Determination of the products of aldolization of trioses

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Received 18 February 1974

Depending upon the conditions of base-catalyzed aldolization of trioses D,L-fructose, D,L-sorbose, and D,L-dendroketose are formed in different proportions. A method has been developed for the determination of thus produced ketohexoses using gas chromatography of per-*O*-TMS derivatives of the sugars themselves and of the polyols derived therefrom. The aldolization products can also be separated by paper chromatography, and, on a preparative scale, by column chromatography on cellulose.

Based on the analytical study, an attempt to explain the nature of dendroketose is also described.

The determination of the products of aldolization of trioses was described by *Schmitz* [1] and *Fischer* and *Baer* [2]. Having found out that the aldolization of glyceraldehyde gives a mixture of fructose and sorbose the quoted authors determined the sugars produced gravimetrically in the form of the corresponding phenylhydrazone derivatives. In their study D(+)-glyceraldehyde was used as a substrate and the course of the reaction was followed polarimetrically. *Meyerhof* and *Schulz* [3] investigated the composition of the equilibrium mixtures of the aldolization of glyceraldehyde and dihydroxyacetone in sodium orthophosphate and found the ratio of hexoses to trioses to be greater than 11 : 1. The incorporation of deuterium into base-catalyzed aldolization products has also been described [4].

Dendroketose — a ketohexose, the branched-chain nature of which was deduced on the basis of the properties of its various derivatives, was described by *Utkin* [5].

Other authors [6, 7] monitored the course of their kinetic aldolization studies spectrophotometrically using the known thiourea-resorcine [8] and anthrone [9] method which is specific for hexoses. Taking advantage of the enzymic conversion of D-fructose it was possible to determine the ratio of fructose to sorbose and dendroketose in the case of the aldolization of D(+)-glyceraldehyde [7].

With the aim to make a complete evaluation of the kinetic study of aldolization reactions of trioses [10] we have attempted to elaborate an analytical method of the determination of all the reaction products in the mixture with the starting trioses. Of the methods tried most satisfactory results have been obtained by gas chromatography.

In view of its great separation power, gas chromatography has been widely used as an efficient tool in the analysis of sugar mixtures [11—13]. Owing to some irreversible adsorption on the column material, low volatility and thermal instability of polyhydroxy compounds having more than three hydroxyl groups unsubstituted, this class of substances is not amenable to direct analysis and is normally chromatographed in the form of suitable derivatives. For this purpose per-*O*-trimethylsilyl ethers (TMS) [14, 15] are most suitable as these are readily formed under mild conditions; they show sufficient thermal stability and, owing to their low polarity, can be separated on a variety of stationary phases.

Experimental

Instruments and materials

Gas chromatography was carried out with a Hewlett—Packard instrument, Model 5754 G, equipped with a flame detector. The temperature of the injection port and that of the detector was kept 50°C higher than that of the column. The following columns were used: *A*. 180 cm × 0.31 cm (o.d., stainless steel) packed with 3% OV-17 (Applied Science Laboratories) on Gas Chrom Q, 100—120 mesh (C. Erba); *B*. capillary column 45 m × 0.2 mm (i.d., stainless steel) coated with OV-17; *C*. 300 cm × 0.31 cm (o.d., stainless steel) packed with 1% XE-60 (Hewlett—Packard) on Gas Chrom Z, 80—100 mesh (Applied Science Laboratories). Isothermal conditions with nitrogen as the carrier gas were used throughout. Quantitative evaluation of the recording was done using a planimeter (Reiss, VEB, Bad Liebenwerda, Model 3005). In the preparation of dendroketose the reaction was followed and the pH-stating measurements were done with an automatic titrator TTT-2 equipped with a magnetic valve MNV 2 (Radiometer, Copenhagen) combined with a glass electrode EA 109 U, calomel electrode EA 404, and an universal thermostatted titration vessel EA 880 (Metrohm, A.G., Herisau). The temperature during aldolization reactions was controlled with the accuracy of ±0.02°C. The u.v. spectra for water solutions were measured at 25°C with an ORD/UV-5 JASCO spectrophotometer in 0.5-cm cells. Polarography was done using an OH 102 instrument (Radelkis, Budapest).

Chemicals

D-Fructose, L-sorbose, D-mannose, and D-glucose (commercial products of reagent grade purity, Lachema, Brno) used as standards for chromatography were recrystallized from ethanol—ether. D,L-Dendroketose was prepared by pH-stating aldolization reaction from dihydroxyacetone and purified by chromatography on powdered cellulose. D,L-Glyceraldehyde purum, dihydroxyacetone, puriss., isobutylamine, purum, and *o*-phenylenediamine, reagent grade were obtained from Fluka A.G. (Buchs) and used without further purification. Solutions of sodium hydroxide, hydrochloric acid, and buffers were standard solutions of the "Titrisol" (Merck, A.G., Darmstadt) series. Trimethylchlorosilane, hexamethyldisilazane, and pyridine, silylation grade, used for making the TMS derivatives as well as sodium borohydride used for the conversion of sugars into the corresponding alditols was obtained from Lachema (Brno).

Working procedures

Aldolizations were carried out by pH-controlled reactions in a temperature-controlled vessel in the manner similar to the one used in previous kinetic studies [10]. In order to simulate the kinetic conditions the reactions were run under nitrogen and with the exclusion of atmospheric carbon dioxide. The decrease in the content of trioses was monitored polarographically. Samples were withdrawn at intervals from the reaction media in such an amount that the concentration of the trioses in the analyzed sample was, taking into account the starting concentration, 5×10^{-4} M. The polarographic analysis was performed in an 0.3 M isobutylamine buffer in the presence of 10^{-2} M *o*-phenylenediamine [16, 17]. The reaction was terminated, or, when complete, neutralized to pH 6 by an addition of 1 M hydrochloric acid (by a pH-stat).

Standard mixtures of D,L-dendroketose, D-fructose, and L-sorbose were also chromatographed. Samples for the analysis were prepared by evaporation under reduced pressure

at 30°C without deionization, silylation of about 5—30 mg of the material [14] and the amount of sorbose was determined on column *A* at 130°C (14 ml N_2/min). The amount of fructose was determined on the capillary column *B* at 190°C using a 1 : 100 inlet splitter and an inlet pressure 0.8 atm. The separation of dendroketose from the mixture of fructose and sorbose as well as the determination of dendroketose in the form of the corresponding alditol TMS derivatives was achieved using column *C* at 130°C (16 ml N_2/min). The samples were prepared in a similar manner after the reduction of ketohexoses with sodium borohydride, neutralization with Dowex 50 W, H^+ form, and the removal of boric acid as its methyl ester.

Paper chromatography on Whatman No. 1 paper was done by the descending technique using an acetone—*n*-butanol—water (7 : 2 : 1) mixture [18]. Acetone and *n*-butanol (reagent grade) were redistilled before use. When trioses were present the chromatograms were allowed to develop for about 5 hrs without allowing the solvent to drip off the paper. In the separation of dendroketose, fructose, and sorbose the elution was allowed to proceed for 20 hrs. The detection was done by spraying with diphenylamine [19].

Preparative chromatography of the products of aldolization reactions was done on columns (5 × 90 cm) packed with powdered cellulose using the above-mentioned solvent system. When the aldolization was complete the sample containing originally about 7 g of trioses in a water solution of sodium hydroxide was neutralized, evaporated, dissolved in the solvent system (undissolved solids were separated by decanting the supernatant liquid), put on the top of the column, and chromatographed for 4 days. The fractions containing the wanted material were collected, concentrated, dried over phosphorus pentoxide, and weighed.

Results

Preparation of dendroketose and of its polyols

pH-Statically controlled (pH ≐ 12) aldolization of a 1 M solution of dihydroxyacetone (7.2 g) in 0.01 M sodium hydroxide was allowed to proceed for 70 min at 25°C and this afforded by chromatographic separation 5.6 g (78%) of pure dendroketose. A mixture of polyols (0.412 g, 81%) was obtained from dendroketose (0.5 g) by reduction with sodium borohydride.

Aldolization of glyceraldehyde (0.5 M solution) was done in a similar manner while the reaction was allowed to proceed for 60 min. Conversion of glyceraldehyde gave 82% of a mixture of fructose and sorbose together with 18% of dendroketose. The amounts of these substances obtained from dihydroxyacetone were 10 and 90%, respectively. Sorbose and fructose could not be separated under the conditions of preparative chromatography. About 100 mg amounts of the two decomposition products of dendroketose were also obtained.

Gas chromatography

Several systems were tried before OV-17 and XE-60 were selected as liquid phases for the analytical determination of dendroketose, fructose, and sorbose as well as for the studies regarding the composition and the behaviour of dendroketose.

Relative retention times on OV-17 of per-*O*-TMS ethers of the products of aldolization reactions of trioses are given in Table 1. Fig. 1 shows a typical example of an analysis on column *A* of the reaction products of aldolization reaction of dihydroxyacetone. When pure dendroketose obtained from dihydroxyacetone was chromatographed, the size

Table 1

Relative retention times of per-*O*-TMS ethers of the aldolization products of trioses and their polyols

Compound	Column		
	A 130°C	*B* 190°C	*C* 130°C
Fructose	0.65	0.70	
	0.73	0.72	
Sorbose	1.00[a]	1.00[b]	
	1.11		
1. Dendroketose	0.58	0.63	
2. Dendroketose	0.70	0.70	
3. Dendroketose	0.76	0.78	
4. Dendroketose	1.08	1.00	
Decomposition product DP_f	0.88		
Decomposition product DP_s	1.25		
Mannitol	0.92		0.98
Iditol	0.98		1.02
Sorbitol	1.00[c]		1.00[d]
Dendroketose-polyol (1)	0.79		0.77
Dendroketose-polyol (2)	1.08		0.81

a) 27.0 min; *b*) 29.4 min; *c*) 23.15 min; *d*) 9.29 min.

of the four peaks (*1*, *3*, *4*, and *6*) characteristic of dendroketose expressed in percent, based on the average area values, corresponds to 18.9, 48.7, 14.9, and 17.4, respectively. Dendroketose prepared from glyceraldehyde gave peaks corresponding to the same isomers in the the ratio of 33.3, 52.6, 10.9, and 3.2% showing thus that the amount of these components was different.

Using the capillary column *B* it was possible to obtain better resolution of the faster moving components so that the separation of fructose was sharper. The slower moving components were, however, still not satisfactorily separated from the last isomer of dendroketose.

TMS derivatives of the reduced dendroketose gave on OV-17 only two peaks.

As can be seen in Fig. 2, showing the separation of the polyols derived from dendroketose, sorbose, and fructose on column *C*, only two complex peaks are eluted from this column, one of which corresponds to dendroketose and the other one to a mixture of sorbose and fructose.

Paper chromatography

The composition of the reaction mixture was monitored by comparing it with a standard mixture of the studied substance. The R_F and R_{sorb} values as well as the colour of the spots produced by spraying with diphenylamine are given in Table 2.

It has been observed that the aldolization of glyceraldehyde affords a substantial amount of sorbose and fructose with fructose predominating, together with a little dendroketose. In the aldolization reaction of dihydroxyacetone the major reaction product is dendroketose; a small amount of fructose and sorbose in an approximate ratio 1 : 1 is also formed.

Table 2

Paper chromatographic mobilities and the colours produced by the detection with diphenylamine of the products of aldolization of trioses

Compound	R_F	R_{sorb}	Colour produced
Glucose	0.19	0.78	Dark-blue
Decomposition product DP_s	0.20	0.82	Brown
Mannose	0.24	0.95	Dark-blue
Sorbose	0.25	1.00	Brown-green
Fructose	0.29	1.12	Purple-red
Dendroketose	0.36	1.43	Yellow-green
Decomposition product DP_f	0.41	1.65	Brown
Glyceraldehyde	0.53	2.20	Gray-red
Dihydroxyacetone	0.65	2.60	Light-brown

The increase of the temperature during the conversion results in an increased amount of the decomposition products (DP_f and DP_s). The amount of these decomposition products is also greater in older samples of dendroketose stored dry or in solutions.

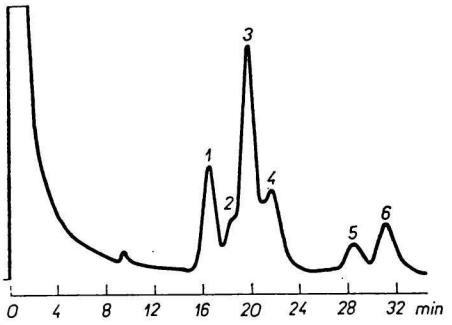

Fig. 1. Separation of the products of aldolization of dihydroxyacetone by g.l.c. in the form of the corresponding per-*-O*-TMS derivatives on column *A*.
Peak numbering: *1.*, *3.*, *4.*, *6.* isomers of dendroketose; *2.* fructose; *5.* sorbose.

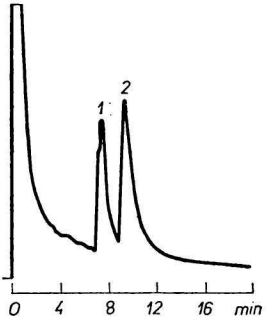

Fig. 2. Separation by g.l.c. of per-*O*-TMS derivatives of polyols derived from the products of aldolization of trioses on column *C*.
Peak numbering: *1.* complex peak of the polyols produced from dendroketose; *2.* complex peak of mannitol, sorbitol, and iditol.

Analytical application

For the analytical application three mixtures containing the following amounts (%) of the components were used:

Mixture	Dendroketose	Fructose	Sorbose
1	50	25	25
2	80	10	10
3	20	40	40

The maximum difference between the amount injected and the one determined was 9.6 relative percent.

It has been found that for the analytical purposes sorbose can be most satisfactorily determined on column *A*, dendroketose (after reduction) on column *C*, and fructose is calculated up to 100%.

Analysis of the reaction mixtures

The analysis of the reaction mixtures of the aldolization of trioses in the concentration range of 0.02—1 M performed in 0.01 M sodium hydroxide at 25°C by gas chromatography is given in Table 3. The decomposition products are included in the amount of dendroketose.

Table 3

The composition of the reaction mixtures of aldolization of trioses in 0.01 M sodium hydroxide at 25°C as found by gas chromatography

Starting trioses		Reaction products		
Glyceraldehyde [M]	Dihydroxyacetone [M]	Dendroketose [%]	Fructose [%]	Sorbose [%]
0.5	—	19	61	20
0.02	—	11	66	23
0.5[a]	—	17	62	21
0.5[b]	—	16	63	21
—	1.0	94.5	2.5	3
—	0.5	90	5	5
—	0.05	86	7	7
—	0.5[a]	90.5	5	4.5
—	0.5[b]	88	5.5	6.5
0.4	0.1	8	69	23
0.25	0.25	11	67	22
0.1	0.4	36	49	15

a) In 0.05 M-Na_2CO_3; *b*) at 50°C.

It has been found by experiments run separately when the reaction was terminated that in 0.01 M sodium hydroxide at 25°C 53% of dihydroxyacetone (determined polarographically) from the starting M solution has reacted within 4 min. The respective value found for 0.5 M glyceraldehyde and the duration of the reaction 9 min was 55%.

The presence of the starting trioses in the analyzed samples does not interfere with the gas chromatographic analysis as this class of compounds when converted to the corresponding per-*O*-TMS derivatives is eluted, under the given conditions of the analysis, together with the solvents. The reaction mixture of the aldolization of dihydroxyacetone contained, in addition to 47% of the starting triose, 51.8% of dendroketose, and 0.6% of both sorbose and fructose. Using glyceraldehyde as a starting material the amounts of the triose, dendroketose, sorbose, and fructose present in the reaction mixture were 45, 1.8, 13.3, and 39.9%, respectively.

Formation of dendroketose decomposition products

A very small amount of decomposition products is formed at 25°C when glyceraldehyde is used as the starting material. From dihydroxyacetone 2—5% of these substances is formed in the range of 15—35°C. The amount of these products increases with the

elevation of the temperature and prolonged time of the reaction. About 4% of the decomposition products could be found in an isolated sample of dendroketose stored in a desiccator over a period of 3 months. The formation of decomposition products is accompanied by discoloration of the samples. When an older sample of dendroketose was purified, these decomposition products were isolated and studied by chromatography and u.v. and i.r. spectrometry. Chromatographic data related to the two decomposition products (one moving faster — DP_f — and the other slower — DP_s — than dendroketose itself) are given in Tables 1 and 2.

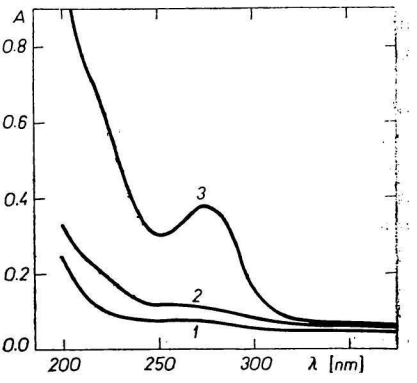

Fig. 3. Ultraviolet spectra of water solutions of dendroketose and its decomposition products.
Curve numbering: *1.* dendroketose; *2.* decomposition product DP_s; *3.* decomposition product DP_f.

The u.v. spectrum of pure dendroketose and of the two decomposition products is given on Fig. 3. The spectrum of pure dendroketose contains a slight peak at 273 nm. The spectrum of DP_s shows three poorly pronounced peaks at 212, 273, and 320 nm. The same absorption bands present in the spectrum of DP_f are stronger by an order, the peak at 273 nm being much more pronounced than the ones at 212 and 320 nm.

On the i.r. spectrum of dendroketose (film) a slight peak at 1740 nm corresponding to the free carbonyl group can be seen. When the spectrum was run on a lyophylized sample in a KBr pellet this absorption was more pronounced.

Discussion

Apart from the determination of the major components in the mixture of aldolization of trioses, the analytical procedure described here makes also possible to determine quantitatively the products of decomposition of dendroketose. Although it is less accurate than the methods based on spectrophotometry [8, 9], it is very specific for the sugars studied. Compared to the combined spectrometric-enzymic method [7] the new method lacks stereospecifity. The enzymic method, on the other hand, is suitable merely for the determination of D-fructose. The main source of errors in the now described method lies in the indirect determination of fructose. The quantitative data given in Tables take into account the response of the standard compounds as well as the inaccuracies of planimetric evaluation. The gas chromatographic method is suitable for the analysis of mixtures of the aldolization of trioses which were allowed to proceed to completion. For the mixtures containing trioses, gas chromatography has to be combined with polarography [17, 18] by which the amount of the starting trioses can be conveniently determined. As only relative values are obtained, the presence of salts does not interfere with

the determination. Neither do trioses in their monomeric form but dimers do and, therefore, the samples should be silylated immediately after concentration and drying. When partially aldolized samples are to be reduced with sodium borohydride pH should be kept at values lower than 10 so that the reaction terminated by neutralization could not proceed.

The suitability of the presented analytical procedure was practically verified in a kinetic study of aldolization reaction of trioses [10]. It has been found that of the changed reaction conditions only the concentration of the starting trioses has an effect upon the ratio of the main to side-reaction products. This is in agreement with the fact that the aldolization of trioses is a system of the first and second order competitive-consecutive reactions.

Dendroketose decomposes on standing even at room temperature. The u.v. spectrum of the decomposed product DP_f contains absorption bands of $n-\pi$-transitions of an isolated double bond and, to a smaller extent, also of a double bond conjugated with the carbonyl group. The substance DP_s having a double retention time shows virtually no such absorptions. It can be assumed that the substance DP_f is a product of dehydration and the substance DP_s of consecutive condensation reactions of decomposition of dendroketose.

Gas chromatography of dendroketose shows four peaks corresponding to the eight possible isomers in their cyclic forms (three chiral centra are formed by cyclization, compared to one in the open-chain form). These are the *erythro-*, *threo-*, -D-, and -L-isomers with each of them possibly forming α and β anomers. Since the gas chromatography does not distinguish between L- and D-forms the number of peaks observed is reasonable provided that the equilibrium between the cyclic and open-chain forms is shifted virtually completely to the cyclic forms. This was confirmed by i.r. and u.v. spectrometry. The conversion to the corresponding alditols reduces the number of chiral centra to two with the formation of *erythro-*, *threo-*, -D-, and -L-configurations. This, consequently, gives two peaks of polyols of dendroketose. The peak-isomer assignment could not be made as yet.

Acknowledgements. The authors are indebted to Drs K. Babor and V. Bílik for helpful discussions, Dr M. Fedoroňko for making possible to carry out this investigation and to Mr A. Sedlák for skillful technical assistance.

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Translated by P. Kováč