## 748. *Phosphorylated Sugars. Part I. The Alkaline Hydrolysis of Methyl α-D-Glucoside 4,6-(Hydrogen Phosphate).**

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Alkaline hydrolysis of methyl α-D-glucoside 4,6-(hydrogen phosphate) is shown to produce methyl α-D-glucoside 4- and 6-phosphate in the ratio of 4 : 1, no other phosphate being formed. Analogous results have been obtained with methyl β-D-galactoside 4,6-(hydrogen phosphate). A chromatographic method for the separation of the isomeric methyl glucoside monophosphates, on a basic ion-exchange resin in the borate form, is described.

The formation of cyclic phosphates during alkaline hydrolysis of diesters of phosphoric acid or pyrophosphates bearing a suitably placed hydroxyl group in one of the organic residues is now widely recognised, *e.g.*, the formation of glucose 1,2-(hydrogen phosphate) from uridine-diphosphate-glucose,[1] pantothenic acid 2′,4′-(hydrogen phosphate) [2] from coenzyme A, nucleoside 2′,3′-(hydrogen phosphate) [3,4] from ribonucleic acid, adenosine

* For a preliminary note see Szabó and Szabó, *Compt. rend.*, 1958, **247**, 1748.

[1] Paladini and Leloir, *Biochem. J.*, 1952, **51**, 426.
[2] Baddiley and Thain, *J.*, 1952, 3783.
[3] Markham and Smith, *Biochem. J.*, 1952, **52**, 552.
[4] Brown, Magrath, and Todd, *J.*, 1952, 2708.

3′,5′-(hydrogen phosphate) [5] from adenosine triphosphate. The cyclic esters formed may be further hydrolysed to monoesters of phosphoric acid. If the course of the hydrolysis can be predicted, important conclusions may be drawn as to the structure of the starting material of natural origin by identification of the relatively stable end-product.

Known cyclic phosphates have five-, six-, or seven-membered rings, of which those with five-membered rings are the best known and most widely investigated. Five-membered are generally less stable than six-membered rings towards reagents such as dicyclohexylcarbodi-imide and hydrolytic agents; [6] only one seven-membered cyclic phosphate has been described,[6] so no general conclusion can be drawn as to the stability of this class.

We have investigated the behaviour of methyl α-D-glucoside 4,6-(hydrogen phosphate) [7] (I). This was transformed almost quantitatively by ~0·5N-barium hydroxide at 100° in 4 hr. into a methyl hexoside monophosphate with only a negligible amount of inorganic phosphate. Baddiley and Thain [8] showed that pantothenic acid 2′,4′-(hydrogen phosphate), in which the phosphoric acid is esterified simultaneously with a primary and a secondary hydroxyl group forming a six-membered ring, yields under similar conditions exclusively the 4′-phosphate; *i.e.*, the phosphate group remains attached to the primary carbon atom. If alkaline hydrolysis of methyl α-D-glucoside 4,6-(hydrogen phosphate) followed the same pattern, it should afford methyl α-D-glucoside 6-phosphate. The formation of a derivative of D-galactose, arising through alkyl–oxygen fission during the hydrolysis is not expected, as it has been shown [9] that diesters of phosphoric acid are hydrolysed in alkaline medium by phosphorus–oxygen fission. As will be seen below, comparison of the behaviour of methyl α-D-glucoside 6-phosphate and methyl β-D-galactoside 6-phosphate on ion-exchange columns makes such an alternative very improbable; it is, however, not rigorously excluded.

Methyl α-D-glucopyranoside 6-phosphate has three vicinal hydroxyl groups and accordingly should consume two equivalents of periodate: the methyl hexoside monophosphate obtained by hydrolysis as above consumed only somewhat more than one equivalent and could not therefore be the 6-phosphate. This behaviour towards periodate would be explained if the methyl hexoside were methyl α-D-glucoside 4-phosphate. In view of the reported behaviour of pantothenic acid 2′,4′-(hydrogen phosphate) and since 1,2-*O*-isopropylidene-D-xylofuranose 3,5-(hydrogen phosphate) [10] gave on alkaline hydrolysis (followed by acid hydrolysis to remove the isopropylidene group) a mixture of 18% of xylose 3-phosphate and 45% of xylose 5-phosphate, it seemed improbable that methyl α-D-glucoside 4-phosphate would be the sole product of hydrolysis. It was therefore necessary to characterise the reaction product further.

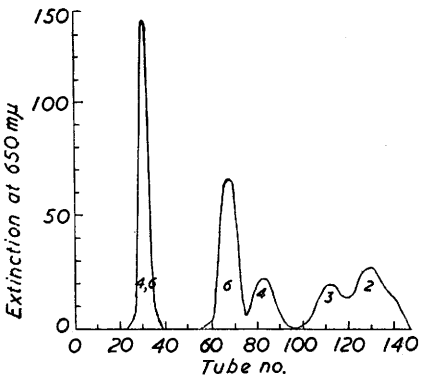
It was considered that during the alkaline treatment of the cyclic phosphate an intramolecular transesterification might have occurred, leading, on hydrolysis, to several or all of the isomeric methyl glucoside phosphates. Such transesterifications are known to occur with diesters of glycerophosphoric acid [11] and involve five-membered rings. To investigate this hypothesis a method of separating the four isomeric phosphates of methyl glucoside was sought.

Khym and Cohn [12] separated glucose 1- and 6-phosphate, fructose 6-phosphate, and ribose 5-phosphate by ion-exchange column chromatography using an eluant containing borate. In all these sugar phosphates, except glucose 1-phosphate, the functional groups of the first and the second carbon atom of the sugar moiety are unsubstituted. The hypothesis that these groups are involved in strongly ionised borate-complex formation [13]

[5] Lipkin, Markham, and Cook, *J. Amer. Chem. Soc.*, 1959, **81**, 6075.
[6] Khorana, Tener, Wright, and Moffatt, *J. Amer. Chem. Soc.*, 1957, **79**, 430.
[7] Baddiley, Buchanan, and Szabó, *J.*, 1954, 3826.
[8] Baddiley and Thain, *J.*, 1951, 3421.
[9] Blumenthal and Herbert, *Trans. Faraday Soc.*, 1945, **41**, 611.
[10] Moffatt and Khorana, *J. Amer. Chem. Soc.*, 1957, **79**, 1194.
[11] Long and Maguire, *Biochem. J.*, 1953, **54**, 612.
[12] Khym and Cohn, *J. Amer. Chem. Soc.*, 1953, **75**, 1153.
[13] MacPherson and Percival, *J.*, 1937, 1920.

was corroborated by the observation that the elution position of glucose 1-phosphate was practically unaffected by varying concentrations of borate. It seemed thus unlikely that a separation of the methyl glucoside phosphates could be achieved by using Khym and Cohn's system; in fact, although methyl glucoside 4- and 6-phosphate were separable from each other, methyl glucoside 2- and 4-phosphate were eluted in the same position. We believe that the separation of methyl glucoside 6-phosphate from the 2- and 4-phosphate is due to differences in the acid strength of the esters.

Using zone electrophoresis, Foster and Stacey [14] showed, by blocking individual hydroxyl groups of methyl glucopyranosides, that under certain conditions the 4- and 6-hydroxyl groups may be involved in complex formation with the borate ion. It is also known that the complex formation increases with increasing pH values.[15] It thus seemed likely that by increasing the pH of the eluant and augmenting the borate-ion concentration in the system, a separation of the four isomeric phosphates might be achieved. Accordingly an anion-exchange resin, Dowex 1, in the borate form was used and the sugar phosphates were eluted with a solution of potassium borate: methyl α-D-glucoside 2-, 3-, 4-, and 6-phosphate [16] and methyl α-D-glucoside 4,6-(hydrogen phosphate) were then all easily separable (Fig.).




*Separation of methyl* α-D-*glucoside* 4,6-(*hydrogen phosphate*) *and methyl* α-D-*glucoside* 6-, 4-, 3-, *and* 2-(*dihydrogen phosphates*) (*fractions each* 11 *ml.*).

Analysis of the alkaline hydrolysate of methyl glucoside 4,6-(hydrogen phosphate) by this method disclosed besides a trace of unhydrolysed material, 4—5 times as much methyl α-D-glucoside 4- as 6-phosphate, no other isomer being present.

These experiments prove that intramolecular transesterification does not take place during the alkaline treatment of the cyclic phosphate.

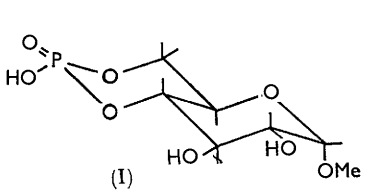
The absence of interference of the 2- and 3-hydroxyl groups with the course of the hydrolysis has been rigorously proved in the following manner. Methyl 2,3-di-*O*-benzyl-α-D-glucoside 4,6-(hydrogen phosphate) has been synthesised [16] and hydrolysed with barium hydroxide. The resulting methyl 2,3-di-*O*-benzyl-α-D-glucoside monophosphate(s) were hydrogenated on a palladium catalyst to remove the benzyl groups (no migration of phosphate groups is to be expected). Again 4—5 times as much 4- as 6-phosphate was ound.

We investigated also the effect of the conformation of the molecule on the hydrolysis. In methyl β-D-galactoside 4,6-(hydrogen phosphate) [16] (II) the pyranose and the six-membered phosphate rings are *cis*-fused and the phosphate ring does not confer rigidity on the sugar moiety, whereas in methyl glucoside 4,6-(hydrogen phosphate) (I) the rings are *trans*-fused and the pyranose ring is rigidly maintained in the C1 conformation.[17] The former ester was therefore hydrolysed as above and the products were analysed on an

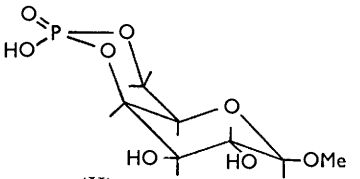
[14] Foster and Stacey, *J.*, 1955, 1778.
[15] Consden and Stanier, *Nature*, 1952, **169**, 783.
[16] Szabó and Szabó, following paper.
[17] Reeves, *J. Amer. Chem. Soc.*, 1949, **71**, 215, 1737.

ion-exchange column standardised with methyl β-D-galactoside 4- and 6-phosphate.[16] Again, about 4 times as much 4- as 6-phosphate was formed, proving that the conformation is without marked influence.

Alkaline hydrolyses of adenosine 3′,5′-(hydrogen phosphate) [18] and of thymidine 3′,5′-(hydrogen phosphate) [19] also follow this pattern, giving 4—5 times as much 3′- as



(I)



(II)

5′-phosphate. However, in these compounds the sugar is in the furanose and not in the pyranose form, and the results are therefore not strictly comparable with ours.

The reasons for this " anomalous " hydrolysis of these compounds are not clear at present. Further experiments are in progress.

## Experimental

*Separation of Methyl Glycoside Phosphates.*—A column of anion-exchange resin (Dowex 1 × 2; 200—400 mesh; 1·85 × 26·5 cm.) was converted into the borate form, the excess of borate removed by washing with water, and the column treated with 0·0185M-dipotassium tetraborate (100 ml.) (solution A). The sugar phosphate or mixture of sugar phosphates was adsorbed on to the column from solution A (10 ml.), and the column washed with a further 11 ml. of solution A before elution with a 0·062M-solution of dipotassium tetraborate (solution B) (11 ml. fractions; flow rate 0·5 ml./min.), the sugars being detected with the anthrone reagent.[20] It was immaterial whether the sugar phosphates were adsorbed on to the column as their barium or cyclohexylammonium salts. The elution positions of each of the four monophosphates of methyl α-D-glucoside and of the cyclic phosphate were determined in successive experiments. A mixture of the five sugar phosphates (10 mg. of the cyclohexylammonium salt of each) was then separated on the same column (Fig.) and finally the alkaline hydrolysate (40 mg. of the barium salt) of methyl α-D-glucoside 4,6-(hydrogen phosphate) was analysed.

The same column was used for the separation of the methyl β-D-galactoside 4- and 6-phosphate. Again, the elution position of each phosphate was determined in consecutive experiments and finally a mixture of the two phosphates was separated. (In a later paper we shall discuss the appreciable influence of the aglycone group of a glycoside phosphate on the elution positions.)

*Alkaline Hydrolysis of Methyl* α-D-*Glucoside* 4,6-(*Hydrogen Phosphate*).—The cyclohexylammonium salt of the cyclic phosphate (1 g.) was heated in saturated aqueous barium hydroxide (30 ml.) at 100° for 4 hr., then cooled and the excess of barium ions was removed with Amberlite IR-120 resin ($H^+$ form). The resin was filtered off and washed with water. The combined filtrates were concentrated *in vacuo* and the barium salts (0·93 g.) of the mixed phosphates precipitated with acetone, filtered off, washed with a little acetone, and air-dried. A sample (40 mg.) of this powder was analysed as above.

Hydrolysis of methyl β-D-galactoside 4,6-(hydrogen phosphate) was accomplished similarly.

*Alkaline Hydrolysis of Methyl* 2,3-*Di*-O-*benzyl* α-D-*Glucoside* 4,6-(*Hydrogen Phosphate*).—The cyclohexylammonium salt of the cyclic phosphate (400 mg.) was heated at 100° with saturated aqueous barium hydroxide (15 ml.) for 5 hr. The cooled solution was brought to neutrality with Amberlite IR-120 resin ($H^+$ form), filtered, and passed slowly through a column of the same resin in the cyclohexylammonium form. The effluent was evaporated to dryness *in vacuo* and taken up in ethanol. To convert the mixture of methyl 2,3-di-*O*-benzyl α-D-glucoside phosphates into the corresponding methyl α-D-glucoside phosphates, the ethanolic solution was brought to pH 4 with Amberlite IR-120 ($H^+$ form) and shaken with hydrogen at

[18] Lipkin, Cook, and Markham, *J. Amer. Chem. Soc.*, 1959, **81**, 6198.
[19] Tener, Khorana, Markham, and Pol, *J. Amer. Chem. Soc.*, 1958, **80**, 6223.
[20] Dreywood, *Ind. Eng. Chem. Analyt.*, 1946, **18**, 499; Morris, *Science*, 1948, **107**, 254.

room temperature and pressure in the presence of a palladium catalyst. When the uptake of hydrogen ceased, the catalyst was filtered off, and the filtrate neutralised with cyclohexylamine and evaporated to dryness *in vacuo*. For the analysis on the anion-exchange column, a portion (43 mg.) of the residue was dissolved in solution A (10 ml.) and adsorbed on to the column as already described.

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