with 75 ml. of ethyl acetate. The reaction mixture was extracted with 2 *N* hydrochloric acid, aqueous potassium chloride and sodium bicarbonate solutions. The organic solution was dried over magnesium sulfate and the solvent removed under reduced pressure. The crystals obtained were recrystallized several times from ethyl acetate–ether solution yielding 0.66 g. (75%), m.p. 127–128°.

Anal. Calcd. for $C_{16}H_{20}N_2O_7$: C, 54.55; H, 5.68; N, 7.96. Found: C, 54.52; H, 5.66; N, 7.80.

Benzyloxycarbonylglycyl-β-methyl-L-aspartyl-glycine Methyl Ester.—Benzyloxycarbonyl glycine (1.4 g., 0.0066 mole) was dissolved in dimethylformamide and the solution cooled to 0°. Triethylamine (1 ml., 0.0066 mole) and isobutyl chloroformate (1 ml., 0.0066 mole) were added, and the reaction was allowed to proceed for 20 min. β-Methyl α-*p*-nitrophenyl-L-aspartate (2.3 g., 0.0066 mole) in dimethylformamide was then added followed by slow addition of triethylamine (1 ml., 0.0066 mole). After 4 hr., glycine methyl ester hydrochloride (0.82 g., 0.0066 mole) was added followed by slow addition of triethylamine (1 ml., 0.0066 mole). After 14 hr., the solution was diluted with ethyl acetate (125 ml.) and extracted with 2 *N* hydrochloric acid, aqueous potassium chloride and saturated sodium carbonate solutions until the yellow *p*-nitrophenoxide color disappeared. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The resulting solid was recrystallized a few times from a minimum of hot ethyl acetate yielding 1.55 g. (57%), m.p. 141°.

Anal. Calcd. for $C_{18}H_{23}N_3O_8$: C, 52.81; H, 5.63; N, 10.25. Found: C, 53.04; H, 5.83; N, 10.21.

Benzyloxycarbonyl-di-(β-methyl-L-aspartyl)-glycine Methyl Ester.—Benzyloxycarbonyl-β-methyl-L-aspartyl-β-methyl-α-*p*-nitrophenyl L-aspartate (0.56 g., 0.001 mole) was dissolved in dimethylformamide and cooled to 15°. Glycine methyl ester hydrochloride (0.25 g., 0.002 mole) was added followed by slow addition of triethylamine (0.14 ml., 0.001 mole). The reaction proceeded for 12 hr. after which time the volume was reduced under vacuum and ethyl acetate (150 ml.) was added. The solution was extracted with 2 *N* hydrochloric acid, aqueous potassium chloride and saturated sodium carbonate solutions until colorless. The organic layer was dried over magnesium sulfate and the solvent removed under reduced pressure. The resulting solid was recrystallized three times from hot ethyl acetate, yielding 0.3 g. (63%), m.p. 115°.

Anal. Calcd. for $C_{21}H_{27}N_3O_{10}$: C, 52.39; H, 5.61; N, 8.73. Found: C, 51.97; H, 5.74; N, 8.53.

Benzyloxycarbonyl-glycyl-β-methyl-L-aspartyl–Diethyl L-Aspartate.—Benzyloxycarbonylglycine (0.29 g., 0.001 mole) was dissolved in dimethylformamide and cooled to 0°. Isobutyl chloroformate (0.14 ml., 0.001 mole) and triethylamine (0.14 ml., 0.001 mole) were added and the reaction allowed to proceed for 20 min. A solution of β-methyl α-*p*-nitrophenyl-L-aspartate hydrobromide was added (0.35 g., 0.001 mole) followed by addition of triethylamine (0.14 ml., 0.001 mole) and the reaction was allowed to proceed for 4 hr. Diethyl L-aspartate hydrochloride (0.23 g., 0.001 mole) was then added followed by slow addition of triethylamine (0.14 ml., 0.001 mole). After 12 hr., the solution was diluted with ethyl acetate and extracted with 2 *N* hydrochloric acid, aqueous potassium chloride and sodium carbonate solutions until colorless. The organic layer was dried over magnesium sulfate and the solvent distilled under reduced pressure. The resulting compound was recrystallized twice from ethyl acetate–ether–petroleum ether to give 0.4 g. (79%) of the desired product, m.p. 115°.

Anal. Calcd. for $C_{23}H_{31}N_3O_{10}$: C, 54.22; H, 6.09; N, 8.25. Found: C, 53.88; H, 6.24; N, 8.10.

Benzyloxycarbonylglycyl-di-(β-methyl-L-aspartyl)-glycine Methyl Ester.—Benzyloxycarbonyl-di-(β-methyl-L-aspartyl)-glycine methyl ester (0.5 g., 0.0012 mole) was treated with 1.5 ml. of dry hydrogen bromide (33%) in glacial acetic acid. After 30 min. the compound dissolved with evolution of carbon dioxide. An oil separated on addition of ether. The ethereal solution was decanted, and the oil triturated several times with ether. The oil was dissolved in methanol, precipitated twice with ether, and dried under vacuum to yield 0.43 g. of product (0.001 mole) which was used immediately in the following reaction.

Benzyloxycarbonylglycine (0.2 g., 0.001 mole) was dissolved in dimethylformamide and cooled to 0°. Isobutyl chloroformate was added (0.14 ml., 0.001 mole) followed by addition of triethylamine (0.14 ml., 0.001 mole). The reaction was allowed to proceed for 20 min. The hydrobromide oil (0.43 g., 0.001 mole) prepared above was added and then triethylamine (0.14 ml., 0.001 mole) was added slowly.

After 4 hr. the solution was diluted with ethyl acetate (200 ml.) and washed with 2 *N* hydrochloric acid, aqueous potassium chloride, and sodium bicarbonate solutions. The ethyl acetate layer was dried over magnesium sulfate and the solvent removed under reduced pressure. The resulting solid was recrystallized from warm ethyl acetate several times to give 0.28 g. (51%) of product, m.p. 181°.

Anal. Calcd. for $C_{23}H_{30}N_4O_{11}$: C, 51.30; H, 5.58; N, 10.41. Found: C, 50.94; H, 5.66; N, 10.19.

[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND NUTRITION, GRADUATE SCHOOL OF PUBLIC HEALTH AND BIOCHEMISTRY SCHOOL OF MEDICINE, UNIVERSITY OF PITTSBURGH, PITTSBURGH, PENNA.)

Gas–Liquid Chromatography of Trimethylsilyl Derivatives of Sugars and Related Substances[1]

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RECEIVED APRIL 5, 1963

The separation and estimation of carbohydrates and related polyhydroxy compounds by gas–liquid chromatography of trimethylsilyl (TMS) derivatives is described. The formation of the TMS derivative, in pyridine containing hexamethyldisilazane and trimethylchlorosilane, occurs very rapidly at room temperature so that analyses can be made within a few minutes. Comparative studies of the reaction product of methyl α-glucopyranoside and authentic methyl (tetra-*O*-trimethylsilyl)-α-glucopyranoside indicate that silylation of all free hydroxyl groups occurs and that the yield of TMS derivative is virtually quantitative. Conditions are described for chromatography of a wide variety of carbohydrates from C_2 (glycolaldehyde) to C_{24} (stachyose) and related substances such as glycosides, deoxysugars, inositols, hexosamines, and N-acetylneuraminic acid. Most of the studies have been made with a silicone column (SE-52) and a polyester column (polyethylene glycol-succinate) but separations of the TMS derivatives are possible on other polar and non-polar columns. Isothermal conditions are usually employed for separations within a narrow range of molecular weight; separations of more complex mixtures, with components of widely differing molecular weights, may be made by linear temperature-programmed analysis. Excellent separations are generally observed with anomeric pairs as well as configurational isomers within a given class such as pentoses, hexoses, disaccharides, etc. The identity of an unknown sugar may be determined by multiple analyses on a number of liquid phases or, alternatively, by analyses of the parent sugar and various derivatives such as methyl glycoside, alcohol, lactone, oxime, and acetal. In all such cases TMS derivatives are prepared prior to gas chromatography. Comparisons are reported for the compositions of aqueous equilibrium solutions of aldoses, by gas chromatographic analysis, with those reported by measurements of optical rotation and bromine oxidation. In several cases unexpected retention times are interpreted in terms of conformational differences of the sugars.

The application of gas chromatography to the separation of carbohydrates and related polyhydroxy compounds has tended to lag behind the development of this technique with other classes of compounds. A major difficulty has been the preparation of volatile derivatives of the polyhydroxy compounds by rapid and general techniques. Investigations so far described have generally made use of either *O*-methyl ethers or acetyl derivatives; a comprehensive review of separa-

(1) This work has been supported in part by the following grants from the United States Public Health Service: A-4307, A-725, and H-2458.

(2) Dr. Makita was on temporary leave from the School of Pharmacy, Osaka University.

(3) During the tenure of this work, Dr. Wells was the recipient of an Established Investigatorship of the American Heart Association.

tions of these derivatives by gas chromatography, covering work to about the end of 1961, has appeared.[4] Columns containing low concentrations of liquid phases (either SE-30 or QF-1) were used to extend the useful range for carbohydrates to the separation of two disaccharides as octaacetates,[5] a logical extension of earlier work with steroids.[6] Similarly, Bishop and Cooper have separated several fully methylated disaccharides.[4] More recently, acetyl derivatives were used for the resolution of amino sugars and for separation of a number of acetal and ketal derivatives.[7]

It appeared to us that trimethylsilyl (TMS) derivatives of sugars would be potentially useful for gas chromatography, especially since a simple and rapid quantitative method for the preparation of *O*-trimethylsilyl derivatives of bile acids had already been perfected by Makita and Wells.[8] This method involved solution or suspension of the material in pyridine, followed by reaction with hexamethyldisilazane and trimethylchlorosilane. The use of pyridine as a solvent for polyhydroxy compounds was an obvious advantage. Our experiments demonstrated that the method was remarkably effective for carbohydrates, with trimethylsilylation taking place rapidly and virtually completely at room temperature. Although previous experiences with the gas chromatography of TMS derivatives of sugars were not encouraging,[9,10] we have found that under appropriate conditions excellent separations may be achieved. The present communication records studies with almost 100 carbohydrates and related derivatives. Since the preparation of TMS derivatives is a rapid and convenient process that can be applied on a micro-scale, analysis of carbohydrates by gas chromatography has become a practical laboratory procedure. A preliminary account of this investigation has appeared.[11]

Experimental

Materials.—Many of the carbohydrates and related substances used in this study were commercially available preparations. We are very much indebted to Dr. N. K. Richtmyer for D-allose and D-altrose; to Dr. H. S. Isbell for the δ-lactones of lactobionic and maltobionic acids; to Dr. S. Roseman for N-acetylmannosamine and N-acetylneuraminic acid; and to Dr. T. Reichstein for sodium L-idonate. Derivatives of idose,[12] cellobiose, and maltose, as well as the methyl glycosides of arabinose and galactose, had been synthesized in connection with other work.[13] An authentic sample of methyl (tetra-*O*-trimethylsilyl)-α-glucopyranoside was prepared by the method of Hedgley and Overend[9]; b.p. 142–143° at *ca.* 1 mm. D-Idosan was prepared by hydrolysis of methyl α-D-idoside.[14] Dr. T. Katagi kindly prepared an authentic sample of glucose oxime, which crystallized from aqueous methyl alcohol; m.p. 137–138° (lit. 138°). Hexamethyldisilazane was obtained from Peninsular Chemresearch, Gainesville, Fla.; trimethylchlorosilane was obtained from General Electric Co., Silicone Products Division, Waterford, N. Y. Solvents were reagent grade and were used as supplied unless specially noted.

For gas chromatography, the column packing containing 15% polyethylene glycolsuccinate on 80–100 mesh Chromosorb W was obtained from Applied Science Laboratories, Inc., State College, Pa. The other column packings were prepared from commercially available liquid phases and acid-washed, silanized Chromosorb W (80–100 mesh), supplied in untreated form by Johns–Manville Corp., New York, N. Y., or Gas-Chrom P from Applied Science Laboratories, using the solution coating technique.[15]

Procedures.—A critical evaluation of the optimal proportions of hexamethyldisilazane and trimethylchlorosilane necessary for maximum yield of the TMS derivative was conducted with methyl α-glucopyranoside as the model compound. The test samples each consisted of 5 mg. of methyl α-glucopyranoside dissolved in 1.85 ml. of pyridine. The hexamethyldisilazane was either omitted from the reaction or added in amounts from 0.03 ml. to 0.15 ml., followed by appropriate complementary amounts of trimethylchlorosilane sufficient to bring the total volume of reagents to 0.15 ml. The yield was determined by gas chromatography (peak area) 5 min. and 24 hr. after addition of the reagents. In each case, the quantity of the product formed was essentially independent of time. Almost no reaction occurred when only hexamethyldisilazane was employed, whereas almost 50% of maximum yield was formed in the presence of trimethylchlorosilane alone. The maximum peak area was obtained with a mixture containing 0.1 ml. and 0.05 ml. of hexamethyldisilazane and trimethylchlorosilane, respectively. Nearly identical yields were obtained when the reagents were varied from 0.12 ml. to 0.075 ml. of hexamethyldisilazane and 0.03 ml. to 0.075 ml. of trimethylchlorosilane. The quantity of pyridine selected was not found to be critical. Thus, adjustment of the final volume with pyridine for quantitative studies may be carried out conveniently. The stability of the TMS derivative in stoppered vials was good for several days when peak areas were compared accurately, although the full life of the products in these mixtures appears to be considerably longer.

The standard conditions for trimethylsilylation adopted as a result of these studies were: when sufficient material was available, 10 mg. of carbohydrate was treated with 1 ml. of anhydrous pyridine (kept over KOH pellets), 0.2 ml. of hexamethyldisilazane, and 0.1 ml. of trimethylchlorosilane. The reaction was carried out in a 1-dram, plastic-stoppered vial, or similar container. The mixture was shaken vigorously for about 30 sec. and was then allowed to stand for 5 min. or longer at room temperature prior to chromatography. The solutions became cloudy on addition of trimethylchlorosilane, owing to precipitation, presumably of ammonium chloride. No attempt was made to remove this fine precipitate which in no way interfered with the subsequent gas chromatography. If the carbohydrate appeared to remain persistently insoluble in the mixture the vial was warmed for 2–3 min. at 75 to 85°. In some cases, in which no rearrangements were likely to occur, the sugar was first brought into solution by warming with pyridine, prior to the addition of hexamethyldisilazane and trimethylchlorosilane. Frequently, the method was applied to proportionately smaller amounts of sugar and reagents. From 0.1 to 0.5 μl. of the resulting reaction mixture was used for injection into the gas chromatograph.

Nature of Product and Yield of the Trimethylsilylation Reaction.—The TMS derivative of methyl α-glucopyranoside was prepared in the usual manner from accurately weighed samples of the glycoside. The reaction mixture was allowed to stand at room temperature and accurately measured aliquots, drawn into a 10-μl. Hamilton syringe, were injected into the chromatograph after 5 min., 0.5, 1.5, and 18 hr. Observed areas were measured by a compensating polar planimeter. The area of the peak obtained at each test interval was identical. These areas were compared with those obtained from known quantities of authentic methyl (tetra-*O*-trimethylsilyl)-α-glucopyranoside. Duplicate analyses were in good agreement and indicated that the trimethylsilylation reaction yielded methyl (tetra-*O*-trimethylsilyl)-α-glucopyranoside in 90% of theory.

Preparation of Derivatives.—Aqueous solutions of aldoses were reduced to the corresponding alcohols as follows. The solution (0.1 ml.) containing from 5 to 10 mg. of sugar was cooled in ice, then treated with 5 mg. of KBH_4. The mixture was allowed to warm to room temperature and then stood for 35 min. before adding 0.1 ml. of 1 *N* HCl. When decomposition of the excess KBH_4 was complete, a further 0.2 ml. of water was added, followed by 200 mg. of a mixed ion exchange resin (MB-2, Rohm and Haas Co., Philadelphia, Pa.). The aqueous solution was removed with a Pasteur pipet and the resin was washed once with about 0.5 ml. of water. The combined solutions were evaporated to dryness in a warm water bath under a stream of air and the residue was dried briefly in a vacuum desiccator prior to trimethylsilylation.

Oximes were prepared from solid aldoses or from aqueous equilibrium solutions. In a typical preparation 10 mg. of a

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(10) H-G. Witsch, Thesis (Doktors der Naturwissenschaften), Technischen Hochschule, Karlsruhe. We are grateful to Professor E. Bayer for sending a copy of Dr. Witsch's thesis.

(11) R. Bentley, C. C. Sweeley, M. Makita, and W. W. Wells, *Biochem. Biophys. Research Commun.*, **11**, 14 (1963).

(12) Unless there are special circumstances, the configurational prefixes D- and L- will be omitted throughout this paper. We obtained identical results with enantiomorphs, *e.g.*, with D- and L-arabinose, D- and L-lyxose. No separation of enantiomorphs would be expected on our columns.

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(14) E. Sorkin and T. Reichstein, *Helv. Chim. Acta*, **28**, 1 (1945).

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mixture of α- and β-glucose and 6 mg. of hydroxylamine hydrochloride were dissolved in 1 ml. of pyridine. The reaction mixture was heated at 70 to 80° for 30 min. after which the solution was cooled and 0.1 ml. of hexamethyldisilazane and 0.1 ml. of trimethylchlorosilane were added.

Gas Chromatography.—Three instruments were used for analyses by gas chromatography. The F & M Model 609, with hydrogen flame ionization detector, employed coiled stainless steel columns, 6 ft. by 0.25 in. o.d. packed with 3% SE-52 and 8 ft. by 0.25 in. o.d. packed with 10% Carbowax 1540. The Barber–Colman Model 10, with argon ionization detector A-4183, utilized U-shaped borosilicate glass columns containing 15% polyethylene glycolsuccinate (EGS), 8 ft. by 0.25 in. o.d., or 15% Apiezon M, 6 ft. by 0.25 in. The Chromalab instrument, made by Glowall Corporation, was equipped with an argon ionization detector and coiled glass columns, 6 ft. by $^1/_8$ in. i.d., containing 4% nitrile silicone (XE-60). In general, flow rates were adjusted for optimal column efficiencies and ranged from 75 to 150 ml. per minute with inlet pressures of 15 to 20 p.s.i.

Results and Discussion

The Trimethylsilylation Reaction.—The use of TMS derivatives for the preparation of volatile derivatives of phenols for use in analyses by gas chromatography was first described by Langer, Pantages, and Wender.[16] The preparation of pentatrimethylsilylglucose was described by two groups of workers[17] and derivatives of galactose, fructose, and sucrose by Henglein, *et al.*,[18] who made use of formamide as a solvent, mixed with trimethylchlorosilane and pyridine, and reaction at room temperature or at 35 to 40° for several hours. Gas chromatography of TMS derivatives of several sugars was investigated recently.[9] Best results were obtained with Apiezon M columns (20–50%) operated at temperatures in excess of 220°; these operating conditions led to a relatively short lifetime for the columns. Methyl (tetra-*O*-trimethylsilyl)-hexopyranosides were separated from methyl (tri-*O*-trimethylsilyl)-pentopyranosides, but resolution of anomeric pairs of derivatives was not possible, and separations of mixtures of configurational isomers were poor. A further disadvantage was the fact that multiple peaks were obtained when free sugars were subjected to the trimethylsilylation procedure. Bayer[19] and Witsch[10] have described similar studies with TMS derivatives of glucose, galactose, and fructose, prepared by the method of Henglein, *et al.*[18] More recently, trimethylsilylation of xylose[20] and other pentoses[21] has been studied and a number of separations were obtained with Apiezon L (20%) as the stationary phase. In these studies the procedure for the preparation of TMS derivatives was similar to that of Hedgley and Overend.[9]

The procedure reported here for trimethylsilylation does not require the inconveniently long reaction times used by previous workers; it has also been found to be generally applicable to a wide variety of polyhydroxy compounds. The products obtained from pure, single anomers of pentoses and hexoses generally show a single peak on gas chromatography though, in some cases, small amounts of another anomeric form may be apparent. In general, anomerizations occurring in pyridine during preparation of the TMS derivative are remarkably minimal; with α-glucose the β-peak amounted to 3.9% of the total and with β-glucose the α-peak was 4.5% of the total.

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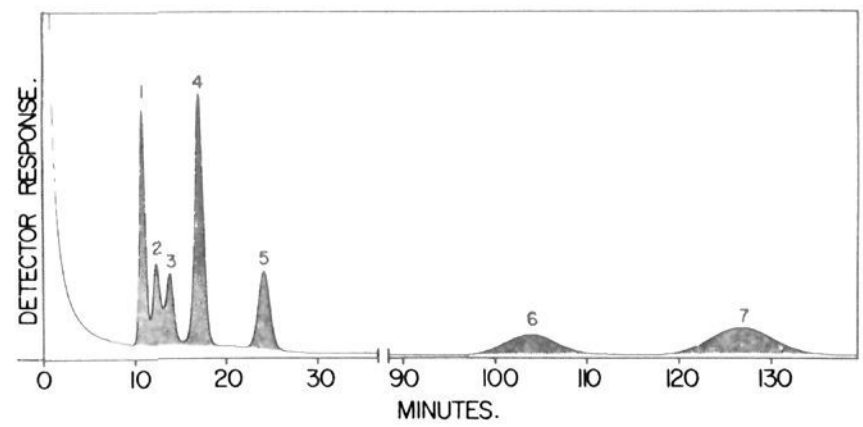


Fig. 1.—Gas chromatography of di- and trisaccharides on SE-52. The column was operated isothermally at 210°. The following are the assignments made to the various peaks in order of increasing retention times: 1, sucrose; 2, α-maltose; 3, β-maltose; 4, β-cellobiose; 5, gentiobiose; 6, raffinose; 7, melezitose.

Since a pure anomer yields a single peak it appears likely that all of the free OH groups of the compounds we have studied are converted to the TMS derivative. This conclusion is supported strongly by the fact that the peak observed by allowing methyl α-glucopyranoside to react according to the standard conditions is indistinguishable from that obtained with an authentic sample of methyl (tetra-*O*-trimethylsilyl)-α-glucopyranoside, and no other peaks were observed on the chromatogram. This conclusion is further reinforced by the excellent separations of α- and β-forms of many free sugars, as well as by the fact that only two peaks are obtained from those aqueous equilibrium aldose solutions decisively known to contain only two components (*e.g.*, glucose, mannose). The effect of heating during the trimethylsilylation reaction will be considered in detail in connection with the discussion of equilibrium solutions of the aldoses.

The Stationary Phases.—Experience in gas chromatography over the past several years has shown that successful identification of complex mixtures of closely related substances, such as occurs in fatty acids (from various lipids), steroids, amino acids, urinary aromatic acids, etc., requires analyses on both polar and non-polar phases. This "two phase" concept applies equally well to carbohydrate TMS ethers. Many cases are observed in which incomplete resolution results on the non-polar, non-selective phase, SE-52, while analyses on polar columns, such as polyesters, polyethylene glycol, and nitrile silicone, are often more satisfactory. On the other hand, polar columns are less useful for analyses of mixtures which may contain substances with a wide range of boiling points. For example, separations of the aldohexoses are more complete on the polar EGS column, but SE-52 is the liquid phase of choice for a complete scan of carbohydrates from the tetroses to polysaccharides.

Much of the previously reported work on gas chromatography of polyhydroxy compounds has been at temperatures from about 170 to 220° or higher.[4] Using SE-52 we found that at 140° reasonable retention times were obtained for C_4 to C_7 sugars. Lower temperatures, *e.g.*, 125°, were useful for C_2 and C_3 compounds; at this temperature the retention time for α-glucose was still reasonable (32–34 min.), but that for β-glucose was unduly long (54–55 min.). Columns of SE-52 can be operated at much higher temperatures for the chromatography of materials of substantially lower volatility than the hexose derivatives. We have used 210° routinely for analyses of disaccharides; trisaccharides may also be analyzed at this temperature but require from 100 to 140 min. Examples of separations of disaccharides and trisaccharides at 210° are shown in Fig. 1. Trisaccharides

TABLE I

GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF MONOSACCHARIDES (ALDOSES)

No. of C atoms	Sugar[a]	SE-52 140°			EGS Polyester 140°			EGS Polyester 150°		
2	Glycolaldehyde	0.044[b]			0.25					
3	Glyceraldehyde	.039 (s)[c]	0.48[b]		3.58			0.68		
4	Erythrose (sirup)	.10	0.12	0.14	0.29	0.35				
5	β-Arabinose	.28			.97					
	Arabinose (equil.)	.28	0.33 (m)	0.38 (s)	.97	1.31 (m)	1.10 (s)			
	β-Lyxose	.26 (t)	.33		1.26					
	Lyxose (equil.)	.26 (m)	.33		0 94 (m)	1.26	1.42			
	Ribose (solid)	.32 (m)	.35		1.22	1.33				
	Ribose (equil.)	.27 (s)	.32 (m)	0.35	1.22 (m)	1.33	1.48 (s)			
	α-Xylose	.43	.54 (s)		1.64					
	Xylose (equil.)	.31 (t)	.43	0.54 (m)	1.64	2.11 (m)				
6	β-Allose	.81						0.91		
	Allose (equil.)	.76	0.81 (m)	0.91				0.78	0.91 (m)	1.20 (s)
	β-Altrofuranose	.94						1.16		
	Altrose (equil.)	.65 (m)	0.68	0.94 (s)				0.63 (m)	0.75	1.16 (s)
	α-Galactose	.88						1.03		
	Galactose (equil.)	.76 (s)	0.88	1.08 (m)				0.91 (s)	1.03	1.38 (m)
	α-Glucose	1.00[d]			5.48			1.00[d]		
	β-Glucose[e]	1.57						1.94		
	Glucose (equil.)	1.00	1.57					1.00	1.94	
	α-Gulose[f]	0.74						0.95		
	Gulose (equil.)[f]	.66	0.74 (m)	0.95 (t)				.84	0.95	2.11 (t)
	Idosan	.27 (t)	0.50					.66		
	β-Mannose	1.08						1.31		
	Mannose (equil.)	0.70 (m)	1.08					0.62 (m)	1.31	
	α-Talose	.86						1.22		
	Talose (equil.)	.86 (m)	1.00 (t)	1.13				1.06 (s)	1.22 (m)	1.51
7	3-*O*-Methylglucose	.56	0.85 (t)					0.53		
	D-*glycero*-D-*galacto*-Heptose[e]	4.27								
	D-*glycero*-D-*gulo*-Heptose[e]	2.29								
8	4,6-Ethylidene-α-glucose	0.54						0.57		

[a] The values given in this and subsequent tables are retention times relative to α-glucose with the SE-52 column and the EGS polyester column at 150° and higher. With EGS at 140°, the retention times are relative to methyl α-arabinopyranoside; the observed value for this reference standard was 10.85 min. at 140°. [b] These compounds were chromatographed at 125°; retention time for α-glucose, 34.4 min. [c] The following abbreviations are used in describing the peaks: (t), trace; (s), small; (m), major. [d] Over a period of several months, the retention time for α-glucose on SE-52 at 140° was 20 ± 2 min.; on SE-52 at 210°, the value was 1.3 ± 0.2 min.; on EGS polyester at 150°, the value was 25.2 min. [e] The following relative retention times were determined for SE-52 at 210°: β-glucose, 1.22; D-glycero-D-guloheptose, 1.62 (m) and 2.92; D-glycero-D-galactoheptose, 1.77, 2.50 (m), 3.08, and 3.54. [f] α-Gulose was obtained as the calcium chloride complex; this solid was treated directly with pyridine and silanes. The equilibrium solution of gulose is that prepared by solution of the calcium chloride complex in water.

are handled more conveniently at 250°, which is the maximum temperature we have used with the SE-52 column. Furthermore, at this temperature the tetrasaccharide, stachyose, had a retention time of 120.5 min. The TMS derivative of stachyose (14 OH groups) has a molecular weight of 1676; it is, indeed, remarkable that a compound with so high a molecular weight may be eluted successfully from a gas–liquid chromatographic column. By carrying out linear temperature-programmed analyses with SE-52 columns, it should be possible to accommodate the entire range of carbohydrate compounds from C_2 to tetrasaccharide (C_{24}) in a single run. An example of the separation of a mixture of 20 components, from C_4 to C_{18}, in 75 min., by this technique, is shown in Fig. 2.

The polyester column (EGS) was operated at one of three temperatures, 140, 150, or 170°, depending on the molecular weight range of the mixture. Since the limit of thermal stability of this column was exceeded at about 200°, analyses were limited to sugars with approximately 6 carbon atoms Separations within a narrow range of molecular weight were generally excellent on EGS; a typical example of the resolution of a multi-component mixture under isothermal conditions is shown in Fig. 3.

Separation of Representative Carbohydrates and Derivatives.—The results with simple aldoses containing from 2 to 8 carbon atoms and with oligosaccharides are shown in Tables I and II, respectively. With both polar and non-polar columns there is a general increase in retention time as the number of carbon atoms is increased. Compounds with an alkyl substituent on one or more OH groups tend, however, to have decreased retention times relative to the parent compound (*e.g.*, α-glucose, 1.00; methyl α-glucoside, 0.92; 3-*O*-methylglucose, 0.56; 4,6-ethylidene-α-glucose, 0.54). Similarly, the replacement of OH by H in the deoxysugars leads to decreased relative retention times as shown in Table III. In the case of glyceraldehyde the major peak is almost certainly to be attributed to the dimeric form of this aldose. On the SE-52 column a small peak with a retention time close to that observed with a C_2 compound, glycolaldehyde, was also present. This small peak in the glyceraldehyde chromatogram is likely to be that of the TMS derivative of monomeric glyceraldehyde.

When aqueous equilibrium solutions were prepared, evaporated to dryness, and the residues subjected to trimethylsilylation, the relative areas of the peaks due to α- and β-anomers agreed well with the known

TABLE II

GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF OLIGOSACCHARIDES AND PLANT GLYCOSIDES

No. of C atoms	Name	Structure	SE-52 210°[a]			SE-52 250°[b]	
12	Sucrose	β-D-Fructofuranosyl-α-D-glucopyranoside		10.4			1.00
	Lactose	4-*O*-β-D-Galactopyranosyl-D-glucose		10.5			
	Turanose	3-*O*-α-D-Glucopyranosyl-D-fructose	10.0 (t)	12.7			
	β-Maltose	4-*O*-α-D-Glucopyranosyl-D-glucose	11.7 (s)	13.1			1.16
	Maltose (equil.)		11.7	13.1 (m)			
	Trehalose	α-D-Glucopyranosyl-α-D-glucopyranose	9.04 (t)	13.5			
	β-Cellobiose	4-*O*-β-D-Glucopyranosyl-D-glucose	11.9 (s)[c]	16.6		1.14 (t)	1.37
	Melibiose	6-*O*-α-D-Galactopyranosyl-D-glucose	15.1 (t)	19.0	20.0		
	Gentiobiose	6-*O*-β-D-Glucopyranosyl-D-glucose		22.6			
18	Raffinose	*O*-α-D-Galactopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside		99.0			6.4
	Melezitose	*O*-α-D-Glucopyranosyl-(1 → 3)-*O*-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside		120.5			7.98
	Plantcose	*O*-α-D-Galactopyranosyl-(1 → 6)-*O*-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside		133.5			8.12
	Gentianose	*O*-β-D-Glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside	10.3 (t)[d]	138.0		1.03 (t)	8.12
24	Stachyose	*O*-α-D-Galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside					52.4
15	Esculin	6,7-Dihydroxycoumarin-6-glucoside					3.2
21	Phloridzin	4,6-Dihydroxy-2-(β-D-glucoside)-β-(*p*-hydroxyphenyl)-propiophenone					7.3

[a] At 210°, retention times are relative to α-glucose (1.1 to 1.3 min.). [b] At 250°, retention times are relative to sucrose (2.3 min.). [c] This small peak almost certainly represents the α-form of cellobiose. [d] Probably a trace of sucrose.

compositions of the equilibrium solutions, established by optical rotations or studies of bromine oxidations. The compositions of a number of these equilibrium solutions, calculated on the basis of the peak areas,

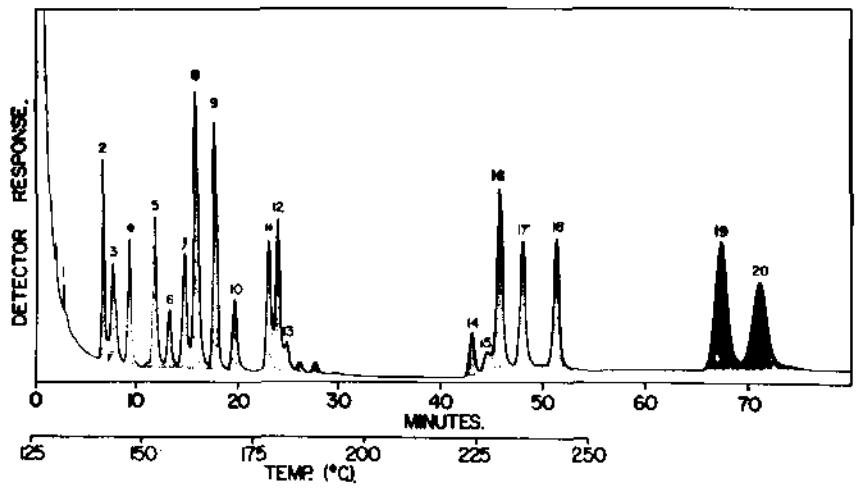


Fig. 2.—Linear temperature-programmed separation of TMS derivatives. The column used was SE-52, with an initial temperature of 125°. The F & M instrument was programmed for a temperature increase of 2.3° per minute, holding under isothermal conditions when 250° was attained. The components are: 1, erythrose (from sirup); 2, β-arabinose; 3, ribose (two ribose components are present in this peak); 4, α-xylose; 5, methyl α-mannoside; 6, α-gulose (from $CaCl_2$ complex); 7, α-galactose; 8, α-glucose; 9, L-ascorbic acid; 10, β-glucose; 11, N-acetylglucosamine; 12, N-acetylgalactosamine; 13, D-glycero-D-gulo-heptonolactone; 14, sucrose; 15, α-maltose; 16, β-maltose; 17, β-cellobiose; 18, gentiobiose; 19, raffinose; 20, melezitose.

are shown in Table IV. The values for arabinose and talose are least satisfactory. Further, the chromatograms indicated other components in the equilibrium solutions of arabinose, xylose, and galactose; it is generally agreed that aqueous equilibrium solutions of these sugars consist almost entirely of α- and β-anomers, with at most small amounts of "γ"-sugars (probably free aldehydes or furanose modifications). To determine whether the discrepancies were associated with the use of pyridine, portions of the sugars were refluxed in pyridine solution for 40 min., then subjected to trimethylsilylation in the usual way. A marked increase in the proportion of the third component was noted with arabinose, xylose, and galactose;

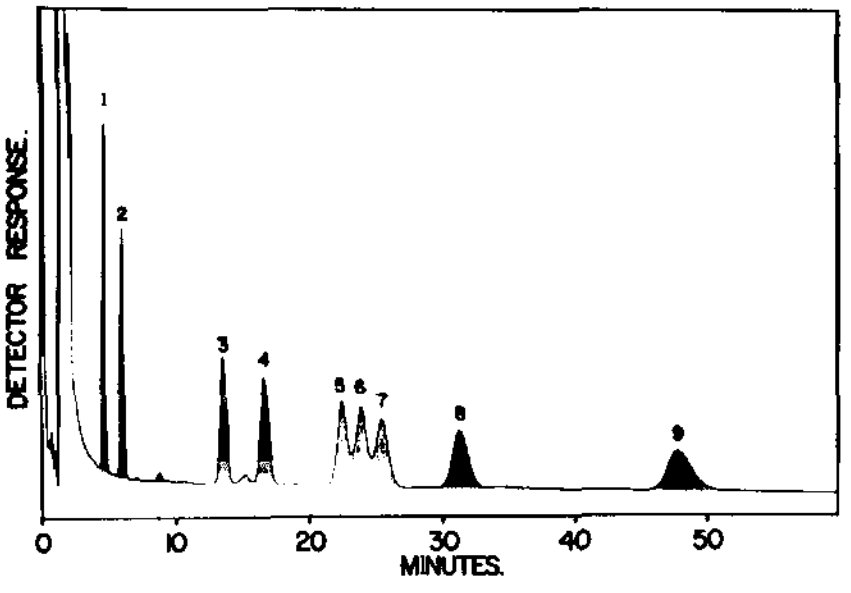


Fig. 3.—The separation of a multi-component mixture of sugar TMS ethers. This sample was chromatographed on 15% EGS polyester at 150°, 15 p.s.i. inlet pressure. The following are assignments made by comparison with chromatograms of the individual sugar derivatives: 1, α-rhamnose; 2, α-fucose; 3, fructose; 4, sorbose; 5, β-allose; 6, α-glucose; 7, α-galactose; 8, β-mannose; 9, β-glucose.

in the latter case, the change was from 5.4% of the total to 24.3%. The arabinose solution now contained more of the β-anomer than of the α and the xylose solution more α than β (the reverse is true in both cases in aqueous solution). With glucose, 3.2% of a third component was found after refluxing with

TABLE III

GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF DEOXY SUGARS

	SE-52, 140°				EGS polyester, 140°	EGS polyester, 150°
2-Deoxyribose	0.16				0.49	
Digitoxose	.18	0.53 (t)			0.51	
Rhamnose	.30					0.20
Fucose	.33	0.38 (m)	0.45			.25
2-Deoxygalactose	.42	.45	.53 (m)			.59
2-Deoxyglucose	.16 (s)	.25	.46	0.64 (m)		

TABLE IV

THE COMPOSITION OF EQUILIBRIUM MIXTURES AS DETERMINED BY GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES

	Aqueous equilibrium solution: Gas chromatography			Aqueous equilibrium solution: Bromine oxidation[b]		Aqueous equilibrium solution: Optical rotation[b]		Pyridine equilibrium[a]: Gas chromatography		
Sugar	α	β	γ	α	β	α	β	α	β	γ
Arabinose	50.8	43.8	5.4	67.6	32.4	73.5	26.5	31.9	53.1	15.0
Lyxose	74.1	25.9	...	79.7	20.3	76.0	24.0	71.8	28.2	Trace (2)
Xylose	41.3	55.2	3.4	32.1	67.9	34.8	65.2	45.9	42.8	11.3
Galactose	31.9	62.6	5.4	31.4	68.6	29.6	70.4	29.9	45.8	24.3
Glucose	39.8	60.2	...	37.4	62.6	36.2	63.8	47.3	49.5	3.2
Mannose	72.0	28.0	...	68.9	31.1	68.8	31.2	78.3	21.7	...
Talose	83.8	16.2	...	55.9	44.1					
Maltose	44.9	55.1	...	37.7	62.3	36.0	64.0			

[a] The pyridine "equilibrium" solutions were prepared by refluxing 10 mg. of sugar with 1 ml. of dry pyridine for 40 min., then treating immediately with the silanes. [b] The values for equilibrium solutions obtained by bromine oxidations and by optical rotations are taken from Bates, *et al.*[22]

pyridine, and lyxose contained traces of two other components.

The results of Ferrier[21] on the trimethylsilylation of pentoses are of interest in this connection. Using conditions for preparing the derivative similar to those of Hedgley and Overend,[9] gas chromatography was used to study the products from mutarotated mixtures. Calculations from the areas of Ferrier's published records show that only in the case of lyxose does the composition of the mixture agree with the known composition of the aqueous equilibrium solution. The order is reversed with arabinose and xylose, as we observed when the sugars were refluxed in pyridine prior to trimethylsilylation. Ferrier's curves also indicate two minor components in the lyxose mixture and one in arabinose.

It is apparent that new mutarotation mixtures of some sugars are established on heating with pyridine; lyxose and mannose appear to be exceptions, at least within the limits of our experiments. The "third components" are likely to be furanose modifications. It is known, for instance, that the proportions of the pyrano- and furanoacetates of galactose, in dry pyridine, vary according to the temperature.[23] Similarly, the rotation of the aqueous equilibrium solution decreases with temperature. The very rapid nature of the trimethylsilylation taking place under our conditions is evidenced by the generally good agreement of the values for aqueous equilibrium solutions determined by gas chromatography as compared to those obtained by other methods. In general, the trimethylsilylation reaction should be carried out at room temperature, particularly in the case of free sugars. Heating, if necessary for solution, should be carried out *after* the addition of hexamethyldisilazane and trimethylchlorosilane. When problems of mutarotation are not likely to be encountered, dissolving of the sugar by warming with pyridine can, of course, be used to advantage.

The excellent resolution of the anomeric forms of the sugars on both polar and non-polar columns is a remarkable feature which, although valuable in some studies, may create difficulties in others. Biological samples of sugars, for example, are almost invariably obtained from aqueous solution, and will thus be present as the equilibrium mixtures unless sufficient material is available for controlled crystallizations. As an example of the separations to be expected in such cases, an aqueous equilibrium solution containing the eight D-aldohexoses was prepared (idose was actually in the form of the anhydride, idosan, in view of the instability of the free sugar). If small trace components are not considered, there are 17 possible components in this mixture, as established by studies with individual equilibrium solutions (see Table I). They are one for idosan, three each for allose and altrose, and two each for the other hexoses. Chromatography on the succinate polyester gave 13 readily distinguished peaks as shown in Fig. 4. Of these, 10 could be assigned to a single anomeric form of one of the hexoses, while two peaks contained two components and one peak contained three components. Separation of this mixture was less effective on SE-52 since only 10 distinct peaks were observed.

In view of the difficulties likely to be encountered in the assignment of peaks to the proper sugars in a biological mixture containing several sugars, methods were examined for converting the sugars to a derivative which, on trimethylsilylation, would yield a single peak on the gas chromatograph. Sugars in aqueous equilibrium could be reduced conveniently with KBH_4 to the corresponding alcohols; following trimethylsilylation, single peaks were obtained for these derivatives. As indicated in Table V, however, the pentitols and hexitols were usually less well resolved than the parent sugars themselves. Arabitol and ribitol were not separated on SE-52; the retention times of the TMS derivatives of these two pentoses were also similar in other systems[21] and they were not resolved as pentaacetates. Dulcitol and sorbitol were poorly resolved on SE-52. Parallel study of the sugars as well as the products of borohydride reduction may have some use in the study of complex mixtures, especially if liquid phases can be found which resolve the TMS derivatives of the reduction products. Although mannitol and dulcitol hexaacetates are not well separated on QF-1, this phase separates either member of this pair from sorbitol hexaacetate.[5,24]

(22) F. J. Bates and Associates, "Polarimetry, Saccharimetry and the Sugars," U. S. Government Printing Office, Washington, D. C., 1942, p. 455.

(23) H. H. Schulubach and V. Prochownick, *Ber.*, **62**, 1502 (1929).

(24) J. A. Hause, J. A. Hubicki, and G. G. Hazen, *Anal. Chem.*, **34**, 1957 (1962).

TABLE V

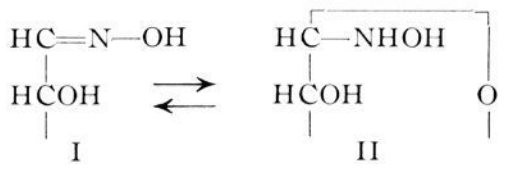
GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF SUGAR ALCOHOLS AND INOSITOLS

Number of C atoms	Compound[a]	SE-52 140°	EGS polyester 140°	EGS polyester 150°
4	Erythritol	0.16	0.49	
5	Arabitol	.46	2.13	
	Ribitol	.46	2.06	
	Xylitol	.42	2.08	
6	Allitol[b]	1.24		1.39
	Altritol[b] (talitol)	1.30		1.59
	Galactitol (dulcitol)	1.28		1.38
	Mannitol	1.21		1.31
	Sorbitol	1.24		1.42
	i-Inositol			2.56
	(+)-Inositol			1.13
	(−)-Inositol			1.13

[a] The C_3 parent of this series, glycerol, was found to have a relative retention time of 0.04 on SE-52 at 125°; under these conditions the retention time for α-glucose was 34.4 min. [b] These samples were prepared from aldohexoses by reduction with KBH_4.

Oxidations of equilibrium mixtures with bromine water was another technique investigated in the hope that multiple peaks for each sugar could be avoided. Oxidation on a small scale was generally less convenient than reduction, however, and, further, the possible formation of several lactone forms (γ, δ) was a complication. More than one peak was observed in a number of cases when the calcium salt of an aldonic acid was brought to "equilibrium" by warming with dilute hydrochloric acid for 30 min. (see Table VI).

A simple procedure was developed for the conversion of an aqueous equilibrium mixture of an aldohexose to the TMS oxime, in two steps, without isolation of the intermediate oxime. By this process a mixture of α- and β-glucose was converted smoothly to TMS glucose oxime which showed a single symmetrical peak on gas chromatography. The observed retention time was somewhat lower, however, than that obtained from the TMS derivative of authentic crystalline glucose oxime. Two isomeric forms of glucose oxime are known[25]; one of these, a cyclic modification (II), is known to be the only form of crystalline oxime



whereas an equilibrium with an acyclic modification (I) probably exists in solution. Since the retention time of the TMS glucose oxime, formed directly, was different from that obtained from the cyclic crystalline oxime, it is probable that direct reaction produces exclusively the acyclic modification (I). This is supported by the fact that the cyclic TMS oxime, in pyridine solution, slowly changed to give a mixture containing the product of direct reaction (isomerization to a mixture of I and II).

Although single peaks were generally obtained for the TMS oximes of a number of aldohexoses, resolution of these derivatives was poor (Table VII). The use of this derivative for routine analysis was complicated further by the possible formation of both cyclic and acyclic modifications of the oxime. The results suggest, however, a convenient method for study of the interconversions of isomeric forms of sugar oximes.

(25) W. W. Pigman and R. M. Goepp, Jr., "Chemistry of the Carbohydrates," Academic Press, Inc., New York, N. Y., 1948, pp. 410–411.

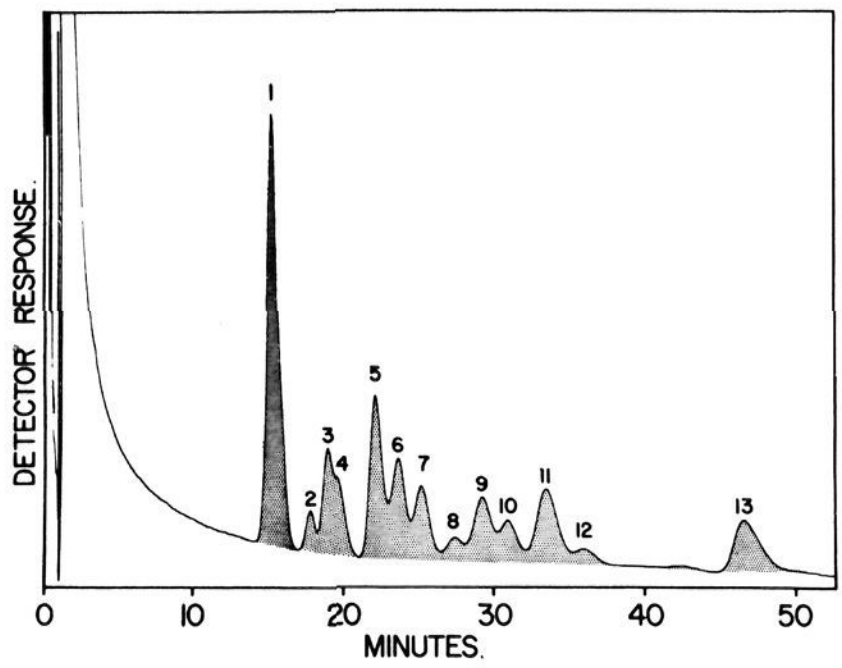


Fig. 4.—Gas chromatography of TMS derivatives prepared from an aqueous equilibrium solution of the eight aldohexoses. The sample was chromatographed on 15% EGS polyester at 150°. The following are assignments made by comparison with chromatograms of the TMS derivatives of individual sugars and equilibrium mixtures. Where anomeric assignments are not entirely certain, the peaks from an individual sugar are differentiated by superscript numbers, the numbers indicating the order of decreasing peak size: 1, idosan, altrose[1], α-mannose; 2, altrose[2]; 3, α-allose; 4, gulose[2]; 5, β-allose, gulose[1]; 6, α-glucose; 7, α-galactose; 8, β-altrofuranose; 9, α-talose, allose[3]; 10, β-mannose; 11, β-galactose; 12, β-talose; 13, β-glucose. The minor peak (no number) before β-glucose is an unidentified impurity.

Additional studies were made of other derivatives, such as acetals and thioacetals, but no derivative, easily prepared on a micro-scale, was found which gave single peaks from anomeric mixtures and which could also be separated completely from other configurational isomers. To our knowledge the special structural properties imparted by the pyranose or furanose ring account for the excellent separation of various stereoisomers, while destruction of the ring by formation of oximes, acetals, alcohols, etc., causes a deterioration of the separations observed with the parent sugars.

Little work has been reported to date on separations of the various ketoses. The results shown in Table VIII indicate that gas chromatography of TMS derivatives may be applied equally well to these compounds. The retention times of the ketoses were somewhat lower than those of the analogous aldoses. Fructose isolated from aqueous solution gave a single peak on the SE-52 column. The relative retention time (0.57) of the simplest ketose, dihydroxyacetone, was almost certainly due to the dimeric form of this compound. The small peak with a relative retention time of 0.48 might be derived from the monomeric form; it is to be noted, however, that this peak has the same retention time as glyceraldehyde (Table I) and may represent an impurity.

With the exception of the idosides (see below) the TMS derivatives of anomeric methyl glycosides showed the same behavior as those of the free sugars; in the anomeric pairs that were studied, the isomer with the anomeric OCH_3 group in the equatorial position had the longer retention time (see Table IX). Although the pairs, methyl α- and β-arabinosides, and methyl α- and β-xylosides, were not separated on SE-52, excellent resolution of these pairs was possible on EGS. Anomeric glucosides, galactosides, and idosides were separated on both columns. It was pointed out by Ferrier[21] that, for pentose sugars, differences between

TABLE VI
GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF LACTONES

Name	SE-52 140°	SE-52 140°	SE-52 210°	SE-52 210°	EGS polyester[a] 170°
Arabinolactone[b]	0.31	0.36 (s)			
Xylonolactone[b]	0.33 (m)	.39 (s)	0.58		
γ-Ribonolactone		.36			
γ-Galactonolactone		.97			
δ-Gluconolactone		.98			
Idonolactone[b]	0.92 (s)	1.05			
γ-Gulonolactone		1.08			3.03
γ-Talonolactone		1.16			3.07
L-Ascorbic acid		1.25			
Mannolactone[b]	0.93 (s)	1.47			
D-Glycero-D-gulo-γ-heptonolactone			1.52 (s)	2.00	
δ-Maltobionolactone			14.38		
Cellobionolactone[b]			14.86	19.1 (t)	
δ-Lactobionolactone			14.92		
Melibionolactone[b]			18.6	30.8 (t)	

[a] Retention times relative to α-glucose (5.35 min.). [b] These were "equilibrium" preparations obtained by treating the calcium or sodium salt with 1 *N* HCl for 40 min. at 100°.

TABLE VII
GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF ALDOSE OXIMES

Oxime	EGS polyester[a] 170°	Oxime	EGS polyester[a] 170°
Glucose	2.16[b]	Altrose	1.98
Galactose	1.98	Allose	2.08
Gulose	2.10	Mannose	2.00

[a] Retention times relative to α-glucose (6.0 min.). [b] Oximes were prepared from aqueous equilibrium solutions; only in the case of galactose was more than one peak apparent (slight shoulder at 2.07).

TABLE VIII
GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF KETOSES

Number of C atoms	Sugar	SE-52 140°	EGS polyester 150°	EGS polyester 170°[a]
3	Dihydroxyacetone	0.48 (t), 0.57[b]		
5	Ribulose	0.23 (t), 0.25, 0.33, 0.35[c]		
6	Fructose	0.69	0.56	
	Sorbose	.85	0.69	
7	Sedoheptulose (anhydride)	1.12		2.22

[a] Retention time for α-glucose, 5.35 min. [b] Dihydroxyacetone was chromatographed at 125° on SE-52 (α-glucose, 34.4 min.). [c] The peaks at 0.25 and 0.33 were approximately equal in size, that at 0.35 was somewhat less (one of these peaks may represent ribose impurity).

the retention times of anomers are significantly smaller in the methyl tri-*O*-trimethylsilylglycosides than in the case of the tetra-*O*-trimethylsilyl compounds. The separation factors for anomeric sugars and methyl glycosides summarized in Table X show that the influence of the stationary phase is critical in the determination of these differences. For example, the methyl arabinosides and methyl xylosides are not separated on the non-polar columns, SE-52 or Apiezon, but are separated on the polar EGS column. Further, although decreased separation factors of glycosides *vis-a-vis* sugars are observed on SE-52 for both galactose and glucose, the separation of methyl galactosides on EGS is as good as that of the free sugars, while the methyl glucosides are less well resolved on this column than on SE-52 (see Table X). In fact, because of overlap of α-glucose with β-galactose on the SE-52 column, and α-glucose with α-galactose on the EGS column, studies of this pair of sugars, from equilibrium solutions, are best carried out as the methyl glycosides, all of which are nearly completely resolved on SE-52.

Gas chromatography of a phenyl glucoside and of a benzylidene derivative as well was possible on SE-52 at 210°. The only previous reports on the separation of benzylidene compounds are for methyl 4,6-*O*-benzylidene-2,3-di-*O*-methyl-α-D-glucoside, 3,5-di-*O*-acetyl-2,4-*O*-benzylidene-D-xylose dimethylacetal, 1,3,-5-tri-*O*-acetyl-2,4,-*O*-benzylidenexylitol, and methyl 4,6-*O*-benzylidene-α-D-glucoside on columns containing two or more stationary phases.[7] With the benzylidene α- and β-idosides, little resolution was achieved. The α-anomer is most probably in 1C conformation, with the OCH_3 group equatorial; however, the conformation of the β-idoside is less certain, but it has been suggested that this compound has the skew conformation referred to as 1B3.[13] In this case, the anomeric OCH_3 group is also equatorial, possibly accounting for the observed similarity of the relative retention times. With the unsubstituted α- and β-idosides, the α-anomer has the longer retention time. This would be expected in view of the equatorial OCH_3 group. For the β-idoside, it has been proposed that the conformation of the free sugar is most nearly represented by a half-chair structure (HC3), with the actual conformation somewhere in the series C1 ⇄ HC3 ⇄ 1B3.[13] The influence of the relatively bulky trimethylsilyl substituents on the mobility of the conformation is unknown; it is possible that a further shift beyond 1B3, more in the direction of the 1C conformation, takes place so that the OCH_3 group becomes axial rather than equatorial as in the conformations C1, HC3, and 1B3. In any event, there is at present no information to provide a basis for discussion of the possible effect of conformations other than C1 or 1C on retention times.

In the disaccharide series, the separation of methyl β-maltoside and methyl β-cellobioside was accomplished on SE-52 at 210°. For comparison, methyl hepta-acetyl β-maltoside (with no free OH groups) was chromatographed on the same column. Marked tailing of this compound occurred as evidenced by the asymmetry of the peak. The methyl hepta-*O*-trimethylsilylmaltoside had a relative retention time of 13.1, compared to 31.85 for the heptaacetyl derivative; this demonstrates the higher volatility of TMS sugars as compared with the polyacetyl derivatives.

To be of general utility, the reagent used to increase the volatility of polyhydroxy compounds should be applicable to as great a variety of structures as possible. In this study it has been shown that, in addition to the simple aldoses and ketoses, glycosides, deoxysugars, alcohols, lactones, and oximes are equally amenable to gas chromatography as TMS derivatives. As shown in Table XI, excellent separations of a number of amino compounds were also obtained. Of particular significance to current research in biochemistry is the resolution of the N-acetyl derivatives of glucosamine, galactosamine, mannosamine, and neuraminic

TABLE IX

GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF GLYCOSIDES[a]

Sugar	SE-52 140°	SE-52 210°	EGS polyester 140°	EGS polyester 150°
Methyl β-arabinoside	0.20		0.78	
Methyl α-arabinoside	.21		1.00	
Methyl α-xyloside	.31		1.29	
Methyl β-xyloside	.34		1.44	
Methyl α-mannoside	.59			0.54
Methyl β-mannoside	.67			.70
Methyl β-idoside	.64			.73
Methyl α-idoside	.75			.89
Methyl α-galactoside	.72			.80
Methyl β-galactoside	.82			1.08
Methyl α-glucoside	.92			0.94
Methyl β-glucoside	.49 (t), 1.07			1.01
Methyl 3-methyl-4,6-benzal-β-idoside		1.93		
Methyl 2,3-dimethyl-4,6-benzal-β-idoside[b]		2.15		
Methyl 4,6-benzylidene-α-galactoside		2.77		
Methyl 4,6-benzylidene-β-galactoside		3.00		
Methyl 4,6-benzylidene-α-idoside		2.96		
Methyl 4,6-benzylidene-β-idoside		2.52 (s), 3.11		
Methyl 4,6-benzylidene-α-glucoside	4.67	2.54 (t), 3.15		
Phenyl β-glucoside	7.16	2.54 (s), 3.23, 3.62 (m)		
Methyl β-maltoside		11.6		
Methyl β-cellobioside		13.1		
Methyl heptaacetyl β-maltoside[b,c]		31.9		

[a] All of the glycosides reported here are pyranosides. [b] These compounds do not contain free OH groups, but are included for comparison; they were not subjected to trimethylsilylation but were simply dissolved in pyridine. [c] Marked tailing was observed with this compound.

TABLE X

COMPARISON OF SEPARATION FACTORS FOR ANOMERIC SUGARS AND METHYL GLYCOSIDES

Compound	Apiezon M Sugar e/a	Apiezon M Glycoside e/a	SE-52 Sugar e/a	SE-52 Glycoside e/a	EGS polyester Sugar e/a	EGS polyester Glycoside e/a
Arabinose	1.20	1.05	1.18	1.05	1.35	1.28
Lyxose	1.36	1.14	1.26		1.34	
Ribose	1.08	1.10	1.06		1.09	
Xylose	1.37	1.12	1.25	1.09	1.29	1.12
Galactose			1.24	1.14	1.33	1.35
Glucose			1.55	1.16	1.94	1.07
Mannose			1.56	1.14	2.11	1.29

The e/a ratios refer to the separation factor for the compound with the equatorial (e) OH or OCH_3 group, compared to that for the compound with the axial (a) OH or OCH_3 group. The separation factor is the ratio of the observed retention times. The results for Apiezon M are calculated from the data of Ferrier.[20]

TABLE XI

GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF AMINO SUGARS

Compound	SE-52 140°	SE-52 160°[a]	EGS polyester 170°[b]
Galactosamine	0.79		
Glucosamine	0.91	1.15	
N-Acetylmannosamine		1.71	5.14
N-Acetylgalactosamine	2.34	2.08	8.45
N-Acetylglucosamine	2.62	2.27	8.39
N-Acetylneuraminic acid[c]		7.25	

[a] Retention time for α-glucose, 6.2 min. [b] Retention time for α-glucose, 5.35 min. [c] This substance was first converted to a methyl ester with methyl alcohol, 12 *N* HCl, and dimethoxypropane (1:0.5:0.1) at room temperature for 30 min.; the solution was evaporated and the residue was converted to the trimethylsilyl derivative in the usual way.

acid. The free carboxyl group in neuraminic acid and related substances is converted to methyl ester prior to trimethylsilylation. All of the various carbohydrates and related substances which may occur as components of glycolipids, such as glucose, galactose, inositol, hexuronic acids, hexosamines, and sialic acid, are subject to this method of analysis.

In some cases it may be advantageous to make use of a number of liquid phases of differing properties. Although most of these studies were made with SE-52 and EGS, preliminary results indicated that other liquid phases could be used for separation of TMS derivatives of polyhydroxy compounds (Table XII). Columns containing a nitrile silicone (XE-60) gave the widest separation of the anomers of glucose that we have yet observed (relative retention time for β-glucose, 1.99). A Carbowax 1540 column was useful for separations of a variety of hexoses, deoxyhexoses, ketoses, and pentitols.

TABLE XII

OTHER LIQUID PHASES FOR GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES

Compound	Carbowax 1540[a] 140°	Nitrile silicone (XE-60)[a] 128°	Nitrile silicone (XE-60)[a] 205°
α-Glucose	1.00	1.00	1.00
β-Glucose	1.73	1.99	1.38
α-Galactose		0.91	
Allose	0.79		
Rhamnose	.33		
Fucose	.42		
Fructose	.60		
Sorbose	.82		
Arabitol	.36		0.46
Xylitol	.36		.44
Mannitol			.84
Allitol			.86
Altritol			.93

[a] Retention time for α-glucose on Carbowax at 140°, 25.9 min.; on XE-60 at 128°, 18.2 min.; on XE-60 at 205°, 9.2 min.

The Effect of Structure on Retention Time.—It has been proposed that "for any methyl glycopyranoside, the anomer in which the C_1 methoxy group exists in *trans* relationship with the C_2 substituent will have the lower retention volume except when the C_2 OH is unsubstituted, in which case the order is reversed."[4] Inspection of the results given in Table I shows that

this generalization cannot be extended to the tetratrimethylsilyl ethers of the pentoses, nor to the pentatrimethylsilyl ethers of the hexoses. For example, in glucose and galactose, the *trans* relationship is found in the *slower* moving β-anomers under our conditions. Furthermore, the generalization does not hold true with the TMS derivatives of the methyl glycosides (see Table IX). Our studies, however, reveal another possible generalization for the TMS derivatives. "When the normal conformation of the sugar is one of the chair forms, C1 or 1C, and when there is no evidence of conformational instability, that anomer in which the anomeric OH group is equatorial has the longest retention time." For the following D-sugars of C1 conformation, the β-anomer moves more slowly on polar and non-polar columns: glucose, galactose, mannose, talose, xylose, and lyxose. Similarly, for D-arabinose of 1C conformation, the α-anomer with the equatorial OH group has the longer retention time. In the case of gulose, the β-anomer has a lower retention time than the α; a possible explanation for this result is that while β-gulose is almost certainly in C1 conformation, α-gulose may well be 1C. In this latter conformation, the two TMS groups on C_2 and C_5 are axial; a $\Delta 2$ instability condition is also present. On the other hand, for α-gulose in C1 conformation, there are three axial groups at C_1, C_3, and C_4; it is therefore possible that the 1C conformation with the $\Delta 2$ condition is energetically more favorable than the C1 conformation with the three bulky groups in axial positions.

With the remaining aldoses, allose, altrose, and ribose, a number of difficulties arise in considering the possible relationships between structure and retention time. The problem stems mostly from the fact that structural assignments for the various forms of these sugars are not well understood. The solid allose available to us was believed to be the β-pyranose form.[26] The equilibrium solution was found on the SE-52 column to contain three components, while about 6% of a fourth was clearly revealed on EGS. The relative retention time of the major peak (54.6%) was 0.91 on EGS and corresponded to the single peak given by the solid sugar. Isbell[27] has recently suggested that the major product to be expected in the equilibrium solution of allose would be the β-pyranose form. This suggestion would, in general, agree with our observation. On the basis of the rates of bromine oxidation of the solid allose, and its equilibrium solution, Isbell has, however, concluded that only a small proportion, possibly 10%, of a modification oxidized *more* rapidly than the rest of the allose is present in the equilibrium solution. On the gas chromatogram there are two additional peaks with retention times lower and higher than that of β-allose. The three other peaks observed on the EGS column had relative retention times of 0.78, 0.84, and 1.20 and amounted, respectively, to 23.9, 5.9, and 15.6% of the total. One peak may be that corresponding to the free aldehyde form of allose, since it was reported by Cantor and Peniston[28] that about 1.38 mole % of material, reducible at the dropping mercury electrode, is present in equilibrium allose solution; this is considerably more than occurs with other common hexoses. If it is assumed that the smallest peak, with relative retention time of 0.84, is due to the aldehyde form, the first peak (23.9%) might then be due to α-pyranose. Furthermore, the slower moving compound with relative retention time of 1.20 may be the furanose form; this possibility is suggested by the results obtained with altrose, discussed subsequently.

Another problem with allose arises from a fact that Ferrier[21] has discussed previously; for equatorial anomeric ethers of lyxose, ribose, and arabinose, there is little difference in relative retention times on Apiezon columns. This generalization is also true for both polar and non-polar columns used in this study. These sugars all have a single axial OH group, at C_2, C_3, and C_4, respectively. Similarly with the hexose sugars, nearly identical retention times are observed with β-mannose and β-galactose, with one axial OH group at C_2 and C_4, respectively (relative retention times are 1.06 and 1.08 on SE-52, and 1.31 and 1.38 on EGS). The hexose with the single axial substituent on C_3 is allose, and the results for the β-anomer of this sugar are markedly out of line, 0.81 on SE-52 and 0.91 on EGS. A better understanding of all these factors would be possible if there were more information about the precise conformation of allose. Barker and Shaw[29] have suggested that α-allose may have the 1C conformation; if this is so, it may offer a rationalization for the fact that the equilibrium solution, with the major component the equatorial anomer, contains a substance oxidized more rapidly than the major component. If this component is α-allose, in 1C conformation, the anomeric OH group of this compound is also equatorial; the generalization that the equatorial β-forms are oxidized more rapidly than the axial α-forms would not then hold.

With altrose, the solid available to us was apparently the β-altrofuranose form, which yielded a single peak on gas chromatography. This peak was a *minor* component of the altrose equilibrium mixture. The major components were not as well separated as the more common α,β pairs; the situation is similar in this respect to that with allose. The fact that the furanose peak of altrose has a longer retention time than the others may indicate that the smallest peak in allose is furanose, as was previously indicated as one possibility. In studies of the rates of bromine oxidation of freshly dissolved solid altrose and altrose equilibrium solutions, Isbell[27] has reported almost identical results in both cases. We had also observed this phenomenon independently.[30] Isbell has therefore concluded that there is no evidence "for a substantial proportion of a second modification in the equilibrium mixture (of altrose)." This conclusion is not supported by the results of gas chromatography. It should be noted that, both for allose and altrose, the residue obtained on drying the equilibrium solution was directly treated with a solution of hexamethyldisilazane in pyridine, to which was then added trimethylchlorosilane. This procedure was used, rather than adding pyridine followed by the silanes, to minimize any possible changes in the equilibrium composition due to the action of pyridine. Further, no heating was used in these preparations.

Difficulties are also encountered in the assignment of anomeric classification for ribose. With ribose recrystallized from ethyl alcohol, we have observed two peaks in the TMS derivative prepared from the solid (contact with pyridine prior to reaction with silane was kept at a minimum). In equilibrium ribose solutions three forms are present, two of which corresponded to those obtained with the solid.

If the major peak observed in the equilibrium mixture is assigned to α-ribopyranose on the grounds that the equilibrium solution is known to contain 89% of the

(26) Personal communication from Dr. N. K. Richtmyer.

(27) H. S. Isbell, *J. Res. Natl. Bur. Std.*, **66A**, 233 (1962).

(28) S. M. Cantor and Q. P. Peniston, *J. Am. Chem. Soc.*, **62**, 2113 (1940).

(29) G. R. Barker and D. F. Shaw, *J. Chem. Soc.*, 584 (1959).

(30) R. Bentley and C. P. Thiessen, unpublished observations.

least reactive compound to bromine (which is likely to be α[13]), then with both polar and non-polar columns there is a definite peak, possibly the β-anomer, with a slightly greater retention time. With Apiezon L columns, Ferrier[21] assigned the middle of three peaks to β-ribose, having a longer retention time than the peak identified as α-ribose. However, as previously discussed, Ferrier's equilibrium mixtures were changed from the normal aqueous pattern owing to the preparative method used for the TMS derivatives; consequently, his "β" peak is considerably larger than the "α" peak. According to Cantor and Peniston,[28] 0.1 M ribose solution at pH 7 and 25° contains 8.5 mole % of reducible sugar (free aldehyde). Again, it is impossible to be certain of the anomeric and conformational assignments with the present information. Although solid ribose is usually regarded as the β-pyranose, Barker and Shaw[29] have cautioned against uncritical acceptance of this assignment. These workers also believe that β-ribose may favor the 1C rather than the C1 conformation.[31]

(31) NOTE ADDED IN PROOF.—After this manuscript was completed, the paper by Gee and Walker (*Anal. Chem.*, **34**, 650 (1962)) came to our attention, in which the chromatography of methylated trisaccharides is described.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE PENNSYLVANIA STATE UNIVERSITY, UNIVERSITY PARK, PENNA.]

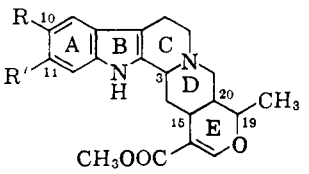
The Stereochemistry of the Heteroyohimbine Alkaloids[1]

BY MAURICE SHAMMA AND JANE MOSS RICHEY[2]

RECEIVED MARCH 15, 1963

The stereochemistry of the heteroyohimbine alkaloids has been elucidated. (1) The compounds can be assigned to their stereochemical groups by measurement of the chemical shifts of the C-methyl doublets of the free bases. An alternate procedure is by the study of the shapes of the infrared bands near 8.4 μ of the free bases. (2) The stereochemistry of the hydrogen at C-3 is defined by the nature of the peaks near 3.4 μ in the infrared. If the absorption is complex with at least two bands above 3.4 μ, the hydrogen is axial; if the absorption is simple, the hydrogen is equatorial. (3) The kinetics for the formation of the methiodide salts reflect the degree of steric hindrance at N_b. The allo-C-19α-methyl series methylates most slowly of any group. The allo-C-19β-methyl series, which undergoes a conformational change, methylates somewhat more slowly than the remaining groups. The pK_a values for the heteroyohimbine alkaloids generally show the same trend as the rates, although the differences are of a smaller magnitude. (4) The conformations of the methiodide salts can be determined by analysis of the chemical shifts of the $^{\oplus}$N-methyl groups. A *trans* C/D N-methylquinolizidinium system gives rise to a peak at about 3.3 p.p.m., whereas a *cis* C/D system shows a peak near 3.5 p.p.m. The raunitidine to isoraunitidine isomerization is thus shown to be an allo to epiallo transformation. (5) With the possession of members of the four possible groups in the allo and epiallo series it was possible by conformational analysis to assign the stereochemistry of the C-19 methyl groups. The assignment was also supported by a comparison of the C-19 methyl chemical shifts in the free bases and the corresponding methiodide salts and by the rates of methylation data. (6) The stereochemistry at C-19 in the normal series was determined by the study of the chemical shifts of the C-19 methyl groups.

The heteroyohimbine alkaloids share in common the skeleton I where R and R′ can be either hydrogen or methoxyl.[3] The first heteroyohimbine to be isolated, ajmalicine (R, R′ = H in I), was obtained in the course of the initial chemical investigation of *Rauwolfia*



I

serpentina by Siddiqui and Siddiqui.[4] In 1951, Goutarel and Le Hir proposed what has now come to be accepted as the correct structure for ajmalicine without dealing with the stereochemistry of the molecule.[5] The conclusions of the French school regarding the presence of the conjugated enol ether function were further confirmed by Bader, who compared the ultraviolet spectrum of ajmalicine with that of conjugated and non-conjugated enol ethers, and who also noted that the difficulty in hydrogenating the double bond of amjalicine is a characteristic property of enol ethers in general.[6]

With the isolation of reserpine and the subsequent rush toward the study of related indole alkaloids, a number of new heteroyohimbines were obtained so that to date sixteen members of this series have been recognized and are listed in Table I. These sixteen bases differ from each other either in stereochemistry or in the nature of the ring A substituent(s).

TABLE I

KNOWN HETEROYOHIMBINE BASES

Non-methoxylated:	Tetrahydroalstonine, akuammigine, raunitidine, mayumbine, ajmalicine, and isoajmalicine
10-Methoxylated:	Aricine and raumitorine
11-Methoxylated:	Reserpinine, isoreserpinine, tetraphylline, raunitidine, and isoraunitidine
10,11-Dimethoxylated:	Reserpiline, isoreserpiline, and rauvanine[7]

Possible Configurations and Conformations.—Before delving into the data which depict various details of the stereochemistry of the heteroyohimbine alkaloids, it is necessary to consider the configurations and conformations of the possible heteroyohimbine stereoisomers. If one considers only those isomers with an α-hydrogen at C-15, there are 2^3 or eight possible stereoisomers. There are in fact four basic stereochemical arrangements or configurations, namely, allo, epiallo, normal, and pseudo, and each of these may have the C-19 methyl group α or β, thus making for a total of eight stereoisomers.

The picture is further complicated, however, by the fact that a number of different conformations are possible for each of the four basic configurations. Thus if one considers only the conformations with the piperidine ring D in the more stable chair form, some of the more

(1) A preliminary account of this work was given in *J. Am. Chem. Soc.*, **83**, 5038 (1961), and **84**, 1739 (1962).

(2) Recipient of pre-doctoral fellowship No. GF-17,292 from the Division of General Medical Sciences of the National Institutes of Health.

(3) For a review of the heteroyohimbine alkaloids see J. E. Saxton in "Alkaloids," Vol. VII, R. H. F. Manske, Ed., Academic Press, Inc., New York, N. Y., 1960, pp. 59–62.

(4) S. Siddiqui and R. H. Siddiqui, *J. Indian Chem. Soc.*, **8**, 667 (1931).

(5) R. Goutarel and A. Le Hir, *Bull. soc. chim. France*, **18**, 909 (1951).

(6) F. E. Bader, *Helv. Chim. Acta*, **36**, 215 (1953).

(7) R. Goutarel, M. Gut, and J. Parello, *Compt. rend.*, **253**, 2589 (1961)