Acid Dissociation Constants of Glycopeptides

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Glycopeptides containing mainly four amino acid residues in the sequence Asn-Leu-Thr-Ser, with small amounts of additional amino acid residues, were isolated from enzymic hydrolysates of hen's-egg albumin. Heterogeneity of the carbohydrate moiety was confirmed. Acid–base titrations showed that the α -amino group has a pK_a value of 6.43 at 25°C. The standard free energy and entropy changes associated with the ionization at 25°C were $37.2\text{ kJ}\cdot\text{mol}^{-1}$ and $-0.014\text{ kJ}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ respectively. The complications arising in the interpretation of titration curves of the glycopeptides, which are heterogeneous with respect to the peptide chain, were considered and discussed in the light of the earlier suggestion that the titration curve of the glycopeptide might be interpreted as being due in part to a structure in which the hydroxyl group of the threonine residue is hydrogen-bonded to the β -aspartamido oxygen atom [Neuberger & Marshall (1968) in *Symposium on Foods – Carbohydrates and their Roles* (Schultz, H. W., Cain, R. F. & Wrolstad, R. W., eds.), pp. 115–132, Avi Publishing Co., Westport, CT]. It is concluded that either the glycopeptides do not contain a hydrogen bond of that type, or, if they do, that it cannot be recognized by acid–base-titration studies.

Some years ago (Johansen *et al.*, 1961) acid–base-titration studies were carried out on a preparation of glycopeptide which had been isolated from hen's-egg albumin. Acid hydrolysates of the glycopeptide contained aspartic acid, leucine, valine, serine and threonine, but in non-integral amounts relative to the aspartic acid, in addition to mannose and glucosamine. The sequence of amino acids is known to be of the form represented by:

Three titrating groups were found, with pK_a values (at about 17°C) of 3.5, 6.7 and also apparently of about 9.5. The first and second of these pK_a values were reasonably assigned to the C- and N-terminal groups respectively of the peptide chain, but the nature of the third group was unexplained.

Glycopeptide that contained aspartic acid as the only amino acid was also found to have a third group ionizing at a relatively high pH, with a pK_a value of about 11.9 at 26°C (Fletcher, 1965; see also Montgomery *et al.*, 1965) and 4-*N*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine had a group with a pK_a value of 12.3 (Fletcher, 1965).

The identity of the group ionizing at about pH 12 is not rigorously established, but it was not un-

reasonable to suggest that it is the imido group linked directly to that *N*-acetyl-D-glucosaminyl residue involved in the carbohydrate–peptide linkage. It was also hypothesized that, in the glycopeptide that contained several amino acids, the hydroxyl group of the threonine residue, which is next-but-one to the asparagine residue, facilitates dissociation of the imido hydrogen atom through hydrogen-bonding (Plate 1). The implications of this deduction were discussed, especially with regard to the glycosylation of asparagine-34 of bovine pancreatic ribonuclease A. From these and other considerations it was proposed that, in order that glycosylation of an asparagine residue might occur, a necessary but not sufficient condition was that the latter must be followed on its C-terminal side by a serine or threonine residue (Marshall, 1967; Neuberger & Marshall, 1968). A sequence of the type ...Asn-X-Thr... or ...Asn-X-Ser... may be described as an asparagine sequon (Marshall, 1974).

At the time the initial studies were done (Johansen *et al.*, 1961), the stability to alkaline conditions of the carbohydrate–peptide linkage was unknown, and for this reason the titration curve was not studied very thoroughly above pH 9. It seemed desirable therefore to examine in more detail the acid–base titration of hen's-egg albumin glycopeptides, to reinvestigate the nature of the groups apparently ionizing at a pH around 9.5.

Methods

Preparation of hen's-egg albumin

Hen's-egg albumin was prepared from 200 fresh non-fertilized eggs (Appleby Farm, Ashford, Kent, U.K.) by $(\text{NH}_4)_2\text{SO}_4$ fractionation of the egg-white (Sørensen & Høyrup, 1915–1917). It was re-crystallized five times. The crystalline product gave two bands on polyacrylamide-gel electrophoresis under the conditions of Davis (1964), the electrophoretic heterogeneity being due to differing extents of phosphorylation (Perlmann, 1955). It exhibited one band when subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Marshall & Zamecnik, 1969). A solution of the crystalline egg albumin was dialysed exhaustively against water in the cold (4°C). The protein solution was poured into 5 vol. of methanol with stirring and the denatured egg albumin was collected by filtration and dried over CaCl_2 . The dried product weighed 114g.

Enzymic digestion of egg albumin

Denatured egg albumin (100g) was suspended in water (2 litres) at 37°C under a layer of toluene. The pH was adjusted to 2.8 with 4M-HCl, and pepsin added (Armour Laboratories, Eastbourne, Sussex, U.K.; 1.6g initially, followed by amounts of 1.6, 0.8 and 0.8g after 26, 48 and 110h respectively). Incubation throughout the total period of 160h was at 37°C and the pH was maintained at 2.8 by adding 4M-HCl as needed, automatically, by using a pH-stat (Radiometer, Copenhagen, Denmark). About 30% of the peptide bonds were split, as shown by ninhydrin assays (Moore & Stein, 1954) with glycine as standard.

The pH was raised to 7.8 with 4M-NaOH, and crystalline trypsin (Armour; 0.27g) and chymotrypsin (Armour; 0.27g) were added. Digestion was continued at 37°C for a further 56h, 4M-NaOH being added periodically for the first 17h and 4M-HCl for the remainder to keep the pH at 7.8. The pH was then adjusted to 5.5 by addition of 4M-HCl.

Isolation of glycopeptides

The digest was concentrated to a volume of 200ml by freeze-drying, and the solution which re-formed was centrifuged (1000g; 10min; 4°C). Propan-1-ol (1800ml) was added to the supernatant, and the mixture was kept at 4°C for 3h. The precipitate was collected by centrifugation (1000g; 10min) at 4°C, and taken up in 100ml of water. Acetic acid (0.6ml) was added to the viscous solution, which was chromatographed at 4°C on Sephadex G-25 (75cm × 6cm) previously equilibrated with 0.1M-acetic acid. Elution (with the latter solvent) was effected at a rate of 50ml/h, and the fractions (vol. 11 ml) shown by the method of François *et al.* (1963) to contain mannose

were combined and concentrated to about 25ml. This material was re-chromatographed on Sephadex G-25 as before, and the mannose-positive fractions were combined and subjected a third time to chromatography under the same conditions (Fig. 1).

Assays

Hexose. This was measured by the procedure of François *et al.* (1963), with mannose as standard.

Glucosamine. This was determined by a modification of the Morgan–Elson reaction (Kraan & Muir, 1957) after hydrolysis of samples in 4M-HCl at 100°C for 4h.

Amino acid analysis. Amino acid composition of hydrolysed samples (6M-HCl; 110°C; 16h) was determined with a Locarte amino acid analyser. The values of threonine and serine were corrected for observed losses of 3.6 and 7.7% respectively.

Total nitrogen. This was measured by the method of Jacobs (1962) as modified by Melamed (1965). The ninhydrin procedure of Rosen (1957) was used to determine the NH_3 released.

Paper electrophoresis (Whatman 3MM)

This was performed on glycopeptide samples (containing 250µg of mannose) in a cooled-plate apparatus (Atfield & Morris, 1961) for 2h at 32V/cm. The pH of the running buffer was 2.0 [12.5ml of 98% (v/v) formic acid, 43.5ml of acetic acid made up to 1 litre with water]. Staining for carbohydrate was effected with the periodate/permanganate reagent of Lemieux & Bauer (1954). The strips were washed with water once brown spots had appeared. Staining was also done with ninhydrin reagent [1% (w/v) ninhydrin (1,2,3-indantrione hydrate) in acetone].

Preparation of samples for acid–base titration

Ethanol (57ml) was added at room temperature (22°C) to 3ml volumes of fractions 104, 105, 106 and 107 (the numbering of the fractions is described in Fig. 1). There was about 40µmol of aspartic acid in acid hydrolysates of the portions taken. The precipitates were collected by centrifugation and each was dissolved in 0.5ml of water before re-precipitation by addition of 9.5ml of ethanol. The precipitates were collected separately, dried over CaCl_2 and dissolved in 4ml of deionized water. Solid KCl was added to each solution in amounts sufficient so that when the volumes were then made up to 5ml by addition of water the concentration of the electrolyte was 1M.

Acid–base titrations

Portions (3.6ml) of the solutions were titrated in a water-jacketed titration cell at a series of controlled temperatures, the surfaces of the solutions being continuously flushed with water-saturated N_2 . The solutions were stirred magnetically during titration. The pH was measured with a Radiometer pH-meter,

EXPLANATION OF PLATE I

Representation of a glycopeptide sequence [Asn(GlcNAc)-Leu-Thr-] found in hen's-egg albumin

Dreiding models were used to construct a model in which the hydroxyl group of the threonine residue (O_T) is hydrogen-bonded to the β -amido oxygen atom (O_{Am}) of the substituted asparagine residue. The ring oxygen atom of the sugar residue is denoted as O_R .

which was standardized with 0.05M-potassium hydrogen phthalate buffer (pH 4.00 at 20°C; Fisons Ltd., Loughborough, Leics., U.K.) and 0.05M-sodium borate buffer (pH 11.01 at 20°C; BDH Chemicals Ltd., Poole, Dorset, U.K.) at the various temperatures used. There was agreement between the two buffers at all temperatures to within 0.02 pH unit.

Titrant (0.1M-, 0.4M-, 1.0M- and 4.0M-KOH or HCl, prepared by dilution with freshly boiled, deionized water of BDH Volucon solutions, followed by standardization) was added from a micro-meter syringe (Burroughs Wellcome, Dartford, Kent, U.K.). A sufficient volume of the appropriate titrant was added to change the pH by about 0.1 unit at a time and the pH was read 30s later. Control titrations were carried out on 3.6ml of 1M-KCl solution and appropriate corrections were made for the volume differences that occurred during titration.

In two cases, after titration was completed (fraction 104 after titration at 60°C and fraction 105 titrated at 2°C), the glycopeptide was recovered. The solution was adjusted to pH 7 by addition of 1M-NaOH, and after concentration the glycopeptide was eluted from Dowex 50 (X8; H⁺ form; 55cm × 3cm) with water (Rosevear & Smith, 1961). In each case an acid hydrolysate of the fractions was analysed for amino acid content and mannose analysis was also done. The analyses of these glycopeptides, 104-G and 105-G, are shown in Table 1. Glycopeptide sample 105-G was re-titrated (see Table 2).

Results and Discussion

Glycopeptides from the third cycle of chromatography on Sephadex G-25 were eluted in a fairly broad band (Fig. 1). Fractions 90–103 were combined, but fractions 104–107 were used individually. Fractions 108–120 were also combined. Portions of the various fractions (250 µg of mannose in each case) were examined by paper electrophoresis (pH 2.0). Pooled fractions 90–103 gave only one ninhydrin-positive spot, which contained carbohydrate (13.1 cm from the origin towards the cathode), fraction 104 gave a single spot (14.8 cm), but combined fractions 108–120 yielded a glycopeptide (15.1 cm) and a second material (1.7 cm) which stained, faintly blue, only with ninhydrin. Thus fractions 90–103, 104, 105, 106 and 107 contained almost wholly glycopeptides, whereas combined fractions 108–120 contained glycopeptides (108–120)-G and a non-sugar-containing peptide also, (108–120)-P. Samples of these last two materials were separated by electrophoresis (Atfield & Morris, 1961) on paper which had been washed previously in the formic acid/acetic acid running buffer (see the Methods section). The peptide and glycopeptide were eluted from the paper with

buffer; the solutions were dried *in vacuo* and analysed.

The composition of the various fractions is of interest. In Table 1 the order in which the amino acids are given is that of the sequence surrounding the glycosylated asparagine residue in hen's-egg albumin, apart from alanine and glycine, whose positions are unknown. The known sequence (Cunningham *et al.*, 1963) in this region of the glycoprotein is:

...Glu-Glu-Lys-Tyr-Asn-Leu-Thr-Ser-Val-Leu...

and the asparagine residue is glycosylated.

The preparations of glycopeptides are heterogeneous with regard to carbohydrate, as may be deduced from the differences in the glucosamine/mannose ratios between one fraction and another. This finding is not unexpected, in view of the known heterogeneity of the carbohydrate moiety of the glycoprotein (Cunningham *et al.*, 1965; Bhoyroo & Marshall, 1965; Cunningham, 1968; Montgomery, 1972). They are also heterogeneous with respect to the polypeptide chain, probably for reasons of a nature which have been discussed (Marshall & Neuberger, 1972). Interpretation of the acid-base titration studies must take the heterogeneity of the peptide chain into consideration, and it is important therefore to discuss further the results presented in Table 1.

It is perhaps relevant to consider the results for the non-glycosylated peptide (108–120)-P first. Its origin in egg albumin was not determined, but there is in hen's-egg albumin (Milstein, 1968) a sequence of amino acids:

1 2 3 4 5 6 7
...Arg-Glu-Val-Gly-Ser-Ala-
8 9 10 11 12 13 14
Glu-Ala-Gly-Asp-Val-Ala-Ala...

Fig. 1. Fractionation of glycopeptide isolated from proteolytic digest of hen's-egg albumin on Sephadex-G25 (76cm × 6cm) at 4°C

Elution was effected with 0.1M-acetic acid. ■, Mannose (François *et al.*, 1963); ▲, nitrogen content (Melamed, 1965).

Table 1. *Molar compositions of acid hydrolysates of glycopeptides*

Combined fractions (90-103), 104, 105, 106, 107 and combined fractions (108-120) were obtained from chromatography on Sephadex G-25 (see Fig. 1). Combined fractions (108-120) were separated by electrophoresis (see the text) into a glycopeptide, (108-120)-G, and a peptide devoid of sugar, (108-120)-P. Samples 104-G and 105-G refer to glycopeptides isolated by Dowex 50 (X8, H⁺ form) chromatography after acid-base titration of portions of fractions 104 and 105. n.d., Not determined.

Component	Fraction ...	Molar composition									
		(90-103)	104	105	106	107	(108-120)	(108-120)-G	(108-120)-P	104-G	105-G
Glu		0.14	0.16	0.31	0.27	0.32	0.50	0.12	2.05	0.08	0.07
Lys		0.07	0.04	0.07	0.07	0.06	0.06	0.06		0.04	0.02
Tyr		0.07	0.06	0.07	0.08	0.07	0.05	0.05		0.08	0.08
Asp		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Leu		0.96	0.95	0.87	0.87	0.81	0.77	0.96	0.03	0.95	0.93
Thr		0.85	0.81	0.78	0.77	0.72	0.69	0.84	0.02	0.88	0.85
Ser		1.02	0.93	0.95	0.89	0.86	1.04	0.91	1.46	0.90	0.89
Val		0.16	0.25	0.48	0.41	0.41	0.56	0.25	2.82	0.19	0.08
Gly		0.14	0.15	0.35	0.30	0.43	0.77	0.15	2.74	0.07	0.06
Ala		0.16	0.15	0.39	0.15	0.42	0.81	0.15	3.19	0.08	0.08
GlcN		4.12	3.42	3.14	3.28	2.23	2.18	2.52		n.d.	n.d.
Man		5.24	4.78	4.55	4.25	3.82	3.21	4.13		4.93	4.85
Man/GlcN		1.27:1	1.40:1	1.44:1	1.29:1	1.71:1		1.64			
Ninhydrin value*		0.61	0.62	0.60	0.61	0.63					
Amount of (108-120)-P present (mol %)		0	3	5	5	5	19				

* Molar colour yield compared with glycine (=1) when the Rosen (1957) method was used.

Table 2. Assignment of titrating groups to those expected to be present from the results of analysis

The substance comprising carbohydrate covalently linked to L-asparagine [(Carb)Asn] is, strictly speaking, not a glycopeptide, and is therefore excluded when the term glycopeptide is used. Treatment of fractions: (a) titrated from pH4.1 to 12 and back-titrated to pH2: values were coincident; (b) two sets of forward titrations on different samples were done, one from pH4.2 to 12.0 and the other from pH4.2 to 2.2: back-titration in the former case did not lead to complete coincidence (see the text); (c) this sample was the glycopeptide which was recovered by procedures described in the text after titration at 2°C; forward titrations were carried out on different samples from pH6.9 to 12.3 and from pH6.9 to 3.0; (d) two sets of forward titrations on different samples were carried out, one from pH4.2 to 11.7 and the other from pH4.2 to 2.4: back-titration of the former did not lead to a completely coincident curve (see the text).

Fraction titrated								Assignment of titrating groups
(a)		(b)		(c)		(d)		
105 at 2°C	107 at 20°C	106 at 40°C	105-G at 40°C	104 at 60°C				
pK _a	Equiv.*	pK _a	Equiv.	pK _a	Equiv.	pK _a	Equiv.	
2.10	0.07	2.10	0.14			2.10	0.13	
3.30	0.95	3.15	0.80			3.26	0.87	
4.25	0.29	4.10	0.26			4.25	0.16	
6.98	0.95	6.25	0.80			5.92	0.81	
8.00	0.08	7.40	0.06					
8.75	0.12	8.60	0.06			7.78	0.17	
10.20	0.06	10.20	0.16			9.30	0.05	
10.75	0.07							

α -Carboxyl group of (Carb)Asn

α -Carboxyl group of glycopeptides and peptide(s) and possibly β -carboxyl group of peptide

γ -Carboxyl group of glutamyl residues

α -Amino groups of glycopeptides in which Asn is *N*-terminal

α -Amino groups of glycopeptides in which Asn is not *N*-terminal, and/or of peptide(s)

α -Amino group of (Carb)Asn

Hydroxyl group of tyrosine residue

ϵ -Amino group of lysine residue

* The values are the numbers of equivalents of the group titrating with the pK_a value shown, based on the presence of 1 mol of aspartic acid residue/mol of hydrolysed glycopeptide.

and the composition of a peptide, which may have arisen during proteolysis, composed of residues 2–13 would be closely similar to that found for peptide (108–120)-P. It seems likely that a number of the other fractions, whose analyses are displayed in Table 1, contain very small amounts of this peptide, although it was not revealed by electrophoresis, and the amounts likely to be present are also given in Table 1. The calculations are based on the relative excesses of glutamic acid in acid hydrolysates of the fractions, and other results discussed below support this assumption.

Acid-base titrations

The results obtained when fraction 105 was titrated at 2°C are shown in Fig. 2. Titration was effected in this case from pH 4.1 to pH 12; back-titration to pH 2 yielded values coincident with those obtained in the forward titration. The results were consistent with the theoretical calculation for the presence of the groups indicated in the first two columns of Table 2. The assignment of the values to the various groups titrated in the glycopeptide fractions was made by comparison of the values with those found in peptides with related structures. The two groups present in by far the largest amounts are ones with apparent pK_a values at 2°C of 3.30 and 6.98. The former is of the order expected for a terminal carboxyl group, although the β -carboxyl group of the small amount of peptide (108–120)-P may have made a small contribution to the amount found. The

other value may reasonably be assigned to the α -amino group of the glycopeptides which have an *N*-terminal asparagine residue, because the latter is the dominant *N*-terminal amino acid residue in glycopeptides prepared by the procedures used (Johansen *et al.*, 1961). Moreover the dissociation constants of the α -amino groups of L-aspartic acid diamide and of L-asparaginyglycine are relatively large [pK_a values of 7.00 at 25°C (Chambers & Carpenter, 1955) and 7.21 at 18°C (Leach & Lindley, 1954) respectively].

There is also evidence of a group, or groups, titrating at a pH higher than can be attributed to the α -amino group of the asparagine residue, as was seen also in the earlier studies (Johansen *et al.*, 1961). The curve is complex, and the tyrosine and lysine residues present in the preparation (0.07 residue of each; Table 1) must contribute those groups titrating with apparent pK_a values of 10.20 and 10.75 respectively. There is likely to be a small amount of carbohydrate covalently linked to L-asparagine in the preparation, in view of the higher amount of aspartic acid in acid hydrolysates of the preparation compared with all other amino acid residues, and the pK_a of 8.75 may be reasonably assigned to the α -amino group of this substance (Marshall & Neuberger, 1964). The remaining group in this region, 0.08 equiv. at pK_a 8.00, is of the order expected for a terminal amino group of a glycopeptide, or peptide, in which asparagine is not *N*-terminal. Interpretation of the titration curve revealed that all the groups titrating up to pH 11.5 could be accounted for by considering the results obtained in the light of the analytical data.

Titration curves were also constructed from the results of measurements made on various fractions at other temperatures (Table 2). It was found that a change was induced in glycopeptides which had been treated at high pH (>10) at temperatures of 40° and 60°C, and the back-titration from high pH was not coincident with the forward titration (Fig. 3). Because of this, apparent pK_a values of the various groups were estimated from the results obtained with fractions which had not been subjected to high pH.

The apparent pK_a values of the α -amino group of glycopeptides of the form Asn-Leu-Thr-Ser..., in which the asparagine residue is glycosylated, were used to calculate the enthalpy change on ionization, ΔH^0 , by application of the van't Hoff equation. The values of ΔG^0 and ΔS^0 were also calculated by using the standard thermodynamic equations:

$$\Delta G^0 = 2.303RT \cdot pK_a$$

and

$$\Delta G^0 = \Delta H^0 - T \cdot \Delta S^0$$

The value of ΔH^0 , assumed to be approximately constant over the temperature range studied (Fig. 4), was calculated to be 33.1 kJ·mol⁻¹ (7900 cal·mol⁻¹).

Fig. 2. Titration curve at 2°C of fraction 105 (see Table 2). The theoretical values are based on the results given in columns 1 and 2 of Table 2.

Fig. 3. Titration curve at 40°C of fraction 106 (see Table 2)

The theoretical values are based on the results given in columns 5 and 6 of Table 2, and those from which the pK_a values were deduced are shown by the solid line, before the glycopeptide fraction had been subjected to high pH (see the text). The back-titration is indicated by ---.

Fig. 4. pK_a values of the α -amino (▲) and α -carboxyl (●) groups, as a function of temperature, of the glyco-tetrapeptide Asn-Leu-Thr-Ser in which the asparagine residue is glycosylated

For details, see the text.

The standard free energy and entropy changes associated with the ionization at 25°C were found to be $37.2 \text{ kJ} \cdot \text{mol}^{-1}$ ($8900 \text{ cal} \cdot \text{mol}^{-1}$) and $-0.014 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ($-3.4 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$).

The values found are of the order that might be expected. The change in entropy is small, as is expected for the transfer of a proton from a charged amino group to a water molecule, an ionization

which does not involve net formation of new ions. It may be predicted that, for ionizations of this type, a group with a relatively low pK_a (6.43 at 25°C) for the group under consideration will exhibit a relatively low enthalpy of ionization ($33.1 \text{ kJ} \cdot \text{mol}^{-1}$) because of the relationship:

$$\Delta H^0 = T(2.303R \cdot pK_a + \Delta S^0)$$

Support for this deduction is given by the knowledge that the enthalpy of ionization of the imidazole group of L-histidine (pK_a at 25°C = 5.97; $\Delta S^0 = -0.017 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) is about $28.8 \text{ kJ} \cdot \text{mol}^{-1}$ and that of L-histidylglycine (pK_a at 25°C = 5.80; $\Delta S^0 = -0.006 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) is $31.3 \text{ kJ} \cdot \text{mol}^{-1}$. On the other hand the enthalpy of ionization of the α -amino group of glycylglycine, with a much higher pK_a value (pK_a at 25°C = 8.17; $\Delta S^0 = -0.016 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$), is greater ($41.9 \text{ kJ} \cdot \text{mol}^{-1}$).

General Conclusions

Glycopeptides isolated from proteolytic digests of glycoproteins are often heterogeneous with respect to the peptide moiety, because of incomplete enzymic splitting of the peptide bonds around a carbohydrate moiety, and the reasons for this have been discussed (Marshall & Neuberger, 1972). The glycopeptides isolated from hen's-egg albumin used in the present series of experiments, like the previous preparations (Johansen *et al.*, 1961), consisted predominantly of a tetrapeptide with *N*-terminal glycosylated asparagine and *C*-terminal serine residues, but smaller amounts of other glycopeptides and of a peptide were also present.

Titration of the preparations revealed the presence of a group titrating in the pH range expected for the *C*-terminal carboxyl group. There was also a group titrating with an apparent pK_a at 25°C of 6.43, and this may be reasonably assigned to the α -amino terminal group of the predominating glycotetrapeptide. We also found further ionization between pH 8 and 11. This was also noted previously (Johansen *et al.*, 1961), but the present results are much more extensive than those previously obtained. At the time the earlier experiments were done, the properties of the carbohydrate-peptide linkage were unexplored, particularly with regard to the stability to alkaline conditions, so that the titration was not continued higher than pH 9, and, moreover, quantitative amino acid analysis of the glycopeptide preparation was not done. It may be deduced from the present results that the release of H^+ ions in the pH range under consideration is due to ionization of small equivalent amounts of α -amino groups of peptides which do not have an *N*-terminal glycosylated asparagine residue, and of tyrosine and lysine residues.

It was assumed previously (Marshall, 1967; Neuberger & Marshall, 1968) that the ionization

occurring between pH 8 and 11 was that of the imido hydrogen of the nitrogen atom linking the *N*-acetyl-D-glucosaminyl moiety to the asparagine residue in the glycopeptide. It was also suggested that the pK_a , suggested to be of the order of 9.5, was lower than that of the corresponding imido group present in 4-*N*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine because of hydrogen-bonding between the hydroxyl group of the threonine residue in the glycopeptide and the β -oxygen atom of the substituted asparagine residue (Plate 1). This interpretation is not supported by the present results. In spite of this, the suggestion played a useful role, in that it was one of the factors which led to the prediction that an asparagine sequon is a necessary but not sufficient condition for glycosylation of an asparagine residue (Neuberger & Marshall, 1968). The fact that glycopeptides do not appear to contain a hydrogen bond of the type suggested or, if they do, that it cannot be recognized by acid-base-titration studies, does not rule out the possibility that the transition state involved in the glycosylation of an asparagine residue may involve assistance by the hydroxyl group of the relevant serine or threonine residue, especially as this would assist in placing the side chain of the intermediate amino acid residue in a favoured position. Alternatively, of course, the serine or threonine residue might help with recognition of active site of the sugar transferase.

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