772. *The Synthesis of Sugars from Simpler Substances. Part III.* Enzymic Synthesis of a Pentose.*

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Glycollic aldehyde and triose phosphate condense in the presence of an aldolase preparation from peas, to give D-xylulose. The metabolism of pentoses is discussed.

THE biogenesis of the pentose sugars in the plant cell is obscure. It has been postulated (Tewfik and Stumpf, *Amer. J. Bot.*, 1949, **36**, 567; Schlenk, *Adv. Enzymology*, 1949, **9**, 455; Hough and Jones, *Nature*, 1951, **167**, 180; *J.*, 1951, 1122, 3191) that they arise by the combination of a diose with a triose, in an analogous manner to fructose and sorbose which are derived by combination of two triose units in the presence of "aldolase," an enzyme present in practically all living cells. This hypothesis is supported by the observations that aldolase preparations from both animal (Meyerhof, Lohmann, and Schuster, *Biochem. Z.*, 1936, **286**, 301, 319; Dische, *Naturwiss.*, 1938, **26**, 252; Schlenk and Waldvogel, *Fed. Proc.*, 1947, **6**, 288; *Arch. Biochem. Biophys.*, 1949, **22**, 185; Racker, *Fed. Proc.*, 1948, **7**, 180; Sable, *Biochim. Biophys. Acta*, 1952, **8**, 687) and bacterial origin (Marmur and Schlenk, *Arch. Biochem. Biophys.*, 1951, **31**, 154; Racker, *Nature*, 1951, **167**, 408; Kaushal, Jowett, and Walker, *ibid.*, 1951, **167**, 949; Rappaport, Barker, and Hassid, *Arch. Biochem. Biophys.*, 1951, **31**, 326; Sable, *loc. cit.*) catalyse the reversible condensation of triose phosphate with glycollic aldehyde or acetaldehyde, to give pentose phosphate or deoxypentose phosphate respectively, although the deoxypentose(s) has not yet been identified. On the other hand, there is little evidence to relate aldolase with the biosynthesis of pentoses in the plant, and, furthermore, there appears to be a second mode of synthesis of pentose operative, which involves oxidative decarboxylation of hexonic acids (see below). A third possibility, involving enzymic decarboxylation of hexuronic acids, has been but little investigated. Tewfik and Stumpf (*loc. cit.*) have observed the occurrence in a wide variety of plants, particularly the pea, of an aldolase which appears to be similar to that isolated from animals and bacteria. The term "aldolase" usually refers to an amorphous enzyme preparation containing, amongst other materials, an enzyme capable of splitting hexose diphosphate into two triose phosphates and possibly pentose phosphate

* Part II, *J.*, 1951, 3191.

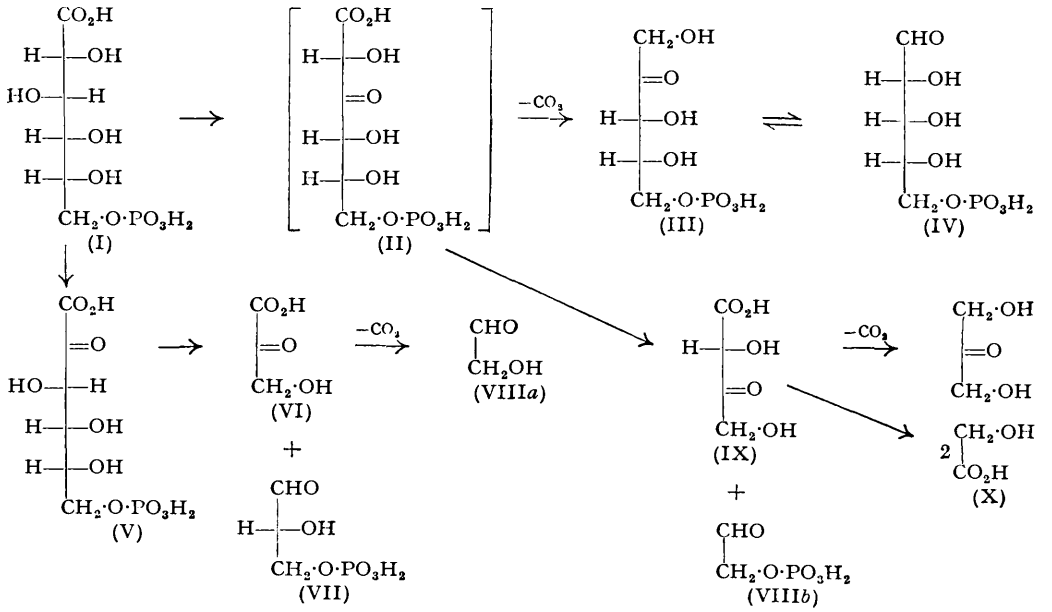
into triose and diose fragments, although it is conceivable that a separate enzyme may be responsible for splitting pentose.

We have attempted the synthesis of pentose from glycollic aldehyde and triose phosphates which were prepared by treating the sodium salt of D-fructose-1 : 6 diphosphate in the presence of sodium fluoride with an aldolase preparation extracted from peas (Stumpf, *J. Biol. Chem.*, 1948, **176**, 233). Glycollic aldehyde was introduced into the reaction mixture and the pH of the solution adjusted to 6·5. This may not be the optimum pH, but was chosen deliberately in order to avoid the possibility of the purely chemical combination of glycollic aldehyde with triose phosphate which is known to occur in the absence of enzyme in alkaline solution (Hough and Jones, *loc. cit.*). At intervals, the reaction mixture was analysed for pentose sugars by the furfuraldehyde method; pentose was rapidly produced. Examination on paper chromatograms suggested the formation of pentose phosphate, followed by dephosphorylation to free ketopentose. The aldolase preparation was observed to contain phosphatase. After 72 hours at 20°, the reaction mixture was heated to inactivate the enzymes, centrifuged, and evaporated. The residue was extracted with alcohol and the extract evaporated to a syrup, which was then subjected to partition chromatography on a column of cellulose to give a fraction containing ketopentose and a little ribose. These pentose sugars were recognised on paper chromatograms from their rates of movement in various solvent systems and from the characteristic red colour given by both with *p*-anisidine hydrochloride and the characteristic blue colour given only by the ketopentose with resorcinol–hydrochloric acid (Hough and Jones, *loc. cit.*). In a control experiment, in which glycollic aldehyde was omitted from the reaction mixture, the products contained ribose, but no ketopentose. The ribose probably arises from D-fructose 1 : 6-diphosphate by conversion into D-glucose 6-phosphate followed by oxidative decarboxylation to D-ribose (Dickens, *Biochem. J.*, 1938, **32**, 1626, 1636; Dickens and Glock, *Nature*, 1950, **166**, 33; *Biochem. J.*, 1951, **50**, 81; Scott and Cohen, *Science*, 1950, **111**, 543; *J. Biol. Chem.*, 1951, **188**, 509; Horecker, Smyrniotis, and Seegmiller, *ibid.*, 1951, **193**, 383), since fluoride inhibits neither glucose 6-phosphate dehydrogenase nor the oxygen uptake of 6-phosphogluconate. The ketopentose was separated from the ribose by sheet-paper chromatography and isolated as a syrup. It moved at the same rate as did xylulose on paper chromatograms. After epimerisation of the ketose in pyridine at 100°, a strong spot corresponding to xylose and weak spots corresponding to arabinose and ribose were detected on paper chromatograms; authentic xylulose gave the same sugars after epimerisation. The arabinose and ribose probably arise by $C_{(3)}$-racemisation, as in the formation of D-tagatose and D-sorbose from D-galactose (Lobry de Bruyn and van Eckenstein, *Rec. Trav. chim.*, 1900, **19**, 5) and the epimerisation of D-fructose to D-glucose, D-mannose, and D-allulose (Wolfrom, Lew, and Goepp, *J. Amer. Chem. Soc.*, 1946, **68**, 1443) and of D-manno-D-*gala*heptose to D-glucoheptulose and D-mannoheptulose (Montgomery and Hudson, *ibid.*, 1939, **61**, 1654). Reaction of the ketopentose with aqueous phenylhydrazine acetate afforded a crystalline pentosazone (m. p. 165—167°) which was optically active and exhibited mutarotation ($[\alpha]_D$ −16° ⟶ −43°), as did D-xylosazone (-lyxosazone). It has been noted that DL-xylosazone has m. p. 205°, approximately 40° above that of the individual D- and L-isomers, whereas that of DL-ribosazone (-arabinosazone) is only slightly (*ca.* 5°) above that of the optically active osazones, thus providing a method of identification (Fischer, *Ber.*, 1894, **27**, 2486; Levene and La Forge, *J. Biol. Chem.*, 1914, **18**, 319). We confirmed these observations and observed that the melting point of our pentosazone was elevated (m. p. 190—192°) on admixture with L-xylosazone, but not with D- or L-ribosazone or D-xylosazone. On this evidence, the osazone is identified as a derivative of D-xylulose.

A preliminary account of these results has been given elsewhere (Forrest, Hough, and Jones, *Chem. and Ind.*, 1951, 1093). It seems therefore that glycollic aldehyde and triose phosphate combine in the presence of the pea enzyme to yield D-xylulose (2-keto-D-xylitol). This is of interest because D-xylulose is a possible precursor of D-xylose, which is of wide occurrence in Nature and is present in the pea as a xylan. Pentose phosphate isomerase remains to be detected in plants, but it is noteworthy that Horecker, Smyrniotis, and Seegmiller (*loc. cit.*) have found D-ribose phosphate isomerase in yeast and in rat liver (Horecker and Smyrniotis, *J. Amer. Chem. Soc.*, 1952, **74**, 2123) and that D-ribulose phos-

phate is the precursor of D-ribose. Benson (*ibid.*, 1951, **73**, 2970) detected a D-ribulose diphosphate in the products formed during the first few seconds of $^{14}CO_2$ photosynthesis. L-Xylulose has been found in human pentosuria (Levene and La Forge, *loc. cit.*) and it would appear that bacteria metabolise D-xylose and L-arabinose by way of ketopentoses (Lampen, Gest, and Sowdon, *J. Bact.*, 1951, **61**, 97; Rappaport, Barker, and Hassid, *loc. cit.*). Reference has been made by Dische and Borenfreund (*J. Biol. Chem.*, 1951, **192**, 583) to the preparation by Dr. E. Racker of xylulose 1-phosphate from glycollic aldehyde and dihydroxyacetone phosphate in the presence of an aldolase.

Early work on pentose metabolism was largely concerned with the oxidation of D-glucose to pentose, and it has been established that D-glucose-6-phosphate is oxidised by yeast and liver preparations to 6-phospho-D-gluconate (I), followed by oxidative decarboxylation to D-ribose 5-phosphate (IV) (Dickens, *loc. cit.*; Dickens and Glock, *loc. cit.*; Cohen and Scott, *loc. cit.*; Horecker and Smyrniotis, *Arch. Biochem. Biophys.*, 1950, **29**, 232; *J. Biol. Chem.*, 1951, **193**, 371). Cohen and Scott (*loc. cit.*) also detected D-arabinose in the oxidation products, but investigations by Horecker, Smyrniotis, and Seegmiller (*loc. cit.*) suggest that arabinose 5-phosphate is not an intermediary in the conversion of 6-phospho-D-gluconic acid (I) into D-ribose 5-phosphate (IV). Since D-ribose is produced from D-gluconic acid, a stereochemical inversion must occur at $C_{(2)}$ of the pentose; simple degradation would give D-arabinose. Horecker, Smyrniotis, and Seegmiller (*loc. cit.*) found that D-ribulose 5-phosphate (III) is a precursor of D-ribose 5-phosphate (IV) and that an equilibrium mixture of the two is formed, the interconversion being catalysed by pentose phosphate isomerase. They postulate oxidation at $C_{(3)}$ in the 6-phosphogluconate with formation of 3-keto-6-phospho-D-gluconic acid (II) (a phosphorylated analogue of the open-chain form of vitamin C), which on decarboxylation could yield D-ribulose 5-phosphate (III) (cf. the oxidative degradation of fats at the β-carbon atom). 3-Keto-6-phospho-D-gluconic acid remains to be detected in this system, and it also remains to separate the oxidative step from the decarboxylation reaction.



This scheme is more feasible than that based on the intermediary formation of an enediol (Dickens and Glock, *loc. cit.*; Cohen and Scott, *loc. cit.*). Bernstein (*J. Amer. Chem. Soc.*, 1951, **73**, 5003), using ^{14}C-acetate, has observed that the direct conversion of hexose into D-ribose is not the major pathway in animal metabolism, and suggests that synthesis is achieved by the combination of two- and three-carbon units which would account for the accumulation of glyceraldehyde 3-phosphate (VII) in the experiments of

Scott and Cohen (*loc. cit.*) and also the stereochemical inversion. Alternatively, it is possible that 6-phosphogluconic acid is oxidised to 2-keto-6-phospho-D-gluconic acid (V), which then splits to give D-glyceraldehyde 3-phosphate (VII) and hydroxypyruvic acid (VI). The latter on enzymic decarboxylation yields glycollic aldehyde (VIII*a*), which on recombination with D-glyceraldehyde 3-phosphate (VII) may yield D-ribose 5-phosphate (cf. Hough and Jones, *Nature*, 1951, **167**, 180; Akabori, Uchara, and Muramatsu, *Proc. Jap. Acad.*, 1952, **28**, 39). If, on the other hand, 3-ketogluconic acid 6-phosphate (II) is produced, a split into glycollic aldehyde phosphate (VIII*b*) and dihydroxyacetoacetic acid (IX) might occur; the latter, losing carbon dioxide, would yield dihydroxyacetone and hence ketopentose 5-phosphate on combination with glycollic aldehyde phosphate (VIII*b*). Alternatively, dihydroxyacetoacetic acid (IX) could be envisaged as splitting into two molecules of glycollic acid (X).

The detection of sedoheptulose amongst the early products of photosynthesis by plants has led Benson *et al.* (*J. Amer. Chem. Soc.*, 1951, **73**, 2970; *J. Biol. Chem.*, 1952, **196**, 703) to the belief that its phosphate esters play a vital rôle in photosynthesis. Horecker and Smyrniotis (*J. Amer. Chem. Soc.*, 1952, **74**, 2123) have observed the enzymic synthesis of sedoheptulose phosphate from pentose phosphate under the combined influence of a pentose-splitting enzyme from rat liver and crystalline muscle aldolase (Taylor, Green, and Cori, *J. Biol. Chem.*, 1948, **173**, 591). Heptulose phosphate was also formed from D-erythrose and hexose 1 : 6-diphosphate in the presence of an aldolase, which suggests that synthesis from pentose phosphate is achieved by fission into triose and diose, two molecules of diose combining to yield a tetrose, a reaction known to occur *in vitro* (Fenton, *J.*, 1900, 129; Hough and Jones, *loc. cit.* and unpublished observations), and combination with dihydroxyacetone phosphate would yield heptulose phosphate.

It is becoming increasingly manifest that the ketopentose phosphates play an important part in plant and animal metabolism, because of their possible transformations into hexose, tetrose, and heptulose sugars, and their rôle as a precursor of the aldopentoses which play such a vital part in living material.

Experimental

Preparation of Aldolase from Pea Seeds (cf. Stumpf, *loc. cit.*).—Dry pea seeds (500 g.; *var.* Onward) were steeped in water (1 l.) and stored in the refrigerator at 2° for 24 hours, by which time they had swollen to their normal size. The peas were disintegrated in a top-drive macerator, the resultant sludge strained through a muslin cloth, and the turbid liquor centrifuged. To the supernatant liquor a saturated (20°) neutral solution of ammonium sulphate was added to 35% saturation, and the precipitate was removed on the centrifuge and discarded. A further portion of saturated ammonium sulphate was added to the supernatant liquid until 70% saturation was obtained. The precipitate, which contains the aldolase, was isolated on the centrifuge and dialysed for 18 hours against tap water to remove ammonium sulphate; coagulated protein was removed on the centrifuge and discarded. The solution thus obtained was used in the following experiments. The aldolase activity of the preparation was found to be 0·04 unit of aldolase per mg. of solid as determined by the cyanide-fixing method of Herbert, Gordon, Subrahmanyan, and Green (*Biochem. J.*, 1940, **34**, 1108), the unit being that defined by Stumpf (*loc. cit.*). The solution also contained phosphatase, since on incubation at 37° for 1 hour with 0·5% of sodium β-glycerophosphate as specified by Bodansky (*J. Biol. Chem.*, 1933, **101**, 33), the enzyme solution (1 c.c.) liberated 1·08 mg. of phosphorus as phosphate at pH 8·6, and 1·7 mg. phosphorus at pH 6·5, the phosphate being determined by the method of Allen (*Biochem. J.*, 1940, **34**, 858).

Formation of Pentose from Triose Phosphate and Glycollic Aldehyde in the Presence of Pea Aldolase (with R. S. Forrest).—A mixture of the enzyme solution (5 c.c.), the sodium salt of hexose diphosphate (0·25 g.) in water (5 ml.), phosphate buffer (pH 6·5; 10 c.c.), and a neutral solution (10 c.c.) of glycollic aldehyde (prepared from 0·5 g. of dihydroxymaleic acid by decarboxylation) was covered with a layer of toluene and incubated at 30°. At intervals, portions of the reaction mixture were removed and the pentose concentration was determined by Youngburg and Pacher's method (*J. Biol. Chem.*, 1927, **73**, 599). This method is a colorimetric procedure depending on the production of colour from furfuraldehyde and aniline acetate; xylose was used for the construction of the calibration curve. The pentose concentration increased until

after 20 hours, when the yield (calculated as xylose) corresponded to 3·6% as derived from dihydroxymaleic acid.

Isolation of Ketopentose.—A mixture of the enzyme solution (100 c.c.), a solution (25 c.c.) of the sodium salt of hexose diphosphate (7·37 g.), glycollic aldehyde solution (200 c.c. prepared by decarboxylation of 10·7 g. of dihydroxymaleic acid), and sodium fluoride (to a concentration of 0·05M) was adjusted to pH 6·5, covered with a layer of toluene and stored at 20°. After 72 hours, the solution (pH 6·8) was heated on the boiling-water bath for 10 minutes to inactivate the enzymes, and coagulated protein was removed on the centrifuge. The clear supernatant liquid was evaporated under reduced pressure at room temperature to a solid mass (A). A small sample of the residue was examined on paper chromatograms for sugar and sugar phosphate, by a variety of solvent systems (for methods see Partridge, *Biochem. J.*, 1948, **42**, 238; Hough, Jones, and Wadman, *J.*, 1950, 1702; Hanes and Isherwood, *Nature*, 1949, **164**, 1107; Benson *et al.*, *J. Amer. Chem. Soc.*, 1950, **72**, 1710). In this manner, ketopentose, hexoses, hexose diphosphate, and free phosphate were detected, but neither heptulose nor pentose phosphate. The ketopentose was recognised on the paper chromatogram from its rate of movement, which is more rapid than that of any one of the aldopentoses, and from the characteristic blue colour obtained on spraying the chromatogram with a solution of resorcinol and hydrochloric acid in butanol, followed by careful heating. Further experiments showed that reaction for 24 hours only at room temperature gave pentose phosphate, since on paper chromatograms a spot was obtained close to hexose diphosphate which gave a positive reaction for phosphate with Hanes and Isherwood's molybdate reagent (*loc. cit.*) and a positive test for pentose (red colour) with the *p*-anisidine hydrochloride reagent (Hough, Jones, and Wadman, *loc. cit.*).

The residue (A) was extracted in the cold by vigorous shaking with absolute alcohol (3 × 200 c.c.). The brown extract was filtered, de-ionised by Amberlite Resins IR-4B and IR-100, and concentrated under reduced pressure at room temperature to a brown syrup (1·01 g.). This syrup was transferred to a column of powdered cellulose (1½ × 16″) and subjected to partition chromatography with butanol nearly saturated with water as the mobile phase (for method, see Hough, Jones, and Wadman, *J.*, 1949, 2511), to give, on evaporation of the appropriate portion of the effluent, a fraction (62·6 mg.) containing the ketopentose and a little ribose (as indicated by paper chromatography). This mixture was refractionated by partition chromatography, but this time on two sheets of Whatman No. 1 filter paper (22½ × 18¼″) with butanol–ethanol–water (40 : 11 : 19 parts v/v) as mobile phase. After separation, the papers were air-dried at room temperature, and the sugars located by the use of test strips (Flood, Hirst, and Jones, *J.*, 1948, 1679), and then extracted with methanol–water (micro-Soxhlet). Evaporation under reduced pressure gave fraction A {43 mg.; $[\alpha]_D$ −12·5° (*c*, 1·3 in water)}, containing the ketopentose, and fraction B (7·5 mg.), a mixture of ribose and ketopentose. The ketopentose moved at the same rate on the paper chromatogram as xyloketose (R_F 0·28; standard, rhamnose R_F 0·30) in both butanol–pyridine–water (10 : 3 : 3 parts v/v), where riboketose (R_F 0·26) moves slightly slower, and ethyl acetate–acetic acid–water (9 : 2 : 2 parts v/v) in which riboketose is slightly faster. Epimerisation of a small portion of the ketopentose in either pyridine at 100° for 3 days or dilute aqueous ammonia at 37° for a week or more gave, as shown by paper chromatography, xylose along with traces of ribose and arabinose. Under these conditions, the same result was obtained with an authentic specimen of D-xyloketose prepared by heating D-xylose in pyridine (Levene and Tipson, *J. Biol. Chem.*, 1930, **115**, 731) and separating the products by partition chromatography. Reaction of the ketopentose (38 mg.) in water (2 c.c.) with phenylhydrazine (0·2 c.c.) and acetic acid (0·2 c.c.) at 60—70° for 3 hours afforded a crystalline pentosazone which was filtered off, washed with a little benzene, recrystallised from ethanol–water, and dried at 60° under reduced pressure (*ca.* 40 mg.; m. p. 165—167°; mixed m. p. with L-xylosazone 190—192°, with D-xylosazone 161°, and with L- or D-ribosazone 161°; m. p. of crystals prepared from equimolecular proportions of the pentosazone and L-xylosazone, 202—203°; authentic DL-xylosazone had m. p. 207°). The pentosazone showed a rise in mutarotation from $[\alpha]_D^{21}$ −19° (*c*, 0·72 in ethanol, 1 hour) to −31°±3° (equil. value; 48 hours); similarly, D-xylosazone exhibits mutarotation in ethanol {$[\alpha]_D^{21}$ −15° (*c*, 0·44) ⟶ −39°±3° (equil. value; 48 hours) (cf. Levene and La Forge, *loc. cit.*; Fischer, *loc. cit.*)}. The crystalline form of the pentosazone was, microscopically, typical of xylosazone and different from that of ribosazone (cf. Hassid and McCready, *Ind. Eng. Chem.*, 1942, **14**, 683). On recrystallisation from benzene, the pentosazone (m. p. 157°) showed $[\alpha]_D^{21}$ −16° (*c*, 0·32 in ethanol; initial value) ⟶ −43°±3° (48 hours; const.) (Found: C, 62·2; H, 7·1; N, 17·4. $C_{17}H_{20}O_3N_4$ requires C, 62·2; H, 6·1; N, 17·1%). Authentic D-xylosazone on crystallisation

from benzene showed $[\alpha]_D^{19}$ $-26° \longrightarrow -46° \pm 3°$ (*c*, 1·98 in alcohol). Attempts to isolate the osatriazole were unsuccessful, presumably because of the small quantity of osazone available. Efforts to prove conclusively the identity of this osazone by *X*-ray analysis proved abortive.

Two control experiments, involving in one the enzyme preparation and hexose diphosphate at pH 6·5, and in the other glycollic aldehyde and the enzyme preparation at pH 6·5, were treated as described above. In neither case was ketopentose produced but, in the experiment involving hexose diphosphate and the enzyme, paper chromatography indicated the presence of ribose, glucose, fructose, and galactose(?).

In another experiment, glycollic aldehyde was kept in aqueous solution with dihydroxyacetone at pH 6·5, and tests for pentose (furfuraldehyde method) were applied at intervals; pentose was not produced. Addition of the enzyme preparation to this mixture did not lead to the formation of pentose.

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