A Comparison of Glycopeptides from the Ovotransferrin and Serum Transferrin of the Hen

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1. Glycopeptides were prepared from proteolytic digests of ovotransferrin and serum transferrin of the hen. The carbohydrate compositions and amino acid sequences of the peptides were studied. 2. The bulk of the carbohydrate of ovotransferrin is present as a single oligosaccharide composed of 4 residues of mannose and 8 residues of N-acetylglucosamine. Transferrin has most of its carbohydrate in a single unit composed of 2 residues of mannose, 2 residues of galactose, 3 residues of N-acetylglucosamine and either 1 or 2 residues of sialic acid. 3. The amino acid sequences of the glycopeptides carrying these different oligosaccharides are the same in ovotransferrin and serum transferrin, showing that the carbohydrate groups are attached to the same site on the protein molecule.

It is well known that in the hen the egg-white protein ovotransferrin (conalbumin) and the serum protein transferrin are very similar in structure and iron-binding properties (for review see Bearn & Parker, 1966). Since it is evident that for the egg-white protein the name 'ovotransferrin' (Komatsu & Feeney, 1967; Azari & Baugh, 1967) is more informative than the classical name 'conalbumin', it is suggested that the latter should not be used. It was proposed previously (Williams, 1962) that the covalent structures of the two proteins differ only in the nature of their oligosaccharide prosthetic groups, and the object of the present work was to discover whether these dissimilar prosthetic groups are attached to the polypeptide chain at the same point or at different points. For this purpose a variety of proteolytic enzymes was used to prepare glycopeptides from transferrin and ovotransferrin. From the overlapping peptides obtained the amino acid sequence in the vicinity of the point of attachment of the carbohydrate was determined, and it is shown below that most of the carbohydrate is attached to the same position in both proteins. A brief account of part of this work has been published (Williams, 1967).

MATERIALS AND METHODS

Enzymes. The pepsin, trypsin and chymotrypsin used in this work were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Subtilisin (type VII), carboxypeptidase A and carboxypeptidase B were obtained

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from Sigma Chemical Co., St Louis, Mo., U.S.A. Pronase (grade B) was obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

Ovotransferrin. The iron complex of ovotransferrin was prepared as described previously (Williams, 1962) except that CM-cellulose was replaced by CM-Sephadex C-50. The protein was adsorbed on the exchanger in 0·1 M-sodium acetate buffer, pH 5·5, and eluted at the same pH with 0·5 M-sodium acetate buffer. The product was inhomogeneous and on starch-gel electrophoresis by the method of Poulik (1957) a major and a minor component were observed. Attempts to isolate these components by gel filtration or by ion-exchange chromatography were not successful, although it was subsequently found that they could be isolated by the method of isoelectric fractionation (Wenn & Williams, 1968).

Transferrin. This protein was prepared from hen plasma. Freshly drawn blood was mixed with $\frac{1}{10}$ vol. of 10% (w/v) trisodium citrate solution, and after removal of the erythrocytes the pH of the plasma was adjusted to 9·4 with 1 N-NaOH. Rivanol (3·5 vol. of a 0·4% solution of 6,9-diamino-2-ethoxyacridine lactate) was added to the supernatant (1 g./100 ml.). After filtration $\frac{1}{4}$ vol. of 1·0 M-sodium acetate buffer, pH 5·0, was added and the mixture was passed through a column of CM-Sephadex (20 cm. × 4 cm./l. of plasma) that had been equilibrated with 0·1 M-sodium acetate buffer, pH 5·0. Transferrin and γ-globulin were adsorbed on the exchanger and were subsequently eluted with 500 ml. of 0·5 M-sodium acetate buffer, pH 5·0. The γ-globulin was precipitated by dialysis against 40%-saturated $(NH_4)_2SO_4$ solution, and the transferrin was finally chromatographed on DEAE-cellulose with a linear buffer gradient from 5 mM-phosphate buffer, pH 7·4, to 75 mM-phosphate buffer, pH 4·4. Starch-gel electrophoresis of the product showed two principal components corresponding to the presence of one and two residues of sialic acid respectively (Williams, 1962).

Preparation of glycopeptides. Glycopeptides were prepared from transferrin and ovotransferrin by digestion

with various enzymes used either singly or in combinations. Pepsin, trypsin, chymotrypsin, subtilisin and Pronase were used, at an enzyme/substrate ratio 1:40 (w/w), and incubation was carried out at 37° for 20hr. Generally 0·5–2·0g. of protein was taken for each glycopeptide preparation, and dissolved in water or buffer solution to give a concentration of 2% (w/v). For pepsin treatment the solution was brought to pH 2·0 with 0·3 N-HCl; other enzymes were used at pH 8·0 in either 0·1 M-tris chloride solution or 0·2 M-N-ethylmorpholine acetate solution. Digestion with carboxypeptidase A or B was carried out in a 1% (w/v) solution of NH_4HCO_3. For chymotrypsin digestion ovotransferrin was heated in 1% (w/v) Na_2SO_4 solution at 90° for 15min. After being washed with water the precipitated protein was suspended in buffer at pH 8·0. After addition of the enzyme the precipitated protein slowly dissolved, leaving a red deposit of $Fe(OH)_3$. For trypsin digestion the protein was heated in 8 M-urea solution at 90° for 15min. After cooling, the solution of denatured protein was slowly added, with stirring, to a solution of trypsin in tris buffer to give a final urea concentration of 2 M. Incubation at 37° was then carried out.

After being concentrated by rotary evaporation the hydrolysates were passed through columns of Sephadex, and the presence of hexose in the fractions was detected by the orcinol–H_2SO_4 reaction (Winzler, 1955). Tryptic hydrolysates were passed first through Sephadex G-50 in 10mM-NH_4HCO_3 solution, and the hexose-containing material was re-filtered through Sephadex G-25 in 0·1 N-acetic acid. Other enzymic hydrolysates were passed directly through Sephadex G-25.

The peptic hydrolysate of ovotransferrin was further fractionated by chromatography of the hexose-containing material on a column of CM-Sephadex (3cm.×1cm.) with a linear buffer gradient from 0·1 M-sodium acetate buffer, pH 5·0, to 0·4 M-sodium acetate buffer, pH 5·0. Two hexose-containing peaks were obtained; they were desalted by passage through Sephadex G-25 before further purification.

Further purification of the glycopeptides was carried out by high-voltage paper electrophoresis under toluene or white spirit (Michl, 1951) with volatile buffer systems giving pH values of 6·5, 3·5 and 2·1 (Ambler, 1963). Peptides were detected on paper by staining with the cadmium acetate–ninhydrin reagent of Heilmann, Barrollier & Watzke (1957). When this method was not sensitive enough the chlorination method of Reindel & Hoppe (1954) was used. Peptides were eluted from the paper strips with water as the solvent.

Performic acid oxidation. Cleavage of disulphide bridges was carried out in two ways. In some cases performic acid (approx. 0·5ml./μmole of peptide) was added to the peptide dried down in a tube, and oxidation was allowed to occur for 1hr. at 0°. The sample was then dried *in vacuo* over NaOH pellets. Alternatively, paper strips cut out from electrophoretograms were exposed to performic acid vapour in a desiccator for 2hr. They were then dried in a fume cupboard and stitched to fresh sheets of paper for further electrophoresis (Brown & Hartley, 1966).

Amino acid analysis. Peptides were hydrolysed with 5·7 N-HCl in sealed evacuated tubes at 105° for 16hr. Amino acid compositions of the hydrolysates were determined, either qualitatively by high-voltage paper electrophoresis at pH 2·1 or quantitatively with the Beckman-

Spinco amino acid analyser. Amino acids were detected on the paper electrophoretograms by staining with a solution of 0·25% ninhydrin in ethanol containing 2% (v/v) collidine. Glucosamine, which was released from the glycopeptides, gave a spot that moved with approximately the same mobility as alanine and stained green with the ninhydrin-collidine mixture.

Amino acid sequence determination. The main method used was the 'dansyl'–Edman method of Gray & Hartley (1963). The cyclization step was performed in trifluoroacetic acid (Konigsberg & Hill, 1962). DNS-amino acids* were identified by electrophoresis at pH 4·4 in a horizontal-plate apparatus, and those regions of the electrophoretogram that were not adequately resolved were stitched to fresh sheets of paper and subjected to electrophoresis at pH 2·1 or chromatography in light petroleum (b.p. 40–60°)–acetic acid–water (10:9:1, by vol.). The main tryptic glycopeptide from ovotransferrin was also examined by the paper-strip modification of the Edman procedure (Schroeder, Shelton, Shelton, Cormick & Jones, 1963). Phenylthiohydantoins were identified by paper chromatography in heptane–pyridine (7:3, v/v) and by thin-layer chromatography (Cherbuliez, Baehler & Rabinowitz, 1964). The position of amide groups in peptides was determined by Offord's (1966) electrophoretic-mobility method. The mobilities of peptides at pH 6·5 are given relative to that of aspartic acid.

Carbohydrate analyses. The orcinol method (Winzler, 1955) was used for the total hexose contents of proteins and peptides. The ratio of galactose to mannose in transferrin was estimated by quantitative paper chromatography (Wilson, 1959). Hexosamine was determined either by the method of Rondle & Morgan (1955) or with the amino acid analyser after hydrolysis with 4 N-HCl at 105° for 4, 8 and 24hr. Sialic acid was determined by the thiobarbituric acid method (Warren, 1959).

For the identification of sugars, proteins or glycopeptides were hydrolysed with 0·1 N-HCl in sealed tubes at 105° for periods from 30min. to 3hr. The hydrolysates were examined by high-voltage paper electrophoresis with 0·02 M-borate buffer, pH 10·0, in a flat-plate electrophoresis apparatus. Alternatively, paper chromatography was used with the solvents butan-1-ol–pyridine–water (6:4:3, by vol.) or butan-1-ol–ethanol–water (4:1:1, by vol.). The hexosamine present in ovotransferrin was further identified by oxidation with ninhydrin (Stoffyn & Jeanloz, 1954). In some cases the oligosaccharides were subjected to methanolysis and trimethylsilylation, and the sugar derivatives determined by gas–liquid chromatography (Sweeley, Bentley, Makita & Wells, 1963).

RESULTS

Sugar analyses of ovotransferrin and transferrin

After hydrolysis of ovotransferrin with 0·6 N-hydrochloric acid at 105° for 2½hr. the only free sugars detected were mannose and glucosamine,

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* Abbreviations: DNS and 'dansyl', 1-dimethylamino-naphthalene-5-sulphonyl; PITC, phenyl isothiocyanate; Asp(CHO), carbohydrate attached to aspartic acid; Asx, aspartic acid or asparagine; $CySO_3H$, cysteic acid; GNac, N-acetylglucosamine.

Table 1. *Carbohydrate compositions of ovotransferrin and transferrin*

	Composition (moles/80 000 g. of protein)	
	Ovotransferrin	Transferrin
Mannose	3·5	3·5
Galactose	—	1·8
Glucosamine	5·6	5·7
Sialic acid	—	1·7

whereas transferrin yielded mannose, galactose and glucosamine; the presence of sialic acid in transferrin was indicated by the thiobarbituric acid method. Experiments on the sugar constituents of glycopeptides from ovotransferrin, described below, indicated that the hexosamine was present as *N*-acetylglucosamine. Table 1 shows the quantitative sugar analyses of the two proteins as determined by colorimetric methods.

Pepsin–Pronase glycopeptides from ovotransferrin

Ovotransferrin was digested successively with pepsin and Pronase and the hexose-containing material obtained by gel filtration on Sephadex G-25 was examined by electrophoresis at pH 6·5. Five main hexose-containing bands were observed. Peptides PP1, PP2 and PP3 were cationic and peptides PP4 and PP5 were approximately neutral at pH 6·5.

Peptide PP1. This peptide gave a pink colour with cadmium–ninhydrin and had the amino acid composition: Asp (1·0), Arg (0·9), His (0·8). By the 'dansyl'–Edman method the amino acid sequence was found to be His-Asp-Arg. Determination of the carbohydrate composition by gas–liquid chromatography showed that each mole of peptide PP1 contained 4·2 moles of mannose and 7·9 moles of glucosamine. Peptide PP1 was resistant to the action of leucine aminopeptidase, but the *C*-terminal arginine residue could be removed either by further treatment with Pronase or by treatment with carboxypeptidase B. These treatments converted peptide PP1 into peptide PP3 (see below).

Peptide PP2. This peptide stained yellow with cadmium–ninhydrin and had the amino acid composition: Asp (1·0), Arg (1·0). The sequence Asp-Arg was given by the 'dansyl'–Edman method. Peptide PP2 was resistant to further digestion by Pronase, but carboxypeptidase B converted it into a new band that stained yellow with cadmium-ninhydrin and migrated slowly towards the cathode at pH 2·1. This product appeared to be an aspartic acid–carbohydrate complex since after hydrolysis it had, by the colorimetric methods, the composi-

tion Asp (1·0), mannose (3·3), glucosamine (4·0). The carbohydrate was therefore attached to the aspartic acid residue.

Peptide PP3. This peptide, which gave a pink colour with cadmium–ninhydrin, had the amino acid composition: Asp (1·0), His (0·9). The *N*-terminal residue by the 'dansyl' method was histidine, giving the sequence His-Asp. Further digestion with Pronase had no effect on peptide PP3, but one treatment with PITC degraded it to the aspartic acid–carbohydrate complex.

Peptide PP4. This band was highly contaminated after electrophoresis at pH 6·5, but after electrophoresis at pH 3·5 and pH 2·1 a hexose-containing peptide was obtained that had the amino acid composition: Asp (1·0), Ala (0·7). The sequence by the 'dansyl' method was Ala-Asp.

Peptide PP5. This band was also impure, but by electrophoresis at pH 2·1 a glycopeptide was obtained in which the only amino acids present were leucine and aspartic acid. The *N*-terminal residue was aspartic acid and after one round of treatment with PITC free leucine was obtained. The amino acid sequence was therefore Asp-Leu.

It is likely that peptides PP1, PP2 and PP3 were overlapping glycopeptides derived from the structure His-Asp(CHO)-Arg. Peptides PP4 and PP5 appeared to be derived from a different part of the molecule. In peptide PP5 the carbohydrate must have been attached to the aspartic acid residue and the same was probably true for peptide PP4. Estimates of hexose recoveries in the different glycopeptide bands indicated that 83% of the total hexose of the hydrolysate was recovered in peptides PP1, PP2 and PP3; peptides PP4 and PP5 accounted for the remaining 17%.

Pepsin–subtilisin glycopeptides from ovotransferrin

After successive treatments of ovotransferrin with pepsin and subtilisin, the glycopeptide fraction gave three hexose-containing bands on electrophoresis at pH 6·5. The cationic peptide PS1 contained 85% of the total hexose and the neutral peptides PS2 and PS3 contained 15%.

Peptide PS1. This peptide, which gave a yellow colour with cadmium–ninhydrin, had the amino acid composition: Asp (1·0), Arg (0·8), Thr (0·6). The 'dansyl'–Edman method gave the amino acid sequence Asp-Arg-Thr. Degradation with PITC removed both the aspartic acid and the carbohydrate, showing that the carbohydrate was attached to the aspartic acid residue. The products of the treatment of peptide PS1 with a mixture of carboxypeptidases A and B were the free amino acids arginine and threonine and the aspartic acid–carbohydrate complex. These products were separated by electrophoresis at pH 2·1. The

aspartic acid–carbohydrate complex gave a yellow colour with cadmium–ninhydrin.

It was shown by Marks, Marshall & Neuberger (1963) that mild acid hydrolysis of the aspartic acid–carbohydrate complex from ovalbumin yielded an aspartic acid–glucosamine complex in the form of 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-β-D-glucose. An attempt was therefore made to isolate this substance from the aspartic acid–carbohydrate complex from ovotransferrin. After hydrolysis with 2N-hydrochloric acid at 100° for 20min., electrophoresis at pH 2·1 was carried out. Cadmium–ninhydrin revealed several bands staining pink and one band that stained yellow. The latter band contained no mannose and after hydrolysis with 5·7N-hydrochloric acid at 105° for 5hr. it gave aspartic acid (0·8), glucosamine (1·0) and ammonia. It seems likely that this compound was a β-aspartylglucosamine complex and represented the linkage between the oligosaccharide and the polypeptide chain.

When peptide PS1 was hydrolysed with 0·1N-hydrochloric acid for different periods of time it was found by paper chromatography that the sugar liberated earliest was N-acetylglucosamine, followed later by mannose and glucosamine. This suggests that the hexosamine was acetylated and that the oligosaccharide may have possessed some terminal non-reducing residues of N-acetylglucosamine. Further evidence that the amino group of the hexosamine was not free was the fact that treatment of peptide PS1 with DNS chloride followed by acid hydrolysis (5·7N-hydrochloric acid at 105° for 20hr.) did not give rise to the 'dansyl' derivative of glucosamine, as shown by electrophoresis of the hydrolysis products at pH 4·40. If peptide PS1 was first subjected to mild acid hydrolysis to remove acyl groups, 'dansyl' treatment led to the production of DNS-glucosamine.

Peptide PS2. Although a single glycopeptide band was observed on electrophoresis of peptide PS2 at pH 2·1, it appears likely that at least two peptides were present. Alanine, aspartic acid, threonine and leucine were present and both aspartic acid and alanine were N-terminal. After one cycle of treatment of peptide PS2 with PITC two main bands were observed on electrophoresis at pH 2·1. Peptide PS2-1 did not contain carbohydrate and was found by the 'dansyl'–Edman method to have the amino acid sequence Leu·Thr. Peptide PS2-2 contained carbohydrate and gave a yellow colour with cadmium–ninhydrin. Its amino acid sequence was found to be Asp(CHO)·Leu·Thr. It is suggested that peptide PS2 contained two glycopeptides with the following structures: Ala·Asp(CHO)·Leu·Thr and Asp(CHO)·Leu·Thr.

Peptide PS3. After purification by electrophoresis at pH 3·5 and pH 2·1 a single hexose-

Table 2. *Compositions of peptides PS2 and PS3*

| | Composition (molar proportions) | |
	Peptide PS2	Peptide PS3
Asp	1·0	1·0
Leu	0·7	0·7
Thr	0·6	0·5
Ala	0·4	—
Tyr	0·4	0·3
Ser	0·3	0·4
Gly	0·3	0·2
Man	2·6	2·4
GNac	4·9	4·6
Gal	0·4	0·4

containing band was obtained. It gave a yellow colour with cadmium–ninhydrin and contained aspartic acid, leucine, threonine, glycine and tyrosine. Aspartic acid was the only N-terminal group detected by the 'dansyl' method. After one cycle of treatment with PITC, however, electrophoresis of the residual peptide at pH 3·5 showed two main products, neither of which contained carbohydrate. Peptide PS3-1 had the structure Leu·Thr·Gly and peptide PS3-2 Leu·Thr·Tyr, as determined by the 'dansyl'–Edman method. It seems likely therefore that two neutral glycopeptides were present in peptide PS3 and that they had the following structures: Asp(CHO)·Leu·Thr·Gly and Asp(CHO)·Leu·Thr·Tyr.

New preparations of peptides PS2 and PS3 were made and although they appeared to be homogeneous by electrophoresis at pH 3·5 and pH 2·1 their compositions, shown in Table 2, suggested that they were heterogeneous. The carbohydrate compositions were determined by gas–liquid chromatography. They both appeared to contain glycine and tyrosine, although the earlier preparation of peptide PS2 contained glycopeptides that terminated at threonine. Unlike the main glycopeptide of ovotransferrin, peptides PS2 and PS3 contained galactose.

Pepsin–trypsin glycopeptides from ovotransferrin

After successive digestion of ovotransferrin with pepsin and trypsin, electrophoresis at pH 6·5 revealed three main hexose-containing bands. Peptide PT1 was cationic and peptides PT2 and PT3 were approximately neutral. As with the pepsin–subtilisin hydrolysate, the cationic glycopeptide accounted for 85% and the neutral glycopeptides for 15% of the total hexose.

Peptide PT1. After electrophoresis at pH 2·1 a glycopeptide was obtained with the qualitative amino acid composition: Asp (+), Arg (+), His (+), Leu or Ile (+). The ratio of mannose to

glucosamine was found by gas–liquid chromatography to be 1·0:2·0. Digestion of peptide PT1 with subtilisin gave two products, which were separated by electrophoresis at pH 6·5. Peptide PT1-1 contained only isoleucine and histidine; peptide PT1-2 was a glycopeptide that gave a yellow colour with cadmium–ninhydrin and whose mobility relative to that of aspartic acid at pH 6·5 was 0·14. The only amino acids found in peptide PT1-2 were arginine and aspartic acid. Since peptide PP1 was His-Asp(CHO)-Arg and peptide PS1 was Asp(CHO)-Arg-Thr, it is likely that peptides PT1-1 and PT1-2 had the structures Ile-His and Asp(CHO)-Arg respectively and that peptide PT1 had the structure Ile-His-Asp(CHO)-Arg. Since peptide PT1-2 had a unit net positive charge and a relative mobility of 0·14, an approximate molecular weight of 2500 can be deduced (Offord, 1966). The most probable numbers of sugar residues in peptide PT1-2 are therefore 4 of mannose and 8 of N-acetylglucosamine, giving a calculated molecular weight of 2561. These values suggest the structure Asp(Man₄,GNac₈)-Arg for peptide PT1-2.

The structure $Asp(Man_4,GNac_8)$-Arg for peptide PT1-2. From the structures of peptides PP1 and PS1 it is very likely that peptide PT1 had the structure Ile-His-Asp(CHO)-Arg.

Peptides PT2 and PT3. Although electrophoresis at pH 2·1 yielded apparently homogeneous glycopeptides, amino acid compositions showed the presence of many neutral amino acids in non-integral proportions in addition to aspartic acid. As with peptides PS2 and PS3 there therefore appeared to be considerable heterogeneity, and there was insufficient material to investigate peptides PT2 and PT3 further.

Trypsin glycopeptides from ovotransferrin

Electrophoresis at pH 6·5 of the glycopeptide fraction from a tryptic digest of ovotransferrin showed three main hexose-containing bands. The total hexose of the hydrolysate was distributed as follows: 60% in peptide T1, 20% in peptide T2 and 20% in peptide T3.

Peptide T1. This peptide gave a yellow colour with cadmium–ninhydrin. Table 3 shows its amino composition as determined after total acid hydrolysis for 20hr. and 66hr. The increase in the values for isoleucine and histidine on prolonged hydrolysis is consistent with the presence of the acid-resistant bond Ile-His. The N-terminal residue of peptide T1 was found to be glycine by the paper-strip Edman method and the N-terminal sequence of peptide T1 was found to be Gly-Leu by the 'dansyl'–Edman method. Partial acid hydrolysis in 12N-hydrochloric acid at 37° for 3 days of peptide T1 gave rise to five main products, which were separated by electrophoresis at pH 6·5. (a) Product

Table 3. *Amino acid composition of peptide T1*

	Composition (molar proportions)	
	20hr. hydrolysis	66hr. hydrolysis
Asp	1·0	1·0
Arg	1·0	0·9
Leu	0·9	0·9
Gly	0·8	0·7
Ile	0·4	0·8
His	0·6	0·8

T1-1 was glucosamine. (b) Product T1-2 was a dipeptide of isoleucine and histidine. Isoleucine was found to be N-terminal by the 'dansyl' method and prolonged acid hydrolysis was necessary to release DNS-isoleucine from the 'dansyl' peptide. The structure of product T1-2 was thus Ile-His. (c) Product T1-3 had the amino acid composition: His (0·8), Ile (0·8), Leu (1·0). The N-terminal residue was leucine, giving the structure of product T1-3 as Leu-(His,Ile). (d) Product T1-4 was the only carbohydrate-containing band. It gave a yellow colour with cadmium–ninhydrin and contained aspartic acid and arginine as its only amino acids. Aspartic acid was N-terminal. The structure of product T1-4 was therefore Asp(CHO)-Arg. (e) Product T1-5 contained only the free amino acids glycine and leucine. These data suggest that the structure of peptide T1 was Gly-Leu-Ile-His-Asp(CHO)-Arg.

Peptides T2 and T3. These peptides were not studied further.

Pepsin glycopeptides from ovotransferrin

Chromatography of the glycopeptide fraction from peptic digests of ovotransferrin on CM-Sephadex gave two glycopeptide peaks P1 and P2 (Fig. 1). On electrophoresis at pH 6·5 peptide P1 was approximately neutral and peptide P2 was cationic. Neither peptide reacted with p-dimethyl-aminobenzaldehyde, indicating the absence of tryptophan. Table 4 shows the amino compositions of peptides P1 and P2 after oxidation with performic acid. From their compositions it seems likely that the greater negative charge of peptide P1 was due to the possession of one extra residue of each of aspartic acid and glutamic acid. After performic acid oxidation peptide P1 gave rise to two new bands, peptides P1-1 and P1-2, which were separated by electrophoresis at pH 6·5. Peptides P1-1 and P1-2 appeared to represent two polypeptide chains that were joined together in peptide P1 by means of a single disulphide bridge.

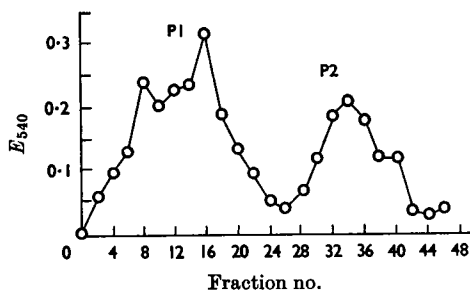*Peptide P1-1.* This peptide contained no carbohydrate. It stained pink with cadmium–ninhydrin

Fig. 1. Chromatography of glycopeptides from a pepsin digest of ovotransferrin on CM-Sephadex with a linear buffer gradient from 0·1 M-sodium acetate buffer, pH 5·0, to 0·4 M-sodium acetate buffer, pH 5·0. The orcinol assay was used.

Table 4. *Amino acid compositions of peptides P1 and P2 (performic acid-oxidized)*

	Composition (molar proportions)	
	Peptide P1	Peptide P2
Lys	0·9	0·9
His	0·6	0·7
Arg	0·9	1·0
Asp	5·0	3·9
Thr	2·9	2·8
Ser	1·0	1·0
Glu	1·0	—
Pro	1·3	1·0
Gly	1·1	1·0
CySO₃H	2·0	2·0
Ile	0·6	0·6
Phe	0·9	0·2

and its relative mobility towards the anode at pH 6·5 was 0·3. The amino acid composition was: Lys (0·9), CySO$_3$H (1·1), Asp (2·0), Thr (1·0), Ser (0·9), Pro (0·9). A net negative charge of 1 was indicated by the mobility and molecular weight of peptide P1-1, and one amide group was therefore probably present. Treatment of peptide P1-1 with PITC gave rise to a new peptide that stained yellow with cadmium–ninhydrin and contained no lysine. Digestion with Pronase also removed the lysine residue but did not degrade the peptide further. The main product of 'dansyl' treatment of peptide P1-1 was ε-DNS-lysine with only traces of α-DNS-lysine and bis-DNS-lysine. When the concentration of DNS chloride was increased there was a greater yield of bis-DNS-lysine and less of ε-DNS-lysine. The 'dansyl'–Edman method showed that the N-terminal sequence of peptide P1-1 was Lys-Thr-CySO$_3$H-Asx.

Three residues were removed from peptide P1-1 by successive treatments with PITC. The remain-

ing peptide stained yellow with cadmium–ninhydrin and after 'dansyl' treatment yielded DNS-aspartic acid. The relative mobility of this peptide at pH 6·5 was 0·55 and its structure was thus probably Asn-(Asp,Ser,Pro). Treatment of this peptide with PITC converted it into a pink-staining peptide with mobility 0·74. The amino acid sequence of this peptide was found by the 'dansyl'–Edman method to be Ser-Asp-Pro.

These results indicate that the structure of peptide P1-1 was Lys-Thr-CySO$_3$H-Asn-Ser-Asp-Pro.

Peptide P1-2. This peptide was a pink-staining glycopeptide that moved with mobility of 0·15 towards the anode at pH 6·5. The following amino acid composition was obtained: His (0·6), Arg (0·9), CySO$_3$H (1·0), Asp (3·0), Thr (1·9), Glu (1·0), Gly (1·0), Ile (0·7), Phe (0·9). Trypsin digestion split peptide P1-2 into two fragments: (i) the glyco-peptide Ile-His-Asp(CHO)-Arg, which has already been identified as peptide PT1; (ii) an acidic peptide, which represented the C-terminal portion of peptide P1-2 and had the expected amino acid composition: CySO$_3$H (1·0), Asp (2·0), Thr (1·9), Glu (1·0), Gly (0·9), Phe (0·9). The structure of this fragment was not analysed further.

Most of the information needed for the determination of the sequence of peptide P1-2 was provided by cleavage with subtilisin. There were four main products: two have already been identified as Ile-His (peptide PT1-1) and Asp(CHO)-Arg-Thr (peptide PS1). Two new acidic bands were also observed, peptides P1-2-1 and P1-2-2.

Peptide P1-2-1. This peptide stained yellow with cadmium–ninhydrin and had a mobility towards the anode of 0·49. It contained: Gly (0·9), Thr (1·0), Asp (1·1), CySO$_3$H (1·0). These results indicate a net negative charge of 1, and hence the aspartic acid must have been derived from asparagine. The amino acid sequence by the 'dansyl'–Edman method was Gly-Thr-CySO$_3$H-Asn. Removal of two residues by treatment with PITC gave a residual peptide that stained yellow with cadmium–ninhydrin and contained only aspartic acid and cysteic acid after total hydrolysis. Cysteic acid was N-terminal. The mobility of this dipeptide was 0·76, which confirmed the structure CySO$_3$H-Asn.

Peptide P1-2-2. This material formed a heterogeneous zone with mean mobility of 0·8 towards the anode at pH 6·5. Electrophoresis at pH 2·1 resolved three components.
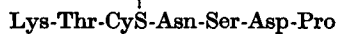
(i) Peptide P1-2-2-1. This peptide contained aspartic acid, glutamic acid and phenylalanine, and the 'dansyl'–Edman method gave the sequence Phe-Asp-Glu. The high mobility of this peptide at pH 6·5 indicated that both acidic residues carried negative charges.

(ii) Peptide P1-2-2-2. The amino acid sequence of this band was found by the 'dansyl'–Edman method to be Asx-Phe-Asp-Glu. Since the peptide stained yellow with cadmium–ninhydrin it is probable that the N-terminus was asparagine.

(iii) Peptide P1-2-2-3. Qualitative amino acid composition showed the presence of cysteic acid, aspartic acid, phenylalanine, glutamic acid, threonine and glycine, and the peptide gave an orange colour with cadmium–ninhydrin. This peptide was not analysed further, but the amino acid composition suggests that it included peptides P1-2-1 and P1-2-2-1 and would therefore have the structure Gly-Thr-CySO₃H-Asn-Phe-Asp-Glu.

The asparagine residue at the N-terminus of peptide P1-2-2-2 served to link together peptides P1-2-1 and P1-2-2-1 to give the sequence Gly-Thr-CySO₃H-Asn-Phe-Asp-Glu. That this is the correct ordering of these peptides is supported by the isolation of a peptide after digestion of ovotransferrin with trypsin and chymotrypsin, in which the amino acid sequence Thr-Gly-Thr-CySO₃H-Asx-Phe-Asx-Glu-Tyr was present (T. C. Elleman & J. Williams, unpublished work).

From the data given above it is concluded that the structure of peptide P1 was:

Peptide C6. After further electrophoresis at pH 3·5 peptide C6 yielded a glycopeptide with the following amino acid composition: CySO₃H (1·0), Asp (2·5), Thr (1·5), Glu (1·2), Gly (1·0), Ile (0·6), Tyr (0·5), Phe (1·0), His (0·6), Arg (1·0). This composition suggested that peptide C6 represented the glycopeptide that had already been identified in pepsin digests as peptide P1-2 with the addition of one residue of tyrosine at the C-terminal end. Treatment of peptide C6 with trypsin gave two products, which were separated by electrophoresis at pH 6·5.

(i) Peptide C6-1. This was a glycopeptide that gave the amino acid composition: Ile (1·0), His (1·0), Asp (1·2), Arg (1·3). This peptide therefore appeared to correspond to the glycotetrapeptide that had already been identified as peptide PT1.

(ii) Peptide C6-2. This stained yellow with cadmium–ninhydrin and migrated to the anode at pH 6·5 with mobility 0·7. It gave the amino acid composition: CySO₃H (0·6), Thr (1·6), Glu (1·2), Phe (1·1), Asp (2·0), Tyr (0·9). The mobility at pH 6·5 and the calculated molecular weight of 1096 indicated a net negative charge of 3, which is consistent with peptide C6-2 having the structure Thr-Gly-Thr-CySO₃H-Asn-Phe-Asp-Glu-Tyr.

Ile-His-Asp(CHO)-Arg-Thr-Gly-Thr-CyS-Asn-Phe-Asp-Glu
                                       |
Lys-Thr-CyS-Asn-Ser-Asp-Pro

Peptide P2. The amino acid composition of this peptide showed that it differed from peptide P1 in the absence of one residue of each of aspartic acid and glutamic acid. After performic acid oxidation of peptide P2, paper electrophoresis at pH 6·5 yielded a band identical in mobility with peptide P1-1. It therefore seems likely that the dipeptide Asp-Glu present at the C-terminal end of the glycopeptide P1-2 was absent from peptide P2, and since peptide P2 also showed a low but constant amount of phenylalanine there appeared to have been some loss of the phenylalanine residue present in peptide P1-2. It is therefore suggested that in the peptic hydrolysis of ovotransferrin incomplete cleavage of the bonds Asn-Phe and Phe-Asp occurred.

Chymotrypsin peptides from ovotransferrin

Paper electrophoresis of the glycopeptide fraction after chymotryptic digestion of ovotransferrin gave a very streaky pattern. Prior oxidation of the peptides with performic acid resulted in a much cleaner pattern. From the amino acid compositions of the bands the only ones that appeared to be relevant to the present study were peptides C6 and C8.

Peptide C8. Electrophoresis of peptide C8 at pH 3·5 yielded six bands. On total acid hydrolysis band C8-1 was found to contain cysteic acid, lysine and proline, in addition to other amino acids, which suggested that it might correspond to peptide P1-1. The amino acid composition showed that peptide C8-1 differed from peptide P1-1 in possessing one residue each of leucine and isoleucine, i.e.: CySO₃H (0·9), Asp (2·0), Thr (0·9), Ser (1·0), Ile (0·8), Leu (1·1), Lys (1·1), Pro (1·0). Lysine appeared to be the N-terminal residue since 'dansyl' treatment yielded a mixture of ε-DNS-lysine and bis-DNS-lysine, and after one treatment with PITC a yellow-staining band was obtained that contained no lysine and possessed a net negative charge of 2 (as deduced from its mobility of 0·66 and a value of 909 for the molecular weight from amino acid composition). These results agree with the structure Thr-CySO₃H-Asn-Ser-Asp-Pro-(Leu,Ile) for the PITC-treated peptide. This peptide was treated with carboxypeptidase A and the following products were obtained: (i) a large amount of leucine; (ii) a trace of isoleucine; (iii) a new peptide that had no leucine as shown by the amino acid composition: CySO₃H (1·2), Asp (2·0), Thr (0·5), Ser (0·7), Pro (1·1), Ile (1·0). It was therefore concluded that in peptide C8-1 leucine was C-terminal
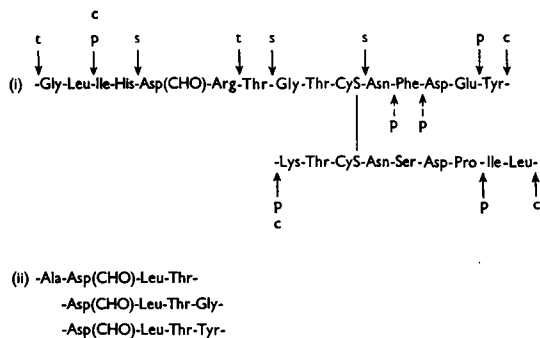
Fig. 2. (i) Basic carbohydrate site of ovotransferrin. ↓ and ↑, Sites of enzymic cleavage, indicated as follows: p, pepsin; c, chymotrypsin; t, trypsin, s, subtilisin. ↑, Sites of incomplete cleavage. (ii) Neutral carbohydrate sites of ovotransferrin.

and isoleucine occurred next to the *C*-terminal position.

From the results given above it appears that most of the carbohydrate present in an ovotransferrin preparation is attached to a single aspartic acid residue, and since this residue is flanked by histidine and arginine residues this structure is referred to below as the 'basic carbohydrate site'. The remaining part of the carbohydrate is attached to aspartic acid residues that are flanked by alanine and leucine, and these are therefore referred to below as 'neutral carbohydrate sites'. The structures of the basic and neutral carbohydrate sites are shown in Fig. 2, which also indicates the bonds that undergo cleavage by the various proteolytic enzymes.

Transferrin glycopeptides

In the second part of this study transferrin was digested with some of the enzymes that had been used in the preparation of glycopeptides from ovotransferrin. In some cases the amino acid sequences of the glycopeptides were determined; in others it was shown that the amino acid compositions or electrophoretic mobilities or both corresponded to those of the appropriate glycopeptides from ovotransferrin. Since the amino acid sequences of transferrin and ovotransferrin are probably the same (Williams, 1962), this was taken as reasonable evidence that the carbohydrate groups were attached to the same positions in both proteins, and no examples of non-corresponding glycopeptides were encountered. To distinguish them from the ovotransferrin glycopeptides, those derived from serum transferrin are marked with the prefix 't'.

Pepsin–Pronase glycopeptides from transferrin

After successive treatments of transferrin with pepsin and Pronase the glycopeptide fraction was subjected to electrophoresis at pH6·5, and four hexose-containing bands were observed.

Band tPP1. This band was approximately neutral at pH6·5 and could be resolved by electrophoresis at pH2·1 into a main component tPP1-1, which was found by the 'dansyl'–Edman method to have the structure Ala-Asp(CHO), and a minor component tPP1-2, which appeared to be an aspartyl–carbohydrate complex, since the only ninhydrin-positive materials present after total hydrolysis were glucosamine and aspartic acid.

Band tPP2. This slightly anionic band stained yellow with cadmium–ninhydrin and was found by the 'dansyl'–Edman method to be the dipeptide Asp(CHO)-Arg.

Band tPP3. This band was more anionic than peptide tPP2 and its amino acid composition was: His (+), Asp (+). There was insufficient material for end-group analysis.

Band tPP4. This strongly acidic band was resolved by electrophoresis at pH2·1 into two glycodipeptides. Peptide tPP4-1 stained pink with cadmium–ninhydrin and had the structure Ala-Asp(CHO); peptide tPP4-2 stained yellow and was found to be Asp(CHO)-Arg.

Thus peptides tPP1-1 and tPP4-1 both had the structure Ala-Asp(CHO), but possessed different electrophoretic mobilities at pH6·5. Similarly peptides tPP2 and tPP4-2 both had the structure Asp(CHO)-Arg.

Pepsin–subtilisin glycopeptides from transferrin

Only one hexose-containing band was observed on electrophoresis at pH6·5 after successive treatments of transferrin with pepsin and subtilisin. This peptide, tPS1, stained yellow with cadmium–ninhydrin and had the amino acid composition: Asp (1·0), Arg (0·9), Thr (0·8). The amino acid sequence was found to be Asp(CHO)-Arg-Thr by the 'dansyl'–Edman method. There was no evidence of the heterogeneity noted with peptides tPP2 and tPP1-1, nor was there clear evidence in this hydrolysate for the presence of the neutral carbohydrate site.

Pepsin–trypsin glycopeptides from transferrin

Two main hexose-containing bands, designated tPT1 and tPT2, were observed on electrophoresis of the glycopeptide fraction at pH6·5 after successive treatments of transferrin with trypsin. The neutral band tPT1 contained 38% of the total hexose of the hydrolysate and the anionic

Table 5. *Carbohydrate compositions of peptides tPT1 and tPT2*

| | Composition (residues/mol. of peptide) | |
	Peptide tPT1	Peptide tPT2
Mannose	2·0	2·0
Galactose	1·6	1·8
Glucosamine	3·1	3·3
Sialic acid	0·9	2·5

Peptide	
tPP3	(His,Asp(CHO))
tPP2	}
tPP4-2	} Asp(CHO)-Arg
tPS1	Asp(CHO)-Arg-Thr
tPT1	Ile-His-(Asp(CHO),Arg)
tP1-1	(Ile,His,Asp(CHO),Arg,Thr,Gly,Thr,CyS,Asx)
tP1-2	(Lys,Thr,CyS,Asx,Ser,Asx,Pro)
tPP1-1	}
tPP4-1	} Ala-Asp(CHO)

Fig. 3. Glycopeptides derived from serum transferrin.

band tPT2 41%. Both peptides gave the following amino acid composition: Ile (0·6), His (0·6), Asp (1·0), Arg (0·8). The partial amino acid sequence Ile-His-(Asp(CHO),Arg) was found for peptide tPT1 by the 'dansyl'–Edman method. In their carbohydrate compositions, as determined by gas–liquid chromatography, peptides tPT1 and tPT2 showed a clear difference in sialic acid content (Table 5), and this was probably the cause of the difference in electrophoretic mobilities.

Pepsin glycopeptides from transferrin

The glycopeptide fraction of a peptic digest of transferrin showed two carbohydrate-containing bands on electrophoresis at pH 6·5, as judged by the presence of glucosamine in the total acid hydrolysates. Peptide tP1, which was approximately neutral, and peptide tP2, which was anionic, had qualitatively the same amino acid compositions after performic acid oxidation and contained lysine, arginine, histidine, glycine, aspartic acid, proline, cysteic acid, threonine, serine and leucine or isoleucine. The oxidized peptides each gave rise to two main products on electrophoresis at pH 6·5. Bands tP1-1 and tP2-1 failed to stain with cadmium–ninhydrin, but were readily detected by the chlorine staining method.

Peptides tP1-2 and tP2-2. These peptides had the same electrophoretic mobility (0·3) at pH 6·5 and the same amino acid composition: Lys (0·9), CySO₃H (1·0), Asp (1·8), Thr (0·9), Ser (0·8), Pro (0·8). Carbohydrate was absent. They therefore appeared to correspond to the peptide identified as P1-1 in the pepsin hydrolysates of ovotransferrin.

Peptides tP1-1 and tP2-1. Peptide tP2-1 (mobility 0·27) carried a greater negative charge than did peptide tP1-1 (mobility 0·15). Total acid hydrolysis of these peptides gave rise to dark-coloured humin, suggesting the presence of sialic acid. The amino acid analysis for peptide tP1-1 is not available, but peptide tP2-1 had the composition: Ile (0·6), His (0·6), Asp (1·9), Arg (1·0), Thr (1·9), Gly (0·9), CySO₃H (1·0). It is likely that peptides tP1-1 and tP2-1 corresponded to the basic carbohydrate site

as identified in pepsin hydrolysates of ovotransferrin, with the qualification that for transferrin the cleavage at the Asn-Phe bond appears to have been complete. Carbohydrate analyses of peptides tP1-1 and tP2-1 were not carried out to test the assumption that the difference in their mobilities arose from a difference in their sialic acid contents.

Thus all the peptides derived from transferrin appear to correspond to glycopeptides already identified in hydrolysates of ovotransferrin (Fig. 3), and both the basic and neutral carbohydrate sites appear to be present in transferrin.

DISCUSSION

The main conclusion that may be drawn from these results is that the ovotransferrin and serum transferrin of the hen yield glycopeptides that differ only in the nature of their carbohydrate moieties. Since these peptides account for the bulk of the carbohydrate content of each protein it can be stated that the different carbohydrate moieties are attached to the same site on the protein molecule. Although it is not yet known how the carbohydrate groups of glycoproteins are constructed, it is clear that for a given protein that is synthesized in more than one tissue the specific structure of the carbohydrate group can vary from tissue to tissue. This type of variation in glycoprotein structure thus differs from that described for ovalbumin (Cunningham, Ford & Rainey, 1965; Levvy, Conchie & Hay, 1966) and myeloma globulin (Clamp, Dawson & Hough, 1966), where a glycoprotein synthesized in a single tissue is associated with a range of carbohydrate groups.

From the gas–liquid-chromatographic analysis of peptide PP1 from ovotransferrin [His-Asp(CHO)-Arg] it seems likely that the oligosaccharide attached to the basic carbohydrate site was composed of 4 residues of mannose and 8 residues of N-acetylglucosamine. The overall carbohydrate composition of ovotransferrin by colorimetric methods was 3·3 residues of mannose and 5·7 residues of glucosamine/unit of molecular weight

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80000. Therefore it is unlikely that more than one oligosaccharide molecule is present, on average, on each ovotransferrin molecule, and if two protein sub-units should be present, as has been suggested for human serum transferrin by Jeppsen & Sjöquist (1967), they cannot both carry oligosaccharide groups at the same time.

Serum transferrin is not homogeneous with respect to its main oligosaccharide group, since the glycopeptides obtained by digestion with pepsin and pepsin–trypsin appeared in two electrophoretically distinct forms depending on the possession of either one or two residues of sialic acid. These forms were probably derived from the two varieties of transferrin observed in starch-gel electrophoretograms, since the effect of neuraminidase treatment suggested that the protein band that had the higher mobility possessed two residues of sialic acid and the slower band only one (Williams, 1962). Three possible explanations of this may be suggested: (i) the addition of the terminal sialic acid residues during synthesis of the protein may be incomplete; (ii) serum transferrin may be synthesized in more than one tissue, each of which may attach a characteristic oligosaccharide group to the protein; (iii) the completed transferrin molecule may be subjected to the action of neuraminidases. It is not possible to choose between these alternatives at present.

Colorimetric analysis of hen serum transferrin gave 3·5 residues of mannose, 1·8 residues of galactose, 5·7 residues of glucosamine and 1·7 residues of sialic acid/unit of molecular weight 80000. The major oligosaccharide as represented by peptides tPT1 and tPT2 had the following molar composition as determined by gas–liquid chromatography: mannose (2·0), galactose (1·6–1·8), glucosamine (3·1–3·3), sialic acid (1 or 2·4). The galactose and sialic acid contents of serum transferrin are therefore accounted for by the presence of one major oligosaccharide per molecule of protein. The values for glucosamine and mannose in the glycopeptide and in the protein are not in such good agreement, and this might be due either to errors in the analyses or to the presence of a second carbohydrate group containing mannose and glucosamine but not galactose or sialic acid. The first alternative seems more likely in view of the recovery of 80% of the total hexose in the peptides tPT1 and tPT2.

In both ovotransferrin and serum transferrin the carbohydrate present at the basic carbohydrate site accounted for 80–85% of the total hexose of the protein. The remainder appeared to be present at one or more neutral carbohydrate sites. Nothing is known about the carbohydrate composition of these glycopeptides from serum transferrin, but in ovotransferrin the neutral glycopeptides differed from the basic glycopeptide in possessing galactose in addition to mannose and glucosamine. There appear to be three possible interpretations of the presence of the neutral glycopeptides. First, they may be derived from unrelated contaminant glycoproteins. For example, Nuenke & Cunningham (1961) proposed that the oligosaccharide of ovalbumin is bound to the aspartic acid residue in the structure Tyr-Asp-Leu-Thr-Ser-(Val,Leu). Some of the neutral glycopeptides encountered in the present work might therefore conceivably have been derived from ovalbumin present in the ovotransferrin preparations, although the level of such contamination must have been low since ovalbumin was not observed in starch-gel electrophoretograms. Secondly, they may be derived from variant types of ovotransferrin in which the amino acid sequence in the vicinity of the carbohydrate site was different from that found in most of the molecules. Thirdly, the transferrin molecule may possess two or more sites where carbohydrate can be attached, but clearly the basic site is utilized more often than the neutral sites. The possibility that the major and minor components of ovotransferrin observed in starch-gel electrophoresis experiments differ in their glycopeptide constituents is attractive and remains to be investigated.

As to the functional significance of the difference in the carbohydrate groups of ovotransferrin and serum transferrin nothing is known and no detailed comparative study of the conformations of ovotransferrin and serum transferrin appears to exist. The proteins are, however, immunologically similar (Kaminski & Durieux, 1956; Williams, 1962), and a comparison of the iron-binding properties of human transferrin and hen ovotransferrin (Aisen, Leibman & Reich, 1966) revealed an overall similarity except for a difference in proton-relaxation rate. Since these proteins presumably differ from one another in both amino acid sequence and carbohydrate structure this difference cannot be interpreted at the moment. There appears to be no gross change in the conformation of human serum transferrin as a result of removing terminal sialic acid residues with neuraminidase (Bezkorovainy, 1966), and Morgan, Marsaglia, Giblett & Finch (1967) found that neuraminidase-treated transferrin behaved normally in ^{59}Fe-exchange experiments in vivo.

Chicken serum transferrin appears to differ from human serum transferrin in both the number and the composition of its prosthetic groups. Jamieson (1965) showed that in human transferrin the total carbohydrate was distributed equally between two carbohydrate groups, each of which was composed of N-acetylneuraminic acid (2 residues), galactose (2 residues), mannose (4 residues) and N-acetylglucosamine (4 residues). The amino acid sequences

to which these apparently identical oligosaccharides are attached were not identical, as was shown by the amino acid compositions of the two glycopeptides obtained after hydrolysis with Pronase. Parker & Bearn (1962) studied the effect of neuraminidase treatment on the mobility of primate and cattle transferrin in starch-gel electrophoresis. Their results suggested that in these groups, as in the hen, transferrin possessed only two residues of sialic acid.

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