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Hexose Phosphate Synthase from *Methylococcus capsulatus* Makes D-*arabino*-3-Hexulose Phosphate

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The product of the reaction catalysed by hexose phosphate synthase prepared from *Methylococcus capsulatus* was dephosphorylated and the sugar moiety purified. The sugar and derivatives were compared by various chromatographic and other methods with authentic samples of allulose (psicose), D-*erythro*-L-*glycero*-3-hexulose and D-*erythro*-D-*glycero*-3-hexulose. The sugar is not allulose, as was previously thought on the basis of less extensive evidence (Kemp & Quayle, 1966), but is in fact D-*erythro*-L-*glycero*-3-hexulose (D-*arabino*-3-hexulose). This identification is consistent with recent studies which have shown that hexose phosphate synthase catalyses the condensation of formaldehyde with D-ribulose 5-phosphate rather than with D-ribose 5-phosphate (Kemp, 1972).

Kemp & Quayle (1966) reported that a crude extract prepared from *Pseudomonas methanica* catalysed the condensation of formaldehyde with D-ribose 5-phosphate to give the phosphate of a compound tentatively identified as allulose (psicose). The reaction was proposed as the first step of a novel pathway for the assimilation of carbon, the ribose phosphate cycle (Kemp & Quayle, 1967). The identification of the product of the reaction as allulose was based on co-chromatography and co-crystallization of ^{14}C-labelled product with authentic allulose or derivatives. Subsequent experiments to detect the product chemically as a 2-hexulose by using tests for authentic allulose were, however, unsuccessful (Kemp, 1966).

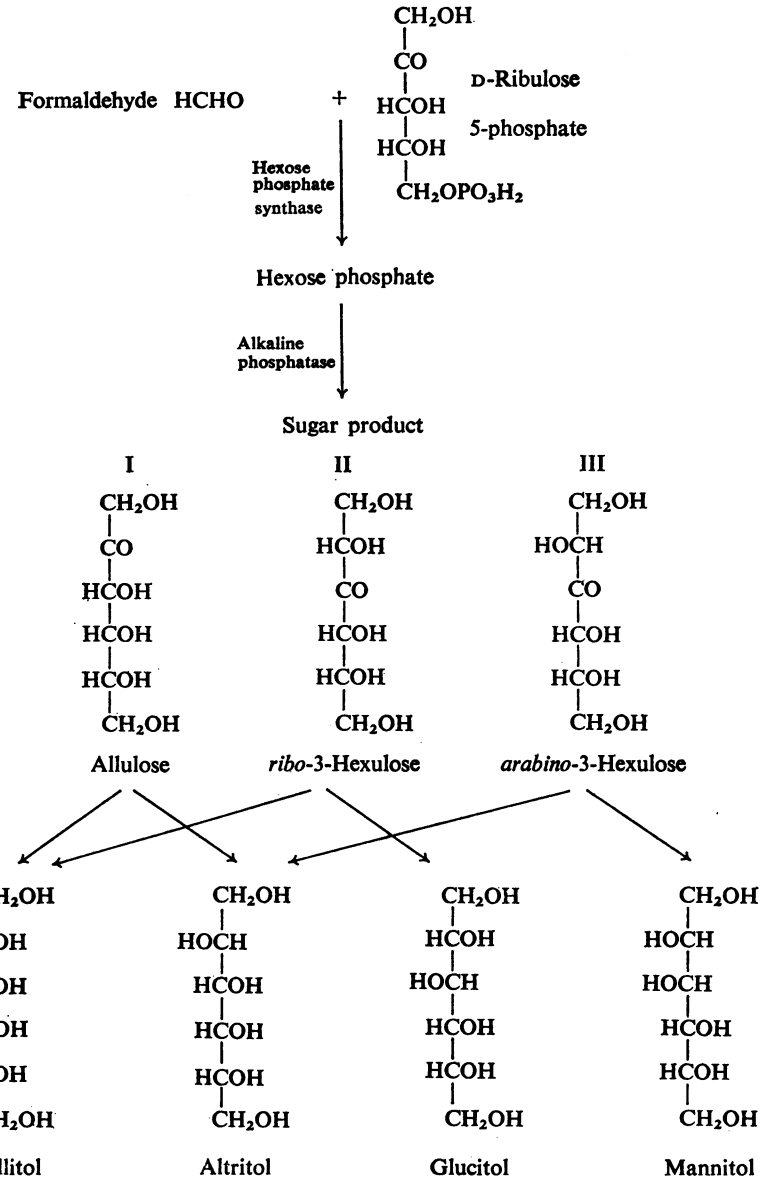
This discrepancy was investigated initially by attempting to purify the enzyme (designated hexose phosphate synthase) from *Methylococcus capsulatus*, in which the same pathway occurred (Lawrence *et al.*, 1970). It was found (Kemp, 1972) that the crude extract could be fractionated into two components; the soluble fraction catalysed the isomerization of ribose 5-phosphate into ribulose 5-phosphate, which then condensed with formaldehyde in the presence of the particulate fraction.

Chemically, it seemed unlikely that allulose (I, Scheme 1) should be the sugar moiety of the product of such a condensation; one of the two 3-hexuloses (II) or (III) seemed more plausible. The recent unambiguous syntheses of all four 3-hexuloses (Angyal & Evans, 1971) has now permitted a comparison of the properties of the dephosphorylated product of the hexose phosphate synthase reaction with those of authentic materials.

Materials and Methods

Preservation and growth of Methylococcus capsulatus

The strain used was obtained initially from Dr. J. W. Foster (Foster & Davis, 1966). It was maintained and grown at 37°C in a medium of composition (per litre): Na_2HPO_4, 0.6g; KH_2PO_4, 0.4g; NH_4Cl, 0.4g; $MgSO_4,7H_2O$, 0.2g; $FeCl_3,6H_2O$, 16.7mg; $CaCl_2,2H_2O$, 0.66mg; $ZnSO_4,7H_2O$, 0.18mg; $CuSO_4,5H_2O$, 0.16mg; $MnSO_4,4H_2O$, 0.15mg; $CoCl_2,6H_2O$, 0.18mg; H_3BO_3, 0.1mg; $Na_2MoO_4,2H_2O$, 0.3mg. The gas atmosphere was methane+air (50:50). Agar plates were made by the addition of 1.2% (w/v) Ionagar (Oxoid Ltd., London S.E.1, U.K.) to the medium. Plates and starter bottles (100ml, containing 10ml of medium) were incubated in desiccators; shaken cultures were contained in conical flasks with ground-glass necks and taps which were shaken at 200cycles·min^{-1} on a rotary shaker. Portions of culture (5ml) from starter bottles were used to inoculate 50ml of medium in 250ml conical flasks; after 24h the contents of one conical flask were poured into a larger flask (3 litres, containing 600ml of medium). The larger flasks were incubated for 24h, by which time the density of the cultures was 0.1–0.2mg dry wt.·ml^{-1} and the cultures were harvested by centrifuging for 20min at 12000*g* and at 2°C. The gelatinous pellet was resuspended in cold 20mM-



Scheme 1. *Possible products of the reaction catalysed by hexose phosphate synthase and the sugar alcohols derived from them*

sodium phosphate, pH7.0 (200ml/litre of culture), and centrifuged again.

Preparation of enzyme

The washed pellet of cells obtained above was resuspended in 3ml of cold 20mM-sodium phosphate, pH7.0, containing 5mM-$MgCl_2$ and 2mM-D-ribose 5-phosphate (Lawrence *et al.*, 1970)/litre of original culture. Portions (3ml) were disrupted for 10min by using an MSE 100W ultrasonic disintegrator at full power equipped with a 6mm probe. The treated suspension was centrifuged for 15min at 12000*g* and the resultant supernatant liquid for 90min at

144000*g*. The resulting pellet was resuspended in the same buffer, centrifuged again, resuspended again (1ml/litre of original culture) and stored in ice.

Preparation of the sugar product

D-Ribose 5-phosphate (2mmol) was preincubated for 60min at 30°C with ribose phosphate isomerase (EC 5.3.1.6; 100EC units) in 20mM-triethanolamine-HCl, pH7.0, containing 5mM-$MgCl_2$; the total volume was 400ml. Assay of samples with cysteine-carbazole (Ashwell, 1957) showed that equilibrium had been reached. [^{14}C]Formaldehyde (2mmol, 10μCi) and hexose phosphate synthase [high-speed pellet (4ml) prepared as described above], were added and after incubation for 1h the reaction was stopped by the addition of ethanol (1.6litres) and barium acetate (5mmol). The precipitate was collected, washed, dried, redissolved in water (20ml), and passed through 10g of Dowex 50 (H^+ form). The eluate was diluted to 400ml, adjusted to pH9.0 with NaOH and incubated for 2h at 37°C with calf intestinal alkaline phosphatase (EC 3.1.3.1.; 125EC units), after which less than 10% of the radioactivity could be precipitated with barium acetate and ethanol (Kemp & Quayle, 1966). The mixture was passed through Dowex 50 (H^+ form) and Dowex 1 (HCO_3 form) and applied to a column (300mm × 30mm) of Dowex 1 (borate form). The sugars were eluted with 3 litres of a linear potassium tetraborate gradient (5–75mM); 10ml fractions were collected and assayed for radioactivity, for pentose (phloroglucinol) and for ketose (cysteine–carbazole) (Ashwell, 1957, 1966). The fractions from the main radioactive peak were pooled, passed through Dowex 50 (H^+ form) and evaporated to dryness (rotary evaporator, 40°C). The boric acid was removed by twice adding methanol and evaporating to dryness. The product was shaken with charcoal, passed through Dowex 50 (H^+ form) and Dowex 1 (HCO_3^- form) and evaporated to a syrup.

A second sample of the sugar product, but with ten times the specific radioactivity, was made by the same method, except that it was scaled down one-hundredfold and the sugar was purified by paper chromatography (see below).

Preparation of derivatives

Sugars were converted into the corresponding alcohols by reduction of the sugar, as an aqueous 1% solution, with 10 times its weight of $NaBH_4$. The mixture was then cautiously acidified with 2M-acetic acid to pH5, passed through Dowex 50 (H^+ form) and the boric acid removed by the repeated evaporation of methanol. The alcohols were acetylated by the method of Angyal & Evans (1972).

Isopropylidene derivatives of the sugar were made and acetylated by the methods of Angyal & Evans (1971).

Separation of sugars and derivatives

Sugars were separated by two-dimensional paper chromatography as described by Large *et al.* (1961). The solvent system of Grado & Ballou (1961) was also used. Sugars and alcohols were separated by electrophoresis on Whatman no. 1 paper in 0.2M-sodium arsenite, pH9.6, for 1h (Frahn & Mills, 1959); the voltage was 50V·cm (length)$^{-1}$ and the current 4mA·cm (width)$^{-1}$.

The urea–phosphate spray reagent of Wise *et al.* (1955) was used to detect hexuloses. Sugar alcohols were detected by spraying first with 0.1% sodium periodate in 0.2M-acetic acid and then after 3min with 0.5% *p*-anisidine in ethanol–acetic acid (4:1, v/v).

Radioactive compounds were located as described by Large *et al.* (1961)

The unknown sugar product was compared with reference compounds by paper chromatography and electrophoresis in two ways. First, chemically indetectable samples of the unknown, prepared with high specific radioactivity, were mixed with chemically detectable amounts of the reference compounds. After the treatment, the positions of the radioactivity and of the chemically detectable material were compared. Secondly, with the use of samples of the unknown prepared with low specific radioactivity, similar, chemically detectable, amounts of both unknown and reference compound were mixed and the spot(s) found after the treatment compared with those from the unknown and reference compounds treated separately.

Sugar alcohol acetates were separated by Dr. P. M. Holligan by g.l.c. on a 1.5m × 6mm column containing 2% (w/w) ECNSS-M on Universal B (85–100 mesh) (Field Instruments Co. Ltd., Surbiton, Surrey, U.K.) operated at 165°C rising at 2°C·min^{-1} to 190°C with N_2 as the carrier gas (45ml·min^{-1}) in a Pye model 64 machine (Pye Unicam Ltd., Cambridge, U.K.).

Di-isopropylidene derivatives of sugars were separated by t.l.c. as described by Angyal & Evans (1971) and by g.l.c. on a 1.2m × 3mm column containing 25% (w/w) silicone oil MS200/200 on Celite operated at 170°C with N_2 as the carrier gas (40ml·min^{-1}) in a Pye model 104 machine. Mass spectra were recorded by Dr. R. K. Mackie with an AEI MS902 machine.

Measurement of radioactivity

The radioactivity of solutions obtained during the purification of the sugar product was determined by liquid-scintillation counting of portions (10μl) mixed with ethanol (1ml) and toluene (5ml)

containing butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; 35mg].

Materials

Ribose 5-phosphate, sugars and enzymes were obtained from Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K., and [^{14}C]-formaldehyde was from The Radiochemical Centre, Amersham, Bucks., U.K. Some rare sugars were most kindly given (see the acknowledgements). Iditol and altritol were obtained (in mixtures) by reducing sorbose and tagatose as described above.

Results

The sugar isolated as described in the Materials and Methods section was judged to be less than 10% contaminated with other sugars; non-radioactive pentoses could easily be detected by g.l.c. of the sugar alcohol acetates and radioactive sugars by paper chromatography and electrophoresis.

Properties of the sugar product

Although the product gave the Nelson and Park-Johnson tests (Ashwell, 1957) for a reducing sugar, it did not give the cysteine–sulphuric acid or cysteine–carbazole tests for a 2-hexulose (Ashwell, 1957, 1966); these tests, however, were positive with authentic allulose. On a paper chromatogram, the product gave a pink colour with the urea–phosphate spray as did authentic *arabino*- and *ribo*-3-hexuloses, whereas allulose and fructose gave a grey–green colour. Fukui *et al.* (1963) reported that 3-oxosucrose gave a pink colour with this spray reagent.

By two-dimensional paper chromatography the product could be separated from allose, altrose, glucose, fructose and mannose. Separate mixtures of radioactive product with allulose, *arabino*-3-hexulose and *ribo*-3-hexulose gave radioactive spots which closely overlapped the chemical material revealed by spraying. The match was perfect with *arabino*-3-hexulose and almost perfect with *ribo*-3-hexulose; with allulose there was a discrepancy, in the second solvent, of typically 3mm in a spot of diameter 30mm which had migrated 200mm. When similar chemical amounts of the product and allulose were mixed and analysed by the same method, closely overlapping pink and green spots were obtained.

The sugars were also analysed chromatographically by using the solvent of Grado & Ballou (1961), which contains borate. The product did not separate from either of the 3-hexuloses but separated clearly from allulose.

Of the various solutions used by Frahn & Mills (1959), that containing arsenite had the greatest resolving power. By this method, the product was found to migrate more rapidly than allulose (Table 1); although the two 3-hexuloses migrated at almost the same rates, the radioactive product coincided completely only with the *arabino* isomer.

Reduction to sugar alcohols

ribo- and *arabino*-3-Hexuloses should be readily distinguishable both from each other and from allulose by the hexitols obtained by reducing the sugars (Scheme 1). A method widely used for separating hexitols utilizes g.l.c. of the acetate derivatives (Holligan, 1971), but Angyal & Evans (1972) failed to separate by g.l.c. the derivatives obtained by the reduction of *arabino*-3-hexulose. Similarly, the six straight-chain hexitols examined here gave only five peaks, because those from mannitol and altritol coincided. Authentic allulose and *ribo*-3-hexulose each gave rise to two peaks on reduction, coincident with those from the expected sugar alcohols (Scheme 1), whereas the product and authentic *arabino*-3-hexulose each gave, on reduction, only one peak, coincident with each other and with those from mannitol and altritol.

Paper chromatography of the sugar alcohols in the solvent of Grado & Ballou (1961) clearly resolved allitol from the other hexitols. The product and authentic *arabino*-3-hexulose gave no allitol, whereas allulose and *ribo*-3-hexulose did. The other hexitols were not so clearly separated, but the radioactivity from the product coincided with a mixture of mannitol and altritol.

Electrophoresis of the alcohols in the arsenite solution of Frahn & Mills (1959) separated all six hexitols. The product gave rise to two major spots, coincident with those from altritol and mannitol. *arabino*-3-Hexulose gave the same spots, whereas allulose and *ribo*-3-hexulose gave the expected alcohols, the allitol spot being particularly well separated (Table 1).

Di-isopropylidene derivatives

The precise stereochemistry of the hydroxyl groups in the ring forms of the sugars influences markedly the structures of the di-isopropylidene derivatives that can be formed and their relative stabilities and hence abundances in an equilibrium mixture. Further, if the free hydroxyl group of a di-isopropylidenehexose is a tertiary hydroxyl group, it cannot be acetylated, whereas a primary or secondary hydroxyl group can be. The presence of the extra rings in the di-isopropylidene derivatives causes their mass spectra to differ much more than the mass spectra of other sugar derivatives, such as trimethylsilyl ethers. These di-isopropylidene derivatives therefore offer a sensitive test for distinguishing isomeric hexoses.

Table 1. *Electrophoretic separation of sugars and sugar alcohols*

The technique used (see the Materials and Methods section) was essentially the same as that used by Frahn & Mills (1959), but no direct comparison of the results obtained in the present study with those obtained by Frahn & Mills (1959) is implied. Rather, their data are included to eliminate also sugars not covered in the present paper. Mobilities are expressed relative to ribose = 100.

Sugar or alcohol	Data of Frahn & Mills (1959)	This study
Allose	75	—
Altrose	77	—
Glucose	16	15
Mannose	35	—
Gulose	53	—
Idose	115	—
Galactose	28	—
Talose	119	—
Allulose	188	165
Fructose	75	75
Sorbose	73	—
Tagatose	103	—
ribo-3-Hexulose	—	184
arabino-3-Hexulose	—	180
Allitol	92	88
Altritol	138	122
Glucitol	161	146
Mannitol	130	113
Iditol	173	158
Galactitol	145	127

Analysis by t.l.c. and g.l.c. of the derivatives from the product showed two major components coincident with the authentic 2,3:4,5-di-*O*-isopropylidene-*β*-*arabino*-3-hexulose and 1,2:4,5-di-*O*-isopropylidene-*β*-*arabino*-3-hexulose. From the relative areas of the peaks obtained by g.l.c., the relative proportions were 66:34 respectively. Authentic *arabino*-3-hexulose gave the same two peaks in roughly the same proportions (70:30). According to Angyal & Evans (1971), *arabino*-3-hexulose gives these peaks in the proportions 60:35, the remaining 5% being a third isomer which, under the conditions used in the present paper, coincided with the major peak. Acetylation of the mixtures from the product and from authentic *arabino*-3-hexulose caused the disappearance of the major peak and the appearance of a new peak migrating more rapidly in t.l.c. and more slowly in g.l.c. Of the four 3-hexuloses, only *arabino*-3-hexulose gives a mixture containing large amounts of more than one di-isopropylidene derivative, and it is the only 3-hexulose to give a derivative with a hydroxyl group resistant to acetylation (Angyal & Evans, 1971).

The column used for separation of the derivatives before determination of the mass spectra did not separate the two major di-isopropylidene derivatives, but the single peak obtained from the product and from authentic *arabino*-3-hexulose gave identical spectral lines of similar intensities, except for one extra line from the authentic compound; this may have arisen because the free sugar was not isolated in the synthesis of the derivatives. The acetylated derivatives did separate from those resistant to acetylation and the new peaks gave identical mass spectra.

Discussion

All of the evidence presented is consistent with the identity of the sugar moiety of the product of the reaction catalysed by hexose phosphate synthase from *Methylococcus capsulatus* and D-*erythro*-L-*glycero*-3-hexulose (*arabino*-3-hexulose). The evidence clearly eliminates all of the other straight-chain hexoses, including allulose (psicose) which had previously been identified as the product made by an extract from *Pseudomonas methanica* (Kemp & Quayle, 1966). Although the product studied in the present paper was made by an extract from *M. capsulatus*, the pathways of carbon assimilation in the two organisms are very similar (Lawrence *et al.*, 1970). The sugar made by the extract from *P. methanica*, like that made by the extract from *M. capsulatus*, gave a pink colour in the spray test for hexulose; it failed to give colour reactions specific for a 2-hexulose and the radioactivity of the product did not coincide completely with that of marker allulose upon chromatography. It is therefore extremely unlikely that the products obtained by using the two organisms are different.

The discrepancy between the present and the previous work may be due to a sample of the sugar or the sugar phosphate partly or completely isomerizing to allulose or allulose phosphate. It is known that the 3-heptulose, coriose, can be isomerized readily to a 2-heptulose in dilute alkali (Okuda & Konishi, 1968). For this reason, care was taken in this investigation to check that the material subjected to mildly alkaline conditions in the hydrolysis with alkaline phosphatase and in the chromatography in a borate gradient had not been altered. Small samples hydrolysed with acid phosphate or purified by paper chromatography gave the same colour tests and movement in two-dimensional chromatography as did the large-scale sample.

No mention has been found in the literature of the natural occurrence of a 3-hexulose or of a derivative, although a 3-heptulose, coriose, has been described (Okuda & Konishi, 1968) and 3-keto sugars (e.g. 3-oxosucrose, i.e. dicarbonylhexose derivatives) have been found in culture filtrates of *Agrobacterium* (Bernaerts & De Ley, 1958; Fukui *et al.*, 1963).

The condensation of formaldehyde with D-ribulose 5-phosphate to give *arabino*-3-hexulose 6-phosphate

is an aldolase reaction analogous to that catalysed by fructose phosphate aldolase (EC 4.1.2.7):

Formaldehyde + dihydroxyacetone phosphate → L-erythrulose 1-phosphate

Reactions catalysed by aldolase enzymes create a new optically active centre at what was C-1 of the acceptor ketose phosphate (Feingold & Hoffee, 1972). Hexose phosphate synthase catalyses a condensation which gives the same configuration at that carbon atom as in the reaction catalysed by rhamnulose 1-phosphate aldolase:

L-Lactaldehyde + dihydroxyacetone phosphate → L-rhamnulose 1-phosphate

This configuration is the opposite of that created at the equivalent carbon atom (C-3) of fructose 1,6-diphosphate in the reaction catalysed by fructose diphosphate aldolase:

D-Glyceraldehyde 3-phosphate + dihydroxyacetone phosphate → D-fructose 1,6-diphosphate

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