Carbohydrates in Protein

3. THE PREPARATION AND SOME OF THE PROPERTIES OF A GLYCOPEPTIDE FROM HEN'S-EGG ALBUMIN*

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In Part 1 of this series (Neuberger, 1938) the preparation of a polysaccharide from hen's-egg albumin was reported, and this was shown to contain mannose, glucosamine and an unidentified nitrogen-containing material. The study was initiated partly in order to investigate the nature of the protein-carbohydrate linkage, but this goal could not be achieved by the methods then available. Some progress has been made in this problem by more recent studies and Johansen, Marshall & Neuberger (1960) have given the most probable values for the mannose, glucosamine and acetyl contents of the whole protein and of a glycopeptide isolated from it. The probability that these are the only sugars present was indicated. It is the purpose of this paper to describe the preparation and some of the properties of this glycopeptide, and to consider the nature of the chemical bond linking the carbohydrate to the protein. A brief description of this work was reported earlier (Johansen, Marshall & Neuberger, 1958). Cunningham, Nuenke & Nuenke (1957) and Jevons (1958) have also given short accounts of their findings on the same subject.

EXPERIMENTAL

Enzymic digestion of egg albumin. A 1% solution of egg albumin was denatured by heating on a boiling-water bath for 10 min. After equilibration at 37° the pH was adjusted to 2.8 with N-HCl and an aqueous solution of crystalline pepsin (Armour Laboratories, Hampden Park, Eastbourne) (pepsin:egg albumin, 1:20) was added. The pH was kept at 2.8 by the addition of further acid, and the rate of hydrolysis was followed by the ninhydrin method as described by Moore & Stein (1954), glycine being used as standard. Digestion was continued for 96 hr. After adjustment of the pH to 7.8 (N-NaOH), trypsin (Armour Laboratories) and chymotrypsin (Armour Laboratories) (enzyme:egg albumin, 1:100 for each enzyme) were added and the progress of the hydrolysis was followed for 24 hr. Finally a portion of an aqueous solution of a purified mould protease produced by *Aspergillus oryzae*

(Crewther & Lennox, 1953) was used (enzyme:egg albumin, 1:250, pH 7.8).

In Table 1, the extent of hydrolysis undergone by the protein with the enzymes used successively is shown, on the assumption that the liberated peptides give rise to the same amount of colour as does glycine in the ninhydrin method of estimation. This assumption is only a crude approximation; many small peptides appear to give molar colour yields of from 0.6 to 1.4 if compared with glycine or leucine (Moore & Stein, 1948; Ottesen & Villee, 1951; Dowmont & Fruton, 1952), whereas others give colour yields of up to 2 (Yanari, 1956). The values obtained are therefore used only for comparative purposes and are devoid of exact quantitative significance.

Solvents for paper chromatography. A number of solvents were used and each has been designated by a letter. Solvent A: butan-1-ol-water-acetic acid (63:17:10). The solvent was allowed to stand for 48 hr. and the upper layer only used (Campbell, Work & Mellanby, 1951). Solvent B: pyridine-amyl alcohol-water (35:35:30) (Edman, 1946). Solvent C: water-saturated phenol in an atmosphere of NH₃.

Table 1. *Rate of liberation of amino groups (ninhydrin values based on glycine) during enzymic digestion of heat-denatured egg albumin*

Enzyme	Enzymes were used successively.	
	Time of hydrolysis (hr.)	Hydrolysis† (%)
Pepsin, pH 2.8	2	4.6
	4	7.5
	8	11.1
	12	12.2
	16	13.3
	24	15.3
	72	23.8
	96	27.3
Trypsin and chymotrypsin, pH 7.8	1	30.2
	2	31.9
	4	32.8
	8	33.0
	20	33.1
Mould protease, pH 7.8	1.5	35.7
	3	36.6
	6	36.8
	12	37.4
	24	37.7

* Part 2: Johansen, Marshall & Neuberger (1960).

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‡ Calculated assuming 387 peptide bonds/45 000 g. (mol.wt.) of egg albumin (Warner, 1954).

(Partridge, 1948). Solvent D: 2-methylbutan-2-ol saturated with 0.05M-phthalate, pH 6.0 (Blackburn & Lowther 1951). Solvent E: butan-1-ol-pyridine-water (3:1:1) (Hough, Jones & Wadman, 1950). Solvent F: ethyl acetate-acetic acid-water (3:1:3) (Jermyn & Isherwood, 1949). Solvent G: prepared by adding ethyl acetate to the monophasic system, ethyl acetate-water-pyridine-acetone (10:10:5:2), until two phases separated; the upper phase was used (Malpress & Hytten, 1958).

The figures given above indicate proportions by volume.

Preparation of glycopeptide

Method 1. The protein was digested successively as described above, first by pepsin, secondly by trypsin and chymotrypsin together and finally by the purified mould protease, each enzymic digestion being stopped after 24 hr. The hydrolysate was applied to a column prepared from a 1:1 (w/w) mixture of charcoal (Nuchar C-190, Kodak Ltd., Kirkby Trading Estate, Liverpool), containing 8% of stearic acid (Asatour & Dalglish, 1956), and water-washed Celite 535 (Johns-Manville Co. Ltd., London S.E. 11). The column was washed with water (about 1 l./76 g. column) and the carbohydrate was recovered by gradient elution with ethanol-water; it was found in the fraction containing between about 16 and 30% of ethanol in water. As a standard method, a 76 g. column (14 cm. $\times$ 4.7 cm.) of charcoal-Celite was used for the hydrolysate obtained by digestion of 2.5 g. of egg albumin. Considerable purification was achieved by this procedure. In general there were between 10 and 30 atoms of nitrogen/5 moles of mannose over the various fractions. We have used 5 moles of mannose as a basis for determining the degree of purification of the glycopeptide because both the purified glycopeptide and the whole protein appear to contain this amount of neutral sugar (Johansen *et al.* 1960). Paper chromatography after acid hydrolysis (5.7N-HCl, 110°, 16 hr.) revealed the presence of aspartic acid, glutamic acid, serine, threonine, leucine, valine, alanine and glycine. Sometimes also lysine, proline and arginine were found. The ethanol concentration in each fraction was measured by the microdiffusion method of Conway (1950). The nitrogen assays were carried out by the micromethod of Conway (1950) and the assays of carbohydrate by the Winzler (1955) orcinol method (see Johansen *et al.* 1960).

The varying nitrogen:carbohydrate ratios over the carbohydrate-containing fractions indicated heterogeneity, which was confirmed by one-dimensional paper chromatography in solvents A, B and C. Separation of the material into a number of distinct bands (ninhydrin) occurred in all of these solvents. The heterogeneity was confirmed by paper electrophoresis.

The recoveries of carbohydrate from these charcoal columns varied between 50 and 100% and were not directly related to the size of the column. They tended to be higher when the same column was used repeatedly.

The carbohydrate-containing solution was evaporated *in vacuo* to small volume and subjected to electrophoresis in a Porath (1956) column with Munktell cellulose powder (V. A. Howe and Co., 46 Pembridge Road, London, W. 11) at pH 7 in collidine-acetic acid buffer (Newton & Abraham, 1954). The carbohydrate-containing material eluted from this column appeared to be homogeneous at the same pH by paper electrophoresis (Whatman 3MM paper, 10 v/cm., 5 hr.). The apparatus used was similar to that described by

Wieland & Fischer (1948) and by Grassmann & Hannig (1952). The material containing the carbohydrate remained at the origin and was also the only ninhydrin-positive spot on the paper. This material, however, exhibited heterogeneity at pH 2.3 (2N-acetic acid) on electrophoresis. Column electrophoresis in 2N-acetic acid was therefore carried out on the material from the pH 7 column. This material was shown to be homogeneous by paper electrophoresis at all pH values from 2.3 to 8.0.

The column size in both these electrophoresis experiments was about 100 cm. $\times$ 3 cm., the amount of Munktell cellulose being of the order of 250 g. Electrophoresis was carried out with a potential difference of 1000 v for about 40 hr. Recoveries of carbohydrate were quantitative. The solution of the glycopeptide was freed from acetic acid by repeated evaporation and dried from the frozen state.

Method 2. The digestion of the protein was carried out in the same way as described above, but with the mould-protease digestion omitted. Applying charcoal-Celite chromatography to this, a somewhat different elution pattern of the carbohydrate was obtained. The digest was applied in water until the column was saturated with carbohydrate, 154 mg. (measured as mannose) being retained by a 100 g. charcoal-Celite column (18 cm. $\times$ 5 cm.). After washing the column with 1.5 l. of water, elution of the carbohydrate was brought about by stepwise increase in ethanol concentration. Carbohydrate was eluted by ethanol concentrations between 20 and 50%. Over the various fractions the eluate contained from 164 to 210 atoms of nitrogen/5 moles of mannose, a much smaller purification being achieved than when the mould protease was used. The total recovery from such a column was about 58% and attempts to elute more with ephedrine and phenol were unsuccessful. All this material was subjected to electrophoresis on Porath (1956) columns at pH 7.0 and at pH 2.3, of similar dimensions to those in the experiments described above.

Dialysis was sometimes used to obtain the initial purification of the enzymic digest after decreasing the volume. This operation was carried out in 2½ in. Visking dialysis tubing which had been previously heated in air at 90° for 24 hr. (Callanan, Carroll & Mitchell, 1957). Distilled water (100 vol.) was used outside the dialysis sac, the latter being gently agitated. After dialysis for 24 hr. purification was achieved as indicated by a decrease in the nitrogen:mannose ratio from approx. 500 to a value between 91 and 146 atoms of nitrogen/5 moles of mannose, for various experiments. Losses of carbohydrate were of the order of 15–20%.

Determination of the carbohydrate. The fractions collected from the column were tested in the first place by spot-testing. A portion (0.1 ml.) of each fraction was placed on a white tile, dried at 37° and 0.1 ml. of orcinol reagent (1 ml. of 1.6% orcinol plus 7.5 ml. of 60% v/v, H₂SO₄) was added. Heating of the tile in the oven at 80° for about 10–15 min. gave rise to a red colour in those containing carbohydrate. The carbohydrate was then determined quantitatively in the orcinol-positive fractions (see Johansen *et al.* 1960).

Carbohydrate spray. For purposes of recognizing carbohydrate on chromatography papers the aniline-diphenylamine-H₃PO₄ spray of Schwimmer & Bevenue (1956) was used. The carbohydrate gave a blue-grey spot with this reagent.

Sialic acid determinations. These were carried out by means of the thiobarbituric acid method after hydrolysis in 0.1N-H₂SO₄ at 80° (Warren, 1959), on egg-albumin preparation M (Johansen *et al.* 1960). As a control for the sialic acid assay ovomucoid (Worthington Biochemical Sales Co., Freehold, N.J., U.S.A.; no. 01529) was used. The latter protein appeared to contain somewhat less than 1 residue/28 800 g.

N-Acetylglucosamine. This was prepared by the method of White (1940). $[\alpha]_D^{25}$ at equilibrium +41.7° (water).

Hydrazinolysis of the glycopeptide. Conditions similar to those employed by Niu & Fraenkel-Conrat (1955) were employed. Hydrazine (British Drug Houses Ltd., Poole, Dorset) was freshly distilled from NaOH flakes under anhydrous conditions twice immediately before use. A portion (0.25 ml.) of this hydrazine was then distilled into a dry tube containing 0.1 μ mole of the dry sample, and the tube was sealed under nitrogen and heated at 100° for 8 hr. The reaction mixture was evaporated to dryness over conc. H₂SO₄ *in vacuo*, dissolved in 0.5 ml. of water and extracted with A.R. benzaldehyde (3 $\times$ 0.4 ml.), shaken well and centrifuged. The aqueous phase, including washings of the benzaldehyde extracts, was evaporated to dryness and subjected to one-dimensional paper chromatography in solvent B. The chromatogram was sprayed with ninhydrin.

Titration curve. A portion (1 ml.) of a solution in 0.1N-KCl of the glycopeptide prepared by the second method and containing 11.9 mg. of mannose was titrated. Hydrochloric acid and NaOH were both 0.2N in 0.1N-KCl. A microglass electrode was used in conjunction with a KCl salt bridge. The solution was stirred magnetically while the acid or alkali was added by means of an Agla syringe. The temperature was 23°. Appropriate corrections were made from results obtained by adding acid or alkali to volumes of 0.1N-KCl similar to those used in the experiments.

RESULTS

Neuberger (1938) gave evidence that all the carbohydrate is present in egg albumin as one unit. This is substantiated in the present work by our obtaining a 100% yield of carbohydrate (estimated as mannose) from the protein on some occasions, when the glycopeptide was prepared by the first method given. Chromatography in various solvents gave no indication of any separation of the material into two or more components. The glycopeptide which was prepared by Method 1 was found to contain 8.7, 9.0, 8.5 and 8.6 atoms of nitrogen/5 moles of mannose in four different preparations, whereas two preparations prepared by the second method contained 9.5 and 9.2 atoms respectively. This difference is probably accounted for by the latter material containing valine, which was never found in the glycopeptide prepared by the first method.

Sugar constituents of the glycopeptide. Some of the glycopeptide was hydrolysed in 0.005N- and 0.01N-HCl at 100° for 1 hr. and the hydrolysate was subjected to paper chromatography (solvent A). All the carbohydrate remained at the origin.

No fucose was released. Hydrolysis in 0.5N-HCl for 5 hr. at 100° followed by paper chromatography in solvent A indicated the presence of glucosamine (Elson-Morgan spray, Partridge, 1948), N-acetylglucosamine (Morgan-Elson spray, Partridge, 1948) and mannose. After hydrolysing the glycopeptide in 4N-HCl at 100° for 8 hr. and chromatographing on paper in solvent B two spots were detected on spraying the chromatogram with the Elson-Morgan spray (Partridge, 1948). The faster one was glucosamine, the slower one having a colour intensity of the order of 5-10% of the glucosamine spot. We do not know the identity of the slower-moving material, but it is possible that it is an oligosaccharide containing glucosamine with free aldehyde and amino groups (see Johansen *et al.* 1960). This might be a product of partial hydrolysis (cf. Gardell, 1953) or as a result of acid reversion of previously liberated N-acetylglucosamine (see Foster & Horton, 1958).

The absence of sialic acid in egg albumin was demonstrated by the Warren (1959) method.

Amino acid constituents. Some of the glycopeptide prepared by the first method was hydrolysed in 5.7N-HCl at 110° for 16 hr. and revealed the presence of aspartic acid, serine, threonine, leucine and glucosamine by two-dimensional paper chromatography in solvents A and C. Traces of tyrosine were observed in some samples. One preparation of glycopeptide was subjected to Moore & Stein analysis (Moore & Stein, 1951; Eastoe, 1955) after hydrolysis in 5.7N-HCl at 115° for both 18 and 36 hr. These results are given in Table 2, together with the values obtained by correcting to zero hydrolysis time. The correction has been applied assuming first-order kinetics for the decomposition (cf. Hirs, Stein & Moore, 1954). These corrected results may be in error if the destruction of a particular amino acid varies with its state of combination, a point which may be particularly relevant when considering the ammonia which we have observed is liberated on acid hydrolysis. This will be discussed below. As well

Table 2. Analytical data for Moore & Stein analysis of hydrolysate (5.7N-hydrochloric acid at 115°) of one sample of glycopeptide (prepared by Method 1)

Hydrolysis time ...	Mole/5 moles of mannose after hydrolysis		
	18 hr.	36 hr.	Corrected to zero time
Amino acid			
Aspartic acid	0.99	0.97	0.99
Leucine	0.78	0.78	0.78
Serine	0.30	0.28	0.32
Threonine	0.33	0.31	0.35
Tyrosine	0.14	0.10	0.20
Glucosamine	1.68	0.79	—

the amino acids listed, alanine in a similar molar quantity to tyrosine, and glycine and glutamic acid in about one-quarter and one-tenth respectively of this amount, were found.

The ultraviolet-absorption spectrum of one sample of the glycopeptide prepared by Method 1 was measured. There was little change in the absorption of the complex over the wavelength range 230–310 m μ , indicating that both tyrosine and tryptophan were absent in this sample.

A portion of the glycopeptide prepared by the second method was shown by hydrolysis and paper chromatography to contain aspartic acid, leucine, valine, serine and threonine, with much smaller quantities of alanine, glycine and glutamic acid. Tyrosine was not found.

'Amide' nitrogen. Hydrolysis of a sample of the glycopeptide (Method 1), containing 0.80 μ mole of mannose, in N-H₂SO₄ at 100° for 4 hr. liberated 0.15 μ mole of ammonia. This was distilled, with half-saturated K₂CO₃ (Tracey, 1952), at 5° and assayed by the photometric ninhydrin procedure of Moore & Stein (1954). Thus under these conditions 1 mole of glycopeptide (containing 5 moles of mannose) gives rise to 0.94 mole of ammonia.

When an amount of the glycopeptide equivalent to 4 μ moles of mannose was hydrolysed in 4N-HCl at 100° for 8 hr., the equivalent of 1.55 moles of ammonia/5 moles of mannose was produced. That this was indeed ammonia was shown by column chromatography on Dowex-50X8 resin (Moore & Stein, 1951; Eastoe, 1954), with 0.2M-buffers (sodium citrate, pH 5.0, sodium phosphate, pH 6.7, and sodium citrate, pH 6.5, successively).

A sample of the glycopeptide (Method 2), containing 1.56 mg. of mannose, was hydrolysed in 2N-HCl for 3 hr. and, after removal of excess of HCl, steam-distilled with saturated sodium borate in saturated Na₃PO₄ (Tracey, 1952). The distillate contained 113 μ g. of ammonia nitrogen, equivalent to 4.7 equiv./5 moles of mannose. This includes ammonia liberated from the alkaline degradation of glucosamine (see Marshall & Neuberger, 1960). The latter probably accounts for three residues (Johansen *et al.* 1960) so that 1.7 residues are present as ammonia in the hydrolysate.

The amount of ammonia produced on acid hydrolysis was directly assayed by precipitating it as phosphotungstate (Berlin, Neuberger & Scott, 1956; Johansen *et al.* 1960). A quantity of glycopeptide equivalent to 3.45 mg. of mannose was hydrolysed in 2N-HCl for 3 hr. and the steam-volatile base arising from the phosphotungstic acid precipitate by decomposition with alkali was determined. There were the equivalent of 1.6 moles of ammonia/5 moles of mannose. The question arose whether a mixture of the constituent amino acids and sugars in the glycopeptide would give rise to

ammonia when hydrolysed in acid. A control experiment was therefore carried out by heating mannose, N-acetylglucosamine, aspartic acid, leucine, serine and threonine in the molar ratios 5:3:1:1:0.5:0.5 in 2N-HCl for 3 hr. The precipitate produced with phosphotungstic acid was negligibly small. It is possible, however, that the constituents of the glycopeptide exhibit a different stability in their combined state.

Carbon dioxide was not liberated during the course of acid hydrolysis in 2N-HCl (Van Slyke apparatus for determination of carbon dioxide).

Paper chromatography of the glycopeptide. With Whatman no. 1 and no. 3MM paper, the behaviour of the glycopeptide in a number of solvents was observed. No movement from the origin was noted with solvents A–F. The glycopeptide, however, did move in solvent G, although accompanied by some streaking, as shown by spraying the paper with ninhydrin. The streaking observed may be attributable to the carbohydrate molecules having differing lengths of peptide chain attached to it, an assumption which has been referred to before.

N-Terminal amino acid. Egg albumin has been reported to have no free N-terminal amino group (Desnuelle & Casal, 1948; Porter, 1950; Fraenkel-Conrat, 1953; Steven & Tristram, 1958). It has been suggested (Porter, 1950) that the carbohydrate moiety in the protein might be blocking the N-terminal amino group and, indeed, our preliminary results on the glycopeptide appeared to substantiate this. More extensive studies, however, indicated that this was not the case. When a sample of glycopeptide was allowed to react with 1-fluoro-2,4-dinitrobenzene (FDNB) in ethanolic trimethylamine at room temperature for 2 hr. (Sanger & Thompson, 1953), no dinitrophenyl (DNP)-amino acid could be detected on acid hydrolysis of a portion of the reaction mixture. That this was not due to destruction during the acid hydrolysis of a DNP-amino acid which had been formed was shown by purifying the remainder of the reaction mixture on a charcoal–Celite column. The resulting carbohydrate was shown on acid hydrolysis (5.7N-HCl, 110°, 16 hr.) to contain the amino acids in approximately the same amounts as in the original material.

Reaction of the glycopeptide with FDNB under slightly more vigorous conditions, namely at 37° for 6 hr. in 0.05M-sodium phosphate, pH 8.0, or 0.05M-ammonium bicarbonate–0.05M-ammonium acetate buffer, pH 8, gave rise to a glycopeptide having an absorption maximum at a wavelength of 3500 Å in 1% NaHCO₃ solution. Assuming that the extinction of a 20 μ M solution of the DNP-glycopeptide is 0.31 at 3500 Å (Sanger, 1949), it was found in two separate experiments that 0.82 and 0.83 dinitrophenyl residues had been taken up per 5 moles of mannose.

That the DNP-peptide was still attached to the carbohydrate was indicated by the following procedures. Paper chromatography in solvent D gave a strong-yellow band at the origin, a band due to dinitrophenol and two faint, fast-moving bands. Carbohydrate was present only in the band at the origin. This material was eluted with 1% NaHCO₃ and was used for measuring the ultraviolet absorption. This material was made 1.5N in HCl and extracted with redistilled ethyl acetate and with ether. No yellow material was extracted into the organic solvent. The yellow due to the dinitrophenyl group and the carbohydrate were inseparable by paper electrophoresis at pH 2 (8v/cm., 2.5 hr.) and pH 7 (10v/cm., 3 hr.) and by paper chromatography in solvents B and G. Only on paper chromatography in solvent G was any movement observed.

These experiments were carried out in general on material that contained no tyrosine, and hydrolysis of the DNP-glycopeptide in acid solution (12 hr., 5.7N-HCl, 100°) gave rise to DNP-aspartic acid as the only DNP-amino acid, in a yield of about 0.4 residue/5 moles of mannose. This would appear to indicate a destruction of about 60% of DNP-aspartic acid, a somewhat greater lability than that of pure DNP-aspartic acid (Porter, 1957) and due presumably to the effect of carbohydrate under the hydrolytic conditions we have used. The peptide therefore seems to have aspartic acid as the *N*-terminal constituent. In one sample of the glycopeptide traces of *ON*-di-DNP-tyrosine were also found as well as DNP-aspartic acid.

During the course of this work with FDNB some evidence of the alkali instability of the carbohydrate-protein bond was found. If the glycopeptide were treated for 3 hr. with aqueous ammonia at pH 9 or above at room temperature, followed by reaction with FDNB in ethanolic trimethylamine at room temperature, then formation of a DNP-derivative was observed. This is in contrast with the negative result obtained when the prior alkaline treatment was omitted. Reaction occurred also when the glycopeptide was caused to react with FDNB in ethanolic trimethylamine at 37° for 16 hr., but paper chromatography in solvent D gave rise to a number of yellow spots. Furthermore, paper electrophoresis in collidine-acetic acid buffer (pH 7) gave rise to a DNP-peptide which separated from the carbohydrate. This is presumably caused by the lability of the glycopeptide under the more alkaline conditions used during treatment with FDNB.

Action of carboxypeptidase. With a sample of glycopeptide that had been prepared by Method 1, carboxypeptidase was found to release amino acids in the order serine, threonine and leucine. In no case was aspartic acid liberated. This would

agree with the sequence of amino acids being Asp. Leu. (Ser. Thr.), as suggested by the analytical data given in Table 2. [For definition of abbreviations used for amino acids see *Biochem. J.* (1957), 66, 6.] Treatment of the glycopeptide with carboxypeptidase for 3 days followed by purification on a charcoal-Celite column yielded material which on paper electrophoresis in 2N-acetic acid revealed a number of ninhydrin-positive spots. Column electrophoresis of similar material gave carbohydrate-containing fractions in the eluate which contained approximately 1 mole of aspartic acid/5 moles of mannose and traces of serine and threonine, but apparently without any leucine. This is not consistent with the deductions made above.

Some of the glycopeptide prepared by Method 2 was subjected to the action of carboxypeptidase [glycopeptide(mannose):carboxypeptidase, 20:1]. Measurement of the rate of reaction indicated that after periods of 1, 6.5, 11, 23 and 32 hr., the amounts of amino acid liberated were 0.56, 0.92, 0.92, 0.89 and 0.88 mole/5 moles of mannose, based on ninhydrin measurements with leucine as standard. Thus the reaction was complete at 6.5 hr. Addition of a further similar quantity of carboxypeptidase did not increase the extent of reaction. The digest was subjected to electrophoresis in a Porath column at pH 3.5, with a four-fold dilution of the pyridine-acetic acid buffer of Michl (1951). After hydrolysis (5.7N-HCl, 110°, 16 hr.) the glycopeptide was found to contain aspartic acid, leucine and serine with very small traces of valine, threonine, glycine, alanine and glutamic acid. The main effect of the carboxypeptidase on this glycopeptide would therefore seem to be in removing valine from the peptide chain. The carboxypeptidase-treated glycopeptide was subjected to the action of crystalline papain (Kimmel & Smith, 1954) that had been prepared by Dr J. R. Kimmel and given to us by Dr Helen Muir. No reaction ensued at 50° during 16 hr. The addition of urea to a final concentration of 8M also failed to cause any enzymic digestion of the glycopeptide by papain so that hydrogen-bonding would not appear to be responsible for its stability to this enzyme.

Hydrazinolysis. Hydrazinolysis of some of the glycopeptide prepared by Method 1 gave rise to leucine, serine and threonine. It is most likely that the three amino acids result from varying lengths of peptide chain attached to the carbohydrate moiety.

Titration curve of the glycopeptide. Some of the glycopeptide which had been prepared by Method 2 was titrated in potassium chloride (*I* 0.1). In Fig. 1 are shown the experimental points obtained when the proton uptake or loss/5 moles of mannose is plotted against pH. Points obtained on changing the pH both from 2.0 to 8.5 and back to 2.0 have

been included. The line has been drawn from the Henderson-Hasselbalch equation assuming 1.05 equiv. each of two groups with respective pK values of 3.5 and 6.7. There is good agreement between the calculated and experimental points between pH 2.0 and 7.8 and the results obtained between 7.8 and 9.0 suggest either that a new acidic group is formed by decomposition of the complex at higher pH values or that the glycopeptide contains a further ionizable group with a pK value of about 9.5.

DISCUSSION

The findings reported in this and the two earlier papers of this series lead to the conclusion that the carbohydrate residues in the albumin of the hen's egg are present as a single polysaccharide unit linked to the peptide chain(s) by one or, less likely, several bonds. In the second paper (Johansen *et al.* 1960) the mannose content of egg albumin as determined by colorimetric methods was reported to be 2%, which is equivalent to 5 mannose residues/mole of egg albumin. This value has been confirmed by isolation of the mannose as phenylhydrazone and by a method based on isotope dilution with carrier [^{14}C]mannose (C. François, R. D. Marshall & A. Neuberger, unpublished work). The glycopeptide, the isolation of which is described in the present paper, was found to have 5 mannose residues/mole of aspartic acid (Johansen *et al.* 1960). The number of amino sugar residues is not established with certainty, but it is highly probable that both the protein and the glycopeptide contain 3 glucosamine residues. With regard to the

acetyl groups the position is different. Hydrolysis of the protein gives rise to 4 molecules of acetic acid, whereas only 3 acetyl residues could be demonstrated in the glycopeptide. As has been stated by Johansen *et al.* (1960), the three acetyl groups in the glycopeptide are almost certainly present as *N*-acetylglucosamine, whereas the fourth acetyl group in the protein is likely to be combined with the terminal group of the peptide chain.

A polysaccharide containing 5 mannose and 3 acetylglucosamine residues has a molecular weight of 1440 and a nitrogen content of 2.9%. The material isolated by Neuberger (1938) was prepared by prolonged hydrolysis of egg albumin with 'pancreatin' and contained between 4.85 and 5.1% of nitrogen and almost certainly contained in addition to the hexoses mentioned 1 residue of asparagine and 1 residue of another nitrogenous compound which could be another amino acid or, what seems less likely, another residue of amino sugar, giving a molecular weight of 1650–1700. The molecular weight of the material then isolated was estimated by an isopiestic method and was found to be, after a small correction for mineral matter, between 1250 and 1275 (Neuberger, 1938). After that paper had been published the molecular-weight estimation was repeated with a sample that had been precipitated three times by addition of ethanol to a concentrated aqueous solution. The molecular weight now obtained was, without correction, between 1450 and 1500. These figures are still lower than those calculated, but the discrepancy of about 10% is most likely caused by the presence of minute amounts of impurities of low molecular weight. These values and the close correspondence between the analytical results obtained with the whole protein and with the glycopeptide render unlikely the possibility that more than one polysaccharide is combined with the peptide chain in egg albumin.

Nitrogenous compounds in the glycopeptide

The glycopeptide contained between 8.5 and 9.5 atoms of nitrogen, the higher values being obtained by a method not involving the use of the mould protease. We must conclude that samples prepared by Method 1, i.e. those not containing valine, have at least 8 nitrogen atoms/5 moles of mannose. The material prepared in 1938 by the action of a crude mixture of proteinases and peptidases had approx. 5% of nitrogen and, judging from the analytical values for non-amino sugar then obtained, contained 5.5–6.0 atoms of nitrogen/5 moles of mannose. Three of the 6 or 8 nitrogen atoms respectively can be definitely accounted for by glucosamine and a fourth nitrogen atom by aspartic acid. Already in 1938 it was shown that appreciable amounts of ammonia are liberated on

Fig. 1. Titration curve of a sample of the glycopeptide. The points represent some of the experimentally determined results with appropriate corrections for titration of the solvent. The continuous line indicates the theoretical values obtained by assuming there are 1.05 equiv. each of ionizing groups with pK values of 3.5 and 6.7 respectively.

hydrolysing the glycopeptide with acid, and in this paper it is shown that hydrolysis with *N*-sulphuric acid gives rise to approximately 1 mole of ammonia. This is likely to arise from an asparagine residue, but as the presence of asparagine has not been directly demonstrated, this must remain an inference. In any case the amount of ammonia obtained increases with the length of hydrolysis and strength of acid used; thus it may be as much as 1.7 moles with 2*N*-hydrochloric acid. The values obtained for amino acids, other than aspartic acid (Table 2), amount to another 1.64 equiv., so that a total of about 7.5 atoms of nitrogen are accounted for by hydrolysis products. There is thus still a deficiency of approximately 1 atom of nitrogen. In addition, the question of the origin of the ammonia, which is liberated in excess of 1 mole on extensive hydrolysis, arises. There are several possibilities to be considered:

(1) The amounts of amino acids obtained by the Stein & Moore method after hydrolysis with 5.7*N*-hydrochloric acid at 115° may give too low an estimate of the quantities of amino acid residues actually present in the glycopeptide. The rate of destruction of these amino acids, when combined with the polysaccharide, might be much greater than is indicated by control experiments in which the free amino acids are heated with acid in the presence of mannose and glucosamine. This destruction would give rise to ammonia and thus explain the high values obtained for this base. If indeed 1 mole each of serine and threonine were present in the glycopeptide the total nitrogen value would be fully explained. It is hoped to explore this possibility further, if enzymes can be found which split the relevant peptide linkages effectively.

(2) In Johansen *et al.* (1960) the difficulties inherent in the hydrolysis of compounds containing amino sugar were discussed and it was pointed out that the yield of free amino sugar is unlikely ever to be fully identical with the theoretical value, as it must depend on the relative rates of splitting of the amide group and that of the glycosidic linkage and the rate of destruction of the amino sugar. The possibility that the glycopeptide contains a fourth residue of amino sugar can therefore not be lightly dismissed. As only three acetyl groups were found in the glycopeptide, such a fourth residue would either have to have a free amino group or the amino group would have to be combined with one of the carboxyl groups of aspartic acid. The latter possibility will be discussed below, but the assumption of a free glucosamine amino group is compatible with the titration results (Fig. 1), which suggest that the glycopeptide has two groups ionizing in the range pH 6.0–10.0, one of which is almost certainly the amino group of aspartic acid, whereas the other may be the amino group of a

glucosaminide. If a non-acylated glucosamine residue were to be present in the glycopeptide, its glycosidic bond would be extremely resistant to hydrolysis and the rate of destruction of the free glucosamine (which would probably yield ammonia) might be equal to its rate of liberation.

(3) The last possibility to be considered is that the glycopeptide contains a nitrogenous component, additional to those identified at present, and that this compound gives rise to ammonia on treatment with strong acid. We have not found any evidence for the presence of an unknown or unidentified ninhydrin-reacting substance in glycopeptide hydrolysates, but the hypothetical substance or grouping may be destroyed during hydrolysis or may be ninhydrin-negative. We have considered the possible presence of an aminouronic acid, but, unless its carboxyl group were blocked, this should show up in the titration curve. There is only one acidic *pK* and this must correspond to the terminal carboxyl group of the peptide chain. The possibility of a ureido group was also considered, but during the hydrolysis of such a linkage the liberation of ammonia should be paralleled by evolution of carbon dioxide. This was not the case. Although there is thus no evidence for a nitrogenous compound in addition to those identified, the possibility of its being present cannot as yet be excluded.

Sequence of amino acids in the glycopeptide

The complete sequence of amino acids cannot be deduced with certainty from the present results. However, in almost all our preparations the amino group of aspartic acid was the only group reacting with fluorodinitrobenzene and it must be concluded that aspartic acid is *N*-terminal. The resulting DNP-peptide was apparently still attached to the carbohydrate and it must therefore be assumed that the linkage to the polysaccharide is through one of the carboxyl groups. As aspartic acid is most probably linked to the adjacent amino acid by an ordinary peptide linkage, it follows that it is the β -carboxyl group which is linked to the polysaccharide. The observation that free aspartic acid is not liberated by carboxypeptidase and the results of the hydrazinolysis experiments are in agreement with this general deduction. In one sample of glycopeptide there was found on hydrolysis *ON*-di-DNP-tyrosine in addition to DNP-aspartic acid. It would thus appear that in the protein the amino group of the carbohydrate-bonded aspartic acid is linked to the carboxyl group of tyrosine.

Material not treated with mould protease contained valine in addition to aspartic acid, leucine, serine and threonine and this valine residue was easily removed with carboxypeptidase. It would

thus appear that valine is *C*-terminal in these preparations. The sequence of the other three amino acids is less certain. The results shown in Table 2 indicate that leucine is present in a hydrolysate in much greater quantity than serine or threonine. If these amounts reflected the relative proportions of the amino acid residues present in the unhydrolysed glycopeptide preparation it would follow that the latter consisted of a mixture of compounds having in common the aspartic acid- or asparagine-carbohydrate structure, but differing with respect to the number of amino acid residues in the peptide chain. It would also follow that leucine follows aspartic acid. However, as pointed out above, the rates of destruction during hydrolysis of the amino acid may vary greatly and the molar amounts of the residues in the glycopeptide may in fact be almost unity. In addition, the observation that the glycopeptide isolated after exhaustive treatment with carboxypeptidase contained some threonine and serine, but no leucine, is not compatible with the assumption that leucine is adjacent to aspartic acid. It would appear therefore that the sequence is

Tyr. Asp (carbohydrate). (Leu. Ser. Thr). Val.

This deduction agrees with the findings of Nuenke & Cunningham (1959), who have also reported that the valine is followed by a second leucine residue. Rosevear & Smith (1958) also obtained evidence that aspartic acid is linked with the carbohydrate in human γ -globulin.

Polysaccharide-peptide linkage

The discussion so far has shown that there are still some gaps in our knowledge of the composition and structure of the egg-albumin glycopeptide. Thus we cannot exclude the possibility of the presence of a nitrogenous component so far unidentified or of a fourth amino sugar residue; nor can we be sure of the origin of the ammonia which appears on hydrolysis. Moreover, we are completely ignorant about the linkages between the hexose and hexosamine units. It may be profitable, however, at this point, to consider likely structures for the bond joining the peptide chain with the polysaccharide unit; this is possible if it is assumed that only identified components, i.e. *N*-acetylglucosamine or glucosamine, mannose, aspartic acid and ammonia have to be taken into account. The glycopeptide has no reducing group and it appears highly probable that the reducing end, which may be either a mannose or a glucosamine unit, is involved in the linkage with the peptide. All the evidence indicates that the amino acid which is closest to the polysaccharide is aspartic acid. The relatively facile evolution on acid hydrolysis of at least 1 mole of ammonia is compatible

with the belief that ammonia originates from an amide linkage, although asparagine itself has not been isolated from a glycopeptide hydrolysate. The titration curve shows only one *pK* in the acid region, and this must be ascribed to the terminal carboxyl group of the peptide, the presence of which is also indicated by the carboxypeptidase experiments. The most probable structure arising from these considerations is that of an acylated glycosylamine in which the acyl group is provided by one of the two carboxyl groups (probably the β -carboxyl) of aspartic acid. Such a structure is not novel amongst natural products as it is closely similar to that of an intermediate in purine biosynthesis, i.e. the glycylamide ribotide (*N*-glycyl-5'-phosphoribofuranosylamine; Peabody, Goldthwait & Greenberg, 1956; Hartman, Levenberg & Buchanan, 1956). The *pK*₂ of 6.7 is also in agreement with that suggestion. The proposed structure is that of a substituted aspartic diamide, and whereas the *pK* of that type of compound is not known, it might be expected to be similar to that of the diethyl ester of aspartic acid, for which *pK* 6.5 has been reported (Edsall & Blanchard, 1933). However, the titration curve (Fig. 1) indicates that the glycopeptide has probably a third *pK* of 9.0-9.5, and this cannot as yet be explained with certainty.

The aspartylglycosylamine structure explains many or most of the known facts, but, as direct proof is lacking, it must be regarded at present as a tentative proposal. We have considered other possibilities such as a linkage between an aspartic acid carboxyl group and an amino group of glucosamine. However, such a structure would require a fourth amino sugar residue, the existence of which is possible but for which no positive evidence exists; it would also increase our difficulties in explaining the origin of the ammonia and it would further raise the problem of how the reducing end group is blocked. Similar objections can be raised against the suggestion that the carboxyl group of aspartic acid is combined with a hydroxyl group in the polysaccharide. The possibility was also considered that the hydroxyl group of serine or threonine is combined with C-1 of a sugar residue. However, the fact that a large fraction of these amino acid residues can be split off by carboxypeptidase without apparently severing the link between the aspartic acid and the polysaccharide is not compatible with this suggestion. It thus appears that the acyl glycosylamine structure is the most reasonable hypothesis that can be put forward at present.

SUMMARY

1. The preparation of a glycopeptide from egg albumin has been described, in which enzymic digestion of the protein is followed by chromato-

graphy on charcoal-Celite columns and electrophoresis on Porath cellulose columns.

2. Evidence is presented showing that the polysaccharide is present in the whole protein as a single unit.

3. The main constituents of this glycopeptide have been quantitatively analysed and found to be mannose, 5 residues; glucosamine, 3; acetyl, 3; aspartic acid, 1; leucine, 0.78; serine, 0.32; threonine, 0.35. The possibility has been discussed that these values may not represent accurately the composition of the isolated glycopeptide.

4. Experiments with fluorodinitrobenzene have shown that aspartic acid is *N*-terminal in the peptide chain (with traces of *N*-terminal tyrosine in some samples). These results, together with those obtained with carboxypeptidase and by hydrazinolysis, are in agreement with the sequence Tyr. Asp (carbohydrate). (Leu. Ser. Thr). Val. The carbohydrate seems to be linked to aspartic acid through one of its carboxyl residues, probably the β -group.

5. Titration-curve data indicate the presence of a group with *pK* 3.5 and another with *pK* 6.7. These represent the ionization of the *C*-terminal and *N*-terminal groups respectively. The possible presence of a third group with *pK* about 9.5 cannot be excluded with certainty.

6. Acid hydrolysis of the glycopeptide gives rise to the formation of ammonia: approximately 1 residue is produced by hydrolysis in *N*-sulphuric acid at 100° for 4 hr., increasing to about 1.7 equiv. after hydrolysis in 2*N*-hydrochloric acid at 100° for 3 hr. The possible sources of this ammonia have been discussed.

7. The nature of the linkage between the polypeptide and the carbohydrate in the whole protein has been considered. The most likely structure from our evidence is that of a β -aspartylglycosylamine, but this suggestion must be regarded as tentative at present.

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Quantitative Studies on Mammary-Gland Enzymes Involved in Lactose Synthesis

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The steps by which lactose is synthesized from glucose in the mammary gland have been tentatively elucidated, and although for some of the stages the evidence is merely the demonstration of a theoretically suitable enzyme in the tissue, a scheme of synthesis has been proposed which probably accounts for about 70 % of the lactose formed under normal conditions. The participation of alternative pathways responsible for the remaining 30 % of synthesis has been indicated by work in which the glucose and galactose moieties of lactose formed from [1-¹⁴C]glucose or from labelled acetate showed a differential labelling; this could not have occurred if all the synthesis had followed the proposed major route. These problems of the biosynthesis of lactose have been discussed elsewhere (Malpress, 1958).

It seemed possible that some confirmation of the mechanism of synthesis might be obtained by studying the variation in the activities of the postulated lactose-synthesizing enzymes in glands removed at different stages of lactation. In common with other enzymes involved in the synthesis of milk constituents (Moore & Nelson, 1952; Greenbaum & Greenwood, 1954) their activities might be expected to vary directly with the amount of milk produced, and in particular to increase for a period after parturition and to fall during a period of lactational decline. Hexokinase, which is the first enzyme required in the synthesis of lactose, has already been investigated in this way for the rat (McLean, 1958) and a clear parallelism has been shown between its activity in the gland and the lactational performance of the animal.

This paper reports similar studies with mammary-gland homogenates on other enzymes of importance

in lactose synthesis: phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase and inorganic pyrophosphatase.

METHODS

Animals. Rats, mainly primiparous and weighing 240–280 g., were killed at 0, 6 and 15 days after parturition; a further group was weaned from the litters 6 days after parturition and killed on the eighth day. Values for guinea-pig glands were obtained for 6- and 15-day intervals after parturition.

After slaughter, the abdominal mammary glands were dissected, weighed and homogenized in a Nelco homogenizer for 2 min. with 10 vol. of 0.1M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer, pH 7.4. This homogenate was strained through muslin and the unhomogenized residue was blotted on filter paper and weighed. The amount of tissue homogenized under these conditions varied with the lactation status of the animal, approaching 100% for individual guinea-pig glands in full lactation and falling to a mean value of 63 % for rat glands during involution. Inspection of the unhomogenized residues showed that they consisted almost entirely of non-secretory tissue.

Lactose in mammary-gland homogenates and correction for 'milk error'. Lactose was determined by the method of Malpress & Morrison (1949) on samples of the homogenate deproteinized with 0.3N-Ba(OH)₂ and 5% ZnSO₄. The lactose contents of rat and guinea-pig milks were derived from the equations of the regression lines of lactose on deoxyribonucleic acid phosphorus (DNA-P) for a series of glands at each lactation interval studied, as suggested by Greenbaum & Slater (1957a). True tissue weights, free from 'milk error', were then calculated in the way outlined by these authors and form the basis on which all results in this paper are expressed.

In rats killed on the eighth day after preliminary weaning, lactose values were frequently zero and no statistical value for lactose in the milk of such animals could be