[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL]

Phosphorylated Sugars. IV.[1] The Synthesis of D-Xylose 3-Phosphate *via* 1,2-*O*-Isopropylidene-D-Xylofuranose-3,5-cyclic Phosphate

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1,2-*O*-Isopropylidene D-xylofuranose-3,5-cyclic phosphate (III), a crystalline substance, has been synthesized in excellent yield by a number of methods. D-Xylose 3-phosphate has been prepared for the first time and has been rigorously characterized. The synthesis was accomplished by alkaline hydrolysis of the six-membered phosphate ring in III, followed by mild acidic treatment of the products (XIII and XIV) and separation of the resulting xylose 3- and 5-phosphates on an ion exchange column. Mild acidic treatment of III gave D-xylose-3,5-cyclic phosphate (XII) in excellent yield. Recent observations[4] of the changes in optical rotations of neutral solutions of D-xylose 5-phosphate have been confirmed and it has now been established that these changes are due to the facile partial conversion of D-xylose 5-phosphate to D-xylulose 5-phosphate.

Levene and Raymond[2] have recorded unsuccessful attempts to synthesize D-xylose 3-phosphate. Their approaches involved the phosphorylation of several suitably protected (1,2-*O*-isopropylidene-5-*O*-acyl) xylose derivatives but the final product invariably obtained was claimed to be D-xylose 5-phosphate. A phosphoryl group migration from the 3- to the 5-hydroxyl group was therefore postulated under the acidic conditions used to remove the isopropylidene group. Recent renewed interest[3,4] in the phosphorylated derivatives of D-xylose, and our own interest in the synthesis and properties biologically important sugar phosphates prompted us to undertake further investigation of the phosphate esters of this pentose. The present communication records several syntheses of 1,2-*O*-isopropylidene D-xylofuranose-3,5-cyclic phosphate and the preparation from this substance of the hitherto inaccessible D-xylose 3-phosphate. During the course of this work the nature of the reaction responsible for the changes[4] in the optical rotations of approximately neutral solutions of D-xylose 5-phosphate was investigated and it has been established that these changes are the result of the partial transformation of D-xylose 5-phosphate to D-xylulose 5-phosphate.

The key intermediate in these studies, namely, 1,2-*O*-isopropylidene D-xylofuranose-3,5-cyclic phosphate (III), was synthesized by three different methods of phosphorylation. In the first method, 1,2-*O*-isopropylidene D-xylofuranose[5] (I) was phosphorylated in anhydrous pyridine with monophenyl phosphorodichloridate.[6,7] The neutral product II, which was obtained in an excellent yield as a viscous oil, was hydrogenated in the presence of a platinum catalyst to give the cyclic phosphate III. The latter substance was isolated, first, as the highly crystalline cyclohexylammonium salt and then as the crystalline free acid.

Prolonged alkaline treatment of the neutral ester II at room temperature also furnished the stable six-membered cyclic phosphate III in a quantitative yield. The tertiary ester II would be expected to be labile and to hydrolyze rapidly to form a diester. The nature of the initial products would, clearly, depend upon the relative labilities of the three P–O linkages involved. When to a dioxane solution of II, 2 *N* sodium hydroxide was added, the initially separated oil redissolved in some ten minutes; a paper chromatographic examination at this stage showed that the six-membered cyclic phosphate was the major product and a slightly faster travelling material corresponding, presumably, to a mixture of the acyclic monophenyl esters (IV and V) was present.[8] The latter products subsequently underwent a slow transesterification reaction[11] to give also after *ca.* 72 hours the stable cyclic phosphate III.

(1) Paper III, G. M. Tener, R. S. Wright and H. G. Khorana, THIS JOURNAL, **79**, 441 (1957).

(2) P. A. Levene and A. L. Raymond, *J. Biol. Chem.*, **102**, 317, 331, 347 (1933); **107**, 75 (1934).

(3) (a) J. L. Barnwell, W. A. Saunders and R. W. Watson, *Can. J. Chem.*, **33**, 711 (1955); (b) P. A. J. Gorin, L. Hough and J. K. N. Jones, *J. Chem. Soc.*, 585 (1955).

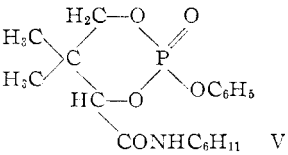
(4) R. W. Watson and J. L. Barnwell, *Chemistry and Industry*, 1089 (1955).)

(5) Prepared according to the procedure of R. Muller and T. Reichstein (*Helv. Chim. Acta*, **21**, 251 (1938)) from 1,2-3,5-di-*O*-isopropylidene D-xylofuranose (H. Grunenberg, C. Bredt and W. Freudenberg, THIS JOURNAL, **60**, 1507 (1938)).

(6) P. Brigl and H. Müller, *Ber.*, **72**, 2121 (1939).

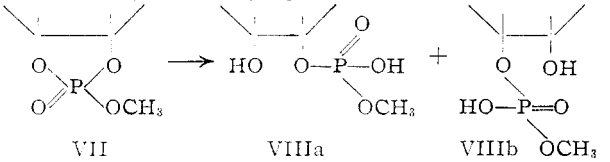
(7) The use of this reagent in the synthesis of the six-membered D-glucose-4,6-cyclic phosphate has been recorded, J. Baddiley, J. G. Buchanan and L. Szabo, *J. Chem. Soc.*, 3826 (1954).

(8) Our own findings on the alkaline hydrolysis of the neutral esters, II, and the corresponding *p*-nitrophenyl analog, XI, which hydrolyses rapidly to give exclusively the cyclic phosphate III, should be compared with those of previous workers.[9,10] The neutral ester VI which must have been the intermediate in Baddiley and Thain's work[9] also hydrolyzed to give the corresponding stable six-membered cyclic phosphate.



VI

(It is not known whether the cyclic phosphate was formed exclusively.) On the other hand the presumed intermediate[10] (partial formula, VII) containing the labile five-membered phosphate ring hydrolyzed to give largely the acyclic monoalkyl esters VIII. Thus, the mode of the alkaline cleavage of neutral phosphate esters containing cyclic structures depends very much on the nature of the compound and the stability of the cyclic structure (*cf.* 10).



VII VIIIa VIIIb

(9) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 903 (1953).

(10) D. M. Brown, D. I. Magrath and A. R. Todd, *ibid.*, 4396 (1955).

(11) While transesterification reactions of acyclic *di-esters* of phosphoric acid, bearing suitably placed hydroxyl groups, to form five-membered cyclic phosphates are well-known,[12] the formation of six-membered cyclic phosphates in an analogous fashion has not been demonstrated previously.

(12) For a comprehensive review of earlier literature see H. G.

In the second method, the crystalline 1,2-*O*-isopropylidene D-xylofuranose-5-diphenyl phosphate (IX), whose preparation in excellent yield has been recorded recently,[3] served as the starting material. This neutral substance would be expected to undergo a rapid intramolecular transesterification reaction (*cf.* ref. 9 and 10 and see below) with the suitably-placed hydroxyl function at C_3 to give the labile II and the latter substance would then yield, as described above, the cyclic phosphate III. Experimentally, the behavior of IX, when treated with a mixture of dioxane and 2 *N* alkali, was, indeed, found to be identical with that of II under these conditions.

The third method for the preparation of III involved the phosphorylation of 1,2-*O*-isopropylidene D-xylose (I) with tetra-*p*-nitrophenyl pyrophosphate,[13] prepared *in situ* by the reaction of di-*p*-nitrophenyl phosphate with di-*p*-tolyl carbodiimide in anhydrous dioxane. The initial product, presumably X, was so labile that transesterification to form the cyclic neutral ester XI, with concomitant release of *p*-nitrophenol, occurred partly during the work-up at neutral *p*H range. The conversion of X to XI was completed by extracting a chloroform solution of the reaction product with extremely dilute sodium hydroxide and the latter substance was then isolated in a crystalline state in 67% yield. Mild alkaline hydrolysis[8] of XI at room temperature gave a nearly quantitative yield of III.

The substance III has been used to prepare the hitherto inaccessible D-xylose 3-phosphate (see below) and, also, to prepare D-xylofuranose-3,5-cyclic phosphate (XII) by selective hydrolysis of the isopropylidene ring. To prepare XII, it was only necessary to heat an aqueous solution of the free acid III under its own *p*H (*ca.* 1.5) for ten minutes at 100°. No hydrolysis of the phosphate ring could be detected under these conditions. The cyclic phosphate XII was isolated as the barium salt in 86% yield and characterized by elemental analysis, periodic acid oxidation (1 mol. of the oxidant consumed, Fig. 1) and electrometric titration, which confirmed the absence of a secondary phosphoryl dissociation in the *p*H range 4–9. The formation of this stable cyclic phosphate directly by the treatment of D-xylose 5-phosphate with dicyclohexyl carbodiimide is discussed below.

With a view to preparing D-xylose 3-phosphate, the alkaline hydrolysis of the phosphate ring in III prior to the removal of the acid labile isopropylidene group, was examined. Although stable at room temperature over long periods, III could be hydrolyzed to a mixture of XIII and XIV by heating it in 1 *N* sodium hydroxide at 100°. The hydrolysis, which was followed conveniently by paper chromatography, was complete in 20 hours. (A quantitative study of this reaction has also been made by following titrimetrically the production of the secondary phosphoryl dissociations in XIII and XIV and has been recorded elsewhere.[12]) No inorganic phosphate was released and, further, the isopropylidene ring was completely stable under these conditions. The products (XIII and XIV) could be quantitatively reconverted, in the form of their pyridine salts, to III by treatment with dicyclohexylcarbodiimide in aqueous pyridine.[12] The removal of the isopropylidene group in XIII and XIV could be effected, again, by simply heating an aqueous solution of the mixture of the free acids (*p*H *ca.* 1.5) at 100° for 10 minutes. The resulting mixture of D-xylose 5- and 3-phosphates was separated on a Dowex 2-formate column and the 3-phosphate was finally isolated as the amorphous barium salt in a yield of 18% (xylose 5-phosphate was recovered in 45% yield).

The synthetic D-xylose 3-phosphate has been characterized in a number of ways. The elemental analysis and pentose determination (after enzymatic dephosphorylation) were in agreement with the formulation of the substance as a pentose monophosphate. It contained xylose as the sole reducing sugar as demonstrated by enzymatic dephosphorylation and paper chromatography in several solvent systems known to separate the pentoses. D-Xylose 3-phosphate could be distinguished from the isomeric 5-phosphate on paper

Khorana, G. M. Tener, R. S. Wright and J. G. Moffat, THIS JOURNAL, **79**, 430 (1957).

(13) J. G. Moffatt and H. G. Khorana, to be published.

chromatograms developed in isopropyl alcohol–ammonia–water (70–10–20 v./v.), through its slightly but consistently higher mobility and by its characteristic pink spot with the aniline hydrogen phthalate spray. The pink spots with this spray, which are characteristic of pentoses appear to be given also by pentose 2- and 3-monophosphates. Thus, in addition to D-xylose 3-phosphate, D-ribose 2- and 3-phosphates also give pink spots. In contrast, D-xylose 5- and D-ribose 5-phosphates form brownish spots.

D-Xylose 3-phosphate and, for comparison, D-xylose 5-phosphate, were reduced either with aqueous sodium borohydride or with hydrogen in the presence of platinum to the corresponding xylitol phosphates. The product XV from D-xylose 3-phosphate was, as expected, optically inactive. D-Xylitol 5-phosphate showed a small but definite levorotation ($[\alpha]^{20}D - 1.2°$).

The study of oxidations with periodic acid of the various phosphate esters (Fig. 1) provided further

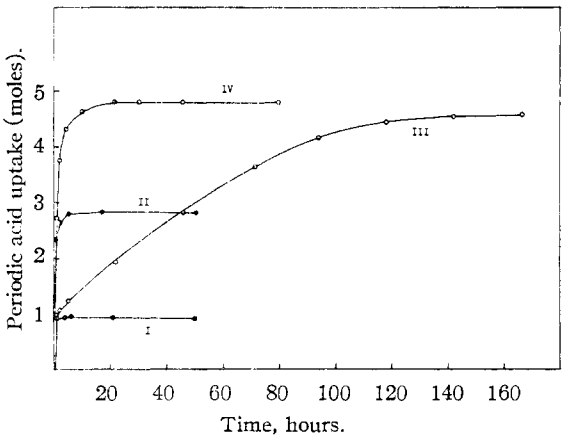
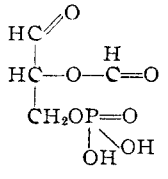


Fig. 1.—Periodic acid oxidation of xylose phosphates: I, D-xylofuranose 3,5-cyclic phosphate; II, D-xylose 5-phosphate; III, D-xylose 3-phosphate; IV, D-xylitol 3-phosphate.

support for their structures. D-Xylose 5-phosphate consumed 2.8 moles of the oxidant in five hours at *p*H 4.5. This result is in general agreement with those reported recently for the same substance[3a] and for D-ribose 5-phosphate from different laboratories.[14,15] The uptake of the third mole of the oxidant in studies with the two pentose 5-phosphates clearly indicates (*cf.* 14, 15) that the presumed intermediate XVI



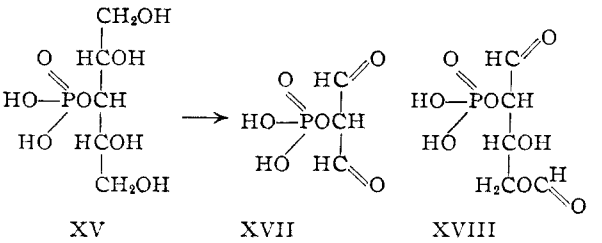
XVI

readily loses[20] the formyl group and is then attacked further to give glycolaldehyde phosphate. The latter substance which has been characterized by Loring, *et al.*, as the final product of the oxidation of ribose 5-phosphate also was obtained from D-xylose 5-phosphate (see below and Experimental).

(14) G. V. Marinetti and G. Rouser, THIS JOURNAL, **77**, 5345 (1955).

(15) H. S. Loring, L. W. Levy, L. K. Moss and J. McT. Ploeser, *ibid.*, **78**, 3724 (1956).

D-Xylitol 3-phosphate readily consumed (Fig. 1) 4.8 moles of periodic acid, clearly due to "over-oxidation"[16] of the intermediate 2-hydroxymalondialdehyde phosphate, XVII.



XV XVII XVIII

This labile intermediate, which was apparently first invoked by Courtois and Ramet[18] in their periodic acid oxidation studies, recently has been mentioned by Khym, *et al.*,[19,20] in connection with the oxidation of D-ribitol 3-phosphate which was found to consume a total of six moles of the oxidant. The discrepancy between the results of these authors[19] and our own results with the analogous oxidation of D-xylitol 3-phosphate is difficult to explain.

The periodic acid oxidation of xylose 3-phosphate itself, although slow (Fig. 1) after the consumption of the first mole, also required a total of five moles of the oxidant (*cf.* slow oxidation of ribose 3-phosphate compared with ribitol 3-phosphate[19]). This result indicates that, again, the intermediate XVII is involved, but its formation is slower possibly because of the relative stability of the initially formed formyl ester, presumably XVIII, as compared with that of XVI.

Further degradative evidence in support of the structure of D-xylitol 3-phosphate was obtained as follows. It was oxidized with two equivalents of periodic acid at neutral *p*H and the product, presumably XVII, was treated with an excess of sodium borohydride for 24 hours. Difficulty was often experienced in effecting reduction and either through over oxidation of XVII during the periodic acid treatment or through hydrolysis, inorganic phosphate frequently was formed (*cf.* 18). However, a stable organic phosphate sometimes was obtained, which after purification on a paper chromatographic sheet and enzymatic dephosphorylation liberated, as expected, only glycerol. Analogous reactions with D-xylose (or xylitol) 5-phosphate, proceeded smoothly and ethylene glycol was identified as the sole product by paper chromatography.

More recently Dr. A. S. Perlin[21] has kindly studied the lead tetraacetate oxidation of our synthetic sample of D-xylose 3-phosphate. This study showed that the substance consumed, as ex-

(16) This is a well-known phenomenon occurring whenever a C–H linkage is activated by two adjacent carbonyl groups. For a recent discussion see ref. 17.

(17) R. A. Edington, E. L. Hirst and E. E. Percival, *J. Chem. Soc.*, 2281 (1955).

(18) J. Courtois and N. Ramet, *Bull. soc. chim. biol. France*, **27**, 610 (1945).

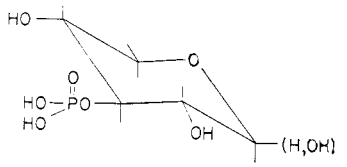
(19) J. X. Khym, D. G. Doherty and W. E. Cohn, THIS JOURNAL, **76**, 5523 (1954).

(20) The lability of this formyl ester may be due to the presence of an adjacent carbonyl group.

(21) Prairie Regional Laboratory (National Research Council of Canada) Saskatoon, Canada.

pected for a 3-substituted aldose,[21a] approximately one mole of the oxidant, no formic acid being liberated. The product, presumably, a formyl ester, was treated with aqueous acid to remove the formyl group and then dephosphorylated enzymatically. Threose was identified by paper chromatography (comparison with an authentic specimen).

The evidence presented above conclusively establishes the identity of the synthetic D-xylose 3-phosphate. One distinctive reaction of this substance deserves mention here. While xylose 5-phosphate reacts with dicyclohexyl carbodiimide[22] in aqueous pyridine to form the six-membered xylose-3,5-cyclic phosphate (XII), the 3-isomer gives reactions indicating five-membered cyclic phosphate formation (involving the hydroxyl groups at C_2 or C_4). From these observations it has been concluded[22] that whereas the former exists in solution in the furanose ring form, D-xylose 3-phosphate assumes the pyranose structure. The conformation (Cl),[23] XIX, appears highly probable for this substance.



XIX

The present synthesis of D-xylose 3-phosphate owes success partly to the mildness of the acidic treatment[24] which was found sufficient to remove the isopropylidene groups from the intermediates (XIII and XIV). It is unfortunate that Levene and Raymond[2] employed unnecessarily severe conditions for this purpose.[25] It may be briefly mentioned here that under the conditions used by Levene and Raymond xylose 3-phosphate is, indeed, converted largely to an isomeric product(s).

A detailed study of the phosphoryl group migration in xylose 3-phosphate and related pentose phosphates and of the influence of phosphoryl group migration upon rates of hydrolysis will be presented in a forthcoming publication. It is also intended to describe there the hydrolytic behavior of the six-membered D-xylose-3,5-cyclic phosphate and to discuss the important question whether the cyclic phosphates are as such the intermediates in acid-catalyzed phosphoryl group migrations.[26]

The Partial Conversion of D-Xylose 5-Phosphate to D-Xylulose 5-Phosphate.—Watson and Barnwell[4] have recently recorded the interesting observation that the optical rotation (initially positive) of an approximately neutral solution of the disodium salt of xylose 5-phosphate decreases slowly at room temperature to attain, finally, a small negative value. These changes in optical rotation were accompanied by increased liberation of formaldehyde upon periodic acid oxidation of the product(s). Since no inorganic phosphate was released, a phosphoryl group migration from the 5 to the 3-hydroxyl group in xylose was postulated. Watson and Barnwell's observations[4] of the above-mentioned optical rotation changes were confirmed by us; however, their interpretation of the phenomenon appeared to us to be highly improbable since phosphoryl group migrations in monoesters of phosphoric acid are known to occur only under acidic conditions and not under neutral or alkaline conditions.[27] That D-xylose 3-phosphate was indeed absent[28] from the solutions of D-xylose 5-phosphate treated according to Watson and Barnwell (heating an aqueous solution, *p*H 6.2, at 50° for two hours) was shown conclusively by the following experiments. (1) Paper chromatography in the solvent system (see above) which clearly distinguished between xylose 3- and 5-phosphates did not reveal the presence of any pink spot corresponding to and characteristic of D-xylose 3-phosphate. (2) The behavior of the solution of D-xylose 5-phosphate, treated as above, toward periodic acid was very similar to that of freshly prepared 5-phosphate and different from that of the synthetic D-xylose 3-phosphate (Fig. 1). (3) Finally, after the degradative series of reactions described above for xylitol phosphates, ethylene glycol was the only product obtained.[29]

It was thought that the data recorded by Watson and Barnwell, especially the formaldehyde release, could be explained by assuming the partial transformation of D-xylose 5-phosphate to D-xylulose 5-phosphate[30] at approximately neutral *p*H's. This

(21a) A. J. Charlson and A. S. Perlin, *Can. J. Chem.*, **34**, 1200 (1956).

(22) For a comprehensive discussion of the cyclization reactions of sugar phosphates, see ref. 12.

(23) R. E. Reeves, THIS JOURNAL, **72**, 1499 (1950).

(24) It is pertinent to enquire if migration (to the 2-, 4- or 5-hydroxyl group) occurred to any extent during the acidic treatment used in the present work to remove the isopropylidene group. After heating an aqueous solution of the free D-xylose 3-phosphate (*p*H 1.5) for ten minutes at 100° no xylose 5-phosphate could be detected by paper chromatography and in fact, the only spot that was present had the pink color and R_f, identical with that of the starting material. In any case, by its method of isolation (ion-exchange chromatography) the synthetic xylose 3-phosphate must be free from the 5-phosphate. However, since the paper chromatographic and ion exchange behavior of xylose 2- and 4-phosphates is unknown, the possibility must be considered whether these isomers contaminate the synthetic 3-phosphate. While the sum of the evidence presented above excludes any gross contamination by these isomers, the following argument may be advanced to show that the contamination cannot be significant. If "xylitol 3-phosphate," prepared from "xylose 3-phosphate," contains the isomeric 2- or 4-phosphates then oxidation using excess of periodic acid should yield a stable organic phosphate. (Ribitol 2-phosphate[19] has been reported to consume 2 moles of periodic acid, the oxidation terminating with the formation of, presumably, glyceraldehyde 2-phosphate). In actual fact, *only inorganic phosphate* (overoxidation and decomposition of XVII) was formed when xylitol 3-phosphate was oxidized with excess of periodic acid.

(25) Gorin, *et al.*, (ref. 3b) also assume that unusually harsh conditions are required to remove the isopropylidene group from XIII.

(26) *Cf.* D. M. Brown, D. I. Magrath, A. H. Neilson and A. R. Todd, *Nature*, **177**, 1124 (1956).

(27) M. Bailly, *Compt. rend.*, **206**, 1902 (1938); **208**, 443, 1820 (1939); P. E. Verkade, J. C. Stoppelenburg and W. D. Cohen, *Rec. trav. chim.*, **59**, 886 (1940); D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

(28) It may also be noted at this stage that both D-xylose 5- and 3-phosphates have small but positive rotations, whereas the changes that are observed with a solution of D-xylose 5-phosphate are in the negative direction.

(29) This evidence definitely establishes that the phosphate group is present exclusively on the terminal carbon atom, since only then can a two carbon fragment be obtained after degradation.

(30) B. Axelrod and R. Jang (*J. Biol. Chem.*, **209**, 847 (1954)) have already noted the partial transformation at room temperature of barium D-ribose 5-phosphate to a ribulose-containing compound. In the present work, samples of D-xylose 5-phosphate precipitated at a neutral *p*H have similarly been found to contain after standing for some time, D-xylulose 5-phosphate.

has been shown to be so by a number of tests performed on the enzymatically dephosphorylated solutions of sodium D-xylose 5-phosphate treated according to Watson and Barnwell. Paper chromatography in three suitable solvent systems (see Experimental), which are known to separate xylulose from xylose revealed the presence of a spot in addition to that of xylose. (Enzymatic dephosphorylation of freshly-prepared D-xylose 5-phosphate gave only the spot corresponding to xylose.) The additional spot was identified as xylulose by comparison of its R_f values with an authentic sample and by its color (characteristic of ketoses) with the orcinol–hydrochloric acid[31] and cysteine–carbazole sprays.[32] Paper chromatography following treatment of the dephosphorylation mixture with bromine water showed the presence of only xylulose. Further evidence was secured by elution of the ketose from a paper chromatographic sheet and measurement of the ferric chloride–orcinol spectrum. The $^{540}/_{670}$ mμ absorption ratio (0.39) found was identical with that of authentic xylulose.[33]

Our above conclusion has been confirmed by Dr. Ashwell and Miss Hickman[34] who have kindly assayed our preparation of the mixture of barium salts obtained by treatment of xylose 5-phosphate according to Watson and Barnwell. Using both the quantitative cysteine–carbazole and enzymatic procedures,[35] they found approximately 13% xylulose 5-phosphate[35] in the above preparations. While freshly prepared D-xylose 5-phosphate was free from the above ketose phosphate, older preparations of the barium salt, which had been standing at room temperature, also showed the presence of significant amounts of this material.

The aldose–ketose transformation at moderate *p*H's established above in the case of D-xylose 5-phosphate and noted previously by Axelrod and Jang[30] in the case of D-ribose 5-phosphate is surprisingly facile and must be ascribed to the presence of the phosphate group on the 5-hydroxyl in these pentoses. In connection with the preservation of the pure pentose phosphates, the point of practical interest may be made that it is advantageous to maintain an acidic *p*H during their preparation and to precipitate the *half* barium salts (at *p*H 4).

Experimental[36]

Paper Chromatography.—The solvent system used successfully for separation of the various phosphate esters described in the present work was isopropyl alcohol–ammonia–water (7–1–2, v./v.),[37] designated below as Solvent system A. The solvents found most useful for separation of sugars and alcohols were pyridine–ethyl acetate–water[38] (2–7 1, v./v.) (solvent system B) and butyl alcohol–ethyl alcohol–water,[39] (5–1–4, v./v.) (solvent C). The R_f values of the various substances are mentioned in the text.

1,2-*O*-Isopropylidene D-Xylofuranose-3,5-cyclic Phenyl Phosphate. (II, R = Phenyl).—Monophenyl phosphorodichloridate[8] (2.3 g., 11 m*M*) was added dropwise to an agitated, ice-cold solution of 1,2-*O*-isopropylidene D-xylose[5] (1.75 g., 9.2 m*M*) in 10 ml. of dry pyridine. The reaction mixture was allowed to stand overnight at 5°, evaporated to dryness and taken up in chloroform (25 ml.). The chloroform solution was extracted eight times with water and evaporated to dryness giving 2.86 g. (95%) of an extremely viscous sirup which distilled in a short path distillation apparatus at a bath temperature of 170° (0.01 mm.). *Anal.* Calcd. for $C_{14}H_{17}O_7P$: C, 51.20; H, 5.22. Found: C, 50.55; H, 5.49.

1,2-*O*-Isopropylidene D-Xylofuranose-3,5-cyclic *p*-Nitrophenyl Phosphate (II, R = *p*-Nitrophenyl).—Dry di-*p*-nitrophenyl phosphate (3.95 g., 11.5 m*M*) was dissolved by warming in anhydrous dioxane (20 ml.) and the clear solution rapidly cooled to room temperature. Di-*p*-tolylcarbodiimide[40] (1.31 g., 5.9 m*M*) was then added and the reaction mixture, which immediately deposited di-*p*-tolylurea, was allowed to stand for ten minutes. 1,2-*O*-Isopropylidene D-xylose (1.0 g., 5.2 m*M*) was then added and the reaction mixture kept overnight at room temperature with exclusion of moisture. Di-*p*-tolylurea (1.24 g., 88%) was then filtered off, washed with a little dioxane and the combined filtrates evaporated to dryness. The resulting sirup was taken up in chloroform and extracted three times with an acetate buffer (*p*H 6.5) to remove di-*p*-nitrophenyl phosphate. *p*-Nitrophenol apparently was liberated during these operations since the extracts were yellow. The chloroform solution then was extracted repeatedly with 10^{-4} *M* sodium hydroxide solution until an extract was no longer yellow. The chloroform solution then was evaporated to dryness and the residual oil crystallized from a mixture of acetone and petroleum ether (65–110° boiling fraction) giving II (R = *p*-nitrophenyl) as stout, colorless needles; yield 1.3 g. (67%), m.p. 148°. *Anal.* Calcd. for $C_{14}H_{16}NO_9P$: C, 45.05; H, 4.32; N, 3.76. Found: C, 45.10; H, 4.40; N, 3.71.

Preparation of 1,2-*O*-Isopropylidene D-Xylofuranose-3,5-cyclic Phosphate (III). (a) From II (R = Phenyl) by Alkaline Hydrolysis.—1,2-*O*-Isopropylidene D-xylofuranose-3,5-cyclic phenyl phosphate (2.85 g., 8.67 m*M*) was dissolved in 8 ml. of dioxane in a polyethylene tube and 24.5 ml. of 1 *N* sodium hydroxide added. The oil which separated redissolved on agitation during the next 15 minutes. A further amount (2 ml. of 8 *N*) of sodium hydroxide was added and the clear solution sealed and kept at room temperature. Paper chromatography in solvent A after the first one hour showed the presence of two spots: one relatively weak spot, R_f 0.72, corresponding, presumably, to the mixture of 1,2-*O*-isopropylidene D-xylofuranose 3- and 5-monophenyl phosphates (V and VI) and the second strong spot, R_f 0.65, corresponding to the cyclic phosphate III. On continued alkaline treatment, the faster travelling spot gradually disappeared and after a total of 72 hours, the cyclic phosphate III was the sole product. The alkaline solution was then neutralized with Dowex 50 (H^+) to *p*H 5, the resin removed by filtration and washed with water. The combined filtrates were extracted five times with ether to remove phenol and the aqueous solution passed slowly through a Dowex 50 (cyclohexylammonium form) column (10 cm. × 1 cm.). The effluent and water wash were evaporated to dryness, the last traces of water being removed by repeated evaporation with absolute ethyl alcohol. The residue, which sometimes crystallized, was dissolved in a small volume of ethyl alcohol and the solution diluted with four volumes of ether. The crystalline cyclohexylammonium salt of III was collected after a few hours and washed with ether giving a yield of 2.08 g. (68%). It was recrystallized

(31) R. G. S. Bidwell, G. Krotkov and G. B. Reed, *Can. J. Botany*, **30**, 291 (1952).

(32) M. W. Slein, THIS JOURNAL, **77**, 1663 (1955).

(33) S. Mitsuhashi and J. O. Lampen, *J. Biol. Chem.*, **204**, 1011 (1953); R. M. Hochster and R. W. Watson, *Arch. Biochem. Biophys.*, **48**, 120 (1954); P. K. Stumpf and B. L. Horecker, *J. Biol. Chem.*, **218**, 753 (1956).

(34) National Institute of Arthritic and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland.

(35) This ester has recently been the focus of much biochemical interest. Its widespread role in carbohydrate metabolism has been established. See G. Ashwell and J. Hickman, THIS JOURNAL, **76**, 5889 (1954); P. A. Srere, J. Cooper, V. Klybas and E. Racker, *Arch. Biochem. Biophys.*, **59**, 535 (1955); B. L. Horecker, J. Hurwitz and P. Z. Smyrniotis, THIS JOURNAL, **78**, 692 (1956).

(36) Melting points are uncorrected. Microanalyses were performed by Mr. W. Manser, Zurich, Switzerland.

(37) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952); D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).

(38) M. Viscontini, D. Hoch and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).

(39) S. M. Partridge, *Nature*, **158**, 270 (1946).

(40) H. G. Khorana, *Can. J. Chem.*, **32**, 227 (1954).

from a mixture of ethyl alcohol, acetone and ether. On heating it decomposed at 217–219°; $[\alpha]^{21}D$ +5.5° (*c* 1.90, water). *Anal.* Calcd. for $C_{14}H_{26}NO_7P$: C, 47.84; H, 7.46; N, 3.99. Found: C, 47.79; H, 7.50; N, 3.61.

An aqueous solution of the crystalline cyclohexylammonium salt of III (200 mg.) was passed slowly through a Dowex 50 (H^+) column (3 cm. × 1 cm.) at 5°, and the column washed with cold water until the effluent was neutral. The total acidic effluent was lyophilized leaving a partly crystalline residue, which was recrystallized from a mixture of ethyl alcohol and ether giving the free acid III as stout colorless needles (100 mg., 70%), which on heating turned brown at 161° and decomposed at 163°. *Anal.* Calcd. for $C_8H_{13}O_7P$: C, 38.11; H, 5.20. Found: C, 37.81; H, 5.11. Potentiometric titration showed the absence of a secondary phosphoryl dissociation in the *p*H range 4–9.

(b) **From 1,2-*O*-Isopropylidene D-Xylofuranose 5-Diphenyl Phosphate (IV).**—The highly crystalline 1,2-*O*-isopropylidene D-xylofuranose 5-diphenyl phosphate prepared in excellent yield according to the method of Jones, *et al.*,[3b] was converted quantitatively to the cyclic phosphate III upon treatment with a mixture of dioxane and 2 *N* sodium hydroxide as described above.

(c) **From 1,2-*O*-Isopropylidene D-Xylofuranose-3,5-cyclic Phenyl Phosphate (II, R = Phenyl).**—The neutral II (R = phenyl) (940 mg.) was dissolved in methyl alcohol (30 ml.) and hydrogenated in the presence of Adams platinum catalyst (200 mg.). Hydrogen uptake (288 ml.) ceased after 25 minutes, after which time the catalyst was removed by filtration and the solvent evaporated at a low temperature. The resulting gum was dissolved in water (5 ml.) and the acidic solution brought to *p*H 8.5 with aqueous cyclohexylamine. The solution was evaporated to dryness and the cyclohexylammonium salt isolated and crystallized as above; yield 850 mg. (85%). Paper chromatography showed the product to be homogeneous and identical with that obtained above under (a) or (b).

D-Xylofuranose-3,5-cyclic Phosphate (XII).—In a preliminary experiment an aqueous solution (*p*H 1.5) of 10 mg. of 1,2-*O*-isopropylidene D-xylofuranose-3,5-cyclic phosphate (free acid) was heated at 100° and aliquots removed at 5-minute intervals. Paper chromatography in solvent system A showed that the removal of the isopropylidene group was complete in ten minutes and that D-xylofuranose-3,5-cyclic phosphate was the sole product. Subsequently, 250 mg. of the crystalline cyclohexylammonium salt of III was dissolved in a few ml. of water, the solution passed through a Dowex 50 (H^+) column (3 cm. × 1 cm.) and the column washed with water until the effluent was neutral. The total effluent (*ca.* 15 ml., *p*H 1.4) was held in a boiling water-bath for 10–15 minutes, rapidly cooled and adjusted to *p*H 4 with aqueous barium hydroxide. The solution was concentrated to a small volume and four volumes of acetone added. The gum which initially separated, solidified on trituration and the resulting powder was then collected by centrifugation, washed with ether and dried *in vacuo* at room temperature, giving 172 mg. (86%) of the barium salt. *Anal.* Calcd. for $C_5H_8O_7P \cdot Ba_{1/2}$: C, 21.47; H, 2.88. Found: C, 21.35; H, 3.14; $[\alpha]^{18}D$ −11.16° (*c* 2.16, water). Potentiometric titration showed the complete absence of a secondary phosphoryl dissociation in the *p*H range 4–9. On treatment with periodic acid (see below) the substance consumed one mole of the oxidant. On paper chromatograms it travelled as a single spot. $R_{f's}$: solvent A, 0.33; solvent C, 0.14.

A paper chromatographic study of the reaction of freshly-prepared D-xylose 5-phosphate with dicyclohexylcarbodiimide[41] showed that D-xylofuranose-3,5-cyclic phosphate was the only product. When samples of barium D-xylose-5-phosphate which had been stored at room temperature for some time were used, a weak spot (R_f 0.88 in solvent A) also appeared. This corresponds, presumably, to the phosphoryl urea derived from the contaminating D-xylulose 5-phosphate and is formed according to the reaction sequence[12] (D-xylulose 5-phosphate → D-xylulose-4,5-cyclic phosphate → D-xylulose-4(5)-N-phosphorylureas).

Preparation of a Mixture of 1,2-*O*-Isopropylidene D-Xylose 3- and 5-Phosphates (XIII and XIV).—Crystalline cyclohexylammonium 1,2-*O*-isopropylidene D-xylofuranose-3,5-cyclic phosphate (1.0 g.) was dissolved in 15 ml. of 1 *N* sodium hydroxide and the solution heated in a polyethylene tube at 100°. Paper chromatography in solvent A at intervals showed that heating the solution for *ca.* 20 hours[12] was necessary to complete the conversion of III (R_f 0.67) to, presumably, a mixture of 1,2-*O*-isopropylidene D-xylose 3- and 5-phosphates. The latter products travelled as a single spot with R_f 0.40. The alkaline solution was then cooled, passed through a Dowex 50 (H^+) column and the acidic effluent neutralized to *p*H 7.5 with aqueous barium hydroxide. The amorphous barium salts of XIII and XIV (1.04 g., theoretical) were isolated by evaporation of the solution and trituration of the residue with acetone.

Reconversion of XIII and XIV to III.—Treatment of the pyridinium salts of XIII and XIV with dicyclohexylcarbodiimide according to the general procedure described previously[12] resulted in the quantitative conversion of XIII and XIV to III in *ca.* two hours.

Preparation of D-Xylose 3-Phosphate.—Crystalline cyclohexylammonium 1,2-*O*-isopropylidene D-xylofuranose-3,5-cyclic phosphate (III, 1.95 g.) was hydrolyzed in 1 *N* sodium hydroxide as above to a mixture of XIII and XIV and the excess of alkali neutralized with Dowex 50 (H^+) resin. The neutral solution was further passed through a Dowex 50 (H^+) column (10 cm. × 1.5 cm.) and the column washed until the effluent became neutral. The combined acidic effluent was heated under its own *p*H (1.5) for 10 minutes in a boiling water-bath and the cooled solution neutralized with barium hydroxide solution to *p*H 7. The mixture of the barium salts of D-xylose 5- and 3-phosphates was precipitated by the addition of two volumes of ethyl alcohol and collected by centrifugation; yield 2.19 g. (96%). The mixture separated on paper chromatography in solvent A, using Whatman No. 4 paper, to give, after spraying with the aniline hydrogen phthalate reagent a brown spot (R_f 0.04) corresponding to xylose 5-phosphate and a pink spot (R_f 0.09) corresponding to xylose 3-phosphate.

The mixed barium salts (1.5 g.) were dissolved in 5 ml. of water and the solution applied to the top of a Dowex 2 (formate form) column (11 cm. × 4 cm.). After a water-wash (1 liter), elution was carried out with 0.1 *M* sodium formate–formic acid buffer (*p*H 3.0), 20-ml. fractions being collected by means of an automatic fraction cutter (flow rate, 4 ml./minute). The elution of the sugar phosphates was followed by spotting (several applications) every fifth fraction and spraying with the aniline–phthalate spray. The first five liters of effluent contained no reducing material. Xylose 5-phosphate appeared in the next 2350 ml. of the effluent and after one more liter of the eluent had passed through, xylose 3-phosphate appeared in the subsequent 2500 ml. The two combined fractions were each concentrated at low temperature under reduced pressure to *ca.* 25 ml. and freed from sodium ions by passage through Dowex 50 H^+ columns (10 cm. × 1.5 cm.). The total acidic effluents were lyophilized, giving viscous sirups which were taken up in the minimum amounts of water and neutralized to *p*H 7.5 with barium hydroxide. The precipitates of barium phosphates (29 and 140 mg. from D-xylose 3- and 5-phosphate, respectively) were centrifuged off and xylose phosphates were precipitated by the addition of two volumes of ethyl alcohol. They were collected by centrifugation and washed with 60% aqueous ethyl alcohol, acetone and ether and dried in a high vacuum at room temperature. Barium xylose 5-phosphate (brown spot with the aniline–hydrogen phthalate spray) was recovered in a yield of 680 mg. (45.4%). *Anal.* Calcd. for $C_5H_9O_8PBa \cdot 2H_2O$: P, 7.82. Found[42]: P, 7.86. Barium xylose 3-phosphate was recovered as a white amorphous solid in a yield of 230 mg. (18%). On paper chromatography in solvent A, it gave a single pink spot with aniline–phthalate spray, travelling faster than xylose 5-phosphate. *Anal.* Calcd. for $C_5H_9O_8PBa \cdot 3H_2O$: C, 14.33; H, 3.60; P, 7.38. Found in an air equilibrated sample: C, 14.70; H, 3.63; P, 7.21. Calcd. for $C_5H_9O_8PBa$: P, 8.47; xylose, 41.0. Found on a sample dried over phosphorus pentoxide: P, 8.40; xylose, 41.5. (Xylose estimations[43] were carried out after enzymatic dephosphorylation, as described below, $[\alpha]^{22}D$ + 1.27° ±0.2° (*c* 5.13, water)).

(41) The general procedure described for the study of cyclic phosphate formation[12] was used.

(42) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(43) Xylose estimation was carried out according to W. Mejbaum (*Z. physiol. Chem.*, **258**, 117 (1939), as modified by R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *J. Biol. Chem.*, **209**, 23 (1954)).

Enzymatic Dephosphorylations.—The enzymatic dephosphorylations of the various compounds were carried out according to the following general procedure. The barium salts of the phosphate esters (2–5 mg.) were dissolved in 0.1 *M* acetate buffer (*p*H 4.5, 0.5 ml.) and magnesium acetate (0.02 ml. of 0.1 *M*) and wheat germ acid phosphatase[44] (1–2 mg.) were added. The solutions were incubated for two hours at 37° and examined by paper chromatography in solvents B and C, the two systems which proved most satisfactory in this work. In this way, D-xylose 3-phosphate and a freshly-prepared sample of D-xylose 5-phosphate (see below for D-xylose 5- → D-xylulose 5-phosphate transformation) gave only a single spot in solvent B (R_f 0.26) as well as in solvent C (R_f 0.26). Other dephosphorylations are mentioned below.

Preparation of D-Xylitol 3- and 5-Phosphates.—Barium D-xylose 3-phosphate (30 mg.) was dissolved in water (2 ml.) and shaken under a slight pressure of hydrogen in the presence of Adams platinum catalyst (20 mg.). Hydrogen uptake was complete in two hours after which the catalyst was removed by filtration and the solution concentrated to about 0.5 ml. A trace of insoluble material was removed by centrifugation and the non-reducing barium xylitol 3-phosphate then precipitated by the addition of 1.5 volumes of ethyl alcohol. One further reprecipitation from a mixture of water and ethyl alcohol gave the pure product (25 mg., 83%); $[\alpha]^{21}D$ 0.00 ± 0.01° (*c* 4.8, water). *Anal.* Calcd. for $C_5H_{11}O_8P\cdot Ba$: P, 8.42. Found: P, 8.44.

In an identical manner the barium salt of xylitol 5-phosphate was obtained in 91% yield; $[\alpha]^{21}D$ −1.21 ± 0.5° (*c* 1.24, water). *Anal.* Calcd. for $C_5H_{11}O_8P\cdot Ba$: P, 8.42. Found: P, 8.38.

The D-xylitol 3-phosphate used in some experiments was prepared by reduction of xylose 3-phosphate with sodium borohydride. To a solution of barium D-xylose 3-phosphate (49 mg.) in water (0.5 ml.) was added sodium borohydride (20 mg.) and the solution allowed to stand for 15 minutes at room temperature. The solution, which was now non-reducing, was acidified with dilute hydrochloric acid and evaporated repeatedly in the presence of methyl alcohol. Water was then added and the *p*H of the solution brought to 8. To the clear solution was added 0.05 ml. of 2 *M* barium acetate solution and barium xylitol 3-phosphate precipitated by the addition of two volumes of ethyl alcohol; weight of the air-dried white powder, 43 mg.

Periodate Oxidations.[17]—The barium salt of the phosphate ester (10–15 mg.) was dissolved in the minimum volume of water and to the solution was added freshly washed IR-120 (H^+) resin (0.2 ml.). After thorough mixing the supernatant was transferred to a 25-ml. volumetric flask and the resin washed ten times with 0.5-ml. portions of water. To the combined aqueous solution was added 0.1 *M* acetate buffer (*p*H 4.5) to a volume of *ca.* 20 ml., then 2 ml. of 0.10 *M* periodic acid (in 0.1 *M* acetate buffer, *p*H 4.5) and finally water to make up to 25 ml. A blank was prepared in the same way, except that the phosphate sample was omitted. The solutions were stored at room temperature in the dark and, at intervals, two-ml. aliquots were removed and sodium bicarbonate (0.5 g.), 0.1 *N* sodium arsenite (2 ml.) and 20% aqueous potassium iodide (1 ml.) were added. The resulting mixtures were titrated after 15 minutes against a 0.0995 *N* solution of iodine in aqueous potassium iodide. The results are shown in Fig. 1.

Degradation of D-Xylitol 3- and 5-Phosphates to, Respectively, Glycerol and Ethylene Glycol. (a) D-Xylitol 3-Phosphate.—Barium D-xylitol 3-phosphate (10 mg.) was treated with sodium-Dowex 50 ion-exchange resin and the resulting aqueous solution of the sodium salt concentrated to 0.1 ml. Sodium periodate (0.6 ml. of 0.1 *M* solution, 2 moles) was then added and, after ten minutes at room temperature in the dark, was followed by 25 mg. of sodium borohydride. The mixture was kept at room temperature overnight, was then acidified to *p*H 4 with acetic acid and concentrated. The total concentrate was applied to a Whatman No. 4 strip (ten inches wide) and the chromatogram developed for *ca.* 30 hours in solvent A (descending technique). The stable organic phosphate band was then located by cutting out a thin strip from one end of the sheet and spraying it with the molybdate spray.[45] (Varying amounts of inorganic phosphate were also formed in different experiments.) The stable phosphate band was then eluted with water, the solution evaporated to dryness and the residue dephosphorylated with wheat germ acid phosphatase as described above. The mixture after incubation was applied directly to a paper chromatogram which was developed in solvent B. Although variable results were obtained during several repetitions of this experiment, glycerol was identified sometimes as the sole final product, as visualized with the periodate–benzidine spray.[38]

(b) **D-Xylitol 5-Phosphate.**—Analogous degradation of this substance using three moles of periodic acid proceeded smoothly and ethylene glycol was invariably obtained as the sole product.

Partial Conversion of D-Xylose 5-Phosphate to D-Xylulose 5-Phosphate. (a) Optical Rotation Changes.—Freshly-prepared barium D-xylose 5-phosphate (100 mg.) was converted to the sodium salt by passing its aqueous solution through sodium IR-120 resin. The effluent and washings were concentrated to 5 ml. and the optical rotation of this solution was observed in a 10-cm. long polarimeter tube at different intervals of days. The initially observed rotation ($[\alpha]^{21}D$ +8.3°) decreased after three days to $[\alpha]^{21}D$ +0.70° and after a total of seven days to $[\alpha]^{21}D$ −1.0°. These changes are in agreement with the observations of Watson and Barnwell.

(b) **Identification of Xylulose after Enzymatic Dephosphorylation.**—The *p*H of a solution of the disodium salt of D-xylose 5-phosphate (prepared from 250 mg. of the barium salt as described above) was adjusted to 6.2 with dilute acetic acid and the solution was heated at 50° for 2.5 hours (*cf.* Watson and Barnwell[4]). The following experiments were performed on this solution. (1) An appropriate aliquot containing 10 mg. of the salt was evaporated to dryness and then taken up in 0.5 ml. of acetate buffer (0.1 *M*, *p*H 4.5) and dephosphorylated enzymatically as described above. Paper chromatography in Solvents B and C showed the presence of xylulose (R_f in solvent B and C, 0.42 and 0.33, respectively) in addition to xylose (R_f in both solvents 0.26). D-Xylulose used for comparison on paper chromatograms was prepared (a) by the partial transformation of D-xylose to D-xylulose in boiling pyridine and (b) by enzymatic dephosphorylation of D-xylulose 5-phosphate kindly supplied by Dr. Horecker. The spots corresponding to xylulose gave the characteristic color tests for ketoses with the orcinol-hydrochloric[31] and cysteine-carbazole[32] sprays. Further, paper chromatography after treatment of the enzymatically dephosphorylated mixture with bromine water showed that, as had been expected, the xylulose spot had survived while xylose had disappeared. (2) An appropriate aliquot of the solution containing 5 mg. of the sodium salt, now was dephosphorylated as has been described above and the entire mixture applied as a streak to a Whatman No. 4 paper sheet. The chromatogram was developed overnight by the descending technique in solvent B. By spraying thin strips cut from the edges of the chromatographic sheet with the aniline–hydrogen phthalate spray two zones corresponding to xylose and xylulose were located and were then eluted with water. The eluted materials were concentrated and rechromatographed as above. After re-elution and concentration of the zones, the concentrates (1 ml.) were added to a solution (4 ml.) of freshly crystallized orcinol and ferric chloride in concentrated hydrochloric acid.[43] The yellow solutions were held in a boiling water-bath for ten minutes, rapidly cooled and their spectra (440–760 mμ) determined using as a blank an aqueous eluate from a strip of Whatman 4 paper, containing the reagent and treated in an identical way. The spectra of the faster and the slower travelling materials were identical with those of authentic xylose and xylulose, the characteristic ratios[33] at $^{540}/_{670}$ mμ being 0.23 and 0.39, respectively, for the eluted materials.

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(44) Worthington Biochemicals, Freehold, New Jersey.

(45) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949); R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).