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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Received March 25, 1963

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

(1)(a) Supported by grants A-2903 (C-3) and A-4315 (C-2) from the U. S. Public Health Service, Bethesda, Md.; (b) Taken in part from a dissertation to be submitted by T. Y. Kim in partial fulfillment of the requirements for the Ph.D. degree from Duke University.

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satisfactory analyses and was adequate for conversion to the next intermediate; yield, 73%; m.p. 161°.

Anal. Calcd. for $C_{14}H_{19}O_6N$: C, 56.56; H, 6.40; N, 4.71. Found: C, 56.45; H, 6.53; N, 4.54.

N-(*p*-Methoxybenzylidene)-tetra-O-acetyl-2-amino-2-deoxy-β-D-galactopyranose (II).—Eight grams of I was mixed in an ice bath with 27 ml. of ice-cold acetic anhydride and 48 ml. of anhydrous pyridine. The mixture was allowed to stand in ice with occasional shaking for 3 hr. and then for an additional 24 hr. at room temperature. The resulting solution was poured onto crushed ice and the tetraacetyl derivative allowed to crystallize for approximately 30 min. The crude crystals were washed with ice–water, recrystallized from methanol, and dried over phosphorus pentoxide *in vacuo;* yield, 75%; m.p., 187°; $[\alpha]^{25}_{578}$ +108.5 (*c* 1, chloroform).

Anal. Calcd. for $C_{22}H_{27}O_{10}$: C, 56.53; H, 5.78; N, 3.00. Found: C, 56.84; H, 6.04; N, 2.98.

2-Aminotetra-O-acetyl-2-deoxy-β-D-galactopyranose (III).—Eight grams of II was dissolved in 400 ml. of acetone and the solution heated in water bath until boiling. Exactly 3.5 ml. of 5 *N* hydrochloric acid was added, resulting in the immediate formation of a white crystalline precipitate. The solution was refluxed for 15 min., cooled to below room temperature, and 500 ml. of ether added. After standing overnight at 4°, the precipitate was harvested, recrystallized from methanol, and dried over phosphorus pentoxide. The yield was essentially quantitative; m.p. 205° dec.; $[\alpha]^{25}_{578}$ +34.7 (*c* 1.5, water).

Anal. Calcd. for $C_{14}H_{22}O_9NCl$: C, 43.80; H, 5.74; N, 3.65; Found: C, 43.75; H, 5.96; N, 3.59.

2-Acetamidotetra-O-acetyl-2-deoxy-β-D-galactopyranose (IV).—Five grams of III was mixed with 700 ml. of chloroform, 1.8 g. of anhydrous sodium acetate, and 27 ml. of water.[7] After stirring until a homogeneous mixture was obtained, 20 ml. of acetic anhydride was added and the solution stirred for an additional 25 min. The chloroform layer was separated and dried over anhydrous magnesium sulfate. Solvent was removed *in vacuo;* crystalline material was obtained with heptane and recrystallized from ethanol–chloroform; yield, 90%; m.p. 240°; $[\alpha]^{25}_{578}$ +2.6 (*c* 1, chloroform). Stacey reports this compound as melting at 235°; $[\alpha]^{20}_{D}$ +7.[8]

Anal. Calcd. for $C_{16}H_{23}O_{10}N$: C, 49.36; H, 5.91; N, 3.59. Found: C, 49.25; H, 5.98; N, 3.43.

Some crystalline material may appear at the chloroform–water interface and is also the β-pentaacetate.

2-Acetamido-2-deoxy-D-galactopyranose-α 1-Phosphate.—Phosphorylation of the fully acetylated amino sugar was carried out according to the procedure of MacDonald.[3] After removal of lithium phosphate, the resulting solution was passed through a 2.5 × 40 cm. column of Dowex 1 × 8 200–400-mesh chloride form resin and the column washed extensively with water until no additional acetyl hexosamine was present in the effluent. The column was then eluted with a lithium chloride gradient consisting of 0.01 *M* of chloride, 0.003 *M* hydrochloric acid in a 1-l. mixing vessel and 0.2 *M* lithium chloride in the same concentration of hydrochloric acid in the reservoir. Column fractions were analyzed for acid-labile acetyl hexosamine,[9] positive fractions combined and adjusted to pH 8 with lithium hydroxide. Solvent was removed *in vacuo* and the lithium salt of the acetyl hexosamine phosphate obtained by precipitation with methanol–acetone (1:9) and washing with the same solvent until the wash was chloride-free; yield, 20–35%.

Anal. Calcd. for $C_8H_{14}O_9NPLi_2 \cdot 2H_2O$: C, 27.51; H, 5.16; N, 4.10; P, 8.8. Found for the galactosamine analog: C, 27.29; H, 5.25; N, 3.88; P, 8.4; $[\alpha]^{25}_{578}$ +197 (*c* 0.7, water); reducing sugar and acetylhexosamine negative; ratio of acid labile acetyl hexosamine to acid-labile phosphorus was 0.89:1. Found for the glucosamine analog: C, 27.06; H, 5.01; N, 3.81; P, 8.6; $[\alpha]^{25}_{578}$ +144 (*c* 0.88, water); reducing sugar and acetyl hexosamine negative; ratio of acid-labile acetyl hexosamine to acid-labile phosphorus was 0.82:1.

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Equilibrium Constants for the Demetalation of Iron Porphyrins

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Received February 25, 1963

Conditions for the equilibration of magnesium with porphyrins and chlorins have been determined by Corwin and Wei.[1] Corwin and Bruck[2] reported that mesoheme appeared to equilibrate with ferrous chloride and mesoporphyrin in a solution containing acetic acid, a little water, and some salt. This led us to search for conditions suitable for measuring the equilibrium constants of metalation–demetalation reactions of porphyrins and hemins.

Method.—The method of study adopted was spectrophotometric. It was first learned that mesohemin and meosporphyrin acetate could be distinguished easily by this method, since the hemin has a strong absorption at 632 mμ and the porphyrin salt at 594 mμ. Using this method, the following preliminary observations were made in an attempt to arrive at suitable conditions for measurement of the equilibrium.

Preliminary Observations on Mesohemin.—(1) Mesohemin in glacial acetic acid was treated with mercury and heated at 114°. No porphyrin formation occurred.

(2) The solution was diluted with water sufficient to make fifty mole per cent. The mixture was heated at 100° for two hours. No porphyrin formation was observed.

(3) A solution was prepared with 2.595 mg. of mesohemin in 25 ml. of glacial acetic acid. A 10-ml. aliquot of this was diluted with 3.3 ml. of water, and the solution was saturated with sodium chloride. It was then treated with metallic mercury and heated to 105° for ninety minutes. It was estimated that 50% of the hemin was converted to porphyrin. This experiment established the need for halide ion in the equilibrium mixture.

(4) Mesohemin (ferric) in fifty mole per cent aqueous acetic acid did not equilibrate to porphyrin even in the presence of sodium chloride.[3] The presence of a reducing agent (for example, mercury) was essential. In this dilute aqueous solution, the reducing agent did not change the spectrum but did permit the equilibration to proceed. This spectral stability indicates that no appreciable quantity of ferrous porphyrin is formed.

(5) The solubility of sodium chloride in glacial acetic acid is so low that even in the presence of sodium chloride and mercury and at elevated temperature, equilibration under these conditions was too slow to be measured conveniently.

(6) The concentration of chloride ion is critical for the position of the equilibrium as well as for the possibility of obtaining equilibriu n.

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