THE ISOLATION OF SIALOGLYCOPEPTIDES FROM HUMAN TRANSFERRIN

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Recent studies on human transferrin have been directed mainly towards the occurrence of genetic variants of the protein (Parker and Bearn, 1962) and the nature of the iron binding site (Ulmer and Vallee, 1963); Windle, et al., 1963). Little attention has been paid to its carbohydrate content as an aspect of its structure since the original studies of Schultze et al. (1958) who showed it to contain galactose: mannose: N-acetylglucosamine and N-acetylneuraminic acid (NANA) in the ratio of 8:4:8:4 and who suggested the possible occurrence of one mole of fucose. The present work suggests that the carbohydrate portion of transferrin occurs as two branched chains terminating in sialic acid and linked to the protein moiety via asparaginyl linkages.

Partially purified human transferrin (CC) (Inman et al., 1961) was further fractionated on TEAE-cellulose at low ionic strength. The product was homogeneous on starch gel and in agar-gel immunoelectrophoresis and in the ultracentrifuge. It gave carbohydrate analyses virtually identical with those obtained on twice recrystallized metal-free transferrin (prepared by Dr. J. K. Inman and kindly provided by Dr. H. D. Anderson) and with the published data of Schultze and co-workers (Table I).

Heat denatured (80°/5 min.) purified human transferrin was treated with "Pronase" (Cal. Corp. for Biochem. Res.) (1:50) in a pH-stat at pH 7.8 until the uptake of base had stopped (usually about six hours) and then kept overnight at 37°. Prior removal of NANA did not affect the rate or extent of digestion. After concentration in vacuo the glycopeptide was precipitated

at 80% alcohol concentration. The digestion process was then repeated for a further eighteen hours. The final glycopeptide precipitate was brought to 6% trichloracetic acid concentration, the supernatant solution fractionated on Sephadex G-25 and the indole-positive fractions combined and concentrated to dryness. This glycopeptide fraction (Sephadex-GP) ran as a single elongated spot on paper chromatography in EtOH: NH_3: water (48:2:50) (R_F 0.79) and in n-PrOH:0.1M HCl (50:50) (R_F 0.82) and on high voltage paper electrophoresis at pH 6.4 and pH 8.6.

Carbohydrate analysis gave an NANA: N-acetylglucosamine: hexose ratio of 2:5:6 with a total carbohydrate content of above 70% (Table I).

	Transferrin	Sephadex-GP	DEAE-GP II	DEAE-GP III
Hexose (Indole)	2.6	28.6	33.6	31.9
Galactose: Mannose	1:2.5		1:2	1:2
N-acetylglucosamine (Elson-Morgan)	2.0	29.0	29.5	29.0
NANA (Aminoff, 1961)	1.3	14.8	16.5	16.6
Total Carbohydrate	5.9	72.4	79.6	76.5

Table I: Carbohydrate analyses. Total carbohydrate is calculated as the sum of the individual sugar analyses. Gal:Man ratios were calculated by the paper chromatographic method of Spiro (1960) and confirmed by the carbazole assay (Seibert and Atno, 1946).

Sialic acid of the Sephadex-GP was readily cleaved by mild acid hydrolysis or by V. cholera neuraminidase and fucose was not detectable by paper chromatography or by the cysteine - sulfuric acid method of Dische and Shettles. Aspartic acid was found to be the principal amino acid (Table II) and DNP-aspartic acid was identified by paper chromatography following acid hydrolysis of the DNP-glycopeptide obtained by treatment of the glycopeptide by the fluorodinitrobenzene procedure. Analysis by sedimentation equilibrium in 0.1M KCl ($\bar{v}$ = 0.71) gave a molecular weight of 3,000.

	Sephadex-GP	DEAE-GP II	DEAE-GP III
Aspartic	1.15	0.98	0.87
Lysine	0.31	0.91	-
Serine	0.55	-	0.80
Glutamic	0.27	-	-
Glycine	0.45	0.62	0.59

Table II: Amino acid analysis of glycopeptide preparations. Values are expressed in moles per 3,000 g.; values less than one tenth of the value of aspartic acid have been omitted.

Ammonia was released from the Sephadex-GP by hydrolysis with 2N HCl and estimated by the phosphotungstic acid procedure (Johansen, 1960). A maximum of one mole of ammonia per mole of aspartic acid was obtained from the sialic acid free glycopeptide (Fig. I). The amino acid composition of the glycopeptide fraction was not significantly changed by further treatment with "Pronase", with carboxypeptidase (either before or after carbobenzyoxylation (Fletcher et al., [1963]), or with papain, trypsin or leucine aminopeptidase.

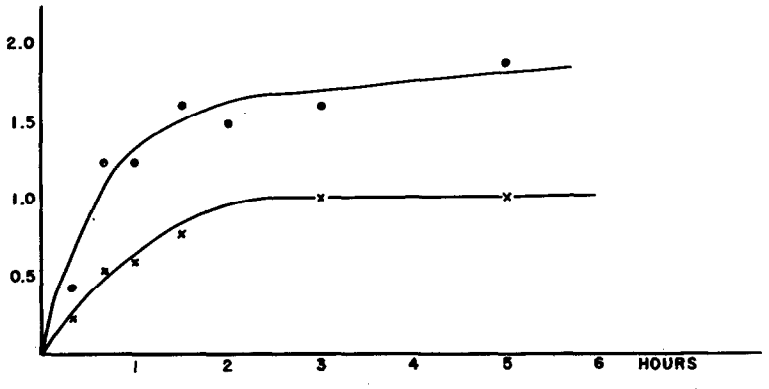

Fig. I: Release of ammonia from glycopeptides by hydrolysis with 2M HCl at 100°. Sialoglycopeptide, ●; sialic acid free glycopeptide, x. The ordinate is expressed in moles NH_3/mole glycopeptide.

Further resolution of the Sephadex-GP fraction was obtained by fractionation on DEAE-cellulose using a linear gradient from 0.002 to 0.15M pyridine acetate at pH 5.3 (Fig. II). Two major indole positive peaks were obtained (DEAE-GP II and DEAE-GP III); these were homogeneous to the periodate-$AgNO_3$ reagent on h.v. paper electrophoresis at pHs 3.5 and 9.4 but showed a single faint ninhydrin positive contaminant in each case. The NANA: N-acetylglucosamine: hexose values (Table I) were close to 2:5:6 in each case and the total carbohydrate content was nearly 80%. Molecular weight determinations by sedimentation equilibrium on DEAE-GP II and DEAE-GP III gave values close to 3,000. Combining the molecular weight and carbohydrate content suggests a carbohydrate molecular weight of approximately 2,400 for the heterosaccharide chains. The aspartic acid content is approximately one mole per mole of glycopeptide (Table II) although glycine and lysine were found in significant amounts in DEAE-GP II and glycine and serine in DEAE-GP III.

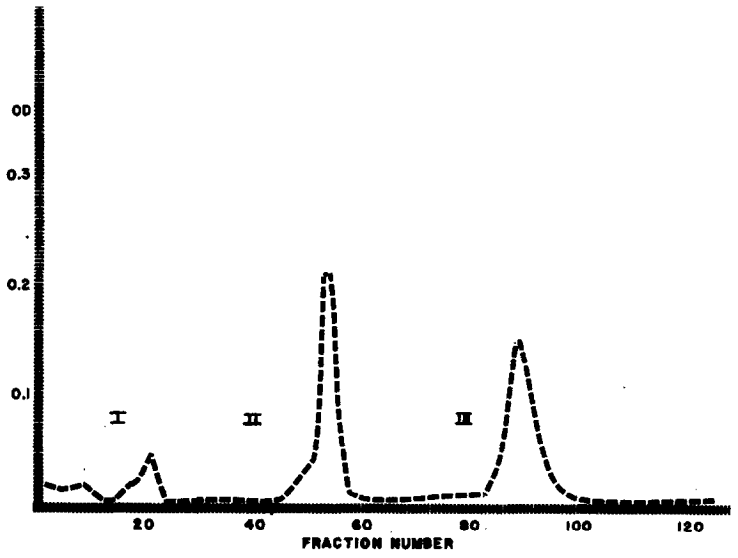

Fig. II: DEAE-cellulose chromatography of Sephadex-GP.

Discussion:

The results of the carbohydrate analyses performed in the present study on human transferrin differ from recorded data principally in having a galactose: mannose ratio of 1:2.5 as against 2:1. This value is confirmed by the analytical figures obtained on the glycopeptide fractions resulting from Pronase digestion followed by fractionation on Sephadex G-25 and DEAE-cellulose. The slight differences in the carbohydrate ratios in the glycopeptides and in transferrin itself probably represent the effect of increased destruction of sugars during hydrolysis in the presence of large amounts of protein. A similar observation has been made for glycopeptides from human ceruloplasmin (Jamieson, 1964). From the combined analytical data on the protein and the glycopeptides it appears that one mole of transferrin contains 4 moles of galactose, 8 moles of mannose, 10 moles of N-acetylglucosamine and 4 moles of NANA.

Several conclusions may be drawn from the analytical results on the glycopeptides: firstly, the occurrence of aspartic acid as the principal amino acid in both the Sephadex and DEAE-cellulose fractions, together with the release of an equimolar amount of ammonia following 2N acid hydrolysis at 100°, suggests the occurrence of an asparaginyl linkage between the carbohydrate and protein moieties. Similar linkages have been clearly demonstrated in, for example, ovalbumin (Fletcher, 1963) and other glycoproteins. Secondly, the rapid and complete cleavage of NANA by mild acid hydrolysis or by V. cholera neuraminidase suggests that it occupies a terminal position; thirdly, the fact that the sialic to aspartic acid ratio is about 2:1, and that the carbohydrate molecular weights of the individual fractions are about 2,400 indicates the presence in human transferrin of two branched heterosaccharide chains terminating in sialic acid.

A preliminary abstract of the analytical results obtained on the Sephadex-GP has already been published (Jamieson, 1963).

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