174. *The Synthesis of Glucosamine 6-(Dihydrogen Phosphate).*

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A crystalline monohydrate of glucosamine 6-(dihydrogen phosphate) has been synthesised from glucosamine *via* 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine. All the intermediate derivatives prepared were crystalline and were characterised. Periodate oxidation confirmed the structure of the 6-phosphate.

Brown [1] records the phosphorylation of glucosamine by hexokinase and the isolation of a small quantity of glucosamine 6-phosphate. He considers that the process may be important in the series of anabolic stages whereby naturally occurring polysaccharides containing glucosamine residues are produced. More recently Leloir and Cardini [2] isolated enzymes from the mould, *Neurospora crassa*, which catalyse the change of hexose 6-phosphate into glucosamine 6-phosphate in the presence of glutamine and of acetylglucosamine 1-phosphate into the corresponding 6-phosphate. This led Kalckar and Klenow [3] to suggest that " the formation of glucosamine 6-phosphate should also be considered as a pathway in the biosynthesis of purine ribosides, the more so since the nitrogen of the *N*-ribosidic linkage in purine ribosides is derived specifically from the amide nitrogen of glutamine." In view of the wide interest in this derivative of glucosamine it appeared to us that an authentic synthesis, whereby supplies of this derivative might be available for more extensive enzymic studies, was desirable.

Direct phosphorylation of glucosamine led to an impure product.[4] In order to prepare glucosamine 6-phosphate from glucosamine it is necessary first to protect the secondary hydroxyl groups and the amino-group with other residues. Preliminary experiments were carried out on crystalline *N*-*p*-methoxybenzylidene-D-glucosamine. It was hoped to prepare 1 : 3 : 4-tri-*O*-acetyl-*N*-*p*-methoxybenzylidene-6-*O*-triphenylmethyl-D-glucosamine and, after removal of the triphenylmethyl residue, to phosphorylate the product. Unfortunately attempts to prepare crystalline derivatives were unsuccessful and these experiments were abandoned. The conditions necessary for the hydrolysis of *O*-acetyl and *N*-acetyl groups in glucosamine were studied, and, as these did not appear to be likely

[1] Brown, *Biochim. Biophys. Acta*, 1951, **7**, 487.
[2] Leloir and Cardini, *Biochim. Biophys. Acta*, 1953, **12**, 15.
[3] Kalckar and Klenow, *Ann. Review Biochem.*, 1954, **23**, 574.
[4] Anderson and Percival, *Chem. and Ind.*, 1954, 1018.

to cause the removal of a 6-phosphate group, a synthesis was begun with 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-6-*O*-triphenylmethyl-D-glucosamine as intermediate.

Crystalline *N*-acetyl-D-glucosamine was prepared, and after tritylation and acetylation 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-6-*O*-triphenylmethyl-β-D-glucosamine with carbon tetrachloride of crystallisation was isolated in 25% yield. From the mother-liquors 5·5% of the α-anomer with mixed solvent of crystallisation was obtained. Further treatment of the mother-liquors gave 2% of 3 : 4-di-*O*-acetyl-*N*-acetyl-1 : 6-di-*O*-triphenylmethyl-D-glucosamine. The phenomenon of solvent of crystallisation is seldom encountered in non-ionic sugar derivatives. Triphenylmethyl ethers, however, appear to be more liable than most derivatives to behave in this way. For example, the preparation of an ethanol solvate of triphenylmethyldulcitol has been reported [5] and Edington, Hirst, and Percival [6] isolated methyl tri-*O*-benzoyl-6-*O*-triphenylmethyl-α-mannopyranoside with various solvents of crystallisation.

When acetic acid at 100° was used to remove the triphenylmethyl residues from the tetra-acetates [7] hydrolysis of the 1-acetyl group also occurred, but by treatment of the β-acetate with a cold saturated solution of hydrogen bromide in glacial acetic acid for not longer than 60 seconds crystalline 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine was obtained in 67% yield. This product could not be isolated by the method used for 1 : 2 : 3 : 4-tetra-acetyl-β-D-glucose.[8] The three times greater solubility of the tetra-acetyl-β-D-glucosamine in water than in chloroform made it necessary to employ exhaustive chloroform extraction and to eliminate the usual water washing of the extract.

In view of the larger yield of the β-anomer of the triphenylmethyl ether, and the fact that all attempts to isolate crystalline 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-α-D-glucosamine were unsuccessful the synthesis was continued in the β-series. As the possibility of acetyl migration [9] during the removal of the triphenylmethyl group could not be ignored the structure of the 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine was confirmed by reconversion into the 6-triphenylmethyl ether, and by toluene-*p*-sulphonylation followed by the replacement of the toluene-*p*-sulphonyl group by iodine under the standard conditions for the replacement of a primary toluene-*p*-sulphonyloxy-group.[10]

Phosphorylation of the tetra-acetyl-β-D-glucosamine was carried out with diphenyl phosphorochloridate and crystalline 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine 6-(diphenyl phosphate) was isolated in 70% yield. Hydrogenolysis of the phenyl groups gave a good yield of the crystalline 6-(dihydrogen phosphate). The acetyl groups were hydrolysed with sulphuric acid and a barium salt was precipitated by ethanol after neutralisation of the hydrolysate with barium carbonate. The difficulty of converting all the phosphate into the barium salt combined with that of removing all the inorganic barium salts made the isolation of a pure salt difficult. Exact neutralisation of the sulphuric acid hydrolysate with barium hydroxide led to the crystallisation of the monohydrate of D-glucosamine 6-(dihydrogen phosphate). In addition a small yield of a crystalline dihydrate was isolated on autohydrolysis of the tetra-acetyl phosphoric acid derivative. Chromatographic analysis of the free acid and of the barium salt (after removal of barium) showed identical spots.

The constitution of the dihydrogen phosphate and of the barium salt was confirmed by periodate oxidation :[1] both took up the theoretical quantity of periodate (Table 1) and gave no formaldehyde (Table 2) :

TABLE 1. *Uptake of periodate in moles/mole of sugar.*

Time (hr.)	22	64	94	Theor.
Glucosamine hydrochloride	4·8	4·9	4·9	5
Glucosamine 6-(barium phosphate)	3·8	3·9	3·9	4
Glucosamine 6-(dihydrogen phosphate)	3·9	3·9	3·9	4

[5] Wolfrom, Burke, and Waisbrot, *J. Amer. Chem. Soc.*, 1939, **61**, 1827.
[6] Edington, Hirst, and Percival, *J.*, 1955, 2281.
[7] Jeanloz, *J. Amer. Chem. Soc.*, 1952, **74**, 4597.
[8] Reynolds and Evans, *Org. Synth.*, 1942, **22**, 56.
[9] Haworth, Hirst, and Teece, *J.*, 1931, 2858; Helferich and Klein, *Annalen*, 1927, **455**, 173.
[10] Tipson, Clapp, and Cretcher, *J. Org. Chem.*, 1947, **12**, 133.

TABLE 2. *Formaldehyde release.*

	Mole of CH_2O/mole of sugar	Theor.
Glucosamine hydrochloride	0·81	1·0
N-Acetyl-α-D-glucosamine	0·98	1·0
Glucosamine 6-(barium phosphate)	0·00	0·0
Glucosamine 6-(dihydrogen phosphate)	0·00	0·0

Estimation of the glucosamine content of the 6-phosphate by the Elson–Morgan technique gave 98% of glucosamine.

The rate of hydrolysis of the phosphate group was compared with that of glucose 6-phosphate.[11] Whilst glucosamine 6-phosphate was 50% hydrolysed during 73 hours, glucose 6-phosphate had undergone 50% hydrolysis in 23 hours under the same conditions.

EXPERIMENTAL

Solvents were removed under reduced pressure below 50°. M. p.s were determined on the Kofler hot-stage microscope. Optical rotations were determined at 20° ± 2° in $CHCl_3$ unless otherwise stated. Light petroleum was the fraction of b. p. 60—80°.

1 : 3 : 4-*Tri*-O-*acetyl*-N-*acetyl*-6-O-*triphenylmethyl*-D-*glucosamine*.—D-Glucosamine hydrochloride (130 g.) was converted into crystalline *N*-acetyl-α-D-glucosamine (115 g., 96%) by the method of Roseman and Ludowieg.[12] After recrystallisation from aqueous ethanol–ether the crystals had m. p. 209—210° (decomp.), $[\alpha]_D^{18}$ +41° (Found: C, 43·4; H, 6·5; N, 6·9. Calc. for $C_8H_{15}O_6N$: C, 43·4; H, 6·8; N, 6·3%).

N-Acetyl-α-D-glucosamine (75 g.) in dry pyridine (500 ml.) in the presence of "Drierite" (8 g.) was heated with triphenylmethyl chloride (98 g.) at 100° until the reactants had dissolved (20 min.).[13] Acetic anhydride (150 ml.) was added to the hot solution and, after cooling overnight, the mixture was filtered and the excess of triphenylmethyl chloride in the filtrate decomposed with ice-water. The product was isolated as a white amorphous solid by pouring the mixture into vigorously stirred ice-water (3000 ml.). After drying, the product (170 g., 85%) had $[\alpha]_D$ +32° (*c*, 1·1). Solution of the crude product in hot carbon tetrachloride gave, on cooling, colourless needles (68 g.) of the carbon tetrachloride *solvate* of 1 : 3 : 4-*tri*-O-*acetyl*-N-*acetyl*-6-O-*triphenylmethyl*-β-D-*glucosamine*. These crystals lost solvent at 100—110° and had m. p. 187—189°, $[\alpha]_D$ +22° (*c*, 1·4) (Found: C, 52·3; H, 4·7; N, 2·3; Cl, 21·9; loss at 100°/15 mm. in 18 hr., 26·2. $3C_{33}H_{35}O_9N,4CCl_4$ requires C, 51·9; H, 4·4; N, 1·8; Cl, 23·8; CCl_4, 25·8%). The *amine* freed from solvent had m. p. 189—191°, $[\alpha]_D$ +33° (*c*, 1·0) (Found: C, 66·8; H, 5·7; N, 2·0. $C_{33}H_{35}O_9N$ requires C, 67·2; H, 6·0; N, 2·4%). When an ethanolic solution of the crystals was heated with Fehling's solution for several minutes a positive reducing test was obtained.

After removal of the β-anomer, evaporation of the carbon tetrachloride from the mother-liquors gave a syrup, which, on dissolution in warm chloroform and treatment with an equal volume of ether, gave crystals (11·0 g.) of a mixed solvate of 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-6-*O*-triphenylmethyl-α-D-glucosamine, m. p. 111—112°, $[\alpha]_D$ +90° (*c*, 0·9) (Found: C, 66·6; H, 6·0; N, 2·6; Cl, 2·7; loss at 100°/15 mm. in 20 hr., 5·6%). Freed from solvent the *amine* had m. p. 154—156°, $[\alpha]_D$ +97° (*c*, 1·2) (Found: C, 66·9; H, 6·0; N, 1·8%). These crystals also reduced Fehling's solution if tested as above.

The residual mother-liquors, when warmed and treated with light petroleum to turbidity, deposited, on cooling, crystals of 3 : 4-*di*-O-*acetyl*-N-*acetyl*-1 : 6-*di*-O-*triphenylmethyl*-D-*glucosamine* (4 g.), m. p. 254—256°, $[\alpha]_D$ −29° (*c*, 1·0), non-reducing to Fehling's solution (Found: C, 75·9; H, 6·0; N, 2·2. $C_{50}H_{49}O_8N$ requires C, 76·0; H, 6·0; N, 1·8%).

3 : 4-*Di*-O-*acetyl*-N-*acetyl*-α-D-*glucosamine*.—To the bistriphenylmethyl ether (1·01 g.) in glacial acetic acid (18 ml.) at 100°, water (12 ml.) was added dropwise and the temperature kept at 100° for 60 min. Cooling, addition of water (60 ml.), filtration, and concentration gave a syrup (390 mg.) which on trituration with chloroform partially crystallised. This 3 : 4-*di*-O-*acetyl*-N-*acetyl*-α-D-*glucosamine* (150 mg., 38%), after recrystallisation from acetone–light petroleum, gave a negative test for the triphenylmethyl group[14] and had m. p. 186—187°, $[\alpha]_D$ +75° (*c*, 0·9), $[\alpha]_D^{18}$ +65° (5 min., *c*, 1·0 in H_2O) → +35° (8 hr., const.) (Found: C, 47·2; H, 6·3; N, 4·5. $C_{12}H_{19}O_8N$ requires C, 47·2; H, 6·3; N, 4·6%).

[11] Robison, *Biochem. J.*, 1932, **26**, 2191.
[12] Roseman and Ludowieg, *J. Amer. Chem. Soc.*, 1954, **76**, 301.
[13] Helferich and Klein, *Annalen*, 1926, **450**, 219.
[14] Valentin, *Coll. Czech. Chem. Comm.*, 1931, **3**, 499.

1 : 3 : 4-*Tri*-O-*acetyl*-N-*acetyl*-β-D-*glucosamine*.—In the following experiments the carbon tetrachloride solvate of the triphenylmethyl ether was used.

(*a*) Treatment as in the previous experiment with glacial acetic acid (36 ml.) of 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-6-*O*-triphenylmethyl-β-D-glucosamine (2·0 g.) gave a syrup (800 mg.), whose cooled solution in ethanol gave crystals of 3 : 4-di-*O*-acetyl-*N*-acetyl-α-D-glucosamine (330 mg., 43%), which on recrystallisation from ethanol had m. p. and mixed m. p. 188—189°, $[\alpha]_D^{19}$ +68° (5 min., *c*, 1·0 in H_2O) → +32° (8 hr., const.) (Found : C, 47·1; H, 6·2; N, 4·7%).

(*b*) A cooled solution of the β-monotriphenylmethyl ether (10 g.) in acetic acid (32 ml.) was shaken with a cooled saturated solution of hydrogen bromide in acetic acid (3·2 ml.) with cooling for 60 sec. The precipitated triphenylmethyl bromide was removed and the filtrate poured into ice-water (200 ml.). This liquid mixture and the washings (300 ml.) from the bromide were extracted with chloroform (8 × 300 ml.). The extracts were dried without washing and on concentration gave a syrup. Residual chloroform and acetic acid were removed by repeated distillation with dry toluene. A crystalline residue (2·9 g., 67%), m. p. 169—171°, was obtained. After two recrystallisations from chloroform–ether, these crystals of 1 : 3 : 4-*tri*-O-*acetyl*-N-*acetyl*-β-D-*glucosamine* had m. p. 175—176°, $[\alpha]_D^{18}$ +5·5° (*c*, 1·6), $[\alpha]_D^{18}$ +18° (*c*, 1·1 in H_2O) (Found : C, 48·1; H, 6·2; N, 4·0. $C_{14}H_{21}O_9N$ requires C, 48·4; H, 6·1; N, 4·0%).

Triphenylmethylation of 1 : 3 : 4-*Tri*-O-*acetyl*-N-*acetyl*-β-D-*glucosamine*.—The foregoing β-tetra-acetate (200 mg.) and triphenylmethyl chloride (160 mg., 1 mol.) in pyridine (2 ml.) at 50° (60 hr.) in the presence of " Drierite " gave, after the usual treatment, the carbon tetrachloride solvate of 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-6-*O*-triphenylmethyl-β-D-glucosamine as needles (150 mg., 33%), $[\alpha]_D$ +24° (*c*, 1·2). The crystals lost solvent at 100—110° and the desolvated material had m. p. and mixed m. p. with the specimen previously prepared 185—187°.

1 : 3 : 4-*Tri*-O-*acetyl*-N-*acetyl*-6-O-*toluene*-p-*sulphonyl*-β-D-*glucosamine*.—A solution of 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine (100 mg.) and toluene-*p*-sulphonyl chloride (110 mg., 2 mol.) in pyridine (1 ml.) in the presence of " Drierite " at room temperature (72 hr.) gave a syrup (105 mg.) which crystallised under methanol. 1 : 3 : 4-*Tri*-O-*acetyl*-N-*acetyl*-6-O-*toluene*-p-*sulphonyl*-β-D-*glucosamine* had m. p. 170—171°, $[\alpha]_D$ +16° (*c*, 1·1) (Found : C, 50·7; H, 5·4; S, 6·1. $C_{21}H_{27}O_{11}NS$ requires C, 50·3; H, 5·4; S, 6·4%).

This material (10 mg.) was heated in a sealed tube with sodium iodide (10 mg.) in acetone at 100° for 2 hr.[10] The mixture deposited characteristic plate-shaped crystals of sodium toluene-*p*-sulphonate.

1 : 3 : 4-*Tri*-O-*acetyl*-N-*acetyl*-β-D-*glucosamine* 6-(*Diphenyl Phosphate*).—1 : 3 : 4-Tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine (11 g.) in dry pyridine (45 ml.) in the presence of " Drierite " at 0° was treated with diphenyl phosphorochloridate[15] (9·35 g., 1·1 mol.) with shaking and cooling during 20 min. The mixture was kept at 0° for a further 15 min.[16] and, after being kept overnight at 10°, was cooled to 0° and filtered. Excess of acid chloride was decomposed by a few drops of ice-water and the mixture kept at room temperature for 30 min. Slow pouring into ice-water with stirring gave a gel which quickly changed to a white crystalline solid. This on recrystallisation from aqueous acetone gave 1 : 3 : 4-*tri*-O-*acetyl*-N-*acetyl*-β-D-*glucosamine* 6-(*diphenyl phosphate*) (11·8 g.), m. p. 144—145°, $[\alpha]_D$ +25° (*c*, 0·7) (Found : C, 53·6; H, 5·3; N, 2·7; P, 5·4. $C_{26}H_{30}O_{12}NP$ requires C, 53·9; H, 5·2; N, 2·4; P, 5·3%). Extraction of the aqueous filtrate with chloroform (2 × 250 ml.) and thorough washing of the extracts yielded on removal of the chloroform a syrup. On trituration with aqueous acetone this gave a further crop of the same crystals (1·1 g.).

1 : 3 : 4-*Tri*-O-*acetyl*-N-*acetyl*-β-D-*glucosamine* 6-(*Dihydrogen Phosphate*).—The diphenyl ester (12·6 g.) (in 3 experiments of 4·2 g. each) in aldehyde-free ethanol (total, 100 ml.) was refluxed for 25 min. with activated charcoal (3·0 g.). The charcoal was removed and the solution added to ethanol (10 ml.) containing the platinum catalyst (from 0·33 g. of Adams platinum oxide reduced *in situ* for each experiment). The solution was shaken in hydrogen at slightly >1 atm. Uptake stopped at 7·6 mol. (95%) in 18, 19, and 7 hr. severally for the 3 experiments. Removal of the solvent at 25° gave a syrup which on trituration with ethanol deposited crystals (7·09 g., 75%) of 1 : 3 : 4-*tri*-O-*acetyl*-N-*acetyl*-β-D-*glucosamine* 6-(*dihydrogen phosphate*), m. p. 166—168° (decomp.), $[\alpha]_D^{19}$ +25° (*c*, 1·0 in H_2O) (Found : C, 38·9; H, 5·0; N, 3·6; P, 7·0. $C_{14}H_{22}O_{12}NP$ requires C, 39·3; H, 5·2; N, 3·3; P, 7·3%).

D-*Glucosamine* 6-(*Dihydrogen Phosphate*).—(a) *Barium salt.* 1 : 3 : 4-Tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine 6-(dihydrogen phosphate) (400 mg.) was hydrolysed with N-sulphuric acid

[15] Foster, Overend, and Stacey, *J.*, 1951, 980.
[16] Lardy and Fischer, *J. Biol. Chem.*, 1946, **164**, 513.

(12 ml.) at 100° until the rotation was constant (2·5 hr.) {$[\alpha]_D^{30}$ +60° (*c*, 2·0 in N-H_2SO_4) for the dipolar ion of the product}. The solution was shaken overnight with finely divided barium carbonate, then filtered, and the barium salts were washed with water. The combined filtrates were treated with ethanol (4 vol.). A white powder was precipitated which was washed with 90% aqueous ethanol, ethanol–ether (3 : 1, then 1 : 3), and ether. The dried precipitate (290 mg.; P, 6·3%) was treated in 0·01N-hydrochloric acid (6 ml.) with ethanol (24 ml.). The precipitate (150 mg., 41%) had $[\alpha]_D^{18}$ (dipolar ion) +53° (*c*, 0·3 in H_2O; pH 2·5). {Brown [17] records $[\alpha]_D^{24}$ +48·5° (*c*, 0·5 in H_2O; pH 2·5)} (Found: C, 19·6; H, 4·4; N, 4·7; total P, 7·8; inorg. P, 0·05. Calc. for $C_6H_{12}O_8NPBa$: C, 18·3; H, 3·1; N, 3·6; total P, 7·9%).

(b) *Free acid.* (i) 1 : 3 : 4-Tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine 6-(dihydrogen phosphate) (2·8 g.) was hydrolysed with N-sulphuric acid as before. The solution was neutralised with a half-saturated solution of barium hydroxide, samples (1 drop) being tested for barium with a solution of rhodizonic acid [18] in water (when all the sulphuric acid had been precipitated a red-brown precipitate of barium rhodizonate was formed). After removal of the barium sulphate by centrifugation the clear solution (pH 4) was concentrated to 50 ml. Addition of ethanol to turbidity gave crystals of the *acid* (0·81 g., 45%) after several days at 0°. This, after drying at 18°/0·1 mm., had m. p. 170—180° (decomp.), $[\alpha]_D$ +54° (*c*, 0·5 of the hydrate in H_2O) (Calc. for the anhyd. compound, +58°) (Found: C, 25·8; H, 5·7; N, 5·2; total P, 11·3; inorg. P. 0·0. $C_6H_{14}O_8NP,H_2O$ requires C, 26·0; H, 5·8; N, 5·1; P, 11·2%).

(ii) A solution of 1 : 3 : 4-tri-*O*-acetyl-*N*-acetyl-β-D-glucosamine 6-(dihydrogen phosphate) (200 mg.) in water (3 ml.) was heated at 100° for 30 hr. Decolorisation with charcoal and concentration gave a glass which on dissolution in water (4 ml.), addition of alcohol, and nucleation with the crystalline monohydrate of D-glucosamine 6-(dihydrogen phosphate) gave crystals (17 mg.) of a dihydrate. These had m. p. 165—175° (decomp.) (Found: C, 24·4; H 6·0; N, 4·6; P, 10·4. $C_6H_{14}O_8NP,2H_2O$ requires C, 24·4; H, 6·1; N, 4·7; P, 10·5%).

Chromatography by the ascending technique [19] with ethyl acetate–pyridine–formamide (6 : 1 : 3) as eluate and ferric chloride–sulphosalicylic acid spray,[20] gave a single identical spot for the glucosamine 6-(dihydrogen phosphate) applied in dilute sulphuric acid solution and the barium salt (freed from barium with sulphuric acid).

Oxidation by Periodate.—(a) *Periodate uptake.* Oxidations were carried out on glucosamine 6-(barium phosphate) and 6-(dihydrogen phosphate) and glucosamine hydrochloride.[1] The weighed substance (100—200 μ moles) was dissolved in water, the solution adjusted to pH 4·5, and the volume made up to 25 ml. A sample (6 ml.) was treated with 3 ml. of 0·1M-sodium metaperiodate, made up to 10 ml. with distilled water, and set aside in the dark at room temperature. At suitable intervals, portions (3 ml.) were treated with sodium hydrogen carbonate (0·6 g.), potassium iodide (0·6 g.), and sodium arsenite (0·1N; 5 ml.). After 10 min. the mixture was titrated with 0·05N-iodine. Barium was removed from the barium salt by the addition of sulphuric acid (0·1N; 3 ml.) before adjustment of the pH to 4·5.

(b) *Formaldehyde release.* Solutions of the sugar derivatives (0·7 μ mole/ml.) were oxidised in buffered solution (pH 7·5, sodium carbonate) with periodate for 21 hr. and the formaldehyde released was estimated under the conditions advocated by O'Dea and Gibbons.[21] Three glucose solutions of different concentrations were used as standards, and the filter used was the Ilford spectrum yellow (606).

Elson–Morgan Estimation of Glucosamine Content.—The glucosamine content of the 6-phosphate was estimated according to the method of Elson and Morgan [22] as modified by Belcher, Nutten, and Sambrook.[23] Glucosamine solutions were used as standards, and measurements made with the Ilford spectrum green (604) filter. The crystalline monohydrate of glucosamine 6-(dihydrogen phosphate) was found to have 98% of its theoretical glucosamine content. The optical densities of the coloured solutions from glucosamine hydrochloride and the 6-(di hydrogen phosphate) (each 0·05 μg. of glucosamine per ml.) were examined at 450—600 mμ with a Unicam SP600 spectrophotometer. It was found necessary to use 2-cm. cells to obtain optical densities equivalent to those Belcher and his co-workers [23] describe for 2-mm. cells, and, although the colours from glucosamine and the 6-phosphate appeared identical to the eye, when the optical

[17] Brown, *J. Biol. Chem.*, 1953, **204**, 877.
[18] Feigl, "Spot Tests," Vol. I, Elsevier, 1954, p. 203.
[19] Horne and Pollard, *J. Bact.*, 1948, **55**, 231.
[20] Wade and Morgan, *Nature*, 1953, **171**, 529.
[21] O'Dea and Gibbons, *Biochem. J.*, 1953, **55**, 580.
[22] Elson and Morgan, *ibid.*, 1933, **27**, 1824.
[23] Belcher, Nutten, and Sambrook, *Analyst*, 1954, **79**, 201.

densities were plotted against the wavelength there was a slight displacement (512 to 518 mμ) in the position of the maximum together with a minor difference in the shape of the curve. This may be due to the production of two similar chromophores in each case, but in rather different quantities.

Hydrolysis of D-*Glucosamine* 6-(*Dihydrogen Phosphate*).—The crystalline monohydrate of glucosamine 6-(dihydrogen phosphate) (67·9 mg.) in N-hydrochloric acid (25 ml.) was heated at 100° and aliquot parts were analysed at suitable times for hydrolysed phosphorus. Robison [11] carried out an identical hydrolysis of glucose 6-phosphate and his figures are given in the Table for comparison.

Time (hr.)	0	4	8	20	45	70	80
Phosphorus (mg./ml.)	0	0·013	0·021	0·047	0·104	—	0·160
Hydrolysis (%) :							
Glucosamine 6-dihydrogen phosphate	0	4·3	6·8	15·6	34·2	—	52·6
Glucose 6-phosphate [11]	—	12·7	21·6	44·9	75·0	88·9	—

Hydrolysis of 1 : 3 : 4 : 6-*Tetra*-O-*acetyl*-N-*acetyl- and* N-*Acetyl-glucosamine*.—4% Solutions were hydrolysed with N-hydrochloric acid at 100° and the hydrolyses followed polarimetrically. (*a*) Tetra-*O*-acetyl-*N*-acetyl-β-D-glucosamine : α_D +1·99° (2 min); +1·36° (42 min.); +1·43° (57 mins.); +1·43° (70 min.). Crystalline D-glucosamine hydrochloride (84%), $[\alpha]_D^{18}$ +86° (3 min.) ⟶ +70° (const.) (*c*, 1·5 in H_2O), was recovered from the hydrolysate. (*b*) *N*-Acetyl-α-D-glucosamine : α_D +1·42° (initial); +2·65 (2 hr.); +2·65° (3 hr.).

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