The Identification of Aldoheptose Sugars

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Ketoheptose sugars have long been known to occur in plants, both free and in phosphorylated form (references cited by MacLennan & Davies, 1957). Aldoheptose sugars have been encountered only during the last few years and have been found as constituents of polysaccharides isolated from Gram-negative bacteria. Only since simple methods have become available for the detection of heptose sugars has it become apparent that they are of frequent occurrence in these polysaccharides. It is also clear that in some instances they have been overlooked in the past, or incorrectly identified as one of the commonly occurring hexoses.

In only three cases have aldoheptose sugars been isolated from natural products and identified. From a *Shigella flexneri* polysaccharide, L-glycero-D-manno-heptose was obtained by Slein & Schnell (1953). The same sugar was isolated by Weidel (1955) from *Escherichia coli* cell walls. A *Chromobacterium violaceum* polysaccharide was described by Davies (1955) and D-glycero-D-galacto-heptose was subsequently isolated from the material (MacLennan & Davies, 1957).

Aldoheptoses have been detected in polysaccharides isolated from the following bacteria: *Shigella sonnei* (Jesaitis & Goebel, 1952), *E. coli* strains (Weidel, Koch & Bobosch, 1954; Fromme, Lüderitz & Westphal, 1954), *C. violaceum* strains (Davies, 1955), *Pasteurella pestis* (Davies, 1956), *P. pseudotuberculosis* and *P. septica* (Davies, 1957), *P. haemolytica*, *Neisseria catarrhalis*, *Haemophilus* spp., etc. (Crumpton, Davies & MacLennan, in preparation). In some, for example, *P. pestis*, the heptose is the major sugar constituent of the polysaccharide.

The simplest method of detection is the sulphuric acid-cysteine reaction described by Dische (1953, 1954), in which polysaccharides without prior hydrolysis give characteristic absorption curves due to their monosaccharide constituents; aldoheptoses give an absorption maximum at 505 m μ .

Paper chromatography can be used for detection; the anisidine-spray reagent of Hough, Jones & Wadman (1950) has been found to give a characteristic colour with aldoheptose sugars (greenish purple) which is quite different from that given by any other class of sugar. Provisional identification can also be made by this means, which is especially

valuable when only small amounts of material are available, as is frequently the case with bacterial products. Unfortunately, the heptose sugars are not readily available for comparison with unidentified components. There are records of *R* values for a few aldoheptoses in the literature (Hirst & Jones, 1949; Isherwood & Jermyn, 1951) but the lack of data for the remainder of the sixteen configurations makes these of only limited value for identification.

In the course of examining the heptose constituents of a number of polysaccharides, twelve different aldoheptoses have been obtained through the generosity of donors acknowledged below. Some chromatographic data which have accumulated are presented in this communication in such a way that possible identifications may be made without using the known heptoses themselves as standards for comparison.

For comparison with isolated samples, infrared-absorption spectra of eight aldoheptoses have already been published (MacLennan & Davies, 1957).

MATERIALS AND METHODS

Sugars. Aldoheptoses and certain other sugars or derivatives from which solutions of the free sugar could be obtained were kindly supplied by Dr Richtmyer, National Institutes of Health, Bethesda, Md., U.S.A. and Dr Isbell, National Bureau of Standards, Washington, D.C., U.S.A. Acetyl derivatives of D-idose, D-glycero-D-gluco-heptose and D-glycero-D-manno-heptose were deacetylated with barium methoxide as described by Isbell (1930); D-glycero-D-altro-heptose was prepared from its aldonic acid lactone by reduction with sodium amalgam according to the method of Isbell (1953).

All of the common sugars were commercial samples; of the large number examined only a few have been found useful as index sugars.

Heptose detection. The H₂SO₄-cysteine reaction of Dische (1953) was used. To amounts of sugar in water (1 ml.), 4.5 ml. of H₂SO₄ (conc. A.R. H₂SO₄ diluted with one-sixth of its vol. of water) was added, tubes being agitated in a cooling bath during the addition of acid. Reaction mixtures were then heated at 100° for 3 min., cooled and 0.1 ml. of 3% cysteine hydrochloride was added. After mixing they were allowed to stand at 20°. For each amount of sugar three tubes were set up, cysteine being added to two of these tubes. Optical densities (*D*) were measured in a Unicam SP. 600 spectrophotometer after 22 hr., with a 1 cm. cell. Figures recorded are *D*₅₀₅-*D*₅₄₀ with cysteine less *D*₅₀₅-*D*₅₄₀ without cysteine, $\times 1000$.

Chromatography. Single-dimensional descending paper chromatography was used with Whatman no. 1 paper and four solvent systems (basic, acidic, neutral and phenolic): butanol-pyridine-water (6:4:3), propan-1-ol-benzyl alcohol-formic acid as used by Giovannozzi-Sermanni (1956), 95% (v/v) acetone-water and phenol-aq. NH_3 soln. For detection anisidine hydrochloride (Hough *et al.* 1950) was used for reducing sugars and ammoniacal AgNO_3 soln. or periodate soln. (Wolfrom & Miller, 1956) for sugar alcohols. Sugars were applied to the paper in 100 μg . amounts in volumes of 10 μl .

EXPERIMENTAL AND RESULTS

Sulphuric acid-cysteine reaction

This has been fully described by Dische (1953). Different aldohexoses give the same absorption peak but the intensity of absorption differs markedly in different cases. It can therefore be used for estimation only when the identity of a sugar is known. Estimations are reproducible and calibrations have not varied over a series of tests. The optical-density values follow a straight-line relationship with weight of sugar over the range 50–250 μg . Typical figures, obtained as described in Materials and Methods, for the two heptoses already isolated are: D-glycero-D-galacto-heptose 340–350 (100 μg .), 675–685 (200 μg .); D-glycero-L-manno-heptose 205–215 (100 μg .), 400–415 (200 μg .).

Paper chromatography

It is becoming generally accepted that *R* values alone are not sufficiently reliable for the identification of sugars on paper chromatograms. The order in which sugars separate, however, is wholly reproducible if an order can be established. It has not been possible to separate some of the slower-running heptoses in some solvents during the course of a single run, but much better results have been obtained by running a solvent to the bottom of the paper, drying thoroughly and running a second or even a third time (Jeanes, Wise & Dimler, 1951). By this method *R* values cannot be reproduced

within sufficiently narrow limits to be of any real value, but a reliable order of separation can be obtained.

The results of Isherwood & Jermyn (1951) showed that the order of separation in different classes of sugars is the same from the point of view of homomorphology. This has now been amplified for the aldohexose series as compared with the aldohexoses and found to be true in a general way though not in detail. The order of separation of the aldohexoses is shown in Table 1.

By using the homomorphology correlation, and by interpolating readily available sugars or hexitols between the positions occupied by the aldohexose sugars (data shown in Table 2) it is possible to deduce the probable identity of aldohexoses without using the authentic sugar for comparison.

In order to obtain a sufficiently good separation, sugars were grouped into those which run faster or slower than glucose. Those running faster were separated in single runs when butanol-pyridine-water or aq. acetone were used and in double runs in phenol-aq. NH_3 soln. or propan-1-ol-benzyl alcohol-formic acid. Those running more slowly than glucose were separated in triple runs with the last solvent system and in double runs in the other three solvent systems.

Aldohexoses generally run more slowly than aldohexoses but overlap the hexose region. In butanol-pyridine-water a number of interpolations were possible, in the other solvent systems fewer common sugars were available in the region of those aldohexoses which ran more slowly than glucose.

In Table 2 *R_F* values are given only to indicate the region in which a sugar travels, and are not on a linear scale since the intervals between successive sugars are not shown.

DISCUSSION

The presence of an aldohexose sugar in a polysaccharide can most easily be demonstrated by the sulphuric acid-cysteine reaction, but possible interference by pentoses should be allowed for (Dische, 1953). In most cases confirmation can be obtained by the appearance on paper chromatograms sprayed with anisidine hydrochloride of a characteristic greenish purple spot, although this can be obscured by the presence of other sugars. To obtain an indication of identity by using the data in Table 2 it is preferable to separate a heptose component in sufficient amount on a thicker paper for elution and re-running in the solvents described.

The 12 D-aldohexoses for which positions are given include all of the C-1 to C-5 configurations; where both members of a pair have been used (-D-gluc- and -L-gluc-, -D-manno- and -L-manno-, -D-galact- and -L-galact-, -D-talo- and -L-talo-) it

Table 1. Separation of D-aldohexoses in different solvent systems on paper chromatograms, in order of increasing *R* value

Butanol-pyridine-water	Propan-1-ol-benzyl alcohol-formic acid	Acetone-water	Phenol-aq. NH_3 soln.
Galactose	Galactose	Galactose	Glucose
Glucose	Glucose	Glucose	Gulose
Allose	Gulose	Mannose	Galactose
Gulose	Allose	Allose	Mannose
Mannose	Mannose	Gulose	Altrose
Altrose	Altrose	Altrose	Allose
Talose	Idose	Talose	Idose
Idose	Talose	Idose	Talose

can be seen that the members of pairs run adjacent to one another in most cases and have suffered only minor displacements in the remainder. Comparing Tables 1 and 2 it will also be seen that the order of separation of hexoses and heptoses do follow the homomorphology pattern in most cases, again with a few minor displacements. The positions given are

the carefully checked results of a large number of runs in each solvent system, and it is considered that the regions in which the four missing aldoheptoses would lie could be judged with reasonable confidence; these four have yet to be synthesized (*D-glycero-L-gulo*-, *D-glycero-L-allo*-, *D-glycero-L-altro*- and *D-glycero-L-ido*-heptoses).

Table 2. Order of separation of aldoheptoses and other sugars on paper chromatograms

R_f values (distance travelled relative to glucose) are given only to indicate the region in which a sugar will be found and not as indices to the exact positions of any particular sugars. Sugars bracketed together do not separate significantly from one another.

R_f	Butanol-pyridine-water	R_f	Acetone-water
0.6	Cellobiose	0.5	D-Glucosamine
	D-Galactosamine		D-Glycero-L-galacto-heptose
	D-Glycero-L-galacto-heptose }		D-Glycero-D-galacto-heptose
	Maltose		D-Glycero-L-gluco-heptose
	D-Glycero-D-galacto-heptose		D-Glycero-D-gluco-heptose
	D-Glucosamine		D-Glycero-L-manno-heptose
	D-Glycero-L-gluco-heptose }	0.8	D-Glycero-D-manno-heptose
0.8	D-Glycero-D-gluco-heptose		D-Glycero-D-gulo-heptose
	Galactitol		D-Glycero-D-allo-heptose
	D-Glycero-D-allo-heptose }		D-Galactose
	D-Galactose	1.0	D-Glucose
	D-Glycero-D-gulo-heptose-		D-Mannitol
	D-Sorbitol		D-Sorbitol
	D-Glycero-D-manno-heptose		D-Glycero-L-talo-heptose
	D-Mannitol		D-Glycero-D-altro-heptose
	D-Glycero-L-manno-heptose }		D-Mannose
1.0	D-Glucose		D-Glycero-D-talo-heptose }
	D-Glycero-L-talo-heptose	1.5	L-Arabinose
	D-Glycero-D-altro-heptose	2.0	D-Glycero-D-ido-heptose
	D-Allose		D-Xylose
	L-Sorbose		L-Fucose
	D-Mannose }		D-Lyxose
	L-Arabinose }		
	D-Glycero-D-talo-heptose		
1.3	D-Xylose		
	D-Glycero-D-ido-heptose }		
	N-Acetyl-D-glucosamine }		
	D-Ribose		
1.5	L-Rhamnose		
R_f	Phenol-aq. NH_3 soln.	R_f	Propan-1-ol-benzyl alcohol-formic acid
0.8	D-Glycero-L-gluco-heptose }		Melibiose
	Melibiose	0.4	Cellobiose
	D-Glycero-D-gulo-heptose }		Maltose
	D-Glycero-D-galacto-heptose }		D-Glycero-L-gluco-heptose }
0.9	D-Glycero-D-gluco-heptose		D-Glycero-L-galacto-heptose }
	Cellobiose		D-Glycero-D-galacto-heptose }
	Maltose		D-Glucosamine }
	D-Glycero-L-galacto-heptose		D-Galactosamine }
	D-Glycero-D-manno-heptose }	0.7	D-Glycero-D-gluco-heptose
	D-Glycero-D-altro-heptose }		D-Glycero-D-gulo-heptose
1.0	D-Glucose		D-Glycero-D-manno-heptose
	D-Glycero-L-manno-heptose		D-Glycero-D-allo-heptose
	D-Glycero-D-allo-heptose }		D-Glycero-L-manno-heptose
	D-Gulose		D-Galactose
	L-Sorbose		D-Glycero-D-altro-heptose
	D-Glycero-D-ido-heptose }	1.0	D-Glucose
	D-Galactose		D-Glycero-L-talo-heptose
	D-Xylose		L-Sorbose
1.2	D-Glycero-D-talo-heptose		D-Glycero-D-talo-heptose }
	D-Glycero-L-talo-heptose }		D-Mannose
	D-Lyxose	1.3	D-Glycero-D-ido-heptose
1.4	L-Arabinose		L-Arabinose }
	D-Ribose	1.6	D-Xylose }

Thus an aldohexose travelling between glucosamine and maltose in butanol-pyridine-water could be none other than *D-glycero-D-galacto-heptose* (or its optical enantiomorph *L-glycero-L-galacto-heptose*) since the missing heptoses could all be confidently expected to travel faster than this. Similarly, *D-glycero-D-talo-heptose* or its optical enantiomorph could be checked between different pairs of common sugars in each of the four solvent systems. By these methods a probable identification or a small number of alternatives could be deduced for any aldohexose sugar.

Whereas the aldohexoses almost always occur in Nature in the *D*-form, the two aldohexoses known thus far from natural sources are one *D*- and one *L*-. The determination of optical form can only be made on the isolated sugars or their derivatives.

SUMMARY

1. The behaviours of 12 of the 16 *D*-aldohexose sugars have been recorded for four different solvent systems on paper chromatograms.

2. The positions of a number of common sugars have been determined in relation to the hexoses so that an idea of the identity of an unknown hexose may be gained without reference to authentic samples.

3. The order of separation of the eight *D*-aldohexoses and their homomorphous *D*-aldohexoses are in tolerably good agreement.

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Heterogeneity of Normal Adult Human Haemoglobin

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Studies with three different techniques have indicated that normal adult human haemoglobin is heterogeneous. These techniques are: solubility in variable solvents (Roche, Derrien & Roques, 1953*a, b*; Roche, Derrien, Reynaud, Laurent & Roques, 1954; Roche & Derrien, 1953); electrophoresis in starch slabs (Kunkel & Wallenius, 1955); and chromatography on columns of ion-exchange resins (Huisman & Prins, 1955; Morrison & Cook, 1955).

In the variable-solvent test a system is set up containing a constant amount of protein at a

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constant pH and varying concentrations of salts. The extent of precipitation of the protein or proteins depends upon the salt concentration in the way described by the Cohn equation, $\ln S = \beta - K(\Gamma/2)$, where S is the solubility in g./l., K is the salting-out constant, $\Gamma/2$ is the ionic strength of the precipitating salt and β is the extrapolated intercept of the linear portion of the curve on the ordinate ($\log S$) axis (Cohn, 1925). Using phosphate buffer, pH 6.5, as the precipitating salt, Roche and his colleagues (Roche *et al.* 1953*a, b*, 1954) described six breaks in the salting-out curve of normal adult carboxy-haemoglobin, and concluded that it contains three adult and three small foetal components. This interpretation has been criticized by Itano (1956), on the grounds that changes in the solid phase, which are