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The Use of [^{32}P] Polyphosphoric Acid* as a Phosphorylating Agent: Syntheses of Phosphorus-32 Labeled Sugar Phosphates**

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SUMMARY

Phosphorylation of D-fructose with [^{32}P]polyphosphoric acid followed by prolonged mild acid hydrolysis at 35° C gave [^{32}P]D-fructose 1, 6-diphosphate. The diphosphate was purified by conversion to the acid-barium salt. [^{32}P]D-fructose 6-phosphate was prepared by two methods : Degradation of the diphosphate by heating with 1N HBr for 1 hour at 88-90° C, or, replacing the prolonged mild acid hydrolysis of the phosphorylation mixture in the case of the diphosphate with a one hour treatment at 88-90° C. [^{32}P]D-glucose 6-phosphate was prepared by dissolving the barium salt of [^{32}P]D-fructose 6-phosphate in water and adding a dialyzed solution of phosphoglucose isomerase. The highly insoluble hepta-hydrate of the barium salt of [^{32}P]D-glucose 6-phosphate readily crystallized from solution. Phosphorylation of D-mannose with [^{32}P]polyphosphoric acid gave [^{32}P]D-mannose 6-phosphate, which upon reduction with KBH_4, yielded [^{32}P]D-mannitol 1-phosphate.

Syntheses of sugar phosphates have always been of importance due to their ubiquity, variety and utilization by living systems. The majority of available synthetic methods are difficult to adapt to large scale or repetitive

* Phosphorus-32 labeled polyphosphoric acid (^{32}PPA). All operations employing the nuclide are carried out in closed glass vessels or behind sufficient glass or transparent plastic shielding to hold the level of beta-radiation to normal background. All spent liquids and solids containing radioactivity are set aside until the level of radiation returns to background, then discarded.

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preparation of phosphorus-32 labeled esters because of the need to use selectively blocked precursors. MacDonald has made two contributions of some significance in this area. The first involves the reaction of anhydrous phosphoric acid with a fully acetylated aldo- or ketohexose to give the corresponding D-glycosyl phosphate [1], and D-fructosyl phosphate [2] respectively. This method has been used by Khan and Ebner to prepare phosphorus-32 labeled sugar phosphates [3]. In a more recent investigation Chatterjee and MacDonald demonstrated that the displacement of a primary sulfonyloxy group on otherwise acetylated sugars by diphenyl phosphate ion gave the corresponding sugar phosphate [4]. Rabinowitz *et al.* [5] have successfully phosphorylated nucleosides using either phosphoric acid or its monobasic salts in spite of the fact that inorganic orthophosphates are not known to be effective phosphorylating agents. They attribute their results to the partial thermal transformation of orthophosphates to condensed phosphates, with the latter serving as the effective phosphorylating agents. With but few exceptions [6, 7] the use of phosphorus-32 labeled polyphosphoric acid (^{32}PPA) as a phosphorylating agent seems not to have been previously exploited. In this communication we report the synthesis of phosphorus-32 labeled mannose and fructose phosphates by an adaptation of the Seegmiller and Horecker procedure [8] whereby primary phosphate esters of sugars can be prepared by indiscriminately phosphorylating the free sugar with labeled polyphosphoric acid followed by partial hydrolysis of the resultant polyphosphorylated material to cleave secondary, hemiacetal and polyphosphates. This approach has the advantage of simplicity, requires no appropriately blocked starting material and yields labeled sugar phosphates of high purity and specific activity. The level of radioactivity desired in the final product is conveniently manipulated by adjusting the mCi content of the orthophosphoric acid in the ^{32}PPA preparation. Since condensed polyphosphate is the effective phosphorylating agent, the method described below ought to be applicable to the preparation of phosphorus-32 labeled glycosyl phosphates when appropriately blocked precursors are used as starting material.

EXPERIMENTAL SECTION.

Thin layer chromatography, carried out on the free acids of the sugar phosphates, prepared by shaking a water solution of the barium salt with Dowex-50 W (Sigma Chemical Company, St. Louis, Mo.) in the hydrogen form, was performed on Eastman Chromagram cellulose sheets (No. 6 064). The solvent systems used were : (*a*) methanol-formic acid-water (8 : 1.5 : 0.5); (*b*) methanol-ammonium hydroxide-water (6 : 1 : 3). Spots were detected by radiochromatography or visually with molybdenum blue [9] or naphthoresorcinol [10]. Specific activities were determined by liquid scintillation counting, using a counting mixture consisting of : Spectrafluor (Amersham/Searle, Des Plaines, Illinois) in toluene (14.0 ml), Triton X-100 (Rohm

and Haas, Philadelphia, Pa., 4.0 ml) and aqueous phase plus sample (1.0 ml).

Phosphorus-32 labeled polyphosphoric acid. — Between 5 and 10 mCi of phosphorus-32 labeled orthophosphoric acid (Cambridge Nuclear Corp., Cambridge, Mass.), accurately measured, was added to 15 ml of 85 % orthophosphoric acid. The orthophosphate was transferred with a 25 ml syringe and mixed into 30 g of reagent grade phosphorus pentoxide (P_2O_5), kept chilled during the mixing process. After the bulk of the P_2O_5 had dissolved, the mixture was heated at 150 ° to 180 °C for two hours with stirring. A thick straw colored solution was obtained.

[^{32}P]D-Mannose 6-Phosphate. — An entire quantity of ^{32}PPA, prepared as described, was cooled in an ice bath to 5-10 °C, then 3.3 ml of deionized water and 5 g of D-mannose was added. After stirring at room temperature for 16 hours the reaction was terminated by the addition of 100 ml of water. The reaction mixture was further diluted by the addition of 250 ml of water and neutralized to pH 7.0 at the glass electrode by the addition of solid sodium carbonate. The removal of CO_2 was hastened by evacuating the mixture until the foaming ceased. The dense slurry of sodium polyphosphate crystals, kept at 4 °C overnight, was cooled in an ice bath for eight hours, then filtered with suction. The filtrate (220 ml) was treated with 48.5 ml of concentrated (48 %) HBr, refluxed for 16 hrs, then cooled to 25 °C. The brown hydrolysis mixture was treated at 25 °C with 45 g of barium carbonate with stirring for 4 hrs (during which time the pH rose to 6.5) then filtered rapidly with suction. The filtrate was washed in the funnel with 2-25 ml portions of water. The filtrate and washings, which contained the barium salt of [^{32}P]D-mannose 6-phosphate, were combined, treated with 4 volumes of ethanol, and the flocculent precipitate was allowed to settle. After 48 hr the supernatant was siphoned off and the precipitate was centrifuged at 25 °C for 15 min at 2 000 × gravity. The packed solid was washed with 70 ml of ethanol and recentrifuged. The pellet was extracted three times with 80 ml portions of water and the combined extract, decolorized for 2 hr at room temperature with 0.75 g of activitated charcoal (Darco G), was filtered, then added in a thin stream with stirring to 4 volumes of ethanol. The flocculent precipitate was allowed to settle, then washed with 100 ml of absolute ethanol and 100 ml of ether. The air dried product weighed 1.2 g. The phenylhydrazine salts of the phenylhydrazone and phenylosazone were prepared [11] and recrystallized from alcohol. The melting points, 144-145 °C and 154-155 °C respectively, and mixed melting points were identical with authentic samples and literature values.

R_f values were identical with a repurified commercial sample, 0.60 in the basic system and 0.75 in the acidic system. Radiochromatography indicated the presence of one spot with no contamination by inorganic phosphate.

Specific activity was 10.7 mCi·mole^{-1}. [^{32}P]D-mannitol 1-phosphate, the reduction product of this sugar phosphate, prepared as described by Wolfe and Kaplan [12], showed pronounced substrate activity when added to a soluble fraction containing mannitol 1-phosphate dehydrogenase, prepared from Absidia glauca [13] (American Type Culture Collection No. 7 852*a*). D-Mannose 6-phosphate showed no enzymic response as substrate with this soluble fraction.

[^{32}*P*]*D-Fructose 1,6-Diphosphate.* — D-fructose (5.0 g) was added to a previously cooled (5-10 °C) quantity of ^{32}PPA prepared as described and containing 3.3 ml of deionized water. After stirring for 16 hr at room temperature the reaction mixture was worked up as described for the mannose phosphorylation. The final filtrate (180 ml) was treated with 25 ml of 48 % HBr and the solution incubated at constant temperature (35 °C) for 5 days. The hydrolysis mixture was brought to a pH of 5.0 with solid $BaCO_3$, then made slightly alkaline by the addition of a fine suspension of $Ba(OH)_2 \cdot 8\ H_2O$. The insoluble residue was filtered off and the clear solution was poured into 4 volumes of ethanol. The precipitate was allowed to settle for 2 hours, collected by centrifugation and washed with 70 ml each of ethanol and ether. The air-dried material (2.5 g) consisted only of inorganic phosphate, fructose 6-phosphate, and fructose 1,6-diphosphate as determined by thin layer radiochromatography. [^{32}P]D-fructose 1,6-diphosphate was isolated by precipitation as the acid-barium salt as follows [14] : To the 2.5 g was added 15 g of ice and 16 ml of 0.44 N HBr. The clear solution was added to 200 ml of ethanol at 3 °C and quickly filtered. The solid, dissolved in a minimum amount of water, was reprecipitated by pouring it into 4 volumes of ethanol, collected by centrifugation and washed with ethanol and ether. The air-dried product weighed 830 mg. (The acid-barium salt is water soluble and from its solution the tetrabasic barium salt can be precipitated by adding a solution of $Ba(OH)_2 \cdot 8\ H_2O$ until the solution is neutral.) Thin layer chromatography showed it to be pure and free of inorganic phosphate. R_f values, 0.55 in the basic solvent, 0.72 in the acid solvent, were identical with known material. The infrared spectrum of a Nujol mull of the acid-barium salt showed characteristic bands at 940 cm^{-1}, 1 070 cm^{-1}, and 1 170 cm^{-1}, whereas the dibarium salt gave a sharp band at 980 cm^{-1} and a broad band at 1 100 cm^{-1} [15]. Specific activity, corrected to the date of synthesis, was 7.5 mCi·mole^{-1}. Product identity was confirmed and enzymic synthesis of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate demonstrated by incubating the material with crystalline aldolase (Sigma Chem. Co., St. Louis, Mo.).

[^{32}*P*]*D-Fructose 6-Phosphate.* — This material was prepared by a procedure similar to that for the diphosphate except the prolonged mild acid hydrolysis of the phosphorylation mixture was replaced by treatment with HBr to give a 1 N solution, then heating this solution at 88-90 °C for 1 hr [16]. Solid $BaCO_3$

was added to a pH of 4.0 after cooling to 25 °C, followed by a fine suspension of $Ba(OH)_2 \cdot 8\ H_2O$ until a weakly alkaline reaction to phenolphthalein was obtained. The solution was bubbled with CO_2 for 0.5 min, filtered and added in a thin stream to 4 volumes of ethanol. The precipitate, collected by centrifugation and washed with ethanol, consisted of fructose 6-phosphate and inorganic phosphate as determined by thin layer radiochromatography. This crude material was extracted with three 70 ml portions of water and the combined extracts stirred with 0.75 g Darco G for 1 hr, filtered and added in a thin stream to 4 volumes of ethanol. The crystalline precipitate was collected by centrifugation and washed with ethanol and ether. The air dried product weighed 800 mg. It gave a positive naphthoresorcinol test with R_f values of 0.45 in the basic solvent system, 0.68 in the acidic system, identical with a repurified commercial sample of unlabeled D-fructose 6-phosphate. Specific activity was 3.75 $mCi \cdot mole^{-1}$ corrected to the date of synthesis.

Alternately [^{32}P]*D-fructose 6-phosphate* was prepared by dissolving [^{32}P]D-fructose 1,6-diphosphate in 1 N HBr and heating at 88-90° C for 1 hr, then working up the material by the same procedure as described above [17].

[^{32}P]*D-Glucose 6-Phosphate.* — The barium salt of [^{32}P]D-fructose 6-phosphate, prepared by either of the two methods described, was dissolved in water and treated with a dialyzed solution of phosphohexose isomerase (Grade III, Sigma Chemical Co.). The solution was seeded, scratched and crystallization of the highly insoluble heptahydrate of the barium salt of [^{32}P]D-glucose 6-phosphate commenced within a few minutes. Crystallization was complete after 18 hrs. The crystalline deposit was collected by centrifugation and washed with water and ethanol. Thin layer chromatography gave results identical with those obtained with an unlabeled repurified commercial sample.

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17. Protocol identical with that described, but eliminating the phosphorus-32 labeled orthophosphoric acid from the preparation of polyphosphoric acid, will give the corresponding unlabeled sugar phosphates.