33. *The Nature of the Carbohydrate Residue in Ovomucoid. Part I. The Glucosamine Constituent.*

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Ovomucoid on suitable treatment with barium hydroxide loses its polypeptide constituent, giving a simpler carbohydrate residue containing glucosamine, mannose, and galactose. The preparation of an acetylated and a methylated derivative is described. From the hydrolysis products of the latter, 2-acetamido 3 : 4 : 6-trimethyl α-methylglucoside, together with partially methylated mannose and galactose compounds, was isolated. It would appear that in the methylated carbohydrate the major portion of the methylated glucosamine residues are "end-groups" linked glucosidically to the rest of the molecule. Moreover, by direct methylation of ovomucoid, it was proved that, in part at least, the amino-groups in the glucosidically linked methylated glucosamine residues are acetylated.

Detailed knowledge of the structure of such carbohydrate residues as are found in association with proteins is scanty, as indeed is the chemistry of the so-called mucoproteins, glycoproteins, mucoids, etc. As an approach to the study of the more general problems an investigation of egg-albumin was undertaken inasmuch as one of the carbohydrate-containing constituents, "ovomucoid," was readily prepared from this source. The presence of carbohydrate in egg-albumin has been known for a considerable time (Pavy, *Proc. Roy. Soc.*, 1893, **54**, 53) and a summary of the literature on the subject has recently been provided (Neuberger, *Biochem. J.*, 1938, **32**, 1435). The ovomucoid used in the present investigation was prepared from coagulated egg-white by extraction with water, a method which would be attended by a minimum change in the carbohydrate residue. The carbohydrate residue in ovomucoid is similar in some properties to the polysaccharide constituent of crystalline egg-albumin (Neuberger, *loc. cit.*), but differs from it in being relatively stable to hydrolysis with acids, in optical properties, and in containing galactose residues in addition to the glucosamine and mannose residues common to both.

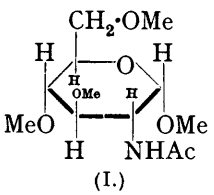
Ovomucoid was a white powder showing $[\alpha]_D$ −57° in water. Fractionation methods indicated that it was essentially homogeneous and free from coagulable protein. In the polypeptide constituent colour reactions indicated the probable presence of tryptophan, tyrosine, arginine, and histidine. The carbohydrate residue was prepared from ovomucoid by a modification of Fraenkel and Jellinek's method (*Biochem. Z.*, 1927, **185**, 392), namely, hydrolysis of the polypeptide constituent with barium hydroxide in an atmosphere of nitrogen. Subsequent treatment by the usual methods for isolating a polysaccharide gave the carbohydrate residue as a non-reducing, ash-free, white powder, $[\alpha]_D$ ±0° in water, and it appeared to be essentially the same product that was obtained by Fraenkel and Jellinek. It dialysed through collodion and parchment membranes, but this indication of a relatively small molecular weight has not yet been confirmed.

It was proved that, in part at least, hydrogen atoms of the amino-groups in the glucosamine residues in ovomucoid were replaced by acetyl groups and since the carbohydrate residue, containing 5·5% of total nitrogen, was shown to have 4·9% as amino-nitrogen, the barium hydroxide treatment caused considerable deacetylation. Apart from this effect it was shown, by comparing the properties of the methylated derivative with those of a methylated derivative prepared directly from ovomucoid, that the method of isolation did not materially alter the nature of the carbohydrate residue.

This residue was partially hydrolysed by being heated at 100° with 5N-sulphuric acid for 70 hours and among the cleavage products glucosamine (as the hydrochloride), mannose (as the phenylhydrazone), and galactose (by mucic acid formation) were detected. The presence of these substances in the carbohydrate residue was confirmed as described below by an examination of the hydrolysis products of the methylated carbohydrate residue. Previous workers have claimed that the carbohydrate residue of serum mucoid is a glucosamine–mannose–galactose complex, and that of ovomucoid is a glucosamine–mannose complex (see Hewitt, *Biochem. J.*, 1938, **32**, 1554). Sørensen (*Biochem. Z.*, 1934, **269**, 271) from colorimetric observations considered, however, that galactose also was a

constituent of ovomucoid. Owing to the resistance of part of the carbohydrate residue to acid a quantitative separation of the hexose constituents was not obtained; it was found with a substance of this type that little reliance could be placed on reducing sugar estimations or on colorimetric analyses of its carbohydrate content.

Treatment of the carbohydrate residue in ovomucoid with the usual methylating reagents failed, as in the case of glucosamine itself, to produce a methylated derivative in satisfactory yield. The same cause, namely, the ionic activity of the free amino-group (Cutler, Haworth, and Peat, J., 1937, 1979), was apparently operating in both cases, for the carbohydrate residue after acetylation gave a methylated product in good yield. The acetate, prepared in 70% yield by means of acetic anhydride and pyridine, had the unusual property of being readily soluble in water. Removal of the *O*-acetyl groups by mild treatment with barium hydroxide gave an *N*-acetyl derivative (acetyl, 11·5%). This fully acetylated derivative was treated under rigidly controlled conditions with methyl sulphate and sodium hydroxide in the presence of carbon tetrachloride and the methylation was completed with Purdie's reagents, whereby the N-*acetyl methyl* derivative was obtained as a white powder ($[\alpha]_D$ ±0° in water), which was hydrolysed by heating with methyl-alcoholic hydrogen chloride. The hydrolysis products were separated into the following main fractions: (*a*) 2-acetamido 3 : 4 : 6-trimethyl α-methylglucoside (I) (Cutler, Haworth, and Peat, *loc. cit.*) (*ca.* 10% yield of the hydrolysate); (*b*) a syrupy fraction (30% yield) of 3 : 4 : 6-trimethyl α-methylglucosaminide, which on acetylation gave (I); (*c*) a syrupy mixture of partly methylated hexoses (10% yield); (*d*) a syrup, which on methylation with silver oxide and methyl iodide gave a light brown powder (*ca.* 50% of hydrolysate) containing silver iodide as a constituent.



(I.)

From the amounts of (*a*) and (*b*) it was possible to state that at least 40% of the methylated carbohydrate residue was built up of methylated glucosamine residues. The amount of (I) in (*a*) showed that at least 10% of these methylated glucosamine residues were " end-groups " joined by glucosidic links to the rest of the molecule, and the yield of (*b*) indicated that at least a further 30% of the methylated glucosamine residues were joined either by glucosidic links or through the amino-groups. Inasmuch as the methylated carbohydrate had an acetyl content of *ca.* 10%, it appears likely that the major part of (*b*) was also linked glucosidically.

Fraction (*c*) contained no nitrogen and gave a Molisch reaction for carbohydrate. It was methylated further with Purdie's reagents and distilled in a high vacuum. The distillate appeared to be a fully methylated methylhexoside. The glucosidic methyl group was removed by hydrolysis, and the resulting syrupy methylated sugar converted into the anilide. This crystallised completely and was separated into tetramethyl mannose anilide and tetramethyl galactose anilide, in the ratio of approx. 4 : 1.

The light brown powder from (*d*) appeared to be a compound of silver iodide with glucosamine derivatives. A similar compound was isolated, as described later, from an authentic specimen of (I) and is being further investigated. Irvine and Hynd (J., 1912, **101**, 1128) had apparently encountered this type of compound in their early investigations on the methylation of glucosamine.

The *N*-acetyl methyl derivative of the carbohydrate residue was also prepared directly from ovomucoid. It was shown by fractionation methods to be mainly homogeneous ($[\alpha]_D$ ±0° in water; acetyl, 9·0%) and appeared to be identical with the product made by the indirect route. It was heated with 2% methyl-alcoholic hydrogen chloride for 24 hours, and from the cleavage products the *N*-acetyl derivative (I) was isolated in 10% yield by extraction with ether. This direct isolation of (I) established the facts that in part at least, hydrogen atoms of the amino-groups in the glucosamine residues in ovomucoid are replaced by acetyl groups and that these 2-acetamido glucose residues are " end-groups " joined by glucosidic links to the rest of the molecule. The amount of (I) isolated (10%) represents a minimum quantity, since (I) is partially deacetylated when it is treated with methyl-alcoholic hydrogen chloride under the foregoing conditions (Cutler, Haworth, and Peat, *loc. cit.*).

This preliminary work shows that the carbohydrate residue in ovomucoid contains mannose, galactose, and glucosamine and, furthermore, that part, and possibly the whole, of the glucosamine is combined with acetic acid as *N*-acetyl glucosamine. In the methylated derivative of the carbohydrate residue it appears that the main part of the glucosamine units occurs as " end-groups " linked glycosidically to the rest of the molecule, a view which is supported by the finding that the mannose and galactose residues are only partially methylated. It is evident that we are dealing with a novel type of carbohydrate structure, but it is felt advisable to defer speculation on possible formulations until the constitutions of the partially methylated units have been determined. The technique of acetylation and methylation has been adapted to suit the peculiarities of this carbohydrate and the groundwork has been laid for a more complete investigation of the quantitative relationships of the three constituent sugars and of the modes in which they are mutually linked. This investigation is now in progress and thereafter the chemical relationship of the carbohydrate residue to the parent ovomucoid and a comparison of the latter with other mucoids, such as serum mucoid, will be made.

Experimental.

Ovomucoid preparations made by several methods and from different samples of egg-albumin had the following properties.

1. Prepared by tryptic digestion of commercial egg albumin (B.D.H.), yield 3%, $[\alpha]_D^{20°}$ $-44°$ in water (*c*, 1·1) (Found : N, 8·0; ash, 2·0%). The preparation was contaminated with a galactan ($[\alpha]_D^{20°}$ $-35°$ in water, *c*, 1·0) which was present in the trypsin.

2. Prepared by Sevag's method (*Biochem. Z.*, **1934**, **273**, 419) from commercial egg-albumin (B.D.H.), yield 4%, $[\alpha]_D^{20°}$ $-57°$ in water (*c*, 1·0) (Found : N, 12·5; ash, 1·0%).

3. Prepared from dried egg-white (Messrs. Wander Bros. Ltd.). The proteins were coagulated and removed by heating an aqueous solution of the egg-white at 100°. Ovomucoid was prepared from the solution by the usual means for obtaining polysaccharide preparations. It was purified by repeated precipitation from aqueous solution by alcohol. Yield 10%, $[\alpha]_D^{20°}$ $-57°$ in water (*c*, 1·0) (Found : N, 12·7; ash, 1·0%). Exhaustive fractionation failed to separate it into fractions having different properties and this method was adopted for large-scale work.

No precipitation resulted when an aqueous solution of ovomucoid was mixed with any of the following reagents : sulphosalicylic acid, trichloroacetic acid, lead acetate, glacial acetic acid, and Esbach's picric acid reagent. A positive reaction was obtained with the following : Molisch reagent, Millon's reagent, phosphotungstic acid, ninhydrin, biuret reagent, Sakaguchi reagent, glyoxylic acid, and diazosulphanilic acid. The aqueous solution did not reduce Fehling's solution and gave a negative test for uronic anhydride residues with naphtharesorcinol. It did not contain phosphate or sulphate ions, although phosphorus and sulphur were constituents of ovomucoid.

Preparation of the Carbohydrate Residue in Ovomucoid.—The preparation made by Fraenkel and Jellinek's method (*loc. cit.*) (7% yield from ovomucoid) had $[\alpha]_D^{20°}$ $\pm 0°$ in water (*c*, 1·2) (Found : N, 3·6; ash, 20·0%). By the modification described below, an increased yield was obtained and the product had a higher nitrogen content : Ovomucoid (80 g.) and barium hydroxide (100 g.) were dissolved in hot water (1 l.), ethyl alcohol (50 c.c.) and amyl alcohol (5 c.c.) added, and the liquid boiled for 12 hours in an atmosphere of nitrogen. The insoluble residue was isolated (centrifuge), washed with water until it no longer gave the Molisch test for carbohydrate, and discarded. The supernatant liquid and washings were combined, and 50% sulphuric acid added until the solution was acid (Congo-red). The barium sulphate was removed (centrifuge) and washed with water and the liquid and washings were combined and again evaporated under reduced pressure. The syrupy residue, to which a few c.c. of dilute hydrochloric acid were added, was poured into alcohol (6 vols.). The carbohydrate residue, precipitated as a glutinous mass, was separated and dissolved in water (200 c.c.), and alcohol (200 c.c.) added. A small deposit, consisting mainly of inorganic material, was removed (centrifuge), the clear liquid poured into alcohol (8 vols.), and the precipitated carbohydrate residue isolated in the usual way as a white powder (13 g.; 16% of the ovomucoid), $[\alpha]_D^{21°}$ $\pm 0°$ in water (*c*, 1·5) [Found : N (Dumas), 5·5; amino-N (van Slyke), 4·9%]. All the usual tests for protein in this material were negative and fractionation methods showed it to be essentially homogeneous. The ninhydrin test was positive and we have made the observation (cf.

Neuberger, *loc. cit.*) that glucosamine itself gives this colour reaction. Further hydrolysis with barium hydroxide failed to alter materially the nitrogen content of the carbohydrate residue. It contained no phosphorus or sulphur and readily dialysed through parchment and collodion membranes. Observations on molecular-weight determinations will be communicated later.

The polysaccharide obtained from crude egg-albumin by Fraenkel and Jellinek (*loc. cit.*) had N 4·5%, $[\alpha]_D \pm 0°$; that isolated by Levene and Mori (*J. Biol. Chem.*, 1929, **84**, 49) from crude or crystalline egg-albumin had N *ca.* 3·7%, $[\alpha]_D +31°$; and that investigated by Neuberger (*loc. cit.*) and prepared from crystalline egg-albumin had N *ca.* 5%, $[\alpha]_D +22°$ in water.

Hydrolysis.—The carbohydrate residue was not readily hydrolysed by hot N-sulphuric acid. A sample (4 g.) was therefore treated with 5N-sulphuric acid (100 c.c.) at 100° for 36 hours; the solution, too dark to observe polarimetrically, was neutralised with barium carbonate, filtered, and evaporated under reduced pressure, giving a viscid syrup (3·5 g.). This was dissolved in water (5 c.c.), and alcohol (20 c.c.) added. A deposit (2·0 g.) consisting mainly of unchanged polysaccharide having a high ash content was removed (centrifuge). The mother-liquid was acidified with hydrochloric acid and evaporated to a syrup, which crystallised (1·3 g.). This material was stirred with warm 90% aqueous alcohol, filtered, and recrystallised from aqueous alcohol (50%). It showed $[\alpha]_D +79°$ (equilibrium value), reduced Fehling's solution, contained ionisable chlorine, gave glucosazone on heating with phenylhydrazine, and was thus identified as glucosamine hydrochloride (0·5 g.). *X*-Ray and crystallographic examinations carried out by Dr. G. A. Jeffrey in this Department confirmed its identity. The material remaining in the mother-liquors after crystallisation of the glucosamine hydrochloride was obtained, after removal of the solvent, as a strongly reducing syrup (0·5 g.). A sample on treatment in aqueous solution at room temperature with phenylhydrazine acetate yielded a pale yellow phenylhydrazone (5% yield), m. p. 180°, which was identified as mannose phenylhydrazone. A further sample (2 g.) of the carbohydrate residue was dissolved in dilute nitric acid (20 c.c., *d*, 1·3) and heated at 95° for 1 hour. The excess of nitric acid was distilled off, leaving a syrup, which was diluted with water (10 c.c.) and kept at 0° for 24 hours. A crystalline deposit (20 mg.) was isolated, m. p. 218° (decomp.), and was probably mucic acid, apparently indicating the presence of a small amount of galactose in the carbohydrate residue of ovomucoid. Owing to the stability of the carbohydrate residue to acid hydrolysis, attempts to determine quantitatively its carbohydrate content by polarimetric observation, reducing sugar estimations, and colorimetric determinations gave inconclusive results.

Acetylation.—After numerous trials, the following satisfactory method was devised. The carbohydrate residue (10 g.) was placed with acetic anhydride (30 g.) and pyridine (50 c.c.) in a bottle containing glass beads, heated for a few minutes at 70°, and then vigorously shaken at 15° for 24 hours. The mixture was centrifuged, the liquid decanted, and the residue treated in an identical manner until almost complete solution was effected. The combined, dark red liquids were concentrated under reduced pressure at 60° to a syrup, which was dissolved in water (20 c.c.), again concentrated to a syrup, and heated at 60° for an hour in a vacuum. A solution in chloroform (150 c.c.) was clarified (centrifuge) and slowly dropped into vigorously stirred light petroleum (600 c.c.). The precipitate was washed by decantation with light petroleum, collected on a filter, and dried to constant weight in a vacuum desiccator. Yield, 12 g. of a light brown, granular powder, $[\alpha]_D^{21°} -20°$ in water (*c*, 2·0) (Found : *O*-acetyl, 29·0; ash, 4·0%). It was readily soluble in water, ethyl and methyl alcohols, acetone, and chloroform.

N-*Acetyl Derivative.*—Purified acetate (8 g.) was heated at 95° for 1 hour with barium hydroxide solution (10%). The barium was precipitated quantitatively with N-sulphuric acid, the filtered solution concentrated to 50 c.c., methyl alcohol (500 c.c.) added, and a small amount of inorganic matter removed (centrifuge). Ether (500 c.c.) was added to the solution, precipitating a white powder, $[\alpha]_D \pm 0°$ in water (*c*, 1·4) (Found : N, 5·5; *N*-acetyl, 11·5%).

Methylation of Carbohydrate Residue.—Numerous attempts to methylate both ovomucoid and the carbohydrate residue with methyl sulphate and sodium hydroxide resulted in an almost complete destruction of the polysaccharide. In a typical experiment the carbohydrate residue (5 g.) was methylated twice in the manner originally used for methylating starch (Haworth, Hirst, and Webb, J., 1928, 2681). Exhaustive extraction of the solution with chloroform and removal of the chloroform by distillation gave a gum (0·4 g.) having OMe, 19%. In a similar manner, methylation of the acetate (above) gave small amounts (*e.g.*, 10% yields) of a methylated product (OMe, *ca.* 16%). Attempted methylation with silver oxide and methyl iodide or with methyl iodide in liquid ammonia also failed to give satisfactory products. Methylation of the acetate by methyl sulphate and sodium hydroxide solution (30%) in the presence of carbon

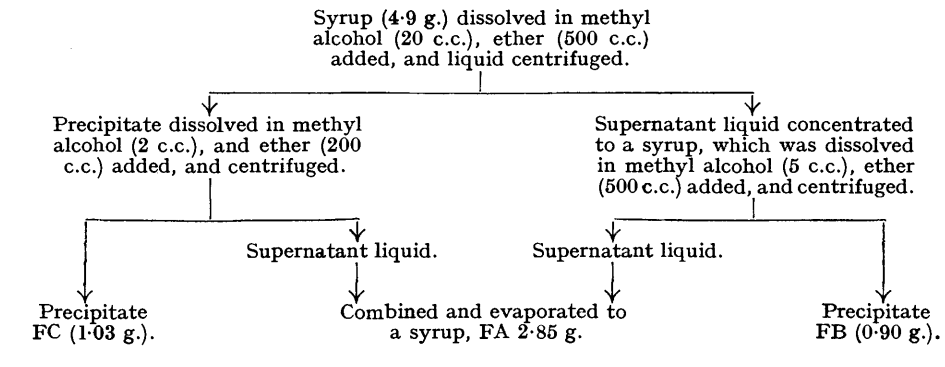
tetrachloride (Holden and West, *J. Amer. Chem. Soc.*, 1934, **56**, 930) gave in 10% yield a methylated product (OMe, 23·8%) which was readily soluble in chloroform.

Eventually the following method, which avoids excess alkalinity in the initial stages of methylation, was developed. The acetate (10 g.) was dissolved in water (100 c.c.), and carbon tetrachloride (300 c.c.) added. Sodium hydroxide (5 c.c. of 35% aqueous solution) was then added, followed by methyl sulphate (80 c.c.), and the solution maintained at 50° with vigorous stirring. Sodium hydroxide (160 c.c. of 35% solution) was added at the rate of one drop per second and finally methyl sulphate (100 c.c.), sodium hydroxide (200 c.c. of 35% solution), and carbon tetrachloride (100 c.c.) were added together during 200 minutes. The mixture was stirred for a further 2 hours at 65° in order to destroy the excess of methyl sulphate. The bulk of the methylated product, which separated on the walls of the flask, together with the deposited sodium sulphate was collected on a cloth filter. The solid was extracted several times with methyl alcohol and the filtrate was extracted with acetone, which separated as an upper layer. The combined acetone and methyl alcohol extracts were cautiously neutralised at 0° with 50% sulphuric acid; sodium sulphate, which slowly separated, was removed and washed with methyl alcohol. The combined filtrate and washings were evaporated under reduced pressure to a viscid syrup, which was dissolved in a mixture of acetone (120 c.c.) and water (30 c.c.) and remethylated in the usual way with methyl sulphate (100 c.c.) and sodium hydroxide (200 c.c. of 35% aqueous solution) in the presence of acetone (100 c.c.) at 45°. The reaction mixture was stirred at 65° for 2 hours and after filtration (cloth) both precipitate and filtrate were exhaustively extracted with chloroform. The chloroform solution was neutralised with carbon dioxide, dried over anhydrous magnesium sulphate, filtered, and evaporated to dryness under reduced pressure at 40°. The resulting brown viscid gum was dissolved in dry chloroform and poured slowly into light petroleum (250 c.c.). The product was precipitated as a voluminous, light brown powder, which was separated (centrifuge) and dried. It was purified by fractional precipitation with light petroleum from a chloroform solution. Yield, 4·0 g. (Found: OMe, 24·5; ash, 5·0%). This partly methylated product was further methylated by means of silver oxide and methyl iodide, with initial addition of a few drops of dry methyl alcohol to assist solution. Two treatments sufficed to give a product with OMe 31·5%, a value which further methylation did not increase.

The product was a light brown powder, soluble in water, chloroform, acetone, methyl alcohol, and ethyl alcohol and insoluble in light petroleum. An average sample showed $[\alpha]_D^{20°}$ $\pm 0°$ in water (*c*, 1·0) or in chloroform (Found: C, 48·1; H, 7·8; N, 4·3; OMe, 31·5; $CH_3 \cdot CO$, 9·7%). It was shown to be essentially homogeneous by fractional precipitation with light petroleum from a chloroform solution. 2·8 G. of methylated product (OMe, 30·5%) were dissolved in chloroform (80 c.c.), and light petroleum added as follows:

Light petroleum added, c.c.	Fraction.	Weight, g.	% OMe.	% Ash.	Time of flow of 1% solution in *m*-cresol at 20°, secs.
30	I	0·32	27·0	3·30	—
50	II	0·75	30·5	0·91	529
80	III	0·90	31·1	1·05	522
300	IV	0·70	30·2	0·39	519

Hydrolysis.—A purified sample (5 g.) having OMe 31·5% was heated for 48 hours with 2% methyl-alcoholic hydrogen chloride (250 c.c.) ($[\alpha]_D$ $\pm 0° \longrightarrow +69°$). The hydrolysate was neutralised with lead carbonate and filtered, and the lead salts washed with methyl alcohol. The combined filtrate and washings were evaporated, leaving a syrup (4·9 g.). This was separated into the fractions shown diagrammatically below:



The ether-soluble fraction FA was exhaustively extracted with boiling dry ether; the extracts gave on evaporation a crystalline residue (10% yield of hydrolysate). This material, recrystallised from ether–light petroleum, had $[\alpha]_D^{20°}$ +120° in chloroform (*c*, 1·0), $[\alpha]_D^{20°}$ +132° in methyl alcohol (*c*, 1·0), m. p. 149° alone or in admixture with 2-acetamido 3 : 4 : 6-trimethyl α-methylglucoside (I) (Found : C, 52·2; H, 8·8; OMe, 44·7; N, 5·1. Calc. for $C_{12}H_{23}O_6N$: C, 52·0; H, 8·3; OMe, 44·8; N, 5·1%).

The portion of fraction FA which remained insoluble in boiling ether contained nitrogen and was acetylated with acetic anhydride in methyl alcohol (a method known to acetylate only amino-groups). By further ether extractions of the acetylated material a second fraction of 2-acetamido 3 : 4 : 6-trimethyl α-methylglucoside (I) (in 30% yield of the hydrolysate) was obtained. This portion of fraction FA which was insoluble in ether was thereby identified as 3 : 4 : 6-trimethyl methylglucosaminide. The possibility that the latter compound could arise by deacetylation of the amino-group in (I) during the hydrolysis with methyl-alcoholic hydrogen chloride was proved by a control experiment in which an authentic specimen of (I) was heated for 48 hours with 2% methyl-alcoholic hydrogen chloride. It was shown by fractional crystallisation of the product that deacetylation had taken place to the extent of *ca.* 60% (cf. Cutler, Haworth, and Peat, *loc. cit.*).

In a second experiment fraction FA (2·4 g.) was fractionally distilled in a high vacuum :

Fraction.	Weight, g.	B. p. (bath temp.).	$n_D^{20°}$.	% N.	% OMe.	Physical state.
1	0·08	140°	1·4530	—	—	Syrup
2	0·20	140	1·4560	4·9	44·5	,,
3	0·37	140	1·4570	4·9	44·4	,,
4	0·14	140—150	1·4590	—	—	Partly cryst.
5	0·20	150—170	1·4680	—	—	Cryst.
6	0·50	170	1·4740	—	—	,,
Residue	0·91	—	—	—	—	Dark brown gum

Fractions 5 and 6 were identified as (I) and fractions 2 and 3 were converted into (I) by acetylation with acetic anhydride in methyl-alcoholic solution. Decomposition during distillation was too great to allow of a quantitative estimation of the proportion of glucosamine derivatives present.

Examination of Fractions B and C.—Fractions B and C and the ether-insoluble residues from the separation of the methylated glucosamine were dissolved in methyl alcohol, combined, and, after removal of the solvent, obtained as a brown gum (fraction FD, 2·1 g.) (Found : N, 3·0; OMe, 26·5%). It gave a positive Molisch test for carbohydrate. The gum was further methylated with silver oxide and methyl iodide. Six treatments gave a hard glass (Found : OMe, 30·8; N, 3·2%). Attempts to hydrolyse this material with methyl-alcoholic hydrogen chloride were only partly successful. In one instance, however, the action of 2% methyl-alcoholic hydrogen chloride on fraction FD (1·0 g.) in a sealed tube at 114° for 24 hours caused some hydrolysis. The hydrolysate, isolated in the usual way, was further methylated with silver oxide and methyl iodide and then distilled in a high vacuum. A fraction (4% yield of fraction FD), isolated at b. p. 120°/0·08 mm., had $n_D^{20°}$ 1·4570 (Found : OMe, 49·0%). It was nitrogen-free, gave a positive Molisch test, and thus appeared to consist of partially methylated methylhexosides.

A sample of the methylated derivative (5 g.) was hydrolysed by boiling with 2% methyl-alcoholic hydrogen chloride for 84 hours ($[\alpha]_D$ ±0° ⟶ +73°); the hydrolysate was separated into two fractions, FA (67% yield) and FD (33% yield).

Fraction FA (3·35 g.) was acetylated with acetic anhydride in methyl alcohol and the product, a syrup, was extracted with boiling ether containing 2% of methyl alcohol, leaving an insoluble residue (1·18 g.) which contained nitrogen and is still under investigation. The ethereal extracts deposited a crystalline sample of (I) (0·90 g.) and the mother-liquors on evaporation gave a syrup (1·2 g.), fraction FE.

Fraction FE (1·2 g.) was methylated with silver oxide and methyl iodide, and the product dissolved in chloroform. Cautious addition of methyl alcohol to the solution gave a precipitate, which was isolated (centrifuge) as a light brown powder (0·2 g.). It appeared to be identical with a product isolated in the same manner from fraction FD (above) and described later. The chloroform–methyl alcohol supernatant liquid was evaporated, leaving a syrup, which was distilled in a high vacuum. The main bulk (0·74 g.) had b. p. (bath temp.) 120°/0·04 mm. and $n_D^{20°}$ 1·4495. A second fraction (0·2 g.), b. p. (bath temp.) 160—170°/0·04 mm., crystallised and was mainly (I). The main fraction (FE_1) was redistilled. It had b. p. (bath temp.) 100—110°/0·04 mm., $n_D^{20°}$ 1·4470, OMe 56·2% (yield, 0·5 g.). A further quantity (0·13 g.) of this

material was isolated as described previously from samples of fraction FD. The combined fractions (0·63 g.) were remethylated with silver oxide and methyl iodide, and the product (0·62 g.) distilled, giving the following fractions:

Fraction.	B. p. (bath temp.)/0·04 mm.	Weight, g.	$n_D^{20°}$.	% OMe.
a	96—105°	0·25	1·4464	58·8
b	105—108	0·24	1·4468	58·2
c	108—120	0·07	1·4470	—

Fraction (a) (0·15 g.) was hydrolysed with 2N-sulphuric acid: $[\alpha]_D$ +58° (initial value); +42° (½ hr.); +37° (1 hr.); +30° (2 hrs.); +26° (3 hrs.); +23° (5 hrs., equilibrium value). The resulting solution reduced Fehling's solution and the methylated sugar was isolated from it in the usual way. It was converted into the anilide by boiling with ethyl-alcoholic aniline. Removal of the alcohol gave a solid mass of crystals (0·20 g.). By fractional crystallisation the crystals were separated into tetramethyl mannose anilide, m. p. 142° alone or in admixture with an authentic specimen, and tetramethyl galactose anilide, m. p. 193° alone or in admixture with an authentic specimen. The isolation was repeated on a further sample (0·3 g.) from fractions (a) and (b). From the amounts of material isolated and from the equilibrium rotation after acid hydrolysis it was estimated that the proportion of mannose to galactose in fraction FE was approximately 4 to 1 (cf. Sørensen, *loc. cit.*).

Investigation of Fraction FD.—This material (2·0 g.) was hydrolysed by heating it at 100° with 4% hydrochloric acid in 50% aqueous acetic acid for 24 hours. The solution was neutralised with lead carbonate and filtered, and the lead salts washed with water. The lead in solution was removed by hydrogen sulphide. The filtrate was evaporated to a syrup (1·6 g.), which was methylated with silver oxide and methyl iodide (containing 5% of methyl alcohol). A vigorous reaction commenced at 15° and the silver salts set to a hard mass on the walls of the flask. After being kept at 15° for 1 hour, the solution was maintained at 40° for a further 3 hours. It was filtered, and the silver salts extracted with boiling chloroform. The combined solution and extracts were evaporated, giving a viscid syrup, which was remethylated as above; the product, now soluble in methyl iodide, was methylated again. The final product was dissolved in chloroform (10 c.c.), and methyl alcohol (90 c.c.) cautiously added. The precipitate (0·67 g.) was isolated (centrifuge). It was a light brown powder (fraction FF) (Found: N, 4·1%) and contained silver iodide (46·2%), which was liberated by boiling with water or with dilute sulphuric acid. When freshly isolated, the powder was soluble in chloroform and methyl iodide, but on drying it became insoluble in these solvents.

Irvine and Hynd (*loc. cit.*) obtained a similar compound from the methylation of glucosamine with methyl iodide and silver oxide. Accordingly control experiments were carried out on an authentic specimen of (I). A sample (0·45 g.) was boiled with methyl iodide and silver oxide for 6 hours. The product (0·41 g.) was soluble in methyl alcohol and in chloroform. It crystallised and consisted entirely of (I). A second sample (0·43 g.) of (I) was heated with methyl-alcoholic hydrogen chloride (20 c.c. of a 2% solution) ($[\alpha]_D$ +131° ⟶ +139° in 48 hours), and the product was isolated as a syrup. This was dissolved in methyl iodide; on addition of silver oxide at 18° a vigorous reaction took place. When this subsided, the solution was heated at 40° for an hour. A syrupy product (0·4 g.) was obtained. It was dissolved in chloroform, and methyl alcohol added, whereby a light brown powder, apparently identical with that isolated above (Fraction FF) was obtained. It appears that fraction FF originated from a glucosamine derivative, probably possessing a free amino-group, present in fraction FD. The mother-liquors remaining after removal of fraction FF contained further amounts of the light brown powder (0·5 g.) together with a small portion (0·20 g.) of syrupy hexose derivatives. Work is being continued on the nature of these fractions.

Direct Methylation of Ovomucoid.—Ovomucoid (25 g.) was dissolved in water (200 c.c.), and carbon tetrachloride (300 c.c.) and methyl sulphate (100 c.c.) added. The mixture was stirred vigorously at 45°, and sodium hydroxide (200 c.c. of a 35% solution) slowly added during 1 hour. Methyl sulphate (100 c.c.) and sodium hydroxide (200 c.c. of the above solution) were added dropwise during 2 hours. The solution was stirred at 60° for a further 2 hours; a deposit of sodium sulphate and methylated product then remained insoluble. These were collected on cloth (the filtrate was rejected) and the methylated product was dissolved in aqueous acetone (1 l.), which was neutralised with dilute sulphuric acid and filtered. The filtrate was evaporated to dryness. The residual, partly methylated carbohydrate was dissolved in water (200 c.c.) and acetone (200 c.c.) and remethylated with methyl sulphate (160 c.c.) and sodium hydroxide (200 c.c. of 35% solution) in the usual manner. The methylated material was extracted from

the insoluble deposit by means of acetone. Removal of the acetone by distillation gave a gum, which was dissolved in chloroform; the solution was dried over anhydrous magnesium sulphate and filtered. Partial removal of the chloroform gave a syrup (100 c.c.), which was poured into light petroleum (400 c.c.). The methylated material was a yellow powder. This was remethylated by three successive treatments with methyl iodide and silver oxide; the product, isolated in the usual manner, was a cream-coloured powder (4 g.), $[\alpha]_D$ $\pm 0°$ in water (*c*, 1·0) (Found: OMe, 30·1%).

A portion was hydrolysed by heating it for 24 hours with 2% methyl-alcoholic hydrogen chloride ($[\alpha]_D$ $\pm 0° \longrightarrow +47°$). From the hydrolysate, by the method of fractionation previously described, 2-acetamido 3 : 4 : 6-trimethyl α-methylglucoside was isolated in 10% yield.

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