417. *Methylation of Glucosamine.*

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It seems probable that aminohexoses appear as part of the structure of some immunopolysaccharides. This being so, it is desirable to investigate suitable crystalline reference compounds which may prove useful in the identification of glucosamine in this type of substance. Methods are given for the preparation of trimethyl glucosamine and several of its derivatives and their properties are described.

Direct attempts to protect the hydroxyl groups of glucosamine by methylation have hitherto achieved little success. For the attainment of this result it would seem to be a necessary condition to diminish the activity of the amino-group. Without this precaution the use of methyl sulphate as methylating agent introduces far-reaching decomposition of the glucosamine which may or may not be accompanied by methylation of the fragments.

In the hope that the required condition might be achieved by the formation of Schiff's bases the properties of three of these compounds were studied, namely *o*-hydroxybenzylidene-, *p*-methoxybenzylidene-, and benzylidene-glucosamine. The last-named has not previously been described. In respect of ease of hydrolysis the three substances were similar, decomposition of each into the original aldehyde and glucosamine being effected by boiling water, dilute acids, and cold dilute alkali. Their instability to alkali rendered these bases unsuitable for the purpose of methylation in aqueous alkali solution, and a fruitless attempt was made to methylate benzylideneglucosamine by treatment in liquid ammonia with potassium and methyl iodide.

On the other hand *N*-acetylglucosamine is known to be comparatively stable in the presence of alkali. It was found from a study of the penta-acetyl glucosamine that the *O*-acetyl groups were easily eliminated by cold alkali with the formation of mono- or *N*-acetylglucosamine. These conditions appeared suitable for the preparation *in situ* of a modified glucosamine of the type required and a successful outcome of the process of methylation in alkaline solution seemed possible. After preliminary trials conditions were found for the conversion of penta-acetyl glucosamine into a crystalline methyl derivative

by the action of methyl sulphate and sodium hydroxide. This product, m. p. 195°, contained one acetyl group and four methoxyl groups. It did not reduce Fehling's solution or give a coloration with Ehrlich's reagent; and a van Slyke estimation showed the absence of a primary amino-group, although nitrogen was present. To this substance is assigned the constitution of N-*acetyl trimethyl* β-*methylglucosaminide* and this is supported by its properties.

The same substance was obtained in much better yield by an alternative route. Acetobromoglucosamine hydrobromide was converted by the method of Irvine, McNicoll, and Hynd (J., 1911, **99**, 260) into the corresponding α-methylglucoside. By treatment of the latter in methyl-alcoholic solution with acetic anhydride in the presence of silver acetate, N-*acetyl triacetyl* β-*methylglucosaminide* (m. p. 159°) was obtained in 90% yield. Methylation of this substance at 50° in carbon tetrachloride solution gives *N*-acetyl trimethyl β-methylglucosaminide in excellent yield. The product was identical with that obtained by the methylation of glucosamine penta-acetate and the over-all yield from glucosamine hydrochloride is three times as great.

The glucosamine penta-acetate used in these experiments was a mixture of α- and β-forms, but the β-configuration is given to the glucoside because of its low specific rotation ($[\alpha]_D$ — 13·1° in methyl alcohol) and because it can be converted by boiling with 2% methyl-alcoholic hydrogen chloride into a substance, m. p. 150°, which, in most of its properties, appeared to be isomeric with the original material and it showed a higher rotation ($[\alpha]_D$ + 135° in methyl alcohol). This substance is considered to be N-*acetyl trimethyl* α-*methylglucosaminide*. When the α-glucoside or the original β-glucoside is boiled with 7% methyl-alcoholic hydrogen chloride, the acetyl group is removed and the product is *trimethyl* α-*methylglucosaminide hydrochloride*, from which, by treatment with sodium bicarbonate, the free *amine* is obtained as a colourless distillable syrup. The process may be reversed by boiling the syrup for a short time with 2% methyl-alcoholic hydrogen chloride, the crystalline α-methylglucosaminide then being re-formed. The hydrochloride and amine are both regarded as α-glucosides, since each is converted by the action of acetic anhydride and sodium acetate into *N*-acetyl trimethyl α-methylglucosaminide unaccompanied by any of the β-form.

Hydrolysis of the β-methylglucosaminide with hydrochloric acid removed both the *N*-acetyl group and the glycosidic methyl group, the crystalline product being *trimethyl glucosamine hydrochloride*. Conditions for the regeneration of the glucoside by treatment with methyl-alcoholic hydrogen chloride were not established.

At this stage it is undesirable to ascribe constitutional formulæ to the substances described inasmuch as experiments are in progress to determine the ring structure and configuration of them. For example, despite the work of numerous authors on the subject we prefer not to regard as settled the question of the configuration of the amino-group. The stability of the *N*-acetyl group in some of these compounds suggests a possible ortho-acetic acid linking, but this also must be the subject of further work.

Experimental.

Methylation of Glucosamine Penta-acetate.—Glucosamine hydrochloride from crab shell was acetylated by acetic anhydride and sodium acetate under the conditions prescribed by Lobry de Bruyn and van Ekenstein (*Rec. trav. chim.*, 1899, **18**, 83) and a crystalline mixture of the α- and the β-form of penta-acetyl glucosamine was obtained. One sample of the penta-acetate showed $[\alpha]_D^{20°}$ + 51·5° (*c*, 0·48 in chloroform). The *N*-acetyl group of glucosamine penta-acetate was not removed by contact with N-sodium hydroxide at room temperature for 2 hours, although hydrolysis of the *O*-acetyl groups occurred under these conditions. At 50°, however, all five acetyl groups were removed by N-sodium hydroxide.

Glucosamine penta-acetate (5 g.) was vigorously stirred at room temperature with carbon tetrachloride (50 c.c.) and water (50 c.c.). To this cold mixture was then added simultaneously, in ten aliquot parts, methyl sulphate (50 c.c.) and 40% sodium hydroxide solution (120 c.c.). The addition was made with extreme slowness, and the temperature kept at 15—20° until the mixture became non-reducing. This stage was reached after the addition of two-tenths of the reagents over the course of 2 days. Thereafter the temperature was raised to 35—40°, and the

remainder of the methylating agents added over a period of 3 hours. The mixture was then boiled for 20 minutes to complete the reaction. The cooled alkaline solution was neutralised in presence of ice with 10% sulphuric acid and thoroughly extracted with chloroform. Removal of the chloroform from the dried extract left a solid residue, which was recrystallised from ethyl acetate. Yield, 1—1·5 g. This product had m. p. 195°; $[\alpha]_D^{21°}$ + 19·6° in chloroform (*c*, 0·306), $[\alpha]_D^{16°}$ − 29·0° in water (*c*, 0·28), and $[\alpha]_D^{20°}$ − 13·1° in dry methyl alcohol (*c*, 0·306). It was non-reducing to Fehling's solution, failed to give the Ehrlich reaction (with *p*-dimethylaminobenzaldehyde) and a van Slyke estimation revealed the absence of free amino-nitrogen. The substance showed a remarkable stability to alkali, being recovered unchanged after boiling with 15% sodium hydroxide solution. These properties, together with others described below, were consistent with the formulation of the substance as N-*acetyl trimethyl* β-*methylglucosaminide* (Found: C, 51·75; H, 8·3; N, 5·05; OMe, 45·0. $C_{12}H_{23}O_6N$ requires C, 52·0; H, 8·3; N, 5·05; OMe, 44·8%).

The yield of methylated product by this method is not good and there evidently occurs complete destruction of a part of the penta-acetate. On the supposition that this destruction is an oxidative process, the methylation was conducted in a number of cases in an atmosphere of nitrogen, but no improvement in yield was effected. Nor was this end achieved by substitution of other solvents such as acetone for the carbon tetrachloride. A slightly increased yield was, however, obtained when the temperature of methylation was not allowed to rise above 40°: that is, when the final boiling process was omitted. In this case, the hydrolysis of the excess of methyl sulphate was effected by continuation of the stirring for 24 hours at room temperature.

Improved Method of Preparation of N-*Acetyl Trimethyl* β-*Methylglucosaminide.*—*l*-Bromotriacetyl glucosamine hydrobromide was prepared by the method of Irvine, McNicoll, and Hynd (*loc. cit.*). This was sufficiently pure after one recrystallisation for the subsequent reactions. A specimen was, however, purified further by repeated crystallisation from chloroform–ether. This specimen had m. p. 149—150° (softening at 144°) and $[\alpha]_D^{19°}$ + 148·0° in acetone (*c*, 0·84). It was soluble in water, methyl alcohol, ethyl alcohol, acetone, and ethyl acetate, and was insoluble in ether, light petroleum and benzene.

Triacetyl β-methylglucosaminide hydrobromide was prepared from bromotriacetyl glucosamine hydrobromide by the method of Irvine, McNicoll, and Hynd (*loc. cit.*). After recrystallisation from methyl alcohol containing a little ether it melted with decomposition at 230—233°.

Acetylation of triacetyl β-*methylglucosaminide.* Three methods of acetylation were tried, namely, by heating with acetic anhydride and sodium acetate; with acetic anhydride and zinc chloride; and by treatment with acetic anhydride in methyl-alcoholic solution. The yields by the three methods were respectively 50, 50, and 90%. The third method was adopted in the routine procedure and conducted as follows. A mixture of triacetyl β-methylglucosaminide hydrobromide (0·4 g.) with silver acetate (0·17 g.; 1 mol.), acetic anhydride (1 c.c.; 2 mols.), and methyl alcohol (8 c.c.) was shaken for 12 hours at room temperature and thereafter filtered. Water was added to the filtrate, and the solution neutralised with sodium bicarbonate. The neutral solution was then extracted repeatedly with chloroform, and the solvent removed from the dried extract. The product was recrystallised from ether–petrol and ethyl alcohol. It was a non-reducing crystalline *substance*, m. p. 159°, $[\alpha]_D^{17°}$ − 21·0° in methyl alcohol (*c*, 0·38). It was soluble in chloroform, the alcohols, and ethyl acetate, and insoluble in ether, petrol, or carbon tetrachloride (Found: C, 50·0; H, 6·5. $C_{15}H_{23}O_9N$ requires C, 49·9; H, 6·4%).

Methylation of N-*acetyl triacetyl* β-*methylglucosaminide.* *N*-Acetyl triacetyl β-methylglucosaminide (2 g.) was treated in aqueous solution (50 c.c.) with carbon tetrachloride (50 c.c.), methyl sulphate (50 c.c.), and 40% sodium hydroxide solution (120 c.c.) at 50°. The methylating agents were added in ten aliquot parts over a period of 3½ hours. The product, extracted in the usual way, was a white crystalline solid (yield, 1 g.). The mother-liquors were subjected to a second methylation treatment and a further quantity (0·5 g.) of the crystalline product was isolated. The total yield was thus 95% of the theoretical quantity. The product was *N*-acetyl trimethyl β-methylglucosaminide, m. p. 195°, identical with that prepared by the methylation of glucosamine penta-acetate (no depression of m. p. by admixture) (Found: C, 51·9; H, 8·3; OMe, 44·4. Calc.: C, 52·0; H, 8·3; OMe, 44·8%).

Behaviour of N-*Acetyl Trimethyl* β-*Methylglucosaminide with Acids.*—(a) *Action of* 5% *hydrochloric acid.* In the cold no action occurred when the crystalline methylated compound (1·0 g.) was dissolved in 5% hydrochloric acid (50 c.c.), but when the temperature was raised to 95—100°, reducing properties developed and the specific rotation changed from −15° to +219° in 3 hours. The solution was then neutralised by keeping it in contact with an excess of lead carbonate for 12 hours. After filtration and removal of the lead by the usual method with

hydrogen sulphide, the solution was treated with the theoretical quantity of hydrochloric acid to form the hydrochloride. The acid solution was taken to dryness under diminished pressure, and the residue crystallised. After recrystallisation from ethyl alcohol–ether, the substance showed the properties of *trimethyl glucosamine hydrochloride*. It decomposed without melting at 210°, contained ionisable chlorine, reduced Fehling's solution, and was unstable to alkali. In methyl alcohol (*c*, 0·42 g.) it showed $[\alpha]_D^{16°}$ + 56·8°. In water (*c*, 1·2) mutarotation was observed, $[\alpha]_D^{19°}$ + 49·2 → +99·4° in 280 minutes (Found: C, 42·1; H, 7·9; N, 5·5; OMe, 36·0; Cl, 14·3. $C_9H_{19}O_5N$,HCl requires C, 42·1; H, 7·8; N, 5·45; Cl, 13·8%). In a van Slyke estimation of amino-nitrogen 4·0 mg. of the substance gave 0·35 c.c. of nitrogen ($C_9H_{17}O_5NH_2$,HCl gives 0·35 c.c.).

The trimethyl glucosamine hydrochloride was boiled for 36 hours with methyl alcohol containing 2% of hydrogen chloride. No glucosaminide was formed, the trimethyl glucosamine hydrochloride being recovered unchanged.

(b) *Action of* 1% *methyl-alcoholic hydrogen chloride.* *N*-Acetyl trimethyl β-methylglucosaminide (0·099 g.) was dissolved in 1% methyl-alcoholic hydrogen chloride (10 c.c.), and the solution heated on the water-bath under reflux until the rotation became constant. The following rotation changes were recorded:

Time of heating (mins.)	0	50	90	150	210	290
$[\alpha]_D$	− 2·0°	+ 24·2°	+ 45·4°	+ 64·6°	+ 80·8°	+ 99·0°
Time of heating (mins.)	345	420	495	555	615	
$[\alpha]_D$	+114·0°	+125·2°	+134·0°	+135·4°	+135·4°	

The reaction was thus complete in 9½ hours and the rotation of the product shows it to be identical with that isolated after treatment with 2% methyl-alcoholic hydrogen chloride (see below).

(c) *Action of* 2% *methyl-alcoholic hydrogen chloride.* *N*-Acetyl trimethyl β-methylglucosaminide (0·5 g.) was heated under reflux for 12 hours with dry methyl alcohol (50 c.c.) containing hydrogen chloride (1 g.). The solution was then neutralised (lead carbonate) and filtered, the solvent evaporated, and the residue extracted with chloroform. The chloroform solution was filtered and evaporated, and the residue once more taken up in a small amount of chloroform. To this solution was added light petroleum until a turbidity appeared. On standing overnight in a refrigerator a crystalline product separated and this was purified by recrystallisation from ethyl acetate. The yield of this compound is 90% of the weight of β-glucosaminide taken. M. p. 150°; $[\alpha]_D^{18°}$ +120·0° in chloroform (*c*, 0·4), $[\alpha]_D^{20°}$ +104·3° in water (*c*, 0·92), and $[\alpha]_D^{21°}$ +135·0° in dry methyl alcohol (*c*, 0·62). It was non-reducing and contained nitrogen, but a van Slyke estimation showed the absence of amino-nitrogen. It gave no coloration with the Ehrlich reagent and was stable to boiling alkali, but was decomposed easily by aqueous acid. The high rotation and the m. p. differentiate this substance from the starting material and the properties suggested that it was N-*acetyl trimethyl α-methylglucosaminide*. This was supported by ultimate analysis (Found for two samples: C, 52·3, 52·3; H, 8·5, 8·5; N, 4·9, 5·0; OMe, 43·5, 44·0. $C_{12}H_{23}O_6N$ requires C, 52·0; H, 8·3; N, 5·05; OMe, 44·8%).

(d) *Action of* 7% *methyl-alcoholic hydrogen chloride.* The expected removal of the acetyl group by hydrolysis was only achieved when more concentrated acid was used. Thus, when *N*-acetyl trimethyl β-methylglucosaminide was refluxed for 24 hours with methyl alcohol containing 7% of hydrogen chloride, a mixture of the glucoside, m. p. 150°, and *trimethyl α-methylglucosaminide hydrochloride* was obtained, the latter constituting 90—95% of the mixture. To isolate the hydrochloride, the product obtained after neutralisation of the mineral acid with lead carbonate and removal of the methyl alcohol was treated in one of two ways: (1) It was shaken with cold ethyl acetate, which dissolved the acetyl compound, leaving the methylated glucosamine hydrochloride, which is practically insoluble in this solvent; or (2), it was dissolved in a small volume of chloroform, and the solution treated with an excess of ether, the hydrochloride being immediately precipitated and the acetyl compound remaining in solution.

The trimethyl α-methylglucosaminide hydrochloride so obtained softened at 210° and decomposed at 237°; $[\alpha]_D^{20°}$ + 129·6° in water (*c*, 0·54) and $[\alpha]_D^{22°}$ + 113·6° in methyl alcohol (*c*, 0·22). It was non-reducing, gave a negative Ehrlich reaction, and contained nitrogen, ionised chlorine, and a primary amino-group. In a van Slyke estimation, 2·56 mg. gave 0·24 c.c. of amino-nitrogen ($C_{10}H_{21}O_5N$,HCl requires 0·21 c.c.) (Found: C, 44·1; H, 8·2; N, 5·3; Cl, 13·0; OMe, 44·8. $C_{10}H_{21}O_5N$,HCl requires C, 44·2; H, 8·1; N, 5·2; Cl, 13·0; OMe, 45·7%).

Deacetylation of N-*Acetyl Trimethyl α-Methylglucosaminide.*—This was effected in the same manner as for the β-glucoside by boiling with 7% methyl-alcoholic hydrogen chloride. The

product was trimethyl α-methylglucosaminide hydrochloride identical in m. p. and rotation with that obtained above.

Acetylation of Trimethyl α-Methylglucosaminide Hydrochloride.—The hydrochloride (48 mg.) was refluxed for 15 minutes with acetic anhydride (2 c.c.) and fused sodium acetate (50 mg.). The mixture was poured into water and neutralised with sodium bicarbonate, and the solution extracted with chloroform. After drying over anhydrous sodium sulphate, the chloroform was removed from the extract, and the residue recrystallised from ethyl acetate. It was *N*-acetyl trimethyl α-methylglucosaminide, m. p. 150°; $[\alpha]_D$ + 135° in methyl alcohol. Yield, 45 mg.

Isolation of the Free Amine.—Trimethyl α-methylglucosaminide hydrochloride (0·38 g.) was dissolved in water (20 c.c.) and treated with a slight excess of sodium bicarbonate. After 3 hours the solution was exhaustively extracted with chloroform, the extract dried, and the solvent removed. The product, *trimethyl α-methylglucosaminide*, was a pale mobile syrup (0·35 g.). The amine was stable to heat and was purified by distillation at 0·004 mm. pressure (bath temperature, 85°). The distillate was a colourless mobile syrup showing $n_D^{23\cdot5°}$ 1·4555 and $[\alpha]_D^{22°}$ + 169·8° in dry methyl alcohol (*c*, 0·79) (Found: OMe, 52·1. $C_{10}H_{21}O_5N$ requires OMe, 52·8%).

Acetylation of the Free Amine.—Trimethyl α-methylglucosaminide (40 mg.) was heated with acetic anhydride (5 c.c.) and fused sodium acetate (50 mg.), and the acetylation product isolated as before. After recrystallisation from ethyl acetate it melted at 150° and had $[\alpha]_D$ + 135° in methyl alcohol. It was thus *N*-acetyl trimethyl α-methylglucosaminide. Yield, 37 mg.

Formation of the Hydrochloride.—Trimethyl α-methylglucosaminide (40 mg.) was refluxed for 10 minutes with 2% methyl-alcoholic hydrogen chloride (30 c.c.). Thereafter the excess of mineral acid was neutralised with lead carbonate, and the filtered solution taken to dryness. The residue was dissolved in chloroform and precipitated by the addition of light petroleum. The crystalline precipitate, separated in a centrifuge, was washed twice with ether and dried in a vacuum desiccator. It was trimethyl α-methylglucosaminide hydrochloride. Yield, 35 mg.

Condensation of Glucosamine with Aromatic Aldehydes.—Salicylideneglucosamine was prepared by the method of Irvine and Earl (J., 1922, **121**, 2376). Similarly, *p*-methoxybenzylideneglucosamine was obtained by the procedure of Bergmann and Zervas (*Ber.*, 1931, **64**, 975). Each of these Schiff's bases was easily hydrolysed by both acid and alkali. It was found possible to prepare a simpler Schiff's base by the condensation of glucosamine with benzaldehyde. Glucosamine hydrochloride (2 g.) was shaken for several hours at room temperature with a mixture of freshly distilled benzaldehyde (1·1 c.c.) and *N*-sodium hydroxide (9·4 c.c.). A crystalline solid which separated during this operation was collected, washed with ice-cold water and with alcohol–ether, and recrystallised from ethyl alcohol. Yield, 2·4 g.; m. p. 156° (decomp.). Benzylideneglucosamine was insoluble in ether, chloroform, acetone, or cold dioxan, and soluble in methyl or ethyl alcohol and hot dioxan. It was also soluble in pyridine, but solution was accompanied by liberation of benzaldehyde. With a view to its subsequent methylation the hydrolysis of benzylideneglucosamine was studied. It was found to be hydrolysed by dilute acids, by boiling water and with particular ease by keeping in the presence of aqueous alkali. Thus despite the presence of alkali during the preparation of benzylideneglucosamine, benzaldehyde was liberated by N-sodium hydroxide and by sodium bicarbonate in the cold, by warm silver oxide in methyl alcohol, by potassium carbonate in boiling ethyl alcohol, and by sodium hydroxide in dioxan. The Schiff's base was not, however, decomposed by sodium bicarbonate in boiling ethyl alcohol. These properties indicated the unsuitability of benzylideneglucosamine for methylation by the usual method, but an attempt was made to methylate the substance by treating it in liquid ammonia successively with potassium and methyl iodide. The product was a dark syrup which contained only 3·2% OMe.

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