A Comparison of Glycopeptides from the Transferrins of Several Species

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1. The carbohydrate compositions of human, pig and cattle transferrins and duck ovotransferrin have been determined. 2. Glycopeptides have been prepared from these transferrins and their carbohydrate compositions and amino acid sequences determined. One of the glycopeptides from human transferrin carries the C-terminal residue of the protein. 3. Each transferrin yielded two glycopeptides that appeared to be identical in carbohydrate composition but different in amino acid sequence. The two glycopeptides have been distinguished as type A, in which the residue following Asn(CHO) (where CHO represents a carbohydrate moiety) is a basic amino acid and type B in which Asn(CHO) is followed by a neutral aliphatic amino acid. Cattle transferrin is exceptional in having two glycopeptides in which this position is occupied by serine. 4. It is suggested that each molecule of human and cattle transferrin and duck ovotransferrin carries an average of two carbohydrate prosthetic groups. Hen and pig transferrins appear to carry only one carbohydrate group per mol of protein. 5. The N-terminal sequences of hen and duck ovotransferrins and of cattle, human and pig transferrins were also determined.

Transferrins probably belong to a family of homologous proteins which characteristically bind two atoms of a variety of metals and consist of a single polypeptide chain of molecular weight approx. 77000 (Greene & Feeney, 1968; Mann *et al.*, 1970; Hudson *et al.*, 1973). The amino acid compositions of different transferrins are similar (Feeney & Komatsu, 1966; Hudson *et al.*, 1973), but there is very little detailed comparative information on the primary structure of transferrins.

All the transferrins studied so far are glycoproteins and there are marked variations in the numbers and compositions of the carbohydrate groups attached to different transferrins. The carbohydrate contents of transferrins isolated from different tissues may be quite different: in the hen the protein moieties of serum transferrin and of ovotransferrin appear to be identical but the carbohydrate prosthetic groups are different (Williams, 1962, 1968). Rabbit milk transferrin and rabbit serum transferrin also differ in carbohydrate composition (Baker *et al.*, 1968). Species differences have also been found. Human serum transferrin differs from hen serum transferrin in the number and composition of its carbohydrate groups (Jamieson *et al.*, 1971). Recently, Hudson *et al.* (1973) have attributed the marked differences that they found in the carbohydrate compositions of cattle, rabbit, horse and pig serum transferrins to variations in the numbers of carbohydrate groups.

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They proposed that there is one group in cattle transferrin, two groups in rabbit and horse transferrin and four groups in pig transferrin, although they did not isolate and study the glycopeptides from these proteins.

The glycopeptides of human serum transferrin, hen serum transferrin and hen ovotransferrin have been studied. Jamieson (1965) showed that Pronase digestion of human transferrin gives the two glycopeptides Asn(CHO)-Lys and Ser-Asn(CHO) (where CHO represents the carbohydrate moiety). Later, Charet *et al.* (1969) studied two tryptic glycopeptides from human transferrin. One of these (glycopeptide A) carried a C-terminal residue of half-cystine and it was proposed that this peptide represents the C-terminal part of the polypeptide chain. The other glycopeptide (B) had C-terminal lysine and was apparently derived from an internal position. Partial sequences were proposed for these peptides. In hen serum transferrin and ovotransferrin about 85% of the carbohydrate is present in the peptide Gly-Leu-Ile-His-Asn(CHO)-Arg and the remainder in the peptide Ala-Asn(CHO)-Leu-Thr-Gly (Williams, 1968). In the hen proteins the different carbohydrate groups are thus attached to the same polypeptide structures.

We report here the amino acid sequences and carbohydrate compositions of glycopeptides from the transferrins of duck, cow, pig and man, and also evidence that confirms the C-terminal position of one of the glycopeptides of human transferrin. The N-terminal sequences of these proteins have also been studied.

Materials and Methods

Ovotransferrin

Ovotransferrin was prepared from the whites of hens' eggs and ducks' eggs by the method described by Williams (1962, 1968). In the case of duck ovotransferrin the protein was adsorbed on CM-Sephadex in 0.1M-sodium acetate, pH 5.5, and eluted by a linear buffer gradient from 0.1M-acetate to 0.5M-acetate at room temperature.

Bovine serum transferrin

All steps in this preparation were carried out at room temperature. Freshly drawn cattle blood was mixed with 0.1 vol. of 10% (w/v) sodium citrate solution and the erythrocytes were removed by centrifugation. Iron was added to the plasma as FeCl_3 (1 mg per 100 ml), 1M-NaOH was added to pH 8.8 and 3 vol. of 5mM-Tris-HCl, pH 8.8, was mixed with the plasma. To this mixture an equal volume of 0.6% (w/v) rivanol (6,9-diamino-2-ethoxyacridine lactate) in the same Tris buffer was added slowly overnight. The supernatant fluid was decanted through a pad of potato starch, previously equilibrated with the Tris buffer, in order to remove rivanol. DEAE-Sephadex A-50 dry beads were added to the filtrate (1 g per 2 litres of filtrate) and the exchanger was then poured into a column (2.5 cm $\times$ 30 cm) and washed with the Tris buffer until the eluate was protein-free. The adsorbed transferrin was eluted with 0.1M-NaCl, dialysed against water and freeze-dried. The protein was redissolved in water to a concentration of 2% (w/v) and solid $(\text{NH}_4)_2\text{SO}_4$ added to 45% saturation. The precipitate was discarded and the red-coloured supernatant dialysed against water and freeze-dried.

Human serum transferrin

The preparation of this protein was very similar to that described above for bovine transferrin and is a modification of the procedure of Roop & Putnam (1967). It was carried out at room temperature. After adjustment of the pH of the iron-saturated plasma to 9.4 with 1M-NaOH, 3.5 vol. of 0.4% rivanol in 5mM-Tris-HCl, pH 9.4, was added. Removal of rivanol from the supernatant and adsorption of the transferrin to DEAE-Sephadex were carried out as described above. The transferrin was eluted with 0.2M-NaCl and dialysed against water. Transferrin was precipitated with $(\text{NH}_4)_2\text{SO}_4$ between 52 and 67% saturation, dialysed again, and freeze-dried. Finally the protein was adsorbed to DEAE-Sephadex in 50mM-Tris-HCl, pH 8.8, and eluted with a gradient from 50 to 500mM-Tris buffer.

Pig serum transferrin

A crude transferrin fraction was precipitated from iron-saturated plasma with $(\text{NH}_4)_2\text{SO}_4$ between 60 and 75% saturation. The precipitate was dissolved in water and dialysed. Rivanol was added to this solution as 0.5 vol. of 0.7% (w/v) solution in 50mM-Tris-HCl, pH 8.8. Rivanol was removed from the supernatant with potato starch and the protein was adsorbed to DEAE-Sephadex (column size 2.5 cm $\times$ 30 cm) in 5mM-Tris-HCl, pH 8.0. Transferrin was eluted with a linear gradient from 5 to 200mM-Tris-HCl, pH 8.0. This procedure was based upon the methods of Laurell & Ingelman (1947) and of Leibman & Aisen (1967).

Enzymes

Trypsin and chymotrypsin were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pepsin, subtilisin (type VII) and carboxypeptidase A and B (di-isopropyl fluorophosphate-treated) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Pronase was obtained from Calbiochem Ltd., Los Angeles, Calif., U.S.A., and neuraminidase was purchased from Behringwerke, Marburg-Lahn, Germany. Thermolysin was obtained through Chugain Boyeki Co. Ltd. (Export Agents), Higashiku, Osaka, Japan.

Preparation of glycopeptides

The digestion of transferrin with various proteolytic enzymes and the purification of glycopeptides from the digests was carried out as described earlier (Williams, 1968).

Carbohydrate analyses

For the identification and measurement of sugars present in proteins and peptides the methods described by Williams (1968) were used. To improve the separations of neutral sugars in paper chromatograms the acid hydrolysates were first passed through Zeo-Karb 225 in water. Transferrins and glycopeptides were treated with neuraminidase (enzyme/substrate ratio 1:40, w/w) in 0.1M-sodium acetate-0.001M- CaCl_2 , pH 5.5, for 20 h at 37°C to remove sialic acid. The molecular weights of glycopeptides were determined by the electrophoretic mobility method described by Wenn (1975). These determinations were carried out after removal of sialic acid. Molecular weights of the asialo-glycopeptides were also calculated from their sugar compositions, determined by g.l.c. For these calculations the analytical values were not rounded off to the nearest integer and the calculated molecular weights represent averages.

In determining the sialic acid contents of transferrins additional evidence was obtained from the

stepwise decrease of mobility in starch-gel electrophoresis on neuraminidase treatment (Parker & Bearn, 1962; Williams, 1962; Stratil & Spooner, 1971). Each step is assumed to correspond to the removal of one residue of sialic acid. At the same time, heterogeneity in the sialic acid content of the original protein may be revealed by this method. In the case of cattle transferrin even the purified protein from homozygous individuals exhibits multiple banding in starch-gel electrophoresis. The cause of this is not known (Stratil & Spooner, 1971; Richardson *et al.*, 1973).

Starch-gel electrophoresis

Horizontal starch-gel electrophoresis was carried out with the discontinuous buffer system of Poulik (1957). The concentration of starch in the gel was 11%.

Identification of N-terminal residues and sequences of transferrins

The *N*-terminal residues of transferrins were identified by dansylation in 8M-urea (Gray, 1967 b) and by the paper-strip modification of the Edman reaction (Schroeder, 1967). In the latter method the phenylthiohydantoin of the amino acids were subjected to alkaline hydrolysis (van Orden & Carpenter, 1964) and the amino acids identified with the amino acid analyser or by conversion into their dansyl derivatives.

For isolating *N*-terminal peptides, the method of Bruton & Hartley (1970) was used. Transferrin (100mg) was treated with maleic anhydride, desalted on a column of Sephadex G-25 in 0.1M-NH₃ solution and freeze-dried. The maleylated protein was dissolved in 0.05M-ammonium acetate, pH 8.5, containing 0.005M-CaCl₂ and digested with thermolysin. The digest was passed through a column of Dowex 50 (X2) (17cm×1cm) in 0.1M-acetic acid in the cold room. The eluate was heated at 60°C overnight to remove the blocking group from the *N*-terminal peptide, which was then purified by paper electrophoresis.

Identification of C-terminal residue and sequence of human transferrin

Before attempting to remove the *C*-terminal residue of human transferrin with carboxypeptidase the protein was reduced and treated with ethyleneimine (Cole, 1967). The resultant aminoethyltransferrin was dialysed, freeze-dried and dissolved in 0.1M-*N*-ethylmorpholine acetate buffer (pH 8.0)–5.6M-urea to give a 0.3% (w/v) solution. Digestion with carboxypeptidase A or carboxypeptidase B was carried out at 37°C with an enzyme/substrate ratio of 1:50 (w/w). After 5h the digestion was stopped by adding acetic acid to pH 4. Suitable amounts of the digests and

enzyme blanks were applied to the amino acid analyser. Carboxypeptidase A crystals were dissolved as described by Fraenkel-Conrat *et al.* (1955). Carboxypeptidase digestion of unmodified human transferrin was also attempted.

The *C*-terminal sequence of human transferrin was isolated by the carboxypeptidase B diagonal-electrophoresis method (Naughton & Hagopian, 1962; Barnett *et al.*, 1970). After heat denaturation (Williams, 1968) transferrin was digested with trypsin (enzyme/substrate ratio 1:50, w/w) at 37°C in 0.1M-sodium phosphate buffer, pH 6.5. This pH was used to minimize possible disulphide exchange reactions. The digest was subjected to paper electrophoresis at pH 3.5. Two side strips (3 cm wide) were cut from the sheet; one was sewn to a second sheet of paper and electrophoresis carried out at right angles to the first electrophoresis. The second strip was sprayed with a solution of carboxypeptidase B (0.01 mg per ml) in 0.07M-ammonium carbonate and suspended in a humid atmosphere at room temperature for 16h. After drying it was sewn to a sheet of paper and subjected to electrophoresis. Those peptides which remained on the diagonal were located on the original pH 3.5 electrophoresis sheet, eluted and further purified by repeating the diagonal electrophoresis this time at pH 2.1.

Purification of peptides

High-voltage paper electrophoresis was used as described by Ambler (1963). Peptides were eluted from electrophoretograms with 1% (v/v) NH₃.

Amino acid analysis

Peptides were hydrolysed with 5.7M-HCl in sealed evacuated tubes at 105°C for 20h. Analyses were carried out with a Technicon TSM analyser.

Peptide sequence methods

The dansyl-Edman method as described by Gray (1967 a) was used. Dansyl-amino acids were identified by thin-layer chromatography (Woods & Wang, 1967; Crowshaw *et al.*, 1967). The electrophoretic mobility method of Offord (1966) was used at pH 6.5 in assigning amide groups. Mobilities are given relative to aspartic acid and the sign used indicates the charge carried.

Results

Carbohydrate compositions of transferrins

Table 1 shows the carbohydrate compositions of duck ovotransferrin and of pig, cattle and human serum transferrins. For comparison, Table 1 also

Table 1. *Carbohydrate compositions of transferrins*

Results are given as mol of sugar/mol of protein. For details see the Materials and Methods section.

	Mannose	Galactose	N-Acetylglucosamine	Sialic acid	Fucose
Human transferrin	6.2	6.0	8.1	3-4	0
Cattle transferrin	7.2	6.2	8.8	2.0	0
Pig transferrin	4.3	3.1	5.6	2.0	1.0
Hen transferrin*	3.5	1.8	5.7	1.7	0
Hen ovotransferrin*	3.5	0	5.6	0	0
Duck ovotransferrin	8.3	0	8.6	0	0

* From Williams (1968).

gives the carbohydrate compositions of hen transferrin and ovotransferrin (Williams, 1968). Pig transferrin alone contained fucose, and the ovotransferrins lacked both galactose and sialic acid.

Human transferrin gave two bands on starch-gel electrophoresis. These bands disappeared on neuraminidase treatment and were replaced by a single slower band. From this it was concluded that the original protein preparations were heterogeneous with respect to sialic acid content and consisted mainly of protein carrying three and four residues of sialic acid. Cattle transferrin showed four bands on starch-gel electrophoresis. Neuraminidase treatment did not diminish the heterogeneity but caused a two-step decrease in the mobility of each band. Two residues of sialic acid were therefore present. Starch-gel electrophoresis of pig transferrin during the course of neuraminidase treatment showed that most of the protein carried two residues of sialic acid but a small amount had only one residue. After neuraminidase treatment pig transferrin was homogeneous on starch-gel electrophoresis.

Glycopeptides from duck ovotransferrin

After successive treatments of duck ovotransferrin with pepsin and subtilisin three glycopeptides, D-PS4.1.1, D-PS5.1.1 and D-PS6.1.1, were isolated. The amino acid compositions of these peptides are given in Table 2.

By the dansyl-Edman method peptide D-PS4.1.1 gave the sequence Asx-Lys-Thr and on further digestion with Pronase and carboxypeptidase B a complex which contained only carbohydrate and aspartic acid was obtained. The structure of this peptide is therefore Asn(CHO)-Lys-Thr.

Peptide D-PS5.1.1 gave the sequence Tyr-Asx-Leu-Thr and peptide D-PS6.1.1 gave the sequence Asx-Leu-Thr-Phe by the dansyl-Edman method. Although both peptides gave low yields of the aromatic amino acids upon analysis, tyrosine and phenylalanine residues were positively identified during the dansyl-Edman procedure. Digestion of peptide D-PS6.1.1 with Pronase and carboxypeptidase A gave a complex of carbohydrate and aspartic acid.

Duck ovotransferrin thus contains two glycopeptides with the structures Asn(CHO)-Lys-Thr and Tyr-Asn(CHO)-Leu-Thr-Phe. The carbohydrate compositions of the aspartic acid-carbohydrate complexes obtained from peptides D-PS4.1.1 and D-PS6.1.1 are given in Table 3. Comparison of these compositions with the carbohydrate composition of the original protein suggests that there are two carbohydrate-accepting sites and that in each molecule of the protein both sites carry carbohydrate. This is supported by the recoveries of the two glycopeptides: from 7.5 μ mol of protein 2.3 μ mol of peptide D-PS4.1.1 and 1.5 μ mol of peptides D-PS5.1.1 and D-PS6.1.1 were obtained.

Glycopeptides from pig transferrin

Seven glycopeptides were isolated from pig transferrin after successive digestion with pepsin and subtilisin. The amino acid compositions of these peptides are given in Table 2 and the amino acid sequences are given in Fig. 1. Digestion of peptide P-PS4.1.1 with trypsin and with carboxypeptidases A and B gave a complex containing carbohydrate and aspartic acid. A similar complex was obtained from peptide P-PS9.1.1 by digestion with Pronase and carboxypeptidase A. These two peptides thus have the structures Lys-Asn(CHO)-Arg-Ser and Asn(CHO)-Leu-Ser respectively. In the case of peptides P-PS9.1.2 and P-PS10.1.2 sequence data suggested the presence of two residues of serine, despite the low recoveries of serine on amino acid analysis. Peptide P-PS13.1.2 appears from its mobility ($m = -0.32$) to have a net negative charge of three. This is consistent with the presence of one residue of aspartic acid and two of sialic acid, although this suggestion is only tentative in view of the heterogeneity of sialic acid content.

After digestion of pig transferrin with chymotrypsin the glycopeptide fraction obtained by gel filtration was treated with neuraminidase before fractionation by paper electrophoresis. Four glycopeptides were isolated and their compositions and sequences are given in Table 2 and Fig. 1 respectively. The *N*-terminal residue of peptide P-C3.1.1 was lysine and after digestion of this peptide with subtilisin a

Table 2. Staining reaction, electrophoretic mobility and amino acid composition of glycopeptides from duck ovotransferrin and pig transferrin

For details see the Materials and Methods section.														
Peptide	Colour with cadmium-ninhydrin reagent	Electrophoretic mobility	Amino acid composition (mol of residue/mol of peptide)											
			Asp	Thr	Ser	Glu	Gly	Ala	Val	Leu	Tyr	Phe	Lys	Arg
Duck ovotransferrin														
D-PS4.1.1	Brown		1.0	0.7									1.0	
D-PS5.1.1	Pink		1.3	0.7						0.8	+			
D-PS6.1.1	Orange		1.2	0.7						0.7		0.4		
Pig transferrin														
P-PS4.1.1	Pink		1.3		0.9								0.7	1.0
P-PS5.1.1	Pink		1.2	0.8	0.9					1.0			1.3	0.8
P-PS8.1.2	Pink	0	3.1	0.8	3.0					1.0			0.9	2.1
P-PS9.1.1	Brown		1.2		0.7					1.0				
P-PS9.1.2	Brown		1.0		1.4					1.0				
P-PS10.1.2	Yellow		1.9		1.4					1.0				
P-PS13.1.2	Yellow	-0.32	2.1		2.4			0.7		1.0				
P-C1.1.1	Pink		1.0	0.9	0.9				1.2	0.4			1.0	0.8
P-C3.1.1	Pink	+0.07	1.3	0.5	0.9	1.0	1.0		0.6	0.7			1.1	0.6
P-C3.1.2	Orange	+0.07	1.4	0.6	2.8	1.9	0.8		1.0	1.1			1.1	1.6
P-C8.1.1	Yellow	-0.17	2.0	1.0	2.4			0.7		0.7		0.8		

For details see the Materials and Methods section.

Table 3. *Carbohydrate compositions of glycopeptides*

Sugar compositions are expressed as mol of sugar/mol of aspartic acid in the glycopeptide, which was treated with neuraminidase to remove sialic acid before examination by g.l.c. Oligosaccharide molecular weights were measured by the electrophoretic mobility method of Wenn (1975) and were calculated from the compositions obtained by g.l.c.

	Mannose	Galactose	N-Acetyl-glucosamine	Fucose	Oligosaccharide molecular weight	
					Measured	Calculated from composition
Duck ovotransferrin						
D-PS4.1.1	3.4	0	3.3	0	1540	1220
D-PS6.1.1	3.7	0	3.3	0	1520	1270
Pig transferrin						
P-PS4.1.1	3.6	2.7	4.2	1.2	2080	2045
P-PS9.1.1	3.6	2.6	4.2	1.2	2080	2029
Cattle transferrin						
C-PS5.2	4.2	3.4	4.3	0	2030	2104
C-PS4.1	4.5	3.6	4.4	0	2040	2209
Human transferrin						
H-PS3.1	3.3	3.0	3.8	0	1830	1790
H-PS4.2	3.2	2.8	3.9	0	1870	1761

further peptide with the structure Val-Gly-Glx was obtained. From the mobility of peptide P-C3.1.1 ($m = +0.07$) the presence of glutamic acid is indicated. Two residues of glutamic acid in peptide P-C3.1.2 are indicated by the mobility ($m = +0.07$) and the mobility of peptide P-C8.1.1 ($m = -0.17$) suggests the presence of one residue of aspartic acid.

Carbohydrate contents of glycopeptides from pig transferrin

Peptides P-PS4.1.1 and P-PS9.1.1 were treated with neuraminidase and digested with proteolytic enzymes, as described above, to give complexes containing only carbohydrate and aspartic acid. The sugar compositions of the asialo-oligosaccharides are given in Table 3. The compositions of the carbohydrate groups attached to the two glycopeptides of pig transferrin appear to be the same. From 10 μ mol of protein 2.3 μ mol of peptides derived from the sequence Asn(CHO)-Leu-Ser and 1.8 μ mol of peptides derived from the sequence Asn(CHO)-Arg-Ser were obtained. Comparison of the carbohydrate composition of pig transferrin with the compositions of the glycopeptides suggests that most molecules of protein carry only one carbohydrate group. Hexose and hexosamine values suggest that one-fifth to one-third of the molecules could carry two carbohydrate groups, although the fucose values indicate that all molecules carry only one such group.

Glycopeptides from cattle transferrin

Four glycopeptides were isolated after successive digestion of cattle transferrin with pepsin and subtilisin. Amino acid compositions and sequence data

for these peptides are given in Table 4 and Fig. 2 respectively. Peptide C-PS1.3 was neutral at pH 6.5, indicating the presence of glutamine and the absence of sialic acid. A complex containing only carbohydrate and aspartic acid was obtained from peptides C-PS4.1 and C-PS5.2 by digestion with Pronase and carboxypeptidase A.

Three glycopeptides were obtained after successive digestion of the protein with pepsin and trypsin. Neuraminidase treatment of the glycopeptide fraction was carried out before purification of the peptides. Table 4 and Fig. 2 give the amino acid compositions and sequence data respectively for these peptides. The fact that peptide C-PT5.1.1 carried no net charge at pH 6.5 could indicate the presence of glutamic acid, since an acidic residue adjacent to histidine would ensure full protonation of the histidine. In peptide C-PT1.3, however, the absence of net charge indicates the presence of glutamine. After removal of leucine from peptide C-PT11.2.1 by the Edman method the remaining tripeptide Glx-Thr-Asx was neutral and therefore contained glutamine and asparagine.

In the case of the tryptic glycopeptides from cattle transferrin sialic acid was removed with neuraminidase, and performic acid oxidation was carried out before purification of the peptides. Table 4 and Fig. 2 give the amino acid compositions and sequence data respectively for the two glycopeptides studied. Peptides C-T2.1 and C-T4.3 were further digested with subtilisin and in both cases three products were obtained. Peptide C-T4.3 was also digested with chymotrypsin and two products were obtained. The specificity of chymotrypsin for aromatic side chains makes it likely that phenylalanine is C-terminal in peptide C-T4.3-C2.

Sequence A

Sequence B

Fig. 1. Amino acid sequences of glycopeptides from pig transferrin

Peptides indicated by a solid line (—) are those for which amino acid analysis results were obtained. Residues positively identified by the dansyl-Edman method are indicated by —. In naming peptides, the letters after the hyphen refer to the enzyme(s) used: C, chymotrypsin; P, pepsin; S, subtilisin; T, trypsin. s indicates the point of cleavage of peptide P-C3.1.1 by subtilisin.

Carbohydrate contents of glycopeptides from cattle transferrin

Carbohydrate-aspartic acid complexes were prepared from peptides C-PS4.1 and C-PS5.2 by digestion with Pronase and carboxypeptidase A. The sugar compositions of the asialo-oligosaccharides are given

in Table 3. The two glycopeptide sequences of cattle transferrin appear to carry oligosaccharides with the same sugar compositions. Comparison of these data with the carbohydrate content of the original protein shows that each protein molecule carries two carbohydrate groups. The yields of the two glycopeptide

Table 4. *Staining reaction, electrophoretic mobility and amino acid composition of glycopeptides from cattle transferrin*
For details see the Materials and Methods section.

Peptide	Colour with cadmium- ninhydrin reagent	Electro- phoretic mobility	Amino acid composition (mol of residue/mol of peptide)																
			Cys(SO ₃ H)	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	Lys	His	Arg	
C-PS1.3	Orange	0		1.2	0.8	0.8	1.0		1.0										
C-PS3.1	Orange			1.1	0.8	0.8	0.7		1.0								0.8		
C-PS4.1	Pink			1.0	0.7	0.7													
C-PS5.2	Pink			1.0		1.9							1.0						
C-PT5.1.1	Pink	0		1.3	1.1	0.5	1.0		1.0	1.0					1.1		1.1		
C-PT7.2.1	Pink			1.8	1.2	1.7	1.0	1.0	0.7	0.5	0.9		0.7	1.0				0.7	
C-PT11.2.1	Pink			2.0	1.1	1.7	0.9						1.0	+					
C-T2.1	Pink			1.2	1.0	0.8	0.9	0.5	1.0	0.6	0.4		0.8			0.8	0.4		
C-T2.1-S1	Pink								0.7	1.0									
C-T2.1-S2	Pink							0.8			0.5		1.0			0.8			
C-T2.1-S3	Orange			1.4	1.3	0.9	1.0	1.0			0.3		0.8			0.4	0.4		
C-T4.3	Orange-brown		2.0	2.0	0.8	2.8	1.6	0.6	1.6	1.8	0.6	1.2	3.0		0.5	1.0			
C-T4.3-S1	Pink									1.0			1.6						
C-T4.3-S2	Pink		0.3			1.0	1.0									0.6			
C-T4.3-S3	Pink		0.5			1.1	1.2		1.2			1.1				1.0			
C-T4.3-C1	Yellow		0.4			1.0	1.2		1.3			0.9				1.0			
C-T4.3-C2	Orange		1.1	2.0	1.1	1.8	0.8	0.7	0.8	2.0	1.2		2.5		0.5				

Sequence A

Arg-Val-Tyr-Asn (CHO) -Ser-Ser-Leu-Gln-Thr-Asn (Pro, Gly, Ala) [(Ala-Leu-Ieu) (CySO₃H-Val) Phe] Gly-Ile-Ser (Glx, CySO₃H) Lys

Sequence B

Phe-Ala-Gly-Asn (CHO) -Ser-Thr-Gln-His-Val-Leu-Pro-Lys

Fig. 2. Amino acid sequences of glycopeptides from cattle transferrin
For explanation see Fig. 1. Peptides labelled with an asterisk were examined with carboxypeptidase B to give C-terminal lysine.

sequences were approximately equal. From $1.87\text{ }\mu\text{mol}$ of protein $0.37\text{ }\mu\text{mol}$ of peptides representing the structure Asn(CHO)-Ser-Thr and $0.42\text{ }\mu\text{mol}$ of peptides representing the structure Asn(CHO)-Ser-Ser were obtained.

Glycopeptides from human transferrin

Six glycopeptides were isolated from human transferrin after successive digestion with pepsin and subtilisin. Amino acid compositions and sequence data are given in Table 5 and Fig. 3 respectively. Peptides H-PS1 and H-PS3.1 had the same sequence, as also did peptides H-PS5 and H-PS3.2 and peptides H-PS6 and H-PS4.2. Thus, each of the three different amino acid sequences occurred in two forms which had different electrophoretic mobilities. This appears to be due to differences in sialic acid content. From the effect of digestion with neuraminidase upon mobility it is deduced that two residues of sialic acid are present in peptides H-PS6 and H-PS3.1 and one residue of sialic acid is present in peptides H-PS4.2 and H-PS1. Complexes containing only carbohydrate and aspartic acid were obtained from peptide H-PS3.1 by digestion with Pronase and carboxypeptidase B and from peptide H-PS4.2 by digestion with Pronase and carboxypeptidase A.

Glycopeptides obtained after successive digestion of human transferrin with pepsin and trypsin also showed heterogeneity of sialic acid content. Two glycopeptide structures were obtained and each occurred with either one or two residues of sialic acid. Amino acid compositions and sequence data are given in Table 5 and Fig. 3. The changes in mobility of peptide H-PT2.1 as a result of removing the first, second and third residues by the Edman method showed that glutamic acid and asparagine were present. Peptide H-PT3.2 contained cystine which, after performic acid oxidation, was replaced by cysteic acid (1.9 mol/mol of peptide). The oxidized peptide gave a single band on electrophoresis at pH 6.5, 3.5 and 2.1 indicating the presence of an internal disulphide bond.

Glycopeptides were also obtained from a trypsin digest of human transferrin from which sialic acid had been removed by treatment with neuraminidase. Performic acid oxidation of the glycopeptide fraction was carried out before purification of the peptides. Four glycopeptides were isolated and the amino acid compositions and sequence data are given in Table 5 and Fig. 3 respectively. Peptide H-T2.1 contained glutamic acid and asparagine as judged by the effect of successive treatments by the Edman method upon mobility. Peptide H-T2.2 had no net charge at pH 6.5 and therefore contained glutamine. Peptide H-T5 had four net negative charges at pH 6.5, ascribed to two residues of cysteic acid and two of aspartic acid. Three products were obtained by subtilisin digestion of peptide H-T5. In the case of peptides H-T5-S1 and

H-T5-S2 two residues of phenylalanine were found by the dansyl-Edman method despite the low recovery of this amino acid in the analyses. After removing the first two residues of peptide H-T5-S2 the remaining tripeptide had mobility $m = -0.45$, indicating the presence of aspartic acid. From the sequence data already described it is likely that peptide H-T3a lies to the C-terminal side of lysine in peptide H-T3.

Carbohydrate contents of glycopeptides from human transferrin

Carbohydrate-aspartic acid complexes were prepared from peptides H-PS3.1 and H-PS4.2 by successive treatments with neuraminidase, Pronase and carboxypeptidase. The sugar compositions of the asialo-oligosaccharides are given in Table 3. The two glycopeptides carry carbohydrate groups with identical compositions. Comparison of this composition with that of the original protein suggests that each molecule carries two carbohydrate groups. From $10\text{ }\mu\text{mol}$ of protein $2.6\text{ }\mu\text{mol}$ of peptides representing the sequence Asn(CHO)-Lys-Ser and $2.3\text{ }\mu\text{mol}$ of peptides representing the sequence Asn(CHO)-Val-Thr were obtained.

Carboxypeptidase digestion of human transferrin

Treatment of transferrin with carboxypeptidases A or B did not yield any free amino acids. Carboxypeptidase A did not release amino acids from aminoethyltransferrin, but carboxypeptidase B released 0.2 mol of aminoethylcysteine per mol of aminoethyltransferrin. The time-course of release of amino acids from aminoethyltransferrin by a mixture of carboxypeptidases A and B is shown in Table 6. These data suggest that the amino acid sequence at the C-terminus is Phe-Leu-Cys(CO_2H).

Isolation of the C-terminal peptide of human transferrin

When a trypsin digest of human transferrin was subjected to a carboxypeptidase B diagonal electrophoresis at pH 3.5 the pattern shown in Fig. 4 was obtained. Spots 1, 2 and 3 remained on the diagonal and after elution from the main sheet they were further purified by diagonal electrophoresis at pH 2.1. They gave rise to four peptides (1.6, 2.4, 3.5 and 3.6) which remained on the diagonal at pH 2.1. Amino acid analysis showed that only peptide 1.6 did not contain basic amino acids.

The amino acid composition of peptide 1.6, after performic acid oxidation, is given in Table 7. It is similar to that of glycopeptide sequence B, although they are not identical. Three peptides were obtained after digestion of peptide 1.6 with subtilisin but none was in sufficient quantity for sequence determination. The composition of peptide 1.6-S1 corresponds to

Table 5. *Staining reaction, electrophoretic mobility and amino acid composition of glycopeptides from human transferrin*
For details see the Materials and Methods section.

Peptide	Colour with cadmium- ninhydrin reagent	Electro- phoretic mobility	Amino acid composition (mol of residue/mol of peptide)													
			Cys(SO ₃ H)	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Leu	Tyr	Phe	Lys	Cys
H-PS1	Pink	0		1.0		0.9										1.1
H-PS3.1	Pink	-0.18		1.0		0.9										0.8
H-PS3.2	Pink	-0.18		1.0	0.9	0.8			0.6		0.9			0.4		
H-PS4.2	Pink	-0.20		1.0	1.0						1.1					
H-PS5	Pink	-0.31		1.0	1.0	0.7			0.7		1.0			0.3		
H-PS6	Pink	-0.37		1.0	0.8						0.8					
H-PT2.1	Pink			2.0			1.3			1.0			0.9		0.8	
H-PT3.2	Orange			3.0	1.4	2.1			2.2		1.0	1.0		1.5		+
H-PT3.2 (oxidized)			1.9	3.0	0.9	2.0			2.0		1.0	1.2		1.4		
H-T2.1	Pink	0		2.0			1.3					0.8	0.7		1.1	
H-T2.2	Pink	0		0.9	0.7	1.1	1.0	1.1	1.1	1.0	0.7	1.1		0.4		
H-T3	Orange		0.5	3.8	0.5	0.8	2.2	1.4	2.4	1.5	3.0	2.2	0.4	1.2	1.3	
H-T3a	Orange			2.0	0.8	0.9	0.9		1.0	0.9	0.7			1.2		
H-T5	Yellow	-0.36	1.4	3.3	1.0	1.9			2.4		1.2	0.6		1.6		
H-T5-S1	Pink		0.6									1.0		1.2		
H-T5-S2	Yellow	-0.45		1.0		0.9			1.0					1.1		
H-T5-S3	Yellow		0.4	1.8	1.0	0.9			1.0		1.0					

Sequence A

CySO₃H-Gly-Leu-Val-Pro-Val-Leu-Ala-Glu-Asn-Tyr-Asn(CHO)-Lys-Ser(Asp₂,Thr,Glu,Gly,Ala,Val,Phe)

Sequence B

Ala-Leu-Pro-Gln-Phe-Gly-Ser-Asn(CHO)-Val-Thr-Asp-CySO₃H-Ser-Gly-Asp-Phe-Phe-Leu-CySO₃H

Fig. 3. Amino acid sequences of glycopeptides from human transferrin

For explanation see Fig. 1. In peptide H-PT2.1, the values refer to the mobility of the remaining peptide after removal of the first, second and third residues respectively.

that of glycopeptide sequence B. Peptide 1.6-S2 appears to correspond to the sequence Gly-Ser-Asn(CHO)-Val-Thr and peptide 1.6-S3 is similar in composition to peptide H-T2.2. The compositions of these peptides thus support the suggestion that glycopeptide sequence B is C-terminal in human transferrin and that half-cystine is the C-terminal residue.

Table 6. Release of free amino acids from human aminoethyltransferrin during digestion with carboxypeptidases A and B

Recoveries are given as mol of amino acid/mol of protein.

Time of digestion (h)	Amino acid released		
	Aminoethylcysteine	Leucine	Phenylalanine
1	0.08	—	—
2	0.16	—	—
5	0.19	0.11	—
10	0.16	0.21	0.18

Fig. 4. Carboxypeptidase B diagonal electrophoresis of a trypsin digest of human serum transferrin

Electrophoresis at pH3.5 was carried out in both directions.

N-Terminal sequences of transferrins

End groups were determined by dansylation in the presence of urea and by the paper-strip modification of the Edman reaction. N-Terminal peptides were also isolated by the maleylation method. The results obtained are summarized in Table 8.

A trypsin digest of hen ovotransferrin was subjected to paper electrophoresis at pH6.5. Ninhydrin-positive bands (23) were eluted and 11 of them contained N-terminal alanine. After removing the first residue by the Edman method two of the bands gave a blue colour when dried on paper and stained with isatin (Smith, 1969), indicating proline in the N-terminal position. After further purification a peptide was obtained with the amino acid composition (mol of residue per mol of peptide) Ala (1.0), Pro (1.9), Lys (1.1), and its sequence was presumed to be Ala-Pro-Lys.

Discussion

The carbohydrate compositions of the serum transferrins of man, cattle, pig and hen show small quantitative differences (Table 1), but they are qualitatively similar in being composed of mannose, galactose, N-acetylglucosamine and sialic acid. Pig serum transferrin contains, in addition, fucose. As Table 9 shows the carbohydrate composition of human transferrin has been variously reported by different authors, but the significance of these differences is not known. The carbohydrate content of cattle transferrin found here is greater than that recently reported by Hudson *et al.* (1973). These authors found three residues of mannose, two residues of galactose, three to four residues of glucosamine and two residues of sialic acid per molecule of protein. On the other hand, Hudson *et al.* (1973) reported much higher values for pig transferrin than those we found. They obtained nine residues of mannose, eight residues of galactose, 14 residues of glucosamine, three to four residues of fucose and seven to eight residues of sialic acid per molecule of protein. All the serum transferrins studied here showed heterogeneity with respect to sialic acid contents. Duck ovotransferrin resembles hen ovotransferrin in possessing carbohydrate groups composed only of mannose and glucosamine.

Table 7. Amino acid compositions of peptides isolated from the C-terminus of human transferrin

Results are expressed as mol of residue/mol of peptide. For details see the Materials and Methods section.

Peptide	Cys(SO ₃ H)	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Phe	Glucosamine
1-6	1.7	2.9	1.0	1.3	2.9	1.1	2.1	1.6	1.5	1.0	2.5	1.4	+
1-6-S1	0.9	2.2	0.5	1.4	0.8	+	2.3	1.5	0.9		1.6	2.1	+
1-6-S2		0.9	0.6	0.4			0.7		0.7				+
1-6-S3		1.5	0.6	0.9	1.3	0.7	1.4	1.0	0.7		0.5	0.5	+

Table 8. Evidence for *N*-terminal sequences of transferrins

For details see the Materials and Methods section.

	<i>N</i> -Terminal residue by dansylation in urea	<i>N</i> -Terminal sequence (paper-strip Edman)	Amino acid composition of <i>N</i> -terminal peptide (maleylation method) (mol of residue/mol of peptide)							Electrophoretic mobility (pH 6.5)	Sequence of <i>N</i> -terminal peptide established by dansyl-Edman method
			Asp	Thr	Ser	Glu	Pro	Ala	Val	Lys	
Human transferrin	Val	Val-Pro	1.0	0.9			0.8		0.8	0.8	Val-Pro-Asp-Lys-Thr →→→→→→→→
Hen ovotransferrin	Ala	Ala-Pro			1.1		2.3	1.0		1.2	Ala-Pro-Pro-Lys-Ser →→→→→→→→
Pig transferrin	Negative	Negative		1.1		1.1	0.9		1.0	1.0	Val-Pro-Glu-Lys-Thr →→→→→→→→
Duck ovotransferrin	Ala	Negative		1.0			1.8	1.0		1.1	Ala-Pro-Pro-Lys-Thr →→→→→→→→
Cattle transferrin	Negative	Ala	1.2		1.0		0.6	0.5		0.6	Ala(Pro,Asp,Lys,Ser) →→→→→→→→

Two glycopeptides have been isolated from enzyme digests of all the transferrins studied here. In all cases the two glycopeptides have different amino acid sequences but appear to have identical carbohydrate compositions. It seems likely that each transferrin molecule possesses two carbohydrate attachment sites, although a complete knowledge of the primary structure would be required to prove this suggestion. The data on recoveries suggest that approximately equal amounts of each glycopeptide are present in duck, pig, cattle and human transferrins, although this conclusion is tentative in view of the large losses of peptide upon purification. In hen transferrin, on the other hand, one glycopeptide accounts for about 85% of the total carbohydrate, the remainder being found in the other glycopeptide (Williams, 1968). R. V. Wenn (unpublished work) has shown that the carbohydrate compositions of the two hen glycopeptides are closely similar. Jamieson *et al.* (1971) have shown that in human transferrin the two carbohydrate groups are identical in composition and probably identical in structure. Each group contained four mannose residues, two galactose residues, four glucosamine residues and two sialic acid residues. In the present study each glycopeptide from human transferrin contained three residues of mannose, three residues of galactose and four residues of glucosamine, sialic acid having been removed before analysis.

The average number of carbohydrate groups per molecule of protein may be deduced from a comparison of the carbohydrate composition of the protein with that of the glycopeptides (Table 10). Pig and hen transferrins appear to be the only ones in which on average only one carbohydrate group per protein molecule is present. In the hen this single carbohydrate group is attached predominantly to one site, but in the pig it is more equally distributed between the two sites. These conclusions differ from the proposal by Hudson *et al.* (1973) that cattle transferrin possesses one carbohydrate group and pig transferrin four.

The amino acid sequences of the glycopeptides found in the present study and also that obtained earlier for the hen transferrin are shown in Table 11. Carbohydrate is linked to the asparagine residue in the sequence Asn-X-Ser (or Thr). The frequent association of this sequence with carbohydrate attachment has been noted by Eylar (1965) and by Neuberger & Marshall (1969). Jackson & Hirs (1970) have observed in many glycoproteins a correlation between the nature of X and the complexity of the carbohydrate group: thus a complex carbohydrate group containing mannose, galactose, glucosamine, fucose and sialic acid occurs when X is a polar residue, and a simple unit composed only of mannose and glucosamine occurs when X is a non-polar residue. In the transferrin glycopeptides, however, X may be polar

Table 9. *Carbohydrate compositions of human transferrin according to various authors*

Results are expressed as mol of sugar residue/mol of protein.						
	Present results	Jamieson (1965)	Schultze <i>et al.</i> (1958)	Montreuil <i>et al.</i> (1965)	Heimberger <i>et al.</i> (1964)	Bezkorovainy <i>et al.</i> (1963)
Sialic acid	3-4	4	4	4	4	4
Mannose	6	8	4	6	4	—
Galactose	6	4	8	6	8	16
Glucosamine	8	8	8	10	8	10
Fucose	—	—	—	trace	1	—
Xylose	—	—	—	1	—	—

Table 10. *Numbers of sugar residues present in transferrins and in transferrin glycopeptides*

	No. of sugar residues per molecule of glycopeptide	No. of sugar residues per molecule of protein	Average number of glycopeptides per molecule of protein
Human transferrin	12	24	2.0
Cattle transferrin	13	25	1.9
Pig transferrin	13	16	1.23
Hen transferrin	9	12	1.33
Duck ovotransferrin	7	17	2.43
Hen ovotransferrin	10	9	1.1

or non-polar irrespective of the nature of the carbohydrate group.

Although from their properties the transferrins are thought to be a family of homologous proteins, there is relatively little similarity between the glycopeptides from transferrins of different species. There is apparently a tendency for position X to be occupied by a basic residue in one of the glycopeptides and an aliphatic residue in the other glycopeptide. In the transferrins of man, pig, duck and hen one glycopeptide has lysine or arginine at position X, whereas the other glycopeptide has leucine or valine. On this basis the glycopeptides in Table 11 have been grouped as type A or type B respectively. In cattle transferrin, on the other hand, position X is occupied by serine in both glycopeptides.

The possession of two apparently identical carbohydrate groups in transferrins was thought by Jeppsson (1967) to support the idea that there are two identical subunits of molecular weight about 40000. More recently, it has been used as evidence of structural duplication in transferrin molecules (Greene & Feeney, 1968; Mann *et al.*, 1970). Although this is an attractive and plausible idea, the structures of the glycopeptides given here show that the two sites in any of the transferrins we have studied are not identical or even closely similar. More data are clearly needed, however, before the possible homology between the

sites can be assessed. The locations of the carbohydrate attachment sites within the transferrin molecule are not known except for the presence of one site close to the C-terminal end of the polypeptide chain in human transferrin. The partial structure that Spik *et al.* (1968) proposed for this C-terminal site is thus confirmed and extended in the present work.

The N-terminal pentapeptide sequence gives some indications of homology. In all five species studied the N-terminus is a neutral aliphatic residue and proline occupies the second position. In the two birds position 3 is occupied by proline and in the mammals by aspartic acid or glutamic acid. The fourth residue is lysine in all five species and the fifth is a hydroxyamino acid.

The preparation of pig transferrin and some preliminary characterization of its glycopeptides were carried out by Mr. P. A. Miall, for whose help we are very grateful. We are indebted to Dr. R. Das for the preparation of duck ovotransferrin used in this work. Financial assistance from the Science Research Council is gratefully acknowledged, and I. G. was in receipt of an S.R.C. Studentship.

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Type A sequences	
Man	Cys-Gly-Leu-Val-Pro-Val-Leu-Ala-Glu-Asn-Tyr-Asn (CHO) -Lys-Ser (Asp ₂ , Thr, Glu, Gly, Ala, Val, Phe)
Cattle	Arg-Val-Tyr-Asn (CHO) -Ser-Ser-Leu-Gln-Thr-Asn- (Pro, Gly, Ala)
Pig	Asp-Asp-Ser-Ser-Arg-Lys-Asn (CHO) -Arg-Ser-Leu-Thr-Val-Gly-Glu-Glu
Duck	Asn (CHO) -Lys-Thr
Hen	Ala-Gly-Trp-Val-Ile-Pro-Met-Gly-Leu-Ile-His-Asn (CHO) -Arg-Thr-Gly-Thr-Cys-Asn-Phe-Asp-
Type B sequences	
Man	Ala-Leu-Pro-Gln-Phe-Gly-Ser-Asn (CHO) -Val-Thr-Asp-Cys-Ser-Gly-Asp-Phe-Phe-Leu-Cys [-----]
Cattle	Phe-Ala-Gly-Asn (CHO) -Ser-Thr-Gln-His-Val-Leu-Pro-Lys
Pig	Thr-Ser-Asp-Asn (CHO) -Leu-Ser-Ser-Ala-Phe
Duck	Tyr-Asn (CHO) -Leu-Thr-Phe
Hen	Ala-Asn (CHO) -Leu-Thr-Gly (Tyr)

Table 11. Summary of the amino acid sequences of glycopeptides from the transferrins of five species

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