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CHEMICAL STUDIES OF BOVINE TRANSFERRIN

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CHEMICAL STUDIES OF BOVINE TRANSFERRIN

CHAPTER I

INTRODUCTION

The isolation of proteins from blood serum has been in the focus of interest for a long time. With each development of new techniques of protein isolation, interest surged, abating again as the limitations of each method became apparent.

The earlier methods of isolation of blood proteins were those of precipitation with salts, for it was shown that different entities of proteins were precipitated at different concentrations of various salts. (1,2) Among the salts used were: Na_2SO_4, $(NH_4)_2SO_4$, $MgSO_4$, phosphates and various acetates. Several systems were developed to fractionate the various serum proteins. The most traditional method is the precipitation with $(NH_4)_2SO_4$. Approximately 0.20-0.25 saturation was used to precipitate fibrinogen, 0.33 saturation precipitated the euglobulin fraction and 0.5 saturation removed the globulin. The remaining solution contained the albumin. Many attempts were made to isolate a single component of plasma protein

using $(NH_4)_2SO_4$ precipitation only. However, except for albumin, which could crystallize out of the albumin rich solution, the general results were disappointing. (3)

The system of Howe used Na_2SO_4 (2), and with this Howe was able to fractionate serum proteins into four fractions: euglobulin, precipitating at 13.5%, pseudoglobulin I, at 17.4%, pseudoglobulin II at 21.5%, and albumin which was soluble in 21.5% Na_2SO_4.

An abbreviated use of this system, that is, use of the 21.5% Na_2SO_4 to separate "globulin" and albumin was widely used in clinical laboratories until recently. Consequently many of the correlations between human disease and changes of serum proteins were based on this method.

The precipitation with potassium phosphate employed by Butler et al. (4) separated plasma into four fractions. The fractions were compared with those obtained from $(NH_4)_2SO_4$ and Na_2SO_4 precipitation. Butler et al. summarized the contents of the fractions as : Fraction I precipitated at 1.25 M contained fibrinogen; Fraction II, probably largely euglobulin but no more than two-thirds of the total amount, started to precipitate at 1.25 M and continued to precipitate up to 1.5 M concentration; Fraction III, representing approximately 70% of pseudoglobulin and 30% euglobulin, precipitated between 1.50 and 2.40 M concentration; and Fraction IV precipitated between 2.40 and 2.58 M concentrations and consisted of albumin with

some pseudoglobulin. The system of Derrien used 3.5 M phosphate solution, consisting of equimolar amounts of monopotassium and dipotassium phosphate. The salt solution was added to plasma diluted to 1 mg N per ml at 24°C. Constant volume was maintained by using 0.7% NaCl solution. The pH was maintained at 6.5. Derrien was able to detect 34 components; 20 of which moved as globulins and 14 as albumins in the electrophoresis. (5)

The salt precipitation yielded only a mixture of proteins. This was clearly shown using ultracentrifugation and electrophoresis. Even with repeated refractionation by this means, homogeneous preparations were not obtained. The different methods of salt precipitation and their results were reviewed by Gutman. (3)

If a nonpolar organic solvent such as ethanol is used, not only the ionic strength, but the pH, protein concentration, as well as the quantity of ethanol used, can be varied to effect a better separation than that of salts only. Cohn and his colleagues (6) worked out several systems, by which they were able to fractionate the plasma proteins into five fractions: Fraction I containing largely fibrinogen, Fractions II and III, β and γ globulins, Fractions IV-1 and IV-4, mostly α and β globulins, and Fraction V, albumin. Several subfractions of Fractions II, III (7), and IV-4 (8) were also obtained. Fraction IV-4 was further fractionated by Surgenor et al. (8) into

Fractions IV-5, IV-6, IV-7, IV-8, and IV-9. Fraction IV-7 contained most of the iron binding β globulin. From this fraction Koechlin was able to crystallize out the transferrin. (9)

Rivanol was first introduced by Horejsi and Smetana in 1956 to isolate γ globulin from blood serum or plasma. (10) A 0.4% Rivanol solution was shown to precipitate the albumin and most of the α and β globulins. The γ globulin could then be precipitated by ethanol. The Rivanol precipitation was later also used to isolate transferrin. The plasma was first treated with 0.4% Rivanol solution, and the precipitate which formed was removed by centrifugation. Activated charcoal was added to the supernatant fraction, which was then filtered. This procedure removed the Rivanol. The γ globulin in the filtrate was then precipitated with ethanol 25%. (11) From the remaining transferrin rich solution, the transferrin could be crystallized (12), using the conditions described by Inman (13), pH 6.3, Γ/2 0.03, alcohol 15%, 0.0°C.

Since proteins are charged molecules, it is natural that the ion exchange absorbents, which had been so successful in the separation of small ions, would be tried on protein separation. However, with a few exceptions, the use of the organic ion-exchange resin was not very successful in the case of proteins. More effective were the cellulosic absorbents, first developed by Sober et al.

The most widely used is the anion exchanger, diethylamino ethyl (DEAE-) cellulose, and the cation exchanger, carboxymethyl (CM-) cellulose. Other useful cellulosic absorbents are: tri ethylaminoethyl (TEAE-) cellulose, sulfomethyl (SM-) cellulose, and sulfoethyl (SE-) cellulose. (14) Whole serum has been fractionated on a DEAE column. (15) However, more success was obtained with the cellulosic absorbents when combined with other methods such as $(NH_4)_2SO_4$ precipitation or electrophoresis.

Gel filtration is another method used extensively. A column of agar or Sephadex is used. Larger molecules will be excluded and go through the column, where as the smaller molecules will enter the gel and be retarded. The separation is thus based on molecule size. Gels of different pore size can be obtained. (16) Sephadex may be combined with one of the ion-exchange celluloses.

Since the development of electrophoresis, the techniques of analytical electrophoresis were modified for the preparation of proteins. The main problem is to recover the separated protein without remixing after the electrophoretic run. Another problem is to develop suitable electrophoretic equipment for working with large quantities of protein.

Attempts have been made to modify moving boundary electrophoresis equipment to make it suitable for the isolation of proteins after they have been separated by

electrophoresis. (17,18) However, only the fastest or slowest moving proteins can be isolated in relative purity. Electrophoresis, using solid supporting media, has been more successfully used for protein fractionation. Here the proteins separate into distinct zones.

Paper electrophoresis has been used for separation of serum proteins. The simplest method of isolation after electrophoresis is to cut up the paper and elute the protein. Only very small samples can be used. Larger samples may be used in continuous paper electrophoresis. Here the application of the sample is continuous while the current is applied. A sheet of paper is hung vertically with the upper end dipped into buffer. The sample is applied continuously on the upper part of the paper. The lower end of the paper is serrated. Current is applied horizontally. As the sample is continuously applied, it will flow down and because of the current the proteins will be separated in a fan like manner. The proteins are collected in test tubes from the serration. (19) This fractionation is comparable to analytical paper electrophoresis.

Starch block electrophoresis uses a moist block of starch granules placed between two glass plates as supporting medium. At the end of the electrophoretic run the starch block is cut and the proteins in the cut pieces extracted with saline or buffer. This is easily done by stirring the starch with the buffer and removing the starch

by centrifugation. The starch block is formed by pouring a starch-buffer mixture into a mold and letting it settle into a solid form. (20,21)

Starch or other poorly adsorbing substances are also put in a column. This allows more sample to be applied in an apparatus taking less space. After the electrophoretic separation, the starch is forced out of the column and cut. The proteins are extracted. (20) Another method of isolating the protein is to elute it from the top of the column. The eluate is collected in a fraction collector. (20) Another elution system has been developed, in which the elution buffer flows in the opposite direction to the electrophoretic migration i.e., the counter flow elution. (22)

A more non-reactive substance than starch is a powdered co-polymer of polyvinyl chloride and polyvinyl acetate, Pevikon C870. This is also used as supporting media in block electrophoresis. The method has been used successfully in conjunction with other methods in the isolation of protein fractions from blood serum of rabbits (23) and rats. (24)

As starch gel electrophoresis introduced by Smithies (25) has very high resolution power, means have been sought to use it as a preparative tool. Several methods were devised to remove the protein from the starch gel. (26,27) After the electrophoresis, a small strip is cut from each side of the starch slab and stained. Using the stained

strip as a guide, the unstained gel is cut. To obtain the protein, the gel is digested by amylase. After the digestion the amylase is separated from the protein by $(NH_4)_2SO_4$ precipitation. The protein should be sufficiently different from amylase as to be easily separated. However, in practice it is not always so, and the yield is low. (27) Another method is to freeze and thaw the gel. This gives the gel a sponge-like structure, from which the protein can be squeezed out. This gives comparatively low yield, and the protein is contaminated with starch constituents. (27)

A better method, that of electrodialysis was developed by Moretti et al. The protein was extracted from the starch into a dialyzing bag by applying an electric current. The starch gel pieces were separated from the dialyzing bag by sintered glass on which was placed insoluble starch. This was supposed to hold the soluble starch from the gel. (27) However, in a later paper the same group of investigators admitted that some of the soluble starch had picked up enough charge during the process as to migrate with the protein into the dialyzing bag. To free the protein from these charged starch molecules an ion-exchange resin column was used. The resin used was Dowex 2X8, 200-400 mesh or Amberlite IR4B. (28) The protein can also be separated from the starch molecules by using the Pevikon C870 block electrophoresis, as suggested by Bocci, who claimed this method to be more convenient than using the

ion-exchange resin. (29)

A simple eluting system for preparative starch gel electrophoresis was reported by Smith et al. (30) A hole was bored on either side of the mold. The liquid starch was poured into the mold as usual with both holes covered. After it had gelled and the sample applied, the electrophoresis was run in the usual vertical position until the protein to be isolated had migrated nearly at the height of the two holes. The electrophoresis was stopped. A strip of gel was cut at the level of the holes. The bottom of the channel left by the strip was covered with dialyzing membrane. The wall of the channel on the opposite side of where the proteins would migrate out of the gel was also covered with dialyzing membrane. The channel was then filled with polyvinyl sponge and the sponge covered with wax. The electrophoresis was continued in a horizontal position. Buffer was led into the sponge at the hole on one side of the mold. It flowed out from the hole at the other side, where it was led into a fraction collector. As the proteins came out of the gel and entered the sponge, they were swept away into the tubes of the fraction collector.

As acrylamide gel also has a high resolution power (because of its sieving effect) it is also used in preparative electrophoresis. The problems also centered around the removal of the protein from the gel. In the electro-

convection method the gel is poured into a rectangular mold to form a slab. The electrophoresis is run in a vertical position. After the run the acrylamide slab is placed in a so called elution-convection cell. It is placed between a dialyzing bag and a grid. Another dialyzing bag is placed against the grid. The cell and the dialyzing bags are filled with buffer. The electrodes are placed in the bags. The current is applied so that the protein will migrate out of the gel into the grid. The specific density of the buffer is chosen, so that it is less than that of the protein. As the proteins enter the grid, they will descend due to gravity. Tubes (for the collection of proteins) are placed at the bottom of the grid. (31) As the tubes are arbitrarily placed, the separation has to be substantial. The proteins have to be separated from one another at least 0.7 cm before a successful separation can be effected.

Another method used in the preparative acrylamide electrophoresis is that of a column. The bottom of the column comes out in an elution chamber. Beneath this elution chamber is the electrode chamber, which is separated from it by a dialyzing membrane. The sample is applied on the top of the column and the proteins migrate downward. The upper part of the column is connected with the other electrode chamber. As the proteins come out of the gel at the bottom, they are swept away by buffer coming in at one

side of the elution chamber. An outlet in the chamber leads the buffer into a fraction collector. A pump circulates the buffer from the electrode chambers into a reservoir, so changes in the buffer are prevented and the electrophoresis can be continued for a long time. (32)

Transferrin

This review will cover chiefly human transferrin. In 1925 Fontes and Thivolle (33) found that iron, not derived from hemoglobin, was present in the ash of horse plasma. Barkan showed later that the serum iron was not dialyzable at physiological pH (34), but that it was present in the ultrafiltrate of serum after acidification. (35) By $(NH_4)_2SO_4$ fractionation of sera, to which iron salts had been added, Starkenstein and Harvalik found that the iron was bound to the globulin fraction. (36) Schade and Caroline (37), and Holmberg and Laurell (38) observed that upon addition of iron salts to serum, the serum color changed from yellow to salmon-pink, which reached a maximun at the iron-saturation limit of the serum. Schade and Caroline (37) showed that a protein component in Cohn's Fraction IV-4 bound the iron to form a salmon-pink colored complex. This fraction also inhibited the growth of certain microorganisms (37), which needed iron as a nutrient. The iron binding component of plasma was first isolated by Laurell and Ingelman (39) from pig plasma and later from

human plasma by Surgenor et al. (8) Surgenor et al. fractionated Cohn's Fraction IV-4 into several subfractions, of which Fraction IV-7 contained most of the iron-binding principle. Starting from Fraction IV-7, Koechlin (9) succeeded in crystallizing the iron-binding protein for which he proposed the term "β_1-metal-combining protein". The name transferrin was proposed by Holmberg and Laurell. (40) Transferrin functions chiefly as the specific iron-transport protein in plasma. (41)

Genetic variation in human transferrin was revealed when the genetic variants of the β globulin demonstrated by Smithies (42,43), were shown to be transferrin. (44) Many different variants were found in different ethnic groups. This polymorphism seems not to be exclusive in human transferrin; it is also apparent in cattle (45), mice (46), rhesus monkey (47), chimpansee (48), and chicken (49) transferrin.

Isolation

Koechlin (9) was able to isolate transferrin in a crystalline state from Cohn's Fraction IV-7. Crystalline transferrin was also obtained by Inman. (13) Schultze et al. (50) reported an ammonium sulfate-aluminum hydroxide crystallization procedure. A much simpler method to obtain transferrin rich solution, from which transferrin could be crystallized out, was that using Rivanol precipitation.

(11,12)

A two step procedure was introduced by Nagler et al. (51) The serum was first treated with Rivanol and then the supernatant was chromatogaphed on a carboxy-methyl-cellulose or diethylaminoethyl cellulose column. Berkorovainy et al. (52) used $(NH_4)_2SO_4$ precipitation instead of the Rivanol. After the $(NH_4)_2SO_4$ was added the pH was adjusted to 4.6 with 6N HCl. The precipitate that resulted at this pH was discarded and the pH of the supernatant was adjusted to 3.7. The precipitate following this procedure was dialyzed and lyophilized before it was chromatographed on a DEAE-cellulose column.

Gordon and Louis (24) described a method for the isolation of rat transferrin. Rat serum was electrophoresed on a Pevikon C870 block electrophoresis. The transferrin, seen as a faint brown band, was cut out and eluted. The filtrate thus obtained was concentrated and chromatographed on a DEAE-Sephadex A50 column.

Transferrin of high purity was prepared by Parker and Bearn using starch block electrophoresis, followed by chromatography on TEAE-cellulose. (53) By this method the different genetic variants could be separated.

Chemical Analysis: Amino Acids

Many reports are found in the literature on the amino acid contents of human transferrin. They do not show much

difference. Amino Acid contents of the different human transferrin do not vary much either. As there is still argument about the molecular weight, the residues per mole of transferrin reported differ considerably. Table 1 summarizes the values of the transferrin from three different species; rat, cynomolgus monkey, and human. The values were recalculated to a g per 100 g and μmoles per mg basis, so that comparison can be made with our preparation.

Chemical Analysis: Carbohydrates

Schultze et al. (54) reported that transferrin is a glycoprotein in 1958.

Studying the glycopeptides isolated from human transferrin, Jamieson (56) concluded that the human transferrin molecule contains 4 moles of sialic acid, 8 moles of N-acetyl glucosamine, 4 moles of galactose, and 8 moles of mannose per 90,000 g of protein. The carbohydrate moiety consists of 2 branched chains, identical in composition and terminating in sialic acid. He further found that the aspartic acid content was 1 mole of aspartic acid per mole of glycopeptide and the amount of amide nitrogen was 1 mole per mole of aspartic acid. From this he concluded that the linkage between the protein moiety and the carbohydrate is most probably an asparaginyl glycosylamine linkage; the O-glycosidic linkage to serine, however, could not be excluded. Table 2 gives the values of human transferrin reported by

Schultze and Jamieson.

Spik et al. (57,58) subjected the glycopeptides isolated from pronase digest of human transferrin to further degradation by leucine-amino peptidase and could isolate a glyco-amino acid with only aspartic acid. Partial hydrolysis of this glyco-amino acid yields a compound consisting of glucosamine and aspartic acid in a 1 to 1 proportion. The compound had the same electrophoretic velocity as 1-(β aspartyl)-2-amino 1,2-di deoxyglucosylamine obtained by partial hydrolysis under the same condition of pure 1-(β aspartyl)-2-acetamide-1,2-di deoxyglucosamine. Further more, it produced the same characteristic color with ninhydrin as 1-(β aspartyl)-2-amino-1,2-di deoxyglycosylamine. With this evidence, Spik et al. concluded that the probable linkage is via N-β aspartyl-glucosylamine.

TABLE 1

AMINO ACID CONTENTS OF TRANSFERRIN FROM HUMAN, CYNOMOLGUS MONKEY, AND RAT

Amino Acids	Human [a]		Monkey [a]		Rat [b]	
	g/100 g	μmole/mg	g/100 g	μmole/mg	g/100 g	μmole/mg
Lysine	10.1	0.691	9.0	0.616	10.9	0.746
Histidine	4.6	0.296	4.6	0.296	4.7	0.303
Arginine	5.6	0.321	5.1	0.293	6.0	0.344
Aspartic Acid	12.5	0.939	12.7	0.954	13.0	0.977
Threonine	4.1	0.344	4.4	0.369	6.0	0.504
Serine	4.7	0.447	5.9	0.561	6.1	0.580
Glutamic Acid	10.4	0.707	11.0	0.748	11.9	0.809
Proline	4.7	0.408	4.7	0.408	5.7	0.495
Glycine	4.4	0.586	4.2	0.559	5.9	0.786
Alanine	6.0	0.673	6.6	0.741	6.8	0.763
Half cystine [c]	4.6	0.380	4.4	0.363	2.7	0.223
Valine	5.6	0.478	6.3	0.538	6.3	0.538
Methionine	1.6	0.107	1.8	0.121	0.9	0.060
Isoleucine	2.2	0.168	2.2	0.168	3.7	0.282
Leucine	9.0	0.686	9.0	0.686	10.8	0.823
Tyrosine	9.0	0.497	9.0	0.497	5.1	0.281
Phenylalanine	5.4	0.327	5.6	0.339	7.6	0.460

[a] From Parker and Bearn (41) recalculated
[b] From Gordon and Louis (24) recalculated
[c] Includes oxidized form

TABLE 2

CARBOHYDRATE CONTENTS OF HUMAN TRANSFERRIN

Carbohydrates	Human Transferrin			
	Results of Schultze et al. (53)		Results of Jamieson (54)	
	g/100 g	μmole/mg	g/100 g	μmole/mg
Hexoses	2.40	0.133	2.6	0.144
Hexosamines	2	0.112	2.0	0.112
Sialic Acid	1.40	0.045	1.3	0.042
Fucose	0.07	0.0004	0	

CHAPTER II

MATERIALS AND METHODS

The isolation of the bovine iron-binding β_1 globulin was done first by a two step precipitation. The bovine serum was treated with an equal volume of 3.4 M $(NH_4)_2SO_4$ solution, by which almost all the γ globulins and part of the α and β globulins were precipitated. The supernatant was dialyzed; 0.2 ml of the Rivanol solution was added to each ml of the dialyzate. The precipitate was removed by centrifugation. After removing the Rivanol with activated charcoal, the supernatant fraction of this last precipitation was lyophilized. (59) Later a transferrin preparation was also purchased from Pentex.

These preparations which still showed albumin, α and γ globulins were then passed through the Porath Column Electrophoresis (LKB Instruments, Inc.). The Porath column was packed with Cellex XF_1 from Bio-rad, an ethanolyzed cellulose powder, which has all ionic groups blocked by cross-linking with formaldehyde. The buffer used was a Tris-HCl buffer, pH 8.6 with a concentration of 0.1 M; 1 g samples were used. The electrophoresis was run with 4°C cooling

water running through the cooling jacket. It took 72 hours before the transferrin, which had a brownish color, reached the elution nozzles. Elution was then begun using the counterflow elution system. The electrophoresis was continued until all brownish colored protein was eluted. The eluates were collected in a fraction collector. The fractions were exhaustively dialyzed and then lyophilized. The transferrin preparations were still contaminated. The first fractions contained albumin, and the last, some γ globulin when tested by disc electrophoresis.

After lyophilization, the protein was passed through a preparative disc electrophoresis apparatus (Canal Industrial Corporation). A 15% separating gel was used. The sample gel was omitted; the sample was mixed with an equal volume of a 14% acrylamide solution. This solution was layered on top of the spacer gel. Up to 50 mg sample was used in the largest column. The initial current applied was 5 ma and after the protein entered the separating gel, the current was increased to 20 ma. The flow rate of the elution was 1.5 ml per minute. The eluate was collected in a fraction collector and monitored with LKB Uvicord. However, the eluted protein could also be detected by the naked eye. Ten ml fractions were collected. The fractions were dialyzed exhaustively against distilled water and lyophilized. They were then checked on the analytical disc electrophoresis apparatus. As a rule the first few fractions and the

last few fractions contained some contaminating protein. These fractions were combined and passed again through the preparative disc electrophoresis. All material which showed a single band in disc electrophoresis was combined; this fraction shall subsequently be referred to as the bovine transferrin fraction.

The homogeneity of this protein was checked with disc electrophoresis, agar immunoelectrophoresis, and ultracentrifugation. The ultracentrifugation was done in a Spinco Model E ultracentrifuge equipped with an RTIC temprature control system. The protein concentration was 5 mg/ml. The buffer used was phosphate buffer pH 7.0, Γ/2 0.3. Rotor speed was 52,640 r.p.m. at 20°C. The agar immunoelectrophoresis was done according to the method described by Schwick and Störiko (60), with the exception that the electrophoresis was run for 2 hours instead of 45 minutes. Antibovine serum of rabbit origin was obtained from Pentex.

Starch gel electrophoresis was done on the Spel System of thin layer starch gel electrophoresis. The buffer used was the discontinuous TRIS-citrate-borate buffer system of Poulik. (61) Starch was obtained from Connaught Medical Research Laboratories.

Chemical determinations were done as described by Shetlar. (62) Hexose was determined by the modified tryptophane method of Shetlar et al. (63) and the anthrone method (64); hexosamine determination was done by the modified

method of Boas. (64) The sialic acid contents were determined by the modified method of Warren. (66) Protein determination was done by Lowry method (66) using Lab-Trol from Dade Reagents Inc. as standard. For the qualitative study of the hexoses the protein was hydrolyzed with 2 N HCl for 16 hours. The hydrolysate was dried in a vacuum desiccator and then dissolved in 1 ml H_2O, after which the solution was applied on a Dowex 50 x 12 column, 100-200 mesh. The hexoses were eluted with distilled water and the eluate taken to dryness in a vacuum desiccator. The dried material was then dissolved in a minimum of water and used for paper chromatography. The developing solvent used was n-butanol: pyridine:0.1N HCl (5:3:2, V/V). The chromatogram was developed twice, i.e., after placing the paper in the chromatographic cabinet for 16 hours, it was air dried and again put in the cabinet for another 16 hours. The paper was then air dried again and dipped into a solution of 2-amino biphenyl according to Gordon et al. (68). After the paper was air dried it was put in an oven at 110°C for 5 minutes.

Preparation of the Glycopeptide

About 100 mg of protein was dissolved in 5 ml of 0.01 M $CaCl_2$ adjusted to pH 8.5 with 1 N NaOH. One Mg of pronase was added, and the solution left for 48 hours at 37°C. The pH was frequently checked and adjusted to 8.5. After 24 hours another 1 mg of pronase was added.

The digestion mixture was mixed with ethyl alcohol

to a concentration of 80% alcohol and left overnight. The precipitate was collected by centrifugation and dissolved in 2 ml H_2O. This solution was then adjusted to 3% trichloroacetic acid to precipitate any protein present. After centrifugation, the supernatant fraction was applied to a Sephadex G25 column. The eluate was monitored with an LKB Uvicord and collected in a fraction collector. A 0.1 ml aliquote was taken from each fraction and the hexose contents determined. The hexose containing peak, which was the first peak, was combined and lyophilized.

The amino acids and the individual hexosamines were analyzed using the Beckman Amino Acid Analyzer. For this purpose the protein and the glycopeptide were hydrolyzed with 6 N HCl in a sealed evacuated tube at 110°C for 21 hours. (69) After hydrolysis the solution was evaporated to dryness in a rotary evaporator at 50°C. The dried material was dissolved in buffer of pH 2.2 and applied to the long and short columns of the Amino Acid Analyzer.

Autoradiogram

Five µl of the bovine transferrin solution containing 0.1 mg was mixed with 5 µl of radioactive $Fe^{59}SO_4$ solution containing 0.03 µc. The resulting mixture was run on the disc electrophoresis. After the electrophoretic run the gel was put in a grid which was wrapped with "Saran Wrap" and placed in contact with a Kodak medical X-ray film.

After 30 hours the film was developed and the gel stained with amido black. The protein had diffused somewhat, but could be seen to carry radioactivity. This procedure was also done with whole bovine serum.

CHAPTER III

RESULTS AND DISCUSSION

After the $(NH_4)_2SO_4$ and the Rivanol precipitation, it could be shown by disc electrophoresis technique, that the transferrin contained albumin, α and γ globulins. Figure 1 shows the results of the disc electrophoresis on bovine serum and the preparation after the two step precipitation. As could be expected, the precipitation procedure resulted in a crude mixture of proteins, and unless the protein could be crystallized out, a homogeneous fraction could not be obtained. The conditions of Inman (13) were tried on our protein preparation, but no crystals were obtained.

For further purification, the preparation was passed through the Porath Column Electrophoresis; however, it still contained traces of albumin, α and γ globulins. The first fractions contained more of the albumin, and the last, more of the γ globulin. This result could be expected, as electrophoresis on a cellulose column does not have a high resolution. Mixing of the separated proteins is also likely to occur during the elution.

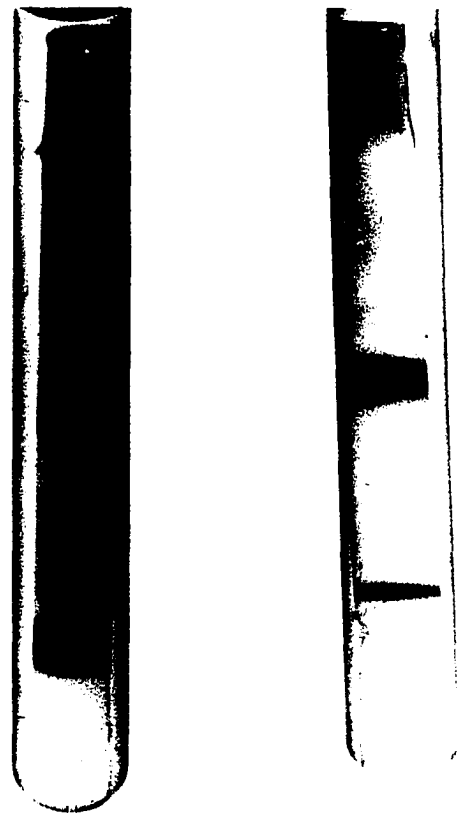

Figure 1. Disc electrophoresis of bovine serum and of the preparation after $(NH_4)_2SO_4$ and Rivanol precipitation.

Better resolution and elution can be obtained with preparative disc electrophoresis. The pore size of the gel can be varied, using different concentrations of acrylamide. The elution flow may also be manipulated; fast moving protein being eluted faster. After several preliminary runs, we were able to obtain a transferrin preparation which showed one band in analytical disc electrophoresis (Figure 2).

The homogeneity of the transferrin was further checked by agar immunoelectrophoresis. Figure 3 shows that there is a single precipitation line which indicates that the preparation is homogeneous by this criterion.

The ultracentrifugation pattern is shown in Figure 4. The first picture was taken 5 minutes after the rotor has attained its speed of 52,640 r.p.m.; the second, 15 minutes after, and the third, 30 minutes after. The peaks were symmetrical and showed the monodispersity of the preparation.

Polymorphism has been shown only with starch gel electrophoresis. Acrylamide gel electrophoresis does not separate the bovine transferrin as does starch gel; neither does agar immunoelectrophoresis separate the genetic variant. We also subjected our preparation to starch gel electrophoresis. The result is shown in Figure 5. The preparation shows a single band with the mobility of the first variant of the bovine transferrin as can be seen comparing it with the electrophoretic pattern of bovine serum.

To show that the protein would bind iron, the

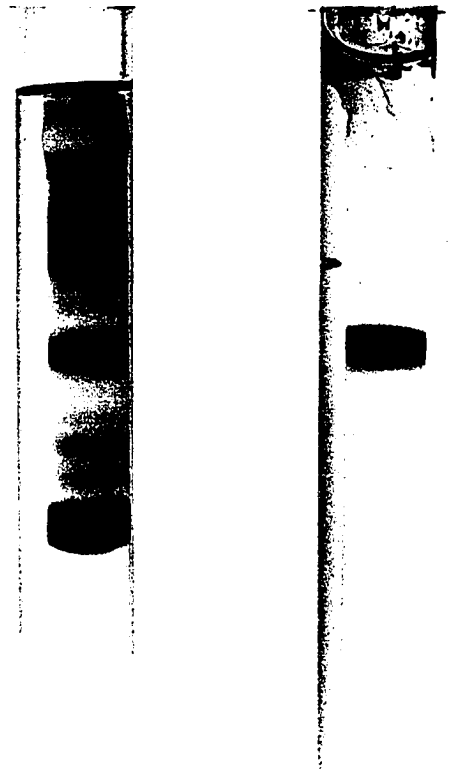

Figure 2. Disc electrophoresis of bovine serum and bovine transferrin.

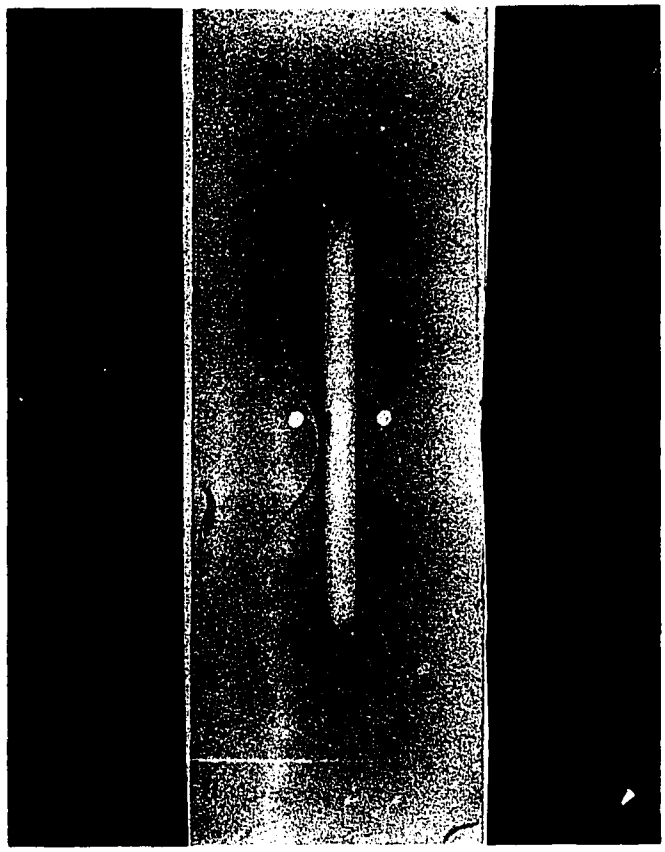

Figure 3. Agar immunoelectrophoresis of bovine transferrin.

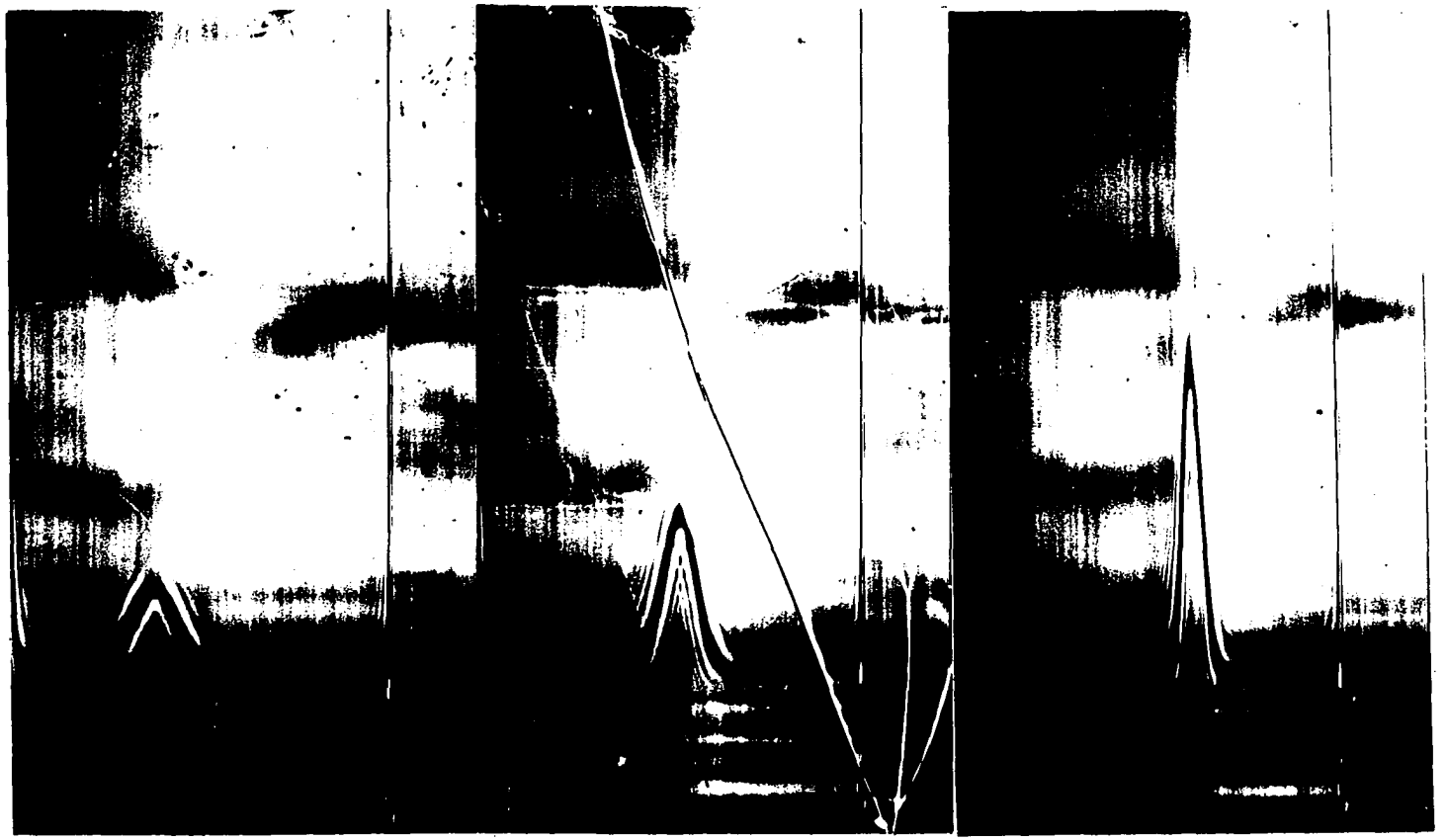

Figure 4. Ultracentrifugation pattern of bovine transferrin. The pictures were taken at 5, 15, and 30 minutes after the rotor had attained the speed of 52,640 r.p.m. Direction of sedimentation is from right to left.

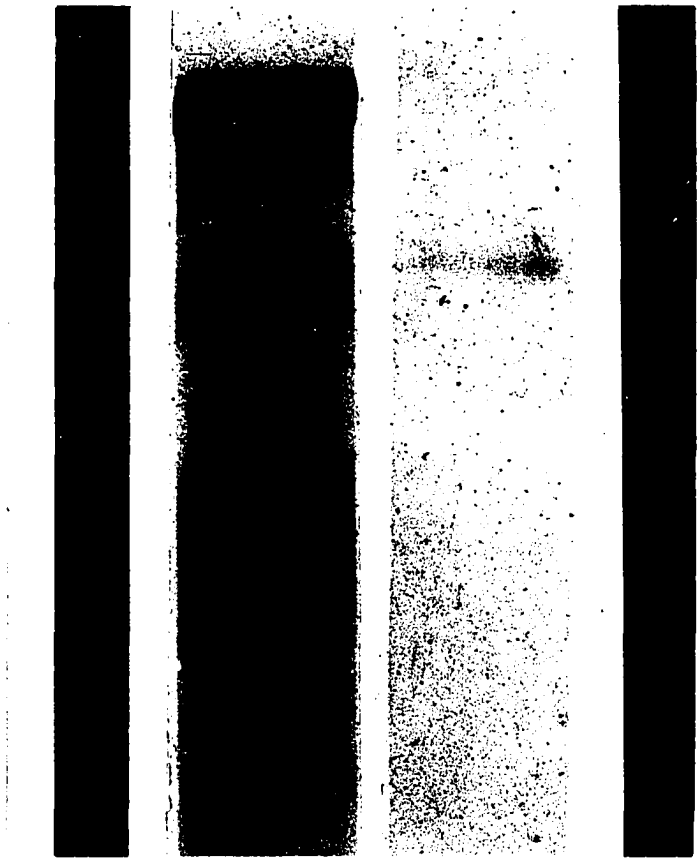

Figure 5. Starch gel electrophoresis of bovine serum and bovine transferrin.

preparation was mixed with $Fe^{59}SO_4$ solution and run on analytical disc electrophoresis. After the run an autoradiogram was made (Figure 6); the protein carried the iron.

Chemical analysis was carried out on this preparation. Table 3 shows the result of the amino acid analysis. Amino acid contents of rat (24), cynomolgus monkey, and human transferrin (53) had been reported. To make a comparison with our results, we recalculated the results to a g/100 g and μmole/mg basis (Table 1). This shows that there are only small differences, except for histidine and tyrosine, which are more than 50% lower in the bovine transferrin than in the human and cynomolgus monkey transferrin. The cystine and methionine contents are much higher than those of the rat; however, histidine and proline contents are much lower.

Table 4 shows the carbohydrate contents of our preparation. The values do not differ much from human transferrin. The hexose is slightly higher, but hexosamine and sialic acid are slightly lower than in human transferrin. For comparison, the carbohydrate contents of the Pentex, preparation is shown in Table 5. The lower values can be explained by the fact that it contains other proteins, especially albumin.

Chemical analyses were also done on the glycopeptide isolated after pronase digestion. Pronase digestion has been used for removing the protein moiety of glycoprotein in

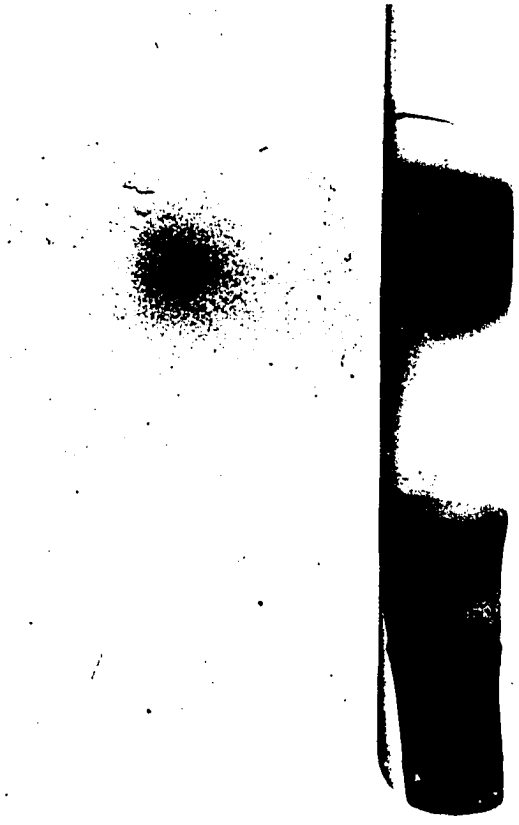

Figure 6. Autoradiogram of bovine transferrin. On the right is the acrylamide gel stained with amido black; on the left, the autoradiogram.

TABLE 3

AMINO ACID CONTENTS OF BOVINE TRANSFERRIN

	g/100 g	μmole/mg
Lysine	9.01	0.62
Histidine	2.53	0.16
Arginine	3.86	0.22
Aspartic Acid	11.75	0.88
Threonine	3.39	0.33
Serine	4.55	0.43
Glutamic Acid	9.68	0.66
Proline	3.08	0.27
Glycine	4.59	0.61
Alanine	4.65	0.52
Half cystine	3.01	0.25
Valine	4.16	0.35
Methionine	1.12	0.07
Isoleucine	2.46	0.19
Leucine	6.50	0.50
Tyrosine	3.65	0.20
Phenylalanine	4.55	0.28

TABLE 4

CARBOHYDRATE CONTENTS OF BOVINE TRANSFERRIN

	g/100 g of protein [a]	μmole/mg
Hexose (Tryptophane Method)	3.41	0.189
(Anthrone Method)	3.42	0.190
Hexosamine (Boas Method)	1.29	0.721
Galactosamine (Amino Acid Analyzer)	0.43	0.024
Glucosamine (Amino Acid Analyzer)	0.68	0.038
Sialic Acid	1.23	0.040

TABLE 5

CARBOHYDRATE CONTENTS OF THE PENTEX BOVINE TRANSFERRIN PREPARATION

	g/100 g of protein [a]	μmole/mg
Hexose (Tryptophane Method	2.63	0.146
(Anthrone Method)	2.25	0.125
Hexosamine (Boas Method)	1.19	0.0664
Sialic Acid	0.84	0.0271

[a] Protein is determined by the Lowry method

order to obtain the carbohydrate with as little amino acid as possible attached to it. This allows us to study the carbohydrate moiety, and the linkage between the heterosaccharide and the protein moiety. Table 6 shows the results. The value found for glucosamine is almost equal to that of aspartic acid, which suggests that the linkage between the protein moiety and the carbohydrate moiety is via these two molecules. The amount of serine and threonine is also significant. To exclude the O-glycosidic linkage through serine and threonine, we subjected the glycopeptide to 0.5N NaOH for 24 hours at 0-4°C. If the threonine and serine are involved in the linkage they would be destroyed by β-elimination. (70) Table 7 gives the result of this procedure. It is clearly seen that the amount of serine and threonine remained the same.

It should also be noted that the glycopeptide did not contain galactosamine. This leads us to suspect that there is more than one glycopeptide; the one with the galactosamine was lost during the procedure.

The qualitative studies on the hexoses were done with paper chromatography. The sugars found were mannose, galactose, and glucose. Glucose was also found by Yang (59) in her impure preparation. She was able to obtain evidence that it was indeed glucose. Figure 7 is a photograph of the chromatogram.

TABLE 6

AMINO ACID AND CARBOHYDRATE CONTENTS OF THE GLYCOPEPTIDE

	g/100 g	μmole/mg
Lysine	0.73	0.05
Histidine		
Arginine		
Aspartic Acid	8.12	0.61
Threonine	1.31	0.11
Serine	2.63	0.25
Glutamic Acid	5.01	0.34
Proline	0.46	0.04
Glycine	1.27	0.17
Alanine	0.71	0.08
Half cystine		
Valine		
Methionine		
Isoleucine		
Leucine		
Tyrosine		
Phenylalanine		
Hexose	20.04	1.11
Glucosamine	8.95	0.50
Galactosamine		

TABLE 7

SERINE AND THREONINE CONTENTS OF THE GLYCOPEPTIDE

BEFORE AND AFTER TREATMENT WITH NaOH

	Before treatment with NaOH	After treatment with NaOH
Serine	0.400	0.402
Threonine	0.102	0.112

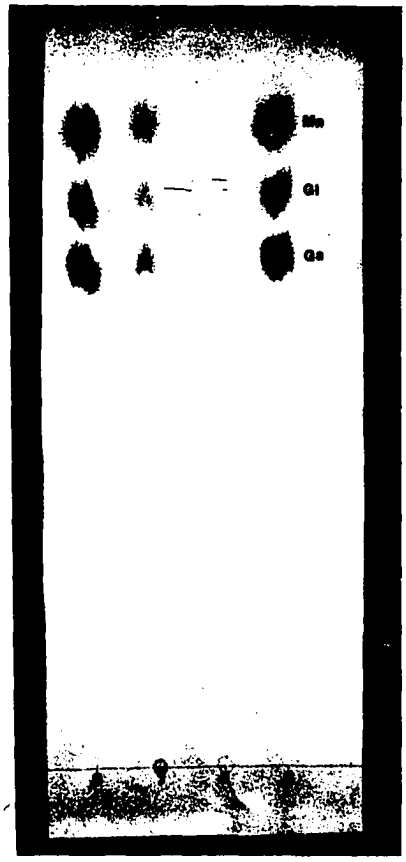

Figure 7. Paper chromatogram of neutral sugars contained in bovine transferrin. A standard solution of sugars was placed at each side of the chromatogram. The sugars are indicated as follows:

Mn - mannose
Gl - glucose
Ga - galactose

The amount of sample placed on position 1 was twice as much as that placed on position 2.

CHAPTER IV

SUMMARY AND CONCLUSION

1. A method of isolating bovine transferrin involving a two step precipitation with ammonium sulfate and Rivanol, followed by preparative disc electrophoresis was described.
2. The transferrin isolated by this method is homogeneous as judged by the criteria of ultracentrifugation, disc electrophoresis, and agar immunoelectrophoresis.
3. The starch gel electrophoresis shows that the preparation is the fastest moving variant of the bovine transferrin.
4. Quantitative analysis indicated the presence of hexose, hexosamine, and sialic acid.
5. Analysis by paper chromatography shows that the hexoses are glucose, galactose, and mannose. The hexosamines were found to be galactosamine and glucosamine.
6. The amino acid contents (g/100 g) are similar to those of human transferrin, with a lower content of histidine and tyrosine in bovine transferrin.
7. Studies of the amino acid contents after pronase digestion

shows the glycopeptide to contain chiefly aspartic acid, glutamic acid, threonine, and serine. The carbohydrates are hexose and glucosamine. The values for glucosamine and aspartic acid are almost equal, which indicates that these two compounds might be involved in the linkage between the protein and carbohydrate moieties.

8. The serine and threonine contents of the glycopeptide show no decrease after treatment with NaOH, indicating they are not involved in the protein-carbohydrate linkage.

LITERATURE CITED

1. Putnam, F. W., The Plasma Protein, Vol. 1, Academic Press, New York, (1960).

2. Howe, P. E., Physiol. Revs., 5, 439 (1925).

3. Gutman, A. B., Adv. Prot. Chem., 4, 155 (1948).

4. Butler, A. M., Blatt, H., Southgate, H., J. Biol. Chem., 109, 755 (1935).

5. Derrien, Y., Compt. Rend. Soc. Biol., 139, 909 (1945).

6. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., Taylor, H. L., J. Am. Chem. Soc., 68, 459 (1946).

7. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., Gros, P. M., J. Am. Chem. Soc., 11, 541 (1949).

8. Surgenor, D. M., Strong, L. E., Taylor, H. L., Gordon, R. S., Jr., Gibson, D. M., J. Am. Chem. Soc., 71, 1223 (1949).

9. Koechlin, B. A., J. Am. Chem. Soc., 74, 2649 (1952).

10. Horejsi, J. K., Smetana, R., Acta. Med. Scand., 155, 65 (1956).

11. Boettcher, E. W., Kistler, P., Nitschmann, H., Nature, 181, 490 (1958).

12. Kistler, P., Nitschman, H., Wyttenbach, A., Studer, M., Niederost, C. H., Mauerhofer, M., Vox. Sang., 5, 403 (1960).

13. Inman, J. K., 10th Conference on Plasma Protein and Cellular Elements of Blood, Protein Foundation and the Commission on Plasma Fractionation and related processes, Cambridge, Mass. (1956).

14. Peterson, E. A., Sober, H. A., The Plasma Protein, Vol. 1, (Putman, Ed.), Academic Press, New York (1960), p. 105.

15. Sober, A., Gutter, F. J., Wyckoff, M. M., Peterson, E. A., J. Am. Chem. Soc., 78, 756 (1956).

16. Porath, J., Adv. Prot. Chem., 17, 209 (1962).

17. Tiselius, A., Methods in Enzymology, Vol. 4, (Colowick and Kaplan, Eds.), Academic Press, New York, (1957), p. 3.

18. Svensson, H., Adv. Prot. Chem., 4, 251 (1948).

19. Pucar, L., J. Chromatography, 4, 261 (1960).

20. Kunkel, H. G., Methods of Biochemical Analysis, Vol. 1, (Glick, Ed.), Interscience, New York, (1954), p. 141.

21. Bloemendal, H., J. Chromatography, 2, 121 (1959).

22. Porath, J., Linder, E. B., Jerstedt, S., Nature, 182, 744 (1958).

23. Bocci, V., J. Chromatography, 8, 218 (1962).

24. Gordon, A. H., Louis, L. N., Biochem. J., 88, 409 (1963).

25. Smithies, O., Biochem. J., 61, 629 (1955).

26. Bodman, J., Chromatographic and Electrophoretic Techniques, (Smith, Ed.), Interscience, New York, (1960), p. 149.

27. Moretti, J., Boussier, G., Jayle, M., Bull. Soc. Chim. Biol., 40, 59 (1958).

28. de Pailleretts, C., Moretti, J., Jayle, M., Bull. Soc. Chim. Biol., 41, 1285 (1959).

29. Bocci, V., J. Chromatography, 6, 357 (1961).

30. Smith, J. C., Bernstein, G. Surks, M. I., Oppenheimer, J. H., Biophys. Biochim. Acta, 115, 81 (1966).

31. Raymond, S., Science, 146, 406 (1964).

32. Radhakrisnamurthy, B., Biophys. Biochim. Acta, 107, 380 (1965).

33. Fontes, G., Thivolle, L., Compt. Rend. Soc. Biol., 93, 687 (1925).

34. Barkan, G., Z. Physiol. Chem., 171, 194 (1927).

35. Barkan, G., Z. Physiol. Chem., 216, 1 (1933).

36. Starkenstein, S., Harvalik, Z., Arch. Exptl. Pathol. Pharmakol., 172, 75 (1933).

37. Schade, A. L., Caroline, L., Science, 104, 340 (1946).

38. Holmberg, C. G., Laurell, C. B., Acta Physiol. Scand., 10, 307 (1945).

39. Laurell, C. B., Ingleman, B., Acta Chem. Scand., 1, 770 (1947).

40. Holmberg, C. G., Laurell, C. B., Acta Chem. Scand., 1, 944 (1947).

41. Bearn, A. G., Parker, W. C., Glycoproteins, Their Composition, Structure, and Function, (Gottschalk, Ed.), Elsevier Publishing Co., Amsterdam (1966), p. 423.

42. Smithies, O., Nature, 180, 1482 (1957).

43. Smithies, O., Nature, 181, 1203 (1958).

44. Horsfall, W. R., Smithies, O., Science, 128, 35 (1958).

45. Giblett, E. R., Hickman, C. G., Smithies, O., Nature, 183, 1589 (1959).

46. Cohen, B. L., Genet. Res., 1, 431 (1960).

47. Ashton, G. C., Nature, 182, 370 (1958).

48. Boyer, S. H., Young, W. J., Nature, 187, 1035 (1960).

49. Ogden, A. L., Morton, J. R., Gilmour, D. G., McDermid, E. M., Nature, 195, 1026 (1962).

50. Schultze, I., Heide, G. K., Schonenberger, M., Schwick, G., Z. Naturforsch., 106, 463 (1955).

51. Nagler, A. L., Kochwa, S., Wasserman, L. R., Proc. Soc. Exptl. Biol. Med., 111, 746 (1962).

52. Berkorovainy, A., Rafelson, M. E., Jr., Likhite, V., Arch. Biochem. Biophys., 103, 371 (1963).

53. Parker, W. C., Bearn, A. G., J. Exptl. Med., 115, 83 (1962).

54. Schultze, H. E., Schmidtberger, R., Haupt, H., Biochem. Z., 329, 490 (1958).

55. Heimburger, N., Heide, K., Haupt, H., Schultze, H. E., Clin. Chim. Acta, 10, 293 (1964).

56. Jamieson, G. A., J. Biol. Chem., 240, 2914 (1965).

57. Spik, G., Monsigny, M., Montreuil, J., C. R. Acad. Sci., 260, 4282 (1965).

58. Spik, G., Monsigny, M., Montreuil, J., C. R. Acad. Sci., 261, 1137 (1965).

59. Yang, H. Y., Dissertation. The University of Oklahoma (1962).

60. Schwick, G., Störiko, K., Lab Synopsis, Modified Laboratoriumsblatter, Behringwerke, Ed. May, 1964.

61. Poulik, M. D., Nature, 180, 1477 (1957).

62. Shetlar, M. R., Progress in Clinical Pathology, (Stefanini, Ed.), Grune and Stratton, New York (1966), p. 419.

63. Shetlar, M. R., Foster, J. V., Everett, M. R., Proc. Soc. Exptl. Biol. Med., 67, 125 (1948).

64. Shetlar, M. R., Analyt. Chem., 24, 1844 (1952).

65. Boas, N. F., Arch. Biochem. Biophys., 57, 367 (1955).

66. Warren, L., J. Biol. Chem., 234, 1971 (1959).

67. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 194, 265 (1951).

68. Gordon, H. T., Thornburg, W., Werum, L. N., Anal. Chem., 28, 849 (1956).

69. Moore, S., Stein, W. H., Methods in Enzymology, Vol. 5, (Collowick and Kaplan, Eds.), Academic Press, New York (1963), p. 819.

70. Anderson, B., Seno, N., Sampson, P., Riley, J. G., Hoffman, P., Meyer, K., J. Biol. Chem., 239, 2716 (1964).