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IDENTIFICATION OF ALDOSES BY USE OF THEIR PERACETYLATED ALDONONITRILE DERIVATIVES: A G.L.C.–M.S. APPROACH*

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ABSTRACT

The g.l.c. retention-times and detector responses have been examined for peracetylated aldononitrile derivatives from aldoses. Correlations have been made between changes in g.l.c. retention-times and changes in the stereochemistry and functional groups of the parent aldose. The mass spectra [electron impact (e.i.), ammonia chemical ionization (c.i.), and methane c.i.] for these g.l.c. peaks were recorded. C.i.-mass spectrometry (m.s.) indicated the molecular weights of the derivatives, and the number of aldehyde and alcohol groups in the parent aldose. E.i.-m.s. indicated the nature and position of functional groups present in the parent aldose. Aldoses containing acetamido, amino, deoxy, and thio substituents were studied.

INTRODUCTION

The use of gas–liquid chromatography (g.l.c.) for the identification and quantitation of glycoses *via* various derivatives has been extensively employed during the past decade[1,2]. Mass spectrometry (m.s.) as a means of identification of glycoses and for the confirmation of the identity of g.l.c. peaks has also undergone rapid development[3]. At present, most of the m.s. studies on glycoses employ electron-impact mass-spectrometry (e.i.-m.s.), which essentially uses a stream of electrons to ionize and fragment the vaporized saccharide under low pressure. More recently, an alternative form of mass spectrometry, using chemical ionization (c.i.), is being used to

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examine saccharides[4,5]. This form of mass spectrometry employs in the ionization chamber somewhat higher pressures than e.i.-m.s., due to introduction of a small, constant pressure of an additional species of ionizing gas into the system. In effect, the additional gas is ionized by the electron stream, and the charge is then transferred to the vaporized glycose. For the glycoses, e.i.- and c.i.-m.s. yield radically different spectra which are complementary in establishing molecular structure.

Underivatized glycoses are not suitable compounds for g.l.c. analysis, owing to their relatively low volatility and their α,β isomerism. This isomerism results in each glycose's yielding an α and a β peak, complicating the analysis of the chromatogram. In addition, the e.i.-m.s. of these compounds is complicated, as furanose and pyranose forms are favored, and, with regard to e.i.-m.s. cleavage, the hemiacetal bond is effectively as strong as the carbon–carbon bonds. Therefore, for a pyranoid ring, cleavage is likely at any point in the ring, with rearrangements occurring[6].

Peracetylation or permethylation of the glycose affords compounds having improved volatility, but the g.l.c. problems associated with α,β isomerism and the complex, fragmentation problem in e.i.-m.s. remain. Considerable success has been achieved by the reduction of aldehyde groups to the alcohols, and subsequent peracetylation to form the peracetylated alditols[7]. The peracetylated alditols can be separated by g.l.c., and these acyclic compounds give e.i. mass spectra much more readily interpretable than those of the parent. However, certain problems persist, even with the peracetylated alditols. The conversion of the aldehyde group into an alcohol makes identification of C-1 difficult. If non-isotopic reduction of the aldehyde group is employed for aldopentoses (arabinose, lyxose, ribose, and xylose), only three pentitols result, as lyxitol and arabinitol are tautomers and therefore have the same g.l.c. retention-times (no distinction will be made in this paper between the enantiomers, except to indicate which compounds were experimentally employed). In like manner, the hexoses yield only five hexitols, as g.l.c. conditions employing an optically inactive, stationary phase cannot resolve the D and L enantiomers.

The peracetylated alditols yield much more interpretable e.i. mass spectra than the corresponding pyranoside and furanoside compounds, because a single, carbon–carbon bond cleavage affords two possible charged fragments (from either end of the molecule), and unrearranged fragments of this type predominate in these spectra. The loss of g.l.c. information is paralleled, although to a lesser extent, when g.l.c.–m.s. is employed. When deuterated reductants are used to reduce the aldehyde group, the resulting, deuterated primary alcohol position serves to identify C-1 in relation to other functional groups present in the saccharide (although it does not affect the g.l.c. retention-time). However, the linear compound resulting, having an acetylated primary alcohol group (perdeuterated and normal) at both ends of the molecule, yields analogous fragmentation-patterns from both ends, and this presents problems of interpretation with complicated spectra.

An alternative method for eliminating the aldehyde group is to convert it into a nitrile group. This procedure has the advantage of eliminating the center of asym-

metry at C-1, and it also readily identifies the position of the aldehyde group in the original glycose. The conversion of an aldehyde group into a nitrile is a reaction that has often been employed in synthetic, saccharide chemistry; a common procedure employs acetic anhydride, and has the advantage of acetylating amino and hydroxyl groups present in the sugar[8]. The peracetylated aldononitrile (PAAN) derivatives resulting have properties similar to those of the peracetylated alditols, and the separation of these PAAN compounds by g.l.c. has been reported for PAAN derivatives of glycoses. Three stationary phases for g.l.c. have previously been employed for the separation of non-methylated PAAN derivatives; neopentyl glycol succinate[9], SE-30 (used with open, tubular, glass-capillary columns[10]), and LAC-4R-886 polyester wax[11]. These compounds (and g.l.c. conditions) have been used for examining products resulting from Smith degradations[12] and in polysaccharide hydrolyzates[13], and for surveying saccharides present in urine[14]. The g.l.c. retention-times for the PAAN derivatives of methyl ethers of xylose[15] have been established. The e.i. mass spectra of some PAAN derivatives of non-methylated saccharides[16] and PAAN derivatives[9] of methyl ethers of glucose have also been recorded. In addition, the g.l.c.–e.i.-m.s. data for PAAN derivatives of methyl ethers of mannose[17] and glucose[18] have been reported.

From published data, and from our studies on glycan hydrolyzates, it has become evident that the PAAN derivatives have certain, distinct advantages compared to other, more commonly employed methods for saccharide survey. The PAAN compounds, compared to peracetylated alditols, provide easier separation by g.l.c., a phenomenon that we ascribe to the greater molecular asymmetry provided by the nitrile group. If α,β isomerism is disregarded, each aldose yields a distinct PAAN derivative which can, in principle, be separated from others by g.l.c. The PAAN derivatives provide readily interpretable e.i. mass spectra, due to two effects. Firstly, for a saccharide containing only C, H, and O, the e.i.-m.s. of the resulting PAAN derivative will contain even *m/e* fragments arising from the nitrile end, and odd *m/e* fragments arising from the non-nitrile end of the molecule. Secondly, the nitrile at C-1 and the adjacent C-2 resist mass-spectral cleavage, allowing larger, and more identifiable, fragments to be observed from the nitrile end of the molecule. Furthermore, the ease of isotopic substitution, through the use of hydroxyl[^{15}N]amine or perdeuterated acetic anhydride, allows convenient identification of the origin of the *m/e* fragment, without the necessity of employing high-resolution, mass spectrometry.

This report is primarily concerned with the use of g.l.c.–m.s. data to identify the saccharide components arising in hydrolyzates. Although m.s. provides precise and extensive structural data, no information can be obtained about the stereochemistry of the molecule. The g.l.c. retention-times can provide certain stereochemical information, if only to differentiate between enantiomers, but this is dependent on the resolution of the columns employed. Therefore, the g.l.c. retention-times of a large number of aldo-pentoses and -hexoses have been determined, in order to establish the g.l.c. retention-times of these compounds and the extent of g.l.c. resolution, and to determine which of the derivatives display mutual interference.

RESULTS AND DISCUSSION

Of specific interest is the employment of a uniform procedure of derivatization applicable to the identification of sugars in glycan or glycopeptide hydrolyzates. We consider that major criteria in selecting survey methods for sugars should include (*a*) the ability of the method to identify a wide variety of possible structures, (*b*) the rapidity and ease of the determination, (*c*) the ease of quantitation of the identified sugars, and (*d*) freedom from interference by non-carbohydrate material. In addition, the capacity of the system to detect, and identify accurately, previously unreported sugars would be a distinct advantage. Data will be presented to show that the PAAN derivatization method can excel as regards each of the foregoing criteria.

The following discussion of PAAN derivatization will be based on an operational procedure that is briefly outlined here, and carefully described in the Experimental section. The polymer hydrolyzate is passed through a small column of an ion-exchange resin, the effluent is evaporated to an oil, this is dissolved in pyridine containing hydroxylamine, the mixture is heated, acetic anhydride is added (with further heating), the mixture is cooled, and partitioned between water and chloroform, and an aliquot of the chloroform layer is injected into a specific, g.l.c. column (*e.g.*, neopentyl glycol succinate) programmed across a specific range of temperature. The compounds emerging may be monitored in any one of four ways: by use of (*a*) a hydrogen-flame detector, (*b*) e.i.-m.s., (*c*) ammonia c.i.-m.s., and (*d*) methane c.i.-m.s. The procedure is then repeated, but using $^{15}NH_2OH$ instead of non-isotopic NH_2OH; it is next repeated, but using acetic anhydride-d_6 instead of non-isotopic acetic anhydride. A combination of relative retention-times in g.l.c. and data from the detectors yields, for each sugar present (with extensive cross-checking of the data), specific information regarding the molecular weight, the number of aldehyde groups, the number of alcohol groups, the presence and position of deoxy and *N*-acetyl groups, and the stereochemistry of the sugar.

G.l.c on columns of neopentyl glycol succinate (NPGS). — Two different stationary phases, NPGS and OV-17, were employed. For convenience, the data from the two stationary phases will be discussed separately. The general observations made in the discussion of NPGS may be considered to be true of the OV-17 conditions also, except for the specific differences discussed in the section on OV-17 columns.

From a number of experiments, both with prepared mixtures and polymer hydrolyzates, we have made several observations concerning the PAAN derivatization procedure; these conclusions may be considered to indicate strengths or weaknesses of the method, depending upon the objective. (*a*) We found in the chromatograms no evidence for derivatives arising from amino acids, ketoses, or sugar phosphates. The derivatives of amino acids are, apparently, rapidly hydrolyzed during the partitioning between chloroform and water, to afford polar, non-chromatographable materials. The ketoses are known to form oximes with hydroxylamine, but the lack of three available carbon valences precludes nitrile formation; the peracetylated oxime, a compound too polar to volatilize under the chromatographic conditions

used, is formed. Similarly, the polarity of the underivatized phosphate group precludes chromatography of sugar phosphates. (*b*) If large proportions of inorganic sulfate or phosphate ions are present, the derivatization process is inhibited. To avoid this inhibition in polymer hydrolyzates, an ion-exchange resin is employed before the derivatization. If large proportions of such ions are known to be absent from the hydrolyzate, use of an ion-exchange resin is unnecessary. (*c*) Large proportions of hydroxylamine hydrochloride inhibit the derivatization of deoxy sugars. Digitoxose (2,6-dideoxy-D-*arabino*-hexose) and 2-deoxy-D-*erythro*-pentose are especially susceptible, but 2-deoxy-D-*arabino*-hexose and 2-deoxy-D-*lyxo*-hexose are affected to a lesser extent. Such problems can be avoided by careful control of the proportion of hydroxylamine hydrochloride added, so that it lies in the range of 60 to 90% (by weight) of the polymer being analyzed. The sugars not containing deoxy groups appear to be totally unaffected by a large excess of hydroxylamine hydrochloride.

With regard to polymers, this derivatization procedure assumes that the hydrolysis had been satisfactory, and this depends on the nature of the polymer under consideration. We have found that this derivatization procedure is completely compatible with formolysis and methanolysis on simple evaporation of the corresponding reagents. A most effective, mild hydrolyst consists[19] of acetic acid containing a trace of sulfuric acid, and this reagent is effectively removed subsequently by treatment with an ion-exchange resin to sequester the sulfate ions, followed by evaporation of the acetic acid.

In actual practice, it is convenient to monitor the hydrolyzate derivatives by g.l.c. employing a hydrogen-flame detector (H-f.d.). The derivatives are then examined by various forms of mass spectrometry. The procedure will, therefore, be first discussed from the standpoint of hydrogen-flame detector g.l.c.; this recognizes that many laboratories are equipped with inexpensive g.l.c. systems and have to have the g.l.c.–m.s. determinations performed at remote locations. C.i.-m.s. will be considered before e.i.-m.s., as knowledge of the molecular weight of the compounds, obtained through the former, is a logical starting-point for identifying an unknown sugar derivative.

The compounds surveyed, and their retention-times, are given in Table I. With the exception of two pairs of PAAN derivatives (D-ribose and L-fucose; D-mannose and D-talose), separation (although not base-line separation) was achieved for all compounds present. The g.l.c. profile of a typical chromatogram of standards is shown in Fig. 1. The sugars studied by employing a column packed with neopentyl glycol succinate are similar to those studied for the SE-30 capillary column[10] and the LAC-4R-886-packed column[11]. For those compounds studied, the order of emergence for the various PAAN derivatives is essentially the same for all columns. It was to be expected that the general order for the two columns (small molecular weight first) would be similar; however, more-subtle factors, such as the order of the pentose isomers, are also faithfully reflected in the chromatograms. A consideration of the similarities of the order of emergence and degree of separation of the PAAN derivatives on such different columns suggests that the primary reasons for the

TABLE I

RELATIVE G.L.C. RETENTION-TIMES, ON A COLUMN OF NEOPENTYL GLYCOL SUCCINATE AND OF OV-17, OF PERACETYLATED ALDONONITRILES FROM ALDOSES

Parent aldose	*Relative retention-time*		
	Condition 1[a]	*Condition 2*[b]	*Condition 3*[c]
DL-Glyceraldehyde[d]	0.21 (1.38)[e]	0 25	0.34
D-Erythrose[d]	1.00[f]	1.00[g]	1.00[h]
D-Digitoxose	1.71 (0.97)	1.65	1.42
L-Rhamnose	1 89 (0.93)	2 15	1.67
2-Deoxy-D-*erythro*-pentose	2.18 (0 86)	1.80	1.49
D-Ribose[d]	2 29 (0.95)	2.25	1.72
L-Idosan[h]	—	2 30	1.76
L-Fucose	2 29	2.35	1.78
D-Lyxose[d]	2 43	2.35	1.78
D-Arabinose[d]	2.54	2 35	1.78
D-Xylose[d]	2.79 (0 96)	2 50	1.84
Levoglucosan[i]	—	2 55	1 94
D-Allose[d]	3.39	3.40	2.30
2-Deoxy-D-*arabino*-hexose	3 42 (0.92)	3.15	2.17
D-Mannose[d]	3.50	3 55	2.36
D-Talose[d]	3.50	3.55	2 36
2-Deoxy-D-*lyxo*-hexose	3 61 (0 94)	3.15	2.17
D-Glucose[d]	3.75 (1.00)	3.65 (1 00)	2.42
D-Galactose[d]	3.89 (1.00)	3.75	2 45
L-Idose[d]	4 03	3.90	2 59
5-Thio-D-glucose[i]	4 39 (0 99)	4.60	2 90
D-*glycero*-D-*gluco*-Heptose	4.79 (1.00)	4.75	2 95
2-Acetamido-2-deoxy-D-glucose	6.11 (0 03)	4 45 (0.72)	2 82
2-Acetamido-2-deoxy-D-galactose	—	4.90 (0.70)	3 01

[a]Condition 1: glass column (1.23 m × 2 mm) with 3% of neopentyl glycol succinate on Chromosorb W (60–80 mesh), nitrogen flow of 32 mL/min, and programmed at 140 to 250° at 3°/min. Injector and detector set at 280°. [b]Condition 2: glass column (1 23 m × 2 mm) with 2% of OV-17 on Chromosorb W HP (80–100 mesh), nitrogen flow of 22 mL/min, and programmed at 130 to 300° at 5°/min. Injector and detector set at 330° [c]Condition 3: same as condition 2, except for programming at 20°/min. [d]Compounds listed in Table II [e]Detector response based on the ratio of the H-f.d. peak area to the weight of parent saccharide (weighed before derivatization). [f]1 00 equals 5.58 min. [g]1.00 equals 4.15 min [h]1 00 equals 2.96 min. [i]Peracetylated derivative (not the PAAN derivative).

separation of compounds are inherent in a very general feature of the structure of the sugar. The g.l.c. retention-time may be considered as a function of the linearity of a compound, and this linearity as being dependent on the number of *cis*-hydroxyl groups in the parent sugar. The dominating factor relating sugar structures to g.l.c. retention-times is the number of hydroxyl groups (or acetoxyl groups for the PAAN derivatives), and, in Table II, the sugars are arranged according to increasing acetyl number.

Each set of sugars having an equal number of acetyl groups is arranged in order of decreasing pairs of *cis*-acetoxyl groups. The order of the unsubstituted sugars in Table I (indicated by letter[d]) is the same as the order of the sugars in

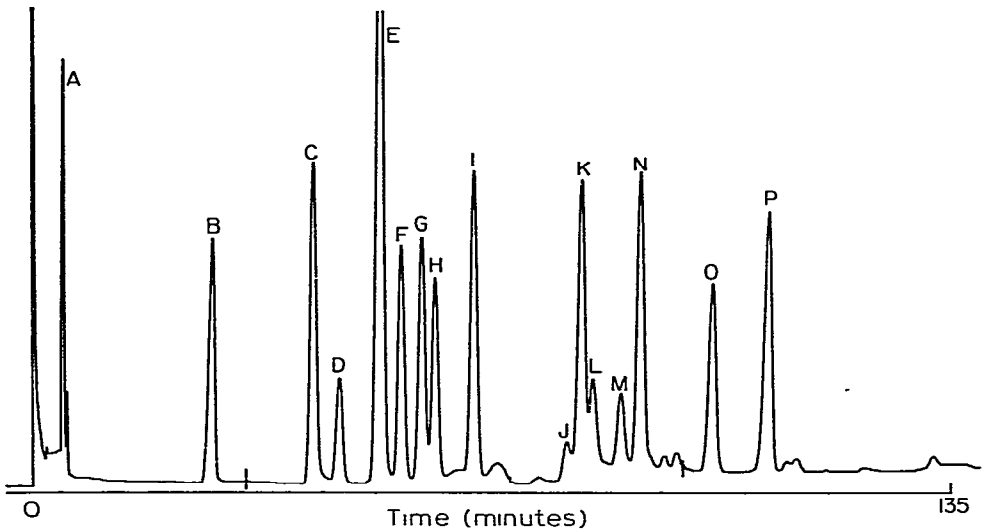


Fig. 1. Gas–liquid chromatograms (hydrogen-flame detector) using neopentyl glycol succinate on Chromosorb W (2 mm × 3 m) at 140°; held for 6 min, then programmed at 1°/min to 250°. [The PAAN derivatives of . A, DL-glyceraldehyde; B, D-erythrose; C, digitoxose; D, L-rhamnose; E, 2-deoxy-D-*erythro*-pentose; F, D-ribose; G, D-lyxose; H, D-arabinose; I, D-allose; J, D-mannose; K, 2-deoxy-D-*lyxo*-hexose; L, D-glucose; M, D-galactose; N, 5-thio-D-glucose (peracetylated); and O, D-*glycero*-D-*gluco*-heptose.]

TABLE II

THE RELATIONSHIP OF ALDOSES IN TERMS OF NUMBER OF HYDROXYL GROUPS AND NUMBER OF *cis*-HYDROXYL PAIRS

Aldose	*Number of hydroxyl groups*	*Number of* cis-*hydroxyl pairs*
Glyceraldehyde	2	0
Erythrose	3	1
Threose	3	0
Ribose	4	2
Lyxose	4	1
Arabinose	4	1
Xylose	4	0
Allose	5	3
Altrose[a]	5	2
Mannose	5	2
Talose	5	2
Glucose	5	1
Gulose[a]	5	1
Galactose	5	1
Idose	5	0

[a]No g l c. retention-time available.

Table II. Table I lists experimental values, and Table II is theoretically constructed on the basis of the number of acetoxyl groups present in a molecule, and their extent of *cis* arrangement. Latitude was permissible in Table II, as the order of the lyxose and arabinose pairs is not defined, as with the altrose–mannose–talose set and the glucose–gulose–galactose set. The PAAN derivatives of mannose and talose are not separated by the g.l.c program. The data from the SE-30 capillary column[10] included the D-threose PAAN derivative (not available for this study), and threose emerged *after* erythrose, as predicted by the results given in Table II. Of the PAAN derivatives of the 15 aldoses comprising aldotriose through the aldohexoses, only altrose and gulose have not yet been studied. Is the coincidence of the data in Tables I and II fortuitous, or does it imply a general principle? A further examination of Table I may indicate the answer.

In Table I, 2-deoxy-D-*arabino*-hexose emerges 1.8 min before the D-glucose PAAN derivative, and 2-deoxy-D-*lyxo*-hexose emerges 1.6 min before the D-galactose PAAN derivative. Similarly, the 6-deoxy-L-mannose (L-rhamnose) PAAN derivative emerges 9.0 min before the D-mannose PAAN derivative, and the 6-deoxy-L-galactose (L-fucose) PAAN derivative likewise emerges 9.0 min before the galactose PAAN derivative. These data, combined with previous observations, support the concept that the PAAN derivatives are retained on the column by interaction of the moderately polar acetyl groups with the stationary phase. The greater the number of acetyl groups, the stronger the interaction, and the longer the retention-times. Likewise, the more readily is the acetate group exposed to the stationary phase, the greater is the interaction. The *cis*-acetate groups, which favor twisting of the carbon (backbone) chain, promote assumption of a less linear molecular shape in which the acetate groups are less exposed to the environment and consequently have shorter retention-times. With PAAN derivatives, functional groups can either be (*a*) more polar than the acetate group, and thus lengthen the retention time (*e.g.*, replacement of an *O*-acetyl by an *N*-acetyl group), or (*b*) less polar, and so shorten the retention time (*e.g.*, replacement of an acetoxyl by a deoxy group). Introduction of a terminal deoxy group (a 6-deoxy group in an aldohexose) decreases the retention time much more than the introduction of a nonterminal deoxy group (*e.g.*, a 2-deoxy group). This is interpreted to mean that the four adjacent acetyl groups of the 6-deoxyhexoses interact analogously to the acetyl groups in pentoses, with the nonpolar 5-methyl group having little effect. For a nonterminal deoxy group in an aldohexose, the resulting methylene unit acts as a chain extender, and the derivative then has a retention time longer than, and distinctly different from, that of the corresponding derivative of the pentose. The introduction of the 2-deoxy group into the PAAN derivative of ribose also results in a decrease (of 0.6 min) in the retention time.

From the foregoing, it is inferred that the introduction of a specific, functional group into any of the sugars listed in Table I would result in a general, uniform displacement of the retention times of all of those sugars. This implies that, should the PAAN derivative of a previously uncharacterized sugar appear on a chromatogram, and should a known analog of this compound be available or capable of being

synthesized, the degree of agreement of the retention times of the two compounds would provide a good indication of the stereochemistry of the unknown compound.

It is also possible that the effects of functional groups may be additive. The retention time of the PAAN derivative of 2,6-dideoxy-D-*arabino*-hexose is the same as that obtained by subtracting the combined effect of the 2- and the 6-deoxy groups (1.7 min + 9.0 min) from the retention time of the PAAN derivative of D-glucose. The retention time calculated is 10.3 min, a value that compares favorably with the experimentally observed value of 9.6 min (see Table I).

It should be noted that, owing to the loss of the center of asymmetry at C-2, 2-deoxy-D-*arabino*-hexose is related to both D-glucose and D-mannose. Similarly, 2-deoxy-D-*lyxo*-hexose is formally derived from both D-talose and D-galactose, and so an accurate difference for the introduction of a 2-deoxy group in D-glucose should include an interpolation of the retention time between the PAAN derivatives of both D-glucose and D-mannose. Such considerations complicate the discussion, and, when the necessary calculations are made, the general thrust of these arguments remains the same. Similar arguments may be made by using the relative retention-times from column 1 of Table I. In general, the tentative nature of these proposals, which are based on selected examples, should be recognized However, the data for the compounds examined show good internal correlation.

The L-idose PAAN derivative described in Table I deserves additional comment. In the normal course of hydrolysis by either 2M hydrochloric acid or H_2SO_4/AcOH (ref. 19), L-idopyranosides yield mainly L-idosan (1,6-anhydro-β-L-idopyranose) instead of L-idose. L-Idosan does not afford an oxime, and it appears in the chromatogram as tri-*O*-acetyl-L-idosan, which is clearly distinguishable from the triacetate of levoglucosan (1,6-anhydro-β-D-glucopyranose); D-glucose affords none of the latter anhydride under identical conditions. Tri-*O*-acetyl-L-idosan is an excellent derivative for quantitatively determining idose in a polymer. A second peak (less than 1% of that of tri-*O*-acetyl-L-idosan) is also present in the hydrogen-flame chromatogram; this peak has an e i. mass spectrum identical to that of the glucose PAAN derivative, but a longer relative retention-time, and is therefore identified as the corresponding L-idose PAAN derivative.

Finally, one carbohydrate class remains to be considered, namely, the thio sugars. 5-Thio-D-glucose was subjected to the PAAN derivatization procedure; the product gave a chromatogram having a single peak (see Table I). However, the mass spectrometry of this peak shows clearly that the resulting compound is not a PAAN derivative, but penta-*O*-acetyl-5-thio-D-glucopyranose. Apparently the reaction conditions are not vigorous enough to cleave the C-1–S bond, and the oxime (and, subsequently, the nitrile) is not formed. Therefore, the derivatization procedure provides a readily identifiable 5-thio derivative, conveniently lying in the chromatographic region between the aldohexose and aldoheptose PAAN derivatives.

An additional g.l.c. peak, appearing before glyceraldehyde, should be mentioned. This strong peak, observed in all PAAN-derivatization extracts, normally makes a large contribution to the g.l.c. injection-front. A much slower program than that

already described, at 90°, with programming up to 3°/min, cleanly separates this peak from the front. The various forms of mass spectrometry (see later) identified this component as di-*N*-acetyl-*O*-acetylhydroxylamine.

The literature contains but little discussion on the relationship of carbohydrate structure to relative retention-times in g.l.c. Ferrier[20] concluded that the retention times of trimethylsilyl (Me_3Si) derivatives of pentopyranosides are dependent on the number of axial and equatorial Me_3SiO groups present. The greater the number of such axial groups in a compound, the smaller the retention time. Bishop[21] independently observed this effect for methyl ethers of methyl α- and β-D-glucopyranoside, and proposed that the number of axial substituents could be used to predict the relative retention-times. However, for pyranosides, the situation can be complicated, as Sweeley *et al.*[22] discovered counter-examples with Me_3Si derivatives, supposedly due to interconversion of the chair conformers. Gheorghiu and Oette[23] recognized the relationship between the availability of a functional group and its interaction with the stationary phase in relating retention times of Me_3Si derivatives of aldopentopyranosides and aldohexopyranosides to the number of equatorial, Me_3SiO groups available. However, for cyclic compounds, an assumption as to the conformation must be made; no such assumption is necessary in using the *cis* effect in connection with acyclic compounds.

For Me_3Si ethers of alditols, the marked shortening of the retention time on introduction of a terminal deoxy group, as contrasted to the introduction of an internal deoxy group, was observed by El-Dash and Hodge[24].

The hydrogen-flame detector-responses in g.l.c. are given in Table I on the basis of weight-response factors relative to D-glucose. The response factor is dependent on both the relative detector-response for each compound and the degree of partitioning during the chloroform–water partition. The saccharides are readily weighed to an accuracy of ± 1 %, which is considered to be the range of accuracy of measurement of the g.l.c. peak. However, after being dried, the saccharides were weighed as supplied by the distributors, and the possibility that impurities were present cannot be excluded. Therefore, the values presented here are considered to be accurate to within a few percent. On a weight basis, the detector responses are quite similar for the PAAN derivatives of the unsubstituted aldopentoses, aldohexoses, and the aldoheptose studied The 2- and 6-deoxyhexoses and 2-deoxy-D-*erythro*-pentose gave considerably lower detector-responses than their non-deoxy analogs; the lower detector-response no doubt reflects the inability of the deoxy group to form an acetate. Therefore, were the detector responses of the aldopentoses, the aldohexoses, and the aldoheptose based on the weight of the resulting PAAN derivatives, all values would approximate unity. However, the value for digitoxose is somewhat larger than would be expected. Replacement of an oxygen atom in D-glucose by a sulfur atom appears to have little effect on the detector response, although penta-*O*-acetyl-5-thio-D-glucose is actually being compared to the PAAN D-glucose derivative. Replacement of the 2-hydroxyl group of D-glucose by a 2-acetamido group profoundly lowers the relative detector-response. For all of the aforementioned derivatives, the

hydrogen-flame detector-response is very similar to the total *m/e* integrated across the g.l.c.–e.i.-m.s. peak.

G.l.c. on columns of OV-17. — A column composed of OV-17 on Chromosorb W may be employed in order to approximate the separation on NPGS already described. Major differences for the OV-17 column are (*a*) a small column-loading factor, and (*b*) the approximately equal response-factor for *N*-acetylhexosamine PAAN derivatives and PAAN derivatives of neutral sugars. OV-17 columns have previously been employed with peracetylated alditols in order to overcome an analogous, low detector-response for *N*-acetylhexosamine derivatives[25]. For the peracetylated alditols, the argument was advanced that OV-17 (on Gas Chrom Q) did not irreversibly adsorb the *N*-acetyl-containing derivatives. Considering the relative detector-responses for the NPGS and OV-17 columns, this argument may be applied to the PAAN derivatives. Although the peracetylated alditols were reportedly separated on 2% of OV-17 on 100–120 mesh Gas Chrom Q, we have concluded that 2% of OV-17 on Chromosorb W is preferable, as it gives a greater loading-factor, more-symmetrical peaks, and better resolution. Owing to the problem of irreversible adsorption, injections of mixtures of the PAAN derivatives of D-glucose and 2-acetamido-2-deoxy-D-glucose were studied in terms of detector response. The detector-response ratio for D-glucose–2-acetamido-2-deoxy-D-glucose remained constant for a wide variety of conditions, with one exception, namely, when small *volumes* [<0.2 μL for the column (1.23 m × 2 mm) described] were injected, the relative detector-response for the 2-acetamido-2-deoxy-D-glucose PAAN derivative, relative to the D-glucose PAAN derivative, fell sharply. This could be remedied by diluting any given sample, and injecting the same amount of saccharide in a larger volume of chloroform. It would be advisable to check any new column with a known mixture of the PAAN derivatives of D-glucose and 2-acetamido-2-deoxy-D-glucose in order to establish the specific limits for this phenomenon.

The general properties of the NPGS and the OV-17 columns are similar, for example, the order of the relative retention-times for the PAAN derivatives of the unsubstituted saccharides is the same (see Table I). However, the OV-17 column gives less resolution. For example, the arabinose and lyxose PAAN derivatives are unresolved, as are the PAAN derivatives of 2-deoxy-D-*arabino*- and -*lyxo*-hexose. For PAAN derivatives on OV-17, the 6-deoxyhexose derivatives have larger relative retention-times than those of the related sugars, and the 2-deoxyhexose and *N*-acetylhexosamine derivatives have smaller relative retention-times Little difference in the relative detector-responses of these PAAN compounds was observed for the two columns, except for the marked difference for *N*-acetylhexosamines. The derivatization procedure produces, on OV-17, chromatogram peaks for ketoses derivatized to their peracetylated oximes; these products have considerably longer g.l.c. retention-times than the corresponding aldose PAAN derivatives, and do not interfere with the aforedescribed chromatogram.

Although 2-amino-2-deoxy-D-glucose is not listed in the Tables or shown in the Figures as a separate entity, the procedure can identify it, and differentiate it

from 2-acetamido-2-deoxyglucose. Production of its oxime proceeds more slowly than that of the oxime of D-glucose or 2-acetamido-2-deoxy-D-glucose, ~6 h of stirring at 70° being required When the resulting 2-amino-2-deoxy-D-glucose oxime derivative, which was not isolated, was treated with acetic anhydride, a product identical to that from 2-acetamido-2-deoxy-D-glucose oxime was obtained, implying that *N*-acetylation had occurred. When deuterioacetic anhydride is employed, mass spectrometry can readily differentiate between derivatives arising from 2-amino- and 2-acetamido-2-deoxy-D-glucose, the hexosamine producing an *N*-deuterioacetyl PAAN derivative, and the *N*-acetylhexosamine, an *N*-acetyl PAAN derivative. The differences between the mass spectra of hexosamine and *N*-acetylhexosamine derivatives are given in Table III for ammonia c.i.-m.s., and in Table IV for e.i.-m.s. In a less quantitative way, a large increase in the 2-acetamido-2-deoxyglucose peak for samples heated for 6 h at 70° (oxime step) compared to that for samples heated for 40 min at 70° would indicate the presence of 2-amino-2-deoxyglucose. The e.i. mass spectra of the hexosamine PAAN derivative differs from that of the *N*-acetylhexosamine PAAN derivative, but this is seen only on close inspection, as the bulk of these mass fragments arise from the non-nitrile end; in fact, it is difficult to differentiate weak mass-spectra. The ammonia c.i. mass spectra are more readily differentiated, but, in general, larger injections (by a factor of ~5) are needed for all ammonia c.i.-m.s. measurements in order to obtain spectra whose quality equals that of the e.i. spectra, so no advantage is gained in this situation.

A simple modification of the OV-17 program described consists in maintaining all conditions the same, except for programming at 20°/min, thus providing an efficient survey-program. This rapid program includes the PAAN derivatives of glyceraldehyde through 2-acetamido-2-deoxygalactose in 10 min, near baseline-separation of the ribose–arabinose–xylose PAAN set, and ample resolution to identify the allose–mannose–glucose–galactose–idose derivative set.

Mass spectrometry. — Previous studies have shown that changes in the centers of asymmetry of carbohydrates do not affect their mass spectra. In the course of examining various sets of stereoisomers (arabinose, ribose, and xylose; galactose, glucose, and mannose), we have confirmed this finding for PAAN derivatives with all of the forms of mass spectrometry described herein. Ignoring differences in stereochemistry, 13 types of compound have been observed in the chromatograms, which, for convenience, are designated as types (for a set of stereoisomers) as follows: type 1 (di-*N*-acetyl-*O*-acetylhydroxylamine), type 2 (*e.g.*, D-glyceraldehyde PAAN derivative), type 3 (*e.g.*, D-erythrose PAAN derivative), type 4 (*e.g.*, 2-deoxy-D-*erythro*-pentose PAAN derivative), type 5 (*e.g.*, D-ribose PAAN derivative), type 6 (*e.g.*, 2,6-dideoxy-D-*arabino*-hexose PAAN derivative), type 7 (*e.g.*, 6-deoxy-L-galactose PAAN derivative), type 8 (*e.g.*, 2-deoxy-D-*arabino*-hexose PAAN derivative), type 9 (*e.g.*, D-glucose PAAN derivative), type 10 (*e.g.*, 2-acetamido-2-deoxy-D-glucose PAAN derivative), type 11 (*e.g.*, D-*glycero*-D-*gluco*-heptose PAAN derivative), type 12 (*e.g.*, penta-*O*-acetyl-5-thio-D-glucose), and type 13 (*e.g.*, per-*O*-acetyl-

TABLE III

FRAGMENT IONS OF PERACETYLATED ALDONONITRILE DERIVATIVES AND RELATED COMPOUNDS[a] IN AMMONIA CHEMICAL-IONIZATION MASS-SPECTROMETRY

Non-isotopically enriched	*^{15}N-Oxime derivative*	*CD_3CO derivative*	*Origin of ion*	*Comments*
Type 1 (di-*N*-acetyl-*O*-acetylhydroxylamine[b])				
177	178	186	M + 18	weak
160	161	169	M + 1	
135	136	141	M + 18 − 42	weak
118	119	124	M + 1 − 42	
Type 2 (DL-glyceraldehyde PAAN)				
189	190	195	M + 18	
112	113	115	M − 59	
Type 3 (D-erythrose PAAN)				
261	262	270	M + 18	
184	185	190	M − 59	
Type 4 (2-deoxy-D-*erythro*-pentose PAAN)				
275	276	284	M + 18	
215	216	221	M + 18 − 60	weak
198	199	204	M − 59	
Type 5 (D-arabinose PAAN)				
333	334	345	M + 18	
256	257	265	M − 59	
115	115	118	e.i. spectra	
Type 6 (2,6-dideoxy-D-*arabino*-hexose PAAN)				
289	290	298	M + 18	
212	213	218	M − 59	
Type 7 (6-deoxy-L-mannose PAAN)				
347	348	359	M + 18	
270	271	279	M − 59	
Type 8 (2-deoxy-D-*arabino*-hexose PAAN)				
347	348	359	M + 18	
287	288	296	M + 18 − 60	weak
270	271	279	M − 59	
Type 9 (D-mannose PAAN)				
405	406	420	M + 18	
345	346	357	M + 18 − 60	weak
328	329	340	M − 59	
Type 10 (2-acetamido-2-deoxy-D-glucose PAAN)				
404	405	416 (419)[c]	M + 18	weak
387	388	399 (402)	M + 1	
327	328	336 (339)	M − 59	
267	268	273 —	M − 59 − 60	
207	208	210 (213)	M − 59 − 120	weak
187	187	193 (193)	e i. spectra	weak
165	166	165 (168)		weak
149	150	149 (152)	background	

TABLE III *(continued)*

Non-isotopically enriched	*^{15}N-Oxime derivative*	*CD_3CO derivative*	*Origin of ion*	*Comments*
Type 11 (D-*glycero*-D-*gluco*-heptose PAAN)				
477	478	495	M + 18	
400	401	415	M − 59	
Type 12 (penta-*O*-acetyl-5-thio-D-glucopyranose)				
424 (100)[a]		439	M + 18	
364 (3)		376		
347 (60)		359	M − 59	
305 (1)		315		
304 (1)		313		
287 (6)		296		
245 (10)		252		
244 (2)		250		
227 (35)		233		
202 (1)		206		
185 (25)		189		
184 (15)		187		
142 (10)		143		
Type 13 (tri-*O*-acetyl-L-idosan)				
306			M + 18	
229			M − 59	

[a]Alternative species arising from PAAN derivatization procedures [b]A specific example of each type of compound examined is listed, but no differences were observed for stereoisomers. [c]The values in parentheses were obtained by performing the PAAN derivatization starting with 2-amino-2-deoxy-D-glucose hydrochloride. [d]Relative intensities are given for this complex spectrum.

levoglucosan). Types 2, 3, 5, 9, and 11 provide a homologous series, allowing clear observance of the effect of lengthening of the carbohydrate chain.

Ammonia c.i.-m.s. — This form of mass spectrometry gave the simplest and most readily interpretable spectra. Horton *et al.*[4] demonstrated that carbohydrates not containing a basic nitrogen atom may, under ammonia-c.i. conditions, give *m/e* values consisting of the molecular weight + NH_4^+ (M + 18) and of the molecular weight + H^+ — a prominent, neutral fragment, *e.g.*, acetic acid (M + 1 — 60). These *m/e* values have been found in the spectra of the PAAN derivatives, to the virtual exclusion of all other mass fragments. The single PAAN derivative that constitutes an exception is the 2-acetamido-2-deoxyglucose PAAN derivative, whose mass spectrum contains (M + H^+), in addition to (M + 18) and (M — 59). This *m/e* value is also in agreement with a finding of Horton *et al.*[4] for carbohydrates containing a basic nitrogen atom (the *N*-acetyl group). The ammonia c.i. spectra of the various types of compound are given in Table III. In addition to the normal derivatization procedure, $^{15}NH_2OH \cdot HCl$ and acetic anhydride-d_6 were employed. The interpretation of the mass spectra of the products appears to be very simple. The two major peaks indicate the molecular weight (M + 18) and (M — 59), but

if the two peaks are not 77 mass units apart, presence of a basic nitrogen atom is indicated. The substitution with nitrogen-15 indicates the number of aldehyde groups originally present, one aldehyde group for each mass-unit change in M (seen in both mass peaks). The use of acetic anhydride-d_6 gives a product that indicates the number of hydroxyl groups originally present; for (M + 18), the increase in mass on deuterioacetylation, divided by 3, gives the number of acetyl groups in the PAAN derivative, and therefore, the number of hydroxyl groups in the original sugar. For the (M — 59) peak, the mass increase on deuterioacetylation, divided by 3, gives a value one acetyl group less than that from the (M + 18) calculation. Through use of the (M + 18) peak, it is possible to determine the molecular weight, the number of aldehyde groups, and the number of hydroxyl groups present in the original sugar molecule. The (M — 59) peak allows confirmation of these data.

The ammonia c.i. mass spectrum of di-*N*-acetyl-*O*-acetylhydroxylamine shows strong (M + 1) and (M + 1 — 42) peaks All of the data accord with a basic nitrogen-containing molecule having three acetyl groups and losing the nitrogen acetate group as ketene (—42). An i.r.- and ^{1}H-n.m.r.-spectral examination of chloroform extracts of selected PAAN derivatives known to contain large proportions of the di-*N*-acetyl-*O*-acetylhydroxylamine yielded spectra identical to those published[26] for this compound, providing further identification of the compound.

The ammonia c.i. mass spectra of the 2-acetamido-2-deoxy-D-glucose PAAN derivative shows additional peaks of (M — 59 — 60) and (M — 59 — 120), indicating further loss of acetic acid, and weak peaks from e.i. spectra. Quite possibly, these additional peaks for the PAAN derivative indicate the relative instability of the molecule. In addition to these data, the gas–liquid chromatogram of the PAAN derivative of 2-acetamido-2-deoxy-D-glucose shows a flat, very broad peak reaching a maximum shortly before the sharp peak for the PAAN derivative. (This broad peak appears only in the *N*-acetylhexosamine derivatization solutions.) The ammonia c.i. mass spectrum of this flat peak indicates a compound of 148 mass units (M + 1) that retains the nitrile nitrogen atom and the *N*-acetyl group, but none of the *O*-acetyl groups (see Table III). The compound may be 1-acetylcyanocyclopentadiene, resulting from extensive loss of *O*-acetyl as acetic acid and final cyclization.

Under ammonia c.i. conditions analogous to those under which the glucose PAAN derivative yielded only two *m/e* peaks, the penta-*O*-acetyl-5-thio-D-glucose derivative gave an ammonia c.i. mass spectrum having two prominent peaks (424 and 347) corresponding to (M + 18) and (M + 59). However, an additional series of *m/e* values is present, indicating the successive stripping of acetic acid and ketene from the thiopyranose ring (see Fig. 3). This mass spectrum clearly indicates that the sulfur is retained during the acetyl-stripping process, implying that it lies in the pyranose ring, as contrasted with the ammonia c.i. mass spectrum published for penta-*O*-acetyl-1-thio-β-D-glucopyranose[4].

E.i.-m.s. — The e.i. mass spectra of types 4, 5, 6, 7, 9, and 11 had previously been examined by use of a probe and of both non-isotopically enriched compounds and derivatives prepared with acetic anhydride-d_6. We have now examined the same

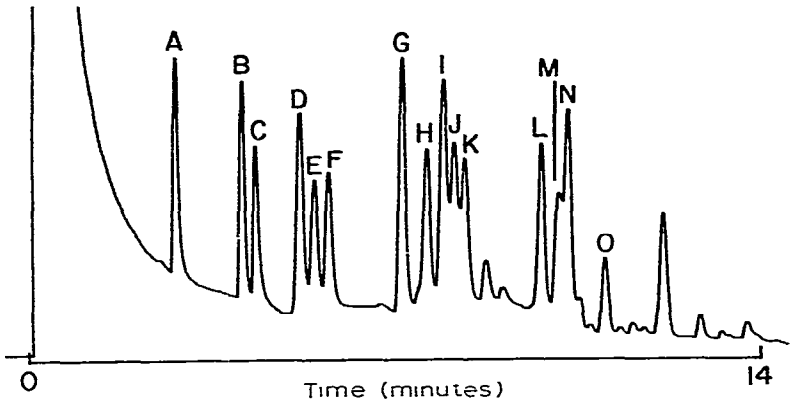


Fig 2. Gas–liquid chromatograms (hydrogen-flame detector) using OV-17 on Chromosorb W (condition 2 of Table I). [The PAAN derivatives of: A, D-erythrose; B, digitoxose; C, 2-deoxy-D-*erythro*-pentose; D, D-ribose; E, D-arabinose; F, D-xylose; G, 2-deoxy-D-*arabino*-hexose; H, D-allose; I, D-mannose; J, D-glucose; K, D-galactose; L, 2-acetamido-2-deoxy-D-glucose; M, 5-thio-D-glucose (peracetylated); N, D-*glycero*-D-*gluco*-heptose; O, 2-acetamido-2-deoxy-D-galactose]

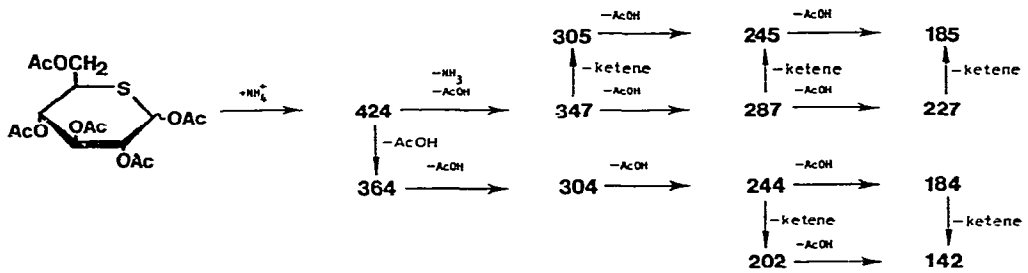


Fig. 3. Ammonia c i -m s. pathways of peracetylated 5-thio-D-glucose, showing the major *m/e* values observed in the spectrum.

compounds by g.l c.–m s.. and find the fragmentation patterns to be essentially identical, both for mass fragments observed and for the relative intensities. In general, our g l.c.–m.s. data do not show mass fragments >300, which may be an indication of lower density of the compound in the ionization chamber, or may reflect the general discrimination of the quadrupole mass spectrometer against higher-mass fragments. In addition, we have examined the ^{15}N-substituted derivatives of PAAN types 4, 5, 6, 7, 9 and 11; for all, the identification, by Szafranek *et al.*[16], of the specific mass fragments with regard to content of nitrogen has been confirmed. In addition to the foregoing mass spectra, the e.i. mass spectra of types 1, 2, 3, 8, 10, 12, and 13 have been recorded by using non-isotopically enriched reagents, and then $^{15}NH_2OH$, and also acetic anhydride-d_6. These data are summarized in Table IV. The e.i.-m.s. data for types 1, 2, and 3 are interpreted as indicating the fragmentation pathways shown in Fig. 4, the structure of the fragments being supported by the isotopic-substitution data. An unusual feature is the structure of the *m/e* 141 fragment, which is clearly shown to contain the nitrogen atom and all six acetoxyl protons. As the

TABLE IV

ELECTRON-IMPACT, MASS-SPECTROMETRY FRAGMENT-IONS OF PERACETYLATED ALDONONITRILE DERIVATIVES AND RELATED COMPOUNDS

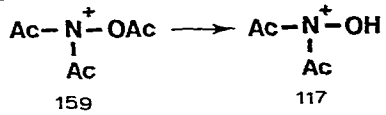
DL-*Glycer-aldehyde PAAN (Type 2)*	D-*Erythrose PAAN (Type 3)*	*2-Deoxy-*D-*erythropentose PAAN (Type 8)*	*2-Acetamido-2-deoxy-*D-*glucose PAAN (Type 10)*	*Penta-*O*-acetyl-5-thio-*D*-gluco-pyranose (Type 12)*	*Tri-*O*-acetyl-*L*-idosan (Type 13)*
141(10[a],1[b],6[c])	170(1[a],1[b],6[c])	256(6[a],1[b],9[c])	289(10[a],0[b],12[c],–[d])	286(1[a],–[c])	228(3[a],6[c])
112(3,1,3)	145(9,0,6)	217(5,0,9)	271(2,1,7,10)	244(3,7)	186(72,4)
99(2,1,4)	141(10,1,6)	214(5,1,7)	253(1,1,–,–)	227(20,6)	157(31,4)
86(3,0,3)	112(3,1,3)	187(5,0,6)	241(1,1,–,–)	226(10,6)	145(90,6)
73(6,0,3)	99(2,1,4)	184(4,1,6)	224(4,1,4,7)	213(7,6)	115(61,4)
	86(3,0,3)	175(3,0,7)	217(3,0,9,9)	185(100,4)	103(42,3)
	73(6,0,3)	167(10,1,4)	211(7,1,6,10)	184(56,4)	98(21,1)
		157(2,0,6)	199(2,1,4,7)	171(9,4)	97(11,1)
		154(18,1,3)	187(100,0,6,6)	155(10,3)	81(17,1)
		145(28,0,6)	182(14,1,–,6)	142(100,1)	
		142(2,1,4)	169(5,1,–,6)	139(15,2)	
		127(2,0,3)	164(5,1,1,4)	125(14,0)	
		125(13,1,4)	145(12,0,6,6)	115(5,–)	
		115(21,0,3)	140(13,1,1,–)	114(60,1)	
		112(2,1,3)	127(21,0,3,3)	113(50,1)	
			115(8,0,3,3)	97(15,0)	
			103(4,0,4,4)	85(10,0)	
			98(18,1,1,2)	73(18,0)	
			85(13,0,1,1)		
			73(2,0,2,–)		

[a]Intensity (percent) of mass fragment relative to that of the most-intense mass-fragment. The *m/e* 43 (or 46, for deuterioacetylations) appeared in all spectra, and was, in most cases, the most intense peak. [b]Hydroxyl[^{15}N]amine was employed in the derivatization procedure. [c]Acetic anhydride-d_6 was employed in the derivatization procedure. [d]Acetic anhydride-d_6 was employed in the derivatization procedure, and the starting saccharide was 2-amino-2-deoxy-D-glucose hydrochloride.

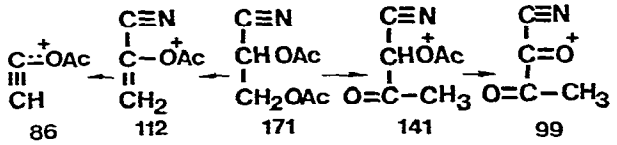
m/e fragment arises from the C_3 chain of the glyceraldehyde PAAN derivative, a rearrangement is indicated.

The e.i. data for type 6 (2-deoxyhexose PAAN) further confirm an observation, first made by Szafranek *et al.*[16], which we further emphasize. These PAAN derivatives have fragmentation modes that contrast markedly with those of their partially methylated PAAN analogs[17]. The backbone carbon atoms of a partially methylated PAAN sugar derivative show (*a*) a marked tendency to be cleaved between adjacent carbon atoms bearing methoxyl groups, (*b*) less tendency to cleave between carbon atoms respectively bearing an acetoxyl and a methoxyl group, and (*c*) considerable resistance to cleavage between adjacent carbon atoms bearing acetoxyl groups. The lack of any functional groups to direct the point of cleavage of the backbone results in a condition where, in general, it is equally possible for cleavage to occur at any carbon–carbon bond of the backbone. The cleavages are indicated in Fig. 5, for a

Type 1



Type 2



Type 3

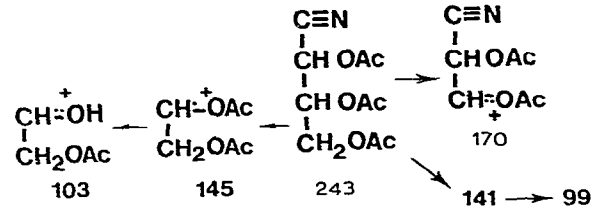


Fig. 4. E.i.-m.s. fragmentation-pathways, and *m/e* values of type 1 (di-*N*-acetyl-*O*-acetylhydroxylamine), type 2 (DL-glyceraldehyde PAAN derivative), and type 3 (D-erythrose PAAN derivative) compounds.

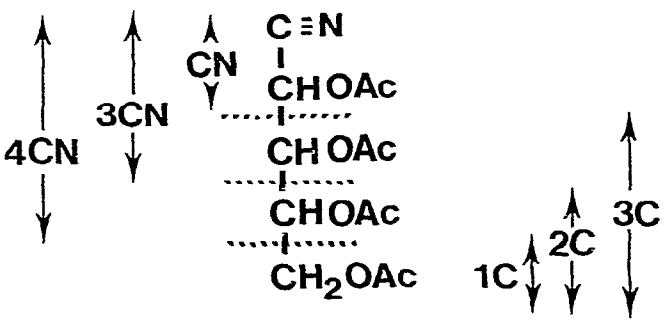


Fig. 5. E.i.-m.s. cleavage positions, and possible mass fragments arising from a pentose PAAN derivative.

pentose lacking non-hydroxyl, functional groups. Fig. 5 illustrates that mass fragments can arise from either end of the molecule, with a generally equal probability of cleavage between any two carbon atoms (the stable bond between the nitrile group and C-2 is an exception). As the carbon chain is lengthened, larger mass fragments become possible; however, in each case observed, the C-series (from the non-nitrile end) well overlaps the CN-series (from the nitrile end). In addition, the specific mass-fragments undergo specific eliminations that are the same, regardless of the origin of the parent mass-fragment. A functional group different from the hydroxyl group will yield an acetoxyl group differing by a specific mass-increment from the normal

acetoxyl group found in the normal PAAN derivative. Fig. 6 shows the progressive generation of mass fragments as the backbone chain of the PAAN derivative of a sugar lengthens. The CN-series represents mass fragments originating from the nitrile end (the number behind the CN designation stands for the number of backbone carbon atoms in the originating mass fragment). The C series represent fragments from the non-nitrile end of the molecule. For carbon–carbon cleavage of the DL-glyceraldehyde PAAN derivative, the largest fragment possible results from a two backbone-carbon fragment for the CN2 series and also for the C2 series, and these fragments are observed. Only the mass fragments actually observed in our g.l c.–m.s. spectra are reported in Fig. 6. The structures of mass fragments are either those described by Szafranck *et al.*[16], or presented in Fig. 5.

Assuming that no extensive rearrangements occur, each mass fragment containing this new functional group (or its functional derivative resulting from the PAAN derivatization process) will also differ by this different increment in mass. By examining the mass fragments arising from the series C1, C2, C3, C4, *etc.*, and the ions arising from CN1, CN2, CN3, *etc.*, the position of substitution can be quickly established. The same ions observed in the pentose PAAN mass spectra will also be found in the mass spectra of hexose PAAN derivatives, with the addition of ions arising from the C5 and CN5, with increasingly larger mass fragments as the chain is lengthened. In contrast to ammonia c.i.-m.s., where all ion-fragments contain the total carbon-chain backbone intact, e.i.-m.s. yields only fragment-ions resulting from the cleavage of the backbone. In Fig. 6, the series on the left, prefixed by D for deoxy, shows the masses arising from deoxy substitution at C-2, or at the terminal position, or both. The e.i. mass spectrum of the D-arabinose PAAN derivative is a composite of the C1 through C4 spectra and the CN2 through CN4 spectra patterns. The mass spectrum of the 2-deoxy-D-*erythro*-pentose PAAN derivative is a composite of C1 through C3 (the normal, non-nitrile end-fragments) and DCN2

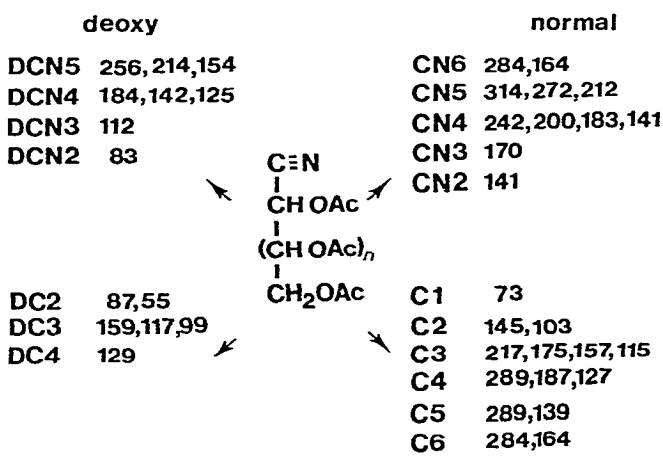


Fig 6. Observed mass fragments in the e.i.-m s. of aldose PAAN derivatives for DL-glyceraldehyde (n = 0) through D-*glycero*-D-*gluco*-heptose (n = 3), and for deoxy analogs.

TABLE V

COMPOSITE FRAGMENT SERIES FORMING THE ELECTRON-IMPACT MASS-SPECTRA OF PERACETYLATED ALDONONITRILES

Compound type	*Specific compound used for PAAN derivative*	*Fragment series comprising the observed spectra*[a]
2	DL-glyceraldehyde	C1, CN2
3	D-erythrose	C1, C2, CN2, CN3
4	2-deoxy-D-*erythro*-pentose	C1, C2, DCN2, DCN3, DCN4
5	D-arabinose	C1, C2, C4, CN2, CN4
6	2,6-dideoxy-D-*arabino*-hexose	DC2, DC3, DC4, CN2, CN4
7	6-deoxy-L-mannose	DC2, DC3, DC4, CN2, CN4, CN5
8	2-deoxy-D-*arabino*-hexose	C1, C2, C3, C4, DCN2, DCN3, DCN4, DCN5
9	D-mannose	C1, C2, C3, C4, CN2, CN3, CN4, CN5
10	2-acetamido-2-deoxy-D-glucose	C1, C2, C3, C4, CNA3, CNA4, CNA5
11	D-*glycero*-D-*gluco*-heptose	C1, C2, C3, C4, C5, C6, CN3, CN5, CN6

[a]Fragment series as defined in Fig. 6.

through DCN4 (the deoxy nitrile end-fragments). Table V shows how the mass-spectral patterns available combine to form the total e.i. mass spectra for each type of compound. For example, the spectra of 2,6-dideoxy-D-*arabino*-hexose (digitoxose) is exactly the sum of the DCN and DC series up to DCN4 and DC4. The identity and mechanism of origin of the fragments from pentoses, hexoses, and heptoses has been discussed by Szafranek *et al.*[16], and the origin of the fragments from the triose and the tetroses has already been discussed. In considering the mass spectra of PAAN derivatives having increasing backbone-length, the contributions from the fragments having shorter chain-lengths decrease in an orderly way.

The e.i. mass spectrum of the 2-acetamido-2-deoxy-D-glucose PAAN derivative is clearly a composite of *m/e* fragments from the non-nitrile end (the C-series) and from the nitrile end, which correspond to the CN-series of glucose but displaced one mass unit smaller. The C-series is strongly represented by the C4 (*m/e* 289, 187, 127), the C3 (*m/e* 217, 115), the C2 (*m/e* 145, 103), and the C1 (*m/e* 73). The CN series (analogous to that of glucose, except that it is displaced by one mass unit) designated CNA is less intense, with many mass fragments not appearing in weak spectra. These are the CNA5 (*m/e* 271, 211), the CNA4 (*m/e* 241, 211, 199, 182, 140, 98), and the CNA3 (*m/e* 169). These CNA fragmentation-pathways are indicated in Fig. 7.

The e.i. mass spectrum of penta-*O*-acetyl-5-thio-D-glucopyranose displays a series of *m/e* values (286, 244, 226, and 184) that are analogous to those in the ammonia c.i. mass spectrum, but one unit smaller. This series of e.i.-m.s. peaks agrees with the structure proposed from ammonia c.i.-m.s. (see Fig. 3), but they do not have the H^+ added by chemical ionization; this provides further evidence for incorporation of the sulfur into the pyranoid ring.

The e.i. mass spectrum of tri-*O*-acetyl-L-idosan (type 13) displays the complicated patterns associated with the pyranoid ring-structure (discussed in detail by

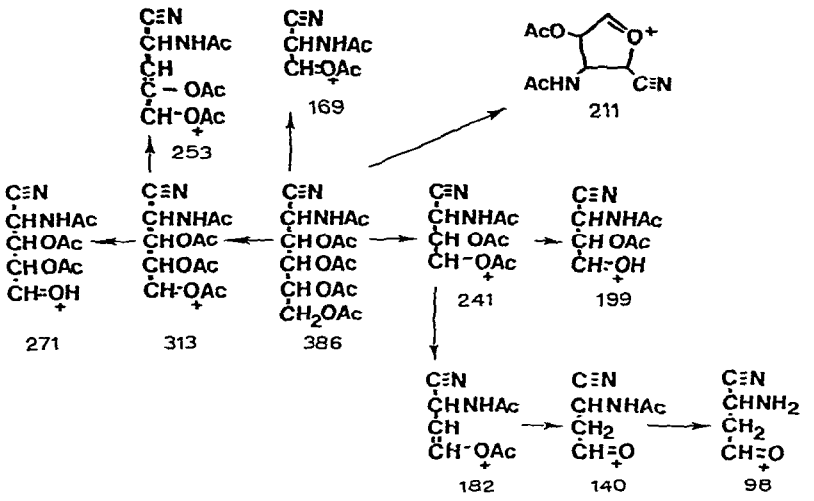


Fig 7. E i.-m s. fragmentation-pathway for ions observed arising from the nitrile end of type 10 (2-acetamido-2-deoxy-D-glucose PAAN derivative) compounds

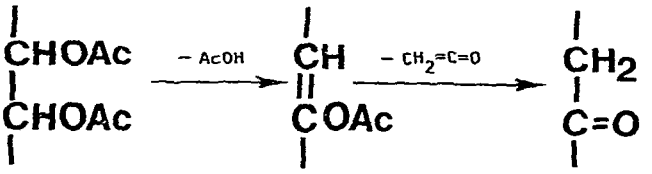


Fig 8. The process dominating for the methane c i mass spectra.

Kochetkov and Chizhov[6]), and is identical to the corresponding mass spectrum for tri-*O*-acetyl-levoglucosan.

Methane c.i.-m.s. — The methane c.i. mass spectra occupy a position intermediate between e.i. and ammonia c.i. mass spectra. The methane c.i. spectra contain many of the prominent e.i. mass fragments (~15 to 20% of the total, integrated *m/e*), but no M, (M + 1), or (M + 18). Previous studies examining the e.i. fragmentation-patterns of the methyl ether PAAN derivatives of mannose indicated that a favored m.s. pathway is the loss of acetic acid, resulting in an alkene bond in the carbon backbone-chain. If this alkene group bears an acetoxyl group, ketene is lost, and a ketone group is formed (see Fig. 8); this process dominates the methane c.i. fragmentation-pathways to the exclusion of most other mechanisms.

Methane c.i.-m.s. yields a large amount of information; however, several factors make it less interesting than the forms of mass spectrometry already discussed. Firstly, methane c.i. spectra provide little information that is not obtained through a combination of e.i.- and ammonia c.i.-m.s. Secondly, under the experimental conditions obtained in our equipment, there was a considerable fluctuation of relative *m/e* intensities. The e.i.-m.s. contribution to the spectra remained constant, but, on a scan-to-scan basis, the relative intensity of the remaining peaks would change quite markedly; this problem can be considerably alleviated by electronically averaging

the *m/e* of successive spectra in a given g.l.c. peak. In view of these limitations, only representative, methane c.i. data are presented, to show the general fragmentation-pathways.

The general procedure for the analysis of these data was to subtract the e.i. mass-fragments from the methane c.i. spectra. Compounds derivatized with per-deuterated acetic anhydride were also examined (except for the 2-acetamido-2-deoxy-D-glucose PAAN derivative), and the number of acetoxyl groups in each fragment was established. Once the basic pattern of loss of acetic acid followed by loss of ketene had been determined, the fragmentation pathways became evident. The *m/e* values shown in the subsequent Figures are all in accord with expected, per(deuterio-acetyl)ation mass-shifts.

The methane c.i. mass spectrum of the PAAN derivative of D-arabinose (see Fig. 9) is typical, showing no *m/e* value near M, a prominent (M + 1 — 60 — 42) resulting from the loss of acetic acid and ketene, and successive *m/e* values indicating further loss of acetic acid and ketene. In general, for this and other methane c.i. mass spectra, the larger *m/e* values are the most intense. The successive stripping of acetoxyl groups from the molecule continues, until an alkane nitrile having alternating ketone groups remains, as indicated for *m/e* 112 in Fig. 9. In addition, there is a tendency for C-1 to be lost, apparently as HCN.

The PAAN derivatives of 6-deoxy-L-galactose (L-fucose) and 2-deoxy-D-*arabino*-hexose yield identical methane c.i. spectra (see Fig. 10); this observation serves to emphasize the necessity of presenting the fragmentation pathways in terms of *m/e* (not in terms of specific structures). There is no reason to suspect that the initial loss of any acetoxyl group is greatly favored over another. Therefore, each *m/e* value

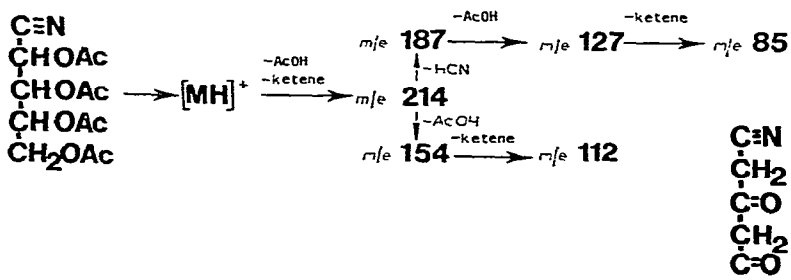


Fig 9 Methane c i -m s pathway of type 5 (D-arabinose PAAN derivative) compounds, and the terminally protonated diketone (*m/e* 112).

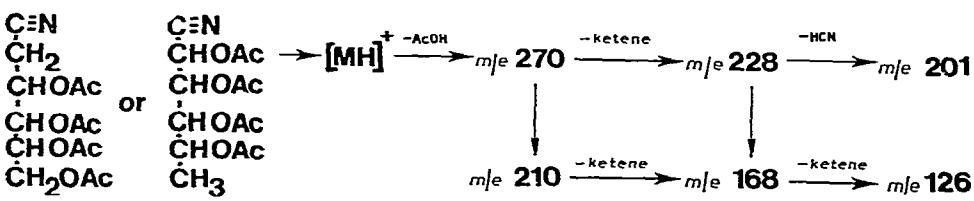


Fig. 10 Methane c i -m s. pathway of type 7 (6-deoxy-L-mannose PAAN derivative) and type 8 (2-deoxy-D-*arabino*-hexose PAAN derivative) compounds.

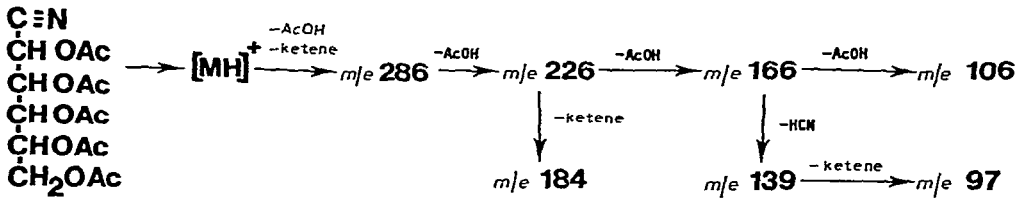


Fig. 11. Methane c.i -m s. pathway of type 9 (D-mannose PAAN derivative) compounds.

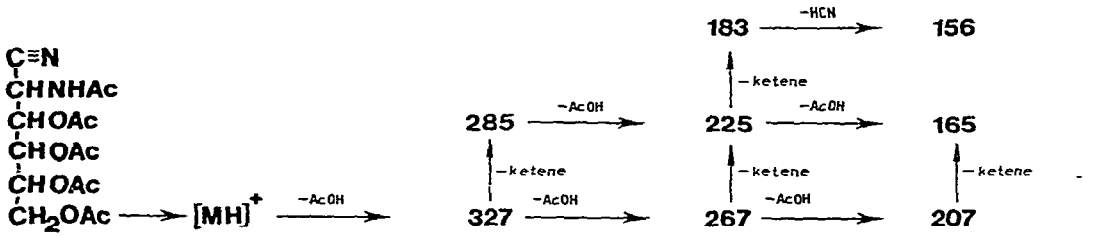


Fig. 12. Methane c i -m s. pathway of type 10 (2-acetamido-2-deoxy-D-glucose PAAN derivative) compounds

probably represents a population of different positional-isomers. For both molecules, the loss of acetoxyl groups proceeds to the production of the dioxoalkane nitrile (*m/e* 126).

The methane c.i. mass spectrum of the D-glucose PAAN derivative (see Fig. 11) also shows the *m/e* value associated with loss of acetic acid and ketene.

The methane c.i. mass spectrum of the PAAN derivative of 2-acetamido-2-deoxy-D-glucose (see Fig. 12) is interesting, as it shows a close parallel to the methane c.i. mass spectrum of the corresponding D-glucose derivative. In the latter, the principal *m/e* values are 286, 226, 184, and 166. For the corresponding 2-acetamido-2-deoxy compound, the principal *m/e* values are 285, 225, 183, and 165, as the *N*-acetyl group contains one mass unit less than the acetoxyl group. This shows clearly that the *N*-acetyl group has a strong tendency to remain intact under these mass-spectral conditions, and, in fact, the entire unit comprising C-1 and C-2 resists fragmentation.

EXPERIMENTAL

Materials. — Reducing sugars were used as obtained: from Sigma Chemical Co., St. Louis, Mo. (2-acetamido-2-deoxy-D-glucose, 2-deoxy-D-*erythro*-pentose, D-digitoxose, DL-glyceraldehyde, D-*glycero*-D-*gluco*-heptose, and D-talose), P-L Biochemicals Inc., Milwaukee, Wis. (D-allose, 2-deoxy-D-*lyxo*-hexose, D-erythrose, and 5-thio-D-glucose), and Pfanstiehl Inc., Waukegan, Ill. (D-arabinose, 2-deoxy-D-*arabino*-hexose, L-fucose, D-galactose, D-glucose, D-lyxose, D-mannose, L-rhamnose, D-ribose, and D-xylose). Reagents were used as obtained from Fisher Scientific Co. (hydroxylamine · HCl, pyridine, and acetic anhydride, all A.C.S. grade). The pyridine was stored over potassium hydroxide. For isotopic substitution, acetic anhydride-d_6 and hydroxyl[^{15}N]amine · HCl (Merck Sharp and Dohme, Canada Ltd., Montreal) were used. Neopentyl glycol succinate (3%) on 60–80 mesh Supelcoport was obtained

from Supelco Inc., Bellefonte, Pa. Methane (Matheson, ultra-high purity) and ammonia (Linde, ultra-high purity) were used as chemical-ionization reagent-gases.

Equipment. — The g.l.c. surveys were performed on a Barber–Coleman Series 5000 dual-channel, g.l.c. instrument equipped with hydrogen-flame detectors and glass columns (2 mm i.d.) with on-column injection, and nitrogen as the carrier gas. The g.l.c.–m.s. determinations were performed with a Hewlett–Packard 5980A GC/MS and an integrated g.l.c.–m.s.–computer system. The gas–liquid chromatograph was equipped with glass columns (2 mm), with on-column injection, and helium as the carrier gas. A membrane separator was used, with the transfer lines held at 250°. The spectrometer was tuned with PTBA, and, for chemical-ionization experiments, the tuning was verified by examining the mass spectrum of the g.l.c. peak of the D-glucose PAAN derivative. For general conditions, the upper *m/e* value was set at 600, and the spectra were scanned at 5-s intervals, the data being stored in the computer-disc memory. The total *m/e* value *vs.* time is displayed in a manner analogous to the hydrogen-flame detector chromatogram. The relative response of the total *m/e* value per peak is identical, insofar as can be measured, to the hydrogen-flame detector. The mass spectrometer is designed to be changed from e.i. to c.i. in a few minutes. For c.i. studies, both methane and ammonia were injected at 1 torr. The system allows the (g.l.c.–m.s.) total *m/e* chromatogram to be examined, and any of the 5-s interval, mass-spectral scans to be printed from the data storage.

Derivatization procedure. — Reducing sugar(s) (1 to 30 mg) and hydroxylamine hydrochloride (60% of the sugar, by wt.) are weighed in a small, screw-capped vial. Pyridine (0.2 mL) and a micro-stirring bar are added to the vial, which is then heated for 20 min in a bath at 70°, with magnetic stirring. Acetic anhydride (0.1 mL) is added, and heating and stirring are continued for a further 20 min. The mixture is then partitioned between chloroform (1 mL) and water (2 mL), and the chloroform layer is removed, washed with water (2 mL), dried (Linde type 3A molecular sieve) for 10 min, and injected directly into the g.l.c. column. Insofar as can be determined, the reaction temperature (in the range of 65 to 80°), the solvent proportions (within ±30%), and the times (20 to 30 min) are not critical, and such changes have no effect on the relative peak-integrals of the derivatives, and do not cause the formation of additional peaks in the gas–liquid chromatogram. The exceptions are found when there are present *N*-acetylhexosamines (40 min for each derivatization step), and hexosamines (6 h for oxime formation; 40 min for PAAN formation). When the chloroform solution of the derivative is to be stored for more than a day, the molecular sieves should be removed. The compounds, either in chloroform solution, or after the solvent has evaporated, are stable for weeks. If, after several months, additional g.l.c. peaks of longer retention-time are observed (presumably due to hydrolysis of acetyl groups), the original chromatogram of the solution may be restored by rederivatizing the solution (but omitting the hydroxylamine hydrochloride reagent).

Gas–liquid chromatography. — All columns were packed by applying a slight vacuum, pouring in the packing, and sharply tapping on the side of the column.

Neopentyl glycol succinate (3%) on columns of acid-washed Chromosorb W (60–80 mesh) was conditioned, with carrier-gas flow, for 24 h at 200°, followed by 4 to 6 h at 250°. Glass columns (2 mm i.d.) of various lengths (1.2, 1.8, and 3.0 m) were employed. Although longer columns gave somewhat improved resolution, the temperature program employed is a major factor for successful separations. A good program (see Table I, column 1) for a glass column (2 mm i.d. × 1.23 m) is 140 to 250° at 3°/min, with a nitrogen flow-rate of 32 mL/min. A second program, for a column (2 mm i.d. × 3.0 m) is: 140°, hold for 6 min, then raise by 1°/min to 250°, with a nitrogen flow-rate of 24 mL/min, this program giving better resolution, but requiring 110 min. In all cases, the detector and injector temperatures were 280°. When 1 mg of a single reducing-sugar was derivatized, and the chloroform phase (~1 mL) used, a 1-μL aliquot gave essentially full-scale deflection (hydrogen-flame detector) on the most-sensitive g.l.c. setting. The peak definition, noise, and base-line drift for such conditions may be seen in Fig. 1. The capacity of the NPGS columns is quite large, as a 100-fold increase of injected PAAN derivative results in little peak-broadening. In general, 1–5 μL of chloroform solution is injected. It is possible to inject the pyridine–acetic anhydride mixture directly onto the g.l.c. column, although somewhat more solvent-tailing occurs than with the chloroform extract. The g.l.c. packing is quite stable, lasting for hundreds of injections with no protection against oxygen or water. On occasion, after many injections of hydrolyzate, the resolution of the column dropped, but, on removing the first few cm of blackened packing, the columns performed normally. Mass spectrometry requires somewhat larger peaks than hydrogen-flame detectors. Peaks representing 3 to 4 μg are necessary for good e.i. spectra, and 10 to 12 μg for good c.i. spectra.

Columns of 2% of OV-17 on Chromosorb W HP (80–100 mesh) were conditioned for 24 h at 300°, followed by 6 h at 340°. Two programs were employed: the first used 130 to 300° at 5°/min with a nitrogen flow-rate of 22 mL/min through glass columns (1.23 m × 2 mm i.d.). The injector and detector were held at 330°. The second program used identical conditions, except that the temperature rise between 130 and 300° was programmed at 20°/min. The relative detector-responses for the columns of OV-17 were essentially the same as those of the NPGS columns, except for the dramatic increase in the response for the hexosamine PAAN derivatives Injection of samples containing more than 10 μg of a specific compound results in loss of peak symmetry.

A series of derivatizations was studied, involving D-glucose and 2-amino-2-deoxy-D-glucose (or its *N*-acetyl derivative) at the 3-mg level in 200 μL of pyridine with 6 mg of NH_2OH, followed by 100 μL of acetic anhydride. The time for formation of oxime was varied from 20 min to 6 h, followed by a 20-min, nitrile-formation step Chloroform extracts (1 μL containing ~0.3 μg of each saccharide) were chromatographed, the peak areas being compared. On employing D-glucose and 2-acetamido-2-deoxy-D-glucose as starting sugars, the ratio of the peak area for 2-acetamido-2-deoxy-D-glucose PAAN to that for the D-glucose PAAN (based on equal weights of starting sugars) was 0.73 for the 20 min through 6 h, oxime-formation step. When

2-amino-2-deoxy-D-glucose hydrochloride and D-glucose were employed in a similar series of derivatizations, the ratio of the peak area for 2-acetamido-2-deoxy-D-glucose to that for D-glucose was as follows: 20 min (oxime step), 0.10; 40 min, 0.25; 1 h, 0.34; 2 h, 0.60; 3 h, 0.83; 4 h, 0.87; and 5 h, 0.87. No change in the hydrogen-flame detector (H-f.d.) ratio for D-glucose: 2-amino-2-deoxy-D-glucose was observed when mixtures of the two sugars were subjected to a 5-h, oxime-formation step and then different nitrile-formation times (20 min to 80 min) were employed. 2-Amino-2-deoxy-D-glucose (mol. wt. 179) and its *N*-acetyl derivative (mol. wt. 221) yield the same PAAN derivative, and the data showed that the amount of product, on a molar basis, was indeed the same.

On comparing 2-amino-2-deoxy-D-glucose to D-glucose on a wt./wt. basis, both compounds having essentially the same molecular weight, the PAAN derivative of the former gave a somewhat lower, H-f.d. response (0.87). The H-f.d. response-ratio for a mixture of D-glucose and 2-amino-2-deoxy-D-glucose hydrochloride was determined through a series of dilutions (the solid mixture being diluted with solid hydroxylamine hydrochloride). Starting with 3 mg of each sugar and 5 mg of hydroxylamine hydrochloride, the solid mixture was progressively diluted with more of the solid hydroxylamine hydrochloride; 3-mg samples of the mixture were then derivatized, and the solutions concentrated to approximately equal concentrations, and subjected to g.l.c. The D-glucose yielded a derivative with no noticeable decrease in the percentage yield as expressed by the H-f.d. response. As the injection front, relative to the peak of the derivative, increased with smaller samples, the lower limit for convenient, quantitative chromatography was a dilution corresponding to 3 μg of starting D-glucose. This involved concentrating the 1-mL chloroform extract almost to dryness with a stream of nitrogen, redissolving in 25 μL of chloroform, and injecting 6-μL portions. Owing to the low volatility of chloroform relative to those of the PAAN derivatives, such concentrations can be employed with little loss of derivative (or selective loss in the case of mixtures).

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