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STUDIES ON THE RING STRUCTURES OF KETOSES BY MEANS OF GAS CHROMATOGRAPHY AND MASS SPECTROSCOPY*

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SUMMARY

Persilylated fructose was separated into five components by means of gas chromatography.

Individual compounds were isolated with the help of micropreparative gas chromatography and characterized by infrared and mass spectroscopy. Components which, because of their small quantity and chemical lability, could not be isolated in this manner were investigated with the instrumental combination of gas chromatography and mass spectroscopy.

Of the five persilylated fructose derivatives two represent the pyranoside, two the furanoside and one the open-chain form.

INTRODUCTION

In a previous study the enzymatic liberation of glucose and fructose from sucrose was investigated using gas chromatography[1]. It was observed that upon persilylation fructose yielded a number of peaks.

In this study it can be shown that after exhaustive persilylation fructose exhibits five components in equilibrium with one another (see Figs. 1a and 2). In addition to these five compounds two incompletely silylated products are observed when a shorter silylation time is used (see Fig. 1b).

Using a gas chromatography–mass spectroscopy combination, the group of peaks 1–3 shown in Fig. 1 resolved into an additional component 3a (see Fig. 2) which appeared after peak 2 and was present in very low concentration.

The individual components were isolated as their trimethylsilyl ether derivatives by means of a micropreparative gas chromatograph, and subsequently characterized, as far as possible, with infrared and mass spectroscopy. Because of their small quantity and ready decomposition, some substances could not be isolated in this manner. These were investigated using an instrumental combination of gas chromatography and mass spectroscopy.

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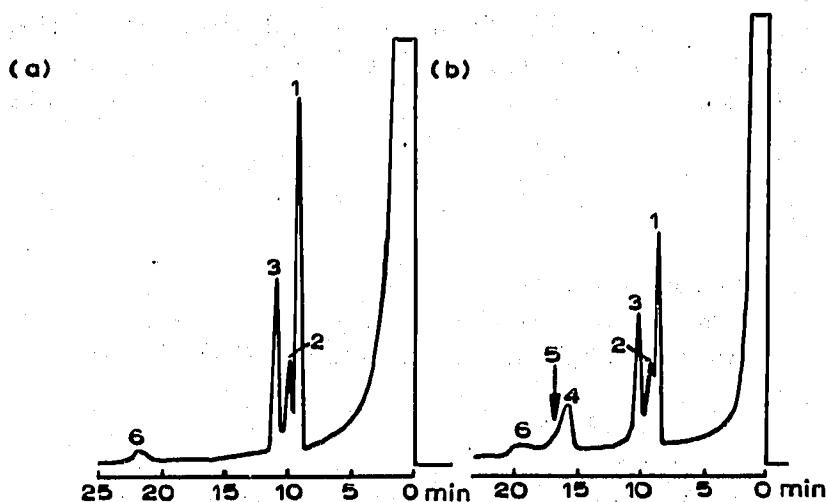

Fig. 1. Gas chromatogram of mutarotated fructose (in water, pH: 5.4) after silylation. (a) 24 h silylation; (b) 1 h silylation. Gas chromatograms were run on an Aerograph "1520" with a split device for preparative work. Column conditions: EGS, 15% on Chromosorb W, 80–100 mesh; glass column 2 m, I.D. 2.7 mm; t_c: 145°, t_i: 255°, t_d: 245°; N_2: 45 ml/min.

Enzymatic hydrolysis of sucrose was performed by incubation of sucrose with sucrase-isomaltase complex*, during which samples were removed at different times and frozen in liquid air. This work is fully described in a previous paper[1]. It had already been suspected earlier that the furanoside and pyranoside forms were involved in the mutarotation of fructose according to ISBELL AND PIGMAN[2]. These authors explained the abnormally fast mutarotation of fructose by a pyranose–furanose

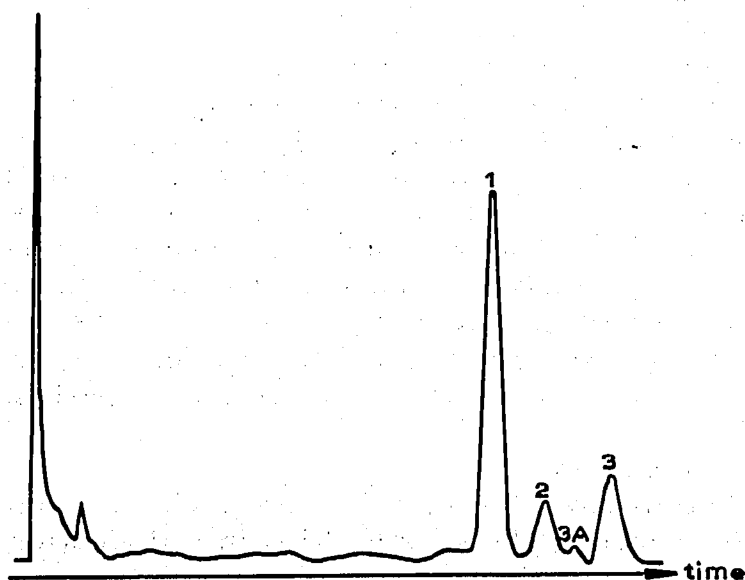

Fig. 2. Gas chromatogram of mutarotated fructose after silylation. Gas chromatograph–mass spectrometer combination: LKB "9000" EGS, 15% on Chromosorb W, 80–100 mesh; glass column 2 m, I.D. 2.5 mm; t_c: 150°; t_i: 255°, t_{sep}: 170°; He: 20 ml/min.

* Isolated from rabbit small intestine; courtesy of Prof. G. SEMENZA.

interconversion. GOTTSCHALK[3] was able to show that in aqueous solutions at 0° the equilibrium mixture contained 12% D-fructofuranose. From the corresponding kinetics he calculated that at 20° about 20% of this hexose existed in the furanoside form.

EXPERIMENTAL

Preparation of the samples for the equilibrium mixture

0.1 mg fructose is equilibrated in water for approximately 12 h. The sample is frozen by immersing the incubation tube in liquid air, lyophilized and silylated with 0.1 ml of silylation mixture.

Silylation

The fructose is converted to its O-trimethylsilyl ethers by the method of BENTLEY *et al.*[4] and completely silylated overnight at room temperature.

Preparation of permethylated fructose

The permethylated fructose is synthesized according to the method of KUHN *et al.*[5].

Isolation of methyl 1,3,4,6-tetra-O-methyl-β-D-fructofuranoside (I) and methyl 1,3,4,5-tetra-O-methyl-β-D-fructopyranoside (II) by means of micropreparative gas chromatography

The permethylated fructose is gas chromatographed on an EGS 15% column. The gas chromatogram obtained agrees with the analytical results of BAYER AND WIDDER[6]. The peaks from methyl 1,3,4,6-tetra-O-methyl-β-D-fructofuranoside and from methyl 1,3,4,5-tetra-O-methyl-β-D-fructopyranoside are isolated by means of micropreparative gas chromatography.

Isolation of peaks 1 and 4 as trimethylsilyl ethers from the reaction mixture

Crystalline fructose (10 mg) is dissolved in 20 ml of the silylation mixture and silylated for 1–2 h. Peaks 1, 4 and 5 are almost the only ones formed by this procedure; these are collected, by means of micropreparative gas chromatography, in an absorption tube about 15 cm long and 3 mm wide (I.D.). The procedure consists of injecting 10 µl samples into the gas chromatograph repeatedly until enough of each substance is collected for analysis by infrared and mass spectroscopy. Since peaks 4 and 5 are not completely separable, only the ascending part of peak 4 is collected for analysis. It is not possible to isolate the shoulder, labelled as peak 5, separately from peak 4.

Isolation of peaks 3 and 6 from the reaction mixture

Crystalline fructose (10 mg) is mutarotated for 14 h in water. The aqueous solution is evaporated to dryness under vacuum, the sample dried in a desiccator, and incubated overnight with 5 ml of the silylation mixture. From this reaction mixture, peaks 3 and 6 can be isolated by means of micropreparative gas chromatography and about 100 µg of each substance was collected for analysis by microinfrared and mass spectroscopy. Not enough of peak 2 can be absorbed for analysis and it is identified using a combination of gas chromatography and mass spectroscopy.

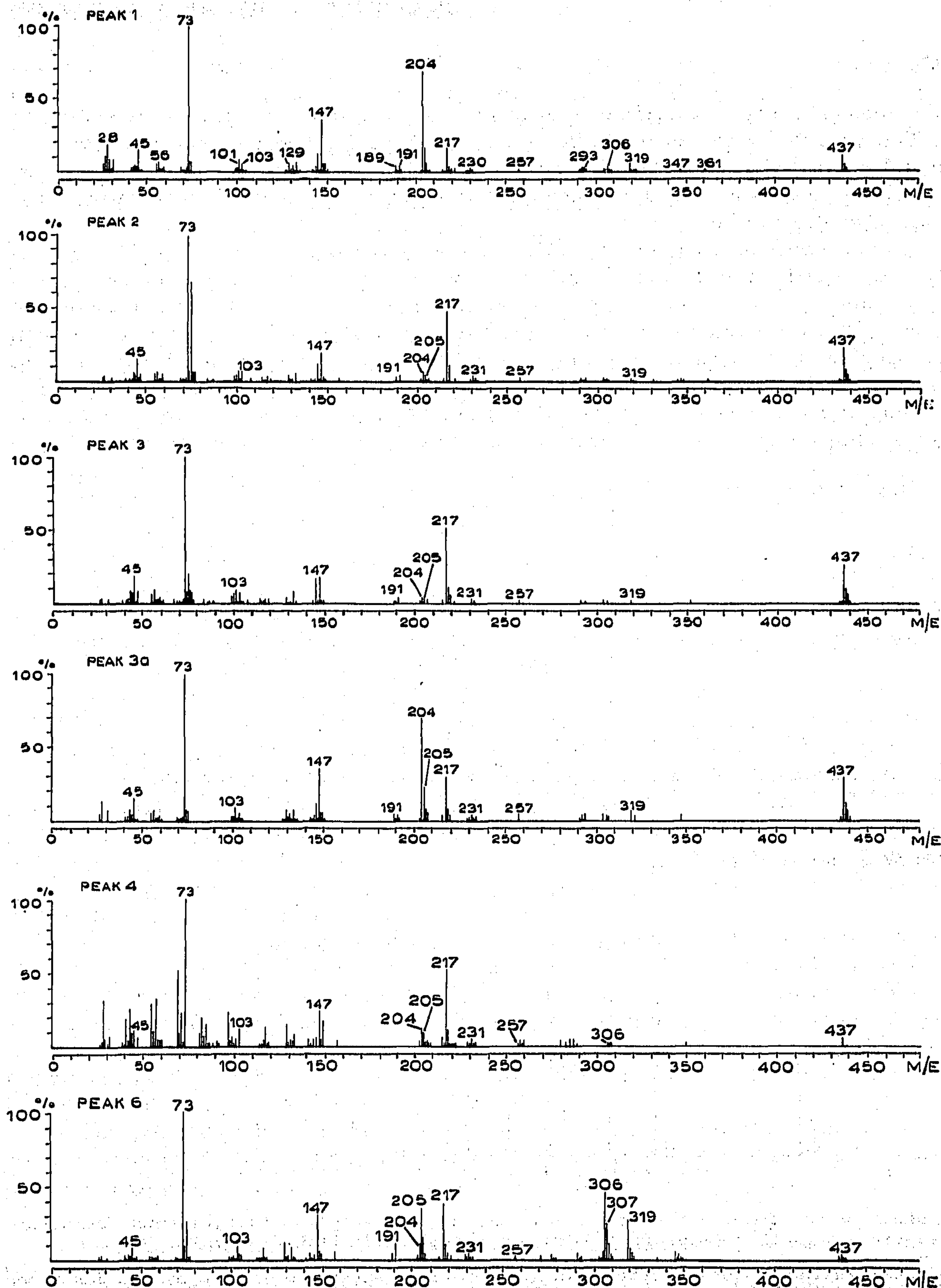

Fig. 3. Mass spectra of the trimethylsilyl ethers of peak 1, 2, 3, 3a, 4, and 6. Peak 1 = M.S.: Hitachi "RMU 6D"; peak 2 = G.C./M.S.: Perkin-Elmer "800"/Hitachi "RMU 6D"; peak 3 = G.C./M.S.: Perkin-Elmer "800"/Hitachi "RMU 6D"; peak 3a = G.C./M.S.: LKB "9000"; peak 4 = G.C./M.S.: Perkin-Elmer "800"/Hitachi "RMU 6D"; peak 6 = G.C./M.S.: Perkin-Elmer "800"/Hitachi "RMU 6D".

RESULTS

The mass spectra of the trimethylsilyl ethers given in Figs. 1 and 2 are shown in Fig. 3. The samples were either collected at the end of the gas chromatographic column and separated, or were led directly into the mass spectrometer. For comparison, two additional mass spectra of permethylated fructose (pyranoside and furanoside forms) are also shown (Fig. 3a).

The mass spectra of the methyl ethers show that the molecular ion ($M^+ = 250$) cannot be detected. Signals of high intensity were *m/e* 219, which could correspond to the cleavage of a methoxy group, *m/e* 88 and 101. It is known from the work of HEYNS *et al.*[7-9] and KOCHETKOV AND CHIZHOV[10] that the major difference between the mass spectra of the pyranoside and furanoside forms of permethylated pentoses and hexoses consists in the relative intensities of the signals at *m/e* 88 ($CH_3O—CH{=}CH—OCH_3)^+$ and *m/e* 101 ($CH_3O{=}CHCH{=}CHOCH_3)^{+}$*. The mass spectrum of the pyranosides was characterized by a very strong signal at *m/e* 88 (the second strongest, 98 %)

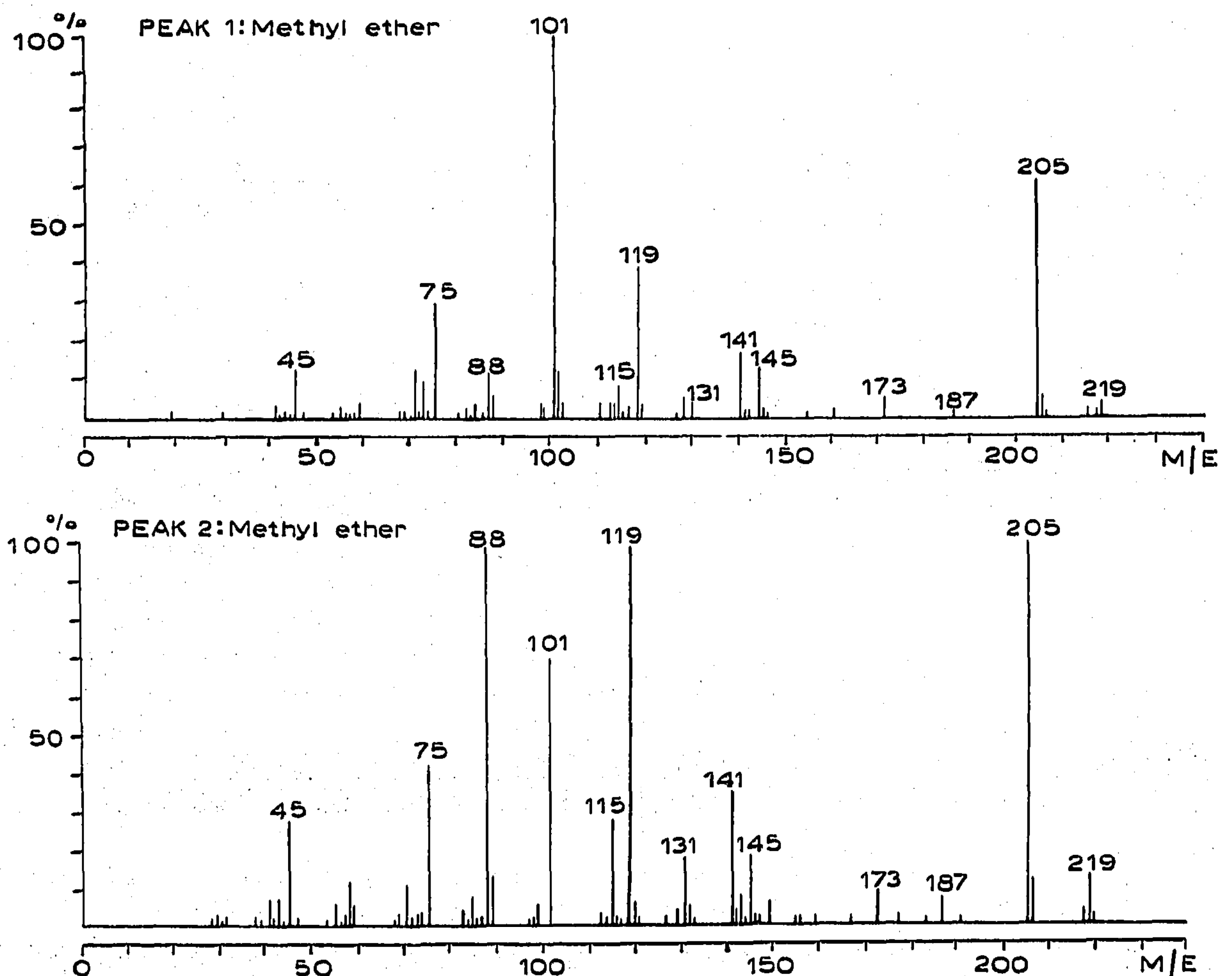

Fig. 3a. Mass spectra of the methyl ethers of methyl 1,3,4,6-tetra-O-methyl-β-D-fructofuranoside (I) and methyl 1,3,4,5-tetra-O-methyl-β-D-fructopyranoside (II). M.S.: Varian "M 66".

* For explanation of this and other formulations of mass spectral fragments used in this paper see H. BUDZIKIEWICZ, D. DJERASSI AND D. H. WILLIAMS, *Mass Spectrometry of Organic Compounds*, Holden-Day Inc., San Francisco, 1967.

and by a signal at *m/e* 101 amounting to 69 %. The spectrum of the furanosides, on the contrary, was characterized by the almost complete lack of the signal at *m/e* 88 (less than 13 %) and by the signal at *m/e* 101 amounting to 100 %.

Similar behavior was exhibited by the trimethylsilyl ethers, as also noted by HEYNS *et al.** The molecular ion ($M^+ = 540$) was not observable. The highest mass number was at *m/e* 437 which could be attributed to the loss of a $-CH_2OSi(CH_3)_3$ group. Intense signals were always found at either *m/e* 204 or 217 (peaks 1, 2, 3, and 3a). These signals correspond to the mass numbers at *m/e* 88 and 101 respectively, observed for the methyl ethers. It was striking that the mass spectra of chromatographic peaks 1 and 3a on the one hand, and those of 2 and 3 on the other, were practically identical. For the former pair, the mass at *m/e* 204 was very intense, while that at *m/e* 217 was weaker. For the latter, the *m/e* 204 signal was almost non-existent, whereas a very strong signal was exhibited at *m/e* 217.

In analogy to the mass spectra of the methyl ethers, the peaks 1 and 3a should be attributed to the pyranoside forms (β- and α-anomers). This assignment was in agreement with the work of HEYNS *et al.*[7–9] and of KOCHETKOV AND CHIZHOV[10], who established that high abundance of mass number *m/e* 88 in methyl ethers indicated a pyranoside form.

Further confirmation of the correctness of the foregoing correlation arises from the following facts:

1. Crystalline fructose exists in the β-pyranoside form[11]. Gas chromatography of persilylated fructose resulted in practically only peak 1. Thus, this peak is very likely to correspond to the β-pyranoside form.

2. In sucrose, fructose exists in the furanoside form. When sucrose was split by intestinal sucrase, which is an α-glucosido-hydrolase, two major peaks of fructose were produced (3 and 1). The peak 1:peak 3 ratio increased as the hydrolysis proceeded. On extrapolation to zero time, only peak 3 was present in the mixture. This indicates that peak 3 is a furanoside.

Fig. 4 shows gas chromatograms of the monosaccharides which were formed during the enzymatic cleavage of sucrose with sucrase at various incubation periods[1].

The results of the cleavage studies and the ratio of the peak areas (peak 1:peak 3) are given together in Table 1.

3. It is known that, generally, the form which contains the least substituents in the axial position is the most stable. From this it would follow that component 1 is in the β-form. This would also explain the vanishingly small amount of component 3a. For the quasi-planar 5 membered ring form, no unequivocal distinction was possible between the axial and equatorial substituents. It would be a reasonable guess to ascribe peak 3 to the β-furanose since this is the initial product of the enzymatic hydrolysis of sucrose. Peak 2 is then the α-furanose.

Components 4 and 5 appeared to be incompletely silylated products. In contrast to components 1 and 3 which did not show an –OH band in the infrared spectrum, components 4 and 5 did have a band at 3460 cm^{-1}**.

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* Personal communication of D. MÜLLER, Chemical Institute, Department of Organic Chemistry, University of Hamburg.

** I.R.: A Perkin-Elmer "257" with a beam condensor and micro-sodium chloride cuvettes with a 2 μl capacity was used. The infrared spectra of the trimethylsilyl ethers were taken in carbon tetrachloride.

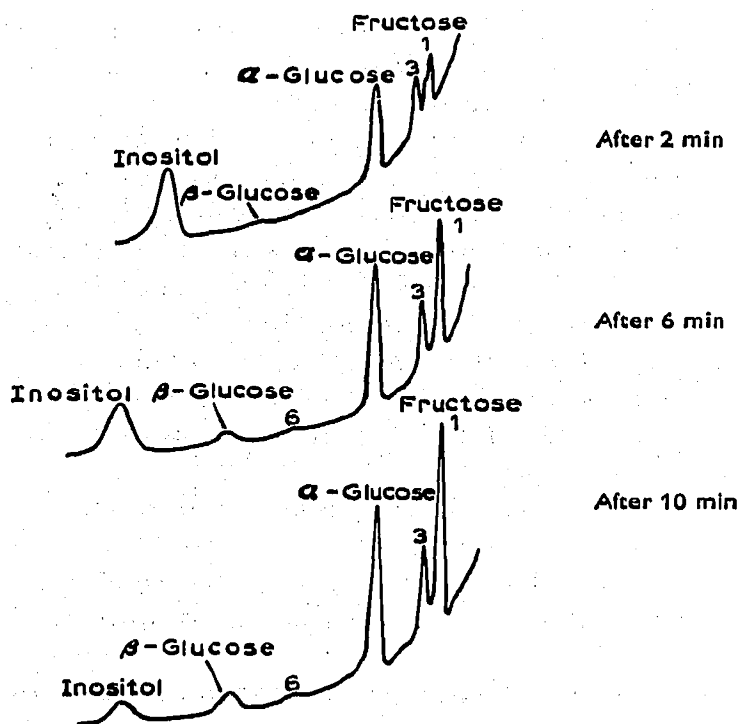


Fig. 4. Gas chromatograms of the monosaccharides formed during enzymatic cleavage after 2, 6, and 10 min. G.C.: Aerograph "1520" with split device for preparative work. EGS, 15% on Chromosorb W, 80–100 mesh; glass column 2 m, I.D. 2.7 mm; t_c: 145°, t_i: 255°, t_d: 245°; N_2: 45 ml/min.

The mass spectrum of component 4 also showed a mass number at 437 indicating that not more than one trimethylsilyl group was missing.

When longer silylation times were employed (24 h), components 4 and 5 disappeared while peak 1 increased. This phenomenon is easily explained by the hypothesis of an incomplete silylation.

TABLE I

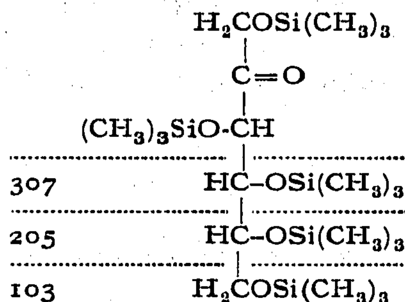
MONOSACCHARIDES: LIBERATED DURING THE HYDROLYSIS OF SUCROSE WITH INTESTINAL SUCRASE

Sample	*Time*	*Glucose (μg)*			*Fructose (μg)*				
		α	*β*	*Total*	*1*	*3*	*1/3*	*6*	*Total*
A	2 min	33.3	3.7	37	15.5	17.5	0.89	—	33
B	6 min	85.1	12.9	98	36.7	31.3	1.17	2.0	70
C	10 min	265.0	44.0	309	194.5	108.6	1.79	7.9	311
D	20 min	283.8	114.2	398	306.8	137.6	2.23	11.6	456

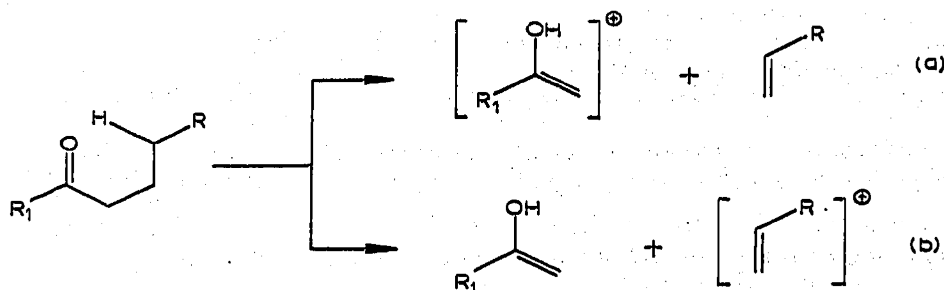
In contrast to the remaining compounds, component 6 showed a keto band at 1735 cm^{-1} in the infrared spectrum. No –OH band was present. This pointed to an open-chain form. The mass spectrum showed a decomposition pattern different from

that of the ring forms. The peak of highest mass number was, once again, 437. Intense signals could be found at *m/e* 103, 205, and 217, while new mass numbers appeared at *m/e* 306, 307, and 319.

The odd mass numbers *m/e* 103, 205, and 307 can be explained through simple cleavage:



The intense fragment of mass number *m/e* 306 must arise from a hydrogen rearrangement. One possibility is the McLafferty rearrangement[12], represented as:



It is known[13] that not both possibilities of charge distribution will necessarily occur; it is rather the stability of the resulting ion as well as of the unchanged fragment which is determinative for the fragmentation pattern.

In the present case, practically only reaction (b) would occur.

DISCUSSION

From the foregoing results it is seen that, of the five persilylated fructose derivatives two are the pyranoside, two the furanoside and one the open-chain form of fructose. This conclusion is drawn from M.S., I.R. and gas chromatographic studies. We find in an equilibrium mixture of fructose about 33 % furanoside and about 67 % pyranoside, corresponding closely to the observations of Gottschalk.[3]

The free, open-chain form of fructose was isolated as its trimethylsilyl ether derivative from aqueous solution at equilibrium. It was judged to be chromatographically pure and the structure was assigned with the aid of I.R. and M.S. spectra. However, this does not unconditionally prove the actual presence of the open-chain form in aqueous fructose solutions at room temperature.

The isolation of a methylated fructose in the keto form was described by Bayer and Widder[14] in 1965; 60 % was the concentration (purity) of the isolated compound. However, the use of more drastic reaction conditions in Bayer's methyl ether

formation may have introduced a bias in the composition of the original equilibrium mixture.

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