Phosphorylated Sugars. Part XVII.[1] Synthesis of 3-Deoxy-D-*arabino*-[1-^{14}C]heptulosonic Acid 7-(Dihydrogen Phosphate)

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The title compound was synthesised by addition of potassium [^{14}C]cyanide to 2-deoxy-D-*arabino*-hexose 6-phosphate and selective oxidation of the resulting 3-deoxyheptonic acid 7-phosphate with chlorate and vanadium oxide.

In many moulds, yeasts, and bacteria, as well as in plants, the synthesis of the aromatic amino-acids tyrosine, tryptophan, and phenylalanine is controlled by specific enzymes, all of which condense phosphoenolpyruvate with D-erythrose 4-phosphate to form the key intermediate 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (IV). This compound has been synthesised by Sprinson *et al.*,[2] by addition of hydrogen cyanide to 2-deoxy-D-*arabino*-hexose, transformation of the

[1] Part XVI, F. Trigalo and L. Szabó, preceding paper.
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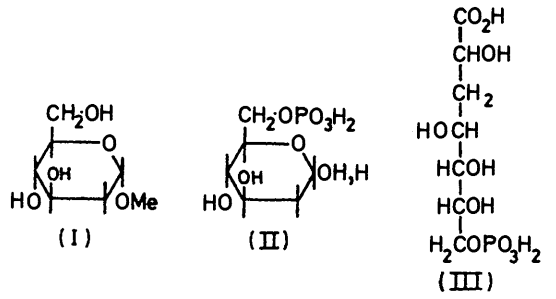
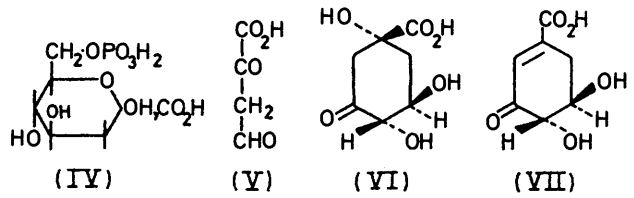
resulting 3-deoxy-D-*gluco*-heptonic acid into its 7-phosphate, and oxidation of this compound to the phosphorylated aldulosonic acid.

In connection with work in progress, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate labelled with ^{14}C was required. Since the reaction sequence described would involve introduction of the labelling in the first stage of a multi-step synthesis, its use was not considered feasible. The following reaction sequence was therefore used: methyl 2-deoxy-α-D-*arabino*-hexopyranoside [3] (I) was transformed into the 6-phosphate [4] and then into the phosphorylated free sugar [5] (II).





The mixture of phosphorylated 3-deoxyheptonic acids (III) was obtained from this by HCN addition and hydrolysis. Selective oxidation of the epimeric hydroxy-groups by Regna and Caldwell's method [6,7] gave the required phosphorylated deoxyaldulosonic acid (IV; the pyranose form is an arbitrary assignment), which was isolated as its calcium salt after ion-exchange chromatography. The original Regna–Caldwell method rather than the more rapid procedure [2] was used, as it has been observed [1] that Sprinson's more reactive catalyst can give rise to secondary products difficult to separate from the main compound.

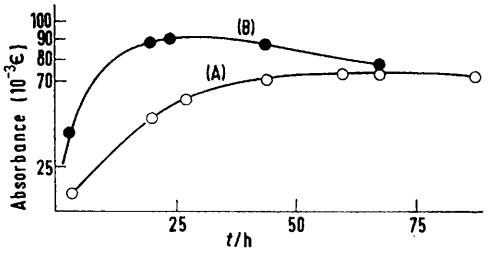
The postulated structure was confirmed by the following reactions. (*a*) With semicarbazide,[8] a semicarbazone having an absorption maximum at 250 nm was formed; the molar absorption coefficient in this test was, as expected, 10,000, but this value was reached only after about 60 minutes of incubation instead of the usual 15 min. Similar slow semicarbazone formation has previously been observed for the non phosphorylated parent acid [9] and for 3-deoxy-D-*manno*-octulosonic acid.[10]

(*b*) Following dephosphorylation with acid phosphatase, the compound reacted rapidly in the periodate–thiobarbiturate test: [11] the theoretical [10] value of 95,000 was attained within 25 h (Figure); in the same test the phosphorylated acid had a maximal value of only about 75,000, attained after oxidation with periodate for about 70 h (Figure). It is likely that the compound's low rate of reaction with periodate involving the concomitant prolonged exposure of the chromogenic fragment, *i.e.* of 2,4-dioxobutyrate (V), to the oxidant, which is known to destroy it, is responsible for this phenomenon.

(*c*) The position of the phosphate group was ascertained by treating the compound successively with borohydride, periodate, and again borohydride, and identifying the polyol phosphate present by paper electrophoresis.[12] Only ethylene glycol phosphate was detected and this proved that the deoxyheptulosonate was phosphorylated on C-7 and, consequently, that no phosphate migration had occurred.

(*d*) The purity of the compound was also checked by sequential enzymic transformation of the deoxyheptulosonic acid phosphate into 3-dehydroshikimic acid * (VI) *via* 3-dehydroquinic acid (VIII) with 3-dehydroquinate synthetase and 3-dehydroquinase, respectively: 0·96 mol of 3-dehydroshikimate were formed per mol of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate treated.

The labelled compound was synthesised by carrying out the same reaction sequence without isolation of the intermediates. From 1·3 mg of potassium [^{14}C]cyanide (specific activity 48·2 mCi mmol^{-1}; 1 mCi) *ca.* 15 mg of calcium 3-deoxy-D-*arabino*-heptulosonate with specific activity 4·5 mCi mmol^{-1} were obtained. About the same amount of labelled calcium 3-deoxyheptonate 7-phosphate (specific activity 3·67 mCi mmol^{-1}) was also isolated.



Kinetic behaviour of (A) phosphorylated and (B) unphosphorylated 3-deoxy-D-*arabino*-heptulosonic acid in the periodate–thiobarbiturate reaction.

* Nomenclature according to *European J. Biochem.*, 1968, **5**, 1.

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EXPERIMENTAL

All evaporations were carried out under reduced pressure below 40°. M.p.s were taken with a Kofler hot-stage apparatus. Optical rotations were measured with a Perkin-Elmer 141 polarimeter.

3-*Deoxyheptonic Acid* 7-*Phosphate* (III).—Methyl 2-deoxy-α-D-*arabino*-hexoside [3] (I) (1·8 g) was transformed into its 6-phosphate by the procedure used to prepare the β-anomer.[5] The *barium salt* obtained (1·5 g) had $[\alpha]_D^{22}$ +56·2° (*c* 1 in 0·1N-HCl) (Found: C, 20·9; H, 3·55; P, 7·5. $C_7H_{13}BaO_8P,H_2O$ requires C, 20·4; H, 3·7; P, 7·5%). The *biscyclohexylammonium salt* had m.p. 171—172°, $[\alpha]_D^{22}$ +46·4° (*c* 1 in H_2O water) (Found: C, 49·65; H, 9·1; N, 6·1; P, 6·7. $C_{19}H_{41}N_2O_8P$ requires C, 50·0; H, 9·0; N, 6·1; P, 6·8%) {lit.,[4] m.p. 216—218° (decomp.) $[\alpha]_D^{22}$ +42·8°}. Hydrolysis of the barium salt under Wolfrom and Franks' conditions [5] gave the phosphorylated free sugar (II), isolated as its lithium salt by adjusting the pH of the acid solution to 6·95 with N-lithium hydroxide, concentration (to *ca.* 5 ml) and addition of ethanol (100 ml); it contained traces of inorganic phosphate and was used in the next step without purification. This lithium salt (1·2 g) was dissolved in water (5 ml) and potassium cyanide (780 mg) was added; the mixture was left at 4° for 48 h, then passed through a column of Amberlite IR120 (H^+) resin (100 ml), and the pH of the effluent and washings was brought to 8·5 with saturated aqueous barium hydroxide. The solution was heated on a boiling water-bath for 15 min, clarified with charcoal, and concentrated (*ca.* 10 ml). The barium salt of the title compound (1·5 g) was precipitated with ethanol (150 ml), centrifuged off, dried, and equilibrated in air; $[\alpha]_D^{22}$ −3·6° (*c* 1 in H_2O) (Found: C, 16·0; H, 3·0; P, 5·7. Calc. for $C_7H_{12}Ba_{1·5}O_{10}P,2H_2O$: C, 15·9; H, 3·0; P, 5·9%).

3-*Deoxy*-D-arabino-*heptulosonic Acid* 7-*Phosphate* (IV).—The lithium salt [prepared by passing a solution of the barium salt through a column of Amberlite IR120 (H^+) resin, raising the pH of the acid effluent to 7 with lithium hydroxide solution, and precipitating the lithium salt by addition of ethanol to the concentrated solution] of the phosphorylated 3-deoxyheptonic acid (350 mg) was mixed with commercial vanadium(v) oxide (3 mg), potassium chlorate (43 mg), and water (1 ml) containing phosphoric acid (0·035 ml; 0·85%; *d* 1·71). The pH of the mixture was adjusted to 4·6—4·8 with pyridine or dilute (1 : 10) phosphoric acid, and the contents of the stoppered tube were stirred for 5 days, and then passed through a column of Amberlite IR120 (H^+) resin (100 ml). The pH of the acid effluent was brought to 7·5 with dilute ammonium hydroxide and the solution was passed through a column of Dowex 1 × 8 resin (100—200 mesh; Cl^- form; 10 ml) which, after being washed with water was eluted with 0·01N-hydrochloric acid (80 ml h^{-1}). The total phosphorus content of the fractions (each 12·5 ml) was estimated.[13] When this was zero or very small (*ca.* 50 fractions), elution was continued with 0·02N-acid and the fractions were tested for phosphorus and α-keto-acid; [8] those containing the phosphorylated heptulosonic acid were pooled and the pH of the solution was brought to 6·9 with aqueous calcium hydroxide. The solution was concentrated (to *ca.* 2—3 ml) below 35° and the pH was adjusted to 7·6 [aq. $Ca(OH)_2$]. The *calcium salt* (150 mg) of the title compound was precipitated with ethanol (100 ml), centrifuged off, washed free of calcium chloride with ethanol (4 × 30 ml), washed with acetone (30 ml), dried *in vacuo* (P_2O_5), and equilibrated in air.[14] It had $[\alpha]_D^{22}$ +18° (*c* 0·5 in water) (Found: C, 21·0; H, H, 4·0; P, 7·6. $C_7H_{10}Ca_{1·5}O_{10}P,3H_2O$ requires C, 21·05; H, 4·0; P, 7·8%).

3-*Deoxy*-D-arabino-[1-^{14}C]*heptulosonic Acid* 7-*Phosphate*.—A solution of potassium [^{14}C]cyanide (1·3 mg; 1 mCi; specific activity 48·2 mCi $mmol^{-1}$) in water (0·5 ml) was added to 2-deoxy-D-*arabino*-hexose 6-phosphate lithium salt dihydrate (45 mg). After 1 h, unlabelled cyanide (7 mg) was added and the mixture was left for 40 h at room temperature and concentrated to dryness.

Commercial vanadium(v) oxide (30 mg), potassium chlorate (430 mg), water (9·65 ml), and phosphoric acid (85%; *d* 1·71; 9·65 ml) were thoroughly mixed; this suspension (0·25 ml) was added to the above dry residue and the mixture (pH *ca.* 5) was thoroughly shaken in a closed vial for 3 days. Water (20 ml) was added, the pH of the solution brought to 7 with N-ammonia, and the volume made up to 50 ml with water (total activity $2·7 \times 10^9$ disint. min^{-1}). The solution was passed through a column (12 × 35 mm) of Dowex 1 × 8 resin (100—200 mesh; Cl^- form) and the column was washed with water (40 ml) and eluted with 0·01N-hydrochloric acid (100 ml h^{-1}). Elution of the 3-deoxyheptonic acid 7-phosphate was followed by measuring the radioactivity of samples (10 μl) of the fractions (10 ml). When the activity had dropped to a very low value, the column was eluted with 0·02N-hydrochloric acid and the elution of 2-deoxy-D-*arabino*-heptulosonic acid 7-phosphate was monitored by thiobarbiturate assay and measuring the radioactivity of samples (20 and 10 μl, respectively). The calcium salt of 3-deoxy-[1-^{14}C]heptonic acid 7-phosphate was recovered from the pooled fractions and washings (162 ml) by adjusting the pH to 8 with saturated aqueous calcium hydroxide and concentration to 8 ml; the suspension was cleared by centrifugation, the sediments were washed with water (4 × 2 ml), and the pooled supernatant solution was concentrated to *ca.* 0·2 ml. The compound (26·2 mg) was precipitated with ethanol (6 ml), collected by centrifugation, washed with ethanol (2 × 6 ml), and dried *in vacuo* (P_2O_5). It contained 63% w/w of phosphorylated deoxyaldonic acid (specific activity 3·67 mCi $mmol^{-1}$), no inorganic phosphate, and a very small amount of radiochemical impurity, probably the lactone.

The pooled fractions and washings (365 ml; 0·1742 mCi) were brought to pH 6·9 with aqueous calcium hydroxide and concentrated to 0·2 ml, the pH being kept constant at 6·9 during the concentration by further addition of the aqueous base. The calcium salt of 3-deoxy-D-*arabino*-[1-^{14}C]heptulosonic acid 7-phosphate (31·1 mg) was precipitated with ethanol (6 ml) and the mixture was centrifuged; the sediment was washed with ethanol (2 × 6 ml), dried *in vacuo* (P_2O_5), and equilibrated in air. On the basis of its phosphorus content (3·93%. Calc. for the pure trihydrate: 7·3%; no inorganic phosphate was detected), the preparation contained about 50% (w/w) of the title compound. The ratio of phosphorus to α-keto-acid [8] to 3-deoxyaldulosonic acid [11] in the preparation was 1 : 1 : 1. The specific activity of the phosphorylated aldulosonic acid was 4·5 mCi $mmol^{-1}$, no radiochemical impurity being detected upon paper electrophoresis (0·5M-pyridinium acetate buffer, pH 3·5).

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