5-Keto-D-fructose

I. CHEMICAL CHARACTERIZATION AND ANALYTICAL DETERMINATION OF THE DICARBONYLHEXOSE PRODUCED BY GLUCONOBACTER CERINUS*

GAD AVIGAD AND SASHA ENGLAND†

From the Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel, and the Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, New York, 10461

(Received for publication, October 13, 1964)

The formation of a dicarbonylhexose by Acetobacter suboxydans growing on mannitol was postulated by Isbell and Karabinos (1) on the basis of indirect evidence. From an Acetobacter culture medium, Weidenhagen and Bernsee (2) subsequently isolated a crystalline dicarbonylhexose which they described as 6-aldo-D-fructose (6-Lyxo-hexose-5-ulose). This identification was based mainly on the fact that the sugar was oxidized by hypoiodite as expected for an aldehyde. However, as pointed out by Theander (3), the susceptibility to hypoiodite oxidation cannot be considered sufficient evidence for the establishment of a 6-aldehyde ketohexose structure. Indeed, other properties found for the isolated sugar could also be compatible with a 5-ketohexohexulose arising from fructose by oxidation at C-5.

Terada et al. (4–6) more recently have isolated a dicarbonylhexose from the culture media of several Acetobacter strains growing on fructose. The structure suggested for the isolated crystalline compound, based mainly on the appearance of fructose and sorbose on partial chemical reduction, was 5-keto-D-fructose (5-dehydrofructose; D-three-2,5-hexodiulose). The formation of this sugar by the oxidation of D-fructose or L-sorbose at C-5 could be predicted by Bertrand rules for the oxidation-reduction of polyols and ketoses by Acetobacter (7). In the culture media in which certain Acetobacter strains were growing on D mannitol and D-sorbitol, 5-ketohexohexulose has recently been detected chromatographically (8).

In this communication, as a prelude to studies of the enzymological pathways of formation and utilization of the dicarbonylhexose by Acetobacter strains, we present specific chemical procedures for the quantitative determination of the sugar. Spectral, chromatographic, and chemical studies were performed and showed the identity of the dicarbonylhexose isolated by Weidenhagen, and Bernsee (2) with that of Terada et al. (4–6). The patterns of reduction of the dicarbonylhexose with sodium borohydride, described in this paper, would favor a 5-keto-D-fructose formulation as proposed originally by Terada et al. (5, 6).

METHODS

Glucobacter cerinus var. ammoniacus Asai, IFO 3267, was obtained from Dr. O. Terada (4) and was grown at 30° on a medium containing 0.5% Difco yeast extract and 5 to 10% fructose.

Reference crystalline samples of 5-dehydrofructose and 6-aldo-fructose were gifts from Dr. O. Terada and Professor Dr. R. Weidenhagen, respectively. D-Erythrose was obtained from Dr. E. Diamant. Crystaline phenylhydrazones were prepared by standard procedures (2, 9). Fructose acid was prepared according to Ashwell, Wahla, and Hickman (10).

Chromatography—Whatman No. 1 filter paper was used in descending or circular fashion with the following solvent systems: Solvent 1, 1-propanol, ethyl acetate, water (7:1:2, v/v/v); Solvent 2, 1-butanol-ethanol-water (5:2:2, v/v/v); Solvent 3, 1-propanol-ethyl acetate-2.5% boric acid (5:2:1, v/v/v); Solvent 4, 1-butanol-pyridine-ethyl acetate-water (5:5:1:3, v/v/v); Solvent 5, 80% phenol.

Spray reagents used for the detection of sugars were: p-anisidine-HCl for reducing sugars (11); urea-H2PO4 for ketoses (12); α-aminobiphenyl for aldohexoses (13); alkaline AgNO3 for both non-reducing and reducing carbohydrates (14).

Quantitative Determination—The various colorimetric, spectrophotometric, and enzymatic methods employed for the characterization of the dicarbonylhexose are described in “Experimental and Results.” Spectrophotometric measurements were made with a Zeiss model PMQ-II spectrophotometer and in several cases with the Cary model 14 recording spectrophotometer.

Growth of Bacteria— Cultures of 600 ml each in 2-liter Erlenmeyer flasks were incubated at 30° on a rotary shaker at 160 rpm. The cells from an agar slant were used to inoculate each flask. Cultures starting with 5% fructose were incubated for 6 to 7 days, and those starting with 10% fructose were incubated 12 to 14 days. Under these conditions, it was found that 60 to 70% of the fructose was converted ultimately to the new sugar, and only a trace or no fructose remained as shown by paper chromatographic examination of the medium.

EXPERIMENTAL AND RESULTS

Isolation of Dicarbonylhexose—One liter of culture medium (pH about 3.8) was processed as follows. The bacteria were removed by centrifugation, 25 g of Norit A and 25 g of Celite 545 were added to the centrifugate, and the solution was filtered through an Amberlite MB-3 resin (H+, OH– form) column (3.2 × 5.0 cm). The colorless eluent at about pH 6.5 was filtered on a Buchner funnel with Super-Cel filter aid, flushed with nitrogen, and then reduced in volume (1 mm at 30°) to a light syrup. Absolute ethanol was added until crystals appeared.
These were collected by filtration, washed with absolute, cold (-10°C) ethanol, then with small amounts of 1-propanol, and dried in a vacuum desiccator. The supernatant was reduced in volume, and fresh amounts of ethanol were added and again evaporated. This procedure was repeated several times until a semisolid material appeared. This material was triturated with ethanol in a Waring Blender and then manually in a mortar until it appeared as a fine, semicrystalline, white material. This substance was collected rapidly by filtration or centrifugation, washed with cold absolute ethanol, and dried as described before. Any unnecessary delay in the procedure would cause rapid browning of the material. The material was recrystallized from hot ethanol. These crystals melted between 157 and 159°C; [α]0° = -85° (c, 3.56 in H2O); the bis-phenylhydrazone obtained, melted at 133-135°C; [α]0° = -104° (c, 1.2 in pyridine).

These values agree with those reported by Weidenhagen (2) and Terada et al. (5, 6), but the optical rotation value for the bis-phenylhydrazone is higher than that found before (2). In one case, a preparation of dicarbonylhexose, after three recrystallizations, yielded a material with a melting point of 172-174°C. Terada has found a melting point of 172-175°C for a sample of dicarbonylhexose after several recrystallizations from ethanol and water in the cold.

Chromatographic Behavior—In all solvent systems tried, the product moved more slowly than fructose (Rf,fructose 0.70 in Solvent 2 and 0.52 in Solvent 5). It gave a yellow-green spot with the o-aminobiphenyl reagent, and brown spots with the urea-phosphoric acid and the p-anisidine·HCl reagents. It should be noted that crude preparations of the isolated compound usually contained trace amounts of two unidentified reducing components which, under these conditions, have a slower chromatographic mobility than the dicarbonylhexose. These contaminants could be removed only by crystallizations. On standing, aqueous neutral solutions of the product slowly acquired a brown color with absorption in the range of 340 to 240 mp. Since this absorption was enhanced by the presence of phosphate, buffers containing this anion were not used for preparation of stock solutions. Also, slightly alkaline solutions or pyridine-containing solvents catalyzed browning of the product, and probably caused the formation of unidentified degradation or polymerization products.

Color Reactions of Dicarbonylhexose—The dicarbonylhexose reacted with Roe's resorcinol reagent for ketoses (15) to yield an absorption curve with a maximum at 425 mp. Fructose yields two peaks with absorption maxima at 405 and 510 mp (Fig. 1).

With the o-aminobiphenyl reagent for aldoses (13), the dicarbonylhexose gives a product with maximum absorption at 415 mp as compared with the 380-mp maximum given by the product with mannose. Inclusion of thiourea in the reagent was found to enhance color formation and to double the specific absorption at 415 mp by the dicarbonylhexose, whereas color formation by aldohexoses was markedly retarded (Fig. 2). A convenient quantitative assay procedure for the new product was thus developed as follows. Reagent, 5 ml (0.4% solution of o-aminobiphenyl and 2% thiourea in glacial acetic acid, freshly prepared), was added to 1 ml of solution containing 0.05 to 1.0 μmole of dicarbonylhexose. Tubes were immersed in a boiling water bath for 25 minutes to allow maximum color development (as compared with 40 to 45 minutes needed for maximum color development by aldohexoses in the original procedure (13)). Absorption is measured at 415 mp if fructose alone is present or at 440 mp when aldohexoses are also present in the sample. By this procedure, the dicarbonylhexose can be estimated quantitatively in the presence of large excesses of aldohexoses and of fructose. Solutions, in which high absorbance in the ultraviolet region developed on standing did not decrease in capacity to produce maximum color with the o-aminobiphenyl reagent.

---
1 O. Terada, personal communication.
The dicarbonylhexose did not yield colors typical for ketoses with either Dische’s cysteine-carbazole (16) or indole-sulfuric acid (16) reagent. With the methylphenylhydrazine sulfate reagent for 5-ketohexonic acids (17), the dicarbonylhexose yielded a deep yellow color with a maximum absorption at 350 m\( \mu \) (\( E_{350}^{\text{max}} \) 3.6 \( \times \) 10\( ^{3} \)), with about 1.2 times the intensity observed with an equimolar amount of 5-ketogluconic acid, 3 times that with glyceraldehyde, and 15 to 20 times that with fructose, glucose, and 2-deoxyribose. Oxidation of the dicarbonylhexose by alkaline iodine solution (17) reduced the color intensity 30 to 40\% without apparent change in the quality of the absorption spectrum.

The \( \alpha \)-aminobenzaldehyde reagent for aliphatic aldehydes (18) reacted with the dicarbonylhexose to give a product with a maximum absorbance at 440 m\( \mu \) as found with other aldehydes. Maximum color was attained after 3 hours at room temperature as compared with 10 minutes for acetaldehyde and 30 minutes for glyceraldehyde and erythrose. The molar absorbance was similar to that obtained with glyceraldehyde and erythrose and about 50\% that with acetaldehyde. As compared with aliphatic aldehydes, aldo- and ketoaldehydes yield only 1\% of the color intensity obtained with this reagent. The dicarbonylhexose reacted with the 3-methyl-2-benzothiazolone hydrazone-FeCl\( _{3} \) reagent for aldehydes of Sawicki et al. (19). The maximum absorption at 665 m\( \mu \) found for this sugar in this colorimetric procedure was 6.6 \( \times \) 10\( ^{3} \) m\( \text{mol}^{-1} \text{cm}^{-1} \); this was about 48\% the value obtained for 2-deoxyribose, about 18\% of that for erythrose and 12\% of that for acetaldehyde. The profile of the absorption curve was similar in all cases (Fig. 3). Color development, read at 665 m\( \mu \), was rapid, as in the case of aldehydes (e.g. acetaldehyde, glyceraldehyde, erythrose), reaching a maximum after 3 minutes at 100\° as compared with 30 minutes needed for maximum development with 2-deoxyribose. Glucose or fructose, when treated with the reagents of Sawicki et al. (19), hardly yielded any absorbance in the 720 to 500 m\( \mu \) region.

Two variations of the original procedure of Sawicki et al. were studied. In the first, 4 ml of glacial acetic acid were added after 500 m\( \mu \) region. After heating and cooling in ice, 2.5 ml of 0.2\% FeCl\( _{3} \) and 6.5 ml of acetone were added. In C, 0.04 \( \mu \) mole of dicarbonylhexose and glyceraldehyde was treated with 0.2 ml of a 0.1\% 3-methyl-2-benzothiazolone hydrazone and 1.0 ml of 0.1 m glycine, pH 4.0, in a total volume of 2.0 ml. Curve 1 for dicarbonylhexose and the glyceraldehyde curve were obtained after 1 hour at 30\°. Curve 2 for dicarbonylhexose was obtained after 5 minutes at 100\°.

A second procedure was suggested by Paz et al.\( ^{2} \). In this case, the reaction with 3-methyl-2-benzothiazolone hydrazone was carried out at pH 4.0 in 0.05 m glycine buffer, and the FeCl\( _{3} \)-acetone reagent was not added. Under these conditions, the dicarbonylhexose showed an absorption peak at 310 m\( \mu \) only after heating with the reagent, and maximum intensity (about 25\% obtained for equimolar amount of glyceraldehyde) was attained after 6 minutes at 100\° (Fig. 3). The appearance of this absorbance after heating presumably indicates, as suggested by Paz et al.\( ^{2} \), that a hemiacetal (probably pyranose) ring was opened in the process.

\( ^{2} \) M. A. Paz, O. O. Blumenfeld, M. Rojkind, E. Henson, C. Furline, and P. M. Gallop, personal communication (manuscript in preparation).
It should be indicated that samples of dicarbonylhexose obtained from G. cerinus IFO 3267 and that obtained from Dr. Weidenhagen behaved identically in all the color reactions described above.

Reduction of Dicarbonylhexose—Hexose (0.2 M) was reduced with sodium borohydride for 1 hour at room temperature and then chromatographed on paper with Solvents 1 and 3. On partial reduction with 0.05 M sodium borohydride, two new reducing ketoses appeared, one with the mobility of fructose and the other corresponding to that of sorbose (Rfructose 0.85, Solvent 3). No aldohexose could be detected with these chromatographic systems. On total reduction with 0.5 M sodium borohydride, no reducing sugars remained, and on the chromatogram, compounds with mobilities of hexitols were detected with the silver nitrate reagent. However, clean-cut chromatographic separation of mannotol, sorbitol, and iditol, possible products of the reduction, was unsuccessful although many solvent systems were utilized including that recommended by Grazi et al. (20). Similar results were obtained when reduction of the dicarbonylhexose was carried out at pH 9.2 in unbuffered media or at pH 6 to 7 in 0.1 M acetate or phosphate buffers. Further evidence that both ketohexoses appear in the course of reduction to hexitols was obtained in a kinetic study summarized in Fig. 4. An immediate appearance of ketoses, 60% of which is sorbose, is evident in the first minute of reaction. A gradual further reduction of fructose, sorbose, and residual dicarbonylhexose then follows at about the same rate.

Crystalline dicarbonylhexose obtained from G. cerinus IFO 3267 cultures and those furnished by Professor Weidenhagen gave the same results on reduction with borohydride.

Reactions Involving Oxidation of Sugar—Iodimetric titration (22) of the dicarbonylhexose consumed 1 mole of oxidant per mole of hexose, as did a control system with glucose. When oxidation by iodine was effected by a different procedure (23), up to 3 times more iodine disappeared with dicarbonylhexose as compared with glucose. This could indicate that overoxidation of unknown nature occurred. As observed by Terada et al. (4), we also have noted that bromine-water while oxidizing aldohexoses at acid pH did not cause marked degradation of the sugar, but oxidation by bromine in a saturated BaCO₃ solution resulted in the formation of unidentified products which could be precipitated as the barium salts by 66% ethanol. Fructuronic acid, however, could not be detected in these precipitates. Many other experiments were conducted to determine whether hypoioidite or bromine oxidation of the dicarbonylhexose would yield fructuronic acid as would be expected from oxidation of a 6-aldofructose molecule. Colorimetric and chromatographic examination of these reaction systems according to the procedures of Ashwell et al. (10) were negative for a ketouronic acid.

Cuprimetrically (24, 25), the dicarbonylhexose yielded nearly the same molar equivalents (1 to 1.1) as glucose or fructose.

Oxidation of the dicarbonylhexose and of its bis-phenylhydrazone by metaperiodate is described in Table I. The moles of periodate consumed and acid equivalents formed in the oxidation of the dicarbonylhexose favor somewhat its being 6-aldo-2-ketohexose rather than 5-ketofructose. However, the differences expected between these two structures are marginal, so that one cannot draw definitive conclusions from these results.

Infrared Spectrum—The isolated sugar in the solid state gave the spectrum shown in Fig. 5. The dicarbonyl hexose sugar obtained from Dr. Weidenhagen has practically the same absorption curve as the G. cerinus product. Of significance is the absence of the absorption peak at 1720 cm⁻¹ which is characteristic of these absorption systems.
Fig. 5. Infrared spectra of dicarbonylhexose. Samples of 0.5 mg each were analyzed in a KBr pellet with the Perkin-Elmer model 21 double beam spectrophotometer. —, G. cerinus product; — — —, 6-aldo-ß-fructose obtained from Dr. Weidenhagen.

Fig. 6. Nuclear magnetic resonance spectrum of the G. cerinus dicarbonylhexose. A fresh 10% solution of the sugar was equilibrated with D₂O and analyzed in a Varian model A-60 proton magnetic resonance spectrometer. The 4.62 ppm peak corresponds to HDO. The instrument was calibrated with trimethylsilane as an external standard.

istic of a free carbonyl group. Strong absorbance is observed at 888, 872, and 820 cm⁻¹ and weak absorbance at 934, 919, 845, 755, and 727 cm⁻¹, all of which might be linked to the presence of the pyranose rings (28, 29). A significant absorption is also found at 706 cm⁻¹.

Nuclear Magnetic Resonance Spectrum (NMR)—The dicarbonylhexose was examined for its NMR spectrum by Dr. D. P. Hollis of Varian Associates, Palo Alto, California. Spectra of 60 and 100 mc in D₂O were taken for the G. cerinus product and a sample of dicarbonylhexose obtained from Dr. Weidenhagen. The two samples gave similar spectra, but the relative intensities of the lines observed differed somewhat. Fig. 6 reproduces a spectrum in D₂O of the dicarbonylhexose obtained in our own laboratory with a Varian 60-mc spectrometer.

DISCUSSION

As was already pointed out by Theander (3), the physical properties of the 6-aldo-ß-fructose isolated by Weidenhagen and Bernsee (2) and of the 5-dehydrofructose described by Terada et al. (5) are closely similar. That these two products are indistinguishable in chromatographic mobilities, colorimetric reactions, absorption spectra, and in chemical patterns of reduction or oxidation, was clearly shown in the present investigation.

The proposal for a 6-aldo-2-ketohexose structure for this dicarbonyl sugar was based mainly on the fact that it could be oxidized by hypiodite to the extent predicted from theory for an aldohexose (2). This observation indeed was confirmed in the present work with the McLeod and Robison titration procedure (22). On the other hand, procedures employing solutions
of higher carbonate and iodide concentration (23), presumably caused further oxidation of the dicarbonylhexose as evident from the consumption of up to 3 moles of iodine per mole of sugar as compared with 1 mole of iodine per mole of aldohexose under the same conditions. Also compatible with the observation of Terada et al. (4), bromine-water, although oxidizing aldohexoses effectively, did not act appreciably on the dicarbonylhexose. Only under slightly alkaline conditions did oxidation occur and then it yielded unidentified products. If the substrate were indeed 6-aldofructose, oxidation by iodine or bromine would be expected to yield 6-fructuronic acid. In the experiments performed, fructuronic acid could not be found among the products. In this case, it must be assumed that oxidation by the halogens leads to products other than the hexonic acid. It should be mentioned that in some cases ketoses, e.g. epi-inosose, are oxidized by hypohalide in a manner similar to an aldehyde (30).

The pattern of reduction of the dicarbonylhexose by borohydride strongly favors a 2,5-diketohexulose structure. After partial reduction, both α-fructose and L-sorbose always were found in the solution. An aldohexose (α-mannose or L-gulose), as expected for the case of a 6-aldoketohexose molecule, was never detected after partial reduction. Total reduction of a 2,5-diketohexulose by borohydride would be expected to lead to D-sorbitol, D-mannitol, and L-iditol. Because of the difficulties encountered in achieving a clear-cut chromatographic separation of the three hexitols, their identification in the reduced dicarbonylhexose reaction mixture was not pursued exhaustively in the present studies.

However, it is of interest that although the 2,5-diketohexulose structure is the only one compatible with formation of L-sorbose and D-fructose on partial reduction, some properties of this sugar, such as susceptibility to hypohalide oxidation, color reactions with o-aminobiphenyl, o-aminobenzaldehyde, and with the reagent of Sawicki et al. (19); this reagent characteristically reacts with compounds bearing free carbonyl groups, aldohexoses in particular, but not with stable hemiacetals of aldo- and keto-sugars. Ring lability is also shown by the fact that solutions of this dicarbonyl sugar rapidly tend to develop browning upon standing, presumably due to polymerization, enolization, or other as yet unknown chemical changes.

5-Keto-D-fructose (α-threo-2,5-hexodiulose) has been obtained as an amorphous powder by chemical synthesis (31). Recently, Grazi et al. (20) have claimed the synthesis of trace amounts of this sugar by a transaldolase-catalyzed transfer between fructose 6-phosphate and hydroxyacrylaldehyde. However, many attempts by us to reproduce this synthesis with crystalline transaldolase have proved unsuccessful. Also, 5-ketofructose synthesized by G. cerinus did not serve as a substrate for transaldolase in the reverse reaction; i.e. fructose 6-phosphate was not formed in the presence of glyceraldehyde 3-phosphate.

SUMMARY

Spectral, chemical, and chromatographic properties of the dicarbonylhexose produced by Gluconobacter cerinus by oxidation of D-fructose were studied. In all respects, it was found to be identical with the dicarbonylhexose isolated by Weidenhagen and Bernsee (2) from an Acetobacter culture medium.

Several specific analytical procedures for the quantitative determination of the dicarbonylhexose are described.

While some color reactions of the compound were suggestive of the presence of an aldehyde group, reduction studies with sodium borohydride more curgently argued that the compound is 5-keto-D-fructose (α-threo-2,5-hexodiulose) as suggested originally by Terada, Tomizawa, Suzuki, and Kinoshita (5, 6).

Acknowledgments—We wish to thank Dr. Olga O. Blumenfeld for her help in the use of the modified 3-methyl-2-benzothiazolone hydrazone method. We are indebted to Dr. Donald P. Hollis and Dr. Robert Lederer for their help in the interpretation of the NMR spectra.

REFERENCES


31. Michelet, F., and Horn, K., Ann. Chem. Liebig's, 155, 1 (1934)
5-Keto-d-fructose : I. CHEMICAL CHARACTERIZATION AND ANALYTICAL DETERMINATION OF THE DICARBONYLHEXOSE PRODUCED BY GLUCONOBACTER CERINUS
Gad Avigad and Sasha England