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Note

Carbohydrates of *Nosema apis* spores*

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The spores from *Nosema apis*, a protozooan parasite of the honey bee, *Apis mellifera*, have been shown to release a number of carbohydrate components during sub-lethal heat treatment in water[1]. The present note deals with the characterization and quantitative determination of these components.

Fractionation of the mixture of products by paper chromatography yielded D-glucose, D-glucitol, and α,α-trehalose (α-D-glucopyranosyl α-D-glucopyranoside), which were identified as crystalline derivatives, but attempts to separate and identify further components by paper chromatography proved difficult. Consequently, an aqueous extract from a larger batch of the heat-treated spores was prepared and yielded 80% alcohol-soluble material which was fractionated by ion exchange into acid (22%) and neutral (78%) fractions. A gluconic acid fraction (32% of the acid fraction) was characterized as the crystalline amide. The neutral fractions, after acetylation and removal of excess α,α-trehalose and D-glucitol as their acetates by fractional crystallization and t.l.c., gave a product which was deacetylated and resolved by paper chromatography to give crystalline D-*glycero*-D-*gluco*-heptitol. The amount of D-fructose isolated was too small to permit isolation of a crystalline derivative.

The approximate percentage composition of the individual neutral components, as determined by g.l.c. of their *O*-trimethylsilyl derivatives, was: D-fructose, <1; D-glucose, 2; D-glucitol, 11; D-*glycero*-D-*gluco*-heptitol, 2; and α,α-trehalose, 64.

The actual significance of these compounds in the physiology of the spores, and in the course of the infection in the honey bee, remains to be determined. However, α,α-trehalose is the storage carbohydrate of the honey bee[2,3] and is present in the haemolymph of many other insects[4]. In the locust, α,α-trehalose arises from conversion of D-glucose during transport from the gut to the haemolymph[5], and in the bee, it is the epithelium cells of the gut which are invaded by the *N. apis* spores. Of further interest is Weinman's observation[6] that addition of trehalose to cultures containing non-infectious trypanosomes conferred infectivity on these organisms.

α,α-Trehalose is widely distributed in nature, in fungi, bacteria, insect blood,

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the trehala manna, algae, lichens, and certain higher plants[7], but honey apparently only contains α,β-trehalose[8]. This is only the second report of the natural occurrence of D-*glycero*-D-*gluco*-heptitol, the first being that of Charlson and Richtmyer[9] from the stems and leaves of *Sedum spectabile*.

EXPERIMENTAL

Analytical methods. — Paper chromatography was carried out by the descending method on Whatman No. 1 paper with the organic phases of (*a*) 8:2:1 ethyl acetate–pyridine–water or (*b*) butyl alcohol–acetic acid–water 4:1:5. Sugars, as their borate complexes, were separated by electrophoresis[10] on Whatman No. 3MM paper in 0.2M borate buffer (pH 10) at 800 volts for 2–3 h. Preparative thin-layer chromatography (t.l.c.) of sugar acetates was performed on glass plates (20 × 20 × 0.37 mm) coated with a uniform layer (250 μm) of Silica Gel G, with detection by spraying with water. Reducing sugars were detected with aniline hydrogen phthalate[11] (*A*), keto sugars with naphthoresorcinol[12] (*B*), non-reducing sugars and alditols with alkaline silver nitrate[13] (*C*), and alditols with Bromocresol purple and borate[14] (*D*). Evaporations were carried out at 35° on a rotary film evaporator. Melting points are corrected. Rotations were measured on a Perkin–Elmer 141 polarimeter and are equilibrium values.

Complete hydrolysis of the sugar samples (2–3 mg) was conducted with M sulphuric acid (0.3 ml) for 3 h at 100°. Partial hydrolyses were similarly conducted with 50 mM sulphuric acid for 45 min. The acetates, prepared in the usual manner by heating the sample with an equal weight of anhydrous sodium acetate and an excess of acetic anhydride for 3 h at 110–120°, were crystallized from methanol. Deacetylations were performed at 0° with 1% methanolic sodium methylate.

Trimethylsilyl (TMS) ethers[15] were prepared by treating the pyridine-dried samples (*ca.* 5 mg) with an aliquot (1 ml) of a solution containing pyridine (10 ml), hexamethyldisilazane (2 ml), and chlorotrimethylsilane (1 ml). After 30 min at room temperature, the reagents were removed by distillation *in vacuo*, and the residue was dissolved in hexane for injection into the column. Unless otherwise stated, g.l.c.[16] was carried out on a Pye 104 chromatograph using 6 ft × 0.25 in stainless-steel columns of 4% SE52 on Gas Chrom Q. The chromatograms were developed by holding for 15 min at 150° and then with temperature programming at a rate of 6°/min to 260°, using a nitrogen flow-rate of 60 ml/min.

Removal and extraction of spores. — Spore suspensions of *N. apis* were prepared from heavily infected bees (25–40 million spores/bee). The procedure for the isolation of spores and the preparation of spore extract has been given in a previous publication[1].

Identification and characterization of carbohydrates. — A sample of dried extract was separated on large sheets of Whatman No. 1 paper by using solvent (*a*). Location and elution of appropriate strips gave the following fractions.

Fraction **1** was tentatively identified as D-fructose from its R_F value (1.32) and colour reaction (spray *B*), and the g.l.c. behavior of its TMS derivative. The R_F value and colour reaction of a syrupy fraction **2** were identical with those of glucose. This

component, after further purification, was converted into its *p*-nitroaniline derivative which, after purification (t.l.c., 1:9 methanol–benzene), yielded crystalline *N*-*p*-nitrophenyl-β-D-glucosylamine dihydrate, m.p. and mixed m.p. 182–183°; lit.[17], m.p. 184°. The fractions **3** and **4** were non-reducing. Paper chromatography (R_G values 0.92 and 0.35, respectively) and paper electrophoresis indicated that the components were identical with D-glucitol and α,α-trehalose. The former component (60 mg) was acetylated, and the acetate, which crystallized from methanol, had m.p. 99–100°, undepressed on admixture with D-glucitol hexa-acetate, $[\alpha]_D^{20}$ +13.3° (*c* 0.21, chloroform); lit.[18], m.p. 99°, $[\alpha]_D$ +10° (chloroform). The latter component, on partial hydrolysis, yielded glucose and the unhydrolyzed fraction. Acetylation of a portion (40 mg) gave α,α-trehalose octa-acetate, m.p. 96–98°, $[\alpha]_D^{20}$ +162° (chloroform).

The two remaining components, which had R_G values of 0.09 and 0.71 (paper chromatography), were characterised by the following procedure.

Ion-exchange chromatography and characterisation of products. — A second batch of material (1.24 g), after removal of material (2.5 mg) that was insoluble in 80% ethanol, was eluted from a column (44 × 1 cm) of Rexyn CGI (CO_3^{2-}) with water (175 ml) to remove the neutral sugars (0.90 g). Gradient elution with 0–M ammonium carbonate, at a flow rate of 0.5 ml/min, then yielded material which was grouped into 4 acidic components on the basis of periodate–formaldehyde[20] and optical rotation analysis. The major formaldehyde-positive fraction (tubes 13–18) (periodate–formaldehyde assay, Fig. 1) was deionized with Rexyn 101 (H^+) resin and evaporated to dryness to yield material (82 mg) designated as the gluconic acid fraction. The remaining three acidic fractions (total yield 170 mg) were not further examined.

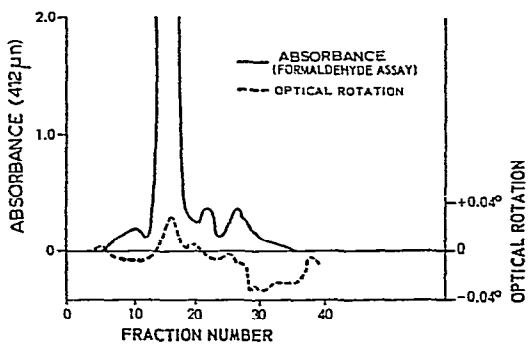

Fig. 1. Fractionation of *Nosema apis* extract on Rexyn CGI (CO_3^{2-}).

(*a*) D-*Gluconic acid.* A portion (2.8 mg) of the major acid component was submitted to a Ruff degradation and, after decolorization with carbon, filtration, and deionization, showed arabinose on paper chromatography [solvent (*a*), spray reagents *A* and *C*]. A second portion (37 mg) in liquid ammonia (5 ml) was held for 2 h at −40°. The ammonia was allowed to evaporate at room temperature under anhydrus conditions, leaving behind a residue which was dried overnight over phosphorus pentoxide and potassium hydroxide. The methanol-soluble portion of the residue, after decolorization with carbon and seeding with authentic D-gluconamide, deposited

crystals which, after three recrystallizations from methanol, showed m.p. and mixed m.p. 142.5–144°, $[\alpha]_D^{27}$ +30° (*c* 0.11, water); lit.[21], for D-gluconamide, m.p. 143–144°, $[\alpha]_D^{20}$ +31° (*c* 5.0, water).

(*b*) *Heptitol.* The neutral fraction (750 mg) was acetylated, and an ethanolic solution of the product was decolorized with carbon to yield a pale-yellow solution from which excess α,α-trehalose octa-acetate was removed by crystallization, the remainder being removed from the mother liquors by t.l.c. (two developments) on twelve plates with 4:96 methanol–benzene. The heptitol acetate band, which also contained acetates of D-glucitol, D-glucose, and D-fructose, was removed from the plates, and the powder was extracted with 1:1 chloroform–methanol to obtain the mixture of acetates from which excess D-glucitol hexa-acetate was removed by crystallization after seeding. A portion of the acetates on t.l.c. (5% methanol in benzene)[22] yielded, *inter alia*, a component having $R_{Glucitol}$ 0.70. G.l.c.[16] of this component was carried out on a Pye-Argon chromatograph fitted with an ionization detector and a straight, glass column (4 ft × 0.25 in), containing 10% neopentyl glycol sebacate on 80–100 mesh Chromosorb W. The column temperature was 225°, and development was made at an argon flow-rate of 150 ml/min. The results showed a major component having a retention time identical with that of D-*glycero*-D-*gluco*-heptitol hepta-acetate.

The remaining syrup (110 mg) from the mother liquors was deacetylated to yield a syrup (57 mg) which was separated on seven sheets of Whatman No. 1 paper (7 × 22 in) by using solvent (*a*). Location and elution of appropriate strips yielded a yellow syrup (23 mg) which was dissolved in hot methanol, and the solution was decolorized with carbon, filtered, and seeded with authentic D-*glycero*-D-*gluco*-heptitol. The resulting crystals (3.5 mg) showed m.p. 131–131.5°, mixed m.p. 131–132°, and $[\alpha]_D^{28}$ +62° (*c* 0.34, 5% ammonium molybdate); lit.[23], m.p. 131–132°, $[\alpha]_D$ +49.6° (5% ammonium molybdate). The authentic D-*glycero*-D-*gluco*-heptitol, kindly supplied by Dr. Richtmyer, showed $[\alpha]_D^{30}$ +52.5° (*c* 0.34, 5% ammonium molybdate) which

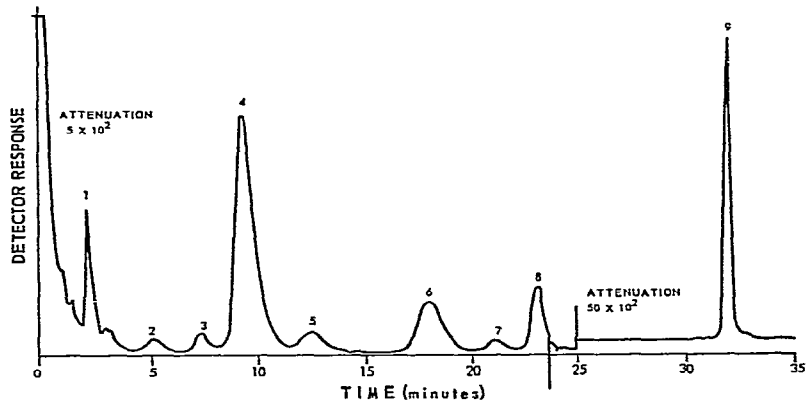

Fig. 2. Gas–liquid chromatogram of trimethylsilyl ethers of *Nosema apis* carbohydrates. (1) Reagent impurity; (2) D-fructose; (3) α-D-glucose; (4) D-glucitol; (5) β-D-glucose; (6) inositol standard and unknown peak 1; (7) unknown peak 2; (8) D-*glycero*-D-*gluco*-heptitol; (9) α,α-trehalose.

was raised to $[\alpha]_D^{27}$ +63° (*c* 0.34, 5% ammonium molybdate) after three crystallizations from methanol at room temperature.

Quantitative determination of components. — The unknown mixture was trimethylsilylated and examined by g.l.c.; a typical result is shown in Fig. 2.

The approximate percentage composition of the mixture shown below was determined by comparison with appropriately prepared, standard solutions of the TMS ethers of D-glucose, D-fructose, D-glucitol, α,α-trehalose, D-*glycero*-D-*gluco*-heptitol, and inositol (internal standard).

D-Fructose	<1
D-Glucose	3
D-Glucitol	14
D-*glycero*-D-*gluco*-Heptitol	2
α,α-Trehalose	80

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