STUDIES ON THE CARBOHYDRATE UNITS OF PORCINE TRANSFERRIN

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CHAPTER I

INTRODUCTION

Statement of Problem

Iron is transported in the body by a serum glycoprotein, commonly referred to as transferrin. Most of the previous work on the transferrins has been performed on rabbit and human transferrin. Studies with the rabbit transferrin have dealt mainly with determining the biological function and the mechanism of iron transport (1). Human transferrin has been extensively studied with regard to the physical and chemical properties (2,3) and the detailed structure of the carbohydrate moiety (4,5).

The carbohydrate composition of transferrins from several species was determined by Hudson, et al. (6). They found that a large species variation occurs in the carbohydrate moiety of the transferrin molecule. They also proposed that this variation is in the number of heterosaccharide units per polypeptide chain with chicken and bovine containing a single unit, rabbit and equine consisting of two units, and porcine containing four. The purpose of this investigation was to unequivocally determine the number, size, and chemical composition of the carbohydrate units present in porcine transferrin and to determine the chemical nature of the glycopeptide linkage.

Survey of Literature

Introduction

The average individual ingests approximately 15 mg of dietary iron per day. The ingested iron is released from the foodstuffs by the combined action of the gastric hydrochloric acid and proteolytic enzymes. Reduction of the released iron to the ferrous (Fe^{2+}) state which can pass through the gut to the mucosal cells is performed by the products of microbial fermentation. After the Fe^{2+} has entered these cells, it is oxidized to the ferric (Fe^{3+}) state which can be absorbed by the mucosal ferritin. The exact mechanism of the Fe^{3+} absorption is unclear, but the amount of this absorption is known to be controlled by the amount of ferritin present. From the 15 mg of ingested iron only 1.5 to 2.0 mg of iron are absorbed per day.

It is from the mucosal ferritin that the iron binding protein of plasma, transferrin or siderophilin, receives the Fe^{3+} for transport to the cells. There is approximately 0.25 grams of transferrin per 100 ml of normal human plasma, of which only one third is saturated with iron. The affinity of transferrin for iron is high with the dissociation constant for the iron-transferrin complex being 10^{29} (7). If small amounts of iron enter the blood stream, the iron is oxidized by ceruloplasmin to the ferric state which can be bound to the transferrin molecules. When the amount of ionic iron entering the blood stream exceeds that required for complete transferrin saturation, a severe toxic reaction results (8,9).

Since the binding constant for the iron-transferrin complex is very high, such physiological reducing agents as ascorbic acid will not reduce

the Fe^{3+} at a pH of 7.0. However, if ascorbic acid is in the presence of ATP, the transferrin bound iron is easily reduced to the ferrous state at a pH of 7.4 and dissociation from the protein occurs with relative ease. In this manner the iron enters the cell and is metabolized, leaving the transferrin free to return to the mucosal cells and bind more iron (10).

Therefore, the function of transferrin is to restrain the iron from reacting with and perhaps causing injury to cells not requiring iron, while at the same time making iron available to cells which will utilize the available iron (10).

Physical Properties

Early physical studies on human transferrin isolated from the blood plasma established that it is a globular protein of molecular weight 86,000 daltons (3). However, more recent studies (2,6) have established that the most correct value for the molecular weight is 77,000 daltons.

Three possible subunit structures were proposed for human transferrin, based on the evidence that each molecule binds two iron atoms and contains two carbohydrate units, as follows (3): 1) two non-identical subunits joined by interactions susceptible to disruption by 6.0 M guanidine HCl, 2) two or more polypeptide chains interconnected by an unusual crosslink which would be resistant to 6.0 M guanidine HCl, and 3) the occurrence of a single polypeptide chain. After rigorous physical studies on native and reduced transferrin, it was concluded that the transferrin molecule is a single polypeptide chain.

Due to the observations that iron had a characteristic effect on the electrophoretic mobility of transferrin in various media (12,13,14), investigations were performed to determine the reason for this change in

electrical charge. Lane (15) used DEAE-Cellulose chromatography to determine possible differences in the three forms of transferrin. He concluded that there is a change in electrical charge on the surface of the protein upon iron binding which is probably conformation dependent. Bezkorovainy and Grohlich (16) found from their ultracentrifugation experiments that the molecular weights of the apo versus metal saturated protein at a pH of 7.0 were similar, but the iron saturated form was more elongated and less extensively hydrated than the apo form. Therefore, it appears that a conformational change takes place upon the binding of the metal to the protein.

Chemical Properties

The most thorough investigation of the carbohydrate moiety of the transferrins has been done by Jamieson, et al. (4,5) on human transferrin. The structure of the carbohydrate unit is shown in Figure 1. Sialic acid, N-acetylglucosamine, galactose, and mannose are the sugars found in the unit. These sugars occur in a ratio of 2:2:1:1, respectively. Both of these units are attached to the protein portion of the molecule by a glucosamine-asparagine linkage.

The carbohydrate composition of the transferrins from a number of other species has been determined by Hudson, et al. (6). They found that all the transferrins have similar molecular weights and amino acid compositions, but differ dramatically in the amount of carbohydrate present. Their findings showed that whereas bovine and chicken transferrins contain approximately one half the total number of monosaccharides per mole of transferrin as do human, rabbit, and equine, porcine contains twice the number of total monosaccharides. As shown in Figure 2, it was postulated

Figure 1. Structure of the Carbohydrate Unit of Human Transferrin

Key to Abbreviations: NANA--Sialic Acid; Gal--Galactose; GlcNAc--N-acetylglucosamine; Man--Mannose; Asn--Asparagine.

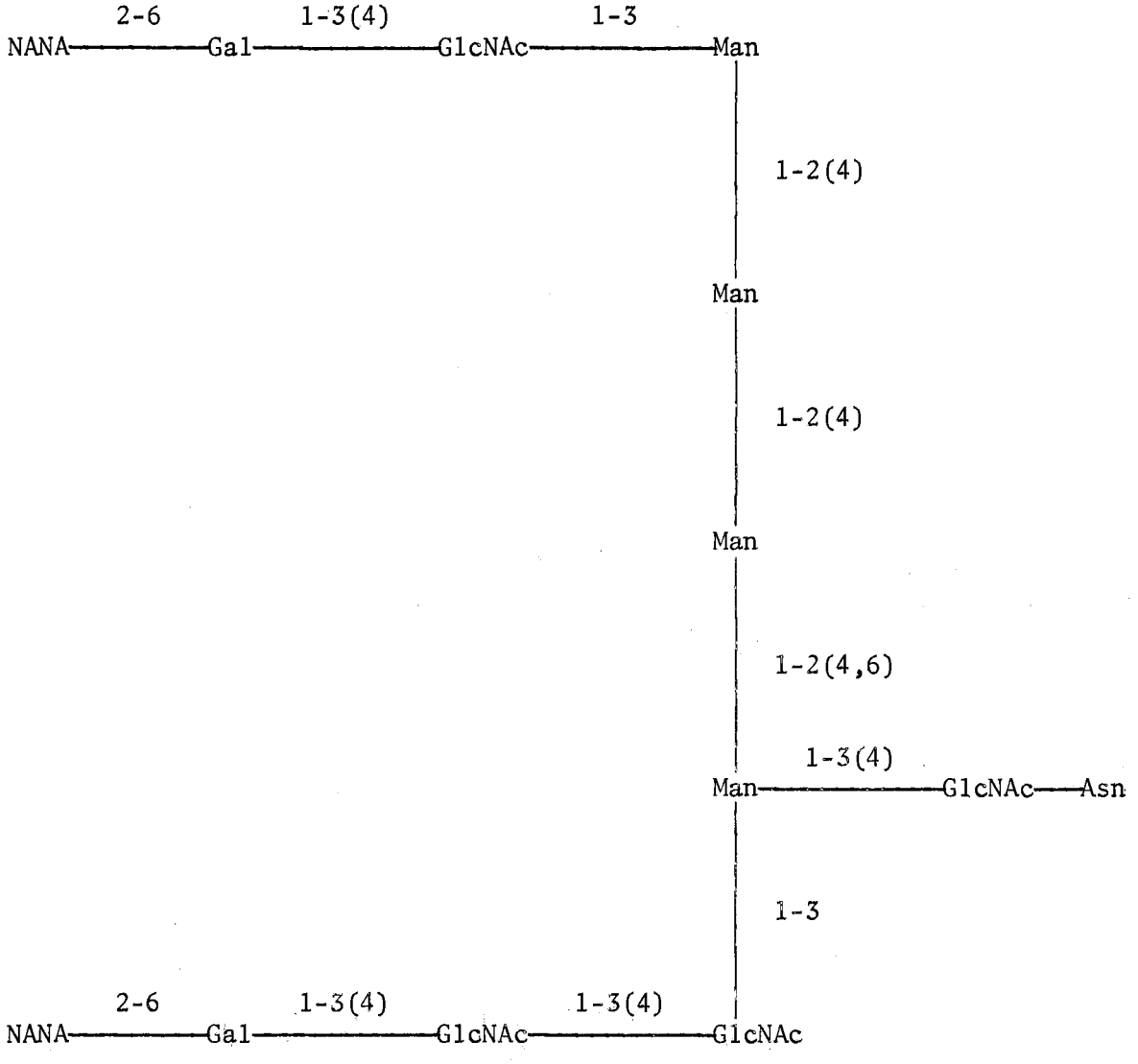
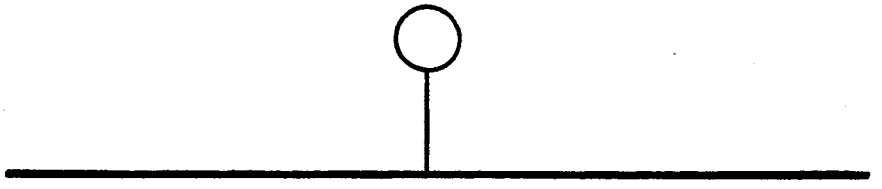
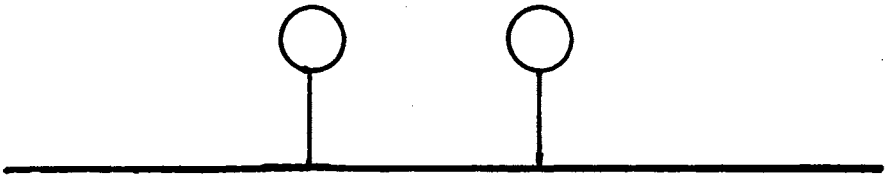

2-6
1-3(4)
1-3
NANA
Gal
GlcNAc
Man
1-2(4)
Man
1-2(4)
Man
1-2(4,6)
1-3(4)
Man
GlcNAc
Asn
1-3
2-6
1-3(4)
1-3(4)
NANA
Gal
GlcNAc
GlcNAc

Figure 2. Carbohydrate Units Proposed for the Various Transferrins

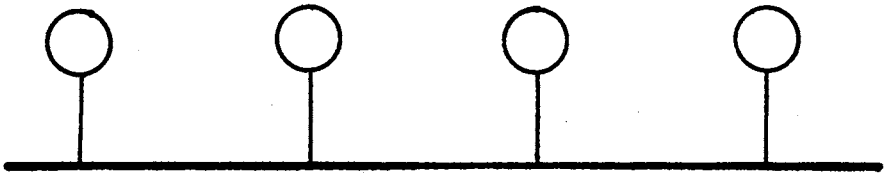
Peptide chain (———); Carbohydrate Moiety (♀).



Bovine, Chicken



Human, Rabbit, Equine



Porcine

that bovine and chicken transferrins contain one carbohydrate unit, human, rabbit, and equine transferrins contain two carbohydrate units, and porcine transferrin consists of four carbohydrate units. Nevertheless, the slight differences that may exist in the numbers and kinds of sugars present within a single carbohydrate unit can probably be explained as a simple deletion or addition of the sugar(s) involved with no apparent alteration in the sequence or linkages proposed by Jamieson. Therefore, the structure proposed by Jamieson is the one most likely found in the transferrins, irregardless of species.

Sialic acid is the most external sugar in the transferrins as it is in most all the circulating glycoproteins. The biological function of sialic acid on this group of glycoproteins is to prevent their uptake by the hepatic parenchymal cells (17). In a recent study by Morell, et al. (18) the uptake of various serum glycoproteins with and without their full complement of sialic acid was investigated. Transferrin was found to be unique in this regard, because the asialo transferrin was not removed from the circulation as were other serum glycoproteins. Therefore, the sialic acid on the transferrin molecule may function only as a termination signal for carbohydrate chains during their assembly in the golgi apparatus.

The amino acid composition of the transferrins from different species has been determined by a number of investigators (2,6,19,20). Approximately 90 to 98% of the native glycoprotein is made up of amino acids with the predominant one being aspartic.

Mechanism of Iron Binding

The mechanism of iron binding has been elucidated by using chelating agents such as nitriloacetic acid (NTA) coupled with ESR-EPR spectra (21, 22). The proposed reaction mechanism is:

$$Fe^{3+}\text{-NTA} + Tf \longrightarrow \text{NTA-}Fe^{3+}\text{-Tf} \xrightarrow[\text{without } HCO_3]{\text{with } HCO_3} \begin{matrix} Fe^{3+}\text{-Tf-}HCO_3 \\ Fe^{3+}\text{-Tf} \end{matrix} + \text{NTA}$$

Bicarbonate (HCO_3) was thought to be necessary for the formation of the Fe^{3+}-Tf complex, but these investigations showed that the Fe^{3+}-Tf complex could be formed just as well in the absence of bicarbonate. Furthermore, it was shown that the bicarbonate is tightly bound to the iron-transferrin complex which strongly suggests a protein-HCO_3 bond and not a metal-HCO_3 bond.

Also of interest with the transferrin molecule have been the amino acids involved in the active site. In order to determine the groups involved in the active site, various chemical modification experiments were performed on the transferrin molecule (23). Since histidine and tyrosine were thought to be the active site groups (24,25), chemical agents known to be specific for tyrosyl and imadazole residues were used.

The tyrosyls were found to have different reactivities. A rapid reacting group consisting of 8-21 tyrosines and containing one or more of the active site tyrosine(s), and a less rapid reacting group consisting of the remaining tyrosines (22-24) and containing the other active site tyrosine(s). These experiments confirmed the implication by Komatsu and Feeney (24) concerning the involvement of three tyrosines per iron atom bound. They further showed that the active site tyrosines are

indistinguishable from the other tyrosines in the molecule.

The chemical modification of histidine showed that after 10-14 histidines had been modified, there was a loss of the iron binding capacity of transferrin. This evidence provided support for the involvement of two histidines per iron atom. In addition they demonstrated that the active site histidines could be differentiated from those not involved.

From the above evidence that tyrosine and histidine were the active site residues, and the questions raised by Buttkus, et al. (26) concerning the loss of iron binding capacity due to amino group modification or tertiary structure alteration; chemical modification experiments were performed to determine the role of these amino groups in iron binding (27). Amino groups which were very reactive were found not to be the iron binding ligands nor were they involved in tertiary structure stabilization. Amino groups which were less reactive were found to be involved in tertiary structure stabilization and to be associated with iron binding, but they were not the iron binding ligands. Therefore, the functional portion of the histidine and tyrosine residues in iron binding have not, as yet, been identified.

Biological Role

In order to elucidate the biological role of transferrin in the mammalian system, the binding of transferrin to the reticulocyte membrane and the placental transfer of iron have been investigated.

The cell requiring the most iron is the reticulocyte. During its development into a mature red cell, the final stages of hemoglobin synthesis are taking place within the cell. In order to fulfill the

requirement of one iron atom per heme molecule, the transferrin binds to the cell surface and releases its iron to the cell. The surface of the reticulocyte has approximately 25,000 to 46,000 transferrin receptor sites. Iron laden transferrin has a four to five times greater affinity (1,3,28) for these sites than do the apo or mono form of the protein. After the cell has matured, there appears to be about a one hundred fold decrease in avidity for transferrin (1), indicating that either the immature cell possesses unique receptors, or that the reticulocyte can more efficiently metabolize the membrane iron which leaves such receptor sites continually free for transferrin binding (10). After the transferrin is bound to the receptors, the release of free, ionic iron which would be toxic to the organism is avoided by a direct transfer of iron to the cells (3). Good evidence for the involvement of metabolic energy at some stage in this direct transfer of iron is provided by the following observations (10): 1) at temperatures of only a few degrees above and below 37°C, there is a significant inhibition of iron uptake by the cells; and 2) whereas glucose stimulates iron uptake, a number of reagents (cyanide, azide, arsenate, etc.) known to block intermediary metabolism also inhibit iron uptake. Therefore, the plasma to cell cycle of transferrin can be summarized as follows (1): 1) a molecule of iron laden transferrin attaches to one of the receptor sites on the surface of the reticulocyte, requiring maintenance of cell surface integrity, 2) some mechanism exists for actively removing the iron from the protein molecule which requires some form of metabolic energy, 3) the now unsaturated transferrin molecule is displaced from the cell by another iron laden transferrin molecule, and 4) the now iron free transferrin acquires more iron from storage sites, and the cycle repeats itself.

Although the above evidence gives strong support for the transferrin binding to the cell surface before releasing its iron to the cell, Morgan and Baker (29) present evidence which supports the idea that the transferrin actually diffuses through the plasma membrane before releasing its iron. According to them the transferrin diffuses through the reticulocyte membrane and binds to the mitochondrial membrane. If the respiratory chain is intact, protons are donated to the transferrin molecule causing the iron to be released into the mitochondria complexed either with ATP and ascorbic acid or with DPNH alone. The Fe^{3+} is reduced to Fe^{2+} while still complexed and is then incorporated into protoporphyrin to form the heme group. After the transferrin has donated its iron to the mitochondria, it is released and diffuses out of the reticulocyte in the unsaturated, apo form.

There are two differences between the above scheme and the plasma to cell cycle scheme. The major difference is that the transferrin diffuses through the cell membrane and is subsequently bound to the mitochondrial membrane. The other exception is that there is no apparent displacement of the unsaturated transferrin molecule by another iron laden molecule from the mitochondrial membrane. Even though these differences exist, the features of the two schemes are basically the same in the way in which iron is delivered to the cell.

Another very important function of transferrin is to deliver iron to the placenta for transfer to the fetus (3). During pregnancy the serum iron level remains the same or falls, and the transferrin content doubles which results in a lower percent saturation of the circulating transferrin. The consequences of this lower saturation are: 1) increased iron mobilization from storage sites (mucosal ferritin), 2) increased iron

absorption in the mucosal cells preventing the depletion of iron reserves in the liver, and 3) all available iron is deferred to the fetus due to the placental absorption sites being more efficient at low iron saturation. Furthermore, the transport of iron from the mother to the fetus is unidirectional, so that the iron received by the fetus cannot be reabsorbed by the mother. Therefore, in order for the mother to avoid iron deficiency anemia during pregnancy, she must ingest enough iron to supply both the needs of the fetus and her own needs.

CHAPTER II

METHODS

Purification of Commercial Transferrin

Porcine transferrin was purchased from Miles Laboratories (Kankakee, Illinois). Analysis provided by the company showed the transferrin to be 77% pure which necessitated further purification. Commercial transferrin (630 mg) was dissolved in 0.05 M Tris-HCl buffer, pH 8.0 (10 ml) and was saturated with iron by adding 27.7 mg of solid ferric ammonium sulfate (Fisher) to the solution. After 24 hours at 4°C, the solution was introduced onto a Sephadex G-100 column (2.25 x 80 cm) which had been equilibrated with the 0.05 M Tris-HCl buffer, pH 8.0 and was eluted with the same buffer. The eluted transferrin was found to be greater than 95% pure by electrophoresis.

Isolation of Porcine Transferrin From Serum

Whole blood obtained from a local slaughterhouse was allowed to coagulate for 24 hours at 4°C. The serum was separated by centrifugation and diluted with an equal volume of water. Saturation of the transferrin with iron was performed by adding solid ferrous ammonium sulfate (Fisher) in excess to the serum solution. The mixture was allowed to stand for 30 minutes at 4°C before ammonium sulfate precipitation was initiated.

Crude isolation of the transferrin was done by ammonium sulfate precipitation. Solid ammonium sulfate was added until 40% saturation

had been reached (30,31), and the resulting precipitate was removed by centrifugation. The supernate was then brought to 70% ammonium sulfate saturation, and the precipitate containing transferrin was removed by centrifugation. This precipitate was dissolved in deionized water, dialyzed against deionized water for 72 hours, and lyophilized.

The dried material (50.8 g) was dissolved in 450 ml of water. A portion (50 ml) of this solution was placed on a small DEAE Sephadex A-50 column (1.5 x 30 cm) for elution with water. The gel was changed after each run due to the high amount of discoloration of the gel by the bound contaminants. The red fraction which was eluted off the column was lyophilized.

In order to obtain a more pure transferrin preparation, the dried material was dissolved in 110 ml of water. A portion (12 ml) of this solution was introduced onto a large DEAE Sephadex column (2.25 x 80 cm) and eluted with water. Fractions found to contain transferrin as the major component by SDS electrophoresis were pooled and lyophilized.

The dried material (2.2 g) was dissolved in 27 ml of 0.01 M phosphate buffer, pH 6.9. After 250 mg of this material had been introduced onto a DEAE Sephadex A-50 column (1.5 x 30 cm) equilibrated with the same buffer, the column was eluted with four to five column volumes of starting buffer which removed some of the contaminating protein. A linear gradient of 250 ml of 0.01 M phosphate, pH 6.9, and 250 ml of 0.2 M phosphate, pH 6.9, was then started for the elution of pure transferrin. Fractions containing pure transferrin, as determined by SDS electrophoresis, were pooled, dialyzed, and lyophilized. The remaining fractions with minor impurities were pooled, dialyzed, and lyophilized for reintroduction onto the same column for further purification.

Electrophoresis

Samples for electrophoresis were prepared by weighing out 1.0 mg of dried protein and adding 0.6 ml of the desired buffer to the protein in order to make a 1.67 mg/ml solution. The samples were made 15% in glycerol and 10 µl were applied to the gels.

Electrophoresis in three different systems was used as the criteria for the purity of the transferrin from the G-100 column. Two different electrophoretic systems were used to determine the purity of the transferrin isolated from the serum. The method of Weber and Osburn (32) was followed for the SDS electrophoresis with 5% gel concentrations being used in all experiments. Brewer and Ashworth (33) described the system which was used for the Tris-HCl electrophoresis. The urea-acetic acid system had been previously described by Panyim and Chalkley (34).

Electrophoresis was termed complete in each case when the tracking dye had reached the end of the gel. The gels were then stained with coomassie blue, fixed, and destained according to the procedure of Fairbanks, et al. (35). Each gel was stored in 7% acetic acid.

Calculation of Extinction Coefficients

A solution of the purified, commercial transferrin (1.895 mg/ml) which had been saturated with iron was used. The extinction coefficients were determined with a 1.0 cm light path at wavelengths of 280 nm, 410 nm, and 470 nm.

Preparation of Glycopeptides by Pronase Digestion

Purified, commercial porcine transferrin (200 mg) was dissolved in 10.0 ml of 0.2 M Tris-HCl, 0.0015 M $CaCl_2$, pH 8.0. The protein solution

was denatured by heating at 85°C for 10 minutes. Pronase (Calbiochem) (2.0 mg) was added initially with subsequent 1.0 mg additions at 48, 72, and 96 hour time intervals. Before each addition of pronase the pH of the solution was readjusted to 7.8. The digestion was termed complete when there was no further uptake of alkali and no further release of ninhydrin positive material. The reaction was terminated by heating the digested mixture in a boiling water bath for 10 minutes, followed by lyophilization.

Glycopeptides of the isolated transferrin were prepared in almost the same manner as described above. The only exceptions were that 500 mg of transferrin were dissolved in 25 ml of the digest buffer, and Pronase (5.0 mg) was added initially with subsequent 2.5 mg additions at the indicated time intervals.

A second pronase digestion was performed on the glycopeptides from the purified, commercial transferrin. The glycopeptide material was lyophilized and then dissolved in 2.0 ml of 0.2 M Tris-HCl buffer, pH 8.0. Pronase (0.6 mg) was added initially with a 1.0 mg addition after 12 hours of digestion. The digestion was terminated after 24 hours and lyophilized.

Isolation of Glycopeptides by Gel Filtration Chromatography

Glycopeptides which had been prepared as described above, were dissolved in 3.0 ml of 0.1 N pyridine-acetate buffer, pH 4.8. Any undissolved material was removed by centrifugation. The greenish solution was placed on a Biogel-P-10 or P-6 column which had been previously equilibrated with the same buffer. The eluted fractions were analyzed for

carbohydrate by anthrone (36), sialic acid by thiobarbituric acid assay (37), and peptides by ninhydrin. The fraction containing carbohydrate was pooled and lyophilized. Glycopeptide fraction P_1 refers to the fraction isolated from the first pronase digestion of the purified, commercial transferrin, and glycopeptide P_2 refers to that isolated from the second pronase digestion.

Carbohydrate Analysis

Neutral sugar content was determined by hydrolysis of 0.45 μmoles hexose equivalents for 4 hours at 100°C in 2.0 $\underline{N}$ H_2SO_4. The hydrolysate was diluted to 4 ml with deionized water, and 0.125 μmoles of xylose were added as an internal standard. The sample was introduced onto a Dowex 50 X 4 (H^+) (200-400 mesh) column coupled to a Dowex 1 X 8 (formate) (200-400 mesh) column (38) and eluted with successive water washes. Rhamnose (0.125 μmoles) was added to the eluted fraction as an internal standard, and the mixture was dried by lyophilization. Subsequent analysis was performed on a Technicon automatic sugar chromatography system as described by Lee (39).

Hexosamines were determined on the short column of the amino acid analyzer, after hydrolysis of protein (1.895 mg) in 4 $\underline{N}$ HCl at 100°C for 6 hours (38).

Sialic acid was determined by the thiobarbituric acid assay of Warren (37), after hydrolysis of the sample in 0.1 $\underline{N}$ H_2SO_4 at 80°C for one hour.

Amino Acid Composition

Amino acid content was determined by hydrolyzing 0.7 mg of protein in 6.0 N HCl for 24 hours, after flushing with nitrogen and sealing under reduced pressure. Subsequent analyses were performed on a Beckman 120C amino acid analyzer.

CHAPTER III

RESULTS

Studies on Carbohydrate Units of Commercial Transferrin

Purification

The elution profile of the Sephadex G-100 column purification of commercial transferrin is shown in Figure 3. The first peak contained the contaminating protein with a small amount of transferrin. The second peak was found by electrophoresis in three different systems to contain transferrin which was greater than 95% pure (Figure 4). Total yield of this purified transferrin was 355 mg or 56.3%. Amino acid and carbohydrate analyses of this material (Table I) are in close agreement with those of Hudson, et al. (6).

Calculation of Extinction Coefficients

Extinction coefficients for porcine transferrin were determined as described in the methods section. The extinction coefficients are shown in Table II along with the extinction coefficients from other species reported by Palmour and Sutton (40).

Figure 3. Sephadex G-100 Purification of Porcine Transferrin Protein (●——●) and Iron (▲——▲).

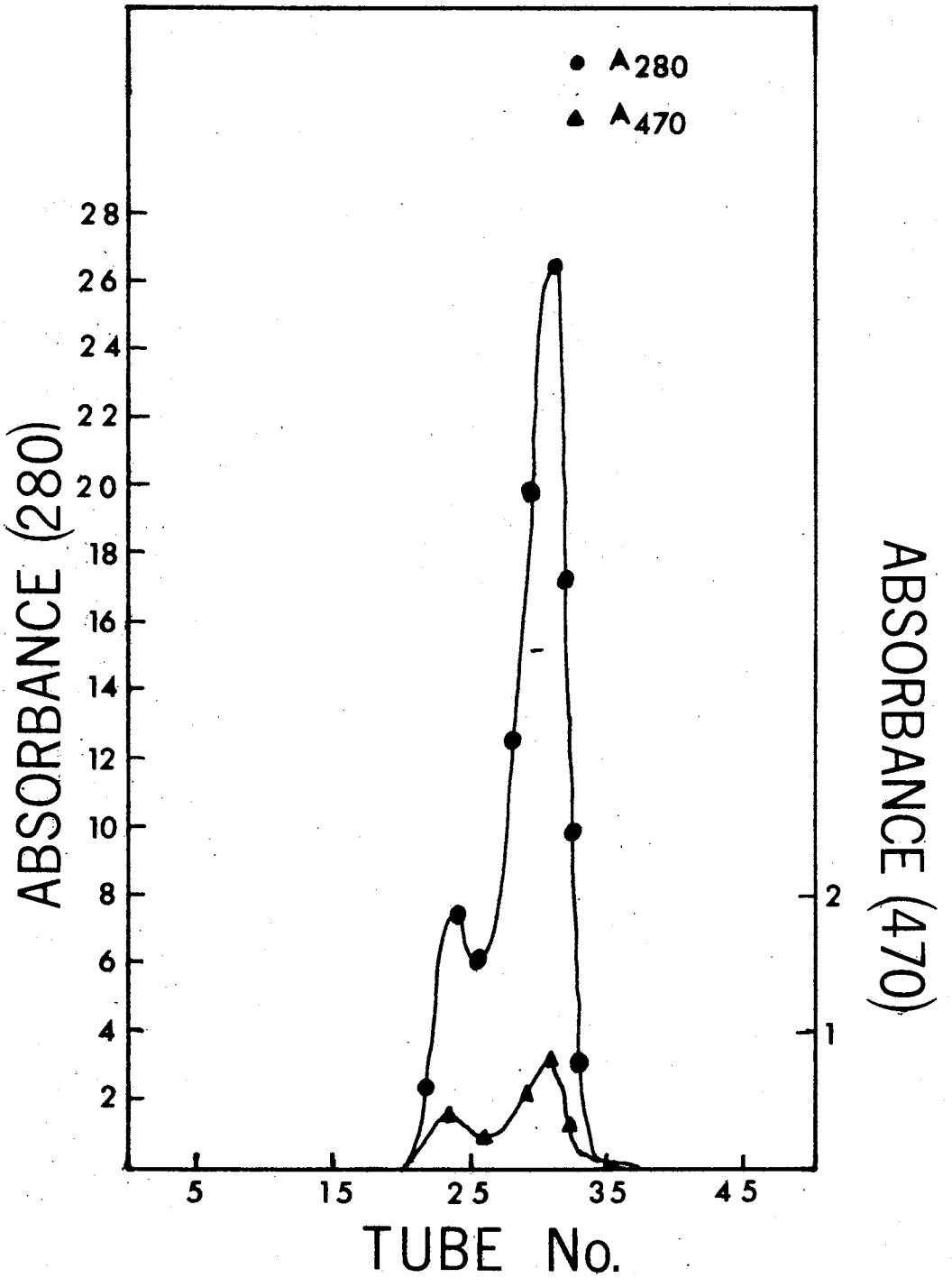

● A280
▲ A470
ABSORBANCE (280)
ABSORBANCE (470)
TUBE No.
28
26
24
22
20
18
16
14
12
10
8
6
4
2
2
1
5
15
25
35
45

Figure 4. Electrophoresis of Purified, Commercial Transferrin in Three Different Systems

Gel A, Urea-Acetic Acid; Gel B, SDS; Gel C, Tris-HCl.

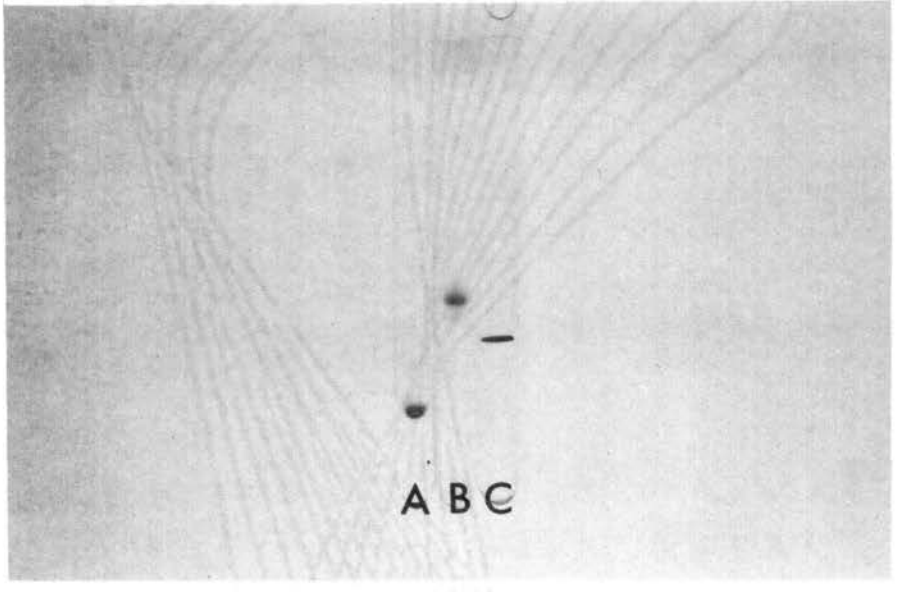

A B C

TABLE I

COMPOSITION OF PORCINE TRANSFERRINS

Composition	Purified, Commercial g/100g	Hudson, *et al.* g/100g
Amino Acids		
Lysine	7.99	6.82
Histidine	2.84	2.06
Arginine	5.29	4.57
Aspartic Acid	11.63	11.70
Threonine	3.97	3.26
Serine	5.06	4.00
Glutamic Acid	11.35	9.86
Proline	4.40	4.81
Glycine	3.36	2.69
Alanine	5.11	4.43
Valine	5.34	5.23
Methionine	0.76	0.91
Isoleucine	2.88	2.55
Leucine	9.08	7.75
Tyrosine	4.21	3.46
Phenylalamine	5.34	4.32
Half-cystine	2.32	4.34
Total	90.90	
Monosaccharides		
Mannose	1.51	1.86
Galactose	1.37	1.64
Fucose	0.52	0.66
Glucosamine	3.03	3.84
Sialic Acid	2.65	2.83
Total	9.10	

TABLE II

EXTINCTION COEFFICIENTS FOR TRANSFERRINS[a]
FROM DIFFERENT SPECIES

Species	$E^{1\%}$ 280 nm	$E^{1\%}$ 410 nm	$E^{1\%}$ 470 nm
Human[b]	14.06	0.315	0.568
Rabbit[b]	13.74	0.308	0.567
Turtle[b]	13.33	1.012	0.473
Frog[b]	11.91	1.083	0.515
Hagfish[b]	10.78	1.166	0.499
Porcine[c]	11.61	0.844	0.606

[a]All transferrins were iron saturated.

[b]Data taken from Palmour and Sutton, Biochem., 10, 4026, 1971.

[c]Values determined from purified, commercial transferrin.

Fractionation of Glycopeptides

Elution patterns for the P-6 and P-10 column fractionations of the glycopeptide are shown in Figures 5 and 6. The hexoses eluted as a single peak and followed that of sialic acid with recoveries of 90% and 95%, respectively. The ninhydrin positive material was well separated from the carbohydrate portion of the profile which permitted the glycopeptide to be isolated free of amino acids cleaved by digestion with pronase.

Data from the two columns indicate that the carbohydrate units involved in the transferrin molecule are of similar molecular weight. If the units have different molecular weights, then an elution profile with more than one carbohydrate peak as is found with basement membranes (41,42) would have been obtained. The resolution of the two carbohydrate units of thyroglobulin by gel filtration chromatography (43) provides additional evidence that carbohydrate units of different molecular weight can be distinguished by this technique. Therefore, from this data the carbohydrate units of porcine transferrin appear to have similar molecular weights. Further support for this conclusion could be determined from ultracentrifuge studies on this glycopeptide fraction.

Composition of P_1 Glycopeptide

The isolated P_1 glycopeptide from the two columns was analyzed for amino acid and carbohydrate content. Results from these analyses are shown in Table III. Amino acid content was reduced from 90% in the native protein to 45% in this P_1 glycopeptide. The predominant amino acids were lysine, aspartic, glutamic, and proline. The carbohydrate content increased from 10% in the native protein to 55% in this

Figure 5. P-6 Fractionation of Pronase Digested Transferrin

Column was eluted with 0.1 N Pyridine-Acetate, pH 5.0. Anthrone (●——●), Thiobarbituric Acid Assay (■——■), Ninhydrin (▲——▲).

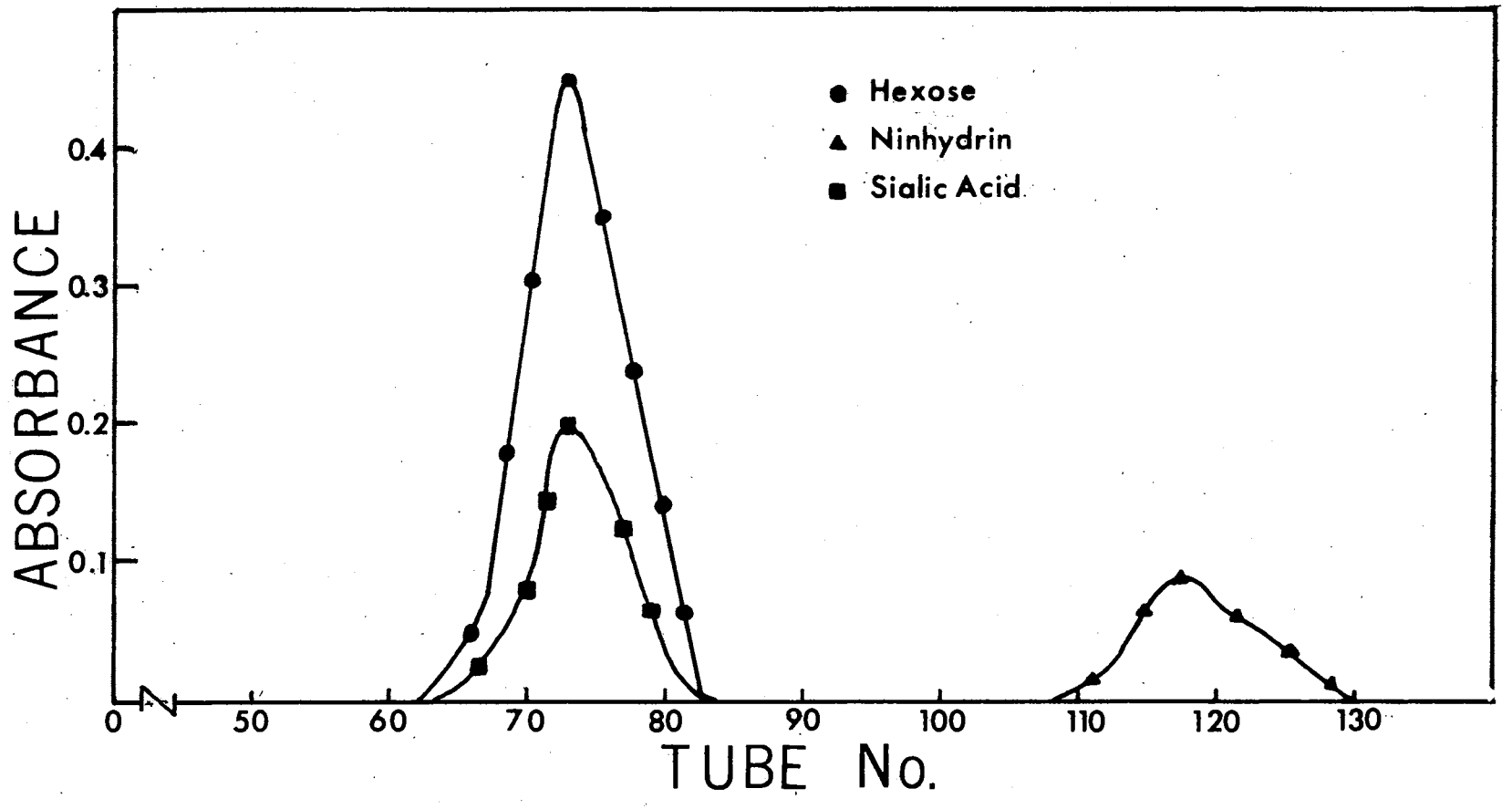

● Hexose
▲ Ninhydrin
■ Sialic Acid
ABSORBANCE
0.4
0.3
0.2
0.1
0
50
60
70
80
90
100
110
120
130
TUBE No.

Figure 6. P-10 Fractionation of Pronase Digested Transferrin

Column was eluted with 0.1 N Pyridine-Acetate, pH 5.0. Anthrone (●——●), Thiobarbituric Acid Assay (■——■), Ninhydrin (▲——▲).

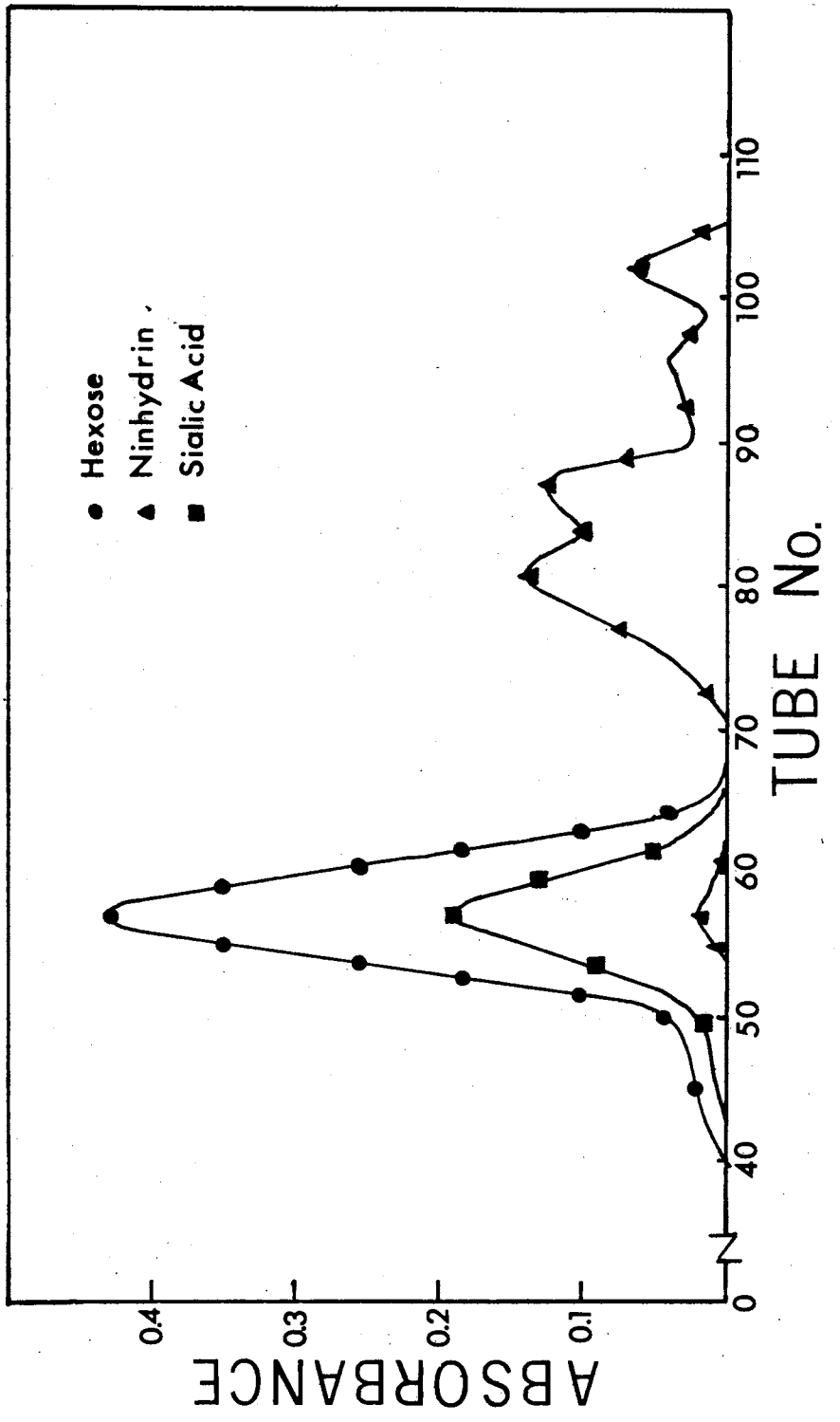

Hexose
Ninhydrin
Sialic Acid
ABSORBANCE
0.4
0.3
0.2
0.1
0
40
50
60
70
80
90
100
110
TUBE No.

TABLE III

COMPOSITION OF GLYCOPEPTIDES FROM PRONASE DIGESTIONS

Component	Pronase Treatment	
	Number 1	Number 2
	g/100g	
Amino Acids		
Lysine	5.90	1.11
Histidine	0.94	----
Arginine	2.23	0.90
Aspartic Acid	8.58	10.19
Threonine	2.04	1.32
Serine	2.27	4.04
Glutamic Acid	8.07	3.97
Proline	5.91	3.03
Glycine	2.04	1.27
Alanine	2.09	1.29
Valine	1.58	0.43
Methionine	Trace	----
Isoleucine	0.77	0.43
Leucine	1.46	0.78
Tyrosine	0.51	0.43
Phenylalanine	0.66	0.17
Total	45.00	29.40
Monosaccharides		
Mannose	8.93	10.37
Galactose	7.13	8.30
Fucose	3.25	4.67
Glucosamine	20.84	24.11
Sialic Acid	14.81	23.16
Total	55.00	70.60

glycopeptide fraction.

Since the amino acid content had been significantly reduced, this P_1 glycopeptide fraction could have been passed through an ion exchange column for further purification and characterization. But, in order to determine the degree to which further pronase digestion would affect the amino acid content of glycopeptide, it was resubmitted to a second digestion.

Isolation and Characterization of P_2 Glycopeptide

The elution pattern for the P-10 fractionation of this second pronase digestion was the same as shown in Figure 6. Carbohydrate and amino acid analyses were performed on this P_2 glycopeptide, and the results are shown in Table III. There was approximately a 30% reduction in amino acid content and a 20% increase in the amount of carbohydrate present in this glycopeptide fraction. Therefore, the transferrin can be submitted to a series of pronase digestions until all of the amino acids except the one involved in the glycopeptide bond are cleaved off. This same procedure was employed by Montgomery, *et al.* (44) in order to isolate the Asn-CHO unit of ovalbumin.

There are several problems involved with such a procedure. First, one must have a large amount of native starting material in order for the proper analyses to be performed throughout the entire course of the investigation. Secondly, after several treatments of the glycopeptide with pronase, one may have to change the type of gel being used for the isolation procedure, due to the decreased molecular weight of the glycopeptide.

However, one rather extensive digestion with pronase is sufficient

for the glycopeptide before its introduction onto an ion exchange column for further isolation and characterization of the individual glycopeptide fractions. Nevertheless, as many amino acids as possible should be removed before initiating the sequence study of the carbohydrate units.

In order to elucidate the composition, size, structure, and linkages of the carbohydrate units, purified glycopeptides are required. Thus, it is necessary to further resolve the glycopeptides from the P-10 column by ion exchange chromatography. However, because all the available material had been exhausted and the sudden discontinuation of commercial transferrin, it became necessary to isolate the transferrin from whole blood.

Studies on Carbohydrate Units of Isolated Transferrin

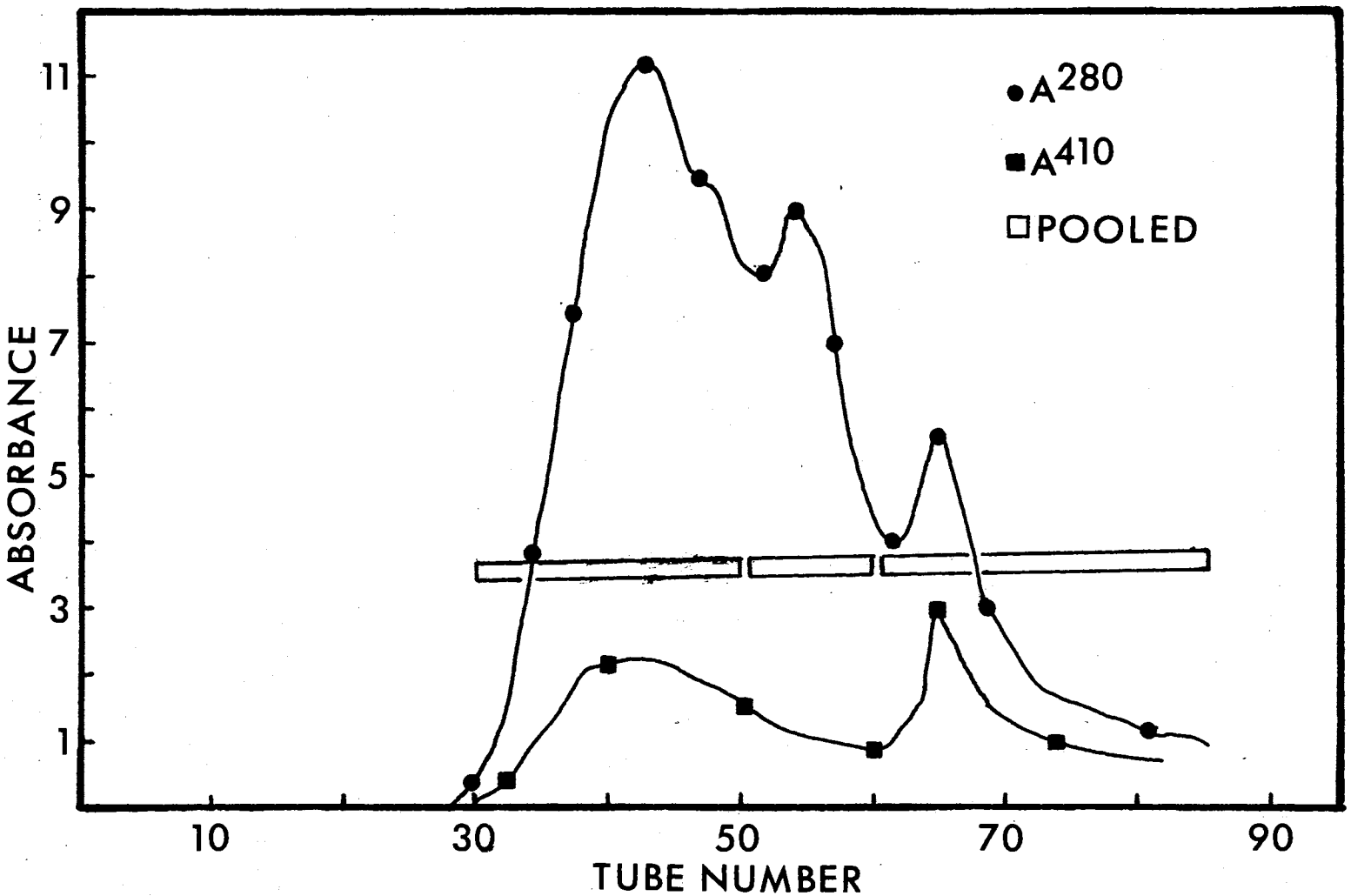
Isolation and Purification of Transferrin From Pig Serum

Several methods have already been established for the isolation and purification of the transferrins based on column chromatography (45,46, 47), and Rivanol precipitation (48), but these are best applied for small scale preparations and are not satisfactory for obtaining large amounts which are required for structural investigations. Therefore, a series of experiments were conducted in order to find a method of isolation which would utilize the previously established chromatographic techniques and would be suitable for large scale purification of the porcine transferrin from serum.

The 40-70% ammonium sulfate fraction containing transferrin, which had been previously passed through a small DEAE Sephadex column to remove some of the major contaminants, was introduced onto the large DEAE Sephadex column; the resulting elution profile is shown in Figure 7. In

Figure 7. DEAE Sephadex A-50 Purification of Porcine Transferrin

Column was eluted with water. Protein (●——●), Iron Binding: Absorbance @ 410 nm (■——■).


ABSORBANCE
11
9
7
5
3
1
10
30
50
70
90
TUBE NUMBER
A280
A410
POOLED

order to determine the fractions which were rich in transferrin, SDS electrophoresis was performed on selected fractions from the column; the results are shown in Figure 8. Fractions 45 and 50 which were representative of 50-75% of the first peak contained transferrin as the major component. The remainder of the first peak (fraction 58) and the second peak (fraction 60) showed an increased amount of a second component which also bound iron as indicated by the absorbance measurements at 410 nm and 470 nm. A third peak (fractions 65, 67, and 70) and the remainder of the fractions (78 and 81) contained a third component which was assumed to be albumin. Fractions 35-50, 51-60, and 61-85 were pooled and treated as described in the methods section.

Material representing fractions 35-50 was introduced onto a DEAE Sephadex A-50 column and eluted with a linear phosphate buffer gradient as described previously. The elution profile is presented in Figure 9. Selected fractions from the peak were analyzed for transferrin content by SDS electrophoresis, and the results are shown in Figure 10. From this analysis it was determined that fractions 105-131 which were representative of approximately 50% of the peak were homogenous with respect to transferrin. These fractions were pooled for further study. The remaining fractions (132-165) were pooled for reintroduction onto the column for further purification of the transferrin. Total yield of the purified component was 750 mg which represents 0.341 mg% of the whole serum.

It is interesting to note the amount of purification obtained by each of the major steps in the isolation procedure (Figure 11). Gel A represents the material which was precipitated from the serum in the 40-70% ammonium sulfate saturation range. Gel B shows the amount of purification obtained after passing the precipitated material through the

Figure 8. SDS Electrophoresis of Selected Fractions From DEAE Sephadex Column

Gel	Fraction No.
A	45
B	50
C	58
D	60
E	65
F	67
G	70
H	78

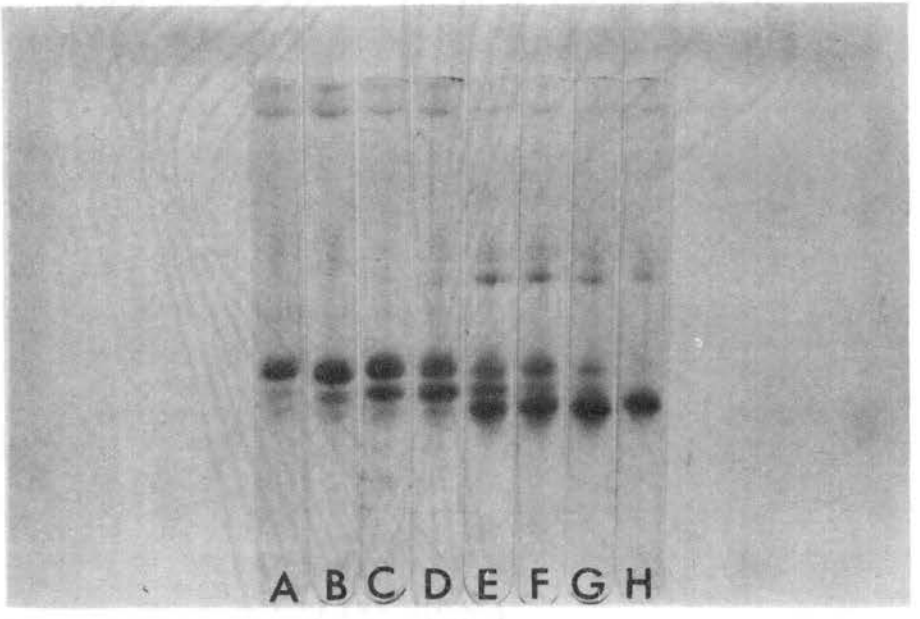

A B C D E F G H

Figure 9. DEAE Sepahdex A-50 Purification of Porcine Transferrin

Column was eluted with a phosphate buffer gradient (250 ml - 0.01 M Phosphate, pH 6.9 and 250 ml - 0.2 M Phosphate, pH 6.9). Protein Protein (●——●), Iron Binding (■——■), Phosphate Gradient (▲——▲).

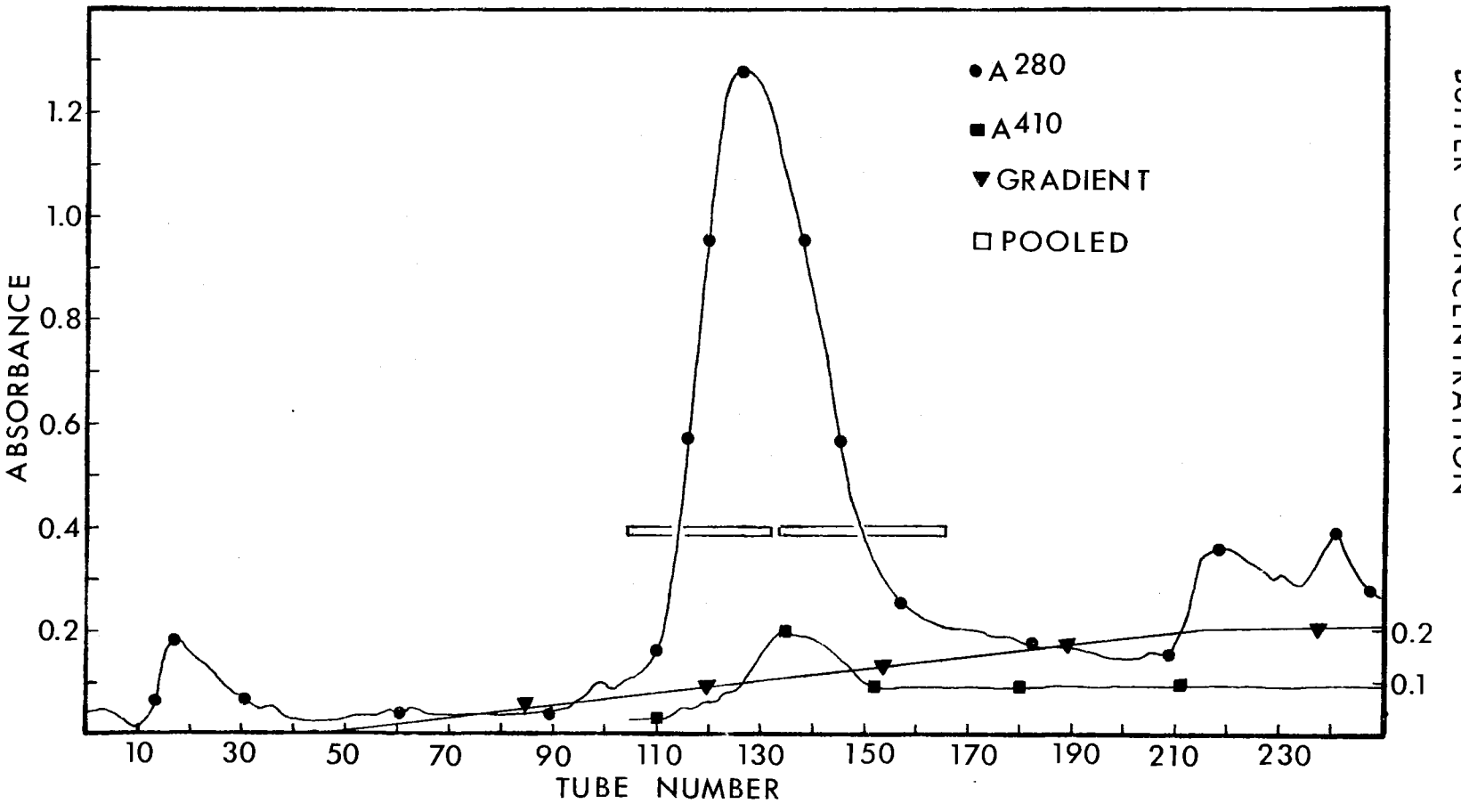

ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
10
30
50
70
90
110
130
150
170
190
210
230
TUBE NUMBER
● A 280
■ A 410
▼ GRADIENT
□ POOLED
BUFFER CONCENTRATION
0.2
0.1

Figure 10. SDS Electrophoresis of Selected Fractions From DEAE Sephadex A-50 Column

Gel	Fraction No.
A	105
B	120
C	127
D	131
E	135
F	140
G	150
H	165

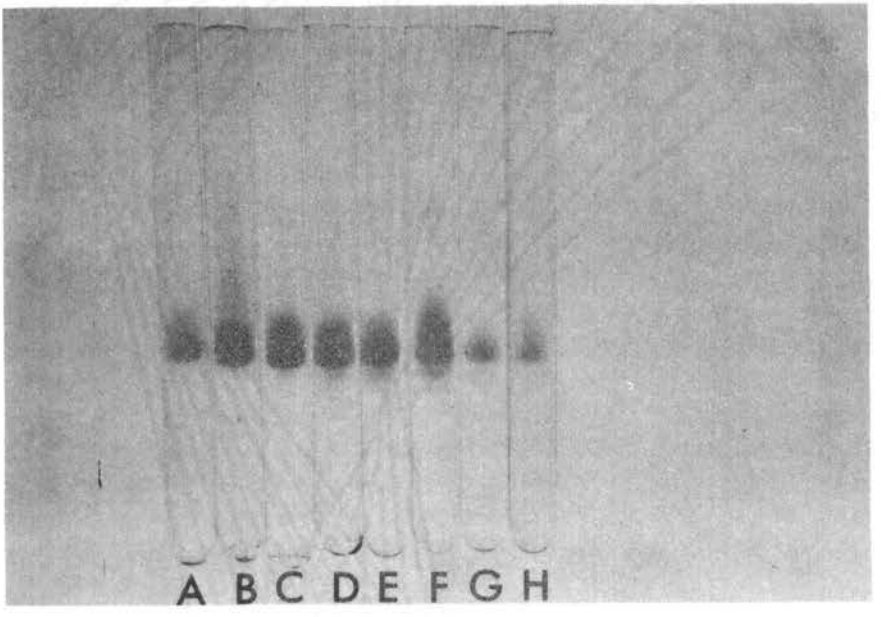
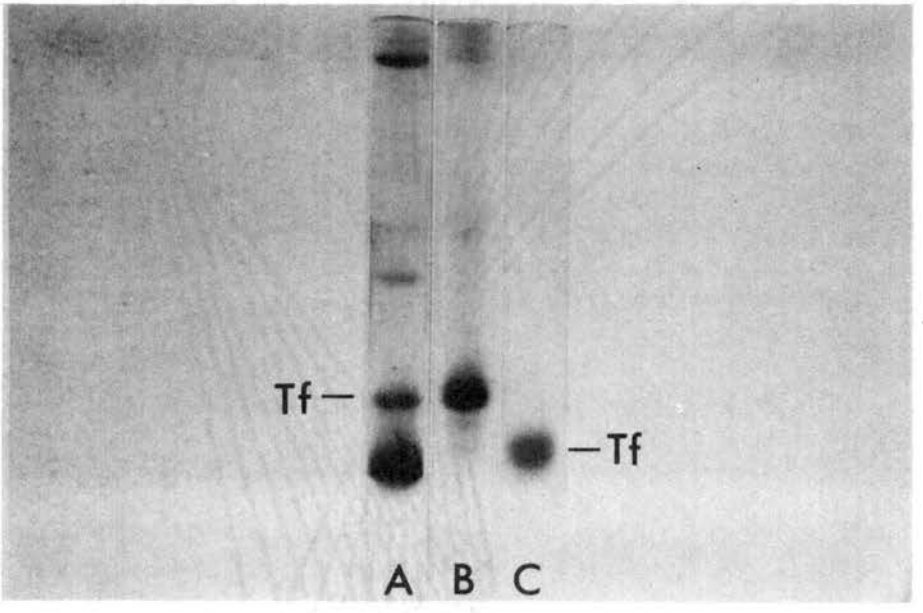

A B C D E F G H

Figure 11. SDS Electrophoresis of Isolated Fractions From Purification Scheme

Gel C is from a separate run which caused the difference in migration of the transferrin.

Gel	Fraction
A	Ammonium sulfate precipitate
B	DEAE Sephadex product (transferrin darkest band)
C	Final product


Tf —
— Tf
A
B
C

DEAE Sephadex columns (water elution) and pooling the previously indicated fractions. Gel C is the purified transferrin which was obtained by pooling the selected fractions from the DEAE Sephadex column to which a phosphate buffer gradient had been applied.

In order to make an accurate determination as to the purity of the final product, the purified transferrin was submitted for electrophoresis in a Tris-HCl buffer system as shown in Figure 12. Gel A was obtained by SDS electrophoresis, and Gel B was the gel obtained from the Tris-HCl system. Since there was a single band in both systems, it was concluded that the material was suitable for further study.

Composition of the Isolated Transferrin

Amino acid and carbohydrate analyses were carried out on the purified material, and the results are shown in Table IV. The amino acid composition is similar to that presented for the purified, commercial transferrin (Table I), but there are significant differences in the amounts of lysine, arginine, aspartic acid, and methionine in this isolated species. The most significant difference is found in the amount of carbohydrate present in this species of transferrin. Whereas, the previous data indicated a carbohydrate content of 10%, this analysis revealed a sugar content of 4%. Thus, the amount of carbohydrate present appears to be 60% lower than the purified, commercial transferrin. Therefore, from this preliminary data it seems that this isolated transferrin may contain two carbohydrate units instead of the four units as is thought to occur in the purified, commercial transferrin. This isolated glycoprotein may represent another species of transferrin in porcine serum as has been found to occur in human serum (49,50).

Figure 12. Electrophoretic Analysis of the Purified Transferrin

Gel	Electrophoretic System
A	SDS
B	Tris-HCl

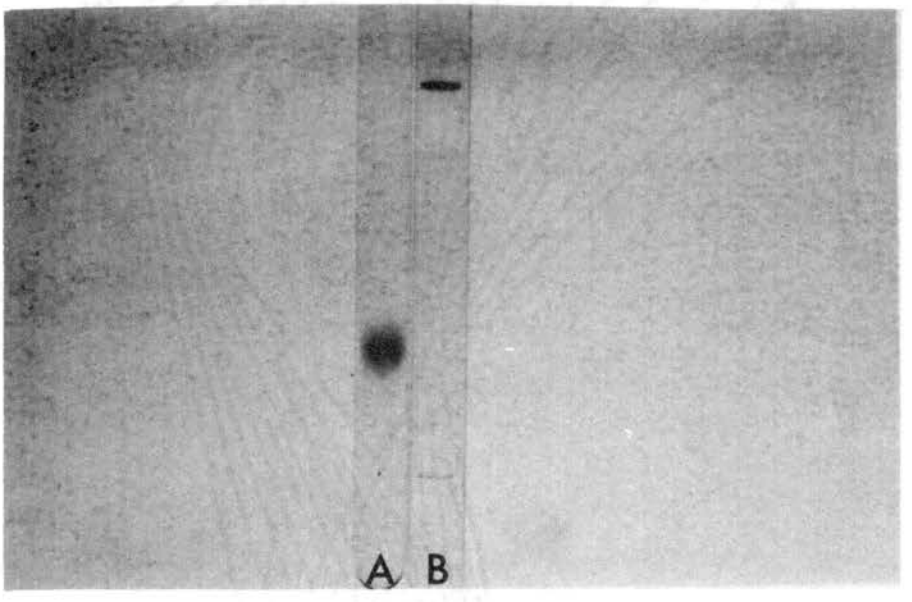

A
B

TABLE IV

COMPOSITION OF TRANSFERRIN ISOLATED FROM SERUM

Component	g/100g
Amino Acids	
Lysine	10.12
Histidine	2.11
Arginine	6.96
Aspartic Acid	14.63
Threonine	3.71
Serine	5.49
Glutamic Acid	10.97
Proline	3.41
Glycine	3.77
Alanine	6.04
Valine	5.38
Methionine	0.36
Isoleucine	2.27
Leucine	7.23
Tyrosine	4.82
Phenylalanine	4.82
Half-cystine	3.91
Total	96.00
Monosaccharides	
Mannose	0.78
Galactose	0.72
Fucose	0.25
Glucosamine	1.02
Sialic Acid	1.22
Total	4.00

Composition of Glycopeptide

The glycoprotein was submitted to pronase digestion, and the glycopeptide was isolated in exactly the same manner as described previously. Amino acid and carbohydrate analyses were performed on the glycopeptide, and the results are presented in Table V. The amino acid content is significantly lower than that found previously for one digestion with pronase. This is probably due to a more complete digestion by pronase. The carbohydrate composition is comparable to that found for the glycopeptide prepared from the purified, commercial transferrin. However, these results do not allow a definitive statement to be made concerning the number of carbohydrate units in this isolated transferrin.

TABLE V

COMPOSITION OF GLYCOPEPTIDE PREPARED FROM ISOLATED TRANSFERRIN

Composition	g/100g
Amino Acids	
Lysine	-----
Histidine	0.45
Arginine	0.94
Aspartic Acid	3.77
Threonine	0.84
Serine	3.23
Glutamic Acid	2.63
Proline	1.76
Glycine	0.90
Alanine	0.66
Valine	0.61
Methionine	-----
Isoleucine	0.88
Leucine	0.45
Tyrosine	0.33
Phenylalanine	0.22
Half-cystine	0.53
Total	18.00
Monosaccharides	
Mannose	15.10
Galactose	12.17
Fucose	2.93
Glucosamine	24.55
Sialic Acid	27.04
Total	82.00

CHAPTER IV

DISCUSSION

From the data presented in the first portion of this investigation, an estimation as to the nature of the carbohydrate units can be made. Composition studies on the native protein and the isolated glycopeptide revealed that there was a sufficient amount of carbohydrate to account for four carbohydrate units per protein molecule, based on the structure presented for human transferrin by Jamieson. Furthermore, the elution of the glycopeptide fraction as a single peak by gel filtration chromatography indicated that the carbohydrate units are similar in molecular weight and perhaps are of identical composition. Proof of a single type carbohydrate unit will come from characterization of the purified glycopeptides from an ion exchange column.

Comparison of the chemical properties of the purified, commercial transferrin to that of the transferrin isolated from whole serum show that a major difference exists in the amount of total carbohydrate, although the relative amount of the individual monosaccharides are approximately the same. The former contained 9.10% carbohydrate, whereas the latter material contained 4.00% carbohydrate. These results may indicate the presence of genetic variants which differ in the carbohydrate content. A difference in the carbohydrate may alter the chemical properties to such an extent that one form is favored in a specific preparative procedure.

The occurrence of genetic variants in the transferrin from the same species is not uncommon. Initial reports by Smithies (58,59) of genetic variants were followed by the discovery of twenty electrophoretic variants in man. Substitutions of single amino acids have been found to be responsible for this variation in the electrophoretic mobility of human transferrin (60,61,62). No inherited variants have, as yet, been studied which appear to depend on altered carbohydrate moieties.

Inherited variants of transferrin are not only found in man. Studies with primate transferrins (63,64,65) have shown that many forms of transferrin coexist in most species. This same type of variation appears to occur in other vertebrate species.

Therefore, two possibilities exist for the isolated transferrin described in the second portion of this investigation. Differences in the amino acid data indicate that there could be a substitution of one or several amino acids. The other possibility for genetic variation is in the amount of carbohydrate present, even though none of these variants have been studied. Thus, from the data available at the present time, it seems that the amount of carbohydrate is the major variation in the isolated transferrin as contrasted to the purified, commercial transferrin.

A unique feature of porcine transferrin as contrasted to the other species of transferrins is the presence of fucose and sialic acid together in the carbohydrate units. However, other investigators have found these two sugars occurring together in the same unit in some of the circulating glycoproteins. Jamieson (51) found the sialic acid to fucose ratio to be 1:0.2 in human ceruloplasmin (copper binding protein of serum). In addition, Dunn and Spiro (52) determined the ratio of sialic acid to fucose to be 1:0.3 in the α_2 macroglobulin of human plasma. This investigator

found the ratio of sialic acid to fucose to be 1:0.4 in porcine transferrin, which is comparable to the other circulating glycoproteins in which fucose and sialic acid occur together.

Even though the present investigation did not elucidate the structure of the carbohydrate moieties of porcine transferrin, several structures for the carbohydrate units can be proposed based on the structure presented by Jamieson for human transferrin (Figure 13). As has been noted previously, porcine transferrin contains one half the amount of mannose as does human. Thus, it seems most likely that mannoses 2 and 3 are deleted from porcine. In addition, the amounts of galactose, glucosamine, and sialic acid are the same in porcine as in human, but porcine transferrin contains fucose which is not found in human (6).

Fucose can be positioned at several locations as shown in Figure 14, based on the known structure of carbohydrate units. It may be attached to the galactose residue in a terminal position for either one or both of the heterosaccharide chains (Structure I), as is found in the H blood group substances (53,54). These possibilities are extremely unlikely due to the amount of sialic acid found in the native protein and the isolated glycopeptides. The most likely points of attachment for fucose are shown in Structure II. It could be attached to one of the internal glucosamines (A, B, or C) as is found in the Le^a blood group substance (55,56); or it could occur attached to the glucosamine involved in the glycopeptide bond (D), as is found in the γG immunoglobulin glycopeptides (57).

Figure 13. Structure for Carbohydrate Unit of Human Transferrin

Key to Abbreviations: NANA--Sialic Acid; Gal--Galactose; GlcNAc--Glucosamine; Man--Mannose; Asn--Asparagine.

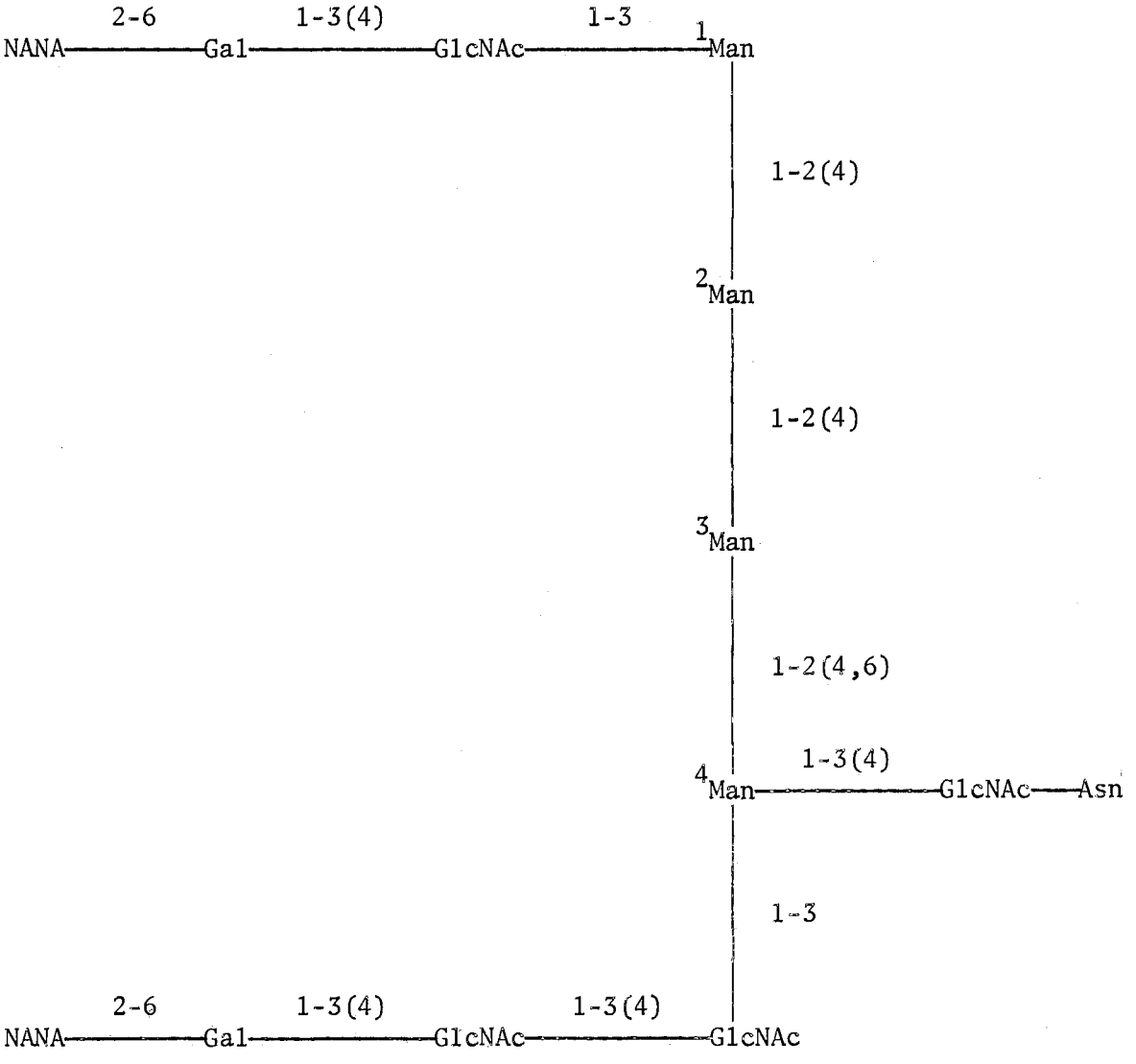
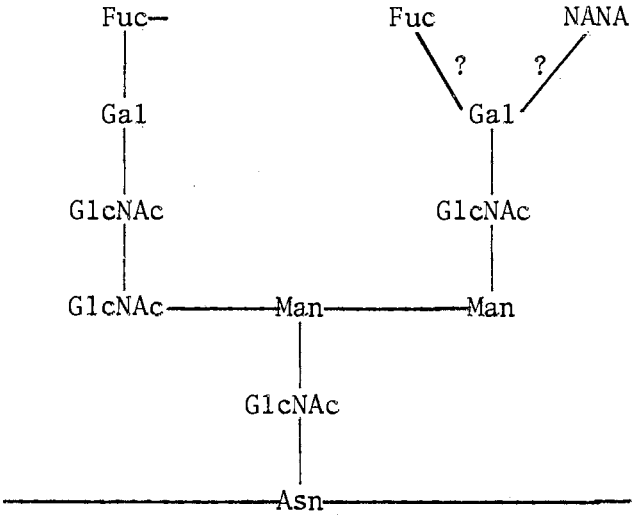
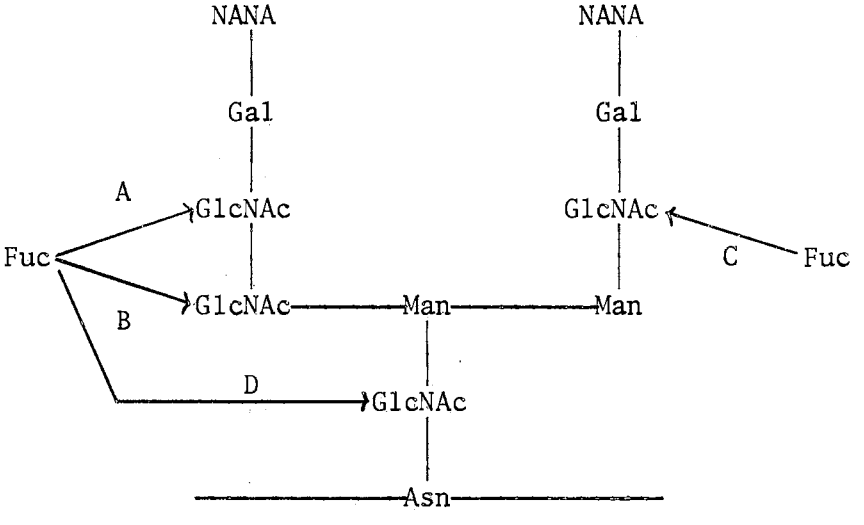

2-6
1-3(4)
1-3
NANA
Gal
GlcNAc
1Man
1-2(4)
2Man
1-2(4)
3Man
1-2(4,6)
1-3(4)
4Man
GlcNAc
Asn
1-3
2-6
1-3(4)
1-3(4)
NANA
Gal
GlcNAc
GlcNAc

Figure 14. Proposed Structures for Carbohydrate Units of Porcine Transferrin

Structure I



Structure II



CHAPTER V

SUMMARY

Chemical studies were performed on porcine transferrin in order to elucidate the number of carbohydrate units, the size of the units, their chemical composition, and the glycopeptide linkage.

Transferrin for a portion of this study was purified from a commercial grade by gel filtration chromatography. Glycopeptides were prepared from the purified, commercial transferrin by pronase digestion and fractionated by gel filtration chromatography on Biogel P-10 and P-6 columns. The glycopeptide fraction eluted from the column as a single peak. Composition of the glycopeptide fraction showed that the amino acid content was greatly reduced from that of the native protein and that the monosaccharide residues existed in the same relative proportion as in the native protein. These data suggest that the carbohydrate moiety of porcine transferrin exists as a single type carbohydrate unit which has a structure similar to that of human transferrin. Unequivocal evidence for a single type unit awaits characterization of the purified glycopeptides.

In order to prepare enough glycopeptide for further purification and characterization, it was necessary to purify a large quantity of transferrin. Since transferrin was no longer available from a commercial source, it was isolated from the pig serum by ammonium sulfate precipitations and conventional ion exchange techniques. Composition studies were performed which showed the carbohydrate content to be significantly

lower than the purified, commercial transferrin. The isolated glycoprotein was submitted to pronase digestion, glycopeptides were isolated, and preliminary composition studies were performed on the glycopeptide. From the preliminary data obtained in this portion of the investigation, it seems most probable that a new species of porcine transferrin has been isolated which contains one half the amount of carbohydrate as the purified, commercial transferrin.

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