THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

PREPARATION, CHEMICAL CHARACTERIZATION AND DEGRADATION STUDIES OF A BOVINE β-GLYCOPROTEIN

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1962

PREPARATION, CHEMICAL CHARACTERIZATION AND DEGRADATION STUDIES OF A BOVINE β-GLYCOPROTEIN

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ACKNOWLEDGEMENT

The author wishes to express her sincere thanks and profound gratitude to Dr. M. R. Shetlar for his guidance and assistance which have made this study possible from the beginning.

Sincere appreciation is expressed to the staff of the Biochemistry Department for their help in the preparation of this dissertation.

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CHAPTER I

INTRODUCTION

The study of protein components of serum began about a century ago; fibrinogen was separated from the rest of the plasma in an insoluble form, fibrin, euglobulin was obtained as precipitate on dilution, and the heat incoagulable fraction was named seromucoid (1). After the recognition that there were proteins of different properties in the blood, numerous procedures were developed for their separation. These methods included salt fractionation, dialysis, ultracentrifugation and chromatographic separation, electrophoresis and selective precipitation. In the early stages of fractionation of the complex mixture of plasma proteins confusion existed in the nomenclature applied by different workers using the various techniques. In 1937 Tiselius (2) developed the moving boundary electrophoresis apparatus and succeeded in separating serum protein into several distinctly different components. The various globulin fractions were named α-, β- and γ-globulin according

to their mobility in an electric field. Svensson (3) by the aid of the electrophoretic technique studied the fractionation of horse, cow, swine and rabbit serum and demonstrated the heterogeneity of many of the globulin fractions obtained through different procedures. At the present time, in order to avoid confusion in nomenclature, physiologically occurring components that are separated by a new technique, or newly discovered abnormal components are frequently named by reference to electrophoretic fractions of normal human serum measured in pH 8.6 barbital buffer.

Among all the fractions of serum, albumin, α-globulin, β-globulin and γ-globulin, relatively few studies have been done on β-globulin glycoprotein.

In this study, a β-globulin was prepared from bovine serum characterized chemically, and a degradative study was carried out on the preparation.

CHAPTER II

LITERATURE REVIEW

Since the study is concerned with the preparation and chemical study of a β-globulin glycoprotein fraction, this review will cover both of the 1) purification of β-globulins and 2) structural studies of the glycoproteins.

Preparation of β-Globulins

Numerous β-globulins have been demonstrated by various centrifugation and electrophoretic techniques. Only the preparative technique will be reviewed here because a large quantity of β-globulin is required for complete chemical studies.

Various salts have been used for the fractionation, especially sodium sulfate, ammonium sulfate, and potassium phosphate.

Five protein fractions were successively separated from plasma by the Howe method (4) with sodium sulfate: fibrinogen by 10.7 % Na_2SO_4, euglobulin by 14.2 %; pseudoglobulin I by 17.8 %: pseudoglobulin II by 21.3 % respectively: the albumin remaining in solution.

Butler et al. (5) succeeded in separating the horse

plasma into 4 fractions. Fraction I containing fibrinogen was precipitated at a molar concentration of phosphate of 1.25. Fraction II, probably largely euglobulin, but no more than two thirds of the total amount, precipitated at a molar concentration of phosphate of 1.25 and it continued to be precipitated without the active precipitation of pseudoglobulin up to a molar concentration of 1.50. Fraction III, representing approximately 80 % of the pseudoglobulin and 30 % of the euglobulin precipitated between the molar concentrations of 1.50 and 2.40. Fraction IV, consisting of albumin 1 with some pseudoglobulin began to precipitate at a molar concentration of phosphate of 2.40 and continued to be precipitated without the active precipitation of albumin 2 up to a molar concentration of 2.58. Fraction V, consisting largely of albumin 2 with a trace amount of pseudoglobulin and albumin 1, began to precipitate at a molar concentration of phosphate of 2.58 and was completely precipitated at a 3.00 M concentration of phosphate.

The traditional ammonium sulfate fraction divided the plasma proteins into fibrinogen, separated at approximately 0.20 - 0.25 saturation with ammonium sulfate; euglobulin, separated by 0.33 saturation; and albumin which remains in solution after all the other fractions have been precipitated (6).

It has long been recognized that this simple scheme

of procedure did not sharply separate a series of homogeneous and uniform fractions. The following evaluation was made by Kugelmass (7): euglobulin = γ-globulin; pseudoglobulin = α_1-and α_2-globulins; supernatant albumin = (α_1 + α_2)-globulins and albumin.

Preparation of an electrophoretically relatively homogeneous β-globulin was accomplished by using salt fractionation with ammonium sulfate and dialysis by Cohn et al. (8). By dialyzing varying quantities of salt into serum through cellulose membrane, they were able to fractionate the horse serum into the following fractions:

FRACTIONATION OF HORSE SERUM

Fraction	Concentration of Ammonium Sulfate		Types of Protein Precipitated
	Moles per Liter	% of Saturation	
I	1.39	0.34	Largely γ-globulin
II	1.64	0.40	γ-, β-and α-globulin
III	2.05	0.50	β-, α-globulin, Mucoglobulin
IV	2.57	0.62	Largely crystalline albumin
V	2.80	0.68	Haemocupreine, choline esterase, glycoprotein, phosphatase

The fraction III was dialyzed against distilled

water and the non dialyzable portion was reprecipitated by slowly adding ammonium sulfate to 1.64 - 1.78 M. The dialysis and ammonium sulfate precipitation were repeated once more. The precipitate was dissolved in water, adjusted to pH 5 and dialyzed. The non dialyzable fraction was found to contain largely β-globulin.

Cohn and co-workers (9), by using ethanol precipitation at controlled temperature, pH, ionic strength and protein concentration, were able to fractionate blood protein into five major fractions, I, II + III, IV - 1, IV - 4 and V. The fraction II + III was demonstrated to be a mixture of γ-globulin and β-lipoprotein. Later the cold ethanol method was extended for subfractionation of the major fractions of II + III. (10)

From Cohn fraction IV-7, the β_1-metal combining protein of human plasma was crystalized from ethanol-water mixtures of controlled pH, ionic strength and temperature by Koechlin (11). When the solution of fraction IV-7 containing 76 % of β_1-metal combining protein with impurities of albumin and α_2-globulin was adjusted to pH 4.45, ionic strength 0.1, the impurity, α_2-globulin, was precipitated by 0.091 M fraction of ethanol at -5° C and protein concentration of 1 %. The supernatant was again adjusted to pH 6.1 ionic strength 0.24 and the β_1-globulin was precipitated by 0.163 M ethanol fraction. The β_1-globulin which had been washed with ethanol to lower the salt con-

centration was crystallized at -3° C, pH 6.2 by adding 53.3 % ethanol very slowly. According to Boettcher (12) the method could not easily be reproduced.

Horejsi et al. (13) observed that 0.4 % aqueous solution of rivanol (2-ethoxy-6,9-diaminoacridine) would quantitatively precipitate albumin, fibrinogen and most of the α- and β-globulins from blood serum or plasma. Gamma globulins were not affected by rivanol and could be recovered by ethanol precipitation. It was also found that the purity of γ-globulin isolated was dependent on the quality of the rivanol used since some of their preparation contained up to 10-20 % β-globulins. According to this experiment the rivanol should be a good precipitant in purifying the commercial β-globulin, Cohn's fraction III, because the commercial β-globulin contains both β-globulin and γ-globulin.

According to Boettcher (12), β-metal binding fraction (transferrin) is not precipitable by rivanol, therefore it is likely that the γ-globulin obtained by Horejsi was contaminated with metal binding β-globulins.

Saifer et al. (14) in studying the interaction of rivanol and protein observed that when an increasing volume of 0.4 % rivanol solution was added to a fixed volume of serum, the facter migrating electrophoretic fractions were progressively removed from the supernatant and appeared in corresponging precipitates. In a 1:4 (Serum, 1 part, :

0.4 % rivanol, 4 parts) mixture the supernatant contains only γ- and β-globulins. Further addition of 0.4 % rivanol solution did not result in separation of any additional fractions. The mixture of γ- and β-globulins (without removal of the rivanol) was injected both intraveneously and subcutaneously to rabbit. The autopsy of the rabbits revealed that rivanol had no deleterious effect on rabbit and resulted in high titer antisera.

Wallenius et al. (15) by using starch block electrophoresis with barbital buffer of pH 8.6, ionic strength 0.1 obtained albumin, α-globulin, β-globulin and γ-globulin fractions from non-lipid human serum. They found that the β-globulin fraction which was believed to be transferrin had a sedimentation constant (S_{20}) of 5.

By using rivanol Boettcher et al. (12) were able to prepare a nearly electrophoretically pure preparation of the metal-binding globulin by a relatively simple procedure. It was shown that at pH between 8 and 10, rivanol precipitated almost all of the proteins except β-globulin and γ-globulin. After removal of rivanol by charcoal absorption from rivanol-free solution the γ-globulin was precipitated by 25 % ethanol at pH 6.8 (-6° C). The β-globulin was precipitated by 40 % ethanol at pH 5.8. The β-globulin thus obtained was claimed to be β-metal combining globulin. No chemical characterization of the fraction was reported.

Steinbuch and Quentin (16) isolated one of the

minor components of α-globulin, ceruloplasmin, by sub-fractionation of fraction III and IV with rivanol. The method was as follows: (1) Precipitation by rivanol of some accompanying globulin at pH 6. (2) Adjustment of pH to 7.8, and addition of another portion of rivanol in order to precipitate the ceruloplasmin. (3) Dissociation of the rivanol-protein complex of step 2 at pH 4. (4) The elimination of accompanying proteins by treatment with 50 % alcohol at pH 4.0. (5) The extraction of the precipitate at pH 6. The fraction isolated was said to be ceruloplasmin. No chemical analyses were reported.

Using calcium phosphate column and stepwise elution by sodium phosphate buffer (0.10 to 1.3 M). Hjertén (17) was able to obtain two fractions from an electrophoretically separated β-globulins. The two fractions were identified to be β-metal combining protein and lipoprotein. The β-metal combining fraction was characterized as a) being reddish-brown colored in solution, b) staining strongly with bromophenyl blue, c) not staining with sudan-black B, and having sedimentation constant of S_{20} 5.5. The characteristics of β-lipoprotein were opalescent in solution, stained strongly with sudan-black B and only weakly with bromophenyl blue. No chemical analysis of the fractions were reported.

Isolation of β_{-2A}-globulin was accomplished by Heremans et al. (18) by combination of zinc sulfate precipitation, ammonium sulfate precipitation and starch block

electrophoresis. The procedure was based on the hypothesis that β_{-2A}-globulin was a substance with high resistance to precipitation with zinc ions. At zinc sulfate concentration of 100 m M per liter at neutral pH, low ionic strength and temperature ranging from 25° C to 29° C, albumin and large part of α-globulin were precipitated. The supernatant was freed of zinc by adding 1 to 2 % glycine and β_{-2A}-globulin was precipitated by 1.8 M ammonium sulfate. The precipitate contained about 75 % β_{-2A}-globulin and had α_2-globulin as a contaminant. The mixture was further resolved into electrophoretic components by starch block electrophoresis.

The composition of the β_{-2A}-globulin precipitation was as follows.

Nitrogen	16.20 %
Hexoses	4.90 %
Hexosamine	3.74 %

The above figure was calculated on the basis of 100 mg of polypeptide as determined by the biuret method.

Partial purification of one of the β-globulins, transferrin, was carried out by Patras et al. (19) as follows. Serum was saturated with respect to iron by addition of 12 mg iron as ferrous ammonium sulfate per ml serum. The mixture was kept at 5° C for one hour. Three volumes of 0.4 % aqueous rivanol was then added to one volume of serum. The final pH was adjusted to 8.5-8.6 with NaOH and the mixture was kept at 5° C for 1 hour. The

supernatant was treated with Norite or dialyzed to remove rivanol. The final product was mixture of transferrin and γ-globulin.

The effect of pH, ionic strength and temperature on the precipitation of bovine serum albumin with rivanol was studied by Kaldor *et al.* (20). The results indicated that at pH 4.0, 5.0 and 6.0 ionic strength 0.1, there was no precipitation at a rivanol concentration of 40 mM or less. At pH 7.0, 70 % of the bovine serum albumin was precipitated by a concentration of 8-20 mM, and 100 % of bovine serum was precipitated by 4.0 mM rivanol solution at pH 8.0, 9.0 or 10.0. Between pH 7 and 8 the interaction between rivanol and bovine serum albumin under the experimental condition used by the authors was stoichiometric in nature. The per cent of bovine serum albumin precipitated by 8.0 mM rivanol solution was determined as a function of the ionic strength of the buffer at pH 7.0, 8.0 and 10.0; increasing the ionic strength reduced precipitation at any pH. Modification of the basic group of bovine serum albumin by means of diazotization, iodination, acetylation or guanidylation caused increased precipitation in the pH range from 6.0 to 8.0. Extensive methylation of bovine serum albumin resulted in diminished or no precipitation. Hence it is concluded that the general nature of the rivanol bovine serum albumin interaction resembles that of bivalent cations to carboxyl groups.

Structural Studies on Glycoprotein

Abundant evidence indicated that carbohydrate and protein are linked in stable chemical bonds in glycoproteins. Numerous structural studies have been carried out of glycoproteins such as egg albumin and ovomucoid because of their ease of preparation. On the other hand, few structural works have been done on other serum glycoproteins. Since the techniques used in studying the egg glycoprotein and serum glycoprotein are similar, this literature review will cover the works done on both egg glycoproteins and serum glycoproteins.

Levene and Mori (21) by repeated $Ba(OH)_2$ hydrolysis obtained a trisaccharide containing one glucosamine and two mannose residue. The egg white was hydrolyzed with $Ba(OH)_2$ and the polysaccharide was precipitated by adding, alternately, $Ba(OH)_2$ and a hot saturated solution of basic lead acetate. The barium and lead were precipitated as carbonates by passing CO_2 through the polysaccharide solution. The non-sugar nitrogeneous material contaminated with the polysaccharide was precipitated by mercuric sulfate. The non precipitable fraction containing excess mercuric sulfate and sugar was treated with hydrogen sulfide and barium hydroxide to remove mercuric and sulfate ions. The polysaccharide was precipitated by methanol from the mercuric sulfate free

solution. After repeating $Ba(OH)_2$ hydrolysis 3 times, the trisaccharide was obtained free of protein.

Fränkel and Jellinek (22) hydrolyzed egg albumin with $Ba(OH)_2$, removed amino acids with neutral lead acetate and precipitated the polysaccharide with ammonical lead acetate. They repeated this procedure several times and finally obtained an optically inactive polysaccharide by precipitation with alcohol. Only mannose and glucosamine were found in the acid hydrolysate of this substance.

Neuberger (23) also isolated a carbohydrate preparation from egg albumin. The egg albumin was digested with trypsin for 4-6 weeks and the undigested protein was precipitated by phosphotungstic acid. The amino acids remaining in the digestion mixture were converted into N-acetyl compounds by reacting with ketene and removed by extraction with chloroform. The polysaccharide, free from amino acids and undigested protein, was acetylated, extracted with chloroform and deacetylated. The biuret negative polysaccharide was finally precipitated by alcohol. Mannose, glucosamine and an unidentified nitrogeneous compound were found in the hydrolysate of this preparation. The unidentified nitrogeneous material is likely to be sialic acid which was not known at the time of Neuberger's report.

Suzuki (24) reported isolation of the prosthetic group of ovomucoid by: 1) hydrolysis of the mucoid either

by heating with barium hydroxide, (3 g of ovomucoid in 30 ml of water, heated at 100° C for 5 to 25 hours with 33 g of barium hydroxide) or tryptic digestion (4 weeks at 37° C); 2) removal of non-sugar nitrogeous material by mercuric chloride precipitation; 3) acetylation and precipitation of the acetylated compound; 4) deacetylation. After the chemical analysis of the "prosthetic group" for nitrogen, glucosamine, mannose, acetyl group, reducing power and ash content, it was concluded that product obtained by the alkali hydrolysis and the one with tryptic digestion were similar. These results seems to indicate that $Ba(OH)_2$ even in such a rigorous condition does not degrade the carbohydrate moiety of the ovomucoid. It is possible that the vigorous $Ba(OH)_2$ hydrolysis and prolonged tryptic digestion both actually cause similar degradation of the carbohydrate portion. It is also possible that the carbohydrate portion contains no reducing end, and therefore it is more stable to alkali hydrolysis. No test for peptides or amino acids was made, therefore, it appears doubtful that the product was completely free of amino acids.

Cunningham *et al.* (25) obtained a glycopeptide from egg albumin by the following procedure. The crystalline egg albumin was hydrolyzed with trypsin and chymotrypsin and a carbohydrate rich fraction was precipitated by 70 or 87 % ethanol. By DEAE-cellulose chromatography a glycopeptide containing glucosamine, mannose, aspartic acid, tyrosine,

threonine, serine, valine and leucine was obtained. Following treatment with carboxypeptidase approximately 0.7 M equivalent of leucine was liberated together with small but substantial amounts of valine, threonine, and serine. The tyrosine was proved to be the N-terminal residue. Hence the oligosaccharide unit was concluded to be attached to aspartic acid.

Neuberger (26) resumed structural studies on egg albumin 20 years after his first preliminary paper (23). The egg albumin was digested with pepsin, trypsin and chymotrypsin. The hydrolysate was fractionated by celite column chromatography and a carbohydrate containing compound was obtained by gradient elution with ethanol-water. The protein-carbohydrate complex was further purified with column electrophoresis. Upon hydrolysis the protein-carbohydrate complex yielded mannose, glucosamine, serine, threonine, leucine and aspartic acid. On treatment with carboxypeptidase, leucine, serine and threonine only were liberated; on fractionation of a two-day digestion product, a carbohydrate containing fragment was obtained, which contained 1 mole of aspartic acid, only traces of serine and threonine, and no leucine. It was therefore concluded that aspartic acid is linked to carbohydrate.

At the same year Jevons (27) digested the egg albumin with pancreatic protease, deproteinized with $CHCl_3$, removed amino acids with Dowex 50 column and obtained a

carbohydrate rich fraction by paper electrophoresis. The glycopeptide obtained aspartic acid, leucine, glucosamine and mannose in molar ratio of 1 : 1 : 2 : 4. Other amino acids were found to be present in trace amount. The unhydrolyzed material gave a weak ninhydrin reaction on paper and reaction with fluorodinitrobenzene showed that the amino group of the aspartic acid residue is free. They concluded that the result would be consistent with combination of an amino acid carboxyl group with flucosamine, but direct evidence for such a bond was lacking.

Thorough studies on chemical structure of egg albumin and ovomucoid was carried out by Bragg and Hough (28). They observed that galactose was completely liberated within 1 hour by hydrolysis with a cation resin (Amberlite I R 120) at 100^{o} C. During this time, only 1/3 of the total mannose appeared free and the remaining mannose required 48 hours for complete liberation. As the result it was suggested that all of the galactose and 1/3 of the mannose are end units of the carbohydrate moiety. This suggestion was confirmed by periodate oxidation data. After periodate oxidation of ovomucoid, 54-52 % of glucosamine, 50 % of mannose and 100 % of galactose was found to be destroyed. The sugar most rapidly destroyed were suggested to be the end units of the carbohydrate. By a methylation technique, 2, 3, 4, 6-tetra-O-methyl galactose and 2, 3, 4, 6-tetra-O-methyl mannose was obtained and these 2 sugar derivatives

were described as occupying the non reducing terminal carbohydrate unit. Unidentified tri-O and di-O-methyl derivatives of mannose were detected, and hence it was suggested that mannose must also occur at other positions in the molecule. The di-O-methyl mannose did not give formaldehyde with periodate either before or after reduction with sodium borohydride and hence it is probably the 2, 6-di-O-methyl derivative. Methylated derivatives of 2-amino-2-deoxy-D-glucose were not detected.

An attempt was made to enrich the carbohydrate moiety of the mucoprotein by hydrolyzing the ovomucoid with 10 % (W/V) $Ba(OH)_2$ under N_2 at 100° C for 17 hours. After removal of barium ion the carbohydrate rich unit was precipitated by alcohol from the hydrolysate, but the carbohydrate unit was not found to be free of amino acids. A small amount of carbohydrate, largely glucosamine and mannose, were degraded. Galactose was not destroyed by alkali due to the fact that it occurs only as the non-reducing end group. By partial acid hydrolysis, small quantities of disaccharides, mannobiose and mannosyl glucosamine were found.

Cunningham (29) published his further structural studies of ovalbumin glycopeptides in detail 4 years after the first preliminary paper (25). The egg albumin was treated with sodium-p-chloromercuribenzoate which was found to enhance the extent of heat denaturation of the protein and to hinder gel formation and allowed denaturation at 32° C

in 20 minutes. This denatured protein was then digested with trypsin. The pH of the digestion mixture was adjusted to pH 5 and the large quantity of precipitate was collected and digested with α-chymotrypsin. The nondialyzable portion was digested with α-chymotrypsin again. The digestion mixture was fractionated by ethanol. The fraction which is not precipitable by 50 % ethanol and precipitable by 87 % ethanol was further fractionated by DEAE cellulose column chromatography. The two major fractions were obtained and one of them containing 39 to 45 % mannose was used for further study. The fraction exhibited heterogeneity as it appeared as a wide peak in column electrophoresis. The fraction was again fractionated with a charcoal-celite column chromatography and again no sharp separation was observed. The glycopeptide fraction was converted into DNP-glycopeptide and fractionated by counter current distribution technique. The chemical analysis and molecular weight determination indicated the molar composition of DNP-glycopeptide as: aspartic acid 1, leucine 2, tyrosine 1, serine 1, threonine 1, valine 1, mannose 5, N-acetyl glucosamine 3. By carboxypeptidase digestion threonine, serine, valine, leucine in molar ratio of 0.59, 0.48, 0.57, 1.25 were liberated. The sequence of amino acids in the peptide portion of the glycopeptide was found to be tyrosine-aspartic acid-leucine-threonine-serine (valine, leucine). The sequence of valine and leucine was unknown. From the carboxypeptidase

hydrolysis it was concluded that aspartic acid is linked to carbohydrate unit.

The glycopeptide was not hydrolyzable in 1 N NH_2OH HCl in 1.75 N NaOH for 40 minutes at room temperature, it was non-reducing, relatively acid labile, and contained 1 mole of ammonia. Consequently, it was suggested that the carbohydrate unit was linked to protein through an aspartic carboxyl group, involving an N-acetyl glycosylamine type linkage, to the reducing end of the polysaccharide.

Some of the first attempts to obtain a carbohydrate rich glycoprotein from the serum protein involved the isolation of the seromucoid complex. Seromucoid was first obtained from the filtrate of the heat coagulated serum albumin and globulin by precipitation with alcohol. The protein thus obtained contained about 25 % carbohydrate (30). Rimington (31) further purified the seromucoid fraction by ethanol fractionation. The main portion of the seromucoid was precipitated by 29 to 50 % of ethanol and found to contain 10.7 % carbohydrate, which consisted of N-acetyl glucosamine, galactose and mannose in equimolecular proportions.

A similar fraction was obtained by Winzler *et al.* and by Weimer *et al.* (32, 33) from 0.6 M perchloric acid filtrates of human plasma by precipitation with saturated ammonium sulfate at pH 4.0. The fraction was demonstrated to consist of at lease three components by boundry electrophoresis. By a series of ammonium sulfate precipitations, an electro-

phoretically homogeneous component which was the major component of seromucoid was obtained. The isoelectric point of the fraction was found to be pH 1.8. This protein has been called orsomucoid by Winzler.

Schmid (34) isolated a crystalline acidic α_1-glycoprotein as the lead salt. Properties of the mucoprotein obtained by Weimer, et al. and the acidic α_1-glycoprotein of Schmid are very similar. The composition of the acidic α_1-glycoprotein is as follows:

Molecular weight	44.100
Isoelectric point	2.8
Hexose	16.4 %
Hexosamine	11.9 %
Sialic acid	10.6 %
Fucose	1.3 %
Protein	64.0 %

Since then considerable chemical investigation has been carried out on the orsomucoid preparation. Winzler et al. (35) was able to show that treatment of the orsomucoid with dilute sulfuric acid (0.01 N) at 80° C caused sialic acid to be split off and markedly reduced its electrophoretic anodic mobility at pH 4.5.

Popenoe (36) has shown that all of the sialic acid could be split from orsomucoid by neuraminidase from Clostridium Birefringens and the isoelectric point rose therewith to pH 5.0. At the same time there occurred a shift in

the titration curve of orsomucoid. This result indicates that sialic acid occupies a terminal position in orsomucoid and that the carboxyl group is free.

According to Winzler (35) the carbohydrate in the serum glycoproteins is firmly bound to the protein and can be extracted from it only after extensive degradation of the protein. They have approached the problem of the chemistry of the glycoprotein by determing the sequence of the liberation of the carbohydrate components from glycoproteins by acid hydrolysis on orsomucoid. The following table on page 22 is the result of their work.

From these results, it was suggested that sialic acid occupies the terminal group and fucose is either second terminal group or is next after sialic acid in a linear sequence. The hexosamine is most stably bound.

The other approach has involved a chromatographic study of the product rendered ultrafiltrable by hydrolysis for one hour at 100° C at various concentration of HCl. They found that sialic acid was liberated by hydrolysis with HCl above 0.001 N. At 0.0025 N a small amount of galactose was liberated, and at 0.005 N and 0.01 N galactose and fucose but not mannose. At hydrochloric acid concentration above 0.1 N a number of poorly resolved reducing substances were liberated. When these poorly resolved reducing substances were hydrolyzed with 2 N HCl for one hour, glucosamine, galactose, mannose were observed. A suggestion was made

LIBERATION OF ULTRA FILTRABLE CARBOHYDRATE FROM ORSOMUCOID BY 1 M-HCL AS A FUNCTION OF TIME

Hydrolysis time Minutes	Hexose		Hexosamine		Sialic Acid		Fucose		Protein	
	mg	%	mg	%	mg	%	mg	%	mg	%
0	40	1.3	0	0	30	1.5	12.5	5.1	100	1.1
5	700	23	150	8.3	1310	66	191	77	180	1.9
15	1080	55	-	-	1890	95	247	100	430	4.5
30	2770	90	910	40	1420	71	-	-	880	9.3
60	3070	100	1040	47	800	40	-	-	1270	13.3
120	2990	97	-	-	440	22	-	-	1640	17.3

that the extension of this work will ultimately permit the conclusion concerning sequence of carbohydrate to be drawn.

The most definite evidence yet available on one type of carbohydrate protein linkage in serum glycoprotein was provided by Rosevear and Smith (37, 38). By papain digestion of human γ-globulin at pH 6.8, removal of amino acids and short peptides by Dowex 50 and starch column electrophoresis, they were able to obtain 3 glycopeptides. The molecular weights of these 3 glycopeptides were determined to be less than 5,000. The composition of the glycopeptide was reported to be as follows.

Composition of Glycopeptides from a Human γ-Globulin

Constituent	Glycopeptide 1	Glycopeptide 2	Glycopeptide 3
Mannose	5	5	5
Galactose	3	3	3
N-acetyl glucosamine	8	8	4
Fucose	2	2	2
Sialic acid	1	1	0
Aspartic acid	2	2	2
Glutamic acid	3	2	1
Tyrosine	1	1	1

The similarity of the three glycopeptides suggested that they are derived from the same structure. The largest, glycopeptide 1, probably represents the prosthetic group of

the intact γ-globulin. Glycopeptide 2 and 3 appeared to be partially degraded. Amino end group analysis by Sanger's method gave DNP-aspartic acid from glycopeptide 3. Glycopeptide 2 yielded DNP-glutamic acid. This suggested that the glycopeptide 1 has the structure; glutamic acid, glutamic acid, aspartic acid (tyrosine, glutamic acid, aspartic acid, carbohydrate), where the carbohydrate group and the residue in parenthesis are of undetermined sequence. Leucine aminopeptidase released 2 residues of glutamic acid, 1 of asparagine and 0.8 of tyrosine from the glycopeptide 1. From this the sequence would be glutamic acid, glutamic acid, aspartic acid, tyrosine, (glutamic acid, aspartic acid, carbohydrate). The aminopeptidase liberated one residue each of aspartic acid, tyrosine and glutamic acid from glycopeptide 3. This suggested the sequence of glutamic acid, glutamic acid, aspartic acid, tyrosine, glutamic acid (aspartic acid, carbohydrate): the only remaining residue was aspartic acid which must be at the C-terminal end of glycopeptide 3 and bound to the carbohydrate.

In order to study the linkage between carbohydrate and amino acid the glycopeptide mixture was treated with carboxypeptidase (recrystallized 4 times) and it was found to remove trace amount of amino acids only. On the other hand the aminopeptidase was found to be able to liberate aspartic acid, glutamic acid and tyrosine. The enzyme hydrolysate was fractionated by two-dimensional electro-

phoresis-chromatography on paper and 3 fractions were obtained. The first fraction contained aspartic acid, glutamic acid, glucosamine and the second and third fractions contained aspartic acid and glucosamine. Consequently it was concluded that aspartic acid was involved in protein carbohydrate linkage.

The carbohydrate was suggested to be attached to the β-carboxyl group of the aspartic acid by an amide or ester linkage. If it were attached by an α-amide bond, both leucine aminopeptidase and papain would hydrolyze such a bond. If it were attached by an α-ester bond, it could probably be split by papain. Incubation of glycopeptide 1 and 3 with carboxypeptidase resulted in release of only trace of amino acids, thus provides additional evidence that the carbohydrate was linked to the terminal carboxyl group of aspartic acid.

Nolan and Smith (39) isolated 5 glycopeptides from a papain hydrolysate of rabbit γ-globulin. The γ-globulin preparation used was obtained from rabbit hyperimmunized to type VII Pneumococcus.

Salt free heat denatured γ-globulin suspended in water was incubated with stirring at 60° C with crystalline mercuripapain activated by 2.3-dimercaptopropanol at pH 6.3 to 6.8. The hydrolysate was treated with Dowex 50 and Sephadex G-25 to remove amino acids. The fraction which was not absorbed by Dowex 50 and Sephadex G-25 was fractionated.

by DEAE-cellulose chromatography. Five glycopeptides were obtained and three of them, the glycopeptides 2, 3 and 4, were chosen for further studies. The sequence of the amino acids was studied by Edman's thiohydantoin degradation and enzyme degradation. Glycopeptide 3 was submitted to the Edman degradation procedure. After the first degradation step, phenylthiohydantoin-glutamine was identified. After the second step, both phenylthiohydantoin-glutamic and phenylthiohydantoin-phenylalanine were present. The peptide residue remaining after the second step contained glutamic acid (0.41 M), phenylalanine (0.65 M), aspartic acid (1.00 M), and glucosamine (4.3 M). The molar ratios determined for those components in the intact glycopeptide were 1.05, 0.91, 1.00 and 4.3 respectively. From this it was suggested the structure of glycopeptide 3 as glutamine, phenylalanine, aspartic acid, carbohydrate. The above suggestion was further confirmed by a Leucine amino peptidase degradation study. After treatment of the glycopeptide 3 with leucine amino peptidase, the major ninhydrin-positive components of the hydrolysate were identified as glutamine, phenylalanine and a component containing glutamic acid (0.6 M), phenylalanine (0.6 M), aspartic acid (1.0 M) and glucosamine (3.0 M). By a similar but more elaborated technique, glycopeptide 4 was identified to be glutamic acid, glutamine, glutamine, phenylalanine, aspartic acid, carbohydrate.

Glycopeptide 2 containing fucose 1 M, aspartic acid

1 M, glutamic acid 2 M, phenylalanine 1 M could not be degraded either by phenylthiohydantoin degradation, leucine aminopeptidase or carboxypeptidase. The reason was suggested to be that the amino-terminus of the glycopeptide was occupied by a pyrolidone carboxylic acid residue formed by cyclization of a glutamyl residue during the fractionation procedure.

Nolan and Smith (40) by using the same technique described above with a slight modification, isolated six glycopeptides from bovine γ-globulin and one of them was characterized in detail. The composition of the glycopeptide was found to be hexose 5 M, glucosamine 4 M, fucose 1 M, sialic acid 0 M, arginine 1 M, lysine 1 M, aspartic acid 1 M, glutamic acid 3 M, proline 1 M, phenylalanine 1 M, and other amino acids in small quantity. By leucine aminopeptidase, lysine, proline, arginine, glutamic acid, glutamine, phenylalanine and a trace of aspartic acid was removed. Of these lysine and glutamic acid were liberated in the largest amounts as indicated by a high voltage paper electrophoresis-chromatography. This suggested the aspartyl residue as the point of attachment between the peptide and carbohydrate. The suggestion was confirmed by further degradative study of the glycopeptide. The glycopeptide was degraded by papain and a glycopeptide containing glutamic acid 0.3 M, phenylalanine 0.9 M, aspartic acid 1.0 M and glucosamine was obtained. By aminopeptidase digestion glutamic acid was

removed from the glycopeptide. This further suggested that aspartic acid is involved in peptide-carbohydrate linkage.

CHAPTER III

METHODS AND EXPERIMENTAL

Three topics, (a) preparation of a β-glycoprotein, (b) characterization of the glycoprotein and (c) degradation studies of the fraction, are included in this section.

Preparation of the β-Globulin

In the initial attempt to purify the β-globulin fraction, bovine Cohn's fraction III from Nutritional Biochemical Corporation was used. This preparation was found to move as a wide band between β- and γ-globulins by paper electrophoretic technique with barbital buffer of pH 8.6. The fraction III was only slightly soluble in distilled water and in 0.9 % NaCl solution.

Since the euglobulin is largely insoluble in salt-free water, a 1 % solution of the fraction III was dialyzed against distilled water in order to remove the euglobulin. None of the protein was found soluble after 24 hours dialysis. Again a 1 % solution of the fraction III preparation was dialyzed against dilute phosphate buffer (41) and all the protein again became insoluble 24 hours later.

The rivanol fractionation of Horejsi *et al.* (13)

was tried without success. According to this procedure, 0.4 % rivanol in proper amount should quantitatively precipitate albumin, fibrinogen, and most of the α- and β-globulin but not γ-globulin. All of the protein in the Cohn's fraction III preparation precipitated with 0.4 % rivanol solution. The use of this fraction was, therefore, abandoned and attempts were made to isolate the β-globulin fraction from fresh bovine serum. At first, the Misco continuous electrophoresis was used (42). The method was time consuming and difficult to obtain enough quantity for structural studies. Continuous electrophoresis with glass beads as the supporting medium was also tried without success.

A nearly homogeneous β-globulin, as indicated by paper electrophoresis, was prepared from fresh bovine serum by the combination of rivanol fractionation (14) and ammonium sulfate fractionation (8). The procedure is described below: In the preliminary experiment, the fresh bovine serum was treated with ammonium sulfate according to the method of Cohn *et al.* (8). The fresh serum was diluted with an equal volume of water and a quantity of ammonium sulfate sufficient to make the solution 1.6 molar was dialyzed into the dilute serum through dialyzing membrane. Meanwhile the serum was stirred continuously by a magnetic stirrer. At the end of 48 hours the supernatant containing albumin, α-globulin, β-globulin and a small

quantity of γ-globulin was separated from the precipitate by centrifugation.

For large scale preparation and further removal of γ-globulin from the supernatant the method was modified as follows. To the fresh serum equal volume of 3.4 molar ammonium sulfate solution was added through a separatory funnel drop by drop and meanwhile the solution was placed in an ice bath and stirred by the magnetic stirrer. The suspension was stirred further for 30 minutes after the addition of $(NH_4)_2SO_4$ solution ended. The suspension was then placed in the cold room for 48 hours. The precipitate containing largely γ-globulin and small quantity of β- and α-globulins was centrifuged off. The supernatant containing albumin, α-globulin and β-globulin was dialyzed against running water to remove ammonium sulfate. Barium hydroxide solution was used to check the extent of dialysis. Without prior removal of ammonium sulfate precipitation by rivanol did not occur. A small amount of precipitate formed during the dialysis was removed by centrifugation or filtration through filter paper No. 50. The supernatant was adjusted to neutral pH 7.0, treated with various quantities of rivanol solution and placed in room temperature for 4 to 5 hours, and finally placed in a cold room at 4° C for 20 hours. The result of the paper electrophoresis indicated that when 0.2 ml of 2.0 % rivanol solution was added to 1 ml of the clear supernatant obtained above, albumin, α-globulin and part of β-globulin were present in the

precipitate and the non precipitable fraction was found to consist largely of β-globulin with trace amounts of α- and γ-globulin. If the rivanol treated serum was placed in cold room right after the addition of the precipitant, less satisfactory results were obtained.

In order to find the optimum pH for the procedure, the ammonium sulfate treated and dialyzed serum was adjusted to various pH values with hydrochloric acid and sodium hydroxide solutions and treated with 2 % rivanol solution in the ratio of 1 volume of the treated serum and 0.2 volume of 2 % rivanol. Twenty four hours later the non-precipitable fraction was treated with activated carbon, dialyzed against distilled water and examined by paper electrophoretic techniques. At hydrogen ion concentrations between pH 7.5 and 8.5 the rivanol precipitated all proteins except a fraction with the mobility of β-globulin.

It was then necessary to determine the quantity of rivanol required to precipitate the albumin and α-globulin at pH 7.5. The same procedure used previously (Page 31) was repeated at 7.5 instead of 7.0.

After the treatment with rivanol, the β-globulin in rivanol solution was dialyzed against distilled water at about 4° C for 48 hours with frequent change of water. The small quantity of rivanol which was not dialyzed was removed by treatment with activated carbon. Enough activated carbon was added to render the filtrate free of greenish

yellow color and then the carbon was removed by either centrifugation or filtration. The β-globulin solution thus obtained was lyophilized.

The schematic diagram of the final procedure is outlined in Figure 1.

Characterization of the β-Globulins

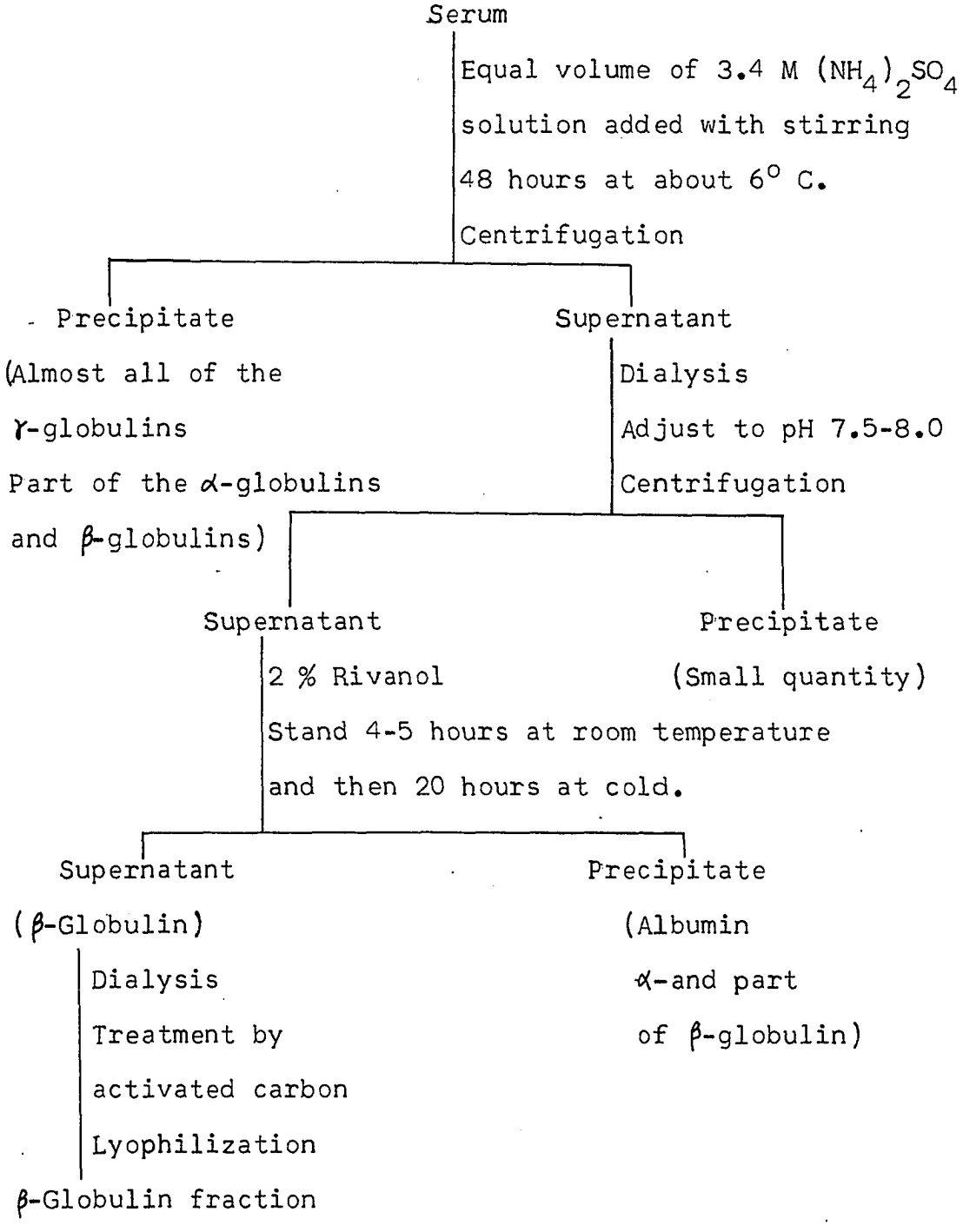
Electrophoretic Study

The β-globulin fraction was studied electrophoretically on paper strips using L K B horizontal electrophoresis cell. Electrophoresis was conducted with 5 milliamperes (for 4 strips) at room temperature for 16 hours in Michaelis buffers pH 2.6, 3.6, 5.1, 6.3, 6.9, 7.5, 8.6 and 9.6 (43). The strips were oven-dried and developed with bromophenol blue. The electrophoresis was also performed with 0.075 M barbital of pH 8.6. The strips were oven-dried and stained with sudan black-B.

In order to further check the homogenity of the prepared β-globulin, cellulose acetate paper electrophoresis (44) was carried out with 0.075 M barbital buffer of pH 8.6 for 4½ hours at 0.5 milliamperes per centimeter width of paper and also with tris-borate buffer of pH 8.9 (45) for 16 hours at constant voltage 180 volt. The strips were oven-dried, stained with ponseau S (44). The strip developed with tris-borate buffer was scanned with the Spinco Analytrol.

Figure 1

PREPARATION OF THE β-GLOBULIN



Chemical Composition

Hexose determination. The hexose content of the β-globulin was determined by Anthrone and Tryptophan methods as described by Shetlar (46, 47). An equimolar mixture of galactose and mannose was used as the standard.

Hexosamine determination. The Elson-Morgan method (48) was used for the determination of hexosamines.

Fucose determination. The cysteine-H_2SO_4 method of Dische et al. (48) was used for fucose determinations.

Protein determination. Biuret or Lowry's methods (49) were used for the determination of protein. Lab-Trol of Dade Reagents Inc., Miami, Florida was used as the standard.

Sialic acid determination. Diphenylamine (48) and thiobarbituric acid method of Warren (50) were used for the determination of sialic acid. A submaxillary mucin preparation containing 19.1 % N-acetyl neuraminic acid from the Nutritional Biochemical Corporation was used as the standard.

Nitrogen determination. Digestion by H_2SO_4 and H_2O_2 followed by Nesslerization was used for the determination of amino nitrogen (51).

Qualitative Study of Hexoses. The β-globulin solution was dialyzed against distilled water for 48 hours with frequent change of the distilled water prior to acid hydrolysis because a preliminary experiment indicated the presence

of glucose in the β-globulin fraction. The dialyzed β-globulin solution was hydrolyzed with 2 N HCl at 100° C for 16 hours, and the hydrolysate was evaporated to dryness in an evacuated oven at room temperature. The material was then dissolved in water, and passed through a Dowex 50 column to remove amino acids. The Dowex 50 column was washed with water and the non-absorbable material was examined by paper chromatography for hexoses. Ascending chromatography developed with n-butanol, pyridine, water (3.0:1.0:1.5) was used. The spots of hexoses were detected by 2-aminobiphenyl.

The hexoses were also studied electrophoretically on paper strips by using the Spinco hanging strip electrophoresis system. Electrophoresis was conducted with constant voltage of 210 volts at room temperature for 5 hours in borate buffer of 0.05 M and pH 9.2. The strip was dried and developed with 2-aminobiphenyl.

The hexose spot having the same R_f value as glucose was reacted with glucose oxidase. The spot was eluted from the paper by distilled water after the paper chromatographic separation of the sugar and removal of pyridine from the paper by handing the chromatographic paper in the hood for 48 hours. To about 30 μg of glucose eluted from the paper, 15 μg of glucose oxidase in 0.5 ml of phosphate buffer of pH 7.4 was added. The incubation mixture was incubated at 37° C for 3 hours and the glucose oxidase was denaturated by heating. The blank was carried out with heat denaturated

enzyme. The incubation mixture was examined again for sugars by paper chromatographic technique.

Quantitative Study of Galactose and Mannose

The β-globulin hydrolysate was prepared by the procedure described in the qualitative study of the hexoses. The hexoses, relatively free of amino acids and glucosamine, were separated by ascending paper chromatographic technique using Whatman #1 filter paper and ethyl acetate, pyridine, water (10:4:3) solvent. The chromatograms were developed with the solvent twice, stained with 2-aminobiphenyl and heated at 100-105° C for 5 minutes. The quantitative measurement of the mannose and galactose was made by measuring the total density of the colored sugar spots on the paper chromatogram using the Spinco analytrol equipped with Klett filter #42. A series of standard sugar solutions was applied on the same sheet of paper on which the sample was applied.

In order to determine the reliability of the method a known quantity of mannose and galactose was added to one of the β-globulin hydrolysate to determine the recovery of the sugar.

Preparation of Glycopeptide

A five per cent solution of β-globulin, adjusted to pH 1.8 with hydrochloric acid, was digested with pepsin (weight ratio of sample to enzyme, 100:1) at 37° C. One-sixth of the total pepsin used was added at every 12 hours

over a period of 3 days. Toluene was added to prevent putrefaction. Aliquots of the digestion mixture were examined every 12 hours electrophoretically on paper strips with the L K B horizontal electrophoresis cell. The electrophoresis was conducted with 5 m. a., (total of 12.5 cm width of paper) in barbital buffer 0.075 M and pH 8.6. The strips were dried and developed with ninhydrin solution (0.25 % ninhydrin solution containing 5 % acetic acid). At the end of 3 days, the acidity of the solution became constant and the electrophoretic pattern of the digestion mixture showed no further change. At the end of digestion a dark colored precipitate, insoluble at any pH, remained. The clear supernatant was collected by centrifugation and dialyzed against 0.05 M barbital buffer of pH 8.6 for 24 hours. The non-dialyzable fraction was determined for protein and hexose. The non-dialyzable clear solution was fractionated by Misco continuous electrophoresis using Whatman 3 mm paper and 0.05 M barbital buffer of pH 8.6. The paper curtain was washed with 0.05 M barbital buffer for 24 hours before the sample was applied. The electrophoresis was carried out at constant voltage, 340 volts, and 9 m.a. The distribution of the fraction was detected by dipping the curtain into ninhydrin acetone solution (0.25 % ninhydrin in acetone solution containing 5 % acetic acid). The two fractions having the lowest mobility were used for further studies. For convenience, these two fractions will be referred hereafter as

glycopeptides I and II.

Electrophoretic Study of the Glycopeptide

The glycopeptides were studied electrophoretically on paper strips (S and S #50 filter paper) by using the L K B horizontal electrophoresis cell. The electrophoresis was carried out at 5 m. a. for 16 hours (1 m. a. per 2.5 cm of paper) in Michaelis buffers of pH 9.6, 8.6, 7.5, 6.9, 6.3, 5.1 and the strips were stained with ninhydrin solution as described above.

Chemical Composition of the Glycopeptides

Qualitative study of the hexoses in glycopeptides were carried out as described for the intact β-globulin except that the treatment of the hydrolysate with Dowex 50 was omitted. The result indicated the presence of the glucose in the glycopeptides. It was first suspected that the glucose might be the hydrolysis product of the paper which was eluted from the curtain during the continuous electrophoretic fractionation. Therefore, the pepsin digested and dialyzed sample prior to electrophoretic fractionation was examined for hexoses by paper chromatographic technique as described for glycopeptides.

Qualitative analysis of the amino acids of the glycopeptides were performed with the high voltage electrophoresis-chromatographic technique. The glycopeptide was hydrolyzed with 6 N HCl for 20 hours and evaporated to dryness in

an evacuated oven at room temperature. The material was dissolved in distilled water and dried again. The same process was repeated twice and then the sample was used for the high voltage electrophoresis. The electrophoresis was carried out with Whatman # 1 filter paper, 3000 volt, 2.5 m.a. for one hour in the solvent of pyridine, acetic acid, water (10:100:890) pH 3.5. The second dimension was developed as an ascending paper chromatograph with n-butanol, acetic acid, water (12 : 3 : 5 ratio in volume). The amino acids were detected with 0.25 % ninhydrin aceton solution.

Partial Acid Hydrolysis of the Glycopeptides

In order to study the nature of the carbohydrate moiety of the glycopeptides, a partial acid hydrolysis was carried out on the glycopeptide I as described below.

To 2 ml solution containing 80 μg of glycopeptide I, a calculated amount of HCl was added to make 0.0010 N, 0.0025 N, 0.0050 N, 0.01 N and 2.0 N. The glycopeptide solution in various acid concentration were heated at 100^{o} C for 2 hours. The hydrolysate was dried in evacuated oven at room temperature. The material was then dissolved in water, centrifuged and the clear supernatant was used for chromatography. The paper chromatographic paper developed with ascending technique in ethyl acetate, pyridine, water (10 : 4 : 3) was stained with $AgNO_3$ and ethanolic sodium hydroxide. The chromatographic paper was also sprayed with Somogyi Nelson spraying reagent

which was used to detect reducing oligosaccharides by French et al. (52).

Further Enrichment of the Carbohydrate Unit

For the study of the linkage between carbohydrate and protein, further removal of protein from the glycopeptide was desirable since there was still considerable quantity of protein in the glycopeptides. The bacterial enzyme pronase (53) was used as the proteolytic enzyme for the further enrichment of the carbohydrate unit of the β-globulin as below.

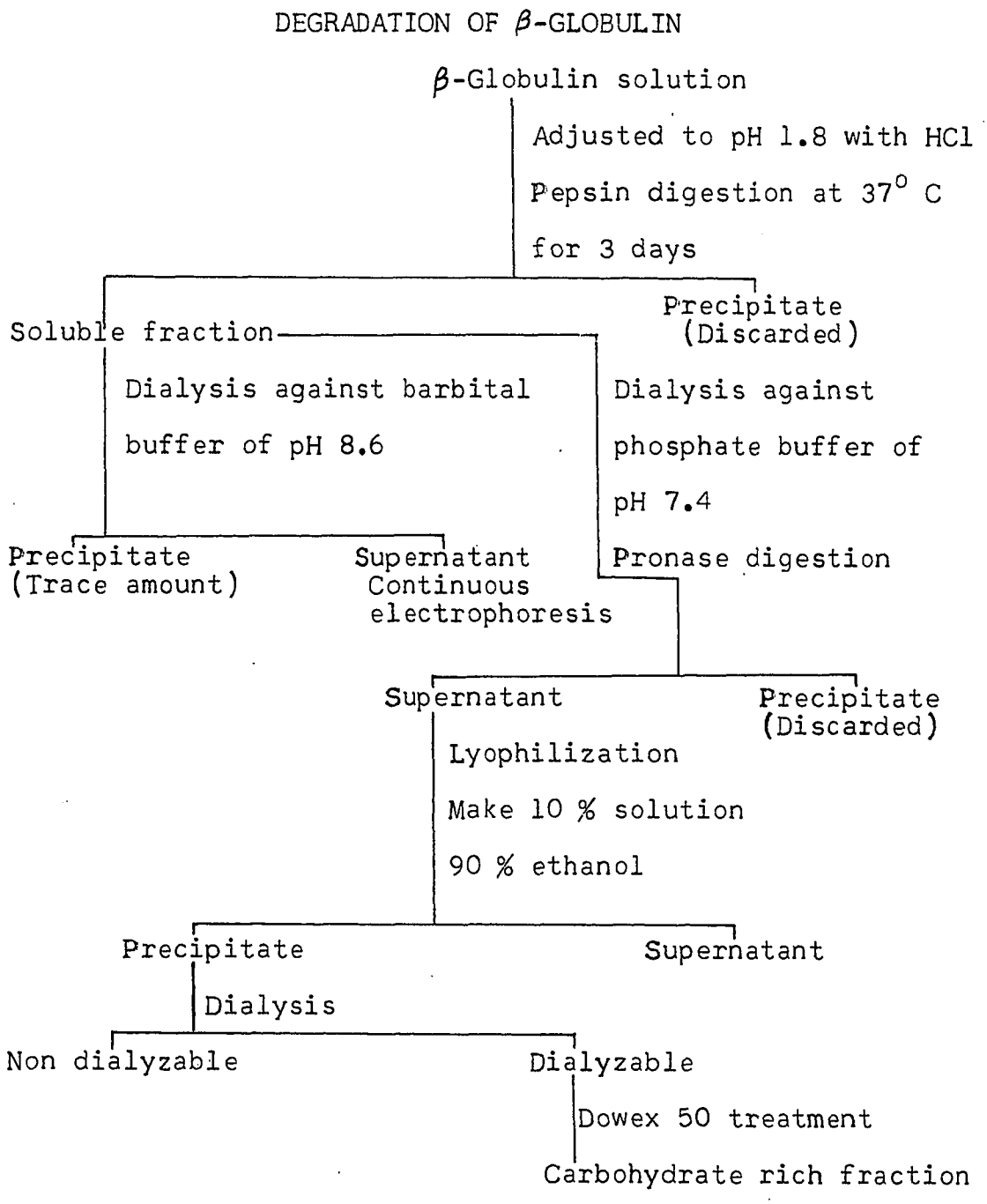
A 5 % β-globulin solution was first digested with pepsin as described before and the digestion mixture was dialyzed against phosphate buffer of pH 7.4 with frequent change of the phosphate buffer for 48 hours at cold room (about 4° C). Pronase was added to the non-dialyzable portion in a weight proportion of original β-globulin, 200 : pronase, 1. The mixture was incubated at 37° C for a period of 2 days. At the end of the digestion there remained a gray colored precipitate which was soluble in alkali solution but not in distilled water. The precipitate was discarded since it contained no detectable amount of hexose (as indicated by the anthrone reaction). The clear supernatant was collected by centrifugation and lyophilized. The lyophilized sample was then dissolved in distilled water to make 10 % solution from which the carbohydrate rich fraction was precipitated by 90 % ethanol. Both the precipitable and non-precipitable fractions were analyzed for hexose and protein by the Anthrone method

(47) and Lowry's methods respectively (49). Further enrichment of the carbohydrate was attempted by dialyzing the precipitable fraction against distilled water and the non-dialyzable fraction was again analyzed for carbohydrate and protein. The dialyzable fraction was passed through the Dowex 50 column to remove the small peptides and free amino acids and the fraction eluted from the column by water was determined for hexose and protein. The dialyzable, Dowex 50 treated fraction was examined for amino acids by the electrophoresis-chromatographic technique as described for qualitative amino acids analysis of the glycopeptide I and II. The dialyzable and Dowex 50 column treated fraction was found to be very rich in carbohydrate and will be hereafter called the unfractionated glycopeptides. The high voltage electrophoresis-chromatographic technique as described for qualitative analysis of amino acid was used to study the homogeneity of the fraction. The schematic diagram of the degradation of the β-globulin is outlined in Figure 2.

Degradation of the Unfractionated Glycopeptides With Amino Peptidase and Carboxypeptidase

The unfractionated glycopeptides solution 0.1 ml containing about 150 μg protein, 250 μg hexose and undetermined quantity of hexosamine in 0.1 ml was incubated with leucine aminopeptidase, 180 μg in 0.23 ml of barbital buffer of pH 8.6, 0.02 M, for 20 hours at 37° C. An additional 180 μg of the enzyme was added after 24 hours. At

Figure 2


DEGRADATION OF β-GLOBULIN
β-Globulin solution
Adjusted to pH 1.8 with HCl
Pepsin digestion at 37° C
for 3 days
Soluble fraction
Precipitate
(Discarded)
Dialysis against barbital
buffer of pH 8.6
Dialysis against
phosphate buffer of
pH 7.4
Pronase digestion
Precipitate
(Trace amount)
Supernatant
Continuous
electrophoresis
Supernatant
Precipitate
(Discarded)
Lyophilization
Make 10 % solution
90 % ethanol
Precipitate
Supernatant
Dialysis
Non dialyzable
Dialyzable
Dowex 50 treatment
Carbohydrate rich fraction

the end of 48 hours, the enzyme was removed by heating at 100° C for 5 minutes followed by centrifugation. The precipitate was washed three times with small quantities of water. The supernatant combined with washings was dried in the evacuated oven at room temperature. The dried material was then dissolved in 0.5 ml of water and pH of the solution was adjusted to about 7.5. To the above solution of 200 µg of carboxypeptidase in 0.2 ml of 0.02 M veronal buffer, pH 7.5, containing 0.1 M NaCl was added. An additional 200 µg of the enzyme was added after 24 hours. The incubation was carried out at 37° C for 48 hours. At the end of incubation, the enxyme was removed from the incubation mixture by heating at 100° C for 5 minutes and centrifugation. The clear supernatant was passed through a Dowex 50 column 3 times and the fraction eluted from the column by water was analyzed qualitatively for amino acid by the same technique used for amino acid analysis of glycopeptide I and II. Prior to acid hydrolysis paper chromatography using butanol, acetate acid, water was used to detect whether the Dowex 50 column treatment did remove all the free amino acids. A blank test was carried out as above except that the heat denaturated enzyme was used instead of active enzyme.

Barium Hydroxide Hydrolysis of the Glycopeptide

The pronase digested, 90 % ethanol precipitated fraction was hydrolyzed with 10 % $Ba(OH)_2$ at 100° C for 12 hours. After removal of barium ion as $BaSO_4$, the carbohydrate

rich fraction was precipitated by 75 % ethanol. The ethanol precipitation was repeated twice. The precipitate was dialyzed against water and the non-dialyzable fraction was analysed for hexose and protein. The dialyzable fraction was treated with Dowex 50 to remove the free amino acids and analyzed for amino acids qualitatively by electrophoresis-chromatography technique.

CHAPTER IV

RESULT AND DISCUSSION

Preparation of β-Globulin

A relatively simple procedure for preparation of the β-globulin fraction from bovine serum was developed by combination of ammonium sulfate with rivanol fractionation.

In the initial ammonium sulfate fractionation studies it was found that at 1.7 molar ammonium sulfate, practically all of the γ-globulin, a fairly large quantity of the β-globulin, and a small amount of α-globulins were precipitated. The distribution of the various protein fractions in the whole serum and the 1.7 molar ammonium sulfate soluble fraction as estimated from the densitometer curve of the paper strip electrophoresis are given in Table I.

According to the result reported by Cohn et al. (8), albumin was not precipitated from horse serum at ammonium sulfate concentration less than 2.05 molar. Therefore the figures in the column III, Table I, was calculated by assuming that no albumin was precipitated at 1.7 M ammonium sulfate, i.e. setting the albumin percentage in the column III equal to 48.2 % and calculating the percentage of other

fractions. From the figures in column III of Table I, it is apparent that 1.7 M ammonium sulfate precipitates most of the γ-globulin, as well as considerable quantities of β- and α-globulins.

By the rivanol precipitation it may be possible to prepare γ-globulin from the 1.7 M ammonium sulfate insoluble fraction since the rivanol at proper concentration and pH should precipitate all of the α-globulins and part of the β-globulins.

Table I

RESULT OF AMMONIUM SULFATE FRACTIONATION OF THE SERUM

	I Serum %	II 1.7 M $(NH_4)_2SO_4$ Soluble Fraction %	III* 1.7 M $(NH_4)_2SO_4$ Soluble Fraction %
Albumin	48.2	76.5	48.2
α-Globulin	8.1	8.1	5.1
β-Globulin	17.2	13.4	8.4
γ-Globulin	26.5	1.9	1.2
Total Protein	100.0	100.0	62.9

* The figures in column III were calculated by setting the albumin percentage in the column III equal to 48.2 % and calculating the percentage of other fractions.

The idea of the ammonium sulfate fractionation was based on the ammonium sulfate fractionation of horse serum

by Cohn et al. (8). They added the ammonium sulfate by dialysis through a rotating cellophane bag immersed in the protein solution to obtain a well packed precipitate. The method proved to be unsatisfactory for a larger scale preparation and the method described in the section III, i.e. adding the same volume of 3.4 M $(NH_4)_2SO_4$ solution into the serum in a fine stream with stirring, was used.

When the rivanol solution was added to the 1.7 M $(NH_4)_2SO_4$ insoluble fraction without previous removal of ammonium sulfate by dialysis, no precipitation was observed regardless of the pH of the solution. This observation is in good agreement with the result reported by Kadlor et al. (20). They observed that precipitation of albumin by rivanol was reduced as the ionic strength of the solution was increased. For rivanol fractionation of human serum, Saifer et al. (14) and Horejsi et al. (13) used 4 volume of 0.4 % rivanol to precipitate albumin, α-globulins and part of β-globulins from 1 volume of serum. The reason for using large quantity of dilute rivanol solution instead of using small volume of concentrated rivanol solution was not stated. It would appear that dilution of the serum was necessary to lower the ionic strength for successful fractionation. When a calculated quantity of $(NH_4)_2SO_4$ was added to the 2 % rivanol solution to make 1.7 molar, the yellow precipitate was observed after a short period of standing in the cold room (about 6° C). Hence the salting out of the rivanol by

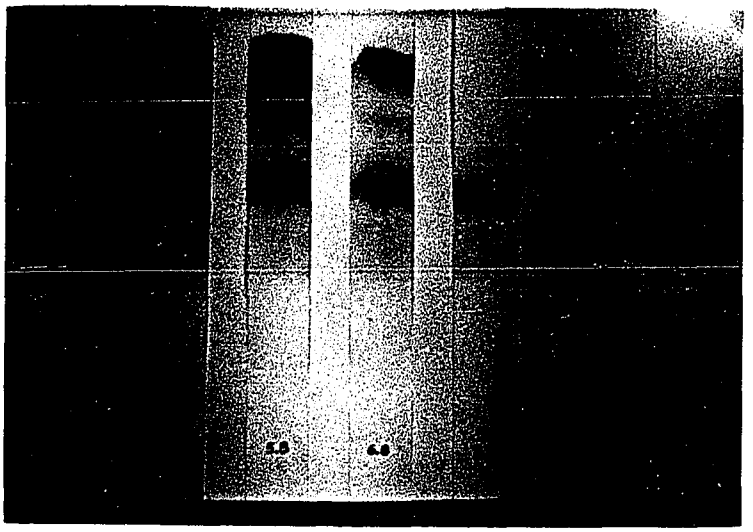
ammonium sulfate at low temperature would appear to be partially responsible for the reduced precipitation of protein by rivanol at high ammonium sulfate concentration. In order to obtain a satisfactory result, the exhaustive dialysis of the ammonium sulfate prior to rivanol fractionation is essential. In this experiment small quantities of rivanol solution was added to a relatively salt free protein solution in order to keep the final volume of the solution within bounds of the available equipment.

It was also foumd that the pH of the solution had a profound effect on the precipitation of the protein. The result of the effect of pH on the rivanol precipitation was shown in Figure 3. At pH 3.9 only very slight precipitation of the protein was observed and the electrophoretic property of the protein was altered by the low pH of the solution. The precipitation of protein increased with the increase of the pH. At pH between 7.5 and 8.5 only β-globulin and trace amount of γ-globulin remained in the filtrate. It was later found that this pH range could be easily obtained if the ammonium sulfate treated serum were dialyzed against tap water.

The quantity of rivanol solution required for complete precipitation of the albumin and α-globulins was determined to be 0.2 ml of 2 % rivanol solution per milliliter of 1.7 M $(NH_4)_2SO_4$ soluble protein fraction freed of ammonium sulfate under the experimental conditions used. The detail of the result is shown in Figure 4.

Figure 3

Effect of Acidity on Rivanol Fractionation



Photographs of the paper strip electrophoresis patterns of the rivanol soluble fraction of the $(NH_4)_2SO_4$ treated serum at various hydrogen ion concentrations.

The figure on each strip is the pH of the protein solution.

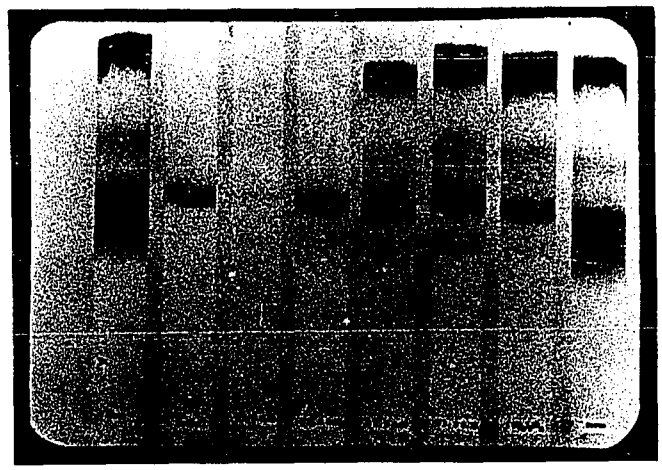
For the electrophoretic pattern of the original beef serum see the Figure 4.

According to Saifer et al. (14), when an increasing volume of 0.4 % rivanol solution was added to a fixed volume of human serum, the faster migrating electrophoretic fractions were progressively removed from the supernatant and appeared in corresponding precipitates. Under the experimental conditions used in this study the same result was not obtained. According to the Figure 4, there was no evidence that albumin was precipitated prior to precipitation of α-globulin. The disagreement might be due to the different experimental conditions used by Saifer and those used in this study. The most apparent difference is ionic strength of the solution which is an important factor in the rivanol fractionation. The different serum used, human serum by Saifer and beef serum by this study, probably is also responsible for the disagreement.

If the rivanol solution were added to the 1.7 M ammonium sulfate soluble fraction which had been previously chilled in the ice bath and the sample were placed in the refrigerator immediately after the addition of rivanol, a less satisfactory result was obtained, i.e. complete precipitation of albumin and α-globulins was not obtained. The yellow crystalline like precipitate appeared different from the usual precipitate of rivanol-protein complex. It is suggested that rivanol precipitated out from the solution in the cold prior to reacting with protein because the solubility of the rivanol is lower at low temperatures (1 part soluble

Figure 4

Rivanol Fractionation of 1.7 M
Ammonium Sulfate Soluble Serum



1 2 3 4 5 6 7 8

Photographs of the paper strip electrophoresis patterns of the rivanol non precipitable fraction.

1. Whole beef serum.
2. Final product.
3. 1 ml $(NH_4)_2SO_4$ treated serum and o.20 ml 2 % rivanol.
4. 1 ml $(NH_4)_2SO_4$ treated serum and 0.15 ml 2 % rivanol.
5. 1 ml $(NH_4)_2SO_4$ treated serum and 0.10 ml 2 % rivanol.
6. 1 ml $(NH_4)_2SO_4$ treated serum and 0.05 ml 2 % rivanol.
7. $(NH_4)_2SO_4$ treated serum.
8. Whole beef serum.

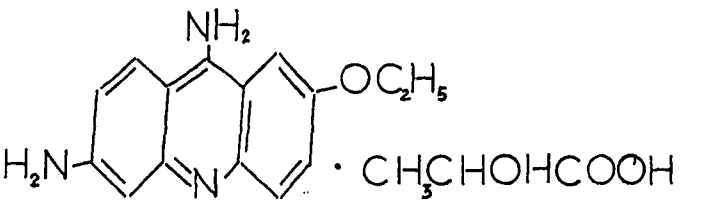
in 15 parts cold water; 1 part soluble in 9 parts boiling water) (54).

It is essential to determine the quantity of rivanol solution necessary for the complete precipitation of albumin and α-globulin in each preparation because there is evidence that rivanol precipitation of protein is effected by protein concentration of the solution and it is rather difficult to control the protein concentration when dialysis is an essential step. Furthermore, the rivanol solution was found to precipitate the β-globulin, which had been obtained as rivanol non precipitable fraction from the serum, from a concentrated β-globulin solution.

The rivanol was removed by dialysis and by absorption by activated carbon. Because of the colloidal property of the rivanol (55) it was difficult to remove the rivanol by dialysis only.

According to Kaldor (20), the general nature of the rivanol serum albumin interaction resembles that of bivalent cations with carboxyl groups of protein under their experimental condition. Rivanol, having a pk of 11.5 is considered as ionized into a bivalent cation at pH below 11.5.

The chemical structure of the rivanol, lactate derivative of 6,9-diamino-2-ethoxyacridine, is shown below.



6,9-diamino-2-ethoxyacridine-lactate

Characterization of the β-Globulin

Electrophoretic Study

The result of the paper electrophoresis (Figure 5) showed that the β-globulin moved as a single band between pH value of 5.1 to 9.6. At pH 2.6 and 3.6 the β-globulin moved as a wide band. The poor mobility of the protein at low pH may be due to the denaturation of the protein or the strong absorption of the positively charged protein by the supporting media, paper. In paper electrophoresis, Tiselius (56) recommends pH values above the isoelectric point of proteins because negatively charged proteins are generally less strongly absorbed on filter paper than the positively charged ones.

The β-globulin preparation was not stained with sudan black B, a lipid staining dye, indicating that the fraction prepared was not a β-lipoprotein.

The result of the cellulose acetate strip electro-

phoresis (Figure 6 and 7) showed that the β-globulin preparation may contain a small quantity of γ-globulin.

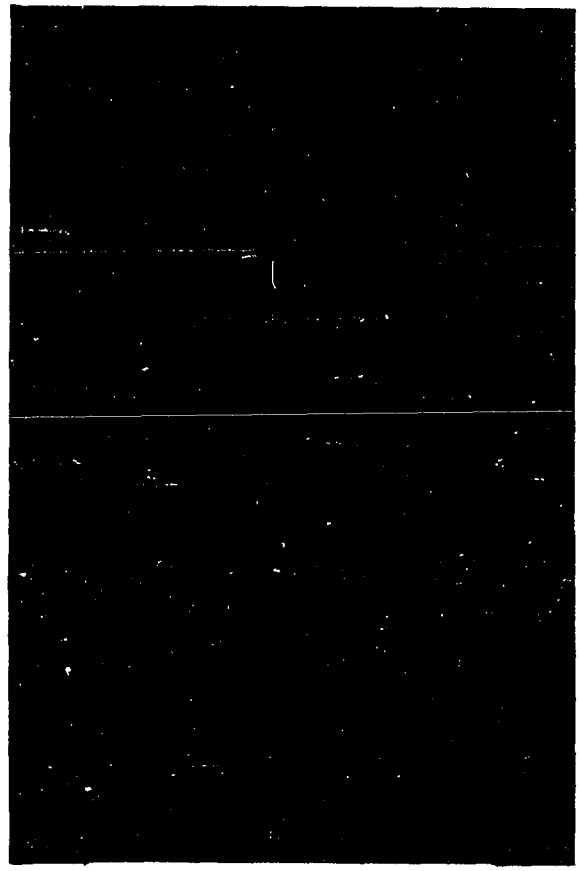
According to Aronsson et al. (45) with the Tris Borate EDTA buffer of pH 8.9, normal human serum was separated into 9 fractions, prealbumin, albumin, α_1-, $2\alpha_2$-, 3β- and one γ-fraction. In this study the beef serum was separated into 5 fractions, albumin, α_1-, α_2-, β_1-, β_2- and γ-globulin. If the mobilities of the fractions in the whole beef serum may be compared with the isolated fractions, then the β-globulin preparation corresponds to β_1-globulin.

The purity of the β-globulin estimated from the densitometer curve of the cellulose acetate strip electrophoresis in a Tris Borate EDTA buffer of pH 8.9 (Figure 8) was 87.7 % β-globulin and 12.3 % γ-globulin. No further purification of the β-globulin fraction was attempted because the quantity of sample required for structural study of the glycoprotein.

The β-globulin prepared was brown colored in solution, stained strongly with bromophenol blue and not with sudan-black B. The β_1-metal combining globulin is described by Hjertén (17) as reddish brown colored in solution, staining strongly with bromophenol blue, but not staining with sudan-black B. Hence, it is likely that the β-globulin preparation was entirely or partly β_1-metal combining globulin. According to the previous studies of

Figure 5

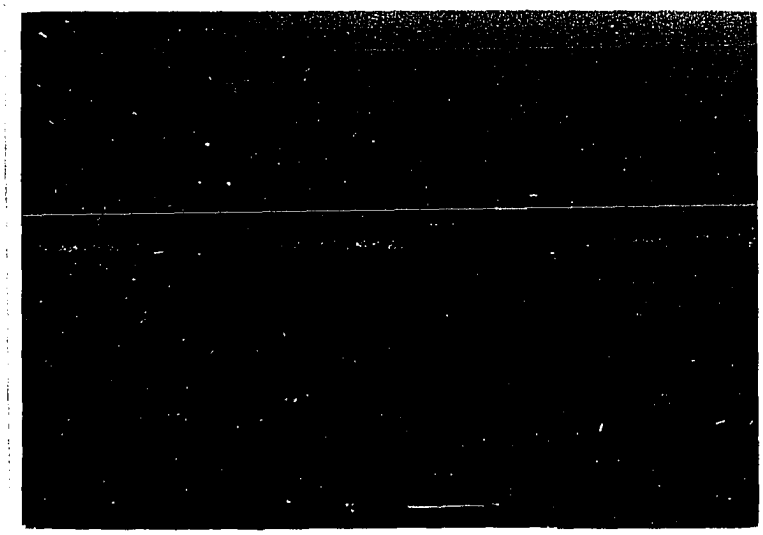
The Electrophoretic Study of the β-Globulin



The lines on the strips are the points of the application of the sample. The right hand side of the figure is positive pole and the left hand side of the figure is negative pole of the electrical field.

Figure 6

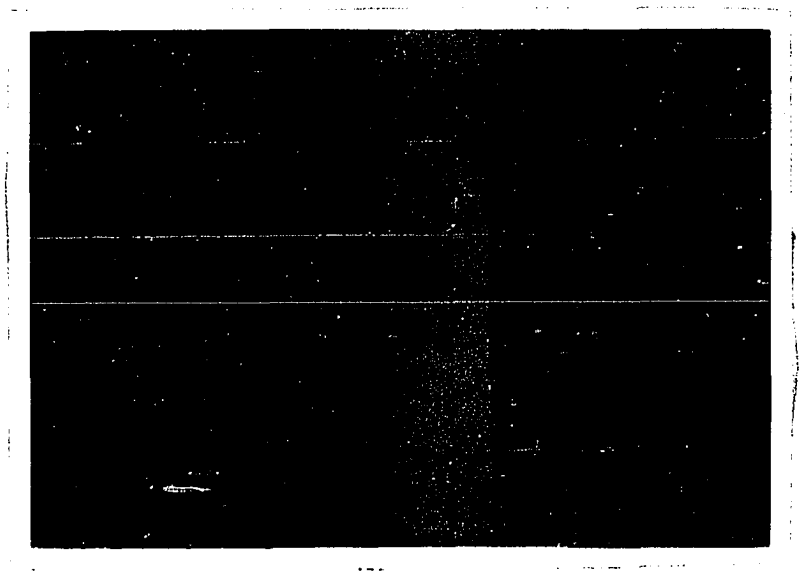
Electrophoresis of the β-Globulin Preparation and of Beef Serum on Cellulose Acetate Strip in a Barbital Buffer of pH 8.6



The lines at the center of the paper strips are the points of the application of the sample. The albumin moves toward the positive pole of the electrical field, the β-globulin and γ-globulin move toward the negative pole of the electrical field and the α-globulin has no mobility.

Figure 7

Electrophoresis of the β-Globulin Preparation and the Beef Serum on the Cellulose Acetate Strip in the Tris Borate EDTA Buffer of pH 8.9



1, Albumin
2, α_1-Globulin
3, α_2-Globulin
4, β_1-Globulin
5, β_2-Globulin
6, γ-Globulin

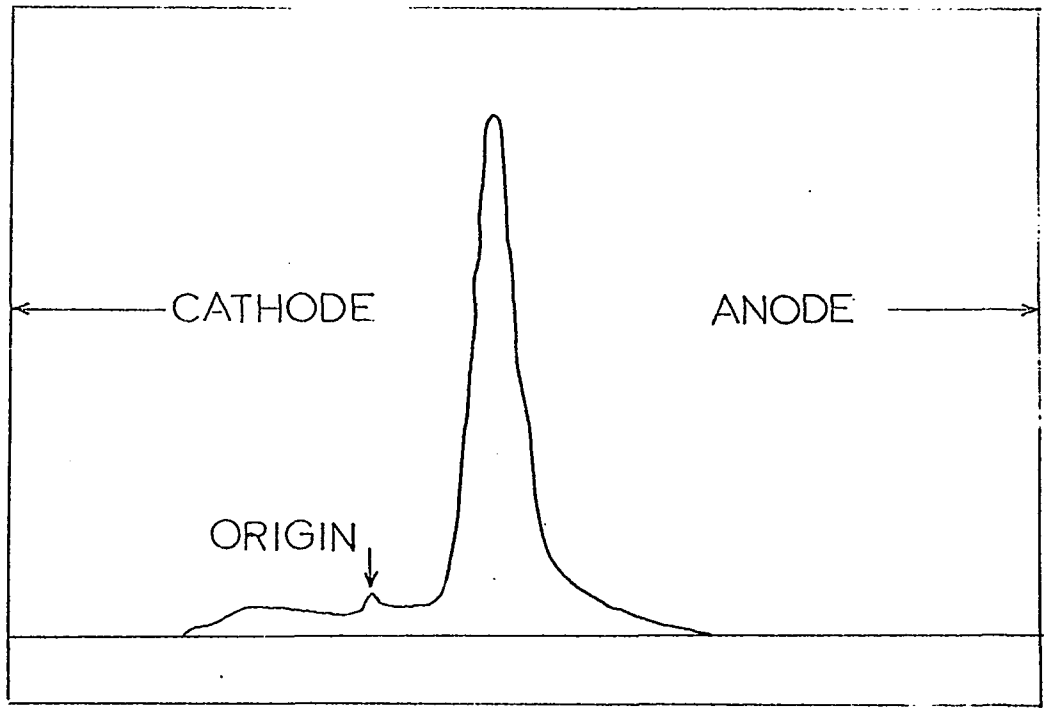
The electrophoretic pattern was sharply visible only under an ultraviolet lamp, therefore the strip was photographed after being marked under the ultraviolet light.

The sample was applied at the center of the strip and the protein band number 5 was about the center of the strip.

As the figure indicated that only small quantity of γ-globulin was detected by the protein dye, azocarmin, in the β-globulin preparation.

Figure 8

Densitometer Curve of the Cellulose Acetate Strip Electrophoresis in a Tris EDTA Borate Buffer of pH 8.9



Horejsi (13) and Boettcher (12), the β-metal binding globulin is not precipitable by rivanol which further suggests the fraction prepared here is likely to be β_1-metal combining globulin.

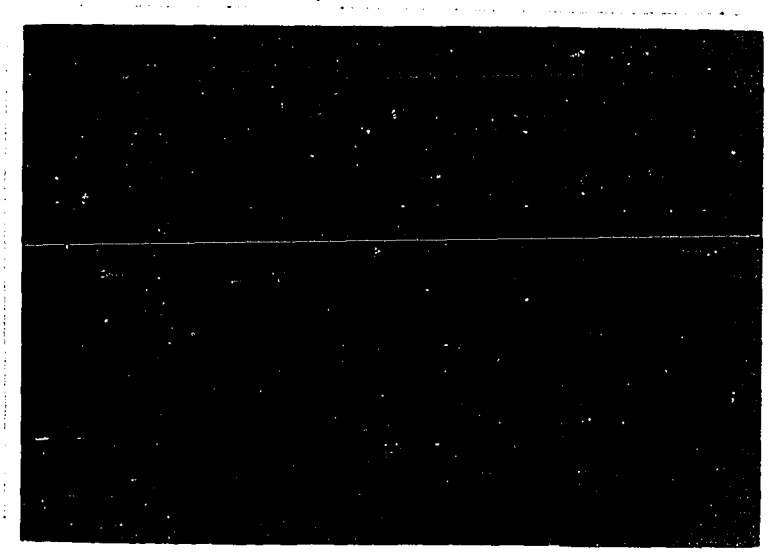
Chemical Composition of the β-Globulin

In the qualitative analysis of the hexoses by paper chromatographic technique, the hexoses detected were galactose, mannose, fucose and a reducing substance having the same R_f value as glucose. The Figure 9 shows the paper electrophoresis with borate buffer on the hydrolysate of the β-globulin. The pattern shows the presence of the glucose in the hydrolysate.

The result of the glucose oxidase experiment (Figure 10). showed the disappearance of the reducing substance after the incubation with glucose oxidase. The evidences stated above suggested the reducing substance as glucose. In considering the high concentration of the free glucose in comparison with the low concentration of the β-globulin in the serum (118 mg glucose per 100 ml calf serum, 85 mg glucose per 100 ml calf serum, 85 mg glucose per 100 ml ox serum (57), 780 mg β-globulin per 100 ml human plasma) it was first suspected that the glucose detected was merely the free glucose firmly absorbed by β-globulin although a long period of dialysis was involved in the preparation procedure.

Figure 9

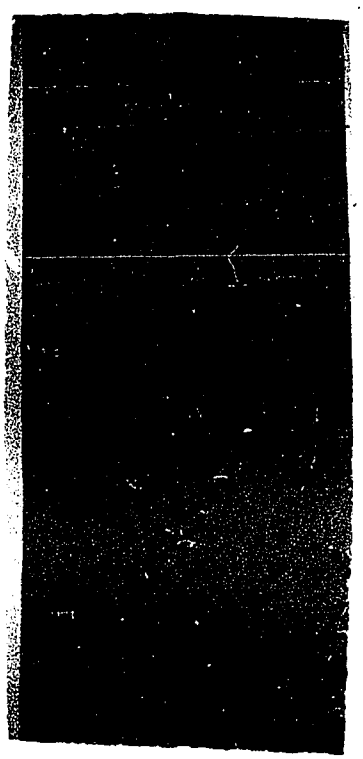
The Paper Electrophoresis of the
β-Globulin Hydrolysate with
Borate Buffer



1. The center strip is the β-globulin hydrolysate.
2. The strips at the both ends are the standard sugars.
3. The sample is applied at the center of the strips.

Figure 10

Result of the Glucose Oxidase Reaction
on the Reducing Substance



1. The reducing substance reacted with denatured glucose oxidase.
2. The reducing substance reacted with glucose oxidase.

In order to clarify the question about the glucose, the qualitative analysis of the hexose was carried out on the non-dialyzable fraction obtained from the pepsin digests of the β-globulin. Again the result showed the presence of the glucose. In the pepsin digestion the β-globulin was incubated at pH 1.8 for 3 days and for the removal of small peptide and free amino acids the digestion mixture was dialyzed overnight, therefore it is not likely that the glucose is merely a contaminant from the free glucose of the serum.

The result of the quantitative analysis of the β-globulin is shown in the Table II. In the sialic acid determination, the value by Warren's (50) thiobarbituric acid method is considered to be more accurate since the diphenylamine method yields high values when applied in direct measurement of sialic acid in unpurified biological materials. In the quantitative measurement of the galactose and mannose (by paper chromatographic technique), when the density of the hexose detected with 2-aminobiphenyl was plotted against the concentration of the hexose, a linear relationship was obtained at hexose concentration of 5 μg to 25 μg. Some typical examples of curves obtained with standard sugars are shown in Figure 11 and 12. The exact, same curve was not reproducible each time primarily due to the difficulty in controlling the experimental conditions, however the linear relationship between the concentration of sugar and

Table II

CHEMICAL COMPOSITION OF THE β-GLOBULIN PREPARATION

Constituent	Method	Weight
Protein	Lowry's	100.0
Hexose	Tryptophan	3.51
	Anthrone	3.91
Hexosamine	Elson-Morgan	2.17
Fucose	Cysteine-H_2SO_4	0.19
Sialic Acid	Diphenylamine	1.67
	Thiobarbituric	1.15
Nitrogen	Digestion followed by Nesslerization	15.43
Mannose/Galactose		1.62

1. The value in the table above was calculated on the basis of 100 g of polypeptide, as determined by the Lowry's method using Lab-Trol from Dade Reagents Inc. as standard.

2. Hexose is reported as free hexose not polysaccharide since the sialic acid, fucose and glucosamine are all reported as free sugars. Equal quantity of galactose and mannose was used as the standard in the hexose determination.

3. A submaxillary mucin preparation (Sialic acid, 19.1 %)from Nutritional Biochemical Corporation was used as the standard.

Figure 11
Density of 2-Aminobiphenyl Hexose vs. Concentration of Hexose

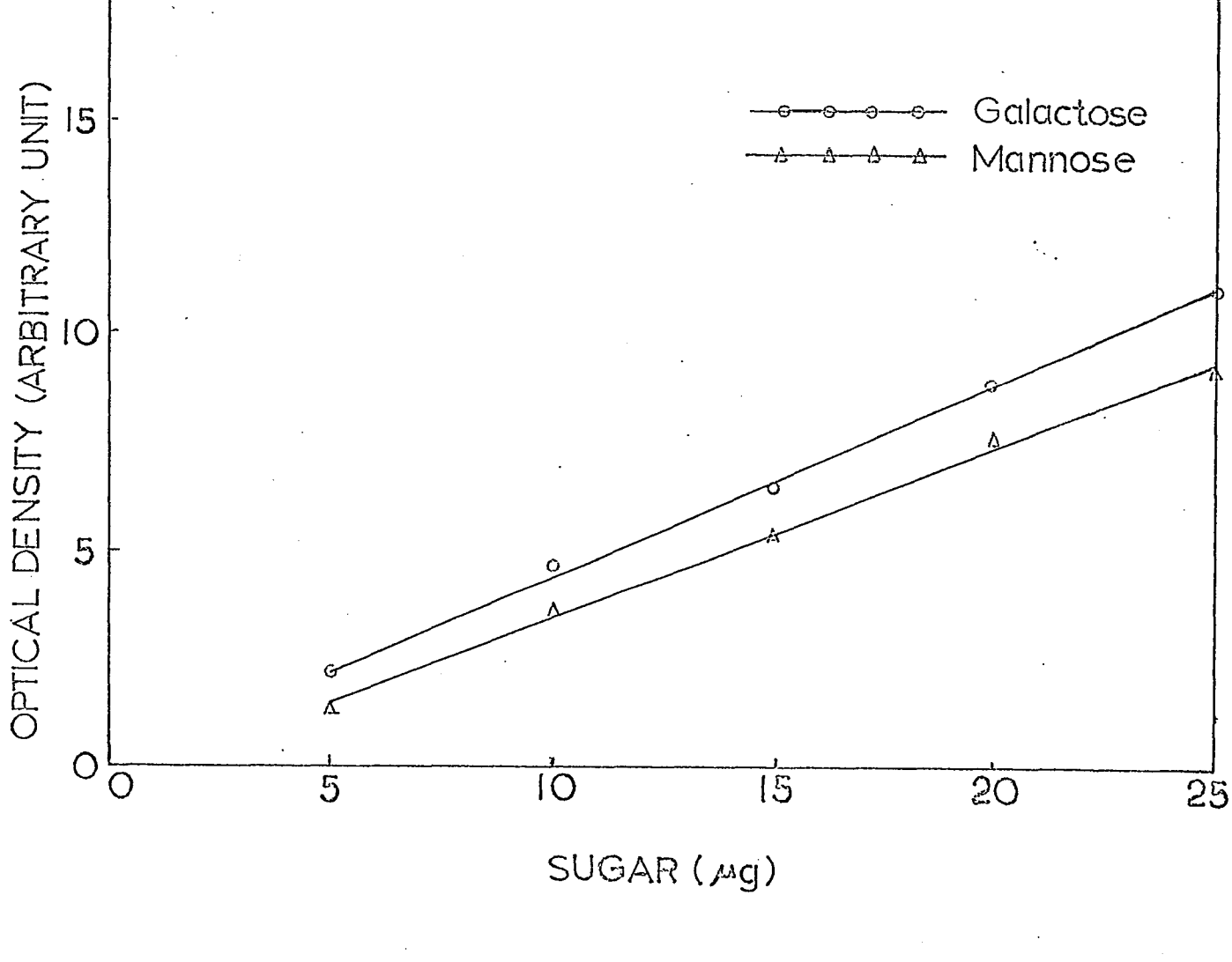
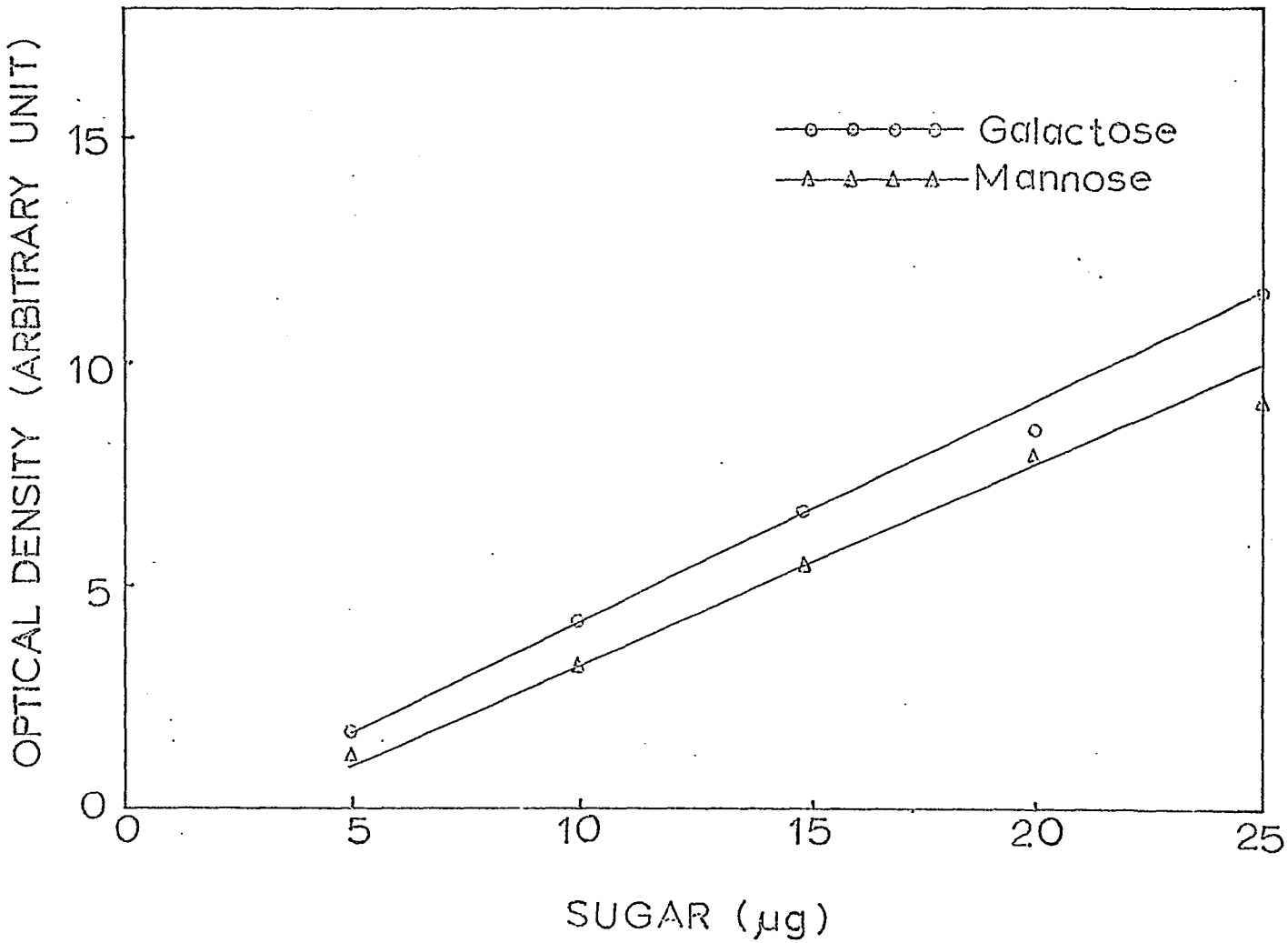


Figure 12
Density of 2-Aminobiphenyl Hexose vs. Concentration of Hexose



density of the 2-aminobiphenyl sugar was always obtainable provided that the 2-aminobiphenyl spraying was done with utmost care. The most important factors to be considered in this experiment are, a) separating the sugars on the paper as far as possible b) spraying the 2-aminobiphenyl as uniformly as possible. The former can be achieved by multiple development of the chromatogram. From the result stated above it is reasonable to determine the galactose and mannose if a series of standard sugar solutions are chromatographed on the same sheet of paper as the sample. In order to test the reliability of the method, a known quantity of the sugar was added to the β-globulin hydrolysate and the recovery of the sugar was examined (Table III).

The difference between the total sugar determined by the Anthrone method and the sum of the galactose and mannose is due primarily to the presence of fucose and glucose in the β-globulin hydrolysate and also due to the standard sugar solution being composed equal amount of galactose and mannose.

In order to compare the composition of the β-globulin preparation obtained in this experiment with the β-globulins prepared by previous workers, the compositions of the β-globulins available so far are described below.

Koechlin (11) reported the composition of the crystalline β_1-metal combining globulin isolated from human plasma as

Table III

ANALYSIS OF GALACTOSE AND MANNOSE

Sample	Volume of Hydrolysate	Total Sugar by Anthrone µg	Chromat. and Analytrol	
			Mannose µg	Galactose µg
β-Globulin Hydrolysate	10	28.8	12.9	8.0
β-Globulin Hydrolysate and 5 µg galactose, 5 µg mannose	10	38.8	18.5	13.3
Sugar Recovered			5.6	5.3

Nitrogen = 0.147 g/g protein

Carbohydrate = 0.0.8 g/g protein

Whether the protein represents the whole β_1-metal combining glycoprotein or the protein determined by the protein reaction was not mentioned in his report.

Schultze (58) reported a slightly different figure for the β_1-metal combining protein of human blood.

Hexose	2.4 %
Galactose	1.6 %
Mannose	0.8 %
N-acetyl hexosamine	2.0 %
Fucose	0.07 %
Sialic acid	1.4 %
Uronic acid	0.05 %
Protein	95 %
Nitrogen	15.4 %

The electrophoretically separated β-globulins was reported to have the following compositions (59)

Hexose	2.7 %
Hexosamine	1.0 %
Sialic acid	1.5 %
Fucose	0.2 %

The figures were reported as grams of sugar per 100 grams of protein.

The composition of the β-lipoprotein was reported as (58)

Hexose	1.1 %
N-Acetyl hexosamine	0.4 %
Sialic Acid	0.3 %
Fucose	0.03 %
Protein	16.5 %

Heremans' (18) study indicated the composition of β_{2A}-globulin as

Hexose	4.90 %
Hexosamine	3.74 %
Nitrogen	16.20 %

The figures were calculated on the basis of 100 mg of polypeptide as determined by the Biuret method.

As stated above, the different workers reported their figures in different terms. For the convenience of comparison, the figures reported by the various investigators are calculated into the terms of gram of sugar per 100 gram of protein and although it is obvious that comparison of the bovine glycoprotein with human glycoprotein may not lead to a valid conclusion, however the comparison is made due to the lack of the information on bovine β-glycoprotein.

Data on bovine β-glycoprotein is not currently available. The chemical composition of β-globulins isolated in this study differed from all of the β-globulin composition listed in the Table (IV). Consequently positive identification of the fraction in the basis of the chemical composition is not possible at present time.

Table IV

COMPOSITION OF THE β-GLOBULINS PREPARED BY PREVIOUS INVESTIGATORS

Type of β-Globulin	Author	Hexose	Gal/ Man.	Hexosa-mine	Sialic acid	Fucose	Nitro-gen
β_1-Metal combining globulin	Koechlin	1.8	-	-	-	-	14.7
β_1-Metal combining globulin	Schultz	2.52	2	1.7	1.5	0.07	16.2
β_{2A}-Globulin	Herman	4.9	-	3.7	-	-	-
β-Globulin	Quoted by Winzler	2.7	-	1.9	1.5	0.1	-
β-Lipoprotein	Schultz	6.7	-	2.42	1.9	0.19	-
β-Globulin preparation	Yang	3.51	0.61	2.17	1.15	0.19	15.43

However the physical appearance and procedure used to prepare the fraction indicate it to be a fraction of entirely or largely β_1-metal combining globulin.

Preparation of Glycopeptides

The β-globulin was readily hydrolyzed by pepsin at pH 1.8. The paper strip electrophoretic technique as described in the chapter III was used to follow the extent of digestion since it was desirable to obtain a reproduceable fraction. Similar electrophoretic patterns of the digestion mixture was always obtained on different batches of pepsin digest. At the end of the digestion the dark precipitate previously described was always found. This precipitate was discarded because of its low solubility.

The results of the analyses of the protein and hexose indicated that the nondialyzable fraction had the ratio of protein to hexose of 6:1 on a weight basis in comparison with the original β-globulin in which the ratio of protein to hexose was 30:1. The enrichment of the carbohydrate moiety by enzymatic hydrolysis has been previously reported by Richmond (58 a). The ratio of 1 part hexose and 6 part protein was always obtainable for the different batches of nondialyzable fraction obtained from the 3 days' pepsin digest of the β-globulin. Further pepsin digestion did not result in further enrichment of the hexose content suggesting the formation of limit product not susceptible to pepsin. The nondialyzable fraction was not coagulable by heat but was

ethanol precipitable.

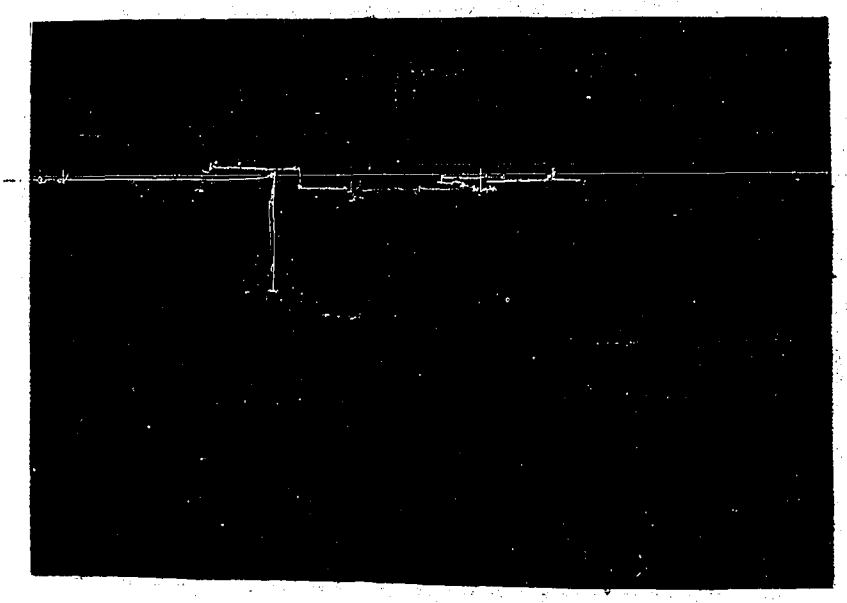
By means of the Misco continuous electrophoresis, the nondialyzable fraction was separated into five ninhydrin positive fractions. The paper curtain of the continuous electrophoresis is shown in Figure 13.

Wenfield and Tunis (60) studying the cleavage of human α_1-acid glycoprotein by pepsin observed that pepsin readily cleaved the α_1-acid glycoprotein to a limited degree and obtained a relatively carbohydrate enriched fraction by acetone precipitation in the presence of NaCl. As the conclusion, he suggested that the pepsin digestion followed by acetone fractionation in the presence of NaCl could be a good tool for the structural study of the α_1-acid glycoprotein.

A similar suggestion can be made in this study as the results stated above indicate that 1) the cleavage of β-globulin is a limited one 2) the extent of the pepsin digestion is reproducible i.e., it is possible to obtain reproduceable glycopeptide fractions 3) the separation of the carbohydrate enriched fraction is possible. Beginning with the further purification of the original glycoprotein, the structural study of the carbohydrate unit of the glycoprotein by using pepsin as a tool may be an ideal procedure. In other words, by studying in detail, the carbohydrate distribution of the various fractions obtained from the pepsin digests of the glycoprotein, whether the carbohydrate unit

Figure 13

The Continuous Electrophoretic Pattern of the Nondialyzable Fraction from the Pepsin Digests of the β-Globulin



-1. The spot on the top is the point of the application of the sample.

2. The right hand side is the cathod and the left hand side is the anode.

The fractions are collected at the botton of the paper curtain.

distributes itself among the protein as one single unit or several different units can be proved provided that the original glycoprotein is fully purified and characterized. This characterization includes the determination of the number of terminal amino acids.

The two fractions having the lowest mobility were used for further study. No further purification of the fractions was attempted because the continuous electrophoresis provides only very small quantities of the glycopeptides.

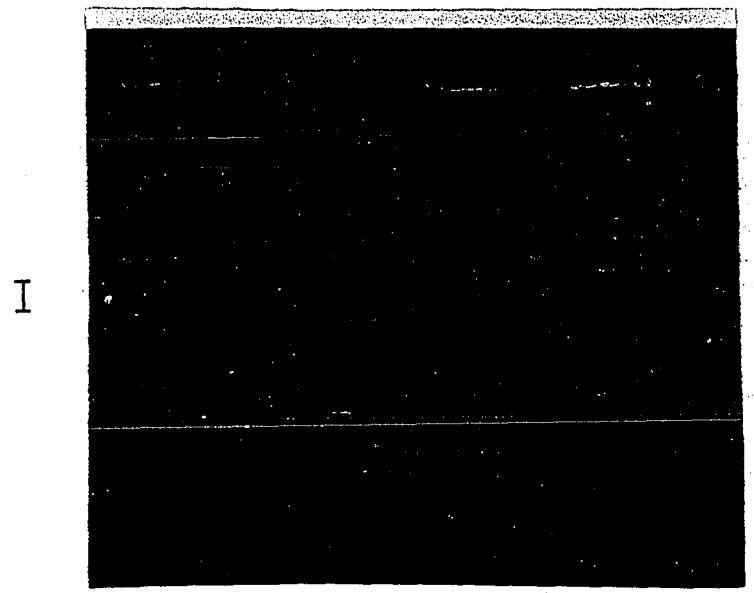
An attempt was made to study the purity of the glycopeptide by paper strip electrophoresis. The electrophoretic pattern of the glycopeptide II (Figure 14) showed the single ninhydrin positive spot at different pH values indicating that this fraction may be a single substance.

Whether the low mobility of the two glycopeptides as indicated in Figure 14 and 15 is due to the low net charge on the glycopeptide or the strong absorption between the paper and the glycopeptides is unknown.

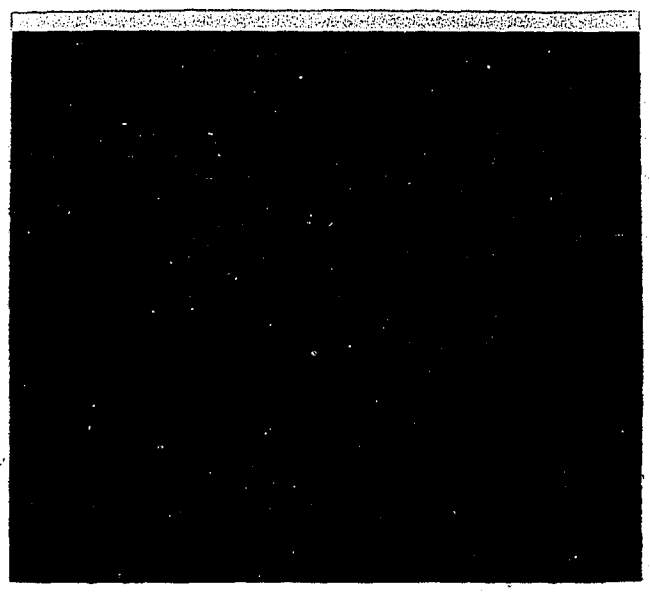
The qualitative study of the carbohydrate was carried out on the glycopeptide which had been freed of barbital buffer by dialysis and trace quantity of white floating material by centrifugation. The result of the paper chromatogram (Figure 16 and 17) indicated the presence of hexosamine, galactose, mannose, fucose and glucose in the glycopeptides.

Figure 14 and 15

Electrophoretic Study of Glycopeptide I and II



I



II

No.	1	2	3	4	5	6
pH	8.6	7.5	6.9	6.3	5.1	3.6

Table V

THE CHEMICAL COMPOSITION OF THE GLYCOPEPTIDES

Constituent	Glycopeptides	
	I	II
Protein	56.8	51.1
Hexose	29.3	31.0
Hexosamine	12.6	16.4
Fucose	1.4	1.4
Sialic Acid	0	0
Glucose	present	present
Mannose/Galactose	1.68	1.70

Sialic acid was not detected in these two particular glycopeptides.

Table VI

CARBOHYDRATE RELATIONSHIPS

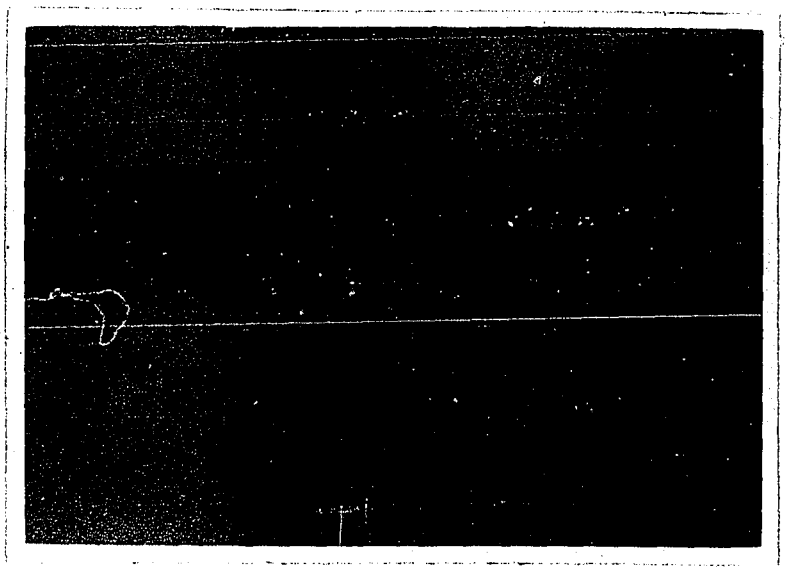
	β-Globulin	Glycopeptides	
		I	II
Galactose/Mannose	1.67	1.68	1.70
Hexose/Hexosamine	1.62	2.33	1.89
Hexose/Fucose	18.8	20.7	21.6
Hexose/Sialic Acid	3.05	----	----

The qualitative analysis of the hexose again indicated the presence of glucose, however, whether the glucose was the

Figure 16 and 17

Carbohydrate Composition of Glycopeptides I and II

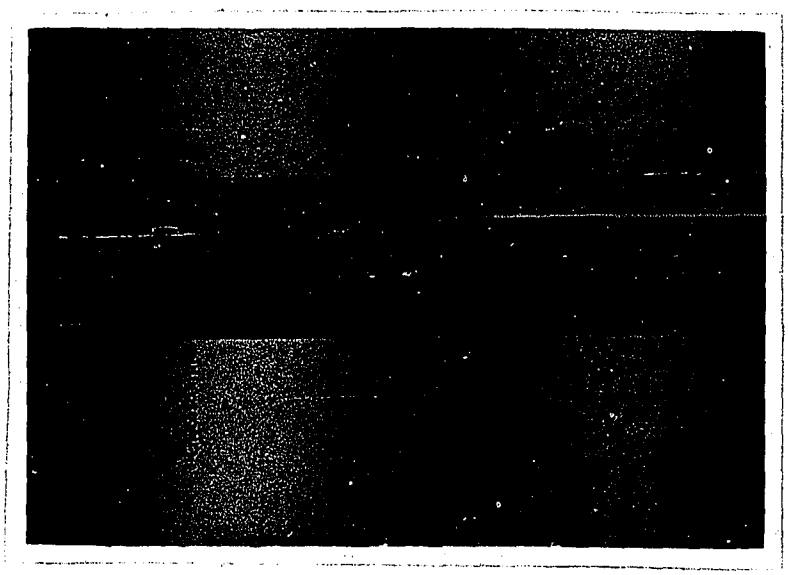
I



Standard

Sample

II



Sample

Standard

constituent of these two glycopeptides isolated by the continuous electrophoresis or the constituent of the paper curtain used as the supporting medium in the electrophoresis is unknown at this time. The Table V shows that enrichment of the carbohydrate unit was achieved by the pepsin digestion followed by dialysis and fractionation. The carbohydrate unit of the β-globulin and that of the glycopeptides are very similar except the hexose/hexosamine ratio in the glycopeptide I and the absence of the sialic acid in the glycopeptides.

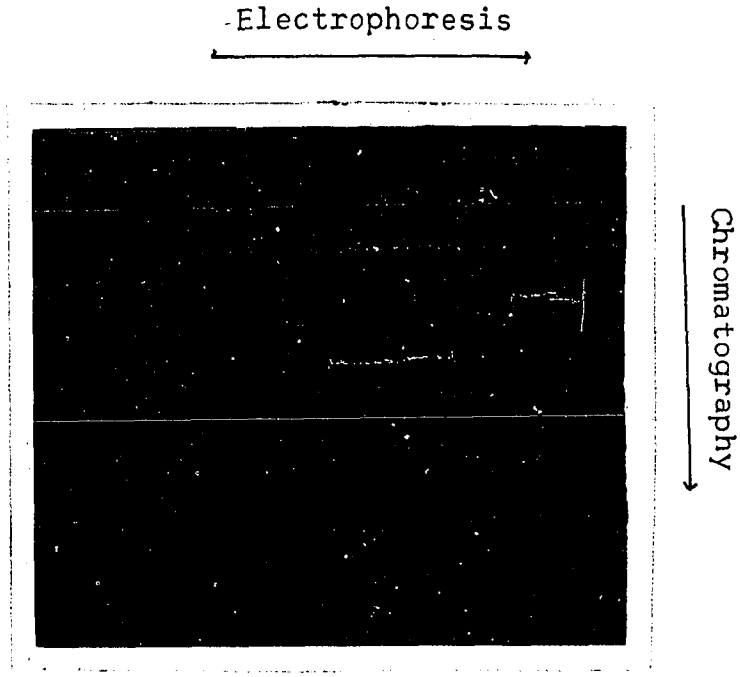
The amino acids composition of the glycopeptides are shown in the Figure 18 and 19. These two figures indicated that all of the amino acids which have been detected in the human β-globulin were detected in the glycopeptides. This further indicates that the attack of the β-globulin by pepsin is a limited one.

In order to study the nature of carbohydrate, the partial acid hydrolysis of the glycopeptide II was carried out. The result was summarized in the Table VII. The result listed in the Table VII seemed to indicate the galactose as the terminal unit of the carbohydrate moiety of the glycopeptides. Quantitative analysis of the various hexoses released under different conditions may eventually supply the information about the sequence of the carbohydrate unit. By the Somogyi reagent no reducing oligosaccharides were detected.

For the study of the nature of the carbohydrate protein linkage, further removal of the polypeptide unit from

Figure 18

Amino Acids Composition of Glycopeptide I



Standards:

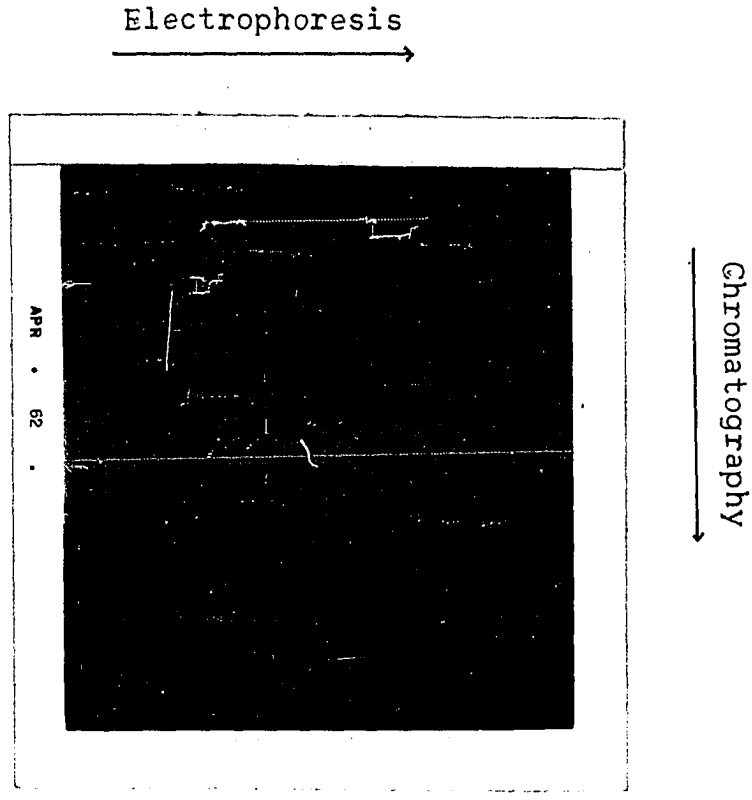
A. Cystine, Cysteine, B. Histidine, Lysine, B. Glucosamine, C. Aspartic acid, Serine, Glycine, D. Glutamic acid, Threonine, E. Alanine, F. Tyrosine, G. Valine, H. Isoleucine, I. Leucine.

Amino Acids in the Figure:

1. Glucosamine, 2. Aspartic acid, 3. Serine, 4. Glutamic acid, 5. Alanine, 6. Valine, 8. Glycine, 9. Threonine, 11. Isoleucine, 12. Leucine, 13. Histidine, 15. Lysine, 14. Unidentified, 16. Unidentified.

Figure 19

Amino Acids Composition of Glycopeptide II



Standards:

A. Cystine, Cysteine, B. Histidine, Lysine, B. Glucosamine, C. Aspartic acid, Serine, Glycine, D. Glutamic acid, Threonine, E. Alanine, F. Tyrosine, G. Valine, H. Isoleucine, I. Leucine.

Amino Acids in the Figure:

1. Glucosamine, 2. Aspartic acid, 3. Serine, 4. Glutamic acid, 5. Alanine, 6. Valine, 8. Glycine, 9. Threonine, 11. Isoleucine, 12. Leucine, 13. Histidine, 15. Lysine, 14. Unidentified, 16. Unidentified.

Table VII

PARTIAL ACID HYDROLYSIS OF THE GLYCOPEPTIDE II

HCl (N)	Time (Hr.)	Sugar Released				
		Galactose	Mannose	Fucose	Glucosamine	Glucose
0.001	2	+				
0.0025	"	+				
0.005	"	++	+			
0.01	"	+++	++			
0.10	"	+++	++	++		+
2.0	"	++++	+++	++	+++	+
0.001	1					
0.0025	"					
0.005	"					
0.01	"	+				
0.10	"	++	+	+		
2.0	"	+++	++	++	++	+

the glycopeptides was desirable. The bacterial proteolytic enzyme, pronase, and $Ba(OH)_2$ were used as the hydrolyzing agents. The degree of enrichment of the carbohydrate unit was expressed in the Table VIII.

From the Table VIII the most carbohydrate enriched fraction was the fraction number 5 having the hexose/protein ration of 1/0.59. The Figure 20 shows the result of the high voltage electrophoresis-chromatography of the fraction number

Table VIII

SUMMARY OF THE DEGRADATION OF THE β-GLOBULIN

No.	Treatment		Hexose/protein (weight basis)
1	Nondialyzable fraction from pepsin digest		1/6
2	Glycopeptide fractionated by continuous electrophoresis from 1	I	1/2
		II	1/1.6
3	Alcohol precipitable fraction from Pepsin and pronase digest		1/4
4	Nondialyzable fraction of 3		1/1.25
5	Dialyzable fraction of 3 freed of short peptides and amino acids by Dowex 50		1/0.59
6	Pepsin, pronase, and $Ba(OH)_2$ hydrolyzed, precipitated with 90 % ethanol		1/0.77

5. Two poorly separated fractions which gave only very faint purple color with ninhydrin were detected near the point where the sample was applied.

The qualitative analysis of the amino acids (Figure 20) of a hydrolysate indicated that the fraction number 5 contained glucosamine, aspartic acid, serine, glutamic acid, alanine, threonine and glycine as major ninhydrin positive spots and valine, leucine, isoleucine, tyrosine and one unidentified spot as the minor ninhydrin positive spots. The amino acids detected in the fraction number 5 which had been digested with carboxypeptidase and leucine aminopeptidase (Figure 21) were aspartic acid, serine, glutamic acid and

small quantities of glycine, threonine and alanine. The fraction digested with carboxypeptidase and leucine aminopeptidase and treated with Dowex 50 column contained no detectable amount of free amino acids.

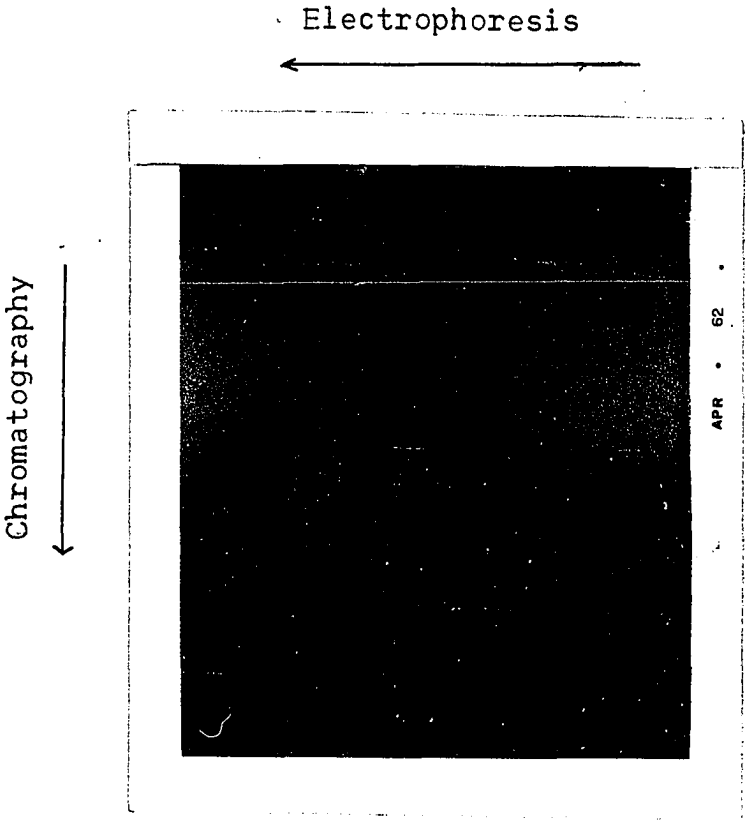
From the result indicated in Figure 21 and 22, it may be suggested that aspartic acid, glutamic acid or serine is the amino acid involved in the carbohydrate protein linkage.

The previous workers have suggested that the carbohydrate unit is linked to the polypeptide unit through the aspartic acid in human γ-globulin (37, 38), rabbit γ-globulin and bovine γ-globulin (39, 40). Spiro (62) prepared two series of similar glycopeptides from papain and nagarase, bacillus subtitis protease, digests of feutin by DEAE cellulose column chromatography and observed that the amino acids occurring most frequently in the vicinity of the three carbohydrate-peptide linkage were aspartic, alanine, serine and proline. Tsugita et al. (63) reported that the serine was the point of attachment of the carbohydrate unit to the polypeptide moiety in the Taka amylase.

The fraction obtained by ethanol precipitate from the $Ba(OH)_2$ hydrolysate, the fraction number 6 in Table VIII, was freed of free amino acids and small peptide and studied for amino acids composition (Figure 23). The result showed that the complete removal of polypeptide unit by the $Ba(OH)_2$ hydrolysis under the condition used was not achieved. This indicates that the protein-carbohydrate bond is alkali stable.

Figure 20

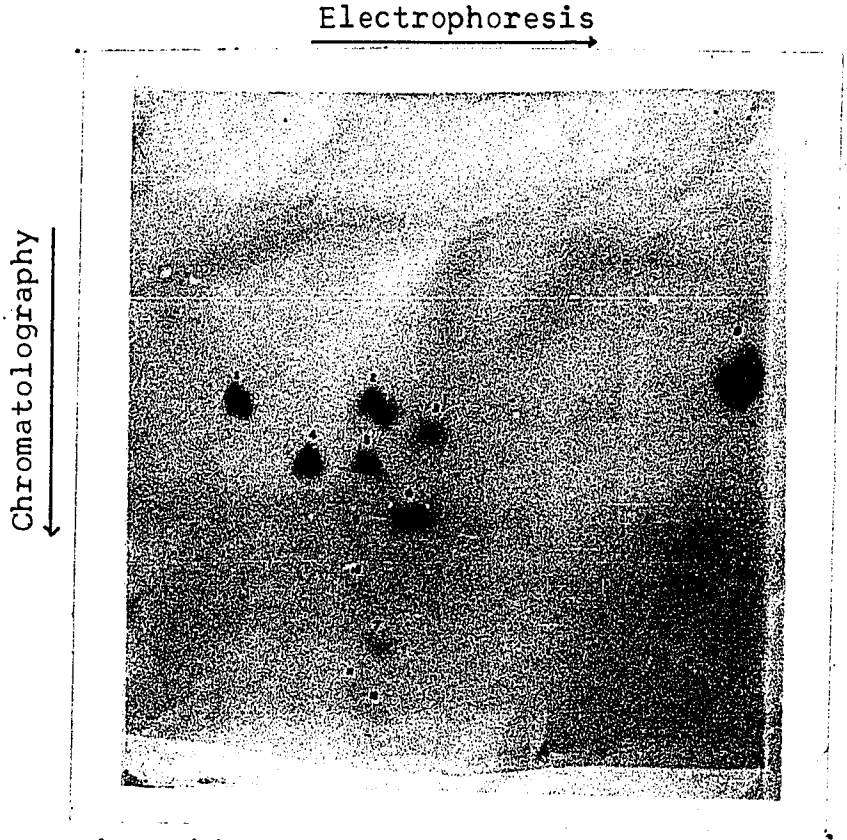
High Voltage Electrophoresis-Chromatography
of the Fraction Number 5



Two poorly separated and diffused spots were detected by ninhydrin. No free amino acids were detected.

Figure 21

Amino Acid Composition of Acid Hydrolysate of Fraction Number 5 Incubated with Denatured Carboxypeptidase and Leucine Aminopeptidase



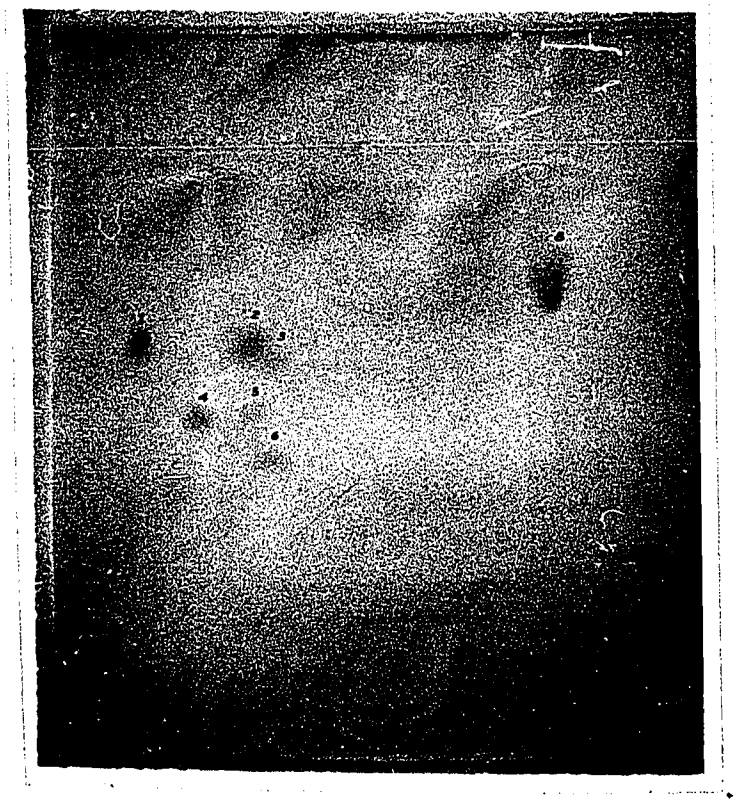
1. Aspartic Acid
2. Serine
3. Glycine
4. Glutamic acid
5. Threonine
6. Alanine
7. Valine
8. Glucosamine
9. Unidentified
10. Tyrosine
11. Isoleucine
12. Leucine
13. Leucine

Figure 22

The Amino Acid Composition of Acid Hydrolysate of Fraction Number 5 Digested with Carboxypeptidase and Leucine Aminopeptidase

Electrophoresis →

Chromatography ↓

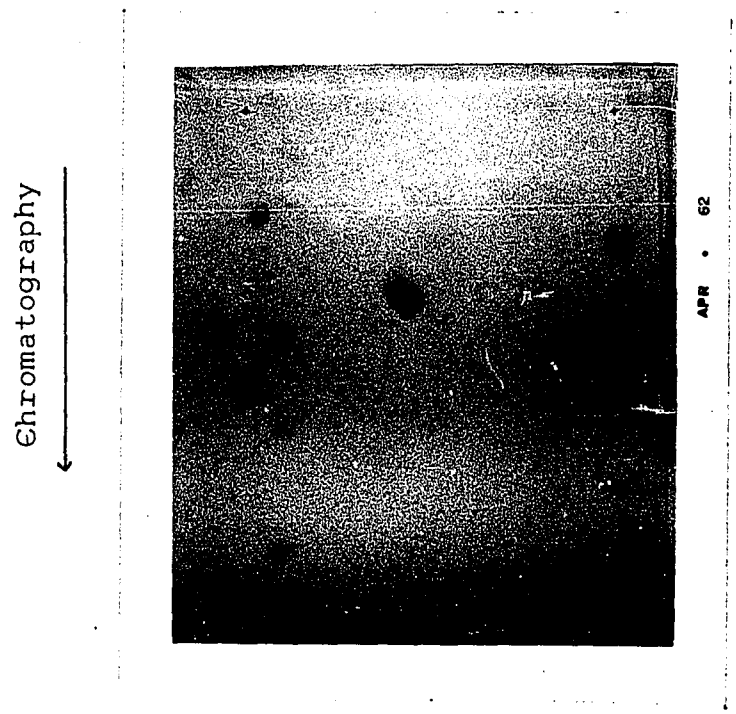


1. Aspartic Acid
2. Serine
3. Glycine
4. Glutamic acid
5. Threonine
6. Alanine
8. Glucosamine

Figure 23

Amino Acid Composition of Acid Hydrolysate of Fraction No. 6

Electrophoresis →



Amino Acids in the Figure:

1. Glucosamine
2. Aspartic acid
3. Serine
4. Glutamic acid
5. Alanine
6. Valine
7. Cysteine

The unmarked spots are not identified.

CHAPTER VI

SUMMARY

1. A β-globulin fraction, nearly homogeneous by electrophoretic analyses, was prepared by the combination of $(NH_4)_2SO_4$ and rivanol fractionation.
2. The cellulose acetate strip electrophoretic studies indicated that the final preparation contains 87 % β-globulin and 13 % γ-globulin.
3. According to the chemical analysis, the β-globulin preparation contained 3.51 % hexoses, 2.17 % hexosamine, 1.15 % sialic acid and 0.19 % fucose. The above figures were calculated on the basis of the protein content as determined by Lowry's method using a serum protein preparation as a standard.
4. The qualitative and quantitative analysis of the preparation showed that the component monosaccharides were glucosamine, galactose, mannose, fucose, sialic acid and glucose.
5. The degradation study of the fraction with proteolytic enzyme, pepsin, indicated that the β-globulin fraction was readily hydrolyzed with pepsin to limit glycopeptides. Relatively carbohydrate rich glycopeptides were

separated from the nondialyzable fraction by Misco continuous electrophoresis.

6. Upon partial acid hydrolysis of the one glycopeptide, galactose was released under the mildest conditions and the glucosamine was released with the most vigorous conditions indicating that the galactose may occupy the terminal position of the glycopeptide and that glucosamine may be the carbohydrate linked to the protein.
7. Further enrichment of the carbohydrate unit was achieved by digestion with the bacteria proteolytic enzyme, pronase. The dialyzable fraction from the pronase digest was found to be very rich in carbohydrate. This highly carbohydrate enriched fraction was digested with leucine aminopeptidase and carboxypeptidase and the residue was analyzed for amino acids content by paper electrophoretic-chromatographic technique. The major amino acids remaining attached to the carbohydrate unit were aspartic acid, serine, and glutamic acid suggesting that one of these amino acid is involved in the protein carbohydrate linkage.

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