be due mainly to its interference with the cell utilization of glucose (62). Recent studies have shown that keto-aldehydes, especially 3-deoxy-D-erythro-hexosulose, have an inhibitory effect on the division of mammalian cells in tissue culture (86). In view of the

possible biological use for alduloses to inhibit cell division in cancer, a study of the effect of D-arabino-hexosulose on the growth of human cells in vitro was undertaken.

$HeLa_{71}$ cells (87) were grown in monolayer culture with Eagle's minimum essential medium (MEM) (88) supplemented by calf serum (10%), glutamine (4 mM/ml), penicillin (50 units/ml), streptomycin (50 ug/ml), and kanamycin (50 ug/ml). Just prior to transfer, the cells were diluted 1 to 4 with MEM and an aliquot was taken for cell counting in a hemocytometer. A 5 ml aliquot of the cell suspension (ca. 6×10^4 cells per ml) was transferred to each of 15 flasks. A solution of D-arabino-hexosulose (500 mM) was prepared in MEM. At this concentration, the test material produced a yellow (acid) color with the phenol red indicator in the MEM. A small amount of 10% sodium bicarbonate solution was added to readjust the pH to neutral. The glucose concentration in the MEM was 5 mM. The test compound was added to yield concentrations which ranged from 0.01 mM to 10 mM. The flasks were incubated at 37°C and inspected under the microscope at 24, 48, and 72 hours. A clear cut effect was observed in flasks containing 10 mM of the D-arabino-hexosulose (two times the concentration of glucose). After a 24 hour incubation many floating, dead cells, were observed while the usual 24 hour cell doubling was observed in the control flasks. The medium was decanted from each flask at the end of its incubation period and the cell monolayer was washed with 2 ml of 0.1 M bicarbonate buffer of pH 10 to remove dead cells. The cell monolayer was then treated with 2 ml of 0.01% commercial trypsin in 1 mM ethylenediamine-tetraacetate (pH 9 to 10, 25°C, for 8 minutes). This treatment yielded a uniform cell suspension which was then diluted with an equal volume of MEM for cell counting in a hemocytometer. The results of 2 such experiments are shown in Table 11. The difference between the lethal and non-lethal dose of D-arabino-hexosulose was very small. At concentrations up to 5 mM (1:1 ratio with glucose) the cell population continued to

TABLE 11

EFFECT OF D-Arabino-HEXOSULOSE ON HeLa$_{71}$ CELLS GROWING EXPONENTIALLY IN MONOLAYER CULTURES

Flask No.	D-Arabino-hexosulose		Cell Count in thousands per ml		
	mM	ratio to glucose	24 hr	48 hr	72 hr
		Experiment 1			
1,2,3	0	0:1	116	293	630
4,5,6	0.01	0.002:1	152	304	633
7,8,9	0.10	0.02:1	144	372	725
10,11,12	1.00	0.2:1	139	344	628
13,14,15	10.00	2:1	78	38	55
		Experiment 2			
1,2,3	0	0:1	177	331	455
4,5,6	2.5	0.5:1	142	328	435
7,8,9	5.0	1.0:1	141	N.D.[a]	452
10,11,12	7.5	1.5:1	61	218	408
13,14,15	10.0	2.0:1	59	8	0

[a] Not determined, flask discarded because of cap leakage.

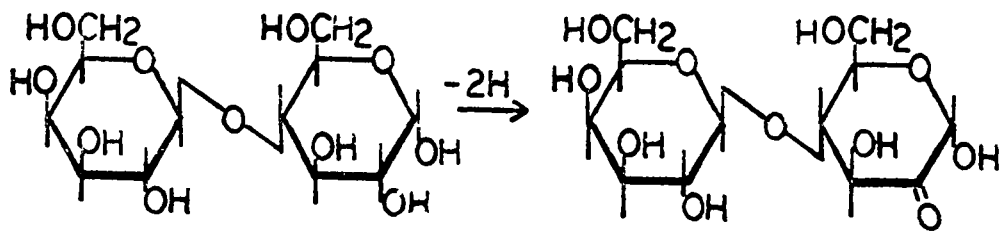
double every 24 hours. At a concentration of 7.5 mM (1.5 times the glucose concentration) a decrease in cell numbers of 66%, 34% and 9% was found at 24, 48, and 72 hours respectively. With the 10 mM concentration of D-arabino-hexosulose (2 times the glucose concentration) practically all cells were dead by 48 hours. The results indicate that this keto-aldehyde compound exerts a lethal effect on HeLa cells only when its concentration exceeds that of glucose in the culture medium.

CHAPTER IV

DISCUSSION

Conversion of Lactose to D-Arabino-hexosulose-containing Oligosaccharides

Temperature dependent changes in structure have been observed when lactose is subjected to ion-exchange chromatography on columns of Dowex 1-X8-(borate). Previous studies showing the alkaline isomerization of lactose to lactulose via the Lobry de Bruyn-Alberda van Ekenstein transformation have been continued and evidence for the oxidation of lactose to the corresponding dicarbonyl derivative has been obtained. Whereas the galactose end of the disaccharide remained intact, the reducing-end moiety was oxidized to D-arabino-hexosulose (D-glucosone) to form lactosone (2-ketolactose):



A new tetrasaccharide which appears to be a product of dimerization of lactosone was also detected among the products obtained in these experiments.

Identification and Structural Studies

The identification of the monosaccharide components of these lactose-derived oligosaccharides was hampered by the extreme instability of D-arabino-hexosulose under alkaline conditions. This unusual lability resulted in loss

of this component during neutralization of acid hydrolysates and in the apparent lack of reducing power observed in the initial studies. The strong reducing power of these compounds was established by employing mild ferricyanide methods at room temperature. The positive reaction obtained in these tests, suggested the possibility of a dicarbonyl sugar. The dicarbonyl function was then established by the ready formation of phenylhydrazine derivatives at room temperature. The problem encountered in the neutralization of acid hydrolysates was solved by using an ion-exchange resin Ag 1-X2(formate) to remove sulfate ions instead of the usual precipitation with barium hydroxide. Another difficulty encountered in the assay of D-arabino-hexosulose, either by quantitative chemical analysis or by paper chromatography methods, was due to the fact that it does not react with most of the common carbohydrate reagents such as anthrone, phenol, and 3,5-dinitrosalicylic acid. However, the monosaccharide moiety derived from glucose was found to react with amine-containing reagents. The yellow colors produced on chromatograms treated with reagents B and C were indicative of a ketose function. The purple color given with reagent A, which contains aniline and diphenylamine, appears to be rather specific for glucosone (89). The fact that this monosaccharide did not react with the usual carbohydrate detection reagents can be explained by its failure to undergo acid degradation to yield specific furaldehyde-type compounds which are requisite for chromogen formation (62). No evidence of 5-hydroxymethyl-2-furaldehyde (HMF) could be found in acid degradation products of this sugar. It was further shown that this sugar was more stable to acid treatment than 3-deoxyglucosone since the latter sugar is rather easily dehydrated and converted to HMF. The first step in the acid degradation of glucose, according to Wolfrom et al. (78), is the elimination of a molecule of water between carbon atoms 2 and 3. The already dehydrated 3-deoxyglucosone easily rearranges under acid conditions to yield the same end-product, HMF. The acid degradation of D-glucosone was found to follow a different path. It does not form HMF by acid treatment, and Sattler and Zerban (90) believe that this is the reason why it does not react with

the anthrone reagent.

Evidence that the new component monosaccharide sugar was D-arabino-hexosulose was shown more definitely by comparison with authentic samples prepared by three different methods. The unidentified monosaccharide component isolated from the new oligosaccharides and the reference compound (D-arabino-hexosulose) were found to have the same ionophoretic migration and paper (or thin-layer) chromatographic mobility in four solvent systems (numbers 1, 2, 7, and 9). D-Arabino-hexosulose, isolated from oligosaccharides prepared by the borate ion-exchange method as well as the reference compound prepared by chemical synthesis, yielded fructose when reduced with sodium borohydride. Fischer (91) demonstrated that glucosone, when treated with zinc and acetic acid, could be reduced to fructose, and this reaction has been used repeatedly for the identification of glucosone. In the presence of two carbonyl groups on a sugar such as this, the aldehyde group is reduced more readily than the keto group (70, 92).

Attempts by us and by others (62) to crystallize glucosone have been unsuccessful. Upon lyophilization all specimens, including the synthetic reference compounds, yielded a syrup. A solid powder was prepared by trituration in, followed by evaporation from, absolute ethanol, and from this preparation it was possible to examine the infrared spectrum in a KBr pellet. The spectrum of this sugar (Figure 41) corresponded more closely to the spectrum of 3-deoxy-D-glucosone than to spectra of D-glucose or D-fructose. A common feature to the spectra of these four sugars is the sharp band at 1630 cm^{-1} indicative of water, but only glucosone and 3-deoxyglucosone produced an appreciable absorption peak at 1730 cm^{-1} which is characteristic of a free carbonyl group. It is interesting to note that a spectrum closely related to those of glucosone and 3-deoxyglucosone has been recorded (93) for D-ribo-hexos-3-ulose, another dicarbonyl sugar, differing in structure from glucosone in that the ketose function is on carbon number three.

The phenylosazone derivatives of glucosone gave elemental analysis

data which compared well with values (94) for the same compound prepared from glucose. The infrared spectra of the phenylosazone derivatives prepared from glucosone, glucose, and fructose, were also identical (Figure 38). The absorption bands which appear in the 1500-1700 cm^{-1} region are typical for phenylosazones. The relative absorption intensities of the bands in this region are different for phenylosazone derivatives of glucose and galactose, also the NH deformation of glucosephenylosazone absorbs at 1540 cm^{-1} whereas that of galactose absorbs at 1535 cm^{-1} (95). This difference is difficult to distinguish, but a definite variation in infrared spectrum was noted in the 1200 cm^{-1} region. Glucosephenylosazone (which is identical to the fructose and glucosone derivatives) exhibits a single band at 1255 cm^{-1}, but galactosephenylosazone spectrum has a double peak in this region with maxima at 1270 cm^{-1} and at 1240 cm^{-1} respectively. Another difference appears in the 800 cm^{-1} region. The galactose derivative shows a double peak (875 and 895 cm^{-1}), while glucosephenylosazone exhibits a single broad peak between 870 and 890 cm^{-1}. The differences discussed here have been reported previously by Kuhn et al. (96) for the infrared spectra of phenylosazones of glucose and galactose.

Although the 2,4-dinitrophenylosazone of glucosone formed easily at room temperature, this compound was difficult to recrystallize; and, hence, the impurities retained may be responsible for the slight departure between the decomposition melting points observed with the two glucosone preparations (Table 6). The infrared spectra of the 2,4-dinitrophenylosazone derivatives of these sugars were not so distinctive as the phenylosazones, but the spectra of glucosone, from the borate ion-exchange column method and the reference compound prepared from the reaction between glucose and cupric acetate, compare well with each other and with spectra obtained with the same derivative prepared from glucose and from fructose. All four of these spectra show a strong similarity to the spectra of 3-deoxyglucosone-2,4-dinitrophenylosazone recorded by Fodor et al. (97).

The molecular sizes (tetrasaccharide and disaccharide) of lactose-derived oligosaccharides were established by Sephadex gel filtration. These

results were confirmed by the data obtained with Darco-Celite fractionation and gas-liquid chromatography. These oligosaccharides could not be crystallized either, but dry samples were obtained by lyophilization. Since these samples were hygroscopic, the results of the elemental microanalyses which corresponded to a lactosone dihydrate and a lactosone dimer pentahydrate respectively were not unexpected and should be interpreted with reservations keeping in mind that it is technically very difficult, if not impossible, to maintain samples of these compounds completely anhydrous. Infrared analysis (KBr pellet) of lyophilized preparations of the disaccharide, tetrasaccharide, lactose, and lactulose shows similar absorption spectra for all four, and, in this regard, was not too informative. All have prominent absorption bands at 1630 cm^{-1} due to water, but only the lactosone spectrum contains the free carbonyl peak at 1730 cm^{-1}.

Standard samples of lactosone, prepared by two different chemical methods, were found to co-chromatograph with the disaccharide isolated from oligosaccharide I mixture in paper chromatograms developed with two solvent systems and with borate paper ionophoresis. Reduction of this disaccharide with sodium borohydride resulted in the production of lactulose as expected since reduction of glucosone gave fructose.

Periodate oxidation of the monosaccharide glucosone for 48 hours showed a consumption of 2.6 moles of periodate per mole of sugar. No data concerning the consumption of periodate by this compound could be found in the literature although formaldehyde, formic acid and glyoxylic acid have been reported among the products of the reaction (98). Other workers (81) have found that 1 mole of glucosone consumed 2 moles of lead tetraacetate in 40 hours and a progressive oxidation continued with an overall consumption of 3 moles at the end of 120 hours. A similar sugar, D-ribo-hexos-3-ulose, was found (93) to consume 2.9 moles of periodate per mole of starting material. The data for glucosone cannot be considered too meaningful at this time due to the difficulty in quantitating a syrupy material. Other problems which complicate the

periodate oxidation studies are the instability of the sugar under various conditions as well as the various possible structural forms of glucosone. The disaccharide lactosone was found to consume between 3.5 and 3.6 moles of periodate per mole of sugar, a value which is close to the calculated value (4.0) and to data available for 3-ketolactose (99) which was found to consume 3.9 moles of periodate per mole of sugar. The consumption of 9.4 moles of periodate per mole of the tetrasaccharide appears to be excessive, but no interpretation can be offered at this time since the linkage of the disaccharide units and other structural information are not yet available. It is possible, however, that the excess periodate consumption may be due to side reactions or that production of uv absorbing materials might introduce an artifact in this determination. It has been concluded that this tetrasaccharide, whose component sugars are galactose and glucosone, probably represents a dimer of the disaccharide lactosone. The simplest osulose (glycerosone or hydroxypyruvic aldehyde) was reported (101) to exist normally as a trimer. In our study the appearance of spots of low chromatographic mobility, having the staining characteristics of glucosone, suggested the possible polymerization of this sugar. This was noted particularly in cases where the sugar was subjected to prolonged purification procedures involving repeated rotary evaporation. Hence, a polymerization involving the hexosulose portion of lactosone appeared to be a plausible explanation for the formation of the observed tetrasaccharide. The tetrasaccharide exhibited decreased staining with aniline-diphenylamine when compared to the same weight of disaccharide, and this is compatible with the involvement of the glucosone moiety in the polymerization.

The elongated spots given on chromatograms by the oligosaccharides precluded any attempts to correlate molecular size with differences in mobility but the elongated spots do suggest that these sugars exist in some type of equilibrium mixture perhaps of several forms. Petuely (100) has concluded that the syrupy nature of glucosone is evidence for its existence in a mixture of isomers or stereo-isomers. The hexosulose part of the oligosaccharides could

appear in a variety open-chain, tautomeric-enediol, hemiacetal, bicyclic or hydrated forms which would vary with the medium. Many structures have been formulated for glucosone (100) and some of them are depicted in Figure 43. In these studies the formation of hemiacetal structures are manifest by the changes in the Rglucose values of D-arabino-hexosulose, when changing from alcoholic (Rglu = 0.6 — 0.8) to non-alcoholic solvents (Rglu = 1.0 — 1.2). This is not so apparent with the hexosulose-containing oligosaccharides where the number and type of isomeric forms may be more restricted.

The multiple peaks observed in glc patterns appear to support the multiplicity of isomeric forms of these sugars. El-Dash and Hodge (102) have pointed out that at least 20 structural isomers have been proposed for 3-deoxy-D-erythro-hexosulose, and they observed 11 peaks in a glc study of this compound. Five of the peaks were unstable and disappeared after the sugar had equilibrated in pyridine for 24 hours; but 6 stable isomers remained. The glc pattern of D-arabino-hexosulose exhibited 7 peaks, one peak disappeared sometime after the sugar had remained in pyridine for 5 minutes; the remaining 6 were stable in pyridine for 17 hours. However, other possible explanations for the multiple peak profile of this sugar should be considered. One is the possibility of incomplete silylation which is a problem for sugars of this type. Another explanation may be the instability of some sugars during the silylation reaction. TMS-ascorbic acid (103) is reported to be unstable due to hydrolysis following exposure to moisture in room air. Quantitative glc analysis of sugars of this type is not really feasible because of the multiplicity of tautomeric forms which give numerous peaks. This problem can be solved by borohydride reduction of these sugars (102) to give hexitols thus reducing the number of peaks from a single sugar.

The ultraviolet absorption studies reported in this investigation suggest the presence of enediol-tautomeric isomers especially in neutral or slightly alkaline conditions. However, the only uv absorbing peak of significant magnitude ($E^{1\%}_{1\ cm}$ of 14 to 15 at 315 mμ) given by D-arabino-hexosulose, the

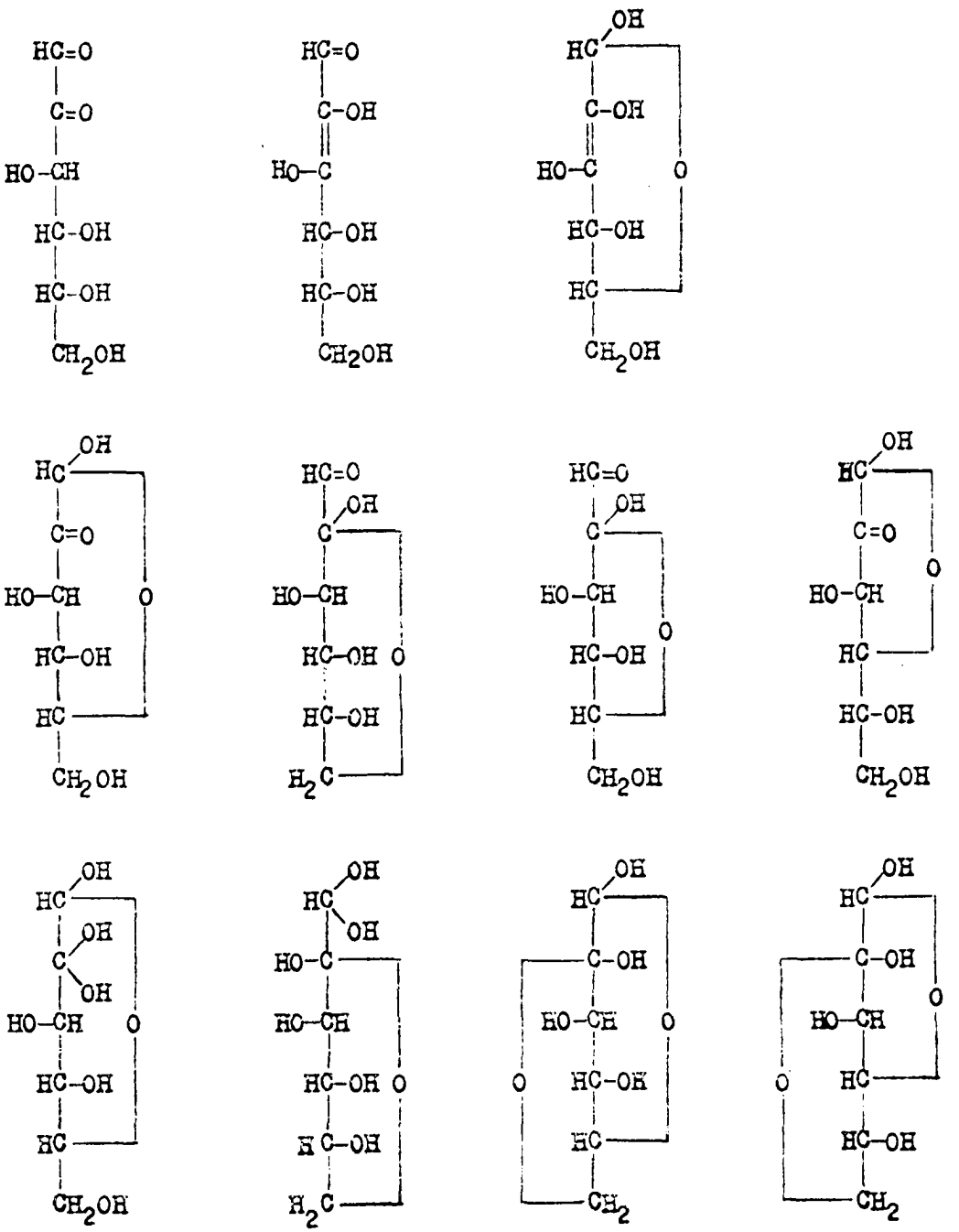


Figure 43. Some of the possible structural forms of D-arabino-hexosulose according to Petuely (100).

disaccharide, and the tetrasaccharide was observed in alkaline solutions. This is in general agreement with data reported by others (62, 89) in studies of glucosone. The uv absorption of these sugars in acid, distilled water, or methanol was very weak with $E^{1\%}_{1\ cm}$ values of 3 or less. The lowest absorption found was with the glucosone reference standard prepared from glucose and copper acetate which had been purified by cellulose column chromatography. Weak uv absorptions of glucosone in acid, aqueous, and methanol solutions have been described (62, 89), however the variability observed in our preparations suggest that these absorptions are probably the result of impurities arising from the different methods of preparation. Some variability might still result from shifts in molecular form known to occur with glucosone. The alkaline instability of D-arabino-hexosulose (and its parent oligosaccharides) produced in this study, is undoubtedly a consequence of the enolic nature of some of its isomeric forms. In the presence of nucleophilic bases proton removal from the sugar would be facilitated and allow shifting of bonds within the structure. Glc studies of the sugar following alkali treatment demonstrated the production of aldonic acids, indicating that the alkaline degradation observed was of the oxidative type. The use of monovalent and divalent cation bases yielded different results. With the monovalent NaOH, arabinonic acid was the main product as a consequence of the scission of the carbon chain. The divalent $Ca(OH)_2$ produced rearrangement of the sugar, rather than chain scission, to yield mannonic acid. These results confirm earlier data by Lindberg and Theander (79) who studied D-arabino-hexosulose under similar conditions. Other alkaline degradative products have been reported for glucosone, e. g. kojic acid (104) but the experimental details of this work are not available.

The presence of acids in the effluent sodium bicarbonate (peak III) from the borate ion-exchange column can be explained, in part, by the aldonic acid degradation products discussed above. In addition, other acidic sugars might be expected from the alkaline degradation of the disaccharide sugars. Rowell et al. (105) have reported the finding of glucose-bound saccharinic acids

when studying the alkaline degradation of cellobiose. Galactose-bound saccharinic acids and galactose-bound aldonic acids could account for some of the acidic products. Such acidic disaccharides might arise directly from lactose or indirectly via lactulose or lactosone. Studies of lactosone under the same alkaline conditions as used for glc studies of D-arabino-hexosulose provided some additional information along these lines. Beta-elimination of galactose was a prominent reaction and lactosone was found to be more stable in the presence of the monovalent base NaOH than in the presence of $Ca(OH)_2$ at 37°C. The still unidentified disaccharide sugar (oligosaccharide II) produced on the borate column operated at 37°C could not be detected on chromatograms containing alkali treated lactosone. This does not rule out the possibility that this sugar might be a degradation product of lactosone, only that this disaccharide cannot be produced by these alkaline conditions.

Formation of Aldosuloses

The conversion of lactose to lactosone was first carried out chemically by Fischer (76) with the same method he used to prepare glucosone. Lactosephenylosazone was formed in the reaction between lactose and phenylhydrazine; the hydrazine groups were then removed by the action of hot hydrochloric acid yielding lactosone. Fischer also prepared other glucosone-containing disaccharides in the same manner starting with maltose, melibiose, and cellobiose. Hexosulose-containing compounds appear to be the first intermediates in the metabolism of many disaccharides by some microorganisms. Feingold et al. (106) isolated 3-ketosucrose from the cultures of Agrobacterium tumefaciens grown on sucrose as the carbon source. Fukui and Hochster (99), using both tumorogenic and non-tumorogenic strains of this organism, demonstrated the conversion of trehalose, maltose, and lactose to the corresponding disaccharides containing a terminal 3-keto-glucose.

Aldosuloses can also be produced from N-glycosides formed in the reaction between sugars and amines. These N-glycosylamines isomerize to

N-substituted 1-amino-1-deoxy-2-ketoses. An intermediate of this Amadori rearrangement (107) is an eneaminol whose structure shows some similarity to the enediol intermediate of the Lobry de Bruyn-Alberda van Ekenstein transformation.

eneaminol enediol

Several products may be formed from the eneaminol depending upon specific conditions, but the main product is usually the Amadori product (1-amino-1-deoxy-2-ketose). The Amadori product of a hexose often dehydrates to a deoxyhexosulose, providing a method for preparing these sugars (47). The non-deoxy type hexosulose, e. g. D-arabino-hexosulose, also appears as a by-product in this reaction with glucose; but the yield is much lower. Kato (63) found that the formation of glucosone in this reaction was dependent especially upon dissolved oxygen. He found that the yield could be increased if air was bubbled through the reaction mixture, and that the yield decreased when nitrogen was used. We found the reaction of glucose with n-butylamine to produce about a 1% yield of glucosone from the eneaminol intermediate. Lactosone could be produced only in low yield by this method probably because of the limited solubility of lactose in methanol, the solvent utilized in this reaction.

Glucosone has been proposed as an intermediate in the direct oxidation of glucose under alkaline conditions, but it has never been isolated in a reaction of this type due to its instability in alkali. In neutral or acidic environment, specific catalysts such as cupric acetate (64), selenium dioxide (65), and manganese dioxide (108) are required for this oxidation. Glucosone has also been found among the degradation products of fructose subjected to ^{60}Co gamma radiation in aqueous solution (109). A new method for the synthesis of aldosuloses utilizes O-isopropylidene derivatives of aldoses as the starting material.

The carbon chain of these hydroxyl-protected sugars is lengthened using a vinyl-magnesium halide (Grignard reagent) to yield a pair of epimeric allylic alcohols. Oxidation with manganese dioxide gives a vinyl ketone which upon ozonolysis produces the corresponding dicarbonyl derivative. Removal of the isopropylidene groups is then accomplished by mild acid hydrolysis to give the free aldosulose with one carbon more than the initial aldose:

$$R \cdot CHO \longrightarrow R \cdot CHOH \cdot CH{=}CH_2 \longrightarrow R \cdot CO \cdot CH{=}CH_2 \longrightarrow R \cdot CO \cdot CHO$$

Glucosone was prepared by this method (80) starting with the O-isopropylidene derivative of arabinose and D-glycero-tetrosulose was prepared from the O-isopropylidene derivative of glyceraldehyde. The latter aldosulose could not be prepared by other methods including the osazone cleavage method from D-erythrosephenylosazone.

It is not yet clear whether the production of glucosone-containing oligosaccharides during borate ion-exchange chromatography of lactose proceeds via oxidation of lactose, lactulose, or enediol intermediates. The conditions of the experiment probably enhance the oxidation and, at the same time, retard the degradation of the glucosone-containing oligosaccharides. Borate solutions provide an alkaline environment which promotes the oxidation of the reducing-end of the disaccharide. The oxidation product may then be stabilized by the formation of borate complexes. The presence of a catalyst, under these conditions, might also accelerate the oxidative process. The Dowex 1-X8, employed in all but the last few experiments, was of "commercial grade quality." This resin was obtained from J. T. Baker Co, and it was supplied without data as to impurities, i. e. metal content. According to data supplied from Biorad Laboratories and shown in Table 12 (110) such a resin contains, on the average, about 4% total ash of which 0.002% is Cu. The analytical grade resin, Ag 1-X8, is purified by Biorad Laboratories in a series of elutions with organic solvents, complexing agents, acids, bases, and water. As a result of this treatment, the analytical grade resin contains only 0.06% ash of which 0.00002% is Cu. Consequently the commercial grade contains about 100 times more Cu than the

TABLE 12

COMPARISON OF SPECIFICATIONS OF AG AND DOWEX ION-EXCHANGE RESINS

Impurities	Percent Found[a]	
	AG 1-X8	DOWEX 1-X8
Fe	0.00005	0.003
Cu	0.00002	0.002
Ni	0.000005	0.0015
Pb	0.0000005	0.002
Al	0.0005	0.08
Total ash	0.06	4.0

[a]Values expressed are maximum percent impurities for the Ag resins and average percent impurities in the Dowex resins (110).

analytical grade. The relative amounts of other contaminants are also shown for comparative purposes. The Dowex 1-X8 commercial grade resin used in the most recent experiments was purchased from Biorad Laboratories and appeared to be more pure than the old product purchased from Baker. This probably accounts for the lower yield of hexosulose oligosaccharides since these metallic impurities, especially Cu, could act as catalyst of the oxidation reaction under alkaline conditions.

Column ion-exchange chromatography methods, particularly those employing borate solutions and elevated temperatures, must be used with caution because these methods can result in structural alterations of the sugars under study. In addition to the aldose-ketose isomerization and to the oxidation to aldosulose, the as yet unidentified oligosaccharide II and sugar acids mentioned above are clear indication of the complexity of these reactions and suggest the existence of other transformations yet to be described. Borate ion-exchange chromatography has been found already to be useful for the production of ketosugars such as lactulose and its possible use in the preparation of aldosuloses is now quite apparent. The application of this experimental procedure to other sugars, e. g. maltose, melibiose, cellobiose, etc. can and should be explored since it would probably lead to similar findings.

Biological Significance of D-Arabino-hexosulose

This compound has been found in nature although rather infrequently. Bean and Hassid (111) demonstrated the enzymatic oxidation of glucose to glucosone in marine red alga, and Berkeley (112) found it (also derived from glucose) in the crystalline styles of Mollusca. The possible function of glucosone in the formation of glucosamine was suggested (113) in 1953, but this could not be substantiated (114) in studies done with a bacterial system. Furthermore, glucosone is not an intermediate in the pathway of glucosamine biosynthesis in mammals.

Interest in aldosuloses has been recently renewed because of their

inhibitory action on enzyme systems of mammalian tumor cells in vitro. A growth inhibitor called "retine" was extracted (86) from steam distilled extracts of such varied sources as mushrooms, calf thymus glands, and human urine. Szent-Gyorgyi et al. (115) demonstrated its growth inhibitor properties in a malignant tumor assay system, Krebs-ascites cells propagated in rats. This inhibitor was later identified as 3-deoxy-D-glucosone (97), but this compound was later found not to be a normal cell constituent as it was first thought but rather an artifact produced during the manipulations of the biological materials from which it was isolated (61).

Glucosone has been known for many years to have a toxic effect on biological systems. Many studies with yeast fermentation (116), ox brain hexokinase (117), yeast hexokinase (118), guinea pig skin slices (119), and Krebs-ascites carcinoma cells propagated in mice (120) have shown that this compound is an antimetabolite of glucose (similar to 2-deoxy-glucose) which competitively inhibits the enzyme hexokinase. The possible toxic effect of glucosone was tested in this investigation with $HeLa_{71}$ cells grown in monolayer cultures. The amount of glucosone required to inhibit the 24 hour doubling rate and subsequently kill these cells was found to be twice the normal concentration of glucose used in the medium. This corroborates studies by others (116, 118) who tested this sugar. The toxic effect of D-glucosone is specific since neither L-glucosone (116) nor lactosone (121) have been found to be toxic.

Other possible biological uses for glucosone include its protective effect in preventing cyanide poisoning in mice (122). However, glucosone appeared to work no better than less toxic antidotes such as cystine. Aldosuloses because of the high reactivity of the dicarbonyl group are also potentially important as starting materials for the chemical synthesis of various compounds. The possible effect of glucosone and lactosone on enzymes other than hexokinase has not been explored yet. The elucidation of the detailed structure of the tetrasaccharide containing glucosone described here appears to be a formidable challenge due to its rather unstable nature and because it might occur under various structural forms.

CHAPTER V

SUMMARY

Formation of 2-ketolactose (lactosone) and its dimerization were observed during borate ion-exchange chromatography of lactose on columns of Dowex 1-X8 (borate). These compounds were isolated from the oligosaccharide mixture eluted by 0.1 M $NaHCO_3$ following the elution of residual lactose and lactulose by borate buffers at toom temperature. These two glucosone-containing oligosaccharides behaved as a disaccharide and as a tetrasaccharide respectively on Sephadex G-15 and Darco 60-Celite chromatography. Lactosone was found to be indistinguishable, by paper and thin-layer chromatography and by borate ionophoresis, from the authentic disaccharide prepared from lactose by two different methods. β-Galactosidase hydrolysis of the lactose-derived oligosaccharides indicated that the galactose moiety was still intact and linked β-glycosidically probably to the C_4 of D-arabino-hexosulose (glucosone) which occupies the reducing-end of the molecule. The latter sugar, isolated from acid hydrolysates of the oligosaccharides, was chromatographically and ionophoretically indistinguishable from the authentic monosaccharide synthesized from glucose and fructose by four different methods. These sugars are extremely alkali-labile and functional group analysis showed them to be strongly reducing dicarbonyl structures able to reduce ferricyanide and cupric ions and to react with phenylhydrazine reagents--all at room temperature. Further characterization included periodate oxidation, borohydride reduction, gas-liquid chromatography, and ultraviolet and infrared spectroscopy. Infrared and elemental analysis of crystalline phenylhydrazine derivatives of D-arabino-hexosulose gave results

which compared favorably to literature values.

The monosaccharide component, D-arabino-hexosulose, was found to inhibit the 24 hour doubling rate of HeLa cells grown in monolayer cultures. This characteristic toxicity is thought to be related to an interference with glucose metabolism by inhibition of hexokinase.

The studies show that borate ion-exchange chromatography of sugars at room temperature can lead to a number of structural changes. In addition to the previously reported isomerization of lactose to lactulose, oxidative reactions have now been shown to occur. Trace amounts of metallic impurities (such as copper) present in the ion-exchange resin are probably essential catalysts for this oxidation process. Other conditions which influence these sugar transformations are temperature, mild alkaline environment, and the sugar-borate complexes.

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