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The Analysis of Gamma-methylfructoside Mixtures by Means of Invertase. II. The Isolation of a New Crystalline Methylfructoside[1]

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In the work an account of which has already been given,[2] fructose was condensed with methyl alcoholic hydrogen chloride and the liquid mixture of non-reducing fructose derivatives so formed was fermented by yeast. As a result of this fermentation a definite chemical individual, hydrolyzed to fructose by invertase and with a specific rotation in water of about $[\alpha]_D^{20}$ $-52°$ when calculated as a methylfructoside, was completely removed while the remaining fructose derivatives underwent no change in specific rotation or in absolute amount. The latter portion was thereby made available for the study which forms the subject of the present communication.

Fructose was condensed with the acid methyl alcohol so that the composition of the product was known from the previous work[2] to be approximately unchanged fructose, 3%, the methylfructoside fermented by yeast, 42%, and the non-reducing remainder, 55%. After removing the two former constituents of the mixture by a fermentation with yeast, the aqueous solution containing the latter was evaporated to a sirup with care to avoid hydrolysis or decomposition. An aqueous methyl alcoholic solution of the product was then precipitated by ethyl acetate and the less soluble portion was twice extracted with amyl alcohol. In this way the whole was divided between ethyl acetate and amyl alcohol mother liquors of very similar composition and a relatively insoluble fraction which still contained derivatives of a gamma nature. The removal of the latter, by acid hydrolysis followed by another fermentation, left a small residue presumably consisting of glycosides of the normal ring structure. The data are summarized in Table I, in which the specific rotations of the liquid fractions were calculated as methylglycosides from their fructose content. As the normal glycosides, resistant to acid hydrolysis with gamma conditions, were weighed as a gum containing the impurities introduced by the fermentations, their relative amount was actually less than 7.5% and their specific rotation more levorotatory than $[\alpha]_D^{20}$ $-20.7°$, the values stated in the table.

TABLE I

PARTIAL ANALYSIS OF THE METHYLFRUCTOSIDE MIXTURE

1. Portion fermented by yeast	% as fructose	$[\alpha]_D^{20}$ in water
Uncondensed fructose	3	$-92.5°$
A liquid γ-methyl derivative	42	$-52 \pm 2°$
2. Residue unfermentable by yeast		
A. More soluble portion		
A crystalline γ-methylfructoside	9.9	$+93.0°$
Liquid γ-methyl derivatives	21.7	$+51.0°$
B. Less soluble portion		
Liquid γ-methyl derivatives	19.8	$+25.2°$
Residue of normal ring type	7.5	$-20.7°$
Total	104% of theory	

With the exception of the residue stable toward aqueous acid under gamma conditions, all of the other derivatives were hydrolyzed with great ease to fructose and were undoubtedly of a gamma type. One of them, to be described in detail later, was isolated as a pure crystalline compound with a specific rotation of $[\alpha]_D^{20}$ $+93.05°$ in water. The remainder of the non-fermentable gamma derivatives could not be crystallized and accounted for some 41% of the ketose used in the original condensation. Their mean specific rotation varied from $[\alpha]_D^{20}$ $+27°$ to $[\alpha]_D^{20}$ $+54°$ according to the details of the fractionation and this fact in itself indicated that the uncrystallized portion contained at least two derivatives of different optical rotation. As the mother liquors, from which the gamma-methylfructoside of specific rotation $+93.05°$ was crystallized, were included in the group, it contained a further amount of the same glycoside which could therefore be regarded as one of the two or more components of the mixture. The simplest but not necessarily the correct conclusion was that these liquid fractions were mixtures of the new crystalline glycoside and an unknown gamma-fructose derivative with a specific rotation not more dextrorotatory than $[\alpha]_D^{20}$ $+28°$. As the latter had not been fermented by yeast, it could not be identified with the levorotatory fructose derivative $[\alpha]_D^{20}$ $-52°$ which had previously been removed in the fer-

(1) Publication authorized by the Surgeon General, U. S. Public Health Service.

(2) Purves and Hudson, THIS JOURNAL, **56**, 702 (1934).

mentation. It followed that when fructose was condensed with acid methyl alcohol the non-reducing product of a gamma type was composed of at least three distinct chemical individuals. These three naturally could not all possess the same ringed structure.

The recent demonstration by Pacsu[3] that both fructose and the fructose residue in turanose were capable of yielding derivatives of the open chain type made it not impossible that a straight chain fructose methyl acetal was a constituent of the gamma-methylfructoside mixture. As such a compound might be expected to undergo ring closure on dehydration, or hydrolysis or other change, with ease, care was taken in the experimental work to avoid drying any solution of the fructose derivatives in an organic solvent and to keep the temperature low. The only occasion on which the temperature of the preparations themselves exceeded that of the room was during the fermentation with yeast, an operation which was known to occasion no change in the unfermentable portion. These conditions were adhered to throughout the preparation of the crystalline methylfructoside, which was accordingly regarded as a primary rather than a secondary product of the condensation of the hexose with methyl alcohol. In addition, the fact that no amount of manipulation increased the yield of the crude crystalline glycoside over 10% of that theoretically possible militated against the view that it was formed, prior to its isolation, from an isomeric compound or by ring closure from a methyl acetal. The crude gamma-methylfructoside crystallized from the ethyl acetate extract with a specific rotation of $[\alpha]_D^{20}$ +88–90°; which was sufficiently close to the value for the pure material, $[\alpha]_D^{20}$ +91.6° in methyl alcohol, to preclude any structural change during the purification.

The new methylfructoside was isolated as shining, stout, pointed prisms or sometimes beveled tablets of the hemimorphic monoclinic system which were often perfectly developed and more than 0.5 g. in weight. On analysis the carbon, hydrogen and methoxyl content were those of a methylhexoside as distinct from a methyl acetal and, on acid hydrolysis, the crystals yielded a molecular proportion of fructose. Although stable for months when kept in a desiccator and not decomposed by heating for hours in a vacuum at its melting point of 69°, the gamma-methylfructoside was hydrolyzed more rapidly than sucrose by aqueous acid and caused slight reduction in the Shaffer–Hartmann alkaline copper reagent. It was not affected by the enzymes of yeast. Raybin[4] has recently found a highly distinctive test for sucrose in the intense green coloration assumed by an alkaline solution of diazouracil containing the disaccharide. The crystalline gamma-methylfructoside gave a clear orange when submitted to the test and the same color was observed when the original gamma-methylfructoside sirup was used. As 43% of the latter consisted of the fructose derivative hydrolyzed by invertase, the negative result which it gave was surprising and suggested that Raybin's diazouracil test was even more specific for sugars of the sucrose group than the action of the enzyme invertase.

A series of experiments, not described below, was also carried out on the original, unfermented fructoside mixture with the object of isolating from it the constituent which was unstable to the enzyme. In this work the relatively insoluble glycosides of normal structure were first removed by fractional precipitation with amyl alcohol and the material recovered from the mother liquor was afterward repeatedly extracted or precipitated with ethyl acetate, amyl or propyl alcohol. The methods already described[2] were used to follow the course of the separation quantitatively and after more than thirty systematic fractionations the composition of each fraction was still within the limits: glycoside or acetal with specific rotation $[\alpha]_D^{20}$ −52°, and hydrolyzed by invertase 20–60%, methylglycoside, $[\alpha]_D^{20}$ +93.0°, stable to yeast and actually isolated in crystalline condition 0–35%, and other non-reducing, non-fermentable derivatives of gamma-fructose 30–40%. In particular, the figures for an ethyl acetate extract of the original were 30, 35 and 35%, respectively. A tendency was noted for the constituent unstable toward invertase to accumulate in the less soluble fractions but the difference in solubilities was not sufficiently marked to render the attempted separation practicable.

The recognition of gamma-methylfructoside sirup as a mixture whose constituents were not all of one ringed structure and were not to be readily separated by means of solvents made it probable that the uncrystallized tetramethylfructose pre-

(3) Pacsu, THIS JOURNAL, **55**, 2451 (1933).

(4) Raybin, *ibid.*, **55**, 2603 (1933).

pared from them[5] was also a mixture of different ringed forms. On the other hand, Haworth and Hirst, with their collaborators,[6] have produced much excellent evidence to show that the liquid tetramethyl-gamma-fructose derived from sucrose consisted of the α and β forms of a uniform methylated derivative to which the 2,5-ringed structure was finally assigned. In spite of the similarity noted by Menzies[5] in the properties of the tetramethylfructose prepared from these two sources, it now appears imprudent to consider them as being identical or to assume that the position of the oxygen bridge linking predominating in the first is the same as that worked out for the second. The researches of these authors, therefore, throw little direct light on the nature of the parent gamma-methylfructoside sirup and the constitution of each of its constituents, including that of the new, crystalline gamma-glycoside, remains a matter for independent investigation.

Experimental

Throughout the work the removal of organic solvents from the preparations took place over a mixture of anhydrous calcium chloride and soda lime in desiccators attached to a water pump. The temperature never exceeded 25° and the acidity of the solutions was always less than P_H 6. Small portions of porous tile were added when necessary to prevent bumping and the material they absorbed was afterward recovered by extraction with methyl alcohol. The gamma-fructose content of the liquid fractions was determined after a Herzfeld hydrolysis by the Shaffer–Hartmann copper reduction method and in the calculation of yield and specific rotation (as methylfructoside) the minute reduction of the unhydrolyzed derivatives was neglected. A 2-dcm. polarimeter tube was used in every case for optical observations, made on a saccharimeter with a conversion factor of 1°V. = 0.3462° circular. The analytical methods have already been described.[2]

Preparation of the Non-fermentable Methylfructosides.—After a solution containing analytically pure fructose (50 g.) and dry hydrogen chloride (3.65 g.), dissolved at 20° in pure dry acetone-free methyl alcohol (1000 ml.) had reached its dextrorotatory maximum ($[\alpha]_D^{20}$ +0.5° in methyl alcohol), it was neutralized in a mortar with silver oxide. A filtration through cellite left a colorless liquid which became brown with colloidal silver when its reaction was changed from P_H 6 to 8–9 with 3 ml. of normal aqueous caustic soda. Another filtration through a little absorbent carbon gave a filtrate, silver-free and again colorless, which was diluted with distilled water and concentrated in a distilling flask (bath 40° at 30 mm.) to eliminate the methyl alcohol. An aqueous suspension of starch-free baker's yeast (2 g.), dihydrogen sodium phosphate (0.2 g.) and ammonium acetate (0.2 g.) was added to the final aqueous solution (volume 500 ml.), previously acidified to P_H 4.5 with dilute acetic acid, and the fermentation was complete in three days at 35°. After a filtration through cellite and absorbent carbon to remove yeast cells, the colorless and almost non-reducing solution was again made alkaline (P_H 8–9) with caustic soda. On the next day it was occasionally found that the alkaline reaction had vanished, presumably owing to dissolved yeast protein, and this was corrected when necessary with added caustic soda before the volume of the solution was reduced in a distilling flask under diminished pressure to 200 ml. In order to avoid discoloration it was essential to conduct this evaporation without permitting local superheating and to keep the temperature of the water-bath below 40°. The final concentration to a nearly colorless, non-reducing sirup containing 70–80% solids took place over a period of days in a desiccator in the way already indicated.

Fractionation of the Non-fermentable Methylfructosides.—The addition, at room temperature, of 500 ml. of acid-free ethyl acetate to the sirupy methylfructosides diluted with 50 ml. of methyl alcohol brought about the precipitation of the bulk of the solute and left a clear supernatant solution A. After concentrating the less soluble portion to a thick sirup, it was dissolved in a little methyl alcohol and reprecipitated with anhydrous, acid-free *n*-amyl alcohol. A repetition of the process left this fraction represented by the amyl alcoholic extracts B_1 and B_2 and a much less soluble, red-yellow residue C. The fractions A, B_1, B_2 and C were then examined in order.

Examination of the Ethyl Acetate Fraction A.—The drop in temperature which the solution underwent during its concentration under diminished pressure caused the deposition of a sirup which was removed by decantation when the supernatant liquid had again become clear. On continuing the evaporation of the mother liquor ($[\alpha]_D^{20}$ +70° in water) in another beaker, large and well shaped crystals commenced to form and after two days at 0°, when the crystallization appeared complete, the crop was separated from the 20 ml. of residual solution: weight, 3.6 g. or 6.7%, with a specific rotation of +88.6° in water after drying in a desiccator at room temperature but prior to recrystallization. The uncrystallized sirup contained in the mother liquor was combined in 10 ml. of amyl alcohol with the sirup deposited from the ethyl acetate before the crystallization and for analysis 0.5 ml. of the mixture was extracted with 10 ml. of water. A Herzfeld hydrolysis changed the reduction of the aqueous extract from an initial value of 0.064 to 2.40% as fructose and the initial dextrorotation of +7.6°V. to a levorotation of −11.85°V. That is, the total non-reducing uncrystallizable portion of the ethyl acetate extract weighed 4.8 g. as fructose and had a specific rotation in water of $[\alpha]_D^{20}$ +50.8°, changed to $[\alpha]_D^{20}$ −85.5° (corr.) by acid hydrolysis with gamma conditions; yield 9.6%.

Examination of the Amyl Alcohol Fractions B_1 and B_2.—Extract B_1, on slow concentration in a vacuum followed by a fractional precipitation with petroleum ether, yielded 1.74 g. of the above crystalline gamma-methylfructoside in a slightly gummy condition. The small amount of thick sirup which comprised the remainder of the fraction could not be crystallized and was added to the amyl alcoholic extract B_2 (total volume 70 ml.). For analysis 2 ml. of this was shaken with 15 ml. of water to give an aqueous solu-

(5) Menzies, *J. Chem. Soc.*, **121**, 2238 (1922).
(6) Avery, Haworth and Hirst, *ibid.*, 2308 (1927).

tion with a dextrorotation of 3.6°V. A Herzfeld hydrolysis increased the reduction of the solution from 0.019 to 1.155% as fructose, thereby indicating the presence in the amyl alcohol of 6.06 g. of non-reducing gamma-fructose derivatives with a specific rotation in water of $[\alpha]_D^{20}$ +50.6°; amount, 12.1% of the theoretical.

Examination of the Less Soluble Residue C.—The whole of the residue from the amyl alcohol extractions was dissolved in 200 ml. of water and was hydrolyzed under gamma conditions by adding 66.6 ml. of normal hydrochloric acid. After eighteen hours at 20° the initial rotation of +4.2°V. had become constant at −21.6°V. and the reduction had risen to 3.72% as fructose. This acid treatment, therefore, had hydrolyzed 9.92 g. of a gamma-methylfructoside with a specific rotation of $[\alpha]_D^{20}$ +25.2°; amount, 19.8%.

When the hydrochloric acid had been removed as the silver salt from the above, the fructose liberated in the hydrolysis was removed in the usual way by a fermentation with yeast and after clarification the solution was evaporated to a golden-brown, uncrystallized, non-reducing gum. The specific rotation of this highly impure residue was $[\alpha]_D^{20}$ −20.7° in water (0.3574 g. in 25 ml. had a levorotation of −1.70°V.) and its weight, 4.0 g., corresponded to about 7.5% of the theoretical. It was not studied in detail.

Examination of the Crystalline Gamma-methylfructoside.—The whole of the crystals, 5.36 g., obtained from the ethyl acetate extract A and the amyl alcohol extract B_1, were combined and recrystallized at 0° by evaporating a methyl alcoholic solution containing 5 ml. of amyl alcohol to small bulk. They separated again as large, well formed, pointed prisms (3.5 g.) which were dried in an evacuated desiccator. A cautious addition of petroleum ether to the mother liquor caused the deposition of a second crop of similar prisms (1 g.) with a specific rotation in methyl alcohol of $[\alpha]_D^{20}$ +91.6° (0.1852 g. in 25 ml., had a dextrorotation of 3.920°V.). As an identical value was found for the first fraction after a second recrystallization (0.3231 g. in 25 ml. had a dextrorotation of 6.84°V.) and further purification made no significant change, a specific rotation of $[\alpha]_D^{20}$ +91.6° in methyl alcohol was accepted as correct for the new gamma-methylfructoside. In water the corresponding figure was $[\alpha]_D^{20}$ +93.05° (0.4911 g. in 25 ml. had a dextrorotation of +10.560°V.). The melting point was sharp at 69°.

Anal. (Sample dried at 40° and 0.1 mm. for nine hours). Calcd. for methylfructoside, $C_7H_{14}O_6$: C, 43.30; H, 7.22; OCH_3, 15.9. Calcd. for a fructose monomethyl acetal, $C_7H_{16}O_7$: C, 39.62; H, 7.55; OCH_3, 14.62. Found: C, 43.05, 43.11; H, 7.42, 7.55; OCH_3, 15.7, 15.6 (Clark's method[7]).

In an aqueous hydrolysis a 1.4733% solution in 0.25 *N* hydrochloric acid gave a final reduction, as fructose, of 1.38% and a final constant levorotation of −7.39°V., corresponding to a final specific rotation of $[\alpha]_D^{20}$ −92.7° which was correct for fructose. The fructose (mol. wt. 180) concentration calculated for a methyl fructoside (mol. wt. 194) was 1.364, and for a fructose monomethyl acetal (mol. wt. 212), 1.251%.

The crystalline gamma-methylfructoside was extremely soluble in water, methyl and ethyl alcohols, dissolved easily in *n*-amyl alcohol and pyridine, was sparingly soluble in cold ethyl acetate and insoluble in petroleum ether and benzene. In the finely divided state it was slightly hygroscopic. The alkaline Shaffer–Hartmann copper reagent was reduced to the extent of less than 0.05 mg. of fructose by 12.0 mg. of the gamma-methylfructoside, the reducing power of which was therefore at least 250 times less than that of fructose under the same conditions. This stability was also marked when the pure crystals were heated for four hours at their melting point of 69° in a vacuum of 0.1 mm. Recrystallization set in on cooling; the methyl fructoside was practically non-reducing and its specific rotation in methyl alcohol was $[\alpha]_D^{20}$ +90.6° (0.4533 g. in 25 ml. gave a dextrorotation of +9.492°V.).

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Summary

1. An unfermentable crystalline gamma-methylfructoside, m. p. 69°, with a specific rotation of $[\alpha]_D^{20}$ +93.0° in water, was isolated in 10% yield from the gamma-methylfructoside mixture obtained by condensing the ketose with acid methyl alcohol.

2. Approximately 41% of the condensation product remained as an unfermentable sirup of variable specific rotation and derived from gamma-fructose.

3. The methylfructoside mixture contained at least three non-reducing derivatives of gamma-fructose; those indicated above together with another, $[\alpha]_D^{20}$ −52°, which was fermented by yeast.

4. Raybin's diazouracil test for sucrose was given by none of the three gamma-fructose derivatives described above although one of them was hydrolyzed by invertase. Raybin's test was therefore more specific for the sucrose linking than the action of invertase.

(7) Clark, *J. Assn. Off. Agr. Chem.*, **15**, 136 (1932).

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