Sugar Esters. II.[1] The Structure of the Alkali-Stable Phosphate Esters Obtained in Alkali Treatment of Sugar Diphosphates and Cyclic Phosphates

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The alkali-lability of certain phosphate esters,[2a] in particular those of some carbohydrates,[2b] contrasts sharply with the stability of most alkyl phosphates.[3] Unlike lability in carbohydrate sulfonates, phosphate ester-lability does not involve anhydro compound formation; in many ways a closer parallel is shown with *O*-alkyl- and *O*-alkylidenehexoses. These points have been reviewed recently and mechanisms discussed.[4] Only preliminary studies have been made of some types of alkali-labile phosphates.[5]

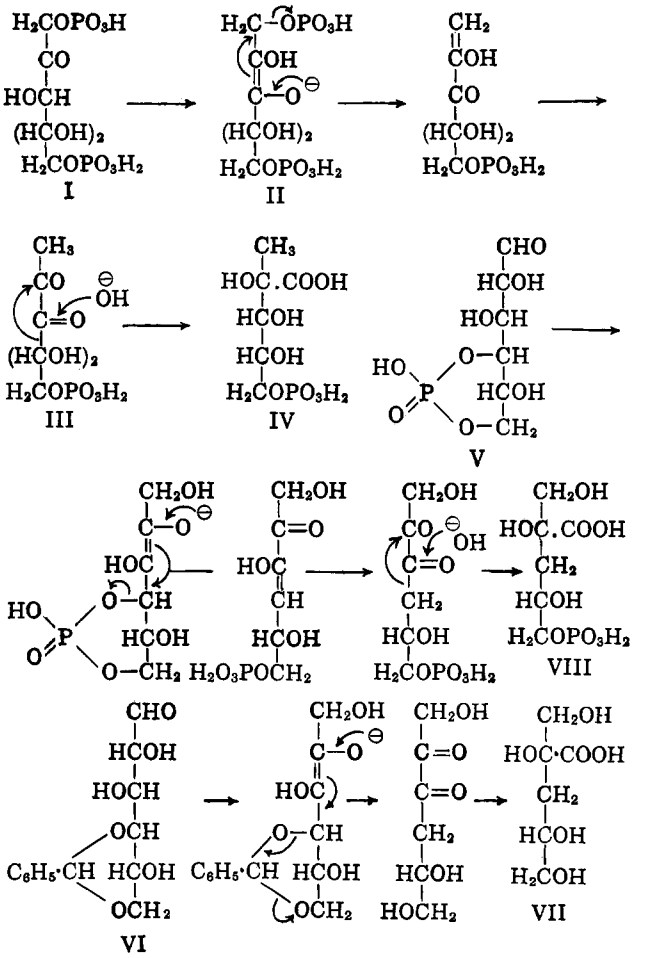
In these laboratories we have been studying the effects of ionizing radiation upon some sugars. The object of some of our work has been to compare the behavior of some normal, branched-chain, and deoxy sugar phosphates, and elucidate the reactions involved. Sugars exemplifying mono- and polyphosphorylation at a number of nonglycosidic positions have been examined. We are at present engaged in a radiochemical examination of mechanisms.

The classes of ester examined include (a) sugar monophosphates, (b) sugar di- and cyclic phosphates, and (c) deoxy sugar phosphates.

So far we find that release of inorganic phosphate on treatment of hexose monophosphates and pentose monophosphates with alkali is almost complete. Similar treatment of hexose diphosphate or aldose cyclic phosphate has been found to produce appreciable amounts of ester-bound phosphate of high alkaline stability. The nature of this stable phosphate interested us, and two examples illustrative of our findings are given. Since complex mixtures form in the presence of oxygen, it was found necessary to exclude oxygen rigorously from these reaction mixtures.

When fructose 1,6-diphosphate (I) was treated with alkali (aqueous sodium or potassium hydroxide, 0.1–0.5 *N*) release of approximately 1.3 to 1.5 moles of inorganic phosphate occurred, and the remaining organically bound phosphate was very stable towards hot alkali. Examination of the mixture of products by two-dimensional paper chromatography showed that numerous components (ten to fifteen according to the conditions) were present.

Paper ionophoresis differentiated acidic and neutral fractions, of which the acidic predominated and included both phosphorus-free material and phosphate esters. The acidic material was separated and further resolved into a portion containing organic acids and one containing phosphate. Finally a phosphate ester was obtained, IV, which behaved as a single substance both on chromatography and ionophoresis. Elementary analysis gave the formula $C_6H_{13}O_9P$. The compound is given structure IV, *i.e.*, glucosaccharinic acid 6-phosphate, on the following evidence. Compound IV reduced hot acidified dichromate and permanganate solutions, but was without action upon hot Fehling, cold Tollens', Schiff, or Brady reagents. It gave positive tests for an α-hydroxy acid[6] (hydroxamic acid test[7] and vanadium oxine test[8]), and formed a mono *p*-bromophenacyl ester, m.p. 157–158°. The phosphate grouping in IV was stable towards alkali, and was only slowly hydrolyzed by acid. No characteristic absorption was observed in the ultraviolet region, but bands in the infrared at 2.9–3.1 (broad), 3.7 (w), 5.65 (s), 8.22 (vs), and 9.65 μ (s) confirmed carboxylic acid (lactonized), free hydroxyl, and P=O of phosphate. One C-methyl group was present (Kuhn–Roth) and probably three adjacent hydroxyl groups, since, on reaction with periodate, IV reduced one mole rapidly and a second fairly rapidly. Thereafter it reacted slowly to consume a third mole of oxidant.



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Treatment of the compound with exactly two moles of the oxidant gave a solution in which some pyruvic acid (2,4-dinitrophenylhydrazone, m.p. 213–214°, *p*-bromophenacyl ester m.p. 117–118°) and some glycolaldehyde phosphate (identified chromatographically) were detected. When only one mole of oxidant was used a second phosphate ester, which rapidly released inorganic phosphate on treatment with dilute alkali, was present in the solution. This suggested a β-carbonyl ester, but the presence of glyceraldehyde 3-phosphate could not be demonstrated.[9]

Compound IV reacted with *o*-phenylenediamine in glacial acetic acid, but the product did not show the characteristic quinoxaline absorption in the ultraviolet region, regenerated *o*-phenylenediamine on treatment with alkali, and appeared to be a salt.

Surprisingly, compound IV gave a weak iodoform reaction. It was, therefore, subjected to oxidation with alkaline hypobromite. D-Erythronic acid 4-phosphate was isolated from the oxidation mixture and identified by comparison with authentic material and conversion to the *p*-bromophenacyl ester, m.p. 182–183°.

These facts are consistent with the structure assigned, and such a structure is in harmony with what is already known of the behavior of glucose 3-phosphate[10] and related compounds.[11] Thus, enolization of the fructose diphosphate (I) to the 2,3-enediol (II), followed by a β-elimination[9,10,11] would produce 1-deoxy-D-*erythro*-2,3-hexodiulose 6-phosphate (III). Unsuccessful attempts were made to isolate this compound by varying the conditions; some evidence has been obtained for similar β-eliminations of alkali treatment of some 3-*O*-substituted 2-deoxy and 2-*O*-alkyl sugars, where the absence or blocking of the C-2 hydroxyl somewhat stabilizes the intermediate, and this evidence will be presented separately.[12]

Rearrangement of the α-dicarbonyl compound to the acid in the present case probably proceeds rapidly.[13] The final confirmation of the structure by synthesis is proceeding.

While this compound results from one mode of degradation of fructose 1,6-diphosphate, the amount of phosphate set free and the complex nature of the products indicate that other reaction pathways exist.

The nature of the other compounds composing this mixture, together with their origin (involving chain scission in some cases), will be discussed in relation to the studies with labelled compounds at present being carried out in this department.

Galactose 4,6-phosphate (V), when subjected to alkaline hydrolysis, also produced an alkali-stable phosphate ester. Since 4,6-*O*-benzylidenegalactose (VI) on similar treatment produces isosaccharinic acid (VII), it was considered that a similar process might be occurring with the cyclic phosphate to give the isosaccharinic acid phosphate (VIII). Comparison chemically and by infrared spectroscopy with an authentic sample confirmed the structure given.

Careful reduction of the lactones of the saccharinic and isosaccharinic acid phosphates produces branched-chain sugar phosphates, and by suitable modification this might prove a useful route to these interesting compounds.

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Experimental

Melting points are uncorrected.

Materials.—Fructose 1,6-diphosphate was a purified commercial sample and still contained traces of inorganic phosphate (<0.9%) and a second phosphate ester (<0.4%).

Other materials were analytical grade where available. Otherwise, purified commercial grade materials were employed.

Analytical Technique.—Inorganic phosphate was determined by the methods of Lowry and Lopez,[14] slightly modified. Periodate uptake was measured by the methods of Aspinall and Ferrier,[15] and of Morrison.[16]

Spectra were measured upon the Perkin-Elmer Infracord spectrophotometer, the Unicam SP.700 spectrophotometer, or the SP.100 spectrophotometer, or the Hilger H-800 Model.

Ascending or descending chromatograms were run on Whatman no. 1 paper without temperature control. Eluting solvents were (a) *n*-butyl alcohol–chloroform–water (4:1:5); (b) *n*-butyl alcohol–toluene–water (4:1:5); (c) *n*-butyl alcohol–ethanol–water (4:1:5); (d) ethyl ether–petroleum ether (60–80°)–water (5:5:1); (e) pyridine–butanol–water (1:5:1); (f) acetic acid–butanol–water (1:8:1). Unless otherwise stated the organic phase was employed.

The following sprays were employed in development: (a) modified Hanes Isherwood reagent[17]; (b) Tollens reagent[18]; (c) bromocresol green (0.4%, 60 parts; Freon, 40 parts)[19]; (d) a solution of potassium permanganate (0.2 *N*) in sodium carbonate (0.5 *N*); (e) aniline–diphenylamine (88 parts of acidified 0.2% aniline–diphenylamine with 12 parts Freon).[19]

Electrophoresis was carried out using Whatman no. 1 paper, and borate buffer (pH 9.5).

Hydrolysis of Fructose 1,6-Diphosphate.—In a series of preliminary experiments the phosphate released from the ester dissolved in aqueous sodium hydroxide or potassium hydroxide was determined at alkali concentrations ranging from 0.1–0.5 *N*, and at temperatures ranging from 50–98°.

In each case release of inorganic phosphate was incomplete. In general, an initial very rapid release of phosphate (*ca.* 1.3–1.5 moles) preceded a very slow hydrolysis of the remaining organically bound phosphate.

Examination of the hydrolysates by two-dimensional paper chromatography–ionophoresis showed the presence of eleven or more separated materials, depending upon the conditions. When no precautions were taken to prevent access of air to the hydrolysates, more complex chromatograms were obtained.

Separation and Identification of the Ester IV.—The separation of products other than compound IV involved slightly modified methods from those subsequently described.

A solution of 1.98 g. of fructose 1,6-diphosphate in 10 ml. of water was degassed and 150 ml. of a degassed solution of 0.46 *N* sodium hydroxide was admixed under an atmosphere of oxygen-free nitrogen. The mixture was heated, under a slow stream of nitrogen, for 15 min. upon the steam bath. The carbon dioxide neutralized mixture was examined by two-dimensional paper chromatography and two-dimensional paper chromatography–ionophoresis using a variety of solvents and developers. At least twelve distinct components were present.

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(18) E. Lederer and M. Lederer, "Chromatography," Elsevier Publishing Co., New York, N. Y., 1953.

(19) Aerosol spray supplied by Sigma Chemical Corp.

The mixture was passed through an anion-exchange column and the column eluted with aqueous boric acid.[20] The phosphate-containing fractions were combined and solvent removed at the pump. The material was subjected to fractionation upon several thicknesses of paper eluted with either solvent e or solvent f, the major fraction being selected on each occasion.

After a final fractionation by electrophoresis, material was obtained homogeneous upon electrophoresis and upon chromatography upon paper (solvents a, e, and f), $[\alpha]^{20}D$ +62° (*c* 0.09, in water), showing bands in the infrared at 2.9–3.1 (broad), 3.7 (w), 5.65 (s), 8.22 (vs), and 9.65 μ (s), indicating the presence of carboxylic acid (lactonized), free hydroxyl, and P=O of phosphate. This material gave a *p*-bromophenacyl ester,[21] m.p. 157–158°.

Anal. Calcd. for $C_{14}H_{18}O_{10}P$ Br: C, 36.76; H, 3.94. Found: C, 37.00; H, 3.94.

Compound IV reduced hot acidified dichromate and permanganate solutions, gave positive tests for an α-hydroxy acid,[6-8] but was negative towards Fehling, Tollens, Schiff, and Brady reagents.

A portion of IV was subjected to further hydrolysis with *N* aqueous sodium hydroxide at 100° for 30 min. Less than 8% of the phosphate was set free as inorganic phosphate. The action of 0.1 *N* aqueous hydrogen chloride under similar conditions yielded approximately 25% of inorganic phosphate.

A portion of material IV reacted with aqueous periodate, and the course of the reaction was followed spectrophotometrically.[15] In the initial rapid reaction 1 mole of periodate was consumed. A further mole of periodate reacted within 24 hr. Thereafter somewhat slower oxidation consumed a 3rd mole of oxidant.

An amount of 0.08 g. of IV in 5 ml. of water was mixed with 0.06 *M* aqueous sodium periodate (2 moles) and the solution set aside in darkness for 24 hr. The solution was freeze dried and the residue extracted with ether. From the ethereal extract pyruvic acid was obtained in small amount, converted to its 2,4-dinitrophenylhydrazone, m.p. 213–214°. In a second experiment the *p*-bromophenacyl ester, m.p. 117–118°, was obtained.

In a similar experiment using one molar proportion of periodate, a second phosphate ester was obtained in solution. Treatment of this solution with 0.4 *N* aqueous sodium hydroxide led to rapid release of inorganic phosphate, suggesting a β-carbonyl ester. Attempts to identify this ester were not successful.

To a portion of 0.09 g. of IV in 3.0 ml. of glacial acetic acid was added 0.25 g. of *o*-phenylenediamine. The mixture was heated for several minutes upon the steam bath. The cooled mixture was triturated with ether and recrystallized from alcohol. This material did not show the usual absorption bands in the ultraviolet characteristic of quinoxalines, and treatment with excess alkali regenerated *o*-phenylenediamine. When insufficient alkali was added sparingly soluble material was obtained, which contained phosphorus, nitrogen and sodium. Recrystallized from aqueous ethanol, this appeared to be a mixed sodium *o*-phenylenediamine salt.

Anal. Calcd. for $C_{18}H_{21}O_{18}P_2N_2Na_2 \cdot H_2O$: C, 31.31; H, 4.93; N, 4.06; residue of $Na_2H_2P_2O_7$ on ignition, 32.18. Found: C, 31.39; H, 4.62; N, 3.72; residue of $Na_2H_2P_2O_7$ on ignition, 32.31.

A portion of IV was treated with iodine in sodium hydroxide forming some iodoform. Compound IV was, therefore, subjected to oxidation with hypobromite. An amount of 0.17 g. of IV was dissolved in 5 ml. of sodium hypobromite solution, and the mixture was set aside for 20 hr. at room temperature. The solution was passed over ion-exchange resin [Amberlite IR 120 (H^+ form)] and the solvent rapidly removed at the pump.

The residue was examined by two-dimensional paper chromatography–ionophoresis and development of the chromatograms (spray a) showed the presence of several phosphate-containing components. One of these components was found to behave identically with D-erythronic acid 4-phosphate[22] under these conditions. Accordingly, the separation was repeated using several thicknesses of paper, and the material was eluted and converted[21] to the *p*-bromophenacyl ester, m.p. 182–183°.

Anal. Calcd. for $C_{12}H_{14}O_9P$ Br·$3H_2O$: C, 30.83; H, 4.28. Found: C, 30.63; H, 4.27.

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Synthesis of Acetylhexosamine 1-Phosphates[1]

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There has been considerable recent interest in the 1-phospho derivatives of N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose) and N-acetyl-D-galactosamine (2 - acetamido - 2 - deoxy - D - galactose). The preparation of the glucosamine analog *via* the one bromo sugar has been previously reported by Maley, Maley, and Lardy.[2] Attempts to prepare the bromo derivative of galactosamine which could be coupled with silver diphenyl phosphate or a similar reagent have not been successful.

We have developed a convenient procedure for the synthesis of N-acetylhexosamine-α 1-phosphates by direct phosphorylation of the fully acetylated amino sugars with anhydrous phosphoric acid according to the procedure of MacDonald.[3] Preparation of the β-pentaacetate of glucosamine was carried out according to Bergmann[4] and this same procedure adapted to yield the corresponding galactosamine derivative. The acetylhexosamine 1-phosphates were purified by chromatography on Dowex-1 ion exchange resin and isolated as amorphous lithium salts.

Although the net change at the anomeric center during the phosphorylation reaction is inversion from the β- to the α-configuration, it is not likely that this reaction is a bimolecular displacement. Recent studies in our laboratories as well as others[5] have indicated that certain α-anomers react under the same conditions, with net retention, thus making it unlikely that an S_N2 mechanism is involved. At present, there is insufficient evidence to permit satisfactory speculations as to the natrue of this phosphorylation reaction.

Experimental

Melting points were obtained on a Fisher microstage and are uncorrected. Microanalyses by Galbraith Analytical Laboratories, Knoxville, Tenn.

N-(*p*-Methoxybenzylidene)-2-amino-2-deoxy-D-galactose (I).—Nine grams of D-galactosamine hydrochloride[6] was dissolved in 42.3 ml. of 1 *N* sodium hydroxide. A 50.3-ml. sample of redistilled anisaldehyde was added rapidly and the solution stirred vigorously for 2 hr. Crystalline material appeared in a few moments and the reaction mixture was nearly solid after 15 min. The suspension of crystalline material was allowed to stand overnight at 4°; the crystals were harvested in a Büchner funnel, washed with ice–water and a 1:1 mixture of alcohol–ether. Recrystallization of the Schiff base is extremely difficult due to the lability of the anisaldehyde group. The product exhibited

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