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SEPARATION OF ENANTIOMERIC SUGARS AS DIASTEREOISOMERIC DITHIOACETALS

The Ohio State University PH.D. 1982

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SEPARATION OF ENANTIOMERIC SUGARS AS DIASTEREOISOMERIC DITHIOACETALS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

The Ohio State University

1982

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PUBLICATIONS

M. R. Little, K. A. Reynolds, C. R. Rauck, G. A. Barber, and E. J. Behrman, "HPLC Separation of Enantiomeric Sugars as Diastereomeric Cyclic Dithioacetals," Abstr. Pap. Am. Chem. Soc. Meet., 179, CARB-28 (1980).

M. R. Little, "Separation, by G.L.C., of Enantiomeric Sugars as Diastereoisomeric Dithioacetals," Carbohydr. Res., 105, 1 (1982).

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I. Introduction

Glycoconjugates comprise a group of glycosylated macromolecules which include glycoproteins, glycolipids, and proteoglycans. It is now well established that glycoconjugates play significant roles in a number of biological processes (1,2,3). Aside from maintaining structural and morphological rigidity in living tissues and cells, the glycoconjugates also are involved in intercellular recognition, cellular adhesion, and specific cellular functions in a number of widely diverse systems. Notable examples include the homing of lymphocytes to various organs (4,5), binding of influenza virus to its host cell (6), species-specific cell aggregation in sponges (7) and in slime molds (8), the sexual mating of compatible yeasts (9), the specific transport of hydrolytic enzymes to lysosomes (10), the antigenic determination of blood groups (11), and the pinocytotic uptake of serum glycoproteins by the liver (12).

Much of the functional specificity characteristic of the glycoconjugates is attributed to the chemical structure of the carbohydrate moiety (13). Typically, the naturally occurring carbohydrate portion of a glycoconjugate is comprised of a number of monosaccharides linked together through glycosidic

bonds. Considerable variation exists, not only in the sugar composition, but also in the manner in which the residues are covalently linked. Therefore, a carbohydrate moiety, at least in theory, can provide greater specificity than can a protein moiety comprised of an equal number of residues (14).

The development of methods used to elucidate the structure of the carbohydrate component of glycoconjugates has grown concurrently with studies on their biological functions. Complete structural characterization of an oligo- or polysaccharide fragment requires knowledge of the composition and sequence of its sugars, the position and anomeric configuration of their linkages, and the ring form of each sugar residue. Such information can be readily obtained on small amounts of material by well-defined chemical and spectroscopic methods (15).

A shortfall in the routine analysis of small amounts of carbohydrate samples is the lack of a straightforward method to determine the absolute configuration of individual sugars. The sugars, obtained by acid hydrolysis, are generally separated and identified as their alditol acetates by gas-liquid chromatography (16). Under these conditions, the researcher cannot distinguish between enantiomers of the same sugar. Therefore sugars cannot be designated with certainty as belonging to the D- or L- family. Only when quantities of material large enough for polarimetric measurements are

isolated, or in a few instances where enzymic procedures have been developed (17), can the absolute configuration of a sugar be designated. There is thus considerable potential for an analytical method by which such assignments can be made. This is particularly important in glycoconjugate research where only small quantities of material can usually be isolated. The following work describes methods in which the absolute configuration of sugars can be assigned. The goal of this research project is to provide a simple, rapid method by which small amounts of enantiomeric sugars can be identified and their relative quantities measured.

Resolution of Enantiomers

In the absence of a chiral environment, optical isomers or enantiomers have identical physical and chemical properties. The resolution of enantiomers then can only be effected in the presence of a chiral agent. In his classical work in 1848, Pasteur performed the first resolution of enantiomeric compounds by physically separating the hemihedral crystals of racemic sodium ammonium tartrate (18). Researchers today, equipped with contemporary chromatographic methods, have exploited the basic principles set by Pasteur. Advances in this field of separation have been the subject of recent reviews (19,20,21).

A convenient approach taken to separate enantiomers is the conversion of a racemic pair into diastereoisomeric compounds. These derivatives, formed by the reaction of each enantiomer with an enantiomer of another optically active compound, can be separated by ordinary chemical means. Direct resolution is an alternative approach to the chromatographic separation of enantiomers. This approach requires the presence of chirality in either the mobile or stationary phase. By this method, separation can be achieved without prior chemical modification of the racemic pair. Numerous examples involving both approaches of separation have appeared in the literature (22). For example, successful resolution of optically active amino acids by gas-liquid chromatography has been achieved both indirectly by the separation of diastereoisomeric derivatives (23) and directly with the use of chiral stationary phases (24). In addition, Hare and Gil-Av have resolved amino acids by liquid chromatography using a chiral eluant (25).

In 1968, Pollock and Jermany described a method by which sugar enantiomers could be separated as diastereoisomeric esters. The procedure required conversion of aldoses into aldonic acids, which, in turn, were converted into the corresponding acid chlorides. The chlorides were treated with optically active alcohols to yield the diastereoisomeric sugar esters, which were separated by gas-liquid chromatography. By this method, they were able to separate enantiomeric tetroses and pentoses (26) and in a later report (27), enantiomeric

hexoses. The difficulties involved in the derivativization of the aldoses set some limit to the practical value of this method.

By related methods, Gerwig et al. (28) and Leontein et al. (29) directly combined optically active alcohols with an enantiomeric pair of sugars to form diastereoisomeric glycosides. The glycosides obtained from each sugar produce α and β anomers of two different cyclic forms. Thus the resulting chromatogram may be complicated by the presence of both anomers for each of the two cyclic forms.

König's research group has studied the direct resolution of sugars using capillary glass columns coated with chiral stationary phases. In their initial report (30), successful separation of enantiomeric trifluoroacetyl hexoses was achieved on a stationary phase coated with XE-60-(S)-valine-(S)-1-phenylethylamide. In later work (31), it was determined that modified phases of XE-60 and OV-225 with the corresponding (S)-valine-(R)- 1-phenylethylamide, were suited for the resolution of pentoses and 6-deoxyhexoses. Leavitt and Sherman (32) have used a commercially available capillary column having a chiral stationary phase to separate some enantiomeric sugars. These direct approaches, although highly desirable, produce chromatograms in which four forms may be present for each sugar. Thus, as many as eight isomeric peaks may be expected in a mixture containing only a single pair of enantiomers.

The synthesis of acyclic sugar diastereomers results in a simplification of the chromatographic pattern. Oshima and Kumanotani (33) separated a number of sugars by reductive amination of the enantiomeric pair with (-)-1-phenylethylamine. This method was not demonstrated to be practicable for routine analyses of microquantities of material. Schweer (34) separated some sugar enantiomers as their (-)-menthyloxime perfluoroacetate derivatives. The resulting chromatograms, as expected, indicated both syn and anti oxime isomers. Zablocki et al. (35), working in this laboratory, showed that enantiomers of galactose and fucose could be separated by gas-liquid chromatography as their peracetylated bis (ethyl-L-lactate) acetals. The peracetylated O-acetals were prepared from the corresponding peracetylated diethyldithioacetals (Figure 1). It was rationalized (36) that a simpler route to acyclic sugar diastereomers would be to treat each pair of enantiomeric sugars with an optically active thiol. Diastereoisomeric dithioacetals are produced in the initial step, thus eliminating the need of subsequent steps. The use of optically active thiols as a simple route to acyclic sugar diastereomers, and their subsequent separation by chromatographic methods, forms the basis of the work described herein.

Figure 1: Separation of D- and L-fucose as their peracetylated bis (ethyl L-lactate) acetals. These diastereomers can be separated by g.l.c. as described by Zablocki, Behrman, and Barber.

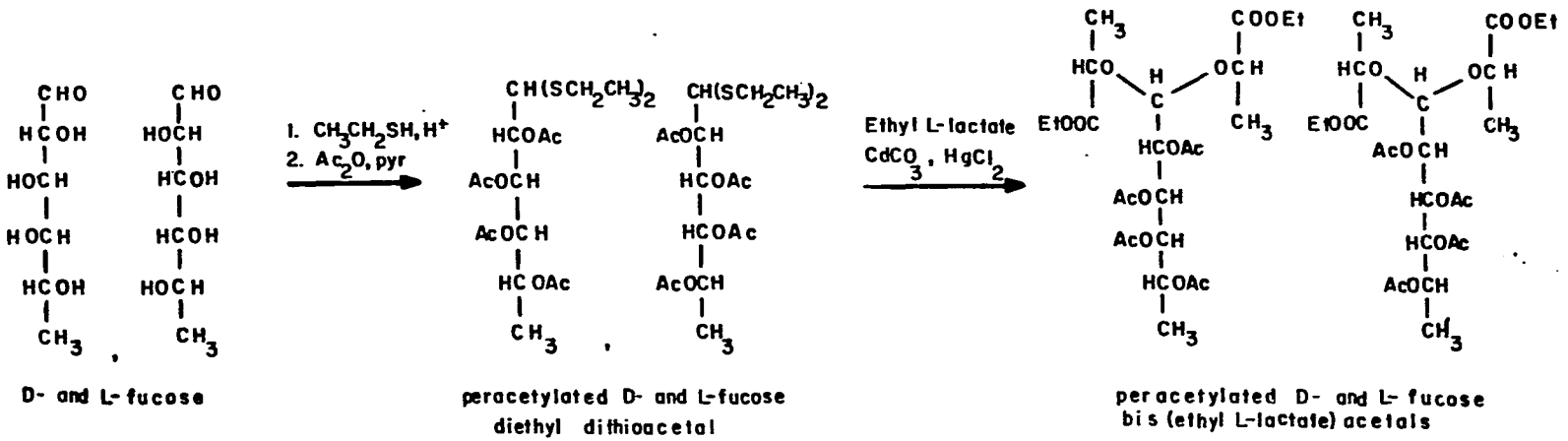

1. CH_3CH_2SH, H^+
2. Ac_2O, pyr
Ethyl L-lactate
$CdCO_3$, $HgCl_2$
D- and L-fucose
peracetylated D- and L-fucose diethyl dithioacetal
peracetylated D- and L- fucose bis (ethyl L-lactate) acetals

Figure 1.

Reaction of aldoses with thiols

Emil Fischer showed that the reaction between aldoses and thiols, in presence of acid, yielded crystalline dithioacetals (37). Lawrence (38) further demonstrated the versatility of this reaction by making ethylene, trimethylene, and dibenzyl dithioacetals of a variety of sugars. An excellent review of sugar dithioacetals and their chemistry has been published (39).

The condensation reaction between sugars and thiols is a particularly useful synthetic route to acyclic sugars. Whereas other routes to acyclic sugar derivatives may modify configurational aspects of the sugar molecule, the reaction of sugars with thiols does not affect any configurational feature inherent to the hypothetical, aldehydo sugar from which it is derived (40). Thus, only one configurational isomeric dithioacetal results when an optically pure thiol condenses with an optically pure sugar. Optically active thiols are therefore ideally suitable resolving reagents for sugars.

The first use of an optically active thiol in a condensation reaction with sugars was reported by Votoček and Veselý (41). They were able to separate enantiomers of arabinose by fractional recrystallization of the corresponding diastereoisomeric bis [(+)-2-methyl-1-butyl] dithioacetals. Reported in this study is the reaction of sugar enantiomers with other optically active thiols. The following section describes experimentation using (+)-2,3-dimercaptosuccinic acid

as a resolving reagent. In section III, the use of (+)-1-phenylethanethiol as a resolving agent is investigated. Biological application of the method develcped in section III are presented in section IV.

II. Resolution of sugars using (+)-2,3-dimercaptosuccinic acid

The ideal resolving reagent should 1) be readily available in optical purity, 2) react equally, if not quantitatively, with the compounds to be resolved, and 3) yield diastereoisomeric derivatives that can be readily separated. In choosing a reagent to carry out a certain resolution, one generally can obtain information in advance regarding the purity in which the reagent can be obtained and its reactivity with the enantiomeric compounds. However, one cannot predict without experimentation how readily the diastereoisomeric compounds can be separated.

Optically active thiols are particularly good resolving reagents for sugars. They react rapidly with sugars to form acyclic dithioacetals in high yield. This section describes the work using (+)-2,3-dimercaptosuccinic acid as a resolving reagent to separate sugar enantiomers. The synthesis of (+)-2,3-dimercaptosuccinic acid, its reactivity with sugars, and the overall usefulness of this reagent in separating sugar enantiomers is discussed.

Preparation of partially active (+)-2,3-dimercaptosuccinic acid. The synthesis of (+)-2,3-dimercaptosuccinic acid as described by Gerecke et al. (42) and Hedblom (43) is outlined

in Figure 2. In principle, recovery of both antipodes in optical purity should be possible since there exists enantioselectivity in the resolution step. However, experimentally the dextrorotatory isomer is more readily obtainable. In the two preparations of 2,3-dimercaptosuccinic acid (44), the dextrorotatory isomer was recovered having indices of specific rotation, $[\alpha]_{589}^{25}$ +81 and $[\alpha]_{589}^{25}$ +56. This corresponds to an optical purity of 82% and 74%, respectively. The levorotatory isomer was recovered having indices of specific rotation, $[\alpha]_{589}^{25}$ -16 and $[\alpha]_{589}^{25}$ -7, which corresponds in optical purity to 56% and 53%. Overall yields of each isomer were less than 2%. For this reason, neither isomer could be obtained in sufficient quantities to allow for routine experimentation. The unresolved mixture of the two isomers,(+/-) 2,3-dimercaptosuccinic acid, was used to study reactivity with sugars and to set certain reaction parameters.

Reaction of Aldoses with (+/-)2,3-dimercaptosuccinic acid. The reaction of an aldose with 2,3-dimercaptosuccinic acid results in the formation of a cyclic dithioacetal (Figure 3). The course of this reaction can be followed by paper electrophoresis. At pH 6.9, the dithioacetal is well separated from each of the reactants. The electrophoretic mobilities of some of the sugar dithioacetals produced by this reaction are given in Table I.

Fischer obtained fair yields of sugar dithioacetals by treating sugars, dissolved in concentrated HCl, with an excess of ethanethiol at 0° (37). Under similar condition (45), the reaction between (+/-)2,3-dimercaptosuccinic acid and D-galactose

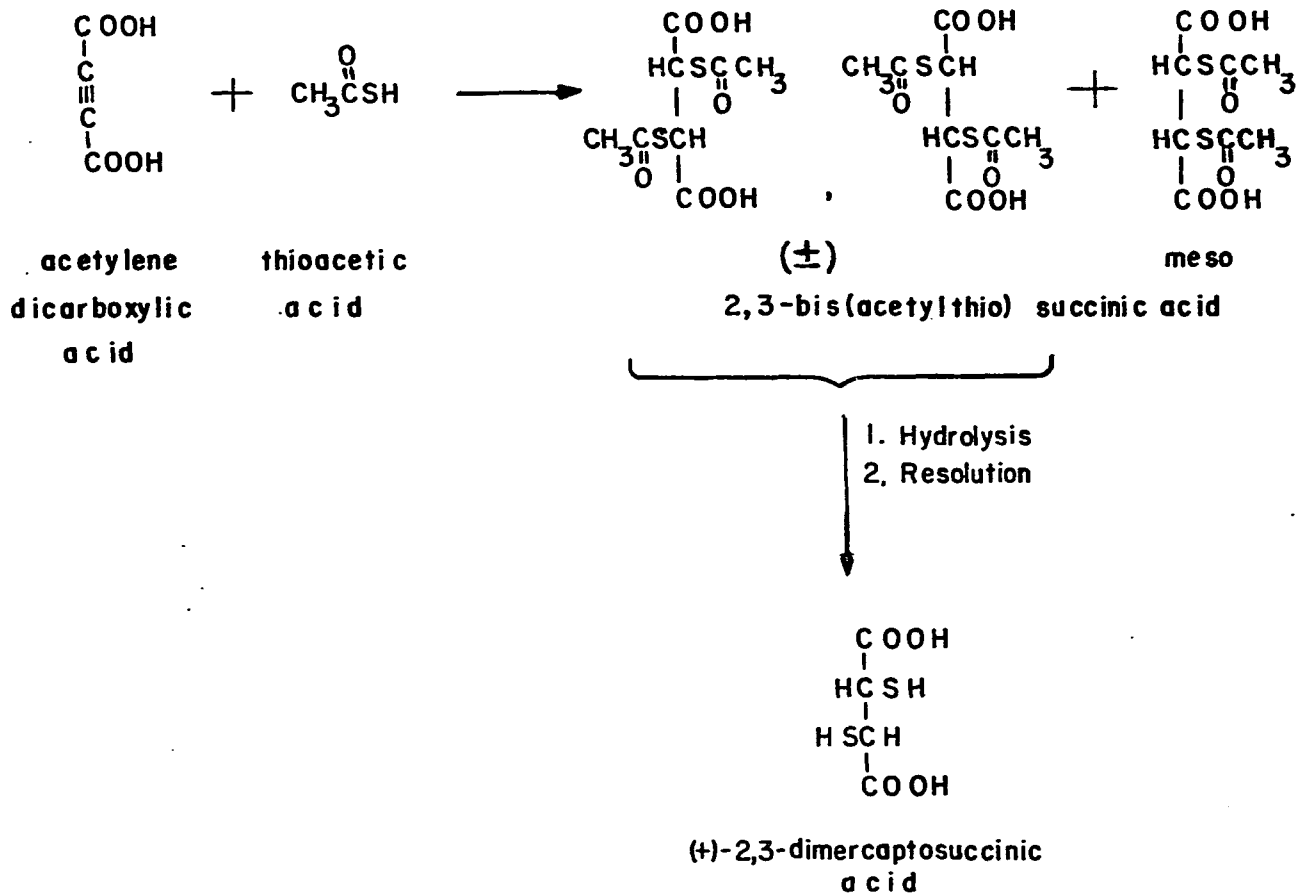


Figure 2: The synthesis of (+)-2,3-dimercaptosuccinic acid.

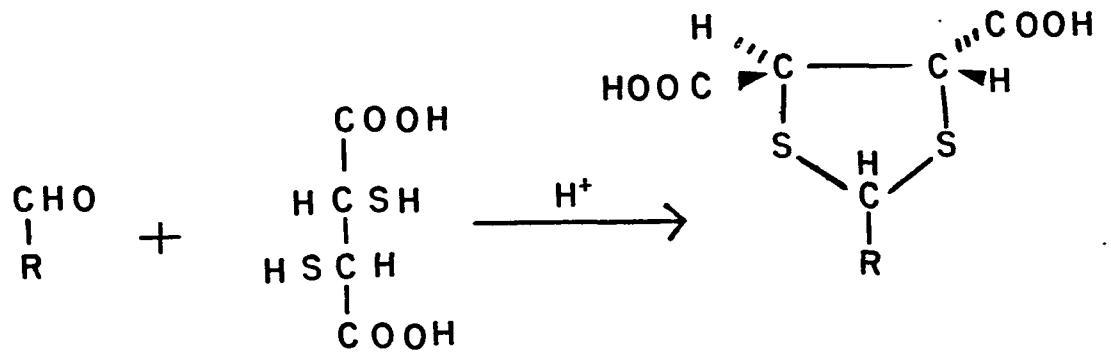


Figure 3: Reaction of an aldose with (+)-2,3-dimercaptosuccinic acid. The product is a substituted 1,3-dithialane derivative. R=polyhydroxyalkyl group.

Table 1: Electrophoretic mobilities of some sugar cyclic dithioacetals. The sugar dithioacetals were produced by reaction with (+/-)2,3-dimercapto-succinic acid. Mobilities are relative to picrate.

Sugar	R_m
D-arabinose	1.96
D-ribose	1.96
D-xylose	1.96
D-fucose	1.98
L-rhamnose	1.87
D-galactose	1.79
D-glucose	1.86
D-mannose	1.86

failed to give any detectable product.

Adequate yields of the dithioacetal were obtained in 6 M HCl at significantly higher temperatures. The course of the reaction between (+/-)2,3-dimercaptosuccinic acid and D-galactose was followed by NMR. The reaction proceded in 6 M DCl at 55°. As may be seen in Figure 4, the anomeric proton resonance at 5.3 p.p.m. of unreacted sugar (a) shifts upfield (b-e) as the reaction progresses. Integration of the NMR signal indicated a 80% yield of the cyclic dithioacetal after a five-hour reaction period. Similar yields of the product were obtained after 16 hours at 37°.

Separation of diastereoisomeric dithioacetals by high pressure liquid chromatography. (+)-2,3-Dimercaptosuccinic acid ($[\alpha]$ = +80) was used to resolve enantiomers of arabinose, galactose, and mannose. The cyclic diastereoisomeric dithioacetals were separated by high pressure liquid chromatography on a Partisil-10 strong anion exchange resin. The diastereomers were eluted at room temperature with 0.05 M potassium phosphate buffer, pH 2.1. Ultraviolet absorbing components were detected at 243 nm. Absorption maxima of sugar dithioacetals have been reported at or near this wavelength (46).

A liquid chromatogram illustrating the separation of D- and L-arabinose achieved by this method is shown in Figure 5. A summary of the retention data, including uncorrected retention times and separation factors, is given in Table 2.

Figure 4: Course of the reaction between (+/-) 2,3-dimercaptosuccinic acid and D-galactose in 6 M DCl as monitored by NMR at 60 MHz. Low-field porrtions of the spectra are shown. Each spectrum was taken at various reaction periods at 55°: a) after initial mixing b) 1 hour c) 2 hours d) 4 hours and e) 5 hours. Chemical shifts shown are downfield from the methyl protons of sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

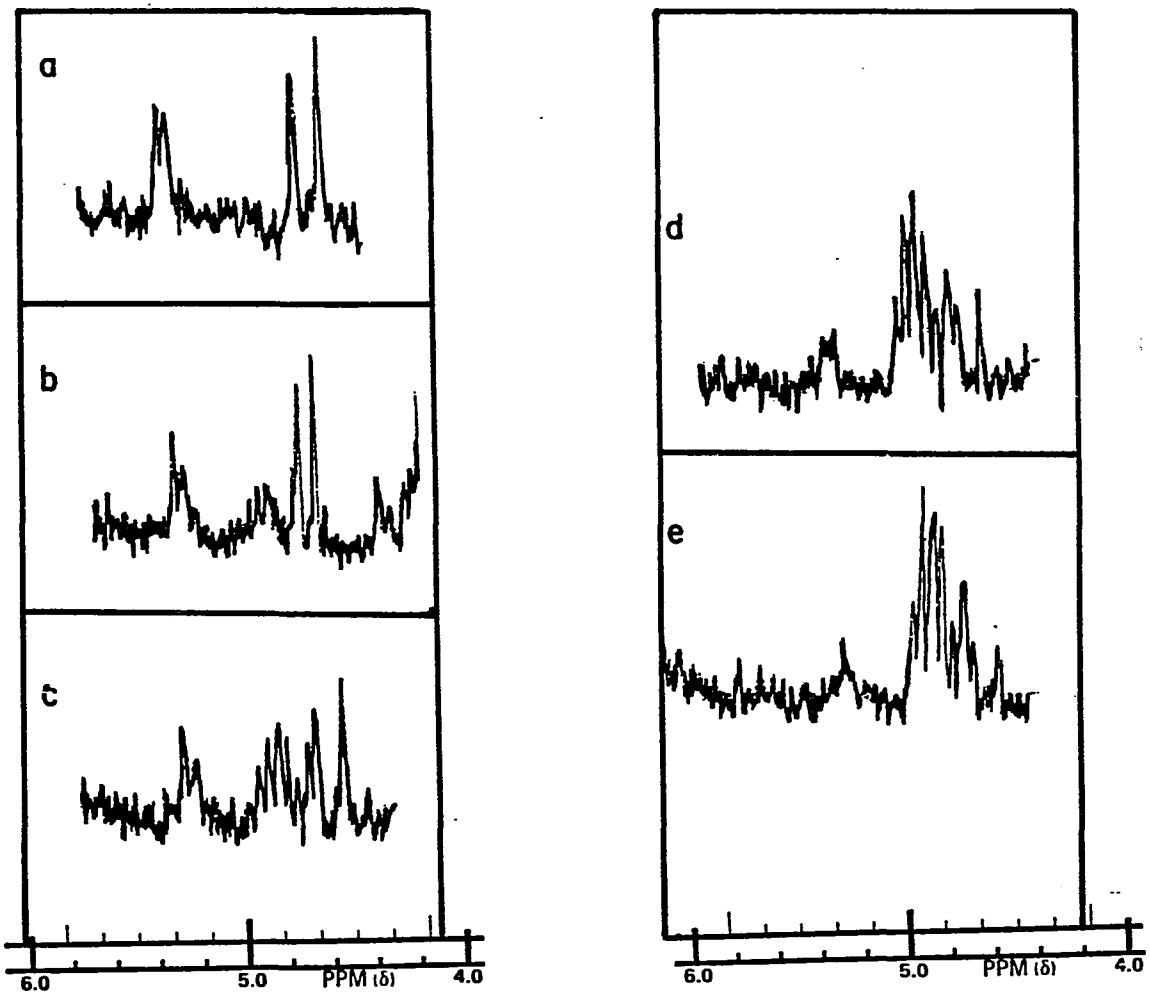


Figure 4.

Figure 5: Liquid chromatogram illustrating the separation of D- and L-arabinose as their diastereomeric cyclic dithioacetals. The derivatives were eluted from a Partisil-10 strong anion exchange resin with 0.05 M potassium phosphate buffer, pH 2.1, and detected at 243nm.

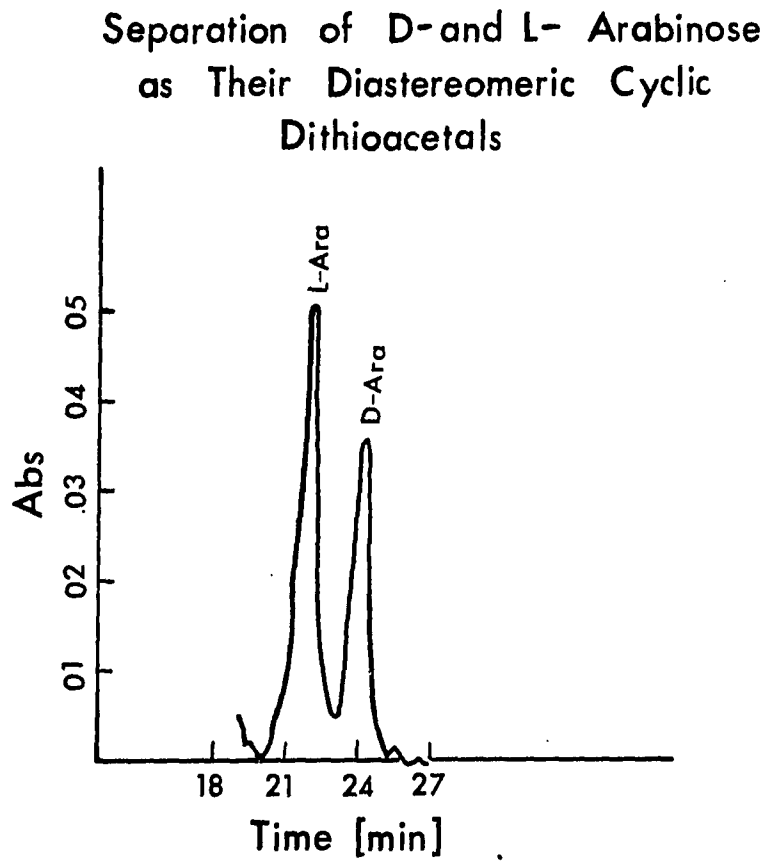

Separation of D-and L- Arabinose
as Their Diastereomeric Cyclic
Dithioacetals
L-Ara
D-Ara
Abs
01
02
03
04
05
18
21
24
27
Time [min]

Figure 5.

Table 2: Retention times and separation factors for diastereoisomeric sugar dithioacetals. Data were taken from a Partisil-10 strong anion exchange resin. Components were eluted with 0.05 M potassium phosphate buffer, pH 2.1. Flow rate was adjusted to 1 ml/min.

Sugar	Retention time (in minutes)	Separation factor
L-arabinose	21.4	1.103
D-arabinose	23.6	
D-galactose	22.4	1.076
L-galactose	24.1	
L-mannose	22.7	1.080
D-mannose	24.5	

Attempted resolution by gas-liquid chromatography. Although the enantiomeric separations using h.p.l.c. were quite good, isomeric hexoses could not be resolved at the baseline since peak widths were exceedingly large. Unfortunately, using h.p.l.c., one cannot expect to separate and identify isomeric aldoses in a mixture containing common monosaccharides. Gas-liquid chromatography was investigated as an alternative means of separating the diastereomers.

Trimethylsilylation (47) is a simple, one-step method by which the diastereoisomeric dithioacetals could be made sufficiently volatile for gas chromatography. This procedure has been used successfully in the separation of aldonic and uronic acids by g.l.c. (48). However, repeated efforts to resolve the diastereomers, by g.l.c., as the per(trimethylsilyl) derivatives failed. No volatile derivative of this type could be detected eluting from a packed glass column, an open-tubular column of borosilicate capillary glass, or an open-tubular column of fused silica. Although the reason for this anomaly was not understood, it is suspected that adsorption or decomposition of the derivatives during the chromatographic process prevented their detection.

Discussion. The use of (+)-2,3-dimercaptosuccinic acid as a resolving reagent has several appealing properties. Condensation with an aldose gives a stable 1,3 dithialane ring system. The cyclization reaction minimizes interference of

thioglycoside formation. In addition, the presence of the five-membered ring necessarily restricts the conformational mobility of groups within the molecule. When this condition exists, chromatographic resolution is generally increased (49,50).

There were however a number of difficulties encountered in using (+)-2,3-dimercaptosuccinic acid as a resolving reagent. Foremost among these is the difficulty in obtaining an optically pure form of the compound. Although absolute purity of the reagent is not necessary to identify and quantitate enantiomeric sugar mixtures, interpretation of resulting chromatograms is much simpler when the resolving is of very high optical purity.

Another, quite unexpected, difficulty was in finding suitable conditions to carry out a small-scale reaction. The conditions eventually set provided for optimal yields of the product with minimal degradation of the reactants. Under these conditions, it was difficult to maintain a high reagent concentration due to the limited solubility of 2,3-dimercaptosuccinic acid in 6 M HCl. This also may account for the unexpectedly slow reaction rate. Use of a more compatible solvent, 6 M HCl in dioxane, gave unsatisfactory results. Ritter and Lover (51) encountered similar difficulties in preparing cyclic dithioacetals of carbonyl compounds using a similar reagent, 1,2-dimercaptopropionic

acid.

Finally, it seems that the chromatographic separation of the resulting diastereomers can be only achieved with the use of high pressure liquid chromatography. Although a powerful analytical tool, h.p.l.c. does not match the resolving power of the modern day gas-liquid chromatography using capillary glass columns (52). Furthermore, detection methods commonly utilized in gas-liquid chromatography allow for far greater sensitivity. It is therefore desirable to develop methods which can be applied to gas-liquid chromatography.

III. Resolution of sugar enantiomers using (+)-1-phenylethanethiol

The difficulties encountered with the use of (+)-2,3-dimercaptosuccinic acid prompted an investigation into other optically active thiols that might serve as suitable resolving reagents. In 1976 Isola, Ciuffarin, and Sagramora (53) published a novel and convenient synthetic route to optically active thiols. Although presented as a general approach, they obtained particularly successful results in the preparation of (+)-1-phenylethanethiol. Hence, this optically active thiol was prepared and studied as a resolving reagent for sugar enantiomers. The following work demonstrates the usefulness of this reagent in separating eight pairs of enantiomeric sugars.

Preparation of (+)-1-phenylethanethiol. The synthesis of (+)-1-phenylethanethiol follows the procedure of Isola et al. (53). An outline of this synthetic route is given in Figure 6. A critical step in the synthesis involves the resolution of the diastereoisomeric dithiocarbonates by fractional recrystallization. In two preparations of the thiol, the menthyl dithiocarbonate containing the dextrorotatory thiol was almost exclusively separated from the diastereomer containing the levorotatory thiol.

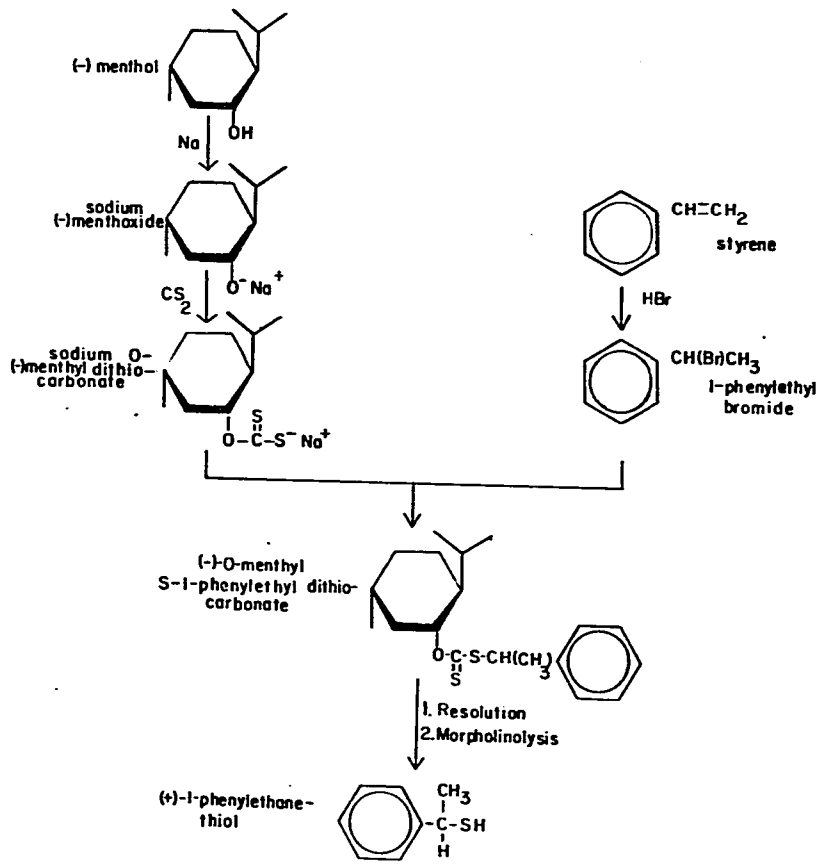


Figure 6: The synthesis of (+)-1-phenylethanethiol.

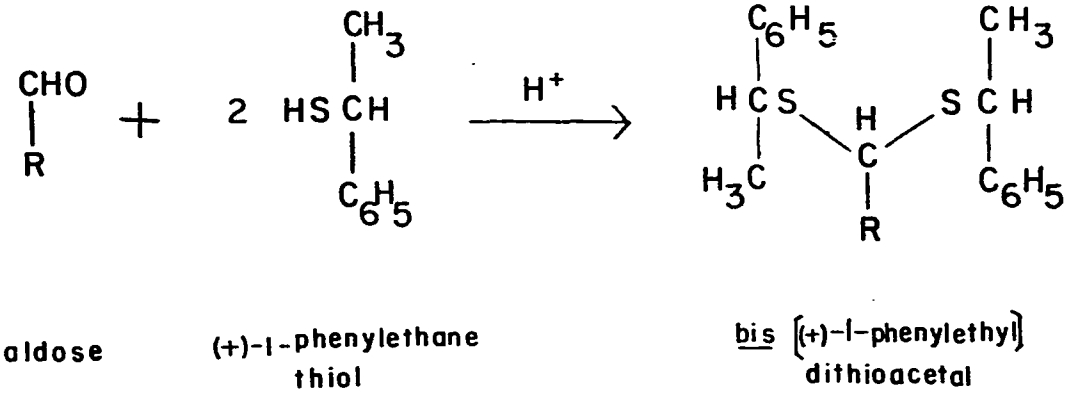
Therefore, thiols of high optical purity were recovered:

preparation I, $[\alpha]_{589}^{25}$ = +90, 98.8 % optically pure;

preparation II, $[\alpha]_{589}^{25}$ = +96, 99.9+% optically pure.

Reaction of aldoses with (+)-1-phenylethanethiol. Reaction of aldoses, in the presence of acid, with (+)-1-phenylethanethiol results in the formation of sugar bis [(+)-1-phenylethyl] dithioacetals (Figure 7). Trifluoroacetic acid, shown by Honda et al. (54) to be effective in converting microquantities of simple sugars into diethyl dithioacetals, was found most suitable in converting aldoses into their bis [(+)-1-phenylethyl] dithioacetals. Conversion of sugars to acyclic dithioacetals in presence of trifluoroacetic was effected at room temperature over a period of 30 minutes.

The course of the reaction can be followed either by thin-layer chromatography or gas-liquid chromatography. Figure 8 illustrates the course of the reaction, as followed by g.l.c., between D-arabinose and (+)-1-phenylethanethiol in the presence of trifluoroacetic acid. As may be seen, at room temperature, the maximum yield of the dithioacetal occurs at 30 minutes. In reaction periods longer than 60 minutes, a noticeable loss in the yield of dithioacetal was observed. A similar course was observed when the thiol was allowed to react with L-arabinose. Figure 9 illustrates the course of this reaction as followed by thin-layer chromatography. After a reaction period of one minute, the unreacted sugar, L-arabinose, was detected only faintly on the thin-layer plate. Only one spot, which



aldose

(+)-1-phenylethane thiol

bis [(+)-1-phenylethyl] dithioacetal

Figure 7: Reaction of an aldose with (+)-1-phenylethanethiol. R=polyhydroxyalkyl group.

Figure 8: Course of the reaction between (+)-1-phenylethanethiol and D-arabinose. Maximum yield of D-arabinose bis [(+)-1-phenylethyl] dithioacetal is found after 30 minutes.

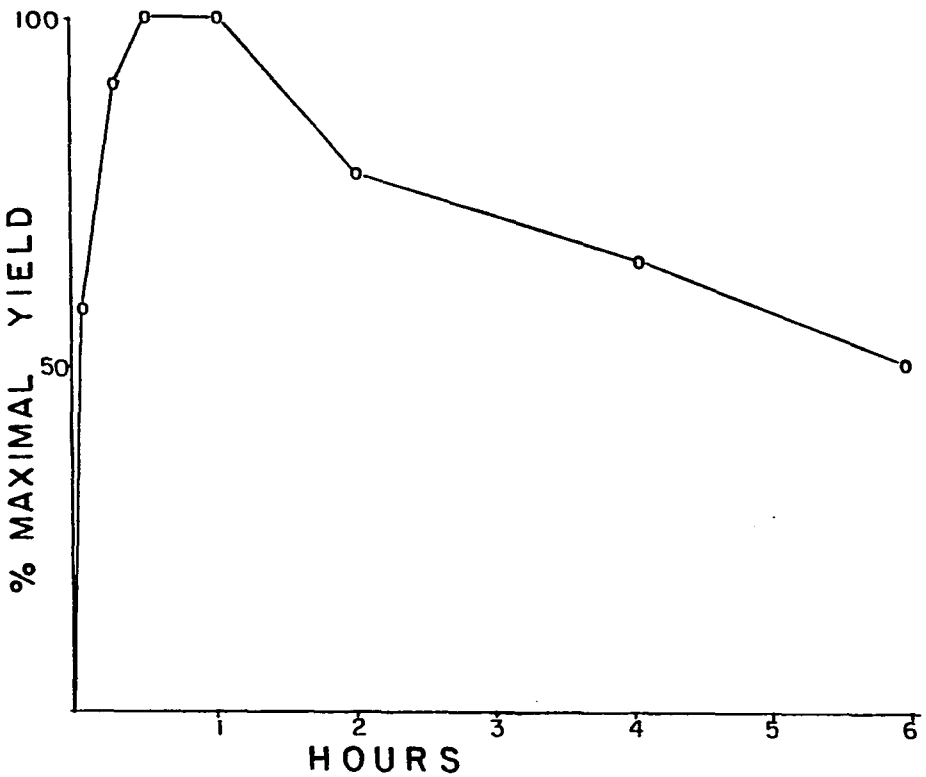


Figure 8.

Figure 9: Course of the reaction between L-arabinose and (+)-1-phenylethanethiol as followed by thin-layer chromatography. The plate was developed with chloroform/butanol (10:1, by volume).

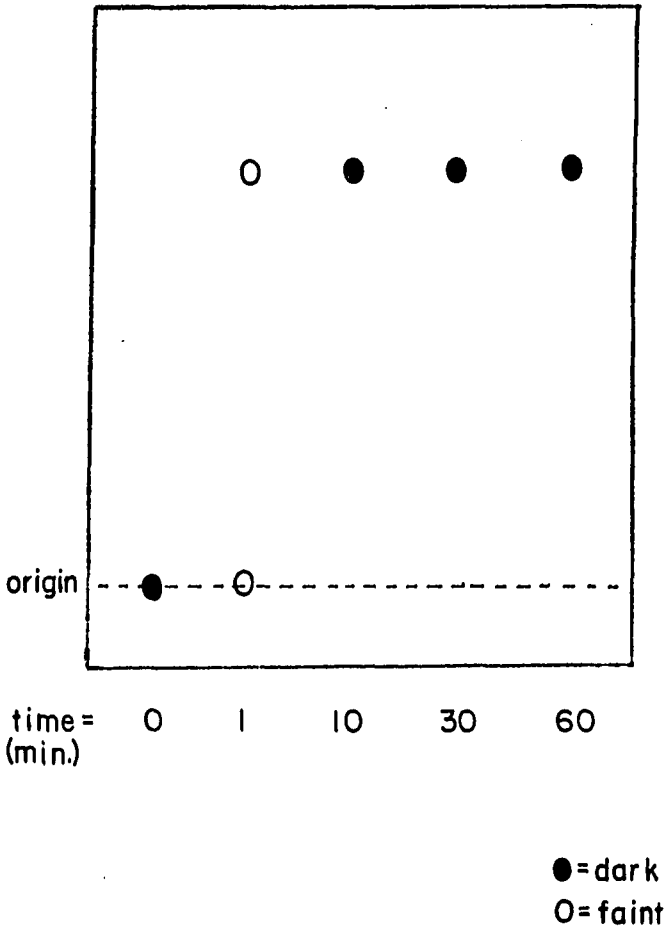


Figure 9.

co-migrated with authentic L-arabinose bis [(+)-1-phenylethyl][33] dithioacetal, was detected after reaction periods of five minutes and up through sixty minutes. These data indicate a good yield of the dithioacetal after short reaction periods with limited degradation after extended periods.

The reaction rates between an optically active reagent and each of a pair of enantiomers may differ. It is important to establish, especially when quantitative work is desirable, that the resolving reagent reacts at similar rates with each of the two enantiomeric compounds. Thus, when equal amounts of the enantiomeric compounds are combined with the resolving reagent, equal amounts of the two diastereoisomeric compounds result. Figure 10 shows a gas-liquid chromatogram of the diastereoisomeric dithioacetals produced when equal proportions of D- and L-arabinose (0.2 micromoles) are condensed with (+)-1-phenylethanethiol. Equal proportions of the dithioacetal products are indicated. This observation implies that reaction rates between the enantiomeric sugars and the optically active thiol are similar, and kinetic resolution is negligible.

Characterization of the dithioacetal product. The ^{1}H NMR spectrum of D-galactose bis [(+)-1-phenylethyl] dithioacetal is shown in Figure 11. Proton resonances showing the expected splitting patterns are shown for the aromatic protons, at 7.2 p.p.m. (m, 10H), and for the diastereotopic methyl protons, at 1.45 p.p.m. (dd, 6H), of the phenylethylthio group. The methine protons of this group are contained within the region of the

Figure 10: Gas-liquid chromatogram of the trimethylsilyl - ated dithioacetals obtained from equal molar proportions of D- and L-arabinose. (SE-54 fused-silica capillary column, column temperature, 260°, programmed at 3°/min to 290°; inlet temperature, 280°.).

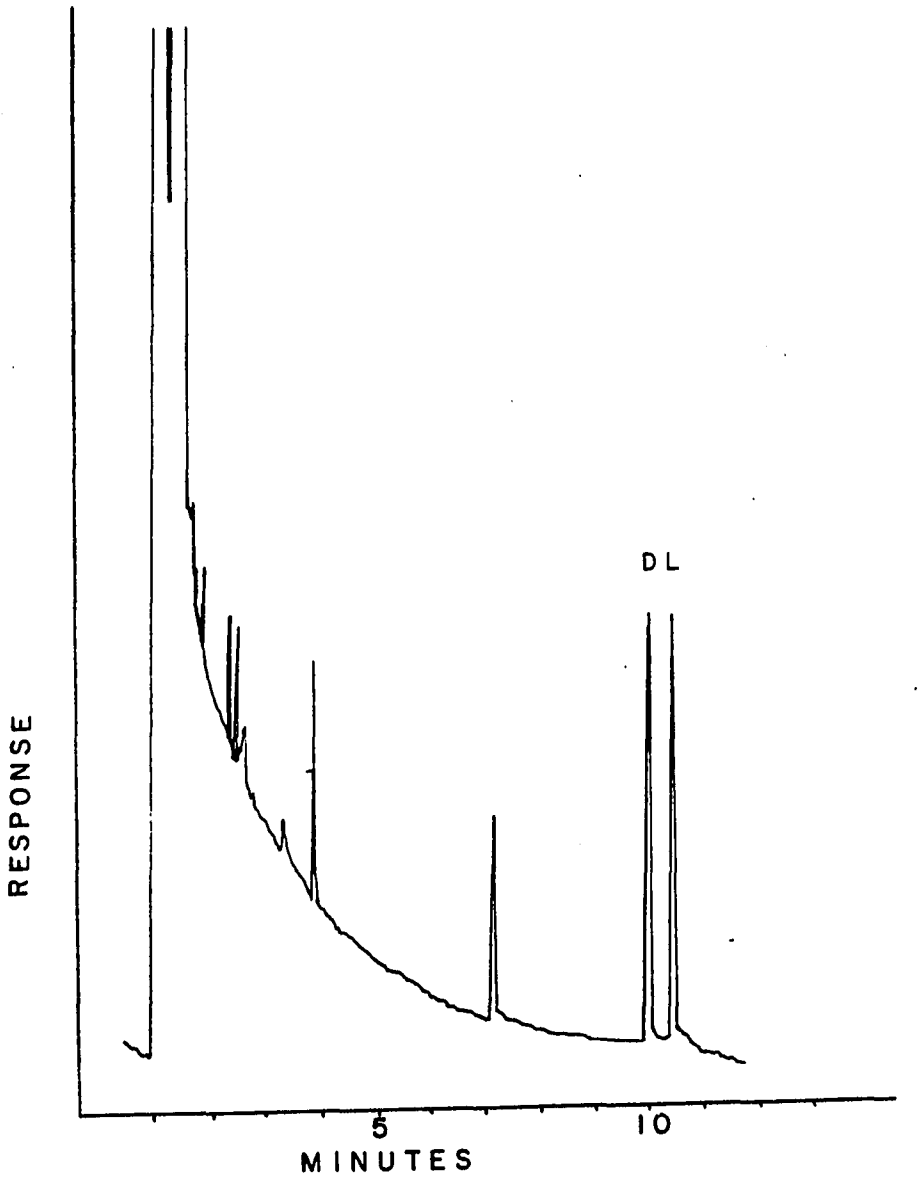


Figure 10.

Figure 11: ^{1}H NMR spectrum of D-galactose bis (+)-1-phenylethyl dithioacetal.

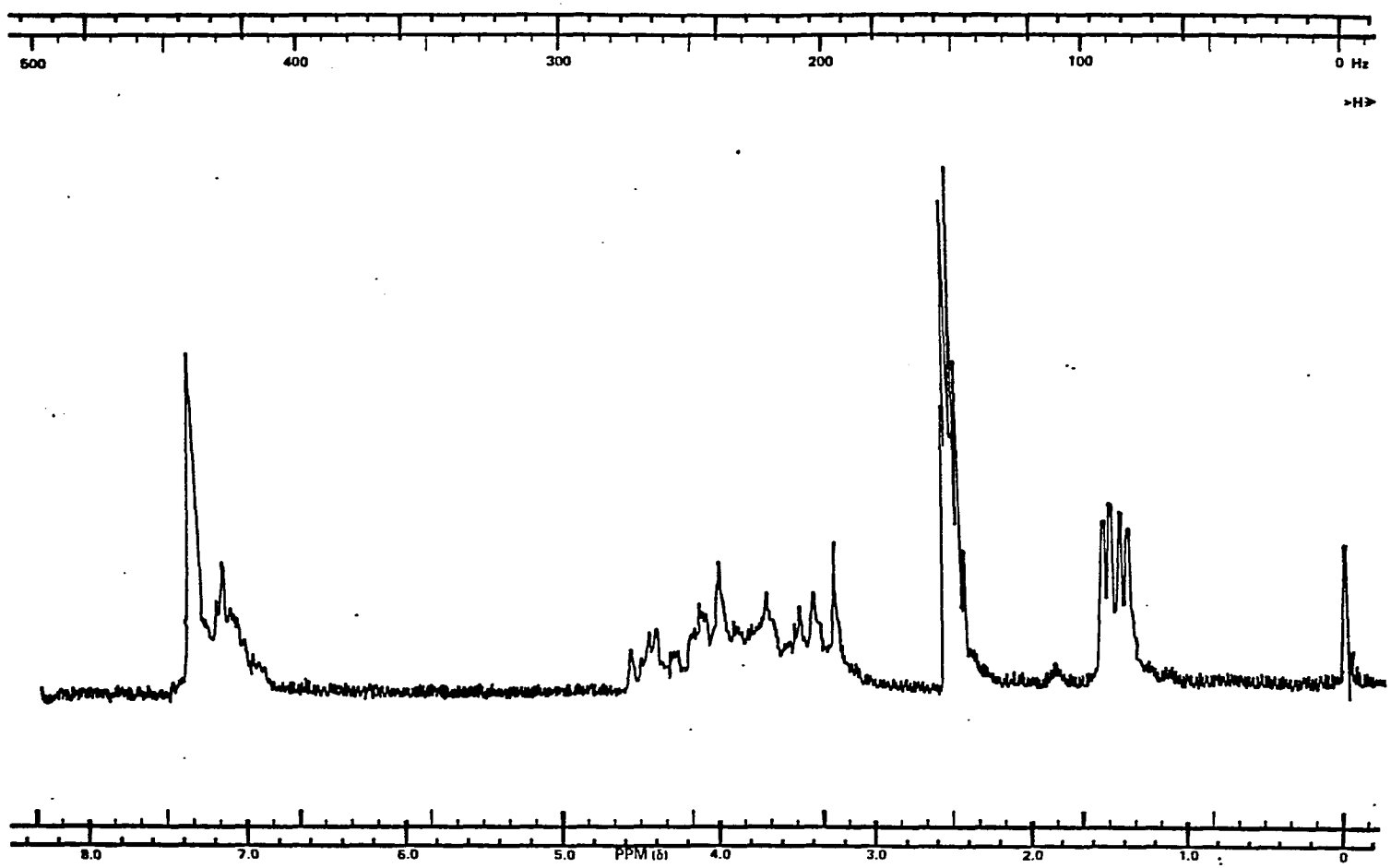


Figure 11.

sugar-proton resonances (3.1-4.8 p.p.m.). Other data obtained from this derivative are included in the Experimental Section.

The mass spectrum of per(trimethylsilyl) L-arabinose bis [(+)-1-phenylethyl] dithioacetal is shown in Figure 12 (panel a). Shown in panel b of this figure, is the mass spectrum of a per(trimethylsilyl) arabinitol derivative. The difference spectrum (panel c) illustrates how well these spectra correlate. As may be seen, the only major discrepancy is at m/e 105. This ratio corresponds to the α-methyl substituted benzyl cation, $C_6H_5\overset{\oplus}{C}HCH_3$, an expected fragment of the sample dithioacetal.

Separation of diastereoisomeric dithioacetals by gas-liquid chromatography. The separation of diastereoisomeric dithioacetals was carried out by gas-liquid chromatography. Acetylated and trimethylsilylated sugar dithioacetals were analyzed on capillary columns of fused-silica coated with either SE-30 or SE-54. A gas-liquid chromatogram, from an SE-54 column, of the acetylated sugar dithioacetals obtained from a mixture of four pairs of sugar enantiomers is shown in Figure 13. A chromatogram, taken from a SE-30 column, of several trimethylsilylated sugar dithioacetals is shown in Figure 14. A complete summary of the retention data is given in Tables 3 and 4.

By this method, the separations of enantiomeric pairs of sugars were good. Separation factors greater than 1.05 were common. Of the enantiomeric sugar pairs tested, only the trimethylsilylated diastereomers of D- and L-mannose could not

Figure 12: Mass spectrum of per(trimethylsilyl) L-arabinose bis [(+)-1-phenylethyl] dithioacetal (panel a). Mass spectrum of per(trimethylsilyl) arabinitol (panel b). A comparison of the two spectra is given in panel c.

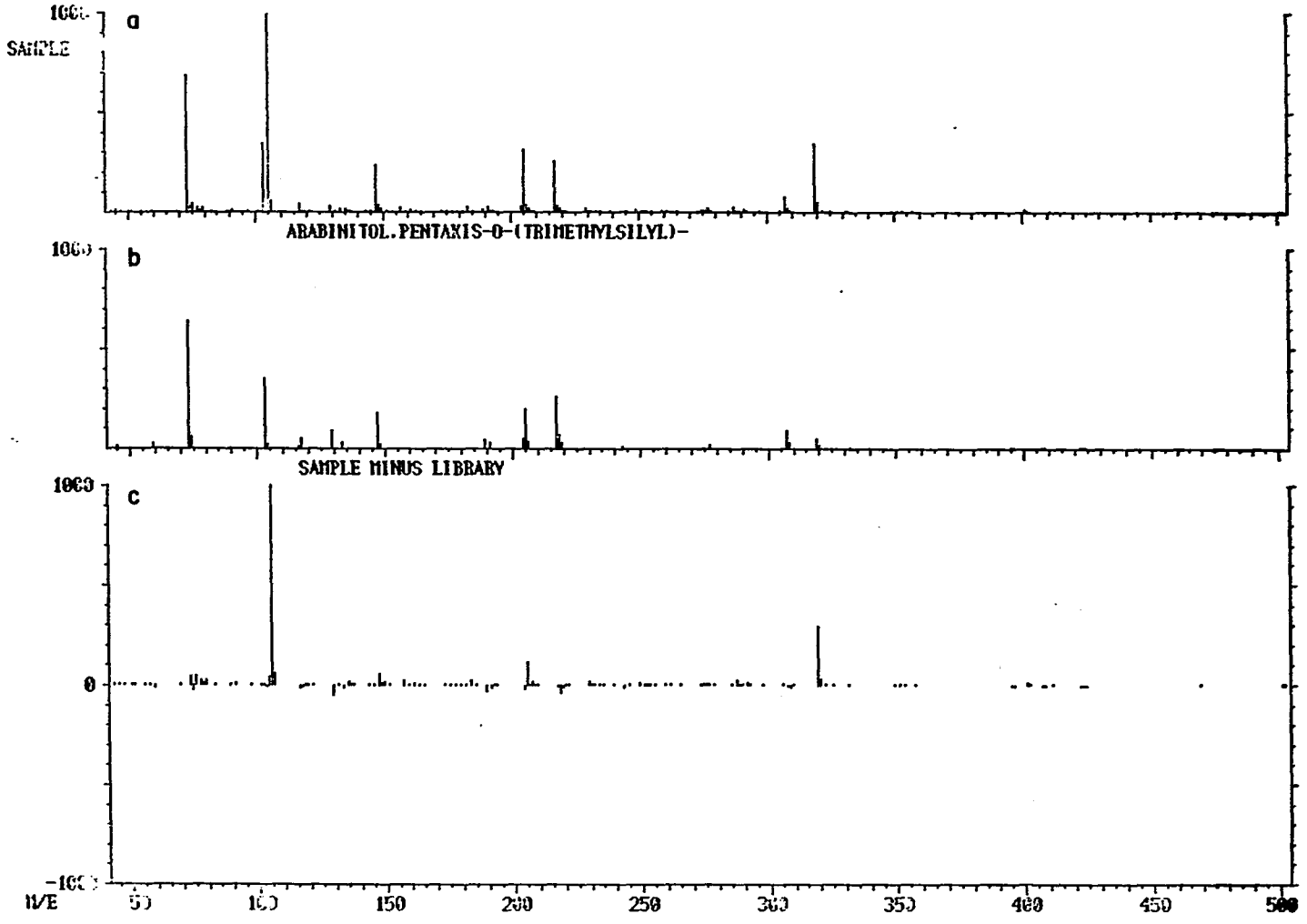

a
SAMPLE
ARABINITOL.PENTAXIS-O-(TRIMETHYLSILYL)-
b
SAMPLE MINUS LIBRARY
c
0
M/E
50
100
150
200
250
300
350
400
450
500

Figure 12.

Figure 13: Gas-liquid chromatogram of some acetylated sugar dithioacetals on an SE-54 fused-silica capillary column. The dithioacetals of D- and L-rhamnose, D- and L-arabinose, D- and L-mannose, and D- and L-galactose are shown. (Column temperature, 275°, programmed at 2°/min to 300°; inlet temperature, 280°.).

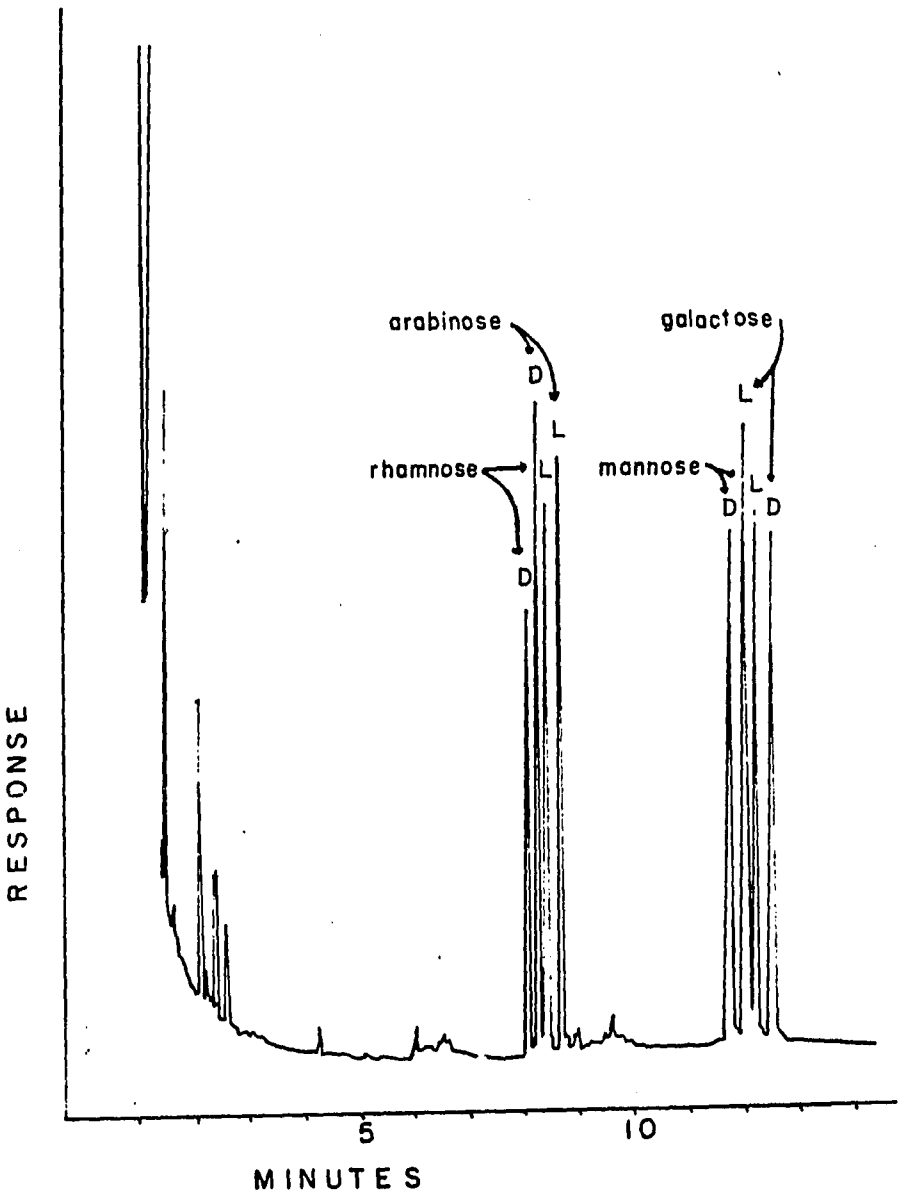

arabinose
D
L
rhamnose
L
D
mannose
L
D
galactose
L
D
RESPONSE
5
10
MINUTES

Figure 13.

Figure 14: Gas-liquid chromatogram of some trimethylsilylated sugar dithioacetals on an SE-30 fused-silica capillary column. The dithioacetals of D- and L-lyxose, D- and L-fucose, and D- and L-glucose are shown. (Column temperature, 270°, programmed at 3°/min to 300°; inlet temperature, 280°.).

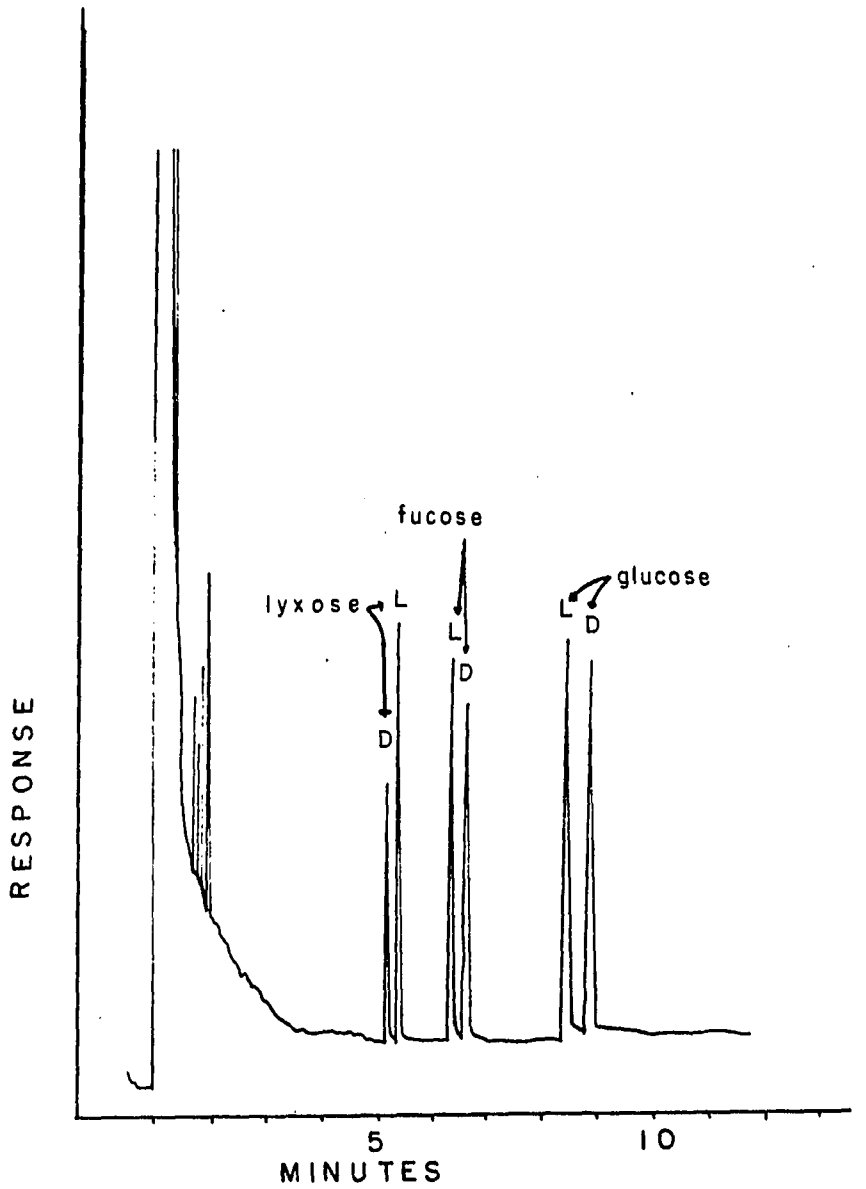


Figure 14.

Table 3: Summary of GLC Data for Acetylated and Trimethylsilyl ated Sugar Dithioacetals on SE-30 fused-silica, capillary column.

Sugar	SE-30 (280°) Acetylated		Silylated	
	k*	α**	k	α
D-arabinose	6.0	1.066	4.8	1.063
L-arabinose	6.4		5.1	
D-lyxose	6.1	1.059	4.9	1.051
L-lyxose	6.4		5.2	
L-fucose	6.1	1.051	6.5	1.057
D-fucose	6.4		6.9	
D-rhamnose	5.9	1.053	6.5	1.047
L-rhamnose	6.2		6.8	
L-glucose	9.6	1.079	9.6	1.071
D-glucose	10.4		10.3	
D-mannose	9.9	1.056	10.2	1.000
L-mannose	10.4		10.2	
L-galactose	10.2	1.055	9.7	1.053
D-galactose	10.8		10.2	

*k, the partition ratio, is obtained by dividing the corrected retention-time (t'_R) by the retention time found for methane (t_m). **α, the separation factor, is the ratio of the corrected retention-times for two components, such that $= t'_{R_2} / t'_{R_1}$, where $t'_{R_2} > t'_{R_1}$.

Table 4: Summary of GLC Data for Acetylated and Trimethylsilyl ated Sugar Dithioacetals on SE-54 fused-silica, capillary column.

Sugar	SE-54 (280°) Acetylated		Silylated	
	k	α	k	α
D-arabinose	8.8	1.075	5.6	1.070
L-arabinose	9.4		6.0	
D-lyxose	8.9	1.066	5.8	1.056
L-lyxose	9.5		6.1	
L-xylose	8.7	1.123	5.9	1.035
D-xylose	9.8		6.1	
L-fucose	8.9	1.042	7.7	1.065
D-fucose	9.2		8.2	
D-rhamnose	8.5	1.060	7.6	1.041
L-rhamnose	9.0		8.0	
L-glucose	14.4	1.088	11.3	1.082
D-glucose	15.7		12.3	
D-mannose	14.8	1.060	12.0	1.000
L-mannose	15.7		12.0	
L-galactose	15.2	1.063	11.4	1.062
D-galactose	16.2		12.1	

be resolved at the baseline. Those diastereomers were, however, well separated as acetylated derivatives.

In general, the acetylated diastereomers were better separated. By comparison, the trimethylsilyl derivatives showed greater volatility and were more readily prepared. For these reasons, they were considered preferable for routine analyses. However, the trimethylsilyl derivatives were found to be more susceptible to adsorption and/or decomposition at the glass inlet. Thus, periodic deactivation of the glass inlet was necessary.

Other volatile derivatives were investigated. Dimethylsilylated or trifluoroacetylated derivatives are expected to form more volatile compounds. Procedures of Vilkas et al. (55) and Sullivan et al. (56) for trifluoroacetylation of sugars and the procedure of Supina et al. (57) for dimethylsilylation of sugars were followed. Neither volatile derivative of the dithioacetal could be detected emerging from the fused silica columns. Both of these derivatives reportedly are more susceptible to adsorption during the chromatographic process (58,59) and in general, are more labile compounds (60).

The order in which the diastereomers were eluted depended upon the stereochemistry at C-2 of the parent sugar. This is consistent for both volatile derivatives. Those sugars having the S configuration at C-2 were eluted prior to those having the R configuration. It follows, then, that a D-arabinose derivative is eluted prior to its L-enantiomer and that an

L-galactose derivative is eluted prior to its D-enantiomer. In contrast, the relative retention times within an isomeric family of aldoses cannot be easily predicted.

Quantitation. The split-injection technique inherently presents problems when quantitative analytical work is desirable. These problems are largely due to disproportionate splitting of the vaporized sample at the inlet and are particularly troublesome in the analysis of high-boiling components (61). For these reasons, reproducible measurements of the quantities of the sugars as dithioacetals were only realized when the analyses were performed at high split ratios, typically 100:1, and when peak areas were standardized against a high-boiling internal standard (α,α-trehalose). A linear calibration curve for L-arabinose was obtained under these conditions (Figure 15).

To illustrate the usefulness of this method, the relative molar ratio of enantiomeric galactose residues present in a galactan from Helix pomatia was determined. G.l.c. analysis (Figure 16) indicated that the polysaccharide was 14% L-galactose. This is in close agreement with the results of Bell and Baldwin (62), who, by polarimetric measurements, determined the molar ratio of D-galactose:L-galactose to be 6:1. It must noted, however, that accurate and reproducible results were only obtained when small amounts ($\leq$ 0.3 μmoles) of sugar sample were analyzed. When larger portions were analyzed, lower ratios were indicated, which presumably, is a consequence of "detector overload" (63).

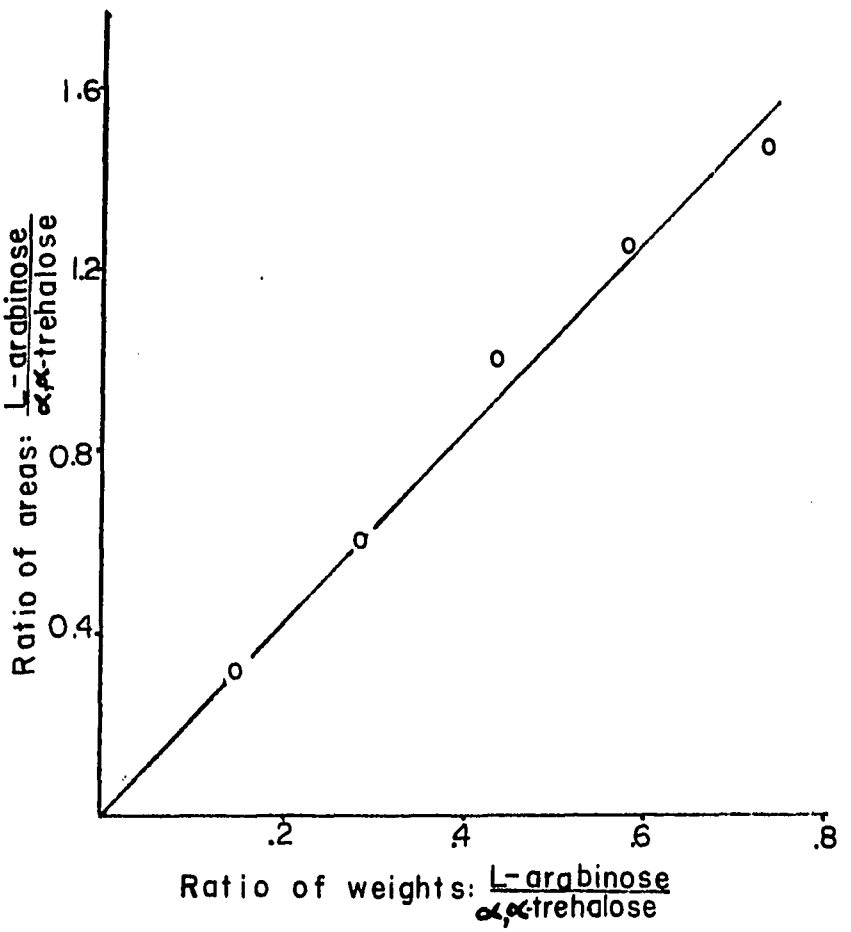


Figure 15: Calibration curve obtained for L-arabinose with α,α-trehalose as the internal standard.

Figure 16: Gas-liquid chromatogram of the trimethylsilylated dithioacetals prepared from the hydrolyzate of a galactan from Helix pomatia. The polysaccharide was found to contain 14% L-galactose. (SE-54 fused-silica capillary column, column temperature, 280°, programmed at 2°/min to 300°; inlet temperature 280°.).

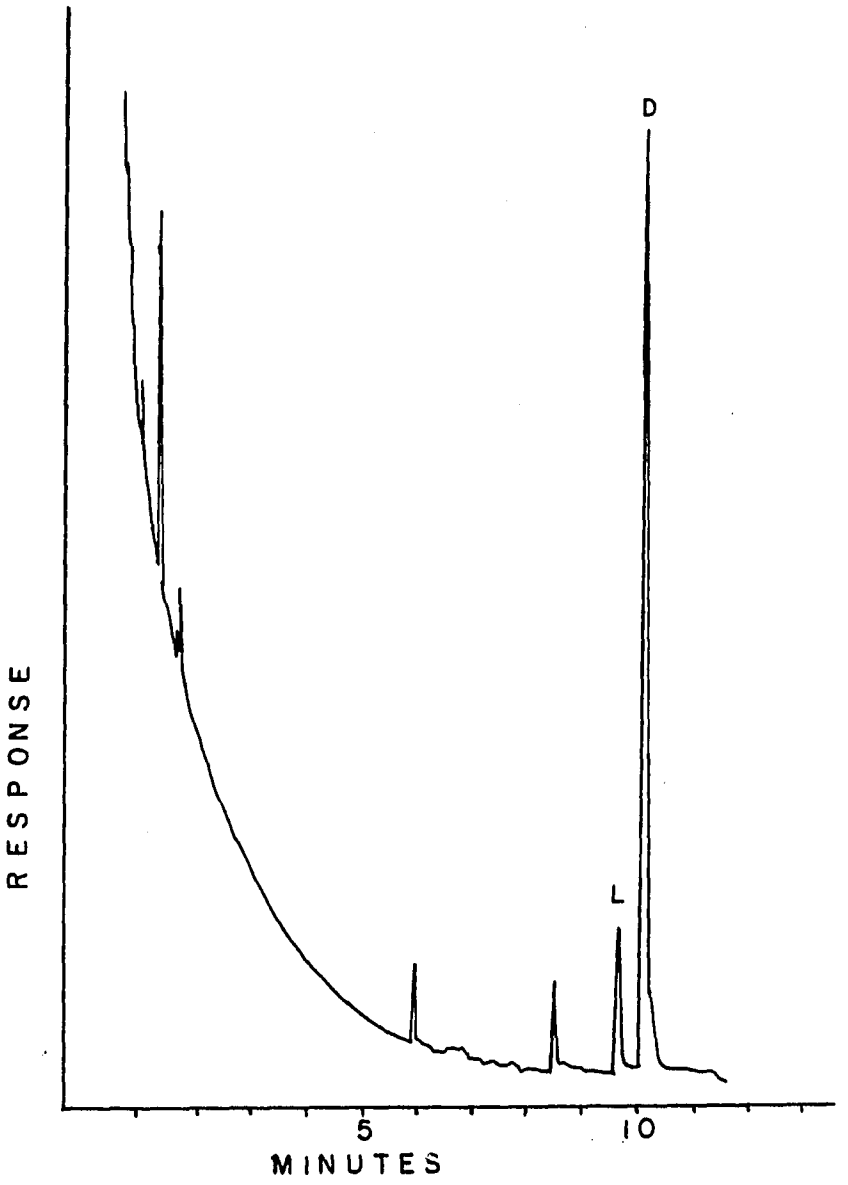


Figure 16.

Sensitivity. The limit of detection was determined for L-arabinose. Under normal g.l.c. operating conditions, in which the split ratio at the inlet is adjusted to 100:1, an original sugar weight of 1 microgram in the reaction mixture can be detected. The actual weight of the derivative introduced into the column is in the range of 10-20 nanograms. At a split ratio adjusted to 10:1, an original sugar weight of 50 nanograms in the reaction can be detected. However, at this split ratio severe solvent tailing occurs. It is expected that a splitless injection would permit greater sensitivity.

Discussion. The usefulness of (+)-1-phenylethanethiol as a resolving reagent for sugar enantiomers has been demonstrated in this section. This reagent is practical for the following reasons: 1) the thiol is readily synthesized in optical purity from inexpensive starting materials, 2) it reacts rapidly with various pairs of sugar enantiomers, and 3) most important, it leads to the formation of diastereomers that can be readily resolved.

The resulting g.l.c. chromatograms taken of a number of sugars showed no trace of thioglycosides. As indicated by the work of Williams and Jones (64), this by-product may be expected particularly at high acid concentration and after extended reaction periods. The absence of these derivatives may be attributed, at least in part, to the use of trifluoroacetic acid. Honda et al. (54) have discussed the advantages of trifluoroacetic acid over a number of other common acids in catalyzing this reaction with sugars. The

formation of these derivatives is also probably prevented by the relative high molar ratio of the reagent thiol. For example, experimentation was carried out on sugar samples in the range of 10^{-5}-10^{-8} mole. This allowed for a 15- to 15000-fold excess of reagent.

IV. Biological Applications

Sugars occur in nature in both D- and L- forms. Those sugars considered most abundant in nature are the D- forms of glucose, mannose, galactose, xylose, and ribose; and the L- forms of rhamnose, fucose, and arabinose (65). However, the enantiomeric forms of several of these sugars also occur naturally. The L- form of galactose is common in plant polysaccharides (66) and also exists in galactans from snails (62) and the gelatinous egg coats of certain sea urchin species (67). D-Fucose occurs naturally in several cardiac glycosides (68) and in other glycosides such as the antibiotic chartreusin (69). L-Mannose has been found in an acidic polysaccharide from the cell wall of an alga (70). The D- forms of rhamnose (71) and arabinose (72), although rare, have also been isolated from natural sources.

Several researchers recently have recognized the possibility that certain carbohydrate material may contain residues of the "rare" enantiomer. In this way, J. Briggs et al. (73) studied the absolute configuration of fucose in fucans isolated from brown seaweed. Roberts and Harrer (17), by an enzymic method, demonstrated the unsuspected presence of L-galactose in a well-characterized polysaccharide from

sycamore. Bretting et al. (74) have characterized in detail four distinct galactans from the snail and shown each to contain residues of both D- and L-galactose. They also studied the immunochemical properties of the four galactans and showed that differences in their cross-reactivity are partially ascribable to the linkage and position of the L-galactose residues.

The presence of L-galactose was confirmed in several samples. The partition ratio (see Table 4) of the acetylated and trimethylsilylated L-galactosyl dithioacetal derivative differs substantially from other common sugar derivatives. Therefore, in most instances, unambiguous identification of this sugar can be made without prior separation of the total galactosyl fraction from other sugars in the sample. Assignment of D- and L-configuration to galactose in various carbohydrate samples. Table 5 lists several carbohydrate samples in which L-galactose was detected in the acid-hydrolyzed material. Gas-liquid chromatograms showing the presence of L-galactose in polysaccharides from corn root, flax seed mucilage, and Bipolaris maydis are shown in Figures 17, 18, 19, respectively. L-Galactose was not detected in the acid-hydrolyzed material recovered from the following sources: gum of locust bean, gum guar, human α_1-acid glycoprotein, earthworm cuticle, Neurospora, and acetone powders of rat brain, porcine brain, porcine lung, and human placenta.

Table 5: Proportion of L-galactose in galactose-containing carbohydrate samples.

Carbohydrate sample	L-gal	D-gal
Flax seed mucilage	99%	1%
Galactan from *Helix pomatia*	14%	86%
Corn root polysaccharide	12%	88%
Cells of *B. maydis*	1%	99%
Gum tragacanth	1%	99%

Figure 17: Gas-liquid chromatogram of the trimethylsilylated sugar dithioacetals prepared from the hydrolyzate of a corn root polysaccharide. (SE-54 fused-silica capillary column, column temperature, 275°, programmed at 1°/min to 290°; inlet temperature, 280°.).

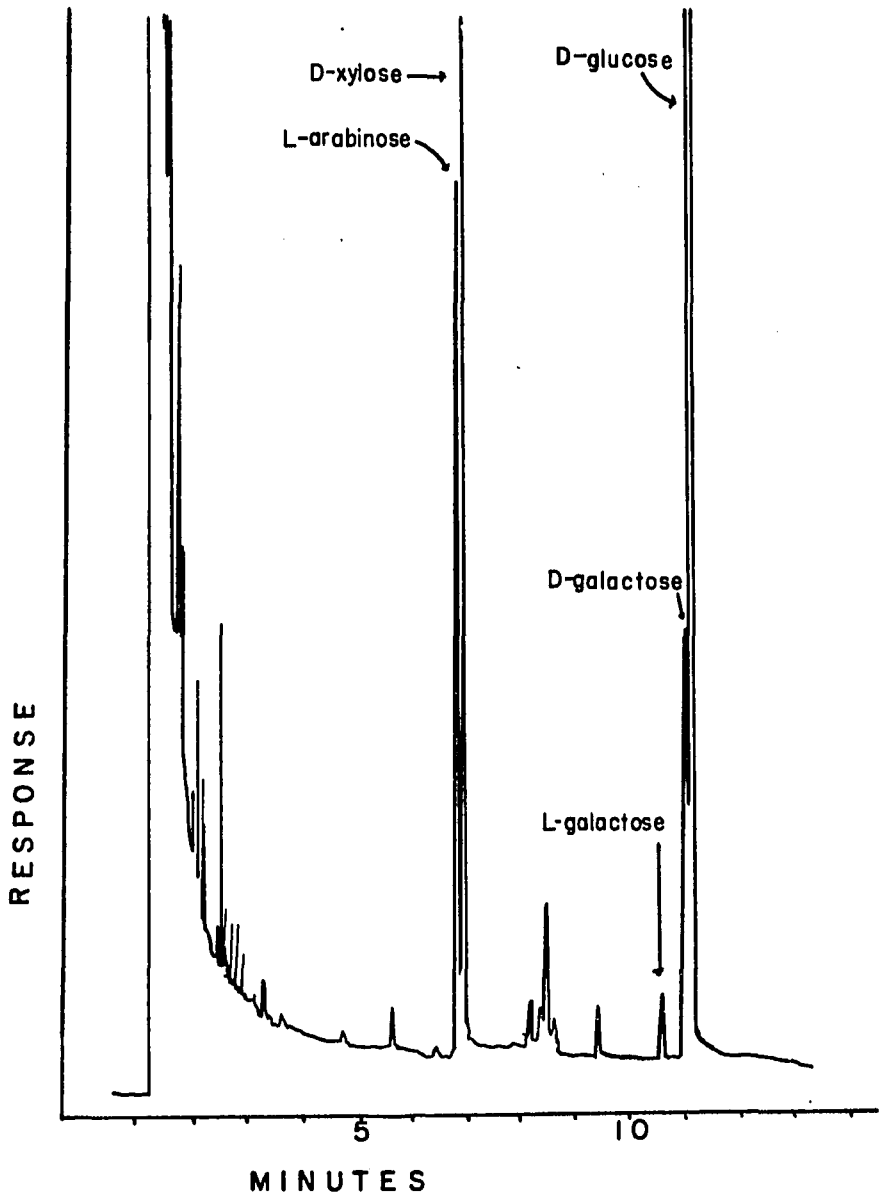

D-xylose
L-arabinose
D-glucose
D-galactose
L-galactose
RESPONSE
5
10
MINUTES

Figure 17.

Figure 18: Gas-liquid chromatogram of the trimethylsilylated sugar dithioacetals prepared from the hydrolyzate of flax seed mucilage. (SE-54 fused-silica capillary column, column temperature, 275°, programmed at 1°/min to 290°; inlet temperature, 280°.).

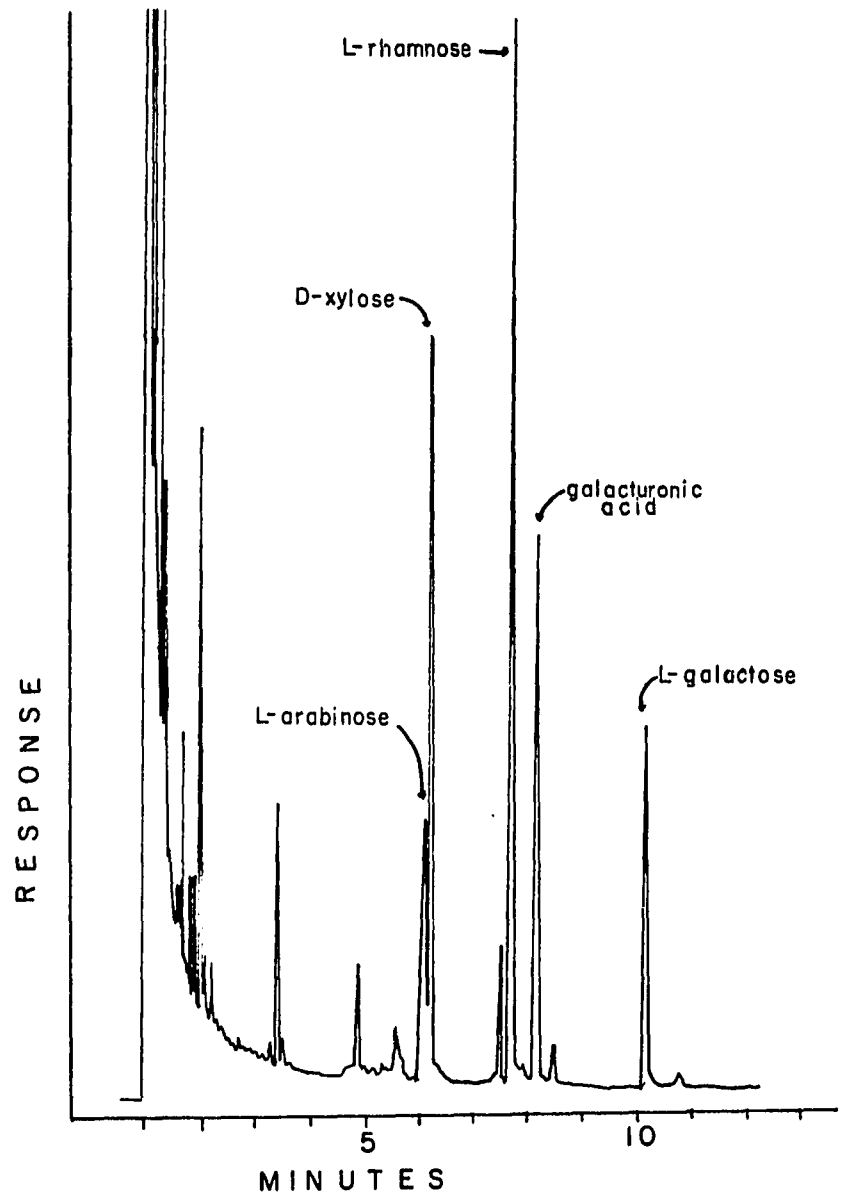

L-rhamnose
D-xylose
galacturonic acid
L-galactose
L-arabinose
RESPONSE
5
10
MINUTES

Figure 18.

Figure 19: Gas-liquid chromatogram of the acetylated hexose dithioacetals prepared from the hydrolyzate of Bipolaris maydis cells. Indicated are the derivatives of D- and L-galactose. (SE-54 fused-silica capillary column, column temperature 290°, inlet temperature 280°.).

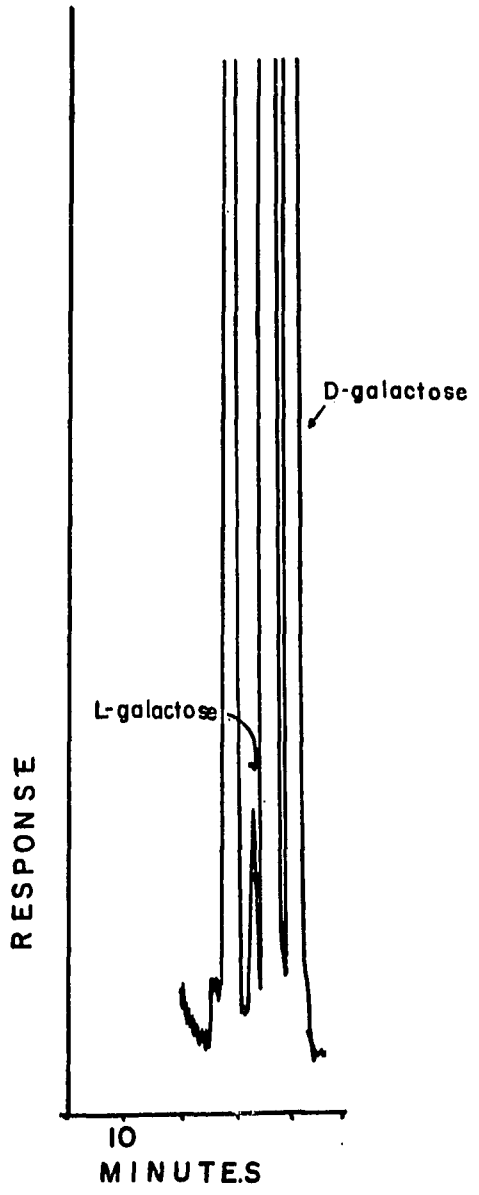


Figure 19.

Discussion. Carbohydrate material from various sources was examined for the occurrence of L-galactose. The presence of this sugar was confirmed in the polysaccharide isolated from corn root and in the flax seed mucilage. Trace amounts of L-galactose were found in gum tragacanth.

The finding of L-galactose in the fungus Bipolaris maydis was most interesting. This fungus is the causal agent of southern corn leaf blight (75). In 1970, a widespread epidemic of this pathogen accounted for a loss of 15-20% of the total corn crop (76). The virulent nature of plant pathogens, in general, has been linked to specific carbohydrate-containing molecules on the surface of the pathogen (77). It is therefore interesting to speculate what significance, if any, the L-galactosyl residues found in B. maydis has on its pathogenesis.

The existence of L-galactose in mammalian tissue remains only speculative. Acid hydrolyses of the acetone powders from rat brain, porcine brain, porcine lung, and human placenta were analyzed by this method and no L-galactose was indicated. However, sugars detected from those crude hydrolyses were those commmon to the proteoglycans. Thus, a more thorough study of the existence of L-galactose and other "rare" sugars awaits the isolation and analysis of specific glycosylated molecules, such as those located within the plasma membrane.

V. Experimental

Materials. Micro-reaction vials (0.3 and 1.0 ml) having Teflon-lined rubber septa were purchased from Supelco, Inc. When very small amounts of sugar (50-1000nanograms) were analyzed, the vials were deactivated with Prosil-28, a product of PCR Research Chemicals. Polypropylene test tubes (1.5 ml) with attached stoppers were obtained from Walter Sarstedt, Inc.

The following chemicals were obtained from the sources indicated: Acetylene dicarboxylic acid, brucine dihydrate, ethanethiol , (-)-menthol, 4-(dimethylamino)-pyridine, styrene, trifluoroacetic anhydride, Aldrich Chem. Co.; dimethylchlorosilane, Analabs, Inc.; trifluoroacetic acid, Eastman Organic Chem.; acetic anhydride, Fisher Scientific Co.; carbon disulfide, Mallinckrodt, Inc.; N,O-bis-(trimethylsilyl)-trifluoro- acetamide (BSTFA), trimethylchlorosilane (TMCS), N-methyl- N-trimethylsilyl-trifluoroacetamide (MSTFA), Pierce Chem. Co.; N,O-bis-(dimethylsilyl)-acetamide, Supelco. Inc. Racemic 1-phenylethyl bromide was prepared by the hydrobromination of styrene as described by Ashworth and Burkhardt (77).

The following sugars were obtained from the sources indicated: D-glucose, Allied Chemicals; D-xylose, D-glucuronic

acid, Calbiochem; L-arabinose, K & K Laboratories Inc.; L-fucose, D-galactose, L-lyxose, D-lyxose, L-mannose, D-mannose, L-rhamnose, Pfanstiehl Laboratories, Inc.; D-fucose, D-galactose acid, N-acetyl D-galactosamine, L-glucose, N-acetyl D-glucosamine, D-ribose, α,α-trehalose, L-xylose, Sigma Chemical Co. L-Galactose was synthesized by Dr. P. A. Hebda in this laboratory from L-galactonolactone, as described by Frush and Isbell (78). D-rhamnose was synthesized by Dr. G. A. Barber and crystallized by use of nucleating crystals of D-rhamnose kindly provided by Dr. N. K. Richtmyer.

The purified galactan from Helix pomatia was received as a gift from Dr. H. Bretting. Corn root polysaccharide was isolated from 7-day old seedlings, as described by Roberts and Harrer (17). The polysaccharide from flax seed mucilage was isolated as described by Araki and Arai (79). Cells of Bipolaris maydis race T were obtained from Dr. M. O. Garroway's laboratory. Cells of Neurospora were obtained from Dr. G. A. Marzluf's laboratory. Gum of locust bean, gum guar, gum tragacanth, human α_1-acid glycoprotein, and acetone powders of rat brain and human placenta were obtained from Sigma Chem. Co. Porcine brain and lung tissues were obtained locally from Ohio Packing Co. Earthworms were obtained from a local bait shop. Cuticles were removed, minced, dried, and pulverized to a powder with the use of a mortar and pestle.

Methods. Elemental analyses were made by Integral Microanalytical Laboratories (Raleigh, N.C.). Optical rotations were measured either in a Perkin-Elmer Model 241 polarimeter or a Lippich Polarizer (Bausch and Lomb Optical Co.). ^{1}H-N.m.r. spectroscopy was performed in a Varian T60 (60 MHz) spectrometer. The mass spectrum of L-arabinose bis [(+)-1-phenylethyl] dithioacetal was taken in a Finnigan Model 400021-C Gas Chromatograph-Mass Spectrometer. Infrared spectra were taken with a Perkin-Elmer 237B instrument.

Thin-layer chromatography was performed on precoated layers (0.1mm) of silica gel on poly(ethylene terephthalate) sheets (no. 13179 Eastman). The sheets were developed with chloroform/butanol (10:1, by volume). Paper electrophoresis was carried out on Schleicher and Schuell acid washed paper (no. 589, orange ribbon) in an apparatus constructed after the design of Crestfield and Allen (80). For electrophoretic isolation of charged sugar derivatives, a buffer of 0.05M potassium phosphate made to pH 6.9 was used at about 40 V/cm. Neutral sugars were isolated by descending paper chromatography on Schleicher and Schuell No. 589 white ribbon paper developed with 1-propyl alcohol/ethyl acetate/water (7:1:2, by volume). Sugars and sugar derivative were made visible with the alkaline silver nitrate reagent (81).

Carbohydrate samples (5-50 mg) were hydrolyzed in 2 Molar trifluoroacetic acid (1-8 ml) for periods of 2-6 hours at 100° in a sealed ampoule. All samples were flushed with N_2 prior to hydrolysis. Sample sizes and periods of hydrolysis

were varied according to the known chemical nature and the solubility of the particular sample. Each sample was then filtered through glass wool into a polypropylene test tube, concentrated under a stream of dry air, and dried overnight in a desiccator containing KOH.

Gas-liquid chromatography was conducted in a Shimadzu Mini-2 Gas Chromatograph equipped with a flame-ionization detector and inlet-splitter assembly. Capillary columns (30 m X 0.25mm) of fused silica coated with SE-54 and SE-30 were obtained from J & W Scientific (Rancho Cordova, Cal.). Hydrogen carrier gas velocity was typically maintained between 33-50 cm/s, as measured by the retention time of methane at 275°. The split ratio was adjusted to 100:1. The glass inlet was periodically removed, cleaned, and deactivated. Deactivation was carried out in situ by injecting a solution of BSTFA/TMCS (2:1, by volume) into the sealed inlet. The inlet temperature during deactivation was set at 175°.

Liquid chromatography was performed using an Altex 100A pump, Altex Model 155-00 analytical flow cell, and a Hitachi variable wavelength UV-Vis detector. A prepacked Partisil-10 strong anion exchange column was obtained from Alltech Associates (Arlington Height, Ill.). Dithioacetal derivatives were eluted with 0.05M potassium phosphate buffer, pH 2.1; and detected at 243 nm. Buffer and samples were filtered through Millipore membrane filters having 0.45μ mean pore size.

Preparation. Preparation of (+)-2,3-dimercaptosuccinic acid. Partially active (+)-2,3-dimercaptosuccinic acid was obtained as described by Gerecke et al. (42) and Hedblom (43). Briefly, three isomers of 2,3-bis (acetylthio)succinic acid were obtained from the treatment of acetylenedicarboxylic acid with thioacetic acid (See Figure 2 of text). The meso isomer was separated from the two optical isomers by fractional recrystallization. (+/-)2,3-Dimercaptosuccinic acid was obtained by hydrolysis of (+/-)bis (acetylthio)succinic acid. (+)-2,3-Dimercaptosuccinic acid was partially resolved by fractional recrystalization of its brucine salt.

Preparation of (+)-1-phenylethanethiol. (+)-1-Phenylethanethiol was synthesized as described by Isola et al (53). This procedure involves the reaction of sodium (-)-O-menthyl dithiocarbonate with racemic 1-phenylethyl bromide and subsequent separation, by fractional recrystallization, of the diastereomers formed. The optically active thiol is released by solvolysis in morpholine and obtained in nearly 100% optical purity. Samples were stored under N_2 at 5°.

Preparation of diastereoisomeric sugar dithioacetals using (+)-2,3-dimercaptosuccinic acid. (S,S)-2,3-(dicarboxy)-ethylene dithioacetals of various sugars were obtained as follows: sugar samples (1.0-5.0 mg) and (+)-2,3-dimercaptosuccinic acid (5.0 mg) were placed in a vial and dissolved in water (0.4 ml). An equal volume of concentrated HCl was added. The vial was sealed and left overnight at 37°. Product formation was typically monitored

by paper electrophoresis at pH 6.9. Samples were taken to dryness by placing the opened vial in a desiccator containing concentrated H_2SO_4 and pellets of KOH and were let stand overnight in vacuo. The dried samples were dissolved in eluting buffer and subjected to analysis by high pressure liquid chromatography.

Preparation of diastereoisomeric sugar dithioacetals using (+)-1-phenylethanethiol. Derivatives of standard sugars were prepared as follows. A sample of sugar (10-100 μg) contained in either a 0.3 ml micro-reaction vial or a 1.5 ml polyethylene test tube was dried overnight in a desiccator containing KOH. Trifluoroacetic acid (5 μl) was added and the vial swirled gently until the residue dissolved. (+)-1-Phenylethanethiol (20 μl) was added, and the vial contents mixed using a Vortex mixer. The reaction was allowed to proceed for 30 minutes at room temperature with occasional mixing of contents. Cold pyridine (50 μl) was added to stop the reaction. For qualitaitve studies, a pyridinium solution (10 μl) of 0.01 M α,α-trehalose was added as an internal standard.

Derivatives of sugars obtained from hydrolyzed carbohydrate samples were prepared similarly. However it was necessary, in some instances, to add additional amounts of trifluoroacetic acid (10-20 μl) to the residue and warm the sample at 37° for 10 minutes. By this procedure, most of the solid residue dissolved.

Formation of volatile derivatives. Trimethylsilyl ethers were obtained by adding BSTFA (50 μl) and TMCS (25 μl) to the reaction

vial and heating in an oven for 2 hours at 65°. Samples (1 μl) of this mixture were analyzed by g.l.c.

Acetyl derivatives were obtained as follows. The excess of thiol and of pyridine were removed by placing the opened vial in a desiccator containing concentrated H_2SO_4, pellets of KOH, and a small beaker of activated charcoal impregnated with $CuSO_4$, and the vial was kept overnight in vacuo. To the pyridinium salt that remained was added a freshly prepared solution (100 μl) of 4-(dimethylamino)pyridine in acetic anhydride (2.5 mg/ml). Samples were heated for 2 hours at 65°, concentrated under a stream of dry N_2, and dissolved in CH_2Cl_2 (50 μl) for g.l.c. analysis.

Characterization of dithioacetal product. D-Galactose bis (+)-1-phenylethyl dithioacetal was prepared in the following manner: D-galactose (60 mg) dissolved in warm trifluoroacetic acid (0.2 ml) was treated with (+)-1-phenylethylthiol (100 μl). Reaction contents were vigorously mixed over a ten minute period. Small portions of cold water were added to induce precipitation. The white preciptiate was collected on a sintered glass funnel and recrystallized twice from EtOH/H_2 to give final yield of 38 milligrams; melting point 152°, $[\alpha]_{589}^{25}$ = +322 (c=0.9, EtOH). Further recrystallization effected no change in melting point or optical rotation.

NMR data: 1.45 (dd, 6H), 7.2 (m, 10H).

Anal. Calc. for $C_{22}H_{30}O_5S_2$: C, 60.24; H, 6.89; S, 14.61.

Found: C, 60.14; H, 6.94; S, 14.74.

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