Glycopeptides from Ovalbumin. Preparation, Properties, and Partial Hydrolysis of the Asparaginyl Carbohydrate*

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ABSTRACT: Ovalbumin has been digested with pronase to give the carbohydrate prosthetic group as a mixture of the asparaginyl(carbohydrate) and asparaginyl(carbohydrate)leucine derivatives. The two components were separated by zone electrophoresis.

The asparaginyl carbohydrate was characterized by group analyses, molecular weight determination, and infrared spectroscopy, and as its 2,4-dinitrophenyl derivative. Partial hydrolyses of the asparaginyl carbohydrate and its dinitrophenyl derivative by acid or emulsin afforded a series of degradation products from which the sequence of sugar residues in the molecule can be written GNAc-Man-GNAc-GNAc-$(Man)_4$-NHAsp.

The initial studies of ovalbumin concerning the structure of the carbohydrate moiety have centered around an analysis of the composition of the carbohydrate, the preparation of simple glycopeptide fragments, and the reactions which might indicate the nature of the protein-carbohydrate linkage (Jevons, 1958; Nuenke and Cunningham, 1961; Bogdanov *et al.*, 1962, 1964; Lee and Montgomery. 1962; Clamp and Hough, 1963; Marks *et al.*, 1962, 1963; Micheel *et al.*, 1963; Yamashina *et al.*, 1963; Tsukamoto *et al.*, 1964). From the standpoint of the carbohydrate content, ovalbumin is one of the simpler glycoproteins, containing only two sugars, D-mannose and 2-acetamido-2-deoxy-D-glucose, which are present as one oligosaccharide. In spite of this and the relative ease with which the pure protein can be prepared in large quantities, the composition of the oligosaccharide has been variously reported in the last few years (Bragg and Hough, 1961; Lee and Montgomery, 1961; Francois *et al.*, 1962; Hoermann and Gollwitzer, 1962; Cunningham *et al.*, 1963; Fletcher *et al.*, 1963a), which reflects the difficulty in obtaining absolute values for small amounts of carbohydrate residues in proteins. The present work was undertaken initially to prepare larger quantities of pure glycopeptide to restudy this problem. Furthermore, it was desired to prepare pure glycopeptides free from tyrosyl residues, which interfere in structural analyses by the periodate procedures (Bragg and Hough, 1961; Lee and Montgomery, 1961; Fletcher *et al.*, 1963a).

This paper describes the digestion of ovalbumin with pronase, a crystalline protease from *Streptomyces griseus* that has been applied previously to ovalbumin (Bogdanov *et al.*, 1964; Fletcher *et al.*, 1963a,b; Yamashina and Makino, 1962). Peptides were obtained that differ only in their amino acid composition. The simplest contains one residue of aspartic acid and the other contains the dipeptide aspartylleucine. These glycopeptides are identified by chemical and physical methods.

Information concerning the structure of the carbohydrate prosthetic group of ovalbumin, which is contained in the two glycopeptides described, is obtained from an examination of the products of hydrolysis, using either emulsin or acid catalysts. Acid catalysis resulted in the usual somewhat random cleavage, whereas emulsin acted principally to cleave the mannoside linkages. A different spectrum of products thus resulted. A further modification was introduced by the action of emulsin upon the DNP derivative of asparaginyl carbohydrate, in which case no hydrolysis of the asparaginyl-carbohydrate bond occurred (Lee *et al.*, 1964a).

Materials

Ovalbumin was prepared by the method of Kekwick and Cannan (1936). The glycopeptide Tyr-Asp(NH·carbohydrate) was prepared from ovalbumin as previously described (Lee and Montgomery, 1962). Sweet almond emulsin was obtained from Worthington Biochemical Corp. Pronase P, a crystalline protease from *Streptomyces griseus*, was obtained from Kaken Chemicals Co., Ltd., Tokyo, Japan; proteolytic activity 45,000 P.U.K.[1] per g (Nomoto and Narahashi, 1959).

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[1] P.U.K. = proteolytic unit of Kaken.

Methods

Chromatographic Analyses. Chromatographic analyses were carried out on Whatman No. 1 paper with one of the following solvent systems: (A) 1-butanol–acetic acid–water, 4:1:5, v/v, upper layer; (B) ethyl acetate–pyridine–water, 2:1:2, v/v, upper layer, or 60:25:20, v/v; (C) ethyl acetate–acetic acid–formic acid–water, 18:3:1:4, v/v.

The sugars were detected on the paper chromatograms by reaction with a silver nitrate–sodium hydroxide dipping reagent (Trevelyan *et al.*, 1950) and a periodic acid–benzidine reagent (Mowery, 1957). Amino compounds were detected with 0.3% ninhydrin in ethanol (Patton and Chism, 1951).

Paper Electrophoresis. An apparatus described by Katz *et al.* (1959) was used for high-voltage paper electrophoresis. The buffer composition was pyridine–acetic acid–water, 5:0.2:95, v/v; *p*H 6.3–6.4.

Amino Acid Analyses. Amino acid analyses were carried out with ion-exchange resin columns at 50° (Hirs *et al.*, 1954; Moore *et al.*, 1958) using an amino acid analyzer (Model K-5000, Phoenix Precision Instrument Co., Philadelphia). To avoid overlapping of the phenylalanine and glucosamine peaks on the 150-cm column, the change of the buffers was programed to take place 380 minutes (190 ml effluent volume) after the elution started (see also Walborg *et al.*, 1963).

Determination of Free Amino Groups. Amino compounds were determined by Rosen's modification of the ninhydrin method (Rosen, 1957).

Determination of D-*Mannose.* Neutral sugars were determined by the phenol–sulfuric acid (Dubois *et al.*, 1956) and the resorcinol–sulfuric acid procedures (Devor *et al.*, 1958). A modification of the phenol–sulfuric acid method was also used, which is sensitive to smaller amounts of sugar. To each aliquot (0.5 ml), containing 3–15 μg of hexose, in optically matched 12 × 75-mm tubes was added the phenol reagent (0.03 ml) and concentrated sulfuric acid (2 ml). For the latter addition, an ordinary 2-ml pipet was cut at the tip, so that 2 ml of acid was delivered in 2–3 seconds. The stream of acid was directed at the liquid surface to achieve maximum mixing as in the macro procedure. The absorbance at 490 mμ was measured after the tubes had cooled to room temperature. By this modification, 10 μg of D-mannose gave an absorbance at 490 mμ of 0.27 as compared to 0.14 in the original method.

Since the determination of D-mannose was sometimes carried out after reduction of an oligosaccharide with sodium borohydride, the effect of borate upon the resorcinol–sulfuric acid method was studied. Sodium borohydride solution (20 mg/ml) was allowed to stand in the cold overnight, and then was decomposed with hydrochloric acid. Aliquots of standard D-mannose solution were mixed with the varying amounts of the decomposed sodium borohydride solution and the resorcinol–sulfuric acid method was applied as usual. The results are shown in Table I.

TABLE I: Effect of Decomposed Sodium Borohydride on Resorcinol–Sulfuric Acid Procedure for D-Mannose.

D-Mannose (μg/tube)	Decomposed $NaBH_4$[a] (mg/tube)	Color Yield (%)[b]
16	0.1	99
16	0.2	99
16	0.3	99
16	0.4	99
16	0.5	100
16	1.0	105
20	5	105
20	10	109
20	15	110
20	20	111

[a] See text. [b] The color in the absence of decomposed sodium borohydride was taken as 100%.

Determination of Reducing Sugar. Reducing sugar was determined by the method of Park and Johnson (1949) with a slight modification. To a 1-ml sample of the aqueous sugar solution in 1.8 × 15-cm Pyrex tubes was added 0.5 ml each of ferricyanide and carbonate-cyanide solutions. A glass bead was placed on top of the tube. After heating for 15 minutes in a boiling-water bath, the tube was cooled and 5.0 ml of an equivolume mixture of the ferric ion solution and water was added. After 15 minutes, the absorbance at 690 mμ was read in a Coleman, Jr. colorimeter. Absorbancies of 0.07–0.42 were obtained for 0.5–3.0 μg of D-mannose. The reducing powers of D-mannose and 2-amino-2-deoxy-D-glucose were equal and about 80% of that for D-glucose.

Determination of Hexosamine. 2-Amino-2-deoxy-D-glucose was determined by the method of Boas (1953) and also by a ninhydrin method using the amino acid analyzer. In the latter case, an acid hydrolysate of the sample was dried *in vacuo* at room temperature and the residue was dissolved in water. An aliquot of the solution, containing approximately 0.1–0.6 μmole of 2-amino-2-deoxy-D-glucose, was added to the 15-cm column of the amino acid analyzer and eluted with *p*H 5.28 buffer. The peak effluent volume of 2-amino-2-deoxy-D-glucose was 34–35 ml at 50°. 2-Acetamido-2-deoxy-D-glucose was determined according to the procedure of Aminoff *et al.* (1952).

*Determination of 2-Amino-2-deoxy-*D*-glucitol and 2-Amino-2-deoxy-*D*-glucose.* 2-Amino-2-deoxy-D-glucitol and 2-amino-2-deoxy-D-glucose were determined on the 50-cm column of the amino acid analyzer. After reduction and decomposition of excess sodium borohydride, aliquots of the reduced oligosaccharides were hydrolyzed with 4 N hydrochloric acid for 5 hours at 100°. The hydrolysate was dried *in vacuo* over sodium hydroxide pellets. The residue was dissolved in 0.2 N sodium citrate buffer, *p*H 2.2, and applied to the column equilibrated with 0.35 N sodium citrate, *p*H 5.28. On

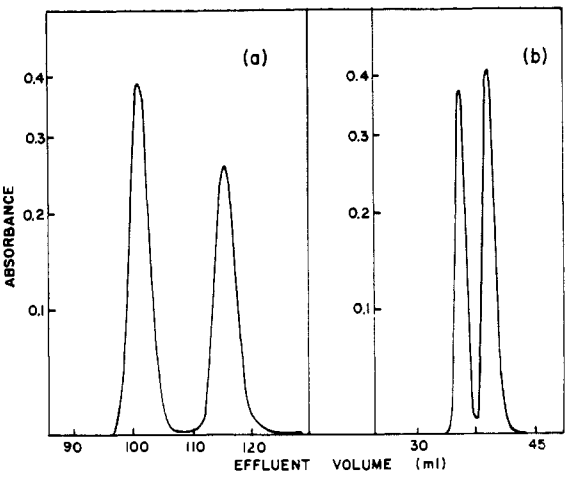

FIGURE 1: Elution diagrams for analyses of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucitol. (a) On 50-cm column, (b) on 15-cm column of the amino acid analyzer.

elution with the same buffer, at a rate of 30 ml/hour, 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucitol appear with peak effluent volumes of 101–102 ml and 115–116 ml, respectively. An example is shown in Figure 1a. The peak positions of these compounds were not influenced by the absence or presence of other salts. Both compounds were estimated by proportionation from known amounts of standard compounds.

If the hydrolysate contains only 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-glucitol, and contaminating ammonia, it is possible to analyze three samples consecutively. After an application of the first sample and elution with 40 ml of buffer, the liquid on top of the column was removed and the second sample was applied. After eluting another 40 ml of buffer, the third sample was applied. 2-Amino-2-deoxy-D-glucitol of the third sample appeared at about 196–198 ml, and the ammonia from the first sample appeared at 245–260 ml (peak volume 251–252 ml), followed by the ammonia of the second and third samples. When all the ammonia was eluted then the column was ready for another series of analyses. It was preferable, however, to wash the column with 0.2 N sodium hydroxide (5–10 ml) as soon as the last 2-amino-2-deoxy-D-glucitol was eluted, and then to equilibrate the column with buffer for an hour.

The color value of 2-amino-2-deoxy-D-glucitol was determined by reducing a chilled solution of 2-amino-2-deoxy-D-glucose (2–5 μmoles) with 5 ml of sodium borohydride (50–75 mg) solution in a 10-ml volumetric flask overnight at 4–5°. After decomposition of the excess sodium borohydride with hydrochloric acid, the solution was diluted to volume and aliquots were analyzed directly. Under these conditions, the reduction was complete as checked by the ion-exchange column. The color yield of 2-amino-2-deoxy-D-glucitol is 71–73% of that of 2-amino-2-deoxy-D-glucose.

Determination of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucitol was also possible using a 15-cm column, with peak volumes at 33–34 ml and 37–38 ml, respectively. Separation was not complete, but enough for quantitative determination by integration of the elution pattern area (Figure 1b). The size of the column did not influence the color values of these compounds. On either a 15-cm or a 50-cm column, 2-amino-2-deoxy-D-galactitol and 2-amino-2-deoxy-D-galactose were not separated from each other or from 2-amino-2-deoxy-D-glucitol.

Determination of the Molar Ratio of 2-Amino-2-deoxy-D-glucose and -D-mannose in Submicro Amounts of Oligosaccharides. The oligosaccharide fragment, equivalent to approximately 0.5 μmole of 2-amino-2-deoxy-D-glucose, was dissolved in 2 ml of 0.03 N hydrochloric acid and heated in an evacuated sealed ampule at 110° for 25 hours. The hydrolysate was evaporated to dryness *in vacuo* at 40° (bath temperature) and the residue was dissolved in 250 μl of water. Aliquots (25 μl) were spotted on Whatman No. 1 paper, together with known reference sugars, and chromatographed for 16 hours with solvent system C. After dipping the strip of paper containing one aliquot of the hydrolysate and the reference sugars with the silver reagent, the 2-amino-2-deoxy-D-glucose and -D-mannose areas were cut out from the other sections and eluted with a known volume of water. An extract of a paper blank was also prepared and the sugars in each extract were determined by the Park-Johnson procedure described above.

By this procedure Asp(NH·carbohydrate), Asp(NH·carbohydrate)-Leu, and Tyr-Asp(NH·carbohydrate) showed molar ratios of D-mannose to 2-amino-2-deoxy-D-glucose as 5:2.6, 5:2.7, and 5:2.7, respectively. Comparison of this submicro method with the more usual colorimetric methods is also seen in Table VI.

Results

Digestion of Ovalbumin with Pronase. Salt-free ovalbumin (47.5 g), as used in previous studies (Lee and Montgomery, 1961, 1962), was dissolved in water (*ca.* 900 ml) and denatured at *p*H 7.8 and 80–82° for 15 minutes. Sodium phosphate and calcium chloride were then added to the cooled solution to make the final concentration 0.05 M in phosphate and 0.001 M in calcium. After adjusting to *p*H 7.4, the enzymic digestion was carried out at 40° for 48 hours with pronase (200 mg), a second addition of enzyme (100 mg) being made after 24 hours. The digest was cooled and centrifuged, and the insoluble residue was washed with water (3 × 20 ml). A 97% recovery of D-mannose was found in the combined supernatants by the resorcinol method. The insoluble residue was suspended in water (about 50 ml), and a minimum amount of dilute alkali was added to obtain a clear solution, which contained a negligible amount of D-mannose.

The combined supernatant was concentrated by freeze-drying to about 550 ml, and 70 to 75-ml portions were fractionated on a column (4 × 65 cm) of Sephadex G-25 (medium), using 1 M acetic acid for elution. A

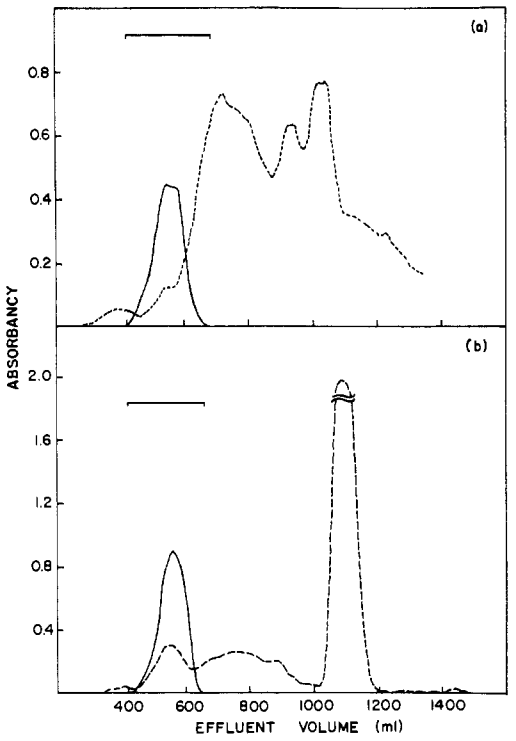

FIGURE 2: Gel filtration of the pronase digest of ovalbumin. (a) Original digest, (b) digest of the glycopeptide fraction obtained in (a) and indicated by the bar. Sephadex G-25 column, 65 × 4 cm; eluent, 1 M acetic acid; ———, carbohydrate; ---------, absorbancy at 230 mμ.

typical fractionation pattern is shown in Figure 2a. All the carbohydrate-containing fractions were combined and freeze-dried to give a white powder, 4.2 g, which was dissolved in 0.1 M Tris (50 ml). The solution was made 0.001 M in Ca^{2+} and the *p*H was adjusted to 7.8. Pronase (100 mg) was added and the mixture was incubated for 42 hours at 50° in the presence of a small amount of thymol. The digest was chromatographed directly in 10-to 15-ml portions on a column (2.5 × 100 cm) of Sephadex G-25 (fine), using 0.1 M acetic acid for elution. An example is shown in Figure 2b. All the carbohydrate-containing fractions were combined and freeze-dried. The recovery of carbohydrate as D-mannose was 98–100%.

Zone electrophoresis of the above glycopeptide mixture was conducted on a cellulose column (3 × 38 cm) in 1 M acetic acid as previously described (Lee and Montgomery, 1962). Samples (3–6 ml, equivalent to 170–335 mg of D-mannose) were applied to the top of the column, washed in with water (3–5 ml), and developed for 21–22 hours at 800 v. A typical separation of a 3-ml sample is shown in Figure 3. When a larger volume was used there was slight spreading of the peaks. The fast- and slow-moving components were glycopeptides Asp(NH·carbohydrate)-Leu and Asp(NH·carbohydrate), respectively. The corresponding

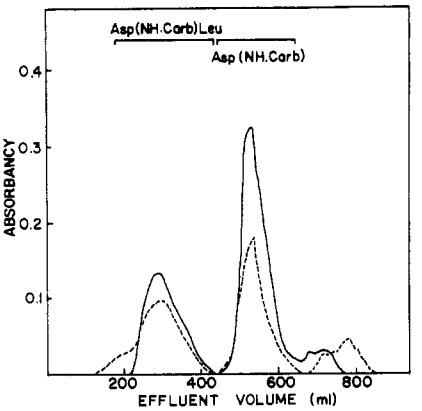

FIGURE 3: Separation of glycopeptides by zone electrophoresis in 1 M acetic acid. ———, carbohydrate; ---------, ninhydrin color.

fractions in each peak were combined and freeze-dried; 749 mg of Asp(NH·carbohydrate)-Leu and 1073 mg of Asp(NH·carbohydrate) were obtained. The total D-mannose recovered in these fractions was 94–96% of the amount present in the starting sample of ovalbumin.

Pronase Digestion of Asp(NH·Carbohydrate)-Leu. Glycopeptide Asp(NH·carbohydrate)-Leu (740 mg) in 0.01 M calcium chloride (15 ml) was digested with pronase (70 mg) at 50° in the presence of thymol. No buffering agent was added. During the reaction, aliquots were withdrawn and examined by paper electrophoresis (1 M acetic acid, 800 v, 2 hours). The position of each compound was detected with ninhydrin and the formation of Asp(NH·carbohydrate) from Asp(NH·carbohydrate)-Leu was readily observed, although after 3.5 days the reaction was still not complete. Zone electrophoresis of the digest gave Asp(NH·carbohydrate)-Leu (57%) and Asp(NH·carbohydrate) (43%), with the relative amounts given being based on the D-mannose contents.

Further Purification of Asp(NH·Carbohydrate) on DEAE-Sephadex. About 22 g of DEAE-Sephadex (A-25, medium, 3.8 meq/g) was successively treated with 0.5 M acetic acid and 0.5 M sodium hydroxide several times, and finally was washed with water before being packed into a column. The size of the column was 24 × 2 cm when washed free of the residual alkali with distilled water. In the presence of salts, the column decreased considerably in volume.

Glycopeptide Asp(NH·carbohydrate) (200 mg) was dissolved in water and added to the column, which was eluted with water (140 ml), followed by ammonium carbonate (160 ml, *p*H 8.6, 0.1 M in ammonia), and then ammonium bicarbonate (*p*H 7.2, 0.1 M in ammonia) at the rate of 50–60 ml/hour, 20-ml fractions being collected. Glycopeptide Asp(NH·carbohydrate) was found in the fractions of 400–500 ml effluent volume, the *p*H of which decreased from 10.2 to 8.9.

These fractions contain ammonium salts, which

TABLE II: Compositions of Asp(NH·Carbohydrate) and Asp(NH·Carbohydrate)-Leu.

Component	Glycopeptide	Hydrolytic Conditions[a] (N)	(°C)	(hours)	Weight (%)[b] Found	Calcd	Method[c]
D-Mannose	Asp(NH·carbohydrate)	No hydrolysis			51.4	52.2	Resorcinol-H_2SO_4
	Asp(NH·carbohydrate)	No hydrolysis			51.4		Phenol-H_2SO_4
	Asp(NH·carbohydrate)-Leu	No hydrolysis			47.0	49.1	Resorcinol-H_2SO_4
	Asp(NH·carbohydrate)-Leu	No hydrolysis			46.6		Phenol-H_2SO_4
N-Acetyl-D-glucosamine[d]	Asp(NH·carbohydrate)	4	100	7.3	38.6	39.2	Boas
	Asp(NH·carbohydrate)	4	100	7.3	38.8		Ninhydrin
	Asp(NH·carbohydrate)-Leu	4	100	5	37.0	36.6	Ninhydrin
$Asp(NH_2)$[e]	Asp(NH·carbohydrate)	6	110	22	7.9	8.5	Ninhydrin
	Asp(NH·carbohydrate)	4	100	7.3	7.9		Ninhydrin
	Asp(NH·carbohydrate)-Leu	6	100	22	7.3	7.9	Ninhydrin
Leucine	Asp(NH·carbohydrate)-Leu				5.9	6.8	Ninhydrin

[a] Hydrochloric acid. [b] Anhydrous residues. [c] See text. [d] Determined as D-glucosamine. [e] Corrected for decomposition (5%) and determined as aspartic acid.

were removed by gel filtration on a column (2 × 27 cm) of Sephadex G-25 using water as eluent. The carbohydrate-containing fractions were combined and freeze-dried, and the product was used for compositional analysis.

In another experiment, Asp(NH·carbohydrate) (1.07 g) was dissolved in water and added to the column (2 × 24 cm) of DEAE-Sephadex prepared as before. It was washed with water (120 ml), followed by elution with CO_2-saturated water at the rate of 5–10 ml/hour, 10-ml fractions being collected. Glycopeptide Asp(NH·carbohydrate) appeared in the fractions from effluent volume 1340 ml, peak volume 1520 ml, with tailing up to 1640 ml. The fractions from 1340 to 1560 ml were combined and freeze-dried to give 876 mg of material, which was used for molecular weight and titration studies. The rest of the carbohydrate-containing fractions, although appearing to be electrophoretically homogeneous, were not investigated.

Digestion of a Glycopeptide Mixture with Pronase. The glycopeptide preparation, equivalent to 115 mg D-mannose, obtained from ovalbumin by papain and carboxypeptidase digestion and gel filtration, but without electrophoresis (Lee and Montgomery, 1962), was digested further with pronase (15.2 mg) at 37° for 24 hours in 0.01 M phosphate buffer (*p*H 8.0). Upon gel filtration on a column (2.5 × 100 cm) of Sephadex G-25, equilibrated and eluted with 1 M acetic acid, only about one-half of the original tyrosine, as measured by the absorbance at 280 mμ, remained with the carbohydrate components, which were determined by phenol–sulfuric acid reagents. The carbohydrate-containing fractions were concentrated by freeze-drying and the digestion and gel filtration procedures were repeated twice more, first with pronase (9.8 mg) at 50° for 58 hours and finally with pronase (37.6 mg) at 45° for 10 hours. The resulting glycopeptide mixture was fractionated by zone electrophoresis, which showed a peak corresponding to glycopeptide Asp(NH·carbohydrate) and a negligibly small peak corresponding to Tyr-Asp(NH·carbohydrate) (Lee and Montgomery, 1962).

Compositional Analysis. Samples of purified Asp(NH·carbohydrate) (98 mg) and Asp(NH·carbohydrate)-Leu (25 mg) were placed in 5-ml volumetric flasks and dried to constant weight at 56° and 0.01 mm pressure for 24 hours over silica gel and then for 10 hours over phosphorus pentoxide. Standard solutions (5 ml) were prepared from the dried samples and aliquots were used for analysis of sugars and amino acids. The results (Table II) are expressed as weight percentage of anhydro sugars and free amino acids. The calculated values are based on the formula $Asp(NH_2)$-$(GNAc)_3$-$(Man)_5$ for Asp(NH·carbohydrate) and Asp-(NH_2)-$(GNAc)_3$-$(Man)_5$-Leu for Asp(NH·carbohydrate)-Leu.

Molecular Weight of Asp(NH·Carbohydrate). The molecular weight of Asp(NH·carbohydrate) was determined by a thermoelectric-type vapor-pressure osmometer (Model 301A Mechrolab, Mountain View, Calif.). A standard aqueous solution containing 23.86 mg/ml showed a molarity of 0.0153 when read against a standard curve of D-glucose. The molecular weight was thus calculated to be 1560. The determination was repeated at concentrations of 13.72 and 8.00 mg/ml, which gave molecular weights of 1512 and 1595, respectively.

Titration Curve of Asp(NH·Carbohydrate). Glycopeptide Asp(NH·carbohydrate) (20 mg) was dissolved in 0.1 M potassium chloride (2 ml) and titrated at 26° with 0.2 N potassium hydroxide and 0.2 N hydrochloric acid, each prepared in 0.1 M potassium chloride. A micro glass electrode and calomel electrode were used

in combination with a Radiometer Model SBR2c/SBUIa Titrigraph. The mixture was magnetically stirred and constantly flushed with nitrogen during titration. The acid and alkali were delivered from a syringe-type microburet.

A blank titration was conducted in the same way using the same volume of 0.1 M potassium chloride and the titre was subtracted from the sample titre to obtain the true acid or alkali uptake. The results are summarized in Figure 4.

Dinitrophenylation of Asp(NH·*Carbohydrate*). A solution of Asp(NH·carbohydrate) (4 ml, 5.38 mg D-mannose equivalent) was made 0.1 M with respect to sodium chloride and dinitrophenylated with 0.133 ml of dinitrofluorobenzene at *p*H 8.0 and 40°. The reaction was followed with a *p*H-stat (Titrator, TTTIa, Radiometer, Copenhagen), using 0.1 N potassium hydroxide delivered from a microsyringe (delivery of 0.2 ml/cm). The surface of the reaction mixture was constantly flushed with nitrogen. After about 90 minutes the reaction was essentially complete and extrapolation of the alkali consumption to zero time indicated a formation of 6.83 μmoles of acid, equivalent to 0.93 mole of dinitrophenylation per 5 moles of D-mannose.

For preparative purposes, 122 mg of Asp(NH·carbohydrate) was similarly dinitrophenylated with appropriate increases in the amounts of the reagents. The final reaction mixture (10 ml) was extracted with peroxide-free ether (2 × 10 ml), acidified with acetic acid, extracted with ether (3 × 20 ml), and freeze-dried. The DNP-Asp(NH·carbohydrate) was desalted by passing through a column (27 × 2 cm) of Sephadex G-25 (medium), using water or 0.1 M acetic acid for elution.

The molar absorbancies of the DNP-Asp(NH·carbohydrate) in various solvents are summarized in Table III.

TABLE III: Molar Absorbances of DNP-Asp(NH·Carbohydrate).

Solvent	Molar Absorbance	Wavelength (mμ)
Glacial acetic acid	15.0×10^3	340[a]
Distilled water	15.1×10^3	355
	15.4×10^3	360
0.1 M Ammonium hydroxide	15.0×10^3	355
	15.1×10^3	360

[a] Fraenkel-Conrat *et al.* (1955).

Partial Hydrolysis of DNP-Asp(NH·*Carbohydrate*). DNP-Asp(NH·carbohydrate) (11.2 mg) was hydrolyzed in 0.1 N hydrochloric acid (1 ml) for 165 minutes at 100°, and the resulting solution was dried *in vacuo* over sodium hydroxide. The residue was dissolved in water (2–3 ml) and washed through a column (3.5 × 1.2 cm) of DEAE-Sephadex (OH′ form) with water (15 ml).

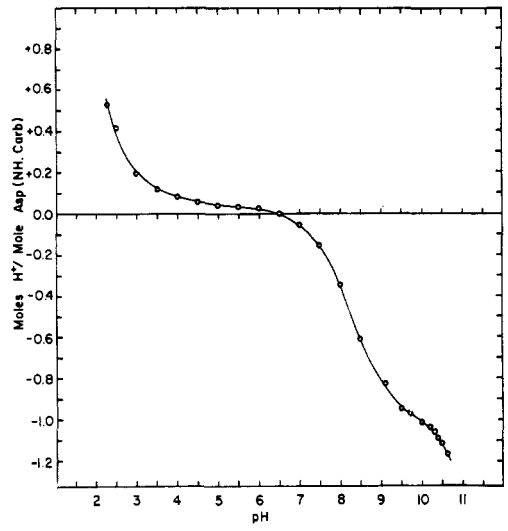

FIGURE 4: Titration curve of Asp(NH·carbohydrate) purified by elution of DEAE-Sephadex with CO_2-saturated water. Titration of 0.1 M potassium chloride at 26° with 0.2 N potassium hydroxide and 0.2 N hydrochloric acid using a syringe-type microburet and a Radiometer Model SBR2c/SBUla Titrigraph.

The aqueous effluent, containing the equivalent of 3.75 mg D-mannose by phenol–sulfuric acid reagents, was evaporated to dryness *in vacuo* and the residue was separated by chromatographic analysis (solvent system C). The components were 2-acetamido-2-deoxy-D-glucose (R_{man} 1.4), D-mannose, components Y (R_{man} 0.80) and X (R_{man} 0.35), the molar ratios relative to D-mannose being 0.85:1.0:0.14:0.55 as estimated by determination of the reducing values using the procedure of Park and Johnson (1949). No free 2-amino-2-deoxy-D-glucose was detected. Acid hydrolysis (0.03 N hydrochloric acid, 110°, 25 hours) of component X followed by paper chromatography (solvent system C) showed the presence of D-mannose and 2-amino-2-deoxy-D-glucose in the molar ratio 1.0:0.96, respectively, by the method of Park and Johnson (1949). After the reduction of X with sodium borohydride, no D-mannose could be detected in its acid hydrolysate.

The yellow substance adsorbed on the column of DEAE-Sephadex was completely eluted with 0.5 N hydrochloric acid. The eluate was dried *in vacuo* over sodium hydroxide and about half of the residue was spotted on a sheet of Whatman No. 3 paper (46 × 57 cm). The paper was first developed with solvent system A for 15 hours, dried at room temperature, and then subjected to paper electrophoresis at a gradient of 35 v/cm for 2 hours. The resulting pattern is shown in Figure 5. DNP-aspartic acid, which was the predominant product, was removed from the paper during elec-

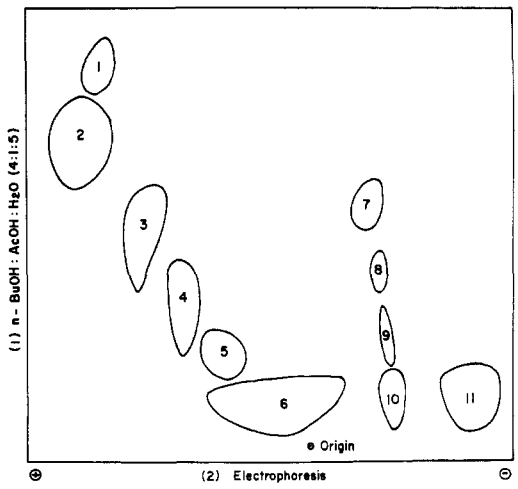

FIGURE 5: Chromatograph of DNP derivatives from the partial acid hydrolysate of DNP-Asp(NH·carbohydrate). Developed in first direction by paper chromatography and in second direction by paper electrophoresis in 0.1 M pyridine acetate, *p*H 6.4, for 2 hours at a gradient of 35 v/cm.

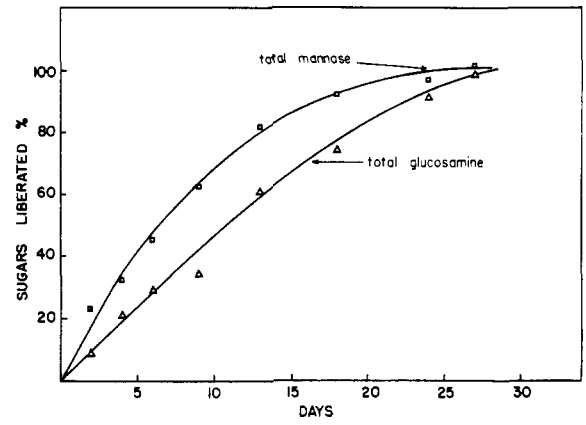

FIGURE 6: Release of carbohydrate fragments from Asp(NH·carbohydrate) by emulsin as described in the text.

trophoresis. Each yellow spot was cut from the paper and extracted with water, and the absorbance of the extract at 355 mμ was measured. On the basis of molar extinction coefficient of 15.1×10^3 the yields of the products in these chromatographic spots were: 2, 21%; 3, 1.4%; 4, 0.7%; 5, 1.7%; 6, 14%; 7, 2.5%; 8, 0.6%; 9, 8%; 10, 1%. In other hydrolysates obtained under the same conditions fraction 2 was isolated in 13 and 17% yields. All the fractions except 2 and 3 contained both D-mannose and 2-amino-2-deoxy-D-glucose. Fractions 2 and 3 contained 1.08 and 2.08 moles 2-amino-2-deoxy-D-glucose per mole of DNP residue, respectively, identifying these products as DNP-AspNH·GNAc and DNP-AspNH·$(GNAc)_2$. In neither fraction 2 nor 3 was any D-mannose detected by the resorcinol–sulfuric acid reagents, it having been separately demonstrated that the DNP residues have negligible influence in this determination.

In order that the mannose-containing fragments could be isolated for further study, a hydrolysis on a larger scale was carried out. DNP-Asp(NH·carbohydrate) (78 mg) was hydrolyzed in 0.1 N hydrochloric acid (3.8 ml) for 3 hours at 100° and dried *in vacuo* over sodium hydroxide. The solution formed by dissolving the residue in water was passed through a column (4 × 1 cm) of DEAE-Sephadex (OH′ form), which was washed with water until the effluent gave a negative test with phenol–sulfuric acid reagents.

The column was then eluted with 0.5 N hydrochloric acid (10 ml) to remove all the DNP compounds, and the effluent was dried *in vacuo* over sodium hydroxide. The residue was applied to a sheet of Whatman No. 3 paper (46 × 57 cm) along a 41-cm line, and subjected to electrophoresis in the *p*H 6.4 buffer for 3 hours at 35 v/cm. DNP-aspartic acid was again removed during the electrophoresis. The dried paper was cut into four sections.

The section that moved between +17 cm and +27 cm from the origin toward the positive electrode corresponded to the movement of components 2 and 3 in the small-scale experiment. This area was not included in the study nor was that around the origin, moving −5 cm to +6 cm. The areas between −5 to −14 cm and +6 to +17 cm were each eluted with water, the yellow eluate was freeze-dried, and the residues were each separated further by paper chromatography using solvent system A. Again each yellow band was cut out and eluted with water to obtain the fractions summarized in Table IV. Blank areas of paper from above the starting line were also cut and eluted similarly to obtain a blank solution.

D-Mannose and 2-amino-2-deoxy-D-glucose were determined in each fraction and, after proper correction for the blank, the molar ratios are tabulated in Table IV on the basis of one DNP group, which was estimated from measurements of absorbance at 355 mμ.

Digestion of Asp(NH·Carbohydrate) with Emulsin. About 45 mg of Asp(NH·carbohydrate) was incubated with 73 mg of emulsin in 20 ml of 0.01 M sodium acetate buffer (*p*H 4.1) at 38–40°. Solutions containing only emulsin and Asp(NH·carbohydrate), respectively, in the 0.01 M acetate solution were similarly incubated. Thymol crystals were added as preservatives. At intervals, an aliquot (0.2 ml) of the digest was added to a column of Dowex 50 X2 (H^+ form, 4 × 1 cm) which was then washed twice with water (2 × 2 ml). Asp(NH·carbohydrate) was completely adsorbed on the Dowex 50 resin and the monosaccharides D-mannose and 2-acetamido-2-deoxy-D-glucose were quantitatively recovered by the same procedure. The effluent was collected in 5-ml volumetric flasks and diluted to volume. Aliquots were used for the determination of total D-mannose (by phenol–sulfuric acid reagents) and 2-acetamido-2-deoxy-D-glucose as determined by the

TABLE IV: Molar Ratios of Sugar Residues in DNP-asparaginyl Oligosaccharides.

Fraction	Electrophoretic Movement (cm)[a]	Chromatographic Movement (cm)[b]	DNP (μmoles)	Yield[c] (%)	Mannose[d,e]	Glucosamine[b,e]
A	+6 to +17	36	203	0.45	1.17	1.11
B	+6 to +17	33	140	0.30	2.96	2.32
C	+6 to +17	29	68	0.15	4.14	2.24
D	−9 to −14	30	343	0.76	1.12	0.84
E	−9 to −14	26	128	0.29	2.12	1.37

[a] Fractions A, B, and C were separated after chromatography for 24 hours using solvent system A, whereas the movements given for fractions D and E resulted after 17.5 hours. [b] Determined by the ninhydrin method. [c] Calculated on a molar basis. [d] Determined by the resorcinol–sulfuric acid method. [e] Moles per mole of DNP residue.

Morgan-Elson reagents (1934). Also aliquots were hydrolyzed for the determination of total 2-amino-2-deoxy-D-glucose. The blanks were treated in the same way and the values so obtained were subtracted from those of the digestion mixture to calculate the amount of sugars liberated.

The results show that the liberation of free 2-acetamido-2-deoxy-D-glucose was negligible. After 13 days all the D-mannose and 2-amino-2-deoxy-D-glucose was found in the ion-exchange eluate.

At this time, aliquots (0.2 ml) from the digest and the enzyme blank were analyzed for aspartic acid with the amino acid analyzer. After correcting for the free aspartic acid in the autodigest of the enzyme blank, 0.87 mole of aspartic acid per 5 moles of D-mannose was found to be liberated.

In another experiment, Asp(NH·carbohydrate), equivalent to 23.6 mg of D-mannose but containing about 10% of Asp(NH·carbohydrate)-Leu, was incubated with 15.3 mg of the emulsin under the same conditions as before. The results are shown in Figure 6. After 32 days, the remaining digestion mixture was passed through a column (15 × 1 cm) of Dowex 50 X2 (H^+ form) and the eluate, containing the equivalent of 13 mg of D-mannose, was investigated by paper and charcoal chromatography.

Examination of a portion of the emulsin digest by paper chromatography (solvent system B, 11 hours) showed at least four sugars besides D-mannose. Again, no 2-acetamido-2-deoxy-D-glucose was found. Quantitative determinations of free D-mannose by paper chromatography combined with the resorcinol–sulfuric acid procedure revealed that 38–43% of the total D-mannose was liberated as the monosaccharide.

Equal weights (20 g) of Darco G-60 and Celite No. 535 were mixed, washed successively with 4 N hydrochloric acid (500 ml) and 95% alcohol (1000 ml), and dried by suction through a Büchner funnel. Charcoal-Celite mixture (7 g) was packed into a column (21 × 1 cm) under slight pressure and washed with 0.01 M formic acid (Taylor and Whelan, 1962).

The emulsin digest (containing 13.0 mg D-mannose in 25 ml) was added to the column, which was eluted with ethanol in 0.01 M formic acid, at the rate of 30 ml/hour, 6-ml fractions being collected. The alcohol concentration was increased stepwise, being 0% up to effluent volume 360 ml, at which point it was increased to 2.5%, then 5% at 720 ml, 7.5% at 1050 ml, and 15% at 1430 ml. The reducing value of the fractions was examined by the Park-Johnson method (1949), and the fractions in corresponding peaks were combined and freeze-dried. The results are summarized in Table V.

TABLE V: Charcoal Chromatography of the Emulsin Digest of Asp(NH·Carbohydrate).

Fraction	Effluent Volume (ml)	Ethanol (%)	Mannose Equivalent[a] (mg)
F-0	30–100	0	2.8
F-25	390–450	2.5	0
F-75	1100–1300	7.5	2.2
F-150	1475–1525	15.0	4.1

[a] By phenol–sulfuric acid. Total recovery was 71%.

Fraction F-150 was separated into its four components by paper chromatography for 40 hours on two sheets (46 × 20 cm) of Whatman No. 1 paper, using solvent system B. The composition of these components is given in Table VI. After reduction of fractions P-23 and P-45 with sodium borohydride and acid hydrolysis the molar ratio of 2-amino-2-deoxy-D-glucose to 2-amino-2-deoxy-D-glucitol was 1.0 in each case. Fraction P-6, however, gave a molar ratio of 3.3, similar to the value obtained under similar condition for authentic chitotriose[2] (Horowitz *et al.*, 1957).

[2] We are indebted to Dr. Saul Roseman of the University of Michigan for the samples of D-glucosamine oligosaccharides.

TABLE VI: Composition of Oligosaccharides from 15% Alcohol Eluate of the Charcoal Chromatography of the Emulsin Digestion of Asp(NH·Carbohydrate).

Oligosaccharide[a]	Chromatographic Mobility (cm)	Mannose (μmoles)[b]	Glucosamine (μmoles)[c]	Glucosamine/Mannose[d]	Glucosamine/Mannose[e]	Yields (%)[f]
P-6	6	7.8	6.3	3:3.8	3:3.7	11
P-23	23	11.1	5.0	2:4.4	2:3.9	17
P-45	45	1.3	0.8	2:3.1	2:3.0	2
P-80	80				2:2	1

[a] The oligosaccharides were extracted with water (10 ml), and the extract was used as the stock solution. [b] The total mannose was determined by the phenol–sulfuric acid method. [c] The total glucosamine was determined by the amino acid analyzer, after hydrolysis in 4 N HCl at 100° for 5 hours. [d] The glucosamine/mannose ratio was calculated from the foregoing data. [e] This glucosamine/mannose ratio was determined by paper chromatography in conjunction with the Park-Johnson method, after hydrolysis in 0.03 N HCl at 110° for 25 hours. [f] The yields of the oligosaccharides were calculated in terms of mannose recovered from the total mannose (13 mg) present in the original Asp-(NH·carbohydrate) (23.6 mg).

TABLE VII: Chromatographic Analysis of the Emulsin Digests of DNP-Asp(NH·Carbohydrate).

Component	R_{man}	Digestion Time (days) 10 (min)	1	2	4	9	11	14	20
1	0.00	Y[a]	Y	Y	Y	Y	Y	Y	Y
2	0.15					Y	Y	Y	Y
3	0.21				Y	Y	Y	Y	Y
4	0.34					Y	Y	Y	Y
5	0.48					Y	Y	Y	Y
6	0.69	C[a]	C	C	C	C	C	C	C
7	1.00	C	C	C	C	C	C	C	C
8	1.53					Y	Y	Y	Y
9	2.28					Y	Y	Y	Y

[a] Y, yellow spot; C, colorless spot detected by the silver nitrate reagent.

Digestion of DNP-Asp(NH·*Carbohydrate*) *with Emulsin.* About 8 mg of DNP-Asp(NH·carbohydrate) was dissolved in 10 ml of 0.1 M sodium acetate buffer, *p*H 4.1, and digested at 38–40° with crude emulsin (20 mg). At the intervals indicated in Table VII an aliquot (1 ml) of the digestion mixture was passed through a column of Dowex 50 X2 (H^+ form) and the effluent was freeze-dried. The residue was analyzed by paper chromatography (solvent system C) and the results are summarized in Table VII. Component 6 was D-galactose, which was present in the crude emulsin autodigest. Component 7 was D-mannose and it was the principal degradation product. Component 1 behaved like unreacted DNP-Asp(NH·carbohydrate), which after 9 days represented 85% of the starting material. Components 8 and 9 were produced in approximately 6 and 1% yield, based on the measurement of the absorbancy of the DNP group at 355 mμ, and upon analysis showed the presence of 2-amino-2-deoxy-D-glucose but no D-mannose. By paper chromatographic comparison with DNP-AspNH·GNAc and DNP-AspNH·$(GNAc)_2$, obtained from the partial acid hydrolysis of DNP-Asp(NH·carbohydrate), component 8 was identified with the former and component 9 with the latter compound. Component 4 upon analysis by the submicro procedure gave a ratio of 1.2 moles of 2-acetamido-2-deoxy-D-glucose to 1.0 mole of D-mannose.

It is most significant that neither DNP-Asp nor DNP-$AspNH_2$ was detected at any stage of the digestion.

Discussion

In the studies on the preparation of glycopeptides from ovalbumin by proteolytic digestion, a number of enzymes and enzyme combinations have been used

(Neuberger, 1938; Cunningham *et al.*, 1957; Jevons, 1958; Kaverzneva and Bogdanov, 1961; Johansen *et al.*, 1961; Lee and Montgomery, 1961; Yamashina and Makino, 1962). It has been generally shown that the peptide bonds around the carbohydrate group are more resistant to hydrolysis than might be predicted, particularly in the isolated glycopeptides (Fletcher *et al.*, 1963a; Lee and Montgomery, 1962). In common with the studies on several other glycoproteins, it is found, however, that the enzyme pronase from *Streptomyces griseus* (Nomoto and Narahashi, 1959) achieves extensive degradation in a few hours under conditions of *p*H (around *p*H 7) that do not destroy glycosidic bonds. In the present work, denatured but soluble ovalbumin was digested with pronase to give two principal glycopeptides, Asp(NH·carbohydrate) and Asp-(NH·carbohydrate)-Leu. These were separated in pure form by the combination of gel filtration and column electrophoresis, procedures which had been successful in previous studies (Lee and Montgomery, 1962; Montgomery and Wu, 1963). The carbohydrate recovered in these two products represented 95% of that in the original ovalbumin and was distributed 60% as the Asp(NH·carbohydrate) and 40% Asp(NH·carbohydrate)-Leu. The extent of the cleavage between the asparaginyl and glucosaminyl residues (Eylar, 1962) must be small in this case. The further digestion of the glycodipeptide with pronase showed that the Asp-Leu linkage was not easily cleaved, only half of the material being hydrolyzed in 3.5 days. Similarly, the mixture of glycopeptides obtained from ovalbumin by digestion with papain and carboxypeptidase (Lee and Montgomery, 1962), in which the Asp-Leu bond had been hydrolyzed and the Tyr-Asp bond was intact, was completely converted to Asp-(NH·carbohydrate) only after repeated digestion with pronase at elevated (45–50°) temperatures. The results of these experiments agree closely with those of Neuberger and coworkers (Fletcher *et al.*, 1963a). Furthermore, it is seen that the ease of hydrolysis of the respective bonds in the whole protein differs from that in the glycopeptides (Lee and Montgomery, 1962). Whether these observations are owing to specificity of the enzymes for macromolecular substrates, to the charged end groups of the glycopeptides in close proximity to the peptide bonds in question, or to steric hindrance by the carbohydrate moiety is a matter for further study.

In all the separations of glycopeptides into pure components, it has not been possible to effect the required result with gel filtration alone (Fletcher *et al.*, 1963a). This is not surprising in view of the similarity in molecular size and chemical composition of the glycopeptides in the mixtures.

The Asp(NH·carbohydrate) and Asp(NH·carbohydrate)-Leu compounds were isolated as colorless solids with the compositions indicated in Table II. The analytical figures clearly support the presence of 5 moles of D-mannose in each compound, in agreement with the results of other workers (Johansen *et al.*, 1961; Kaverzneva and Tsi, 1961; Nuenke and Cunningham, 1961; Clamp and Hough, 1963; Yamashina and Makino, 1962) for the D-mannose content of ovalbumin. It will be recalled that an earlier analysis of ovalbumin (Montgomery, 1961), which indicated 5 moles of D-mannose, was modified when it was found that a higher value of hexose was obtained if the protein was previously hydrolyzed (Lee and Montgomery, 1961). The latter observation was interpreted to indicate the presence of 6 moles of mannose but from the increasing body of evidence the figure must now be placed at 5 moles/mole. The experience serves to caution against the reliance of general colorimetric analyses when applied to unusual and frequently unknown mixtures (Fletcher *et al.*, 1963a,b) and may well explain the divergent analyses for D-mannose in various fractions from the enzymic digestion of ovalbumin (Cunningham *et al.*, 1963). Unfortunately, however, the amount of material for analysis does not always permit an application of the more specific macromethods, for example, the radioisotope-dilution procedure (Francois *et al.*, 1962), so that in the present study two colorimetric methods have been used, the one using resorcinol–sulfuric acid reagents being more specific for D-mannose. Similarly, two procedures were applied to the analyses for 2-amino-2-deoxy-D-glucose, that involving the amino acid analyzer (Moore *et al.*, 1958) being more specific. The latter procedure was also applied to the analysis of hydrolysates that contained 2-amino-2-deoxy-D-glucose and its reduced product, 2-amino-2-deoxy-D-glucitol, whereby structural information was gained from the reduction of oligosaccharides with a reducing sugar end group. It has been noted, using authentic samples of D-glucosamine oligosaccharides from chitose (Horowitz *et al.*, 1957), that the expected molar ratios of hexosamine to its reduced product were not obtained except for chitobiose. This is also seen in some fragments from the emulsin digestion of Asp-(NH·carbohydrate). In a qualitative way the information serves to demonstrate the nature of the reducing-sugar residue; the quantitative aspects are being studied further.

In spite of the knowledge that the analysis of carbohydrates requires conditions and procedures that differ for each monosaccharide residue being determined, and, in the case of 2-amino-2-deoxy-D-glucose, demanding conditions of hydrolysis that differ from that for D-mannose (Haab and Anastassiadis, 1961; Hartree, 1964), it is necessary in some instances to devise methods for submicro amounts of materials, such as might be separated by paper chromatography. In such cases the amount of material does not permit the determination of separate sugar residues by their more specific procedures. It has been found, however, that the molar ratio of D-mannose to 2-amino-2-deoxy-D-glucose in oligosaccharides and glycopeptides, at least those described in this paper, can be ascertained after hydrolysis at 110° for 25 hours using 0.03 N hydrochloric acid. The hydrolysate was chromatographed on paper and the D-mannose and 2-amino-2-deoxy-D-glucose spots, which were located by a guide strip of known sugars, were eluted with water and the reducing sugars were determined at 0.01- to 0.05-μmole

levels by the method of Park-Johnson (1949). The analyses of all the glycopeptides or oligosaccharides except one (Table VI) reported in the present study were carried out by the more macro procedures of phenol–sulfuric acid and resorcinol–sulfuric acid described earlier. In some cases they were compared with the results obtained by the above submicro procedure (Table VI). The molar ratios by these procedures differed by no more than 10%.

The composition of Asp(NH·carbohydrate) was in agreement with the molecular weight of 1560, which was found by vapor-pressure osmometry (Brady *et al.*, 1951). A solution of the compound, prepared by elution from DEAE-Sephadex with carbonic acid, followed by freeze-drying, in neutral potassium chloride showed *p*H 6.5 which has been taken as the isoelectric point, although the slow initial change of *p*H upon the addition of H^+ (Figure 4) suggests that this preparation may still contain some extraneous titratable base. The titration curve indicates that the isoelectric point may be closer to *p*H 5.5. There are two principal ionizable groups, with *pK* values of about 2.3 and 8.2 which are close to those reported for asparagine (pK_1 2.18, pK_2 8.87). A further ionization is suggested above *p*H 9.5, as was also noted by Johansen *et al.* (1961), with the possibility, however, that it may be due to some hydrolysis or other degradation at this high alkalinity.

On the basis of the purity of Asp(NH·carbohydrate), indicated by its electrophoretic mobility as a single component at *p*H 2.6, 6.3, 7.8, and 8.7, its elution as a single peak from DEAE-cellulose and Sephadex G-25, the compositional analysis, molecular weight, and titration curve, the nature of the carbohydrate prosthetic group of ovalbumin was well established. Attention was then turned to structural aspects of the octasaccharide, using the classical procedures of partial hydrolysis. The composition of the products of partial hydrolysis lead first to the sequence of monosaccharides in Asp(NH·carbohydrate). The deductions are facilitated by the presence in most cases of the DNP-Asp-residue which identifies the "reducing end" of the oligosaccharide. This is even more soundly based since the isolation by Neuberger and co-workers (Fletcher, *et al.*, 1963a,b), confirmed by Yamashina *et al.* (1963), of the fragment Asp-NH·GNAc from the hydrolysis of ovalbumin glycopeptides and its identification by chemical synthesis. From this information and the analysis of fragments summarized in Tables IV and VI, one sequence of monosaccharides may be

$$\begin{array}{l}\text{GNAc-Man-GNAc-GNAc-NH-Asp}\\ \qquad\qquad\qquad\quad\ \ |\\ \qquad\qquad\qquad\quad (\text{Man})_4\end{array}$$

This represents a modification of the structure of Clamp and Hough (1963) in the light of the identification of GNAc-GNAc-NH-Asp-DNP. Such a sugar sequence is also consistent, however, with the results of periodate oxidation (Lee *et al.*, 1964b), with the early release of D-mannose in the digestion of Asp-(NH·carbohydrate) or its DNP-derivative with emulsin, and with the appearance of approximately 1 mole of 2-acetamido-2-deoxyglucose when ovalbumin glycopeptides were treated with *N*-acetyl-D-glucosaminidase (Clamp and Hough, 1963; Kaufman and Marshall, 1964). However, from results of partial hydrolysis presented in this paper, little more can be deduced of the structure of the carbohydrate prosthetic group.

A most significant and unexpected observation was the inhibitory effect of the DNP group on the hydrolysis of the asparaginyl residue from DNP-Asp(NH·carbohydrate) by emulsin. After prolonged digestion no DNP-Asp or DNP-AspNH_2 were produced and the degree of digestion of the other parts of the molecule was very low, in contrast to the effect of similar treatment on Asp(NH·carbohydrate) shown in Figure 6. Such a modification in the action pattern of emulsin may enable a wider spectrum of oligosaccharide cleavage fragments to be obtained from glycopeptides with the "reducing-end" of the fragments labeled with the DNP-Asp residue. This presents a great advantage in identifying the origin of the fragment in the original glycopeptide and, furthermore, permits the calculation of the molar quantities of the sugar residues once their molar ratios are known.

References

Aminoff, D., Morgan, W. T. J., and Watkins, W. M. (1952), *Biochem. J. 51*, 379.

Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.

Bogdanov, V. P., Kaverzneva, E. D., and Andreyeva, A. P. (1962), *Biochim. Biophys. Acta 65*, 168.

Bogdanov, V. P., Kaverzneva, E. D., and Andreyeva, A. P. (1964), *Biochim. Biophys. Acta 83*, 69.

Brady, A. P., Huff, H., and McBain, J. W. (1951), *J. Phys. & Colloid Chem. 55*, 304.

Bragg, P. D., and Hough, L. (1961), *Biochem. J. 78*, 11.

Clamp, J. R., and Hough, L. (1963), *Chem. Ind. (London)*, 82.

Cunningham, L. W., Clouse, R. W., and Ford, J. D. (1963), *Biochim. Biophys. Acta 78*, 379.

Cunningham, L. W., Nuenke, B. J., and Nuenke, R. B. (1957), *Biochim. Biophys. Acta 26*, 660.

Devor, A. W., Conger, C., and Gill, I. (1958), *Arch. Biochem. Biophys. 73*, 20.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Eylar, E. H. (1962), *Biochem. Biophys. Res. Commun. 8*, 195.

Fletcher, A. P., Marks, G. S., Marshall, R. D., and Neuberger, A. (1963a), *Biochem. J. 87*, 265.

Fletcher, A. P., Marshall, R. D., and Neuberger, A. (1963b), *Biochim. Biophys. Acta 71*, 505.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Analy. 2*, 359.

Francois, C., Marshall, R. D., and Neuberger, A. (1962), *Biochem. J. 83*, 335.

Haab, W., and Anastassiadis, P. A. (1961), *Can. J. Biochem. Physiol. 39*, 671.

Hartree, E. F. (1964), *Anal. Biochem. 7*, 103.

Hirs, C. H. W., Stein, W. H., and Moore, S. (1954),

J. Biol. Chem. 11, 942.
Hoermann, H., and Gollwitzer, R. (1962), *Ann. 655*, 178.
Horowitz, S. T., Roseman, S., and Blumenthal, H. J. (1957), *J. Am. Chem. Soc. 79*, 5046.
Jevons, F. R. (1958), *Nature 181*, 1345.
Johansen, P. G., Marshall, R. D., and Neuberger, A. (1961), *Biochem. J. 78*, 518.
Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234*, 2897.
Kaufman, H. H., and Marshall, R. D. (1964), Abstracts of the Sixth International Congress of Biochemistry, New York, July 1964, Vol. II, p. 92.
Kaverzneva, E. D., and Bogdanov, V. P. (1961), *Biokhimiya 26*, 105.
Kaverzneva, E. D., and Tsi, D. F. (1961), *Biokhimiya 26*, 420.
Kekwick, R. A., and Cannan, R. K. (1936), *Biochem. J. 30*, 227.
Lee, Y. C., and Montgomery, R. (1961), *Arch. Biochem. Biophys. 95*, 263.
Lee, Y. C., and Montgomery, R. (1962), *Arch. Biochem. Biophys. 97*, 9.
Lee, Y. C., Wu, Y. C., and Montgomery, R. (1964a), *Biochem. J. 91*, 9c.
Lee, Y. C., Wu, Y. C., Wood, T. M., Wu, T. C., and Montgomery, R. (1964b), Abstracts of Papers, 147th Meeting, American Chemical Society, Philadelphia, Pa., April 1964, p. 6C.
Marks, G. S., Marshall, R. D., and Neuberger, A. (1963), *Biochem. J. 87*, 274.
Marks, G. S., Marshall, R. D., Neuberger, A., and Papkoff, H. (1962), *Biochim. Biophys. Acta 63*, 340.
Micheel, F., Ostmann, E. A., and Pielmeier, G. (1963), *Tetrahedron Letters 2*, 115.
Montgomery, R. (1961), *Biochim. Biophys. Acta 48*, 591.
Montgomery, R., and Wu, Y. C. (1963), *J. Biol. Chem. 238*, 3547.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.
Morgan, W. T. J., and Elson, L. A. (1934), *Biochem. J. 28*, 988.
Mowery, D. F., Jr. (1957), *Anal. Chem. 29*, 1560.
Neuberger, A. (1938), *Biochem. J. 32*, 1435.
Nomoto, M., and Narahashi, Y. (1959), *J. Biochem. (Tokyo) 46*, 653.
Nuenke, R. H., and Cunningham, L. W. (1961), *J. Biol. Chem. 236*, 2452.
Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.
Patton, A. R., and Chism, P. (1951), *Anal. Chem. 23*, 1683.
Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.
Taylor, P. M., and Whelan, W. J. (1962), *Chem. Ind. (London)*, 44.
Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature 166*, 44.
Tsukamoto, H., Yamamoto, A., and Miyashita, C. (1964), *Biochem. Biophys. Res. Commun. 15*, 151.
Walborg, E. F., Jr., Cobb, B. F., Adams-Mayne, M., and Ward, D. N. (1963), *Anal. Biochem. 6*, 367.
Yamashina, I., Ban-i, K., and Makino, M. (1963), *Biochim. Biophys. Acta 78*, 382.
Yamashina, I., and Makino, M. (1962), *J. Biochem. (Tokyo) 51*, 359.