DISTRIBUTION AND STRUCTURAL FORM OF PHOSPHATE ESTER GROUPS IN COMMERCIAL STARCH PHOSPHATES[1]

ROBERT E. GRAMERA, JACOB HEEREMA, AND F. W. PARRISH[2]

ABSTRACT

Starch phosphate containing 0.016 phosphate ester groups per D-glucose unit was oxidized with periodate to the dialdehyde derivative, subsequently hydrogenated with sodium borohydride, and hydrolyzed. Two phosphorylated components isolated from the hydrolysate were identified as D-erythritol 4-phosphate and 2-*O*-(α-D-glucopyranosyl 2-phosphate)-D-erythritol. Another component, isolated in trace amount, is suggested to be D-glucose 3-phosphate. From the total amount of phosphorylated components isolated, the percent distribution of phosphate groups esterified in the C-2, C-3, and C-6 positions of the starch phosphate molecule was calculated to be 28, 9, and 63%, respectively. Phosphate ester groups chemically bound to the starch molecule exist in a dibasic structural form.

Starch phosphate, prepared by a simple and economical method (1,2), possesses desirable properties, particularly for use in foods. Specific examples which utilize starch phosphate are dessert compositions, baby foods, fruit pie fillings, salad dressings, and cream-style corn. The unique physical properties of low-D.S. starch phosphate and its suitability for food use prompted an investigation into the site of substitution and structural form of the bound phosphate ester groups. Information of the type developed here proved to be of major value in discussions with the Food and Drug Administration which has approved the use of such modified starches in foods (*Federal Register*, October 20, 1964).

A convenient technique developed by Smith and co-workers (3,4,5) for the chemical degradation of a polysaccharide was used in this work for degrading the starch phosphate molecule to its isolable constituents. Percent distribution of phosphate groups esterified with the C-2, C-3, and C-6 hydroxyls of the starch molecule was obtained from a quantitative estimation of the phosphorylated components isolated. The chemical structure of bound phosphate ester groups was deduced from results obtained by potentiometric titration of the free acid form of starch phosphate.

Materials and Methods

Starch Phosphate. The starting material used in this investigation

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[2]Present address: U.S. Army Natick Laboratories, Natick, Mass.

was a granular corn starch phosphate, prepared by heating starch with sodium tripolyphosphate at 150° (1,6). The bound phosphorus content of derivatized starch, determined by the molybdivanadophosphoric acid method (7), was 0.30%.

Reaction Sequence

Periodate Oxidation. Corn starch phosphate (115 g., I; see Fig. 1) was pasted in 4,000 ml. of water. After the paste had cooled at room temperature (27°C.), periodic acid (228 g.) was added. Immediately after the addition of oxidant, two aliquots (5 ml. each) of starch phosphate solution were withdrawn and treated with potassium iodide (2 g.) in 3*N* sulfuric acid (10 ml.). Liberated iodine was titrated with 0.1*N* thiosulfate solution to establish the zero time of oxidation. After the mixture was stirred at room temperature (27°C.) for 24 hr., two more aliquots (5 ml.) were withdrawn and treated as described above. Calculations from the amount of thiosulfate required corresponded to approximately 1 mole of periodate consumed per anhydro-D-glucose unit of the starch phosphate molecule. The insoluble dialdehyde starch phosphate (II) was filtered and washed free of periodate and iodate ions.

Hydrogenation. Dialdehyde starch phosphate (II), obtained in near quantitative yield (112 g.), was suspended in 3,000 ml. of ice water, to which was added sodium borohydride (25 g.). After the reaction mixture had been stirred in the cold for 16 hr., it was carefully neutralized with dilute hydrochloric acid to pH 6.0. The insoluble, hydrogenated dialdehyde starch phosphate (III) was filtered, washed with cold water, solubilized by acidification with cold 1*N* sulfuric acid, and then placed on a column of Amberlite IR-120 (H) resin to remove sodium ions.

Mild Hydrolysis. Reduced dialdehyde starch phosphate (III) was hydrolyzed at room temperature (27°C.) for 16 hr. in 0.1*N* sulfuric acid. Since the glycosidic linkages of reduced dialdehyde starch are extremely labile in acid (4,8), mild hydrolytic degradation occurred without significant loss of phosphate ester groups (9). After neutralization, the hydrolysate was fractionated on a (4.0 × 40 cm.) column of Amberlite IRA-400 (formate form) resin. The column was sequentially eluted with water and solutions of 0.2, 0.4, 0.6, 0.8, and 1*N* formic acid. At specific intervals collected fractions (40 ml.) were analyzed for total phosphorus (10) and chromatographically examined, and similar fractions were recombined. Fractions 18 to 24, 26 to 30, and 31 to 37 were evaporated to main fractions F-1 (2.020 g.), F-2 (0.178 g.), and F-3 (0.218 g.), respectively. Although F-1 contained most solids, F-2 contained 60% of total phosphorus.

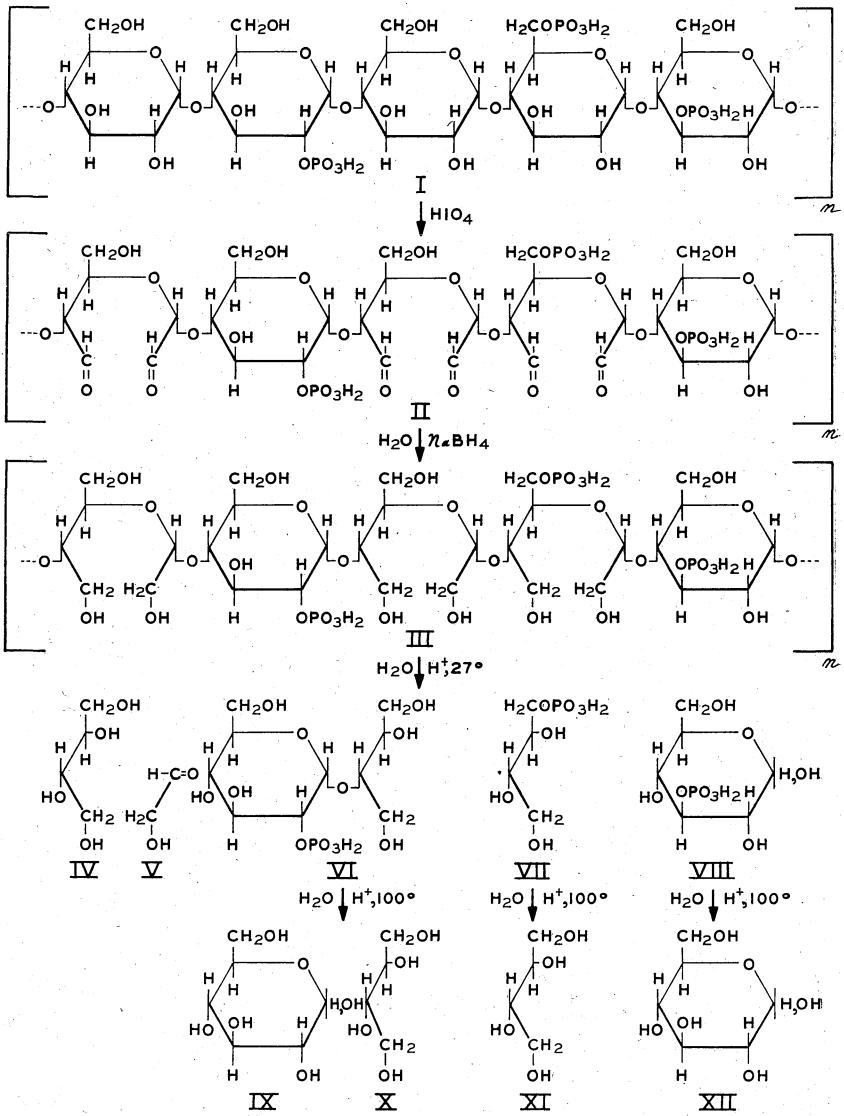

Fig. 1. Reaction sequence.

Analytical Methods. Chromatographic identification and quantitative estimation of sugar derivatives were made on Whatman No. 1 filter papers, developed in irrigants (A) ethyl acetate:pyridine:water (10:4:3 v./v.), (B) 1-butanol:1-propanol:water (6:9:5 v./v.), and (C) methanol: ammonium hydroxide:water (6:1:3 v./v.). Spray indicators employed

were (D) an acetone solution of silver nitrate, followed by an ethanolic sodium hydroxide solution (11) and (E) the Hanes-Isherwood reagent (9). A calibrated Fisher-Jones apparatus was used for melting-point determinations. Evaporations were done at reduced pressure.

Results and Discussion

Separation and Identification of Hydrolysis Products. Main fractions F-1, F-2, and F-3 were carbon-treated, filtered, evaporated to colorless syrups, and diluted to 5 ml. A portion of each fraction was chromatographed with irrigants A and B by the descending method. Spray reagent D revealed that erythritol (IV) and glycolaldehyde (V) were major neutral components in F-1 and F-2. Erythritol was isolated and crystallized; m.p. 120°, undepressed when admixed with an authentic sample. The major neutral component present in F-3, chromatographically identified as D-glucose, was attributed to the anhydro-D-glucose units (1.0–1.5%) which are not oxidized when starch is converted to the dialdehyde derivative with periodate (4,5,12). Other than glycolaldehyde, F-1 and F-2 did not contain any detectable free reducing substances. However, after portions of F-1 and F-2 were refluxed in boiling 1*N* sulfuric acid to completely hydrolyze the phosphate ester groups, small amounts of D-glucose (IX) and significant increases in the amounts of erythritol (X and XI) were observed chromatographically. Since F-2 contained the highest percentage of total phosphorus (60%) and after hydrolysis showed a significant increase in erythritol, it is suggested that the major phosphorylated component present in F-2 was D-erythritol 4-phosphate (VII).

Portions of main fractions F-1 and F-2 were chromatographed with irrigant C, and the phosphorylated components were detected with spray indicator E. Both fractions contained components with R_F values of 0.68 and 0.76, with F-1 containing more of the slower component (R_F 0.68) and F-2 containing more of the faster component (R_F 0.76). The faster component, which migrated at the same rate as D-erythritol 4-phosphate, was isolated and converted to dicyclohexylammonium-D-erythritol 4-phosphate; m.p. 189°. A mixed melting point of this compound with an authentic sample[3] remained undepressed. The slower-moving nonreducing component (R_F 0.68) present in F-1 and F-2 was isolated and concentrated to a syrup, but could not be induced to crystallize. A portion of this syrup was hydrolyzed in boiling 1*N* sulfuric acid and chromatographed with irrigant A. Detection of D-gluose (IX) and erythritol (X) suggested that the slower component was either 2-*O*-(α-D-glucopyranosyl 3-phosphate)-D-erythritol, or 2-*O*-

[3] Courtesy of C. E. Ballou, Dept. of Biochemistry, Univ. of California, Berkeley 4, Calif.

(α-D-glucopyranosyl 2-phosphate)-D-erythritol. The slower-moving component (R_F 0.68) consumed nearly 2 moles of periodate; this was consistent with a structure of the latter compound. Production of glycerol, without the formation of D-glucose, after subsequent hydrogenation and hydrolysis of the periodate-oxidized component, confirmed the structure to be 2-*O*-(α-D-glucopyranosyl 2-phosphate)-D-erythritol (VI).

When F-3 was chromatographed with irrigant C, only one phosphorylated component was present. This component, detected in trace amount, reduced Fehling's solution; and after hydrolytic dephosphorylation, D-glucose (XII) was chromatographically detected as the only neutral sugar. This evidence suggested that F-3 contained an intact D-glucose unit with a phosphate group esterified with the C-2 or C-3 hydroxyl group. The component present in F-3 had a specific rotation $[\alpha]_D^{25} + 37°$ (in water), close to a value reported (13) for known D-glucose 3-phosphate (VIII).

The negative inductive effect of an electron-withdrawing phosphate group at C-2 would probably tend to stabilize the glycosidic linkage in the 2-*O*-(α-D-glucopyranosyl 2-phosphate)-D-erythritol portion of reduced dialdehyde starch phosphate toward mild acid hydrolysis. If, on the other hand, the phosphate group were at C-3, the electron-withdrawing effect would no longer have a stabilizing influence on the glycosidic linkage in a 2-*O*-(α-D-glucopyranosyl 3-phosphate)-D-erythritol structure, and subsequent mild hydrolysis of reduced dialdehyde starch phosphate could conceivably give D-glucose 3-phosphate.

Percent Distribution of Phosphate Ester Groups. Aliquots of main fractions F-1, F-2, and F-3 (500 λ each) were chromatographed with irrigant A to separate the neutral components from the phosphorylated components. Phosphorylated components, all of which remained near the origin of the chromatograms, were eluted, hydrolyzed in 1*N* sulfuric acid, and diluted to 5 ml. Micro aliquots (75 γ) each of the dephosphorylated hydrolysates of F-1, F-2, and F-3 were chromatographed with irrigant A and the sugars eluted from their respective zones. D-Glucose (IX), found in all three hydrolysates, and erythritol (X and XI), detected only in hydrolysates of F-1 and F-2, were quantitatively estimated (14,15).

The D-glucose (IX) in hydrolysates of F-1 (197 γ) and F-2 (113 γ) was the hydrolysis product of 2-*O*-(α-D-glucopyranosyl 2-phosphate)-D-erythritol (VI). Erythritol (X and XI), also estimated in hydrolysates of F-1 (107 γ) and F-2 (579 γ), was the hydrolysis product of both 2-*O*-(α-D-glycopyranosyl 2-phosphate)-D-erythritol (VI) and D-erythritol 4-phosphate (VII). An amount of erythritol (210 γ), equivalent to the amount

of D-glucose (310 γ) previously estimated in hydrolysate of F-1 and F-2, was subtracted from the total erythritol (686 γ) found. Thus, the remaining erythritol (476 γ) was the hydrolysis product of D-erythritol 4-phosphate (VII). D-Glucose (IX, 310 γ) found in hydrolysates of F-1 and F-2, calculated to be 28% of the total D-glucose estimated in all three hydrolysates, represented the percentage of phosphate ester groups located on the C-2 position of starch phosphate. Similarly, D-glucose (XII, 100 γ) found in the hydrolysate of F-3 was 9% of total D-glucose found and represented the percentage of phosphate groups esterified on the C-3 position. The amount of erythritol (XI, 475 γ) resulting from the hydrolysis of D-erythritol 4-phosphate (VII) was equivalent to 702 γ of D-glucose. The weight equivalent of D-glucose (702 γ) calculated to be 63% of total D-glucose found, represented the percentage of phosphate ester groups on the C-6 position.

Structural Form of Phosphate Ester Groups. Twenty-gram samples of granular starch phosphate (sodium salt form) were converted to the free acid by Winkler's procedure (16) and potentiometrically titrated with 0.1*N* sodium hydroxide. The change in pH with respect to volume of titrant was plotted against total volume of alkali consumed to determine the equivalence points for the primary and secondary ionization states of the phosphate ester groups. The pH values (Fig. 2) corresponding to the equivalence points for the primary and secondary

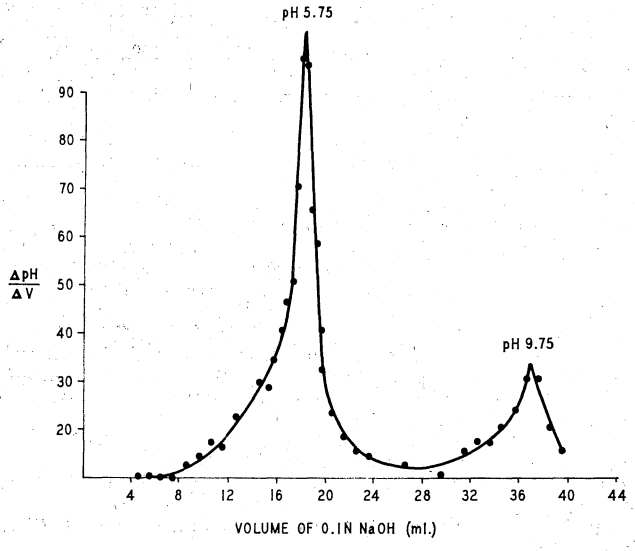

Fig. 2. Curve illustrating a potentiometric titration of a 20-g. sample of starch phosphate with 0.1*N* sodium hydroxide.

VOLUME 0.1N NaOH CONSUMED BY STARCH PHOSPHATE (20g.)	PRIMARY IONIZATION STATE	17.95 ml.
	SECONDARY IONIZATION STATE	18.30 ml.
RATIO OF SECONDARY TO PRIMARY IONIZATION STATES		1.02
PHOSPHORUS BY TITRATION OF SECONDARY IONIZATION STATE		0.28 %
PHOSPHORUS BY MOLYBDIVANADOPHOSPHORIC ACID METHOD		0.30 %

Fig. 3. See discussion in text.

ionization states were 5.75 and 9.75, respectively. Alkali consumption (Fig. 3) revealed that the ratio of secondary to primary ionization states was 1 to 1, indicating that phosphate ester groups were present in a dibasic structural form. Agreement in values for percent phosphorus determined by potentiometric titration of the second ionization state of starch phosphate, and by the molybdivanadophosphoric acid method (7), was further proof for a dibasic structural form of chemically bound phosphate ester groups (Fig. 3).

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