[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF OREGON]

The Synthesis of Derivatives of Glucose-4-phosphoric Acid[1]

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During the course of an investigation of biochemical mechanisms forming glucosidic linkages it became desirable to secure glucose-4-phosphoric acid for use as a substrate. In our hands Raymond's[3] synthesis resulted in very poor yields. The synthesis described by the present authors utilizes a novel rearrangement first described by Helferich and Klein[4] and the convenient phosphorylating agent diphenylchlorophosphonate.[5]

Experimental

1,2,3,6-Tetraacetyl-β-D-glucopyranose.—Pure β-D-glucose-1,2,3,4-tetraacetate[6] was prepared utilizing the crystallization procedure of Lardy and Fischer.[7] In accordance with the method of Helferich and Klein[4] 11.2 g. of the above compound was dissolved in 110 ml. of 95% ethanol, the specific rotation of the solution being $[\alpha]^{25}D$ +4.49°. The addition of 2.8 ml. of 0.1 *M* aqueous potassium hydroxide catalyzed a change in rotation in one minute to $[\alpha]^{25}D$ −1.03°. The alcohol solution was acidified with 0.5 *N* acetic acid, concentrated to one-half the original volume, seeded and cooled.[8] The crystal crop, filtered cold, was recrystallized from ether containing 10% pyridine and then from 95% ethanol. This procedure yielded 3.9 g. of pyridine-free 1,2,3,6-tetraacetyl-β-D-glucose, m. p. 131.5–132.5°; $[\alpha]^{22}D$ −28.6° (chloroform).[9]

1,2,3,6-Tetraacetyl-4-diphenylphosphono-β-D-glucose.—A solution of 2.0 g. of purified 1,2,3,6-tetraacetyl-β-D-glucose in 25 ml. of pure dry pyridine was warmed to 35° until clear and then cooled to 10°. To it was added 1.7 g. (1.33 ml.) of pure diphenylchlorophosphonate.[5] If turbidity developed during the first half hour after addition of the reagent the solution was warmed until it became clear, and again cooled. After a half hour reaction time the temperature of the reaction mixture was allowed to rise to 20° and was maintained at that figure for about twenty-four hours. Any solid material which had deposited was redissolved by the addition of a few drops of water and the whole was poured, with vigorous agitation, into 170 ml. of ice water. The white granular precipitate thus produced was dissolved in 10 ml. of chloroform, washed with dilute hydrochloric acid and water, and dried over anhydrous sodium sulfate. When the chloroform solution was evaporated *in vacuo* there was obtained a flaky white product soluble in acetone, alcohol and benzene, and insoluble in water or petroleum ether. Recrystallization from hot alcohol or aqueous acetone yielded 1.5 g. of silky needles, m. p. 146.5–147.5°; $[\alpha]^{25}D$ −34.4° (chloroform).

Anal. Calcd. for $C_{26}H_{29}O_{13}P$: C, 53.80; H, 5.04; P, 5.34; 4 acetyl,[10] 6.47. Found: C, 53.77, 54.00; H, 5.27, 5.23; P,[11] 5.34; acetyl, 6.35, 6.40.

1,2,3,6-Tetraacetyl-4-phosphono-β-D-glucose.—An 850-mg. sample of pure 1,2,3,6-tetraacetyl-4-diphenylphosphono-β-D-glucose was dissolved in 15–20 ml. of absolute ethyl acetate, 0.1 g. of Adams catalyst added, and hydrogenated at a pressure of 2–3 cm. After two and one-half hours reaction ceased; hydrogen used, 270 ml.; calcd., 262 ml. (8 moles). The catalyst was removed by filtration and the solution evaporated to a volume of about 5 ml. Cooling to −10° caused the deposition of 550 mg. of a crystalline compound, m. p. 168°; $[\alpha]^{20}D$ −0.42° (*c*, 1.2, H_2O).

Anal. Calcd. for $C_{14}H_{21}O_{13}P$: C, 39.2; H, 4.90; P, 7.24. Found: C, 39.04, 39.16; H, 4.81, 4.93; P, 7.26.

Di-sodium Salt of Glucose-4-phosphoric Acid.—Pure crystalline 1,2,3,6-tetraacetyl-4-phosphono-β-D-glucose was neutralized (phenolphthalein end-point) with aqueous sodium hydroxide. Water was removed *in vacuo*, the residue was dissolved in pure anhydrous methanol, and a catalytic amount of potassium methylate in methanol was added. The deacetylated di-sodium salt began to precipitate at once. After twelve hours at 0° the precipitate was centrifuged down, washed with anhydrous methanol and with ether and dried *in vacuo*. A white hygroscopic solid was obtained which decomposed at about 155° and reduced alkaline copper solutions; $[\alpha]^{20}D$ +51.5° (*c*, 2.1, H_2O).[12]

Anal. Calcd. for $C_6H_{11}O_9PNa_2$: P, 10.2. Found: P, 10.2.

1.00 ml. of an aqueous solution containing 20.7 mg. of the disodium salt was added to 1.00 ml. of 2 *N* hydrochloric acid and the mixture heated at 100° for three hours. At the beginning 0.0325 mg. of inorganic phosphorus was present. During hydrolysis an additional 0.262 mg. of inorganic phosphorus was produced. This increase corresponds to 12% hydrolysis.

Dibrucine Salt of Glucose-4-phosphoric Acid.—Pure crystalline 1,2,3,6-tetraacetyl-4-phosphono-β-D-glucose was dissolved in pure dry methanol. To this solution 0.4 *N* barium methylate in methanol was added until a drop of the solution, when added to water, turned phenolphthalein a deep pink. A voluminous white precipitate of the barium salt appeared. After standing twelve hours at 0° the mixture was again tested for excess barium methylate. If an excess was still present the barium salt was centrifuged down, washed with dry methanol and with ether and dried *in vacuo* briefly.

The barium salt of glucose-4-phosphoric acid thus obtained was dissolved in a minimum amount of water and the barium precipitated as barium sulfate by adding the proper amount of sulfuric acid. The resulting solution of glucose-4-phosphoric acid was neutralized (brom thymol blue) with a solution of brucine in methanol. After concentration *in vacuo* to about one-third of the original volume, an amount of acetone equal to the final volume was added, and the mixture cooled to −10°. Crystals slowly appeared forming clusters of rosettes. Several recrystallizations from 50% acetone–water followed by thorough drying *in vacuo* yielded a product which reduced alkaline copper solutions, m. p. 173–

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(3) A. L. Raymond, *J. Biol. Chem.*, **113**, 375 (1936).

(4) Helferich and Klein, *Ann.*, **450**, 219 (1926).

(5) Brigl and Müller, *Ber.*, **72**, 2123 (1939).

(6) "Organic Syntheses," **22**, 56 (1942).

(7) Lardy and Fischer, *J. Biol. Chem.*, **164**, 515 (1946).

(8) Material for seeding was obtained in the original preparation by concentrating the alcohol solution to a thick sirup and adding a small amount of pure dry pyridine. Crystallization occurred immediately.

(9) Helferich and Muller, *Ber.*, **63**, 2142 (1930).

(10) E. P. Clark, "Semimicro Quantitative Organic Analysis," Academic Press, New York, N. Y., 1934, p. 74.

(11) Fiske and SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(12) The concentration of salt was calculated from phosphorus analyses rather than actual weight per ml. Analyses indicated the purity of the sample to be 98.5%.

174°; $[\alpha]^{20}D$ −43.3° (*c*, 1.7, pyridine), $[\alpha]^{20}D$ −16.1° (*c*, 2.1, 20% ethanol).[13]

Anal. Calcd. for $C_{52}H_{65}O_{17}N_4P$: P, 2.96. Found: P, 2.75.

Discussion

In an effort to prove that the phosphate group was actually attached to carbon number four of the glucose, Raymond[3] compared the osazones and the rate of glucoside formation of his product with those of glucose-3- and glucose-6-phosphoric acids and found marked differences.

Steric considerations point to interaction between groups on carbons four and six as the most likely to be involved in rearrangement. The following data, when compared with values for analogous compounds to be found in the present paper, indicate that there has not been a shift of the phosphate group to carbon number six. Lardy and Fischer[7] report 1,2,3,4-tetraacetyl-6-diphenylphosphono-β-D-glucopyranose, m. p. 64–66°, $[\alpha]^{22}D$ 16.5° (*c*, 1.37, pyridine) and 1,2,3,4-tetraacetyl-β-D-glucose-6-phosphoric acid, m. p. 126–128° $[\alpha]^{25}D$ 18.7° (*c*, 1, pyridine). Robison and King[14] report the dibrucine salt of glucose-6-phosphoric acid, $[\alpha]^{20}D$ 20.6° (*c*, 0.84, water).

Both physical properties and presence of reducing power rule out a transfer of the phosphate group to carbon number one. Presumably the pyranose ring would remain intact in such a synthesis as the one presented and it would seem unlikely that carbon number five would be involved. However, until such time as glucose-5-phosphate can be studied this possibility cannot be ruled out and the nomenclature in this paper avoids specifying ring structure.

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Summary

Several derivatives of glucose-4-phosphoric acid have been obtained by an improved synthesis.

The properties of two new compounds have been reported.

(13) It should be noted that the rotation in pyridine is in good agreement with that reported by Raymond[3] but the rotation in alcohol is not. The value given here is that exhibited by several samples. For comparison, Raymond's values were −45.3 and −9.8°, respectively.

(14) R. Robison and E. J. King, *Biochem. J.*, **25**, 323 (1931).

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Phosvitin, the Principal Phosphoprotein of Egg Yolk

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A phosphoprotein preparation containing 10% phosphorus has been isolated from egg yolk in yield sufficient to account for at least 60% of the total protein phosphorus in yolk. The proposed name *"phosvitin"* indicates both its high phosphorus content and its source in the egg yolk. Details of the isolation procedures and the results of various chemical and physical studies will be described in this paper.

A number of investigators have separated polypeptides rich in phosphorus following enzyme digestion of both vitellin and casein.[2–13] The presence of such peptides was attributed to the occurrence of groupings in these proteins resistant to digestion. In the case of vitellin at least it now appears that such polypeptides were formed from a phosphoprotein of as high phosphorus content as any of the polypeptides isolated, and that most of the phosphorus was present in the form of the phosphoprotein to be described.

Isolation

Phosphoprotein fractions containing 7% or more phosphorus[14] were first obtained by the following procedure. Fresh liquid egg yolk was extracted with chloroform to remove lipids. The residual suspension was then washed with water to remove the "livetin" fractions, and finally extracted with 10% sodium chloride in the presence of chloroform to obtain the phosphoprotein. Salt was removed by dialysis. Apparently most of the other yolk protein components were insolubilized

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Miescher, *Medizinische-Chemische Untersuchungen*, **4**, 502 (1870), cited in Jukes and Kay.[40]

(3) Bunge, *Z. physiol. Chem.*, **9**, 49 (1885).

(4) Hugounenq and Morel, *Compt. rend. acad. sci.*, **140**, 1065 (1905).

(5) Posternak, *ibid.*, **184**, 306 (1927).

(6) Posternak and Posternak, *ibid.*, **184**, 909 (1927).

(7) Rimington, *Biochem. J.*, **21**, 1179 (1927).

(8) Damodaran and Ramachandran, *ibid.*, **35**, 122 (1941).

(9) Lowndes, Macara and Plimmer, *ibid.*, **35**, 315 (1941).

(10) Rimington, *ibid.*, **35**, 321 (1941).

(11) Posternak and Pollaczek, *Helv. Chim. Acta*, **24**, 1190 (1941).

(12) Nicolet and Shinn, Abstracts, 110th Meeting, American Chemical Society, September, 1946.

(13) Mellander, *Upsala Läkareforenings Förhandlingar*, **52**, 107 (1947).

(14) Unless otherwise stated, phosphorus analyses are in terms of non-lipid phosphorus not removable by dialysis. Since Plimmer (*J. Physiol.*, **38**, 247 (1909)) cited in Needham[39]) and Schmidt and Thannhauser (*J. Biol. Chem.*, **161**, 83 (1945)) found in yolk considerably less nucleic acid phosphorus than phosphoprotein phosphorus (the latter report 11 mg. and 116 mg., respectively, per 100 g. yolk), no attempt was made to distinguish between the two in most yolk fractions. As described in the text, there is no detectable nucleic acid in phosvitin.