QUANTITATION OF RELATIVE MOBILITIES OF SERUM TRANSFERRIN IN QUARTER HORSES AND THOROUGHBREDS USING CROSSED IMMUNOELECTROPHORESIS

BY

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INTRODUCTION

CHAPTER I

Transferrin, which is also referred to as siderophillin, is an iron binding, beta-1 globulin; a glycoprotein which is synthesized and stored in the liver and released into the blood. It is responsible for transporting plasma iron to the bone marrow and to tissue storage areas. When iron is adsorbed from the small intestine, it immediately combines with transferrin. The loose binding between iron and transferrin facilitates the release of iron to the tissue cells at any point in the body. Ferric iron is bound by two binding sites on the transferrin molecule.

Although the primary function of transferrin is to transport iron to the blood plasma and to the developing red blood cells in the bone marrow, it is also thought to act as an iron buffer by regulating the influx of iron through the intestinal mucosa by the saturation of transferrin in the blood (1). Transferrin is reported to have a bacteriostatic function by lowering the availability of iron for microbial growth (2).

The literature indicates that individual transferrin variants are present in man as well as in various species of animals and that these variants are distinguished from one another by differing rates of migration of the transferrin molecules in an electrophoretic field.

The presence of individual types of transferrin has been explained by various theories in the literature, most of which, however, utilize the concept that autosomal, allelic genes of equal dominance determine the type of transferrin which is present. Although amino acid sequencing has been carefully carried out and reported for various species' transferrin molecules, it does not appear that the structural components of the individual variants have been chemically studied.

The nomenclature system used to describe the individual transferrin variants in humans and in animals, employs the alphabet and numerical subscripts. Transferrin variants are named according to order of electrophoretic mobility, where the fastest moving transferrin type would receive the first letters of the alphabet (such as transferrin type A) and the slower moving transferrin variants would be designated by the latter letters in the alphabet (such as transferrin type F). If there are variants of type A or F, these are then designated as transferrin type A-1, A-2, etc., where A-1 would be the fastest component of the transferrin type A category. Transferrin is noted in genetic description as Tf.

The molecular weight, carbohydrate content and isoelectric point of serum transferrin have been studied extensively since 1945 when Holmberg and Laurell were the first to recognize transferrin as a blood serum glycoprotein which was capable of binding iron (3). In 1946, Shade and Caroline noted that the addition of iron to this colorless glycoprotein resulted in a pink color with an absorption peak of 470 nm (4). Transferrin has a reported molecular weight range of anywhere from 76,000 to 90,000 daltons, which appears to be species dependent. For example, Laurell and Ingleman (5) reported that pig transferrin has a molecular

weight of 88,000 daltons, whereas Laurell in 1947 (6) reported that human transferrin has a molecular weight of 90,000 daltons. Rabbit transferrin has a reported molecular weight of 78,000 daltons (7). Winzler in 1950 and Schultz in 1957 (8, 9) demonstrated that the total carbohydrate content of human transferrin was approximately 5% and that most of the carbohydrate components were hexose, hexosamine, and sialic acid. In 1969, Keller et al. (10) reported that human transferrin has an isoelectric point of 5.6 when fully saturated with iron and 5.80 when iron-free.

Various methods for the isolation and purification of transferrin have been described in the literature. Laurell and Ingleman (5) first isolated pig transferrin in 1957 by using ammonium sulphate precipitation and fractionation with cold ethanol. Inman (11) in 1956, first crystallized human transferrin after ethanol fractionation. The most common method appears to be treatment of whole serum with Rivanol (2-ethoxy-6, 9 diaminicridine lactate) at a pH of 8.0 to 10.0 with subsequent removal of gammaglobulin by alcohol precipitation or diethylaminoethyl (DEAE) cellulose column fractionation. Preparation of small quantities of transferrin has been accomplished by isolating the beta globulin in starch grain gel electrophoresis, first employed by Smithies (12, 13). Following methods using starch gel electrophoresis, transferrin has been recovered and further isolated by column chromatography on DEAE cellulose by Parker and Bearn in 1961 (14).

The stability of the iron-transferrin complex in different pH environments, its properties of solubility and resistance to heat denaturation, and the iron binding capacity of the transferrin molecule alone, have been studied as early as 1955. Ehrenberg and Laurell (15)

demonstrated that transferrin can bind 2 atoms of ferric iron per molecule. Subsequent studies demonstrated that the iron-transferrin complex is stable in alkaline solutions but disassociates at neutral or acidic pH and particularly in the presence of iron chelates such as citrate and phosphate (16). Inman et al. (11) showed that the solubility and resistance to heat denaturation of the iron-transferrin complex are dependent upon the degree of iron saturation. During normal physiologic conditions, the iron binding capacity of transferrin in the serum is only utilized to one third of its capacity as noted by Wintrobe in 1961 (17).

Under normal conditions, human serum transferrin concentration levels are 200-300 mg per 100 ml as reported by Jager and Gubler in 1952 (18). The concentration of transferrin in horse serum has been reported to be approximately 4 mg per ml by Makimura et al. (19).

Transferrin can be identified in starch gel electrophoresis, in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and by immunoelectrophoresis in agarose gel, by the addition of radiolabeled iron to serum or plasma prior to electrophoresis; or by staining the gels after electrophoresis with Nitroso R compound (1-Nitroso-2 Napthol-3, 6-disulfonic acid disodium salt) as demonstrated by Smithies in his early investigations of transferrin in starch gel electrophoresis (20).

In 1961, Parker and Bearn (14) investigated the sialic acid components of human transferrin and postulated that different electrophoretic mobilities of transferrin molecules might be due to differing numbers of sialic acid residues per molecule of transferrin. They showed that treatment of purified transferrin type C (the most prevalent type observed in humans) with neuraminidase, split the single protein band

into four additional slower bands on starch gel electrophoresis. The untreated transferrin type C, the single band, was the fastest type and contained 4 negatively charged sialic acid residues per molecule of transferrin, whereas the slowest one contained none, indicating a step-wise removal of these residues with neuraminidase treatment, and a subsequent change in the rates of migration in starch gel medium. In more recent literature, this is still a popular theory used to explain differing electrophoretic mobilities of transferrin molecules.

What is the purpose in studying transferrin in horses? As Lindstrom indicates in a concise review (21), there has been an increase in the past two decades in the study of measurable biochemical compounds which would facilitate prediction of animal performance. For example, Kiddy in 1979 (22) suggested that cattle having what he refers to as transferrin type E were more resistant to climatic and/or nutritional stress and have better breeding performance.

Kingsbury (2) suggests that recording and identifying genetic variants in blood serum transferrin in horses would constitute a simple and reliable method for determining parentage and lineage in thoroughbred horses.

Osteroff et al. (23) investigated the relationship of transferrin types in thoroughbred race horses in South Africa, to their ability to win races. Out of 206 horses investigated, it was found that those animals which had transferrin type FF (homozygous) were consistent winners at the race track.

From the literature reviewed, it does not appear that the relative electrophoretic mobilities of transferrin types have been quantified or statistically analyzed thus far. Only the difference in

the position of the transferrin types has been noted by visual inspection of stained starch gels, polyacrylamide gels or agarose gels, following different types of electrophoresis.

Although there are many possible future applications for studying transferrin, it is the purpose of this investigation to attempt to quantify, classify and compare the electrophoretic mobilities of transferrin in quarter horses and thoroughbred horses of Oklahoma, using crossed immunoelectrophoresis in agarose gel. Such a study involving quarter horses has not yet been done in this geographical area.

CHAPTER II

REVIEW OF THE LITERATURE

Identification of the Protein Components in Serum by Electrophoretic Techniques

Studies of Human Transferrin Variants

Before transferrin was classified into variant types, it was referred to simply as beta globulin C. This nomenclature was suggested by Holmberg and Laurell in 1945 when they investigated the presence of an iron binding glycoprotein in the beta globulin fraction of human sera (3).

Just as haptoglobins were found to be located primarily in the alpha-2 fraction of human sera, and were later identified and separated into three variant types by Smithies and Walker (24), transferrins all appear in the beta-1 globulin component of human sera and in other species sera.

In 1955, Smithies investigated group variations in serum proteins of healthy, adult humans by means of zone electrophoresis using starch gel as a supporting medium. It was determined that there was greater separation and resolution of the serum proteins in starch gel electrophoretic methods than in the classical filter paper electrophoretic techniques (12). Smithies found that components of the beta globulin fractions and albumin fraction could be further separated

using starch gel electrophoresis. Proteins of similar electrophoretic mobilities migrated at the same rate in free solution in filter paper electrophoresis, whereas they were separated in the starch gel medium according to molecular size as well as charge. The larger complexes such as albumins, migrated at different rates and hence, the terminology arose for prealbumins and postalbumins. These two components migrated at different rates according to their molecular size through the pore medium or starch polysaccharides but they all migrated together in the filter paper electrophoresis method. Smithies also noted that the beta globulin fraction of human sera which appeared as one major component using filter paper electrophoresis, was separated into three bands using starch gel electrophoresis.

In 1958, Poulik and Smithies (25) further compared starch gel electrophoresis to filter paper electrophoresis methods and discovered that the hemoglobin-haptoglobin complexes migrated faster in the filter paper method than in the starch gel medium, indicating that the starch gel allowed for dispersion of the molecules according to size and not charge alone. It was at this time the three bands found in the beta globulin fraction were designated as transferrin types A, B, and C, named according to decreasing order of electrophoretic mobility.

Since the introduction of starch gel electrophoresis by Smithies in 1955 (12), seventeen different variants of human transferrin have been identified, the most common one designated as CC in genetic notation, indicating a homozygous individual for that particular type of transferrin (26). The other variants are now referred to in the literature as B and D. Transferrin types B, C, and D are thought to be controlled

by three allelic, codominant genes, and any variations of these types result in individuals heterozygous for transferrin phenotypes (27).

In 1959, Giblett, Hickman and Smithies, (28) demonstrated through means of vertical starch gel electrophoresis and autoradiography of iron-59 bound proteins, that the variable electrophoretic bands found in the beta 1 fraction of human sera, were, infact, iron-binding proteins, and they concluded that they were transferrin variants.

These investigators stated that only one major type of transferrin was present in a majority of human subjects of different races, and that they thought most all these individuals were homozygous for transferrin type C. Their experiments indicated that other variants of transferrin do exist. Bands of beta 1 globulin proteins migrated at different rates through the starch gel medium and also bound to the iron-59. Their results indicated the presence of what they referred to as transferrin types CD-1, CD-3, CD-0, BC-2, and BC-0. The letters and numerical subscripts D-1, D-2, and D-3, indicated the order of electrophoretic mobilities, from the fastest to the slowest beta globulins (D-1 being the most rapid protein) and all in this group migrated slower than transferrin types B and C. Type B-0 was the fastest migrating protein of all the categories and D-3, the slowest. They concluded that these individuals were heterozygous for transferrin genes and they summarized their findings by stating that eight different transferrin variants were now identifiable in human sera: B-0, B-1, B-2, C, D-0, D-2 and D-3.

In 1967, Jeppsson partially characterized and isolated three human transferrin variants designated as B-1C, B-2C and D-1C. The notation indicated the following: These types of transferrin represented

heterozygous individuals, each containing the C allele, but also the allele for B and D types of transferrin. Type B-1C indicated that this particular variant migrated the fastest in the C zone in starch gel medium, whereas B-2C was the next fastest in the C category. Type D-1C migrated the fastest in the D zone (29).

Also, transferrin types were isolated by chromatography on DEAE Sephadex and Sephadex G-200. The individual genetic variants were separated by means of starch gel electrophoresis and by isoelectric focusing techniques. These isolated variants were tested for purity by immunoelectrophoresis, polyacryalmide gel electrophoresis and absorption spectra.

The peptide characteristics of the variants were further analyzed through fingerprinting tryptic peptide chains. The results indicated that there were single amino acid substitutions in each variant and it was suggested that these substitutions were responsible for the differences in electrophoretic mobility of each variant.

Jeppsson also indicated that hemopexin, a protein synthesized and stored in the liver which is responsible for the transportation of heme, (30) has a molecular weight range very close to that of human transferrin and that recycling chromatography was necessary to fully separate the transferrin from the hemopexin (29).

Since Smithies investigation of zone electrophoresis in starch gel medium, the literature indicates that polyacrylamide gel electrophoresis is a greatly improved method as compared to starch gel electrophoresis for separating serum proteins with greater accuracy and resolution.

Atland et al., (31) found six variants of transferrin, three of type C, two of type B and the presence of type D. Sera from a German

population of unrelated individuals were analyzed by means of double one dimensional electrophoresis. This method employed a consecutive combination of two polyacrylamide slab gel electrophoresis procedures, where one fraction of the initial electrophoresis of many samples was used as a sample for the second electrophoresis. The samples were saturated with iron and solubulized in either 4.5 or 8 molar urea prior to electrophoresis. The intensity of the transferrin bands was increased with iron saturation and the transferrin variants were identified by differences in relative mobilities through the polyacryalmide gel medium. The following types of transferrin were reported in this study: B-1, B-2, C-1, C-2, C-3 and D.

Davies' (32) investigations of human sera proteins revealed 20-30 different proteins using a 7% polyacryalmide gel medium. Davies noted that the fastest proteins were prealbumins, then albumin and then transferrin. There were 4 to 8 fractions between albumin and transferrin which were referred to as postalbumins. Between the origin and transferrin, the following proteins were noted: Beta-1 lipoproetin, S alpha-2 globulin and haptoglobin types 2-2.

Clarke (33) was able to identify 18-25 protein bands in human sera by means of disc polyacrylamide gel electrophoresis, using a 5% gel and solubulizing the samples in 2-5% urea. He found that transferrins were located in what he referred to as the beta 1 position and that ceruloplasm preceded the beta 1 position, and thus was referred to as a beta 2 protein.

Wright et al. (34) used gradient polyacryalmide gels, containing different concentrations of the monomer, as an even more effective means of separating serum components from human blood samples. Four different

gradients were used: 5%, 4.75%, 7% and 10%. A combined gradient polyacrylamide/agar gel immunodiffusion technique was used to identify stained proteins. They found there were anywhere from 29-60 proteins present in normal human sera and that the variation in the total number of components was dependeant upon genetic variation in the number of haptoglobin bands. As many as 16 haptoglobin types were detected from the 3% gel to the 7% region and these were found on either side of the albumin protein. One transferrin band was noted in the 10% region of the gel and was interpreted as being transferrin type C. On either side of this transferrin band were thin bands which were interpreted as hemopexin and ceruloplasm. However, it was stated in this work that it was not possible to thorougly separate the transferrin from the ceruloplasm or hemopexin by means of polyacrylamide gel electrophoresis.

Felgenhaur (35) used polyacrylamide disc gel electrophoresis, agarose immunoelectrophoresis, and densitometric recording of amido black stained proteins to detect as many as 26 different proteins found in dilute cerebrospinal fluid samples from normal human adults. Following electrophoresis, the proteins were subjected to immunoelectrophoresis utilizing specific antisera to known serum proteins in an attempt to identify the location of the separated proteins. The disc gels from the PAGE were subsequently stained with amido black. These discs were run through a densitometer and the scanning profile indicated one major peak behind two smaller ones which were adjacent to the albumin peak. This major peak contained transferrin, ceruloplasm, hemopexin, haptoglobin type 1-1 and inter-alpha trypsin inhibitor proteins. Thus, these components of the sera were not separated by the disc PAGE method. Other staining techniques were employed to identify the presence of ceruloplasm, but not hemopexin.

Hemopexin appears to be a difficult component to separate from transferrin by conventional purification procedures and electrophoresis (30).

Albumin and Haptoglobin Variants in Humans

Just as transferrin has been classified and identified into variant components, so have haptoglobins and albumins in man and many species of animals.

Harris (36) reports of Smithie's and Walker's findings (24). They studied haptoglobins using starch gel electrophoresis and found that there were 3 basic haptoglobin phenotypes present in human sera which they referred to as types 1-1, 2-1, and 2-2. Type 1-1 had a 1 band pattern in starch gel medium, and types 2-2 and 2-1 both and 5 band patterns, but each with different electrophoretic mobilities.

In 1965, Fagerhol and Braend (37) suggested using letters as symbols for the alleles which they classified in the prealbumin system of human sera. The letters chosen indicated the relative mobilities of the allele's products. The position in the alphabet corresponded to either fast or slow moving serum prealbumin proteins. The presence of these prealbumins was explained by a series of codominant alleles described as Prf, Pri, Prm, Prs, and Prv.

By 1967, however, Fagerhol and Laurell (38) instituted more experiments in this area because it was suspected that these prealbumin proteins were actually serum globulin alpha-1 anti-trypsin proteins, protease inhibitors. They showed by means of starch gel electrophoresis, agarose electrophoresis and antigen (alpha-1 anti-trypsin) antibody crossed electrophoresis that these prealbumin proteins were, infact, alpha-1 anti-trypsin.

They discovered that the series of what were thought to be prealbumin protein bands in the starch gel electrophoresis, were antigens to the antibody contained in agarose gel, for alpha-1 anti-trypsin. Their results indicated the prealbumin proteins (Pr) corresponded to alpha-1 anti-trypsin and they proposed that the Pr symbol be replaced by the symbol Pi for protease inhibitor, since this protein is the major protease inhibitor in human serum and since trypsin is the only protease which is inhibited. They concluded that each allelic pair of genes is responsible for the synthesis of one molecular protein pattern distributed in electrophoresis in the form of three major bands, each migrating at a different rate and representing both homozygous and heterozyogous patterns for alpha 1 anti-trypsin protein (38).

The previous discussion of variants of serum proteins is an attempt to illustrate the complexity of the terminology as well as some of the discrepancies that exist in the literature.

Concentration of Transferrin in Human and Animal Sera

Rolf et al. in 1983, (39) used isoelectric focusing techniques on thin polyacrylamide gels to selectively determine the concentration of transferrin in the dog, rabbit, hamster and the human. The purpose of this study was to demonstrate that their techniques could be used to monitor changes in transferrin concentration in the sera since it has been reported that during infection the concentration of plasma transferrin increases noticeably (40). These investigators used commercial, chromatographically pure transferrin for each species as standards to extrapolate their data. The transferrin bands were saturated with a 0.1 molar potassium hexacyanoferrate III solution in phosphate buffer,

pH 8.6, and following isoelectric focusing and staining of the gels, a quantitation of these transferrin bands was prepared using a concentration range of 0.08 - 6.72 mg/ml. The bands were evaluated by scanning at a wavelength of 552 nm. They reported that the hamster, rabbit, human and dog all had four iron binding protein bands, whereas the mouse had five such bands. In all the tested species, two main dark bands of iron binding protein were present accompanied by two lighter bands. Each transferrin was evaluated on a calibration curve as to its concentration in the plasma in mg per liter and the following concentrations of transferrin present in all the bands for each species were reported: man 2130, dog 4620, rabbit 6880, hamster 3,600 and mouse 2100. Presumably, all the tested species were in a healthy state at the time of this investigation.

Losonczy et al. (41) noted that the concentration of transferrin in the blood plasma varies depending upon species, sex, and various physiological conditions. Their work investigated the concentration of transferrin in bovine sera and they report that the normal concentration in this species is usually 3-5 percent.

In their work they quantified bovine serum transferrin by using rocket immunoelectrophoresis. The authors state the problem with developing a sensitive, reproducible method for quantifying serum transferrin lies in the fact that commercially available transferrin is immunologically only 85% pure and the preparation contains other proteins as well as albumin.

Iron Binding Studies of Transferrin

C. Van Der Heul et al. (42) investigated the binding of iron to transferrin as well as to other serum components. Their studies revealed that at greater than 30% saturation iron is distributed throughout other serum proteins and low molecular weight components, and not just exclusively bound to transferrin. They investigated the sera of normal humans, rats and rabbits and also found that the binding of iron to transferrin appears to be independant of pH change. They noted that nearly the same distribution of iron occurred regardless of the iron compound which was used, e.g. FeIII chloride or FeIII citrate. It was also discovered that 10-20% of iron is bound to other components other than transferrin.

H.G. van Eijk et al. (43) isolated 2 monoferric human transferrin components by methods of preparative isoelectric focusing, and reported that there are two distinct binding sites for iron on the human transferrin glycopeptide chain: The B site located on the N terminal domain and the A site located on the C terminal domain. This work suggested that these sites have different physiological roles in the transport and metabolism of iron and that each site may infact have a different iron binding capacity.

Characterization of Transferrin Glycopeptides and Determinations of Transferrin Molecular Weight Ranges

Palmour and Sutton in 1971, (44) studied the molecular weight and chemical composition of transferrin from human, rabbit, frog, turtle and hagfish sera. The transferrins were identified by radiolabeling with

iron-59 and autoradiographic techniques. All of the different species' transferrins were purified in the same manner; by an initial purification with Rivanol, followed by chromatography on DEAE Cellex column. The proteins were eluted with a linear concentration gradient from 0.01 to 0.45 molar Tris-Hcl buffer, with a pH of 7.8. All transferrins were saturated with iron in the form of ferric ammonium citrate.

Their results indicated that both human and turtle transferrin contained four sialic acid residues per molecule of transferrin, whereas the rabbit, frog and hagfish contained two. The hagfish transferrin was considered to be the most primative because it bound only one atom of iron. The following molecular weight determinations were reported: human, 81,000 daltons, rabbit 79,000 daltons, turtle 85,000 daltons, frog 81,000 daltons, and hagfish 41,500 daltons.

The visible absorbance spectra were analyzed for each of the species' transferrins. The human and rabbit transferrins showed one peak at 465 nm, where the frog, turtle and hagfish showed one major peak at 410 nm and also a secondary peak at 455-465 nm. The authors state that the human transferrin contained less than five percent hemopexin after purification, again illustrating the difficulty in isolation of a purified transferrin.

Graham et al. in 1974, (45) compared the structure of glycopeptides of human, pig, cattle, duck and hen transferrins and their studies revealed that each transferrin within these species was composed of two gylcopeptides, identical in carbohydrate composition, but different in the sequence of amino acids.

Human transferrin showed two bands on starch gel electrophoresis but these bands were not present following treatment with neuraminidase, and only one slower band appeared. It was concluded that the original human transferrin protein which contained three to four sialic acid components, had only two, upon treatment with neuraminidase. It was also noted that cattle, pig, duck and hen transferrins all contained two sialic acid moieties.

The authors identified the two isolated glycopeptides in each species as type A and type B. Type A contained the following amino acid sequence: Asn (CHO) followed by a basic amino acid and type B was identified as Asn (CHO) followed by a neutral aliphatic amino acid. The symbol CHO signifies a carbohydrate moeity. Cattle transferrin revealed an exception: The two glycopeptides contained Serine (CHO). It was suggested that each molecule of human, cattle and duck transferrin contained an average of two carbohydrate prosthetic groups, but the pig and hen transferrin molecules contained only one carbohydrate group per molecule of protein.

The carbohydrate composition of human, cattle, pig and hen transferrin was as follows: N-acetylglucosamine, mannose, galactose, and sialic acid, and in addition the pig transferrin contained fucose. However, there were different quantities of these sugars present in each species.

The location for the attachment sites of the carbohydrate moieties are not known except for the presence of one site at the C terminal end of the human transferrin polypeptide chain (45).

According to Graham and Williams, (45) there are marked variations in the composition of the carbohydrate groups attached to the different

transferrin molecules. They found that in the hen the protein moieties were identical to those of the duck transferrin, but the carbohydrate prosthetic groups were markedly different.

Strikland and Hudson (7) isolated and characterized the glycopeptides of rabbit transferrin in 1978 and their studies revealed that rabbit transferrin is composed of two heteropolysaccharide units of identical size and monosaccharide composition. There are two sialic acid residues, two galactose residues and three residues of mannose and four of N-actylglucosamine per molecule of transferrin.

They compared the structure of these heteropolysaccharide units to those found in human transferrin and noted some similarity between them as did Graham et al. in 1975 (45). However, the exact structure of these units of human transferrin is not yet known.

They investigated the iron binding capability of rabbit transferrin and found that it bound two atoms of ferric iron per molecule of transferrin.

The ratio of absorbance at 460 nm and 405 nm was studied and their results indicated that if there was no absorbance at either of these wavelengths, then heme-containing proteins were absent from the purified transferrin preparations (7).

Isolation of Transferrin and Problems with Contamination by Other Serum Proteins

Various methods have been employed in the attempt to purify transferrin from the sera of different species. All the methods which are described in the literature use some form of precipitation procedure, either with ammonium sulphate in varying percentages, or with

Rivanol. This precipitation is followed by either ion exchange chromatography with DEAE Sephadex and/or molecular exclusion chromatography. The elution buffers used on the ion exchange chromatography or the molecular exclusion columns vary in their ionic strength and composition according to the individual methods used for isolation of transferrin from different species.

The purity of transferrin has been tested in a variety of ways: immunoprecipitation procedures have been employed with monospecific antigen components, or radiolabeling with iron-59 in the form of ferric citrate, and various wavelength scans ranging from 400-460 nm have been used to test for the presence of heme-binding proteins in the purified preparations of transferrin.

Makimura et al. (19) investigated immunoglobulins and transferrins in equine serum. The horse serum was precipitated with 0.4 percent Rivanol and then the supernatant was concentrated and then dialyzed against 0.05 molar Tris-Hcl buffer at a pH of 8.0. It was then eluted by gradient elution chromatography on DEAE Sephadex A-50 with 0.05-0.5 molar Tris-Hcl buffer at a pH of 8.0. The fractions which corresponded to the third peak were pooled, concentrated and dialyzed and then subjected to gel filtration chromatography using a Sephadex G-200 molecular weight exclusion column. The first half of the second peak reportedly contained pure transferrin. The purity of the immunoglobulins and the transferrin was tested by using disc PAGE for molecular weight determination and by immunoelectrophoresis with prepared specific antisera in agarose gel.

Letendre and Holbein (46) reported that mouse serum was precipitated with saturated ammonium sulphate and the supernatant fractions were dialyzed against 3 liters of distilled water, followed by dialysis

against 0.01 molar sodium acetate buffer at pH of 5.9. The resulting dialyzate was applied to a carboxy methyl (CM) Sepharose column and transferrin was eluted with 0.09 molar sodium acetate buffer at a pH of 5.9. The presence of pure transferrin was determined through the use of the Cuchterlony double diffusion test in agar against rabbit antiserum to mouse transferrin. Following SDS PAGE, the molecular weight of mouse transferrin was determined to be 81,000 daltons. Subsequent elution from DEAE resulted in 2 peaks which bound radiolabeled iron. These two peaks were identified as transferrin.

Hatton et al. (47) described hemopexin, which is a beta-1 glycoprotein responsible for binding and transporting heme (30), as being a major contaminant in the purification process of transferrin.

Efremov et al. (48) also stated that the complete separation of these two proteins (transferrin and hemopexin) is very unsuccessful when using the common methods of precipitation and ion exchange chromatography.

In 1982, Majuri (30) isolated hemopexin from pig serum by using affinity chromatography and ion exchange chromatography. The initial step of purification involved precipitation with Rivanol. The unprecipitated Rivanol was removed by filtration of the supernatant through a Sephadex G-25 heme AH-Sepharose affinity column which did not absorb the heme-hemopexin complex but free hemopexin was completely bound to the gel. The bound hemopexin was then eluted with 0.1 molar citrate buffer. Hemopexin was identified by its affinity to heme and by spectral characteristics of the complex. Its main absorption band was at 413 nm. The identification of hemopexin was substantiated by immunoelectrophoresis. SDS PAGE was used to determine the progress of the purification as well well as to determine molecular weight which was reported to be 57,000

daltons. However, the author notes that a dimer of hemopexin which had a greater molecular weight than transferrin also was found after SDS SDS PAGE.

Stratil et al. (55) partially characterized and identified variant types of hemopexin in sheep, mouflon and goats. These authors mention that during the purification procedures, hemopexin components were eluted before transferrin but that some of the electrophoretically faster components of transferrin (which they refer to as Tf type A) were also eluted along with hemopexin.

In 1971, Efremov et al. (48) separated hemopexin from transferrin in bovine serum by treatment with neuraminidase followed by chromatography of the resulting products on DEAE Sephadex. Under these conditions it was reported that the contaminant, hemopexin, passed through the ion exchanger but that the transferrin without its Sialic acid components, was retained and was subsequently removed by gradient elution.

Hatton et al. (47) suggests yet another method of purification of transferrin from bovine serum which they report leads to a hemopexin-free preparation of transferrin. In this work, following ammonium sulfate precipitation, transferrin and hemopexin were both adsorbed by Sephadex CM-50 equilibrated with 0.1 molar sodium citrate buffer with a pH of 5.9, which allowed the albumins to pass through the column. The sodium citrate gradient eluted four peaks or fractions. The first peak was the smallest and was subsequently not investigated further. The second peak was immunochemically identified as transferrin and the third peak which had a maximum adsorbance of 414 nm was identified as hemopexin. The fourth peak didn't adsorb at 414 nm and was reported to be free hemopexin due to the characteristic absorbance from heme-hemopexin complexes being 414 nm.

Studies of Transferrin and Haptoglobin Variants in Pigs

In 1960, Kristjansson (50) used two dimensional starch gel electrophoresis and investigated nine serum protein fractions of pigs. He showed that the protein fractions were composed of two or more components which could be further separated when subjected to electrophoresis in a two buffer system. He classified transferrin types in pigs by observing the relative migration rates of the proteins in the first phase of electrophoresis and by subsequently subjecting them to Nitroso R salt reagent in the second phase to test for transferrins. He designated the phenotypes as Tf1/Tf1 and Tf1/Tf7.

In 1963, Kristjansson (51) investigated transferrins as well as haptoglobins and prealbumins in pigs. He changed the nomenclature which he used in the 1960 study of pig serum proteins for pig transferrin Tf-7 to TfA and Tf-1 to TfB. In the 1963 study, horizontal starch block electrophoresis was used as a means of studying serum proteins in pigs and it was reported in this work that pigs which were homozygotic for transferrin had 3 transferrin bands whereas the heterozygotes had 6 transferrin bands. All of these bands were reported to bind iron-59.

Kristjansson (52) studied haptoglobin fractions from Yorkshire and Landrace pigs using starch gel electrophoresis and found that these animals had six phenotypes with respect to haptoglobins type 1, 2 and 3. Three of the phenotypes contained only one of each haptoglobin type, and three contained two of the three basic haptoglobin types. He hypothesized that the genetic synthesis of these basic three types of haptoglobins was under the control of the alleles designated as Hp-1, Hp2-2, and Hp-3. A pig which had only one of these types was homozygous

for the allele controlling the synthesis of that haptoglobin, whereas a pig which had two of the haptoglobin types was heterozygous for the alleles controlling these haptoglobins. A homozygotic animal showed a 2 band pattern in starch gel electrophoresis but the heterozygotic animal had a 3 band pattern. As a means of identifying haptoglobins, the gels were stained with benzidine reagent which binds to the hemoglobin component and visualizes the presence of haptoglobins.

Stratil and Kubek (53) investigated the heterogenity of pig transferrin in homozygous transferrin type B animals in both adult pigs and 79-109 day old pig fetuses. Homozygous type transferrin B was isolated and fractionated by DEAE Sephadex chromatography. In this study it was found that after purification of the pig transferrin, starch gel electrophoresis revealed 12 iron-binding protein bands which were subsequently divided into 6 pairs by the authors. Iron-59 in citrate was used in autoradiography to demonstrate that all the 12 bands seen in the starch gel electrophoresis bound iron.

This literature (53) reported that there were not any differences in electrophorestic mobilities between the transferrin types of the adult pigs and the fetal pigs.

Where transferrins were treated with neuraminidaes 10 of the 12 bands changed electrophoretic mobility in both adult and fetal pigs but two did not.

Studies of Transferrin Variants in Cattle

Liberg and Carlstrom (54) studied transferrin polymorphism in swedish cattle using agarose gel electrophoresis. Some of the serum samples

were radiolabeled with iron-59. They compared the radiolabeled bands with those stained on the agarose gel electrophoresis and identified six transferrin phenotypes referred to as AA, DD, EE, AD, DE, and AE. Types A, D, and E represented three alleles and any three electrophoretically different beta globulin fractions. Types A, D, and E and 3 band patterns. Any variations from the three established band patterns found in the stained agarose gels, indicated that the animals were heterozygous for the type of transferrin.

Spooner et al. (56) also investigated transferrin patterns in adult cattle and fetal cattle. They noted that there were striking differences in the migration rates of the transferrin bands between the adult and fetal animals.

In 1971, Stratil and Spooner (49) studied the properties of the individual sialic residues in cattle transferrin. Their study revealed that homozygous type AA cattle transferrin showed a total of 12 peaks or 6 pairs of peaks, one main one accompanied by a smaller one, on DEAE Sephadex chromatography and that these 12 peaks corresponded to 12 bands seen on starch gel electrophoresis; one major band followed by a smaller thinner band. All the bands bound radiolabeled iron-59 in citrate. According to their investigations, cattle transferrin sialic acid residues vary in number from 0-5 and they hypothesized that this varying number of sialic acid residues may be what causes the differences in the electrophoretic mobilities of the 6 pairs of bands found in the starch gel electrophoresis.

However, according to Makarechian and Howell, (57) the bovine electrophoretic patterns of serum transferrin seen in starch gel electrophoresis in general, showed 10 main heavy bands and 2 weaker bands for each variant present.

Ashton (58) in 1961, studied serum transferrin of dairy cattle and stated that there were only two sialic acid residues per molecule of transferrin.

Hatton et al. (47) who studied the transferrin variants AA, D1D1, D2D2, and EE of homozygotic cattle reported that following gradient elution of transferrin from DEAE Cellulose chromatography, that the four peaks for each of these variants showed little or no difference in sialic acid residue number or in the content of hexose, or hexoasmine. They reported the molecular weight of bovine transferrin to be 76,000 daltons and that the carbohydrate composition was two residues of sialic acid, six of hexose and five of hexosamine per molecule of transferrin.

Studies of Serum Protein Variations in Horses

Ashton (59) used starch gel electrophoresis methods described by Smithies' studies of human sera proteins, (20) to study the components of sera from normal horses. His study indicated that there were 6 bands of beta globulins present, none of which were radiolabeled or identified as transferrins. There were also two prealbumins bands and one postalbumin fraction present. Preceding the post-albumin fraction, Ashton noted the presence of three haptoglobin bands. Preceding the beta globulin bands, there was a large fraction which he referred to as the slow alpha globulins. The gamma globulins appeared as the slowest migrating proteins of all the serum components.

In 1969 Bierer (60) investigated the serum from normal horses and reported the percent concentration of ten serum proteins using methods of cellulose polyacetate strips immersed in an electrophoretic chamber

containing sodium barbital buffer with a pH of 8.8. His findings presented as percent concentration in normal horse sera, provide some indication of the relative amount of different sera proteins: Albumin, 43.0; Alpha 1a,2.2; Alpha 1b, 3.12; Alpha 2a, 12.8; Beta-1, 11.9; Beta 2a, 3.54; Beta 2b, 4.69; Gamma 1, 12.1; Gamma 2, 2.18. Albumins appeared as the fastest migrating proteins and the gamma 2 globulins as the slowest.

Studies of Albumins and Prealbumins in Horses

In 1966, Gahne (61) detected 4 zones in starch gel electrophoresis which migrated ahead of albumin designated as prealbumin zones and belonging to one genetic system which he designated as Pr. This system was said to be controlled by four, autosomal codominant alleles designated as PrF, PrI, PrL, and PrS, F being the fastest in migration. Gahne also described the presence of two albumin homozygous phenotypes: SS and FF. These types showed 2 band patterns on starch gel electrophoresis; a thick band followed by a thinner one. Subsequent electrophoresis of these albumins in a buffer system with a pH of 8.8 rather than 5.4 of the initial system used, elicited better resolution and separation of these components into 4 band patterns. He designated the 4 band patterns to be types LL and II.

Braend (62) in 1969, used starch gel electrophoresis and noted the presence of four additional phenotypes of prealbumins which were designated as: PrN, PrT, PrU, and PrW. These types had slower migration rates than those described by Gahne, and hence, the nomenclature employed the latter letters of the alphabet. These types showed up with 3 band patterns in the starch gel medium.

Ek (63) characterized prealbumin proteins in horses of mixed breeds by using starch gel electrophoresis and antigen-antibody crossed immunoelectrophoresis in agarose gel. This study indicated that the previously noted patterns of prealbumins through starch gel electrophoresis, were actually more complex and Ek was able to discern a total of 8 immunoprecipitation peaks for each one of the three phenotypes, FF, II, and LL. Heterozygous patterns were identified as variations of the 8 peaks found in each of the homozygous patterns.

Initally starch gel electrophoresis was carried out and the prealbumin zones were stained with amido black. This gel was kept as a marker. Again starch gel electrophoresis was employed and a piece of the horizontal gel was cut out according to where the prealbumin zones were supposed to be as designated by the stained marker gel. This piece was suspended in phosphate buffer and used as an immunization in white rabbits. The antibody was tested for its purity against whole horse serum and only one immunoprecipitation peak resulted.

Antigen-antibody electrophoresis was carried out in agarose gel and 8 immunoprecipitation peaks were observed which corresponded to 8 bands found on the starch gel electrophoresis.

Brand and Efremov (64) described 3 albumin phenotypes present in Dole and Fjording horses which they explained by the presence of two alleles. Horizontal starch gel electrophoresis revealed albumin types FF, FS, and SS. The FF type showed 2 major bands with a faint one preceding these. The FS type had 4 bands with the same thickness and staining intensity with a faint trailing fifth band. The SS type was essentially the same as the FS type but the rates of migration all 5 of these bands were slower and thus designated as type SS. They hypothesize that the 3

albumin phenotypes are explained by two autosomal, codominant alleles referred to as A1F and A1S.

As is evident, the results of these investigators have little agreement as to total numbers of bands that are present following electrophoresis, or as to the genetic control of phenotype expression.

Studies of Hemoglobins and Haptoglobins in Horses

Braend and Efremov (64) studied hemoglobin and haptoglobin components in sera selected from Dole, Fjording and thoroughbred horses of Norway using methods of horizontal starch gel electrophoresis. They found that the Fjording and thoroughbred horses all had two hemoglobin types of two banded patterns, whereas the Dole horses had only a one band pattern of hemoglobin. This study indicated that there was no variation in haptoglobins in all three breeds of horses. All the haptoglobins showed up as one thick band followed by a thin trailing band in starch gel electrophoresis.

Braend and Stormont (65) employed horizontal starch gel electrophoresis and staining with benzidine reagent following electrophoresis in a study of 45 horse hemoglobin types. This work indicated that all the serum samples showed the same two band pattern on starch gel electrophoresis.

Studies of Transferrin Variants in Horses

In 1964, Braend and Stormont (65) studied transferrin variants in 45 horses using horizontal starch gel electrophoresis and radiolabeling with iron-59 in citrate. They proposed a nomenclature system using the letters of the alphabet to denote relative migration rates of different transferrin types in the starch gel medium during electrophoresis.

They were able to discern basic two band patterns of transferrin, each pair having a different electrophoretic mobility. These two band patterns were said to represent homozygous transferrin types and were designated as DD, FF, HH, MM, OO. RR. The fastest migration rate was found in type DD and the migration rate decreased to the slowest type RR. Any variations and combination of these two band patterns resulted in heterozygous patterns. They hypothesized that sixteen possible phenotypes of transferrin variants were present and were possible, arising form the six basic autosomal, codominant alleles; DD, FF, HH, MM, OO and RR. Five of the sixteen phenotypes had a two band pattern, one had a three band pattern and the remaining ten had four band patterns.

IN 1964, Braend (66) studied transferrin variants in Norwegian Dole and Fjording horses using starch gel electrophoresis. Standard gels with the observed phenotypes from the previous 1964 study (65) were used as markers to identify the phenotypes in the Norwegian horses serum patterns. Radiolabeling with iron-59 in citrate was not employed in this study. Braend was able to discern that the two breeds of horses differed in their transferrin types. The type D allele was found in the Fjording breed but not in the Dole breed. Transferrin allele type H was seen in the Dole horses only, but the type R allele was the most common in this breed. The type F allele was the most common in the Fjording breed.

Gahne (61) in 1966, studied transferrin types in Salernitana horses in Italy also using methods of starch gel electrophoresis. Radiolabeling was not employed in Gahne's work and the identification of the transferrin variants was carried out by comparison with those already observed by Braend (66) in 1964. Gahne observed all the same phenotypes in

this breed of horses as did Braend, except for one difference: He noted that the type F allele in the Selernitana horse breed separated out into two components in starch gel electrophoresis. One migrated slightly faster than the other in the F zone and thus, he proposed the nomenclature F1 and F2 to designate two different variants within the transferrin F allele category. These variants were noted in heterozygous patterns where F1 was present with type H transferrin and type D and F2 were found with type D and type R transferrin.

Makimura et al. (19) investigated immunoglobulins and transferrin concentrations in equine serum. These studies employed electrophoretic and immunoelectrophoretic techniques. Seven different protein bands were located by cellulose acetate electrophoresis and 20 protein fractions using polyacryalamide gel electrophoresis and a total of 20 antigenic components were located through immunoelectrophoresis developed with antiserum to normal adult horse whole serum.

Makimura and his co-workers found that the concentration in normal horse sera of transferrin was 4 mg/ml. They studied the concentration of transferrin in colostrum, serum and milk whey of normal horses as well, and found that transferrin was present in greater concentration in colostrum than in serum but much less was present in the milk whey of the mare than in the serum of the mare. It was also noted that the serum transferrin level of the newborn foal rose slightly for a short period of time following the initial ingestion of the colostrum, and then declined, remaining constant in the normal adult level range. The authors suggest that transferrin can be transmitted from the colostrum to the serum of suckling foals across the intestinal wall.

Ek (67) investigated transferrin types in sera of the warmblooded Norwegian trotter horse breed. The methods which were employed in this study were essentially the same as those which Ek used to investigate the prealbumins in horse sera. Ek used a sample of transferrin type RR as a standard by which to measure the position of the transferrin phenotype bands in the starch gel electrophoresis. Two starch gels were run and one was stained with amido black as a reference gel while the other was solubulized in phosphate buffer and used to immunize a rabbit to produce antisera. The antigen was tested for purity against monospecific antibody to transferrin and was radiolabeled with iron-59 in citrate. Only one iron-bound precipitation peak resulted. Ek discerned that there were three homozygous phenotypes of transferrin present in these horses, type DD, FF, and RR. He also observed three heterozygous types, DF, HR, and FR. The homozygous transferrin types had three band patterns in the starch gel electrophoretic studies and had three corresponding precipitation peaks in the agarose gels from the immunoelectrophoresis procedure. The heterozygous transferrin types had 6 bands corresponding precipitation peaks in the immunoelectrophoresis gel. The molecular weight of serum horse transferrin was estimated to be 70,000 daltons.

McGjuire et al. (68, 61) in 1979, confirmed Gahne's investigation of two types of transferrin F. Using alkaline polyacryalmide gel electrophoresis, this study indicated that the F variant could be separated into type types, F1 and F2, where F1 had the faster migration rate of the two. They studied the sera from both standardbred and thoroughbred horse breeds and discovered that the F1 and F2 variants were present in the thoroughbred horses but not in the standardbreds.

Stratil and Glasnak (69) observed an atypical type of transferrin in horse breeds of Czechoslovakia, which they called type C. They state that most of the transferrin variants which they observed contained two zones or bands as seen in starch gel electrophoresis; a strong anodal band followed by a weaker cathodal band. Transferrin type C in these breeds of horses exhibited two bands of equal intensity whereas types O and D did not. Each of the individual bands of the variants were isolated in a pure state and treated with neuraminidase and the sialic acid residues were studied in this manner. In all of the three variants, C, D, and O, the mobility of the slower of the two bands was decreased. The authors concluded that there were two sialic acid residues in the slow band of each variant but four in the faster band.

Scott (70) investigated a new type of transferrin D variant which was observed in the Shire breed of horses in England. Using starch gel electrophoresis in a buffer system with a pH of 8.6, Scott observed two bands located between the position of transferrin types D and F. He referred to these bands as transferrin type D2, since they occurred after D.

Rogoni et al. (71) in a short communication describes the presence of H2 transferrin variants in Breton horse breeds. Methods of investigation are not reported in this communication. However, the authors indicate that they noted a single band with a slightly slower electrophoretic mobility than the transferrin type H band and they referred to this band as type H2.

Arellano (72) studied sera from English, Spanish, Arab and Breton breeds of horses using methods of horizontal starch gel electrophoresis described by Kristsansson in 1963, (51) and Braend and Efremov in 1964 (65).

The author observed that the most common and frequent phenotype in all breeds of horse studied was type DF and the least frequent was type DR which was only present int he Spanish horse breed. Out of what is reported to be twenty one possible phenotypes, fifteen were observed: Three homozygotes, DD, FF, and RR and the rest were heterozygotes, DF, DH, DM, DO, DR, FH, FM, FO, FR, HM, HO, and HR. The author states that typing transferrin variants is a reliable method of determining horse lineage and parentage.

In these studies of serum components such as prealbumins, albumins, hemopexins, haptoglobins and transferrin, the accepted terminology appears to be largely based on Braend and Stormont's (65) pioneering work with transferrin. This work was based entirely on electrophoretic mobility of serum proteins in starch gel and appears also to have provided a reference to which all horses have been compared and classified.

CHAPTER III

MATERIALS AND METHODS

Collection and Preparation of Horse Serum for the Isolation of Transferrin

The horse blood sample for the initial isolation of transferrin, was obtained from a thoroughbred horse belonging to the College of Veterninary Medicine at Oklahoma State Unviersity in Stillwater, Oklahoma. The blood was collected from the jugular vein, allowed to clot at room temperature for approximately 4 hours, and was centrifuged at 10,000 x g for 20 minutes. The serum was then removed.

Procedure for the Isolation of Horse Transferrin

The procedure for the isolation of horse transferrin was modified from methods described by Makimura et al. (19). Approximately 150 milliliters of horse serum were precipitated with 0.4% Rivanol (2-ethoxy-6, 9 diaminiacridine-lactate) which was obtained from Sigma Chemical Company in St. Louis, Missouri. The supernatant was concentrated in an Amicon pressure filter (Amicon Company, Danvers, Maine) which contained a membrane filter with a molecular weight exclusion of 30,000 daltons. The concentrated supernatant was dialyzed against 0.05 molar Tris-HCL buffer (Sigma Chemical Company, St. Louis, Missouri)

at a pH of 8.0, and then fractionated by chromatography on DEAE-Sephadex (diethylaminoethyl-Sephadex from Pharmacia Fine Chemicals in Piscataway, New Jersey). A linear gradient of .05 molar to 0.5 molar Tris-HCL at a pH of 8.0 was used for elution. Three ml fractions were collected and the protein concentration was determined spectophotometrically at a wavelength of 280 nm. The third peak was pooled, concentrated as before in Amicon pressure filter, and then dialyzed against 0.5 molar NaCl and 0.1 molar Tris-HCL buffer at pH of 8.0. The fractions corresponding to the third peak were filtered by molecular exclusion chomatography with Sephadex G-200 obtained from Pharmacia Fine Chemicals in Piscataway, New Jersey. They were then eluted with 0.5 molar NaCl and 0.1 molar Tris-HCL buffer at a pH of 8.0.

Three milliliter fractions were collected and read spectophotometrically at a wavelength of 280 nm. The first half of the second peak reportedly contained pure transferrin.

Determination of Protein Concentration in the Antigen

The protein concentration of the isolated transferrin was determined by means of a BCA protein assay (bicinchinonic acid) obtained from Pierce Chemical Company, Rockford, Illinois. This method was modified from the Lowry protein assay (74).

Antisera Production

Antisera was produced by means of 4 subcutaneous injections of the transferrin isolate into sheep with equal volumes of Freunds complete

adjuvant, at weekly intervals. Freunds complete adjuvant was obtained from Difco Laboratories, Detroit, Michigan.

Blood was collected from the jugular vein of sheep and allowed to clot for approximately 4 hours at room temperature. The clotted blood was centrifuged at 27,000 x g for 30 minutes. The serum was removed following centrifugation and the volume was measured.

Precipitation of IgG Antibodies

IgG precipitation was carried out according to the recommendations of Herbert et al. (76). The final concentration of ammonium sulfate was 35%, starting with equal volumes of serum and 70% ammonium sulfate. An equal volume of 70% saturated ammonium sulfate was added to the serum, drop-wise, with continuous stirring. This was allowed to stand at room temperature for at least 4 hours. It was centrifuged as described previously. The pellet was collected and resuspended in distilled water to the original serum volume. Seventy percent saturated ammonium sulfate was again added as previously described and the resuspended pellet was collected and redissolved in distilled water to one tenth of the original serum volume. The redissolved pellet was placed in dialysis tubing and dialyzed against cold tap water for 24 hours. The dialyzed serum was examined for the presence of ammonium sulfate by the addition of saturated barium hydroxide. If no precipitate formed, dialysis was continued against one litre of barbital buffer which was prepared according to methods described by Axelson et al. (77) The dialysis was carried out for 24 hours in a cold room.

Determination of the Purity of the Antibody

The purity of the antibody was determined by means of the Ouchetrlony double diffusion technique in 1% agarose containing phosphate buffer with a pH of 7.5 (75).

Chromtographically pure horse albumin and IgG were reacted with the antibody and precipitation developed between the antibody and albumin, between the antibody and IgG and between the antibody and transferrin. The pure horse IgG and albumin were supplied by Cooper Biomedical Corporation of Malvern, Pennsylvania.

The antibody was then adsorbed with both albumin and IgG overnight at 4^{o} centigrade. Following incubation, the preadsorbed antibody preparation was centrifuged with an Eppendorf Microcentrifuge at full speed for 2 minutes. The supernatant containing the transferrin antibody was stored at 4^{o} centigrade until use. This supernatant was retested for purity by means of the Ouchterlony double diffusion technique in 1% agarose containing phosphate buffer with a pH of 7.5 (75). This test did not reveal any precipitation between the antibody and the horse albumin or between the antibody and the horse IgG, but reactivity between the antibody and transferrin was confirmed.

The antibody was then stored in 1 milliliter aliquots at -20^{o} centigrade.

Determination of Antibody Reacitivity

The antibody was tested for reactivity with the antigen at dilutions of 1:5, 1:10, and 1:20 by means of antigen-antibody crossed immunoelectrohpretic procedures in 1% agarose in barbital buffer according to methods modified from studies done by Ek (67) in 1981.

The antibody was found to be reactive at a dilution of 1:20 and was subsequently stored at -20° centigrade in 0.69 milliliter aliquots.

Antigen-Antibody Crossed Electrophoresis

Crossed electrophoresis encompassed two phases: Electrophoresis in 1% agarose of the antigen to be examined in order to separate the various components, and secondly, electrophoresis of the separated antigenic components into 1% agarose containing an antibody to the respective antigen; in this instance, transferrin.

This procedure was modified from methods described by Ek (67) in 1981. One percent agarose in barbital buffer with a pH of 8.6 was used as the electrophoretic media for both phases. In the second phase the agarose gel included a 1:20 dilution of antibody. Electrophoresis from the first agarose gel into the second agarose gel containing the antibody, was carried out at right angles. An antigen-antibody precipitation interaction occurred and a peak was visible upon staining the gels (Figure 1).

Collection and Preparation of the Horse Serum Samples

Jugular vein blood was collected from 15 healthy, registered quarter horses and 15 healthy, thoroughbred horses from the College of Veterinary Medicine and the Department of Animal Science at Oklahoma State University in Stillwater, Oklahoma. The samples were kept at room temperature for approximatley 4 hours to enhance clotting, and then centrifuged in a clinical centrifuge for 5 minutes. The serum was removed and stored in approximately 1 milliliter alliquots at -20° centigrade.

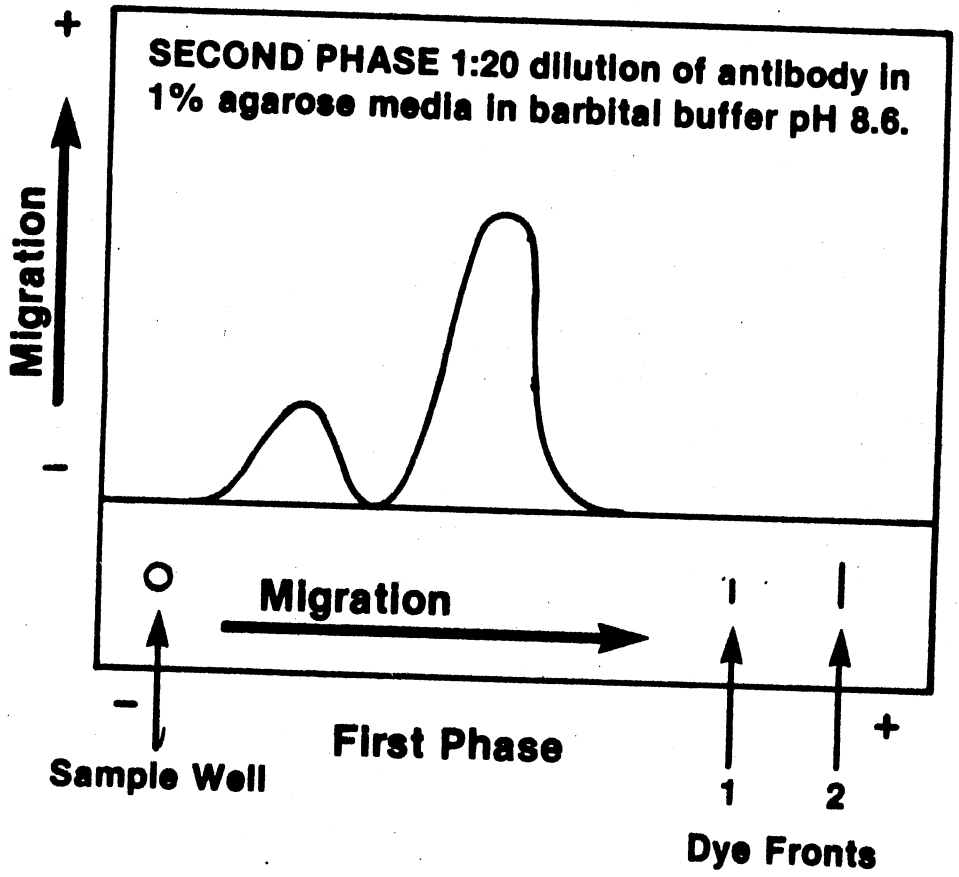

Figure 1. Two Phases of Antigen-Antibody Crossed Electrophoresis. Twenty microliters of horse serum sample containing the antigen, transferrin, were placed in the sample well, and electrophoresed at 70 volts for 4 hours in the first phase. Subsequently, the serum sample was electrophoresed at right angles to the second gel containing the antibody at 20 volts overnight. The immunoprecipitation peaks were visible upon staining the gels with Crocien Scarlet. The methods were were adapted from Ek (67).

Preparation the Barbital Buffer

Barbital buffer with a pH of 8.6 and an ionic strength of 0.1 M was prepared according to methods described by Axelson et al. (77).

Forty grams of barbital were added to 1 litre of distilled water and boiled with continuous stirring. Once the barbital dissolved, 206 grams of sodium barbital and 10 grams of sodium azide were added. Then distilled water was added to a total volume of 10/1. A pH range of 8.5 to 8.7 can be tolerated. This solution was stored at room temperature.

Electrophoresis Apparatus

The electrophoresis cell model number 1405 was supplied by Bio-Rad Laboratories, Richmond, California, and the power supply model number PS 1200 by Hoefer Scientific Instruments, San Francisco, California. A water bath and circulator, model number 2095 was supplied by Forma Scientific of Marietta, Ohio.

Temperature Regulation

The temperature of the cooling plate of the electrophoresis cell was maintained by the water bath and circulator at 8^{o} centigrade for all the electrophoretic procedures. This temperature was recommended as suitable for proper cooling during electrophoresis of this type by Axelson et al. (77).

Buffer Volume

Both the anode and cathode troughs of the electrophoresis cell were filled with 450 milliliters of barbital buffer. This amount of buffer was used for both phases of the electrophoretic procedure.

First Phase of Electrophoresis: Preparation and Pouring the First Agarose Gel

Both gelbond film and agarose media were supplied by FMC Corporation of Rockland, Maine.

It was calculated that 1 milliliter of barbital buffer was needed to adequately cover 5 cm^2 of gelbond film. Seventeen milliliters of barbital buffer were mixed with 0.17 grams of agarose with continuous stirring over heat until the solution was clear. This 1% agarose solution was set aside in a 55° centigrade water bath to maintain solvent condition prior to pouring.

The 1% agarose in barbital buffer was poured onto an 85 cm^2 surface of gelbond film which was kept warm on a glass plate covering a 55° centigrade water bath. The glass plate was cut of slightly larger dimensions so that the gelbond overlapped at the corners of the glass plate. The pouring of the agarose was done as quickly as possible on a warm, level surface.

After the agarose gel solidified, 2 x 8 cm strips were cut and sample wells were punched on each strip with a template, 2 mm in diameter, one cm from the base of the strips.

Electrophoresis of the Horse Serum (Antigen) in the First Phase Agarose Gel

Two serum samples were electrophoresed at the same time. The 2 x 8 cm agarose strips were placed on a thin layer of glycerol in order to maintain adequate contact betweent the gelbond and the cooling plate of the electrophoresis cell.

Electrophoresis wicks, supplied by Bio-Rad Laboratories of Richmond, California, were cut in the following manner: Four strips, 2 cm long and approximately 1 cm wide, with the plastic backing removed, and four 2 x 10 cm strips with the plastic backing intact. The 2 cm strips were immersed in barbital buffer and placed over each of the ends of the agarose gel strips. Then the 2 x 10 cm wicks were immersed in barbital buffer and placed over each of the ends of the agarose gels. The longest piece of each wick was dipping into the troughs of the electrophoretic cell.

Twenty microliters of horse serum sample were placed in each sample well, along with approximately 3 microliters of bromophenol blue tracking dye which was supplied by Sigma Chemical Company of St. Louis, Missouri. These samples were electrophoresed for approximatley 4 hours at 70 volts, or until the second dye front reached a point 6 cm from the sample well.

Preparation of the Second Phase Agarose Gel Containing Transferrin Antibody

The second phase gel also contained 1% agarose in barbital buffer, as well as the transferrin antibody.

Since the first agarose gel was cut in 2 x 8 cm strips or 16 cm^2, and was placed along the base of the second gelbond film (85 cm^2), this amount of area 16 cm^2) was subtracted from the original calculation of 85 cm^2. The remaining surface area was 69 cm^2 (Figure 2).

Therefore, 69 cm^2 required 13.8 milliliters of 1% agarose and 0.138 grams of agarose were mixed with 13.8 milliliters of barbital buffer to

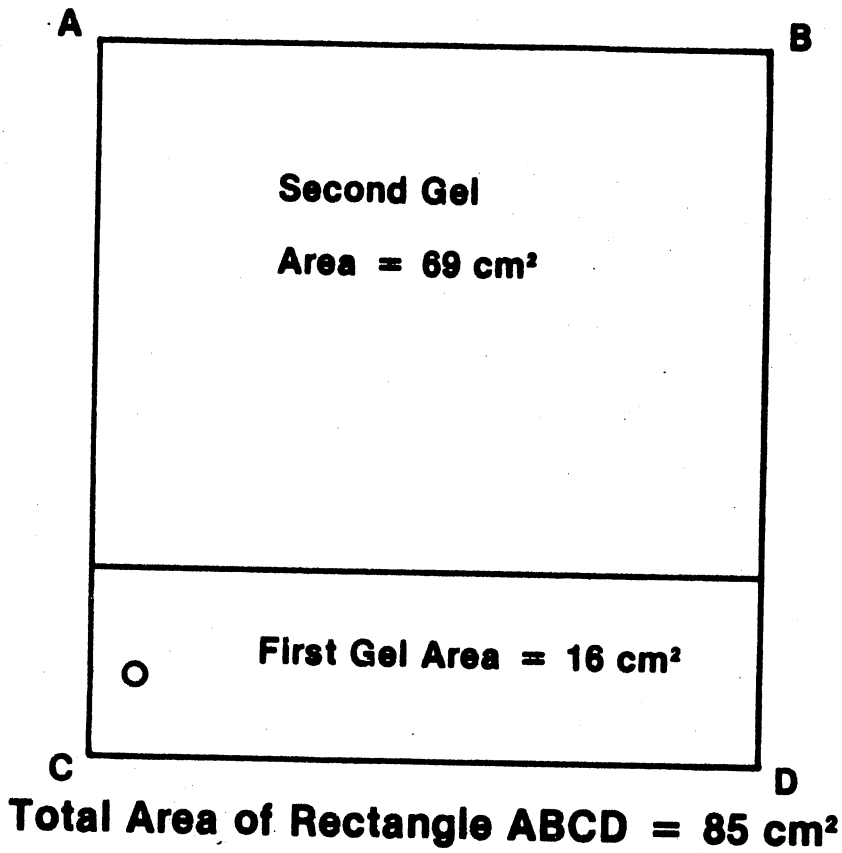

Figure 2. Calculation of Dimensions of Agarose Gels. Since the first agarose gel was cut in 2 x 8 cm strips or 16 cm^2 and was placed along the base of the second gelbond film which had a total area of 85 cm^2, the area of the first gel strip (16 cm^2) was subtracted from the total area of the second gelbond film (85 cm^2). The remaining surface area was 69 cm^2 and required 13.8 milliliters of agarose.

arrive at a 1% solution. The antibody was added in a 1:20 dilution in 0.69 milliliter aliquots. Thus, 10 milliliters of barbital buffer were mixed with 0.138 grams of agarose and heated with continuous stirring until the solution was clear. Barbital buffer (3.11 milliliters) was warmed in a 55° centigrade water bath along with 0.69 milliliters of antibody. This mixture was poured into the 1% agarose solution and stirred over a warm hot plate for approximatley 1 minute. The first phase agarose gel containing the electrophoresed antigen from the horse serum sample was placed along the base of the second phase gelbond film. The entire gelbond film and first phase agarose gel were placed over a warm glass plate of slightly larger dimensions, on a level surface, and the agarose containing the antibody was poured immediately onto this gelbond film.

Electrophoresis of the Second Phase Agarose Gel

Wicks were cut in the following manner: Four strips for two gels of 8 x 2 cm dimensions with the plastic backing removed; four wicks with the dimensions of 8 x 2 cm, with the plastic backing intact. These wicks were immersed in barbital buffer and were placed along the width of each gel in the same manner as the wicks were on the first phase agarose gels.

The second phase agarose gels were placed on a thin layer of glycerol in order to maintain adequate contact between the gelbond film and the cooling plate. They were electrophoresed overnight at 20 volts.

Washing and Pressing the Gels

This procedure was adapted from methods described by Axelson et al. (77). The gels were pressed for a minimum of 15 minutes for up

to several hours, using blotting paper supplied by Bio-Rad Laboratories Corporation of Richmond, California. They were pressed at 15 pounds per square inch. The gels were then placed in a 154 millimolar NaCl solution which contained 0.02% sodium azide to prevent bacterial growth, and soaked overnight in order to remove extraneous proteins from the gels. The gels were repressed as described previously, followed by a wash in distilled water for 15 minutes after which they were pressed again by the same method.

Staining the Gels

Staining the gels was carried out according to methods described by Crowle et al. (78). The staining solution was Crocien Scarlet and was prepared in the following manner: 250 milligrams of Crocien Scarlet, supplied by Sigma Chemical Company of St. Louis, Missouri, and 15 milligrams of Brilliant Blue R also supplied by Sigma Chemical Company, were placed in 100 milliliters of 5% acetic acid 3% trichloracetic acid at 60^{o} centigrade. The solution was allowed to cool at room temperatrue and can be reused for staining many gels.

The gels were stained for a minimum of 30 minutes and then rinsed in distilled water for 5 minutes. They were destained in a 0.3% acetic acid solution overnight, with approximatley 3 changes of this solution. After removing the gels from the destaining solution, they were airdryed for approximately 10 hours.

Quantification of Anigen-Antibody Crossed Electrophoresis: Relative Mobility

Relative mobilities of the transferrin peaks were calculated by measuring the first dye front on the first phase agarose gel in cm, and then measuring the distance from the sample well to the leading edge of the peaks on the second phase agarose gel, as described by Wieme (79).

$$mr = \frac{D_x}{D_t}$$

D_x is the distance from the sample well to the edge of the leading peak and D_t is the distance from the sample well to the leading edge of the first dye front. The distance from the sample well to the edge of the leading peak on the second gel is divided by the distance from the sample well to the leading edge of the first dye front (Figure 3).

Statistical Analysis of Transferrin Peaks

All of the relative mobilities were calculated according to methods described by Wieme (79) or Weber B. Osborne (73), and the coefficients of variability were calculated for each range of relative mobilities according to the statistical methods outlined by Steele and Torrie (80). By using these statistical methods and by accepting a 95% degree of comparability, a separation of the transferrin peaks into specific categories was accomplished.

Electing to use a 95% degree of comparability, a separation of the transferrin peaks into specific categories was accomplished.

Electing to use a 95% degree of commparability, each category of relative mobilities was compared involving two sample means and a student t-test, to show significant variation between populations or categories.

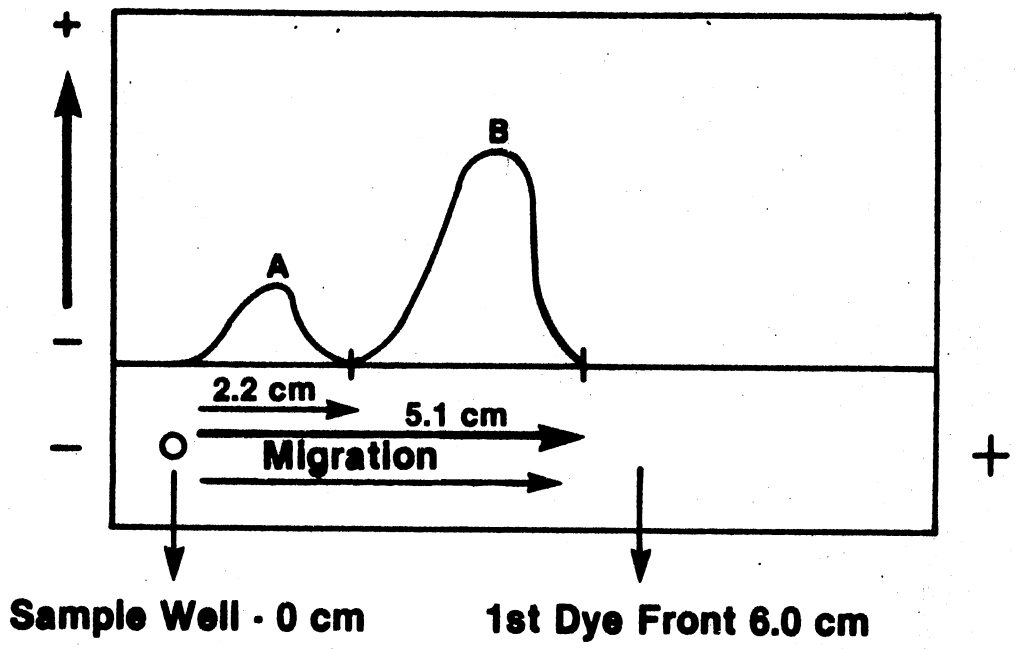

Figure 3. Calculation of Relative Mobility in Antigen-Antibody Crossed Electrophoresis. The relative mobility of both peaks A and B was calculated according to methods of Wieme (79). The relative mobility of peak A was calculated in the following manner: $\frac{2.2\ cm}{6.0\ cm} = 0.366$ cm. The relative mobility of peak B was calculated in the same fashion: $\frac{5.1\ cm}{6.0\ cm} = 0.850$ cm.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The Apparatus

Model SE 600 Vertical Slab Gel Eelctrophoresis unit was supplied by Hoefer Scientific Instruments of San Francisco, California. Model number PC 1200 DC Power supply was also obtained through Hoefer Scientific Instrumetns of San Francisco, California. The water bath and circulating cooling syustem model number 2095 was supplied by Forma Scientific of Marietta, Ohio. A full description for setting up and casting the gels in this apparatus is available from Hoefer Scientific Instruments, San Francisco, California.
Francisco, California.

Preparation of the Gels

This procedure is adapted from methods described by Laemmli (81). Gels which were 1.5 mm thick containing 3% stacking gel, and 3%, 7.5%, and 5% acrylamide were prepared from a stock solution of 30% acrylamide by weight of 0.8% of N, N' bis-methylene acrylamide, by weight. Both the acrylamide and the N, N' bis-methylene acrylamide were supplied by Sigma Chemical Company of St. Louis, Missouri.

The stock solution for the separating gel buffer contained 0.750 molar Tris-HCL, pH 8.8, and 0.2% sodium dodecyl sulfate (SDS). The final concentrations in the separating gel were: 0.375 molar Tris-HCL, pH 8.8 and 0.1% SDS.

The stock solution for the stacking gel buffer contained 0.250 molar Tris-HCL, pH 6.8 and 0.2% SDS. The final concentrations in the stacking gel were 0.125 molar Tris-HCl, pH 6.8 and 0.1% SDS.

The sample buffer contained the following final concentrations: 10% glycerol, 3% SDS, and 0.025 molar Tris-HCL.

The stacking and separating gels were both polymerized chemically be adding 0.025% by volume of teramethlethylenediamene (TEMED) and ammonium persulphate.

Tris-HCL buffer, SDS, TEMED, and ammonium persulphate were all supplied by Sigma Chemical Company of St. Louis, Missiouri.

After using both the 3% and 7.5% acrylamide separating gels, it was determined that the best concentration of acrylamide to use was 5%, as it elicited good separation of both the serum proteins and transferrin protein.

Concentration of Transferrin and Total Protein in the Serum Samples Used in SDS Page

It was determined that the best resolution was achieved by adding 5 microliters of serum sample to each stacking gel well at a 1:5 dilution of serum to sample buffer. Prior to loading the stacking gel wells with the dilute serum samples, each sample received approximatley 3 microliters of bromophenol blue tracking dye. The samples were placed in a boiling water bath for 2 minutes to solubulize the protein. The bromophenol blue tracking dye was supplied by Sigma Chemical Company of St. Louis, Missouri.

The concentration in the serum of transferrin was determined to be 4 micrograms per microliter (19) and at a 1:5 dilution, the concentration was 0.8 micrograms per microliter. Since 5 microliters were added to each well, the concentration of transferrin was 4 micrograms per microliter.

The concentration of total protein in the serum was 50 micrograms per microliter as determined by a BCA protein assay from Pierce Chemical Company of Rockford, Illinois. At a 1:5 dilution, it was 10 micrograms per microliter. Using 5 microliters in each well, the total protein concentration was 50 micrograms per microliter.

Electrophoresis of the SDS Slab Polyacrylamide Gel

The slab gels were electrophoresed at 20 milliamps per 1.5 mm thick gel for 4 hours, or until the bromophenol blue tracking dye reached the bottom of the gel. Once the gel was removed from the glass plates, a mark with a small needle containing India ink was made at this line of tracking dye as a front from which to calculate relative mobilities of the proteins.

Staining and Drying the Gels

The protocol and reagents required for staining the polyacrylamide slab gels by the Silver Stain Method were supplied by Bio-Rad Corporationof Richmond, California.

The gels were fixed in 50% methanol and 12% acetic acid for 20 minutes. They were rinsed in 10% ethanol and 5% acetic acid 3 times at 10 minute intervals, after which they were placed in the oxidizing reagent for 5 minutes, followed by rinsing with 400 milliliters of distilled water 3 times at 5 minute intervals. The gels were then placed in the silver reagent for 30 minutes after which they were soaked in 400 milliliters of distilled water for 2 minutes and subsequently placed in the developing reagent with 2 to 3 changes of this solution or until the protein bands became visible and darkened.

The gels were dryed overnight by a gel dryer supplied by Bio-Rad Corporation of Richmond, California.

Radiolabeling of Horse Serum Samples

Radiolabeling was carried out according to methods described by Tolling et al. (84). The horse serum samples were radiolabeled with iron-59 in citrate which was obtained from Amersham Corporation, Arlington Heights, Illinois. The specific activiity of the iron-59 was was 3-20 microcuries per milligram of iron and the iron-59 was contained in 0.1 molar hydrochloric acid.

The samples were radiolabeled in the following manner prior to crossed immunoelectrophoresis, SDS PAGE and prior to performing the Ouchterlony double diffusion test (75). Five microliters of iron-59 were added to 200 microliters of horse serum sample and to 200 microliters of purified horse transferrin and subsequently incubated at 37^{o} centigrade for 15 minutes.

Following crossed immunelectrophoresis, electrophoresis and the Ouchterlony double diffusion test (75), the gels were exposed to X-omat x-ray film which was obtained from Eastman Kodak Company of Rochester, New York and they were incubated in a light tight film box at 4^{o} centigrade for approximatley 36 hours. The SDS polyacrylamide gels were incubated in the same manner, but for 2 weeks.

Electrophoresis of the Radiolabeled Horse Serum Samples and Purified Transferrin

Antigen-antibody crossed electrophoresis was carried out as described previously and done according to modified methods of Ek (67). SDS polyacrylamide slab gel electrophoresis was carried out as previously described and according to methods described by Laemmli (81).

The Ouchterlony Double Diffusion Test of Radiolabeled Samples and Purified Horse Transferrin

This test was carried out as described previously, in 1% agarose containing phosphate buffer with a pH of 7.5 (75). Twenty microliters of radiolabeled horse serum sample and 20 microliters of radiolabeled purified horse serum transferrin were loaded in wells on either side of the well which contained the antibody.

Delipidization of Horse Serum Samples

Two methods were employed in an attempt to remove lipids from some of the horse serum samples.

1. Centrifugation--Following the initial centrifugation of the horse blood and removal of the serum, the serum was subsequently centrifuged at 35,000 x g for 30 minutes and lipid layer was pipetted off of the serum sample.
2. Extraction of Lipids--This procedure was adapted from methods described by Cham et al. (85). Lipid extractions were performed at room temperature in 11 x 2.5 cm glass tubes, fitted with polyethylene stoppers.

To one-half milliliter (0.5) of serum aliquots were added 0.05 milligrams of ethylenediamene tetra-acetate (EDTA) and 0.1 milliliter of organic butanol-di-isopropyl ether 40:60 (v/v). EDTA was supplied by Sigma Chemical Company, St. Louis, Missouri. Organic butanol-di-isopropyl ether was supplied by Aldrich Chemical Company of Milwaukee, Wisconsin.

The tubes were placed on a serum rocker and extracted for 30 minutes at 23° centigrade. Following 3 extractions, the mixture was centrifuged for 2 minutes at 2000 x g to separate the aqueous and organic phase.

The aqueous phade was transferred to a vacume flask and the residual organic phase removed by a water pump aspirator at room temperature for 5 minutes.

Visualization of Immunoprecipitins in Agarose Gels with Manganese Chloride and Sodium Phosphotunstate

Some of the antigen-antibody crossed electrophoresis gels were stained using a method described by Hueck et al. (86). The gels were removed from the electrophoresis cell at the end of a run and were placed in a solution of 154 millimolar NaCl containing 20 milligrams of sodium azide per litre, overnight. They were then placed in a solution of manganese chloride and sodium phosphotunstate for 1 hour at room temperature, after which they were washed in distilled water for several hours.

The manganese chloride and the sodium phosphotunstate solution was prepared in the following manner: Four grams of phospohotunstate acid were dissolved in 50 milliliters of distilled water, and the pH was

adjusted to 7.6 with 15 milliliters of 1 molar NAOH. The solution was made up to 100 milliliters with distilled water. Manganese chloride (40.6 grams) was dissolved in distilled water and made up to 100 milliliters. Ten milliliters of the phosphotunstate solution were mixed with 2 milliliters of the manganese chloride solution and made up to 100 milliliters with distilled water. This mixture was filtered to separate the precipitates from the clear yellow supernatant which was used to stain the gels.

Phosphotunstate acid was supplied by Sigma Chemical Company of St. Louis, Missouri, and manganese chloride was supplied by Aldrich Chemical Company of Milwakee, Wisconsin.

CHAPTER IV

RESULTS

Isolation of Transferrin

The precipitated concentrated serum sample which was eluted from DEAE Sephadex produced three peaks of activity as determined by spectophotometric evaluation at 280 nm (Figure 4).

The third peak, following concentration and dialysis with 0.5 molar Nacl-0.1 molar Tris-Hcl buffer at pH 8.0, eluted from Sephadex G-200 as two protein peaks of activity. The first peak was small followed by a much larger peak (Figure 5).

SDS Polyacrylamide Slab Gel Electrophoresis of the Standard and Purified Horse Transferrin

The silver stained polyacryalmide slab gel of the isolated transferrin and human transferrin standard revealed a single band in the human transferrin standard and at least at least 6 bands in the purified horse transferrin isolate (Figure 6). Thus it was suspected that the antigen was not entirely pure and radiolabeling procedures were subsequently carried out.

Radiolabeling of the Standard and the Purified Horse Transferrin in SDS Polyacrylamide Gel

Due to the concentration of the transferrin being so dilute (4 micrograms per microliter) and due to having to use this dilute a

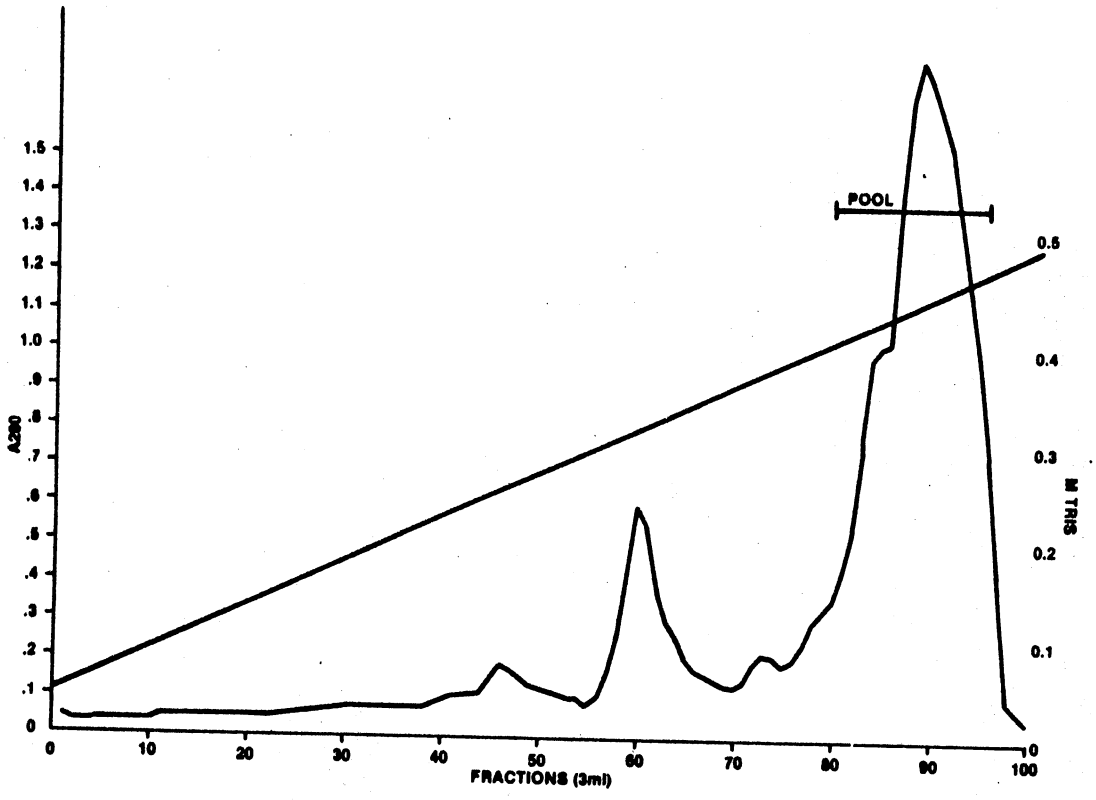

Figure 4. The concentrated supernatant from the Rivanol precipitation was dialyzed against 0.05 molar Tris-HCL buffer at a pH of 8.0 and then fractionated by gradient elution chromatography from DEAE Sephadex. The linear gradient was 0.05 molar to 0.5 molar Tris-HCL at a pH of 8.0. The precipitated, concentrated serum sample which eluted from DEAE Sephadex produced three peaks of activity as determined by spectophotometric evaluation at 280 nm. The fractions which corresponded to the third peak were subjected to molecular exclusion chromatography with Sephadex G-200 (see figure 5). The methods were adapted from Makimura et al. (19).

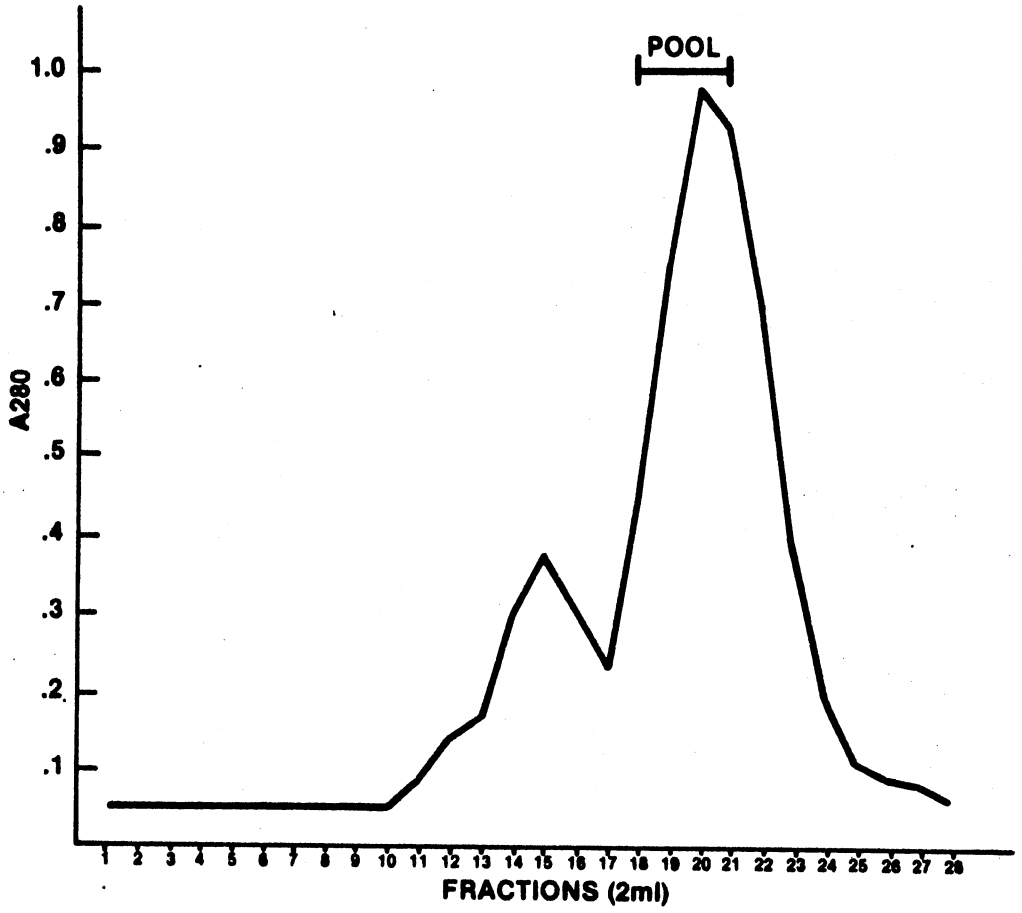

Figure 5. Molecular Weight Exclusion Chromatography on Sephadex G-200. The third peak obtained from the DEAE A-50 Sephadex anion exchange chromatography was pooled, concentrated and dialyzed with 0.5 molar NaCl and 0.1 molar Tris-HCL buffer at a pH of 8.0. The fractions corresponding to this third peak were eluted from Sephadex G-200 as two protein peaks. The first peak was small followed by a much larger peak and three milliliter fractions were collected and read spectophotometrically at a wavelength of 280 nm. The first half of the larger second peak reportedly contained pure transferrin. The methods were adapted from Makimura et al. (19).

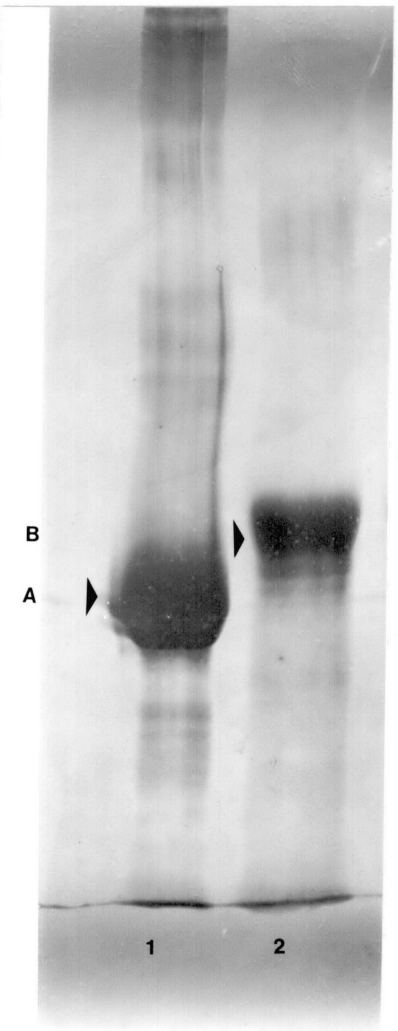

Figure 6. Silver Stained PAGE Containing Transferrin Isolate and Human Transferrin Standard. (1) The horse transferrin isolate as recovered from anion exchange chromatography and molecular weight exclusion chromatography. The transferrin isolate revealed at least 6 bands in addition to the heavy, major band designated as A. (2) The human transferrin standard revealed one large band designated as B. It was suspected that the antigen was not pure.

sample for best resolution in protein separation, it was not possible to discern whether any of the bands bound iron-59.

SDS Polyacrylamide Slab Gel Electrophoresis of Ten Quarter Horse Serum Samples

Upon staining the slab gel with silver stain, it was found that there were a minimum of 5 and a maximum of 6 distinct thin bands appearing in the region where horse transferrin was estimated to be (Figure 7). In order to determine if these bands were transferrin, radiolabeling was subsequently carried out.

Radiolabeling SDS Polyacrylamide Slab Gels Containing Ten Quarter Horse Serum Samples

It had been previously determined that the best resolution of serum proteins was achieved by using a 5% acrylamide separating gel and by using 5 microliters of serum sample in each stacking gel well at a 1:5 dilution of serum to sample buffer. Due to this particular level of serum concentration there was not enough available transferrin to be labeled by the iron-59 and after incubation with the x-ray film for 2 weeks at 4^{o} centigrade, it was not possible to discern whether any of the bands suspected to be transferrin, bound the iron-59.

Determining the Antibody Purity

The antibody was reacted in 1% agarose by means of the Ouchterlony double diffusion procedure with purified horse transferrin, and with commercially purchased, chromtographically pure horse albumin and IgG.

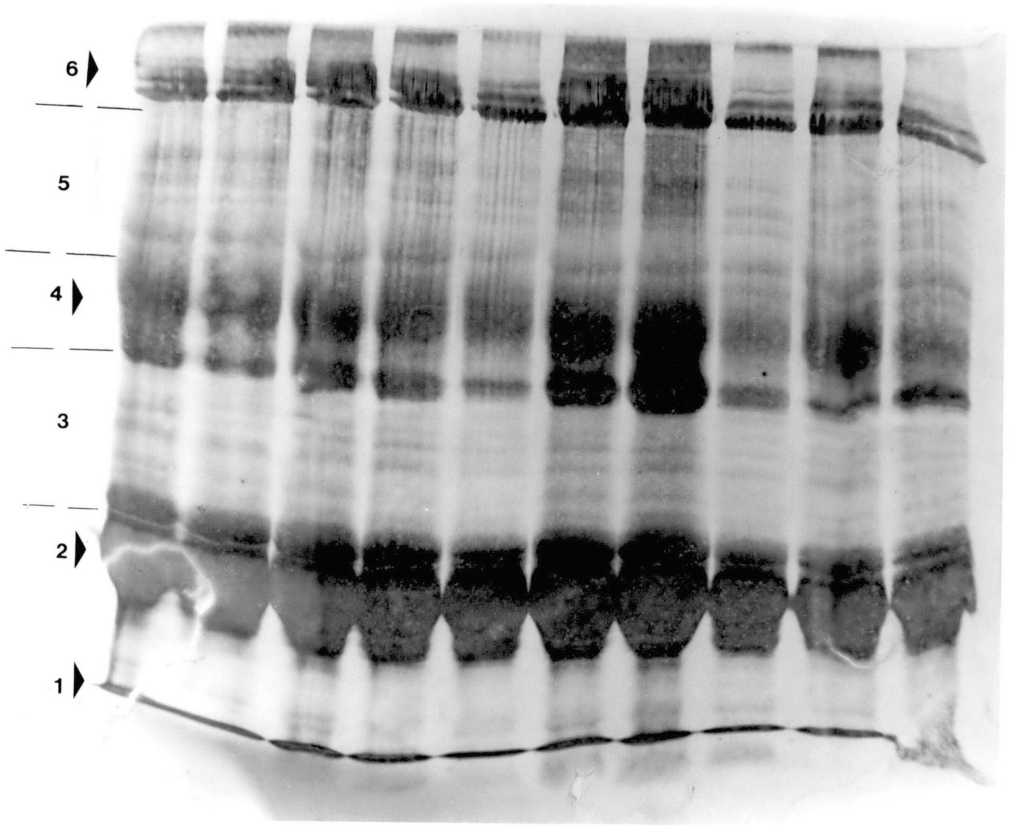

Figure 7. Silver Stained PAGE of Ten Quarter Horse Serum Samples. The band designated as 1 represents the dye front on the 5% polyacrylamide separating gel. Band number 2 represents albumin and the subsequent bands appearing behind albumin designated as 3, were thought to represent transferrin variants. However, the literature indicated that haptoglobins, ceruloplasm and hemopexin were sometimes present in this region of serum proteins. The band designated as 4 represents the beta lipo protein fraction, and the bands designated as 5 represent the S-alpha-2 globulins, while the heavy, slowest bands designated as 6, represent the large molecular weight gamma globulins.

It was discovered that the antibody not only reacted with the whole horse serum and the purified horse transferrin, but also with the albumin and the IgG.

Following these results, the antibody was adsorbed with both albumin and IgG and its purity was retested under the same conditions, using the same procedure. This revealed no reactivity or precipitation lines between the antibody and albumin, or between the antibody and the IgG, but confirmed the antibody's reactivity to horse serum and to the purified horse transferrin preparation.

Radiolabeling in the Ouchterlony Double Diffusion Gel

Whole horse serum, and the purified horse transferrin preparation were both labeled with iron-59 in citrate. The labeled horse serum and pure transferrin preparation were subjected to the Ouchterlony double diffusion procedure in 1% agarose followed by an incubation with x-ray film for 36 hours at 4^{o} centigrade. Both the precipitation lines between the antibody and the pure transferrin, and between the antibody and the whole horse serum transferrin, contained bound iron-59 (Figure 8).

Determining the Antibody's Level of Reactivity

Using varying dilutions of antibody preparation in barbital buffer, 1:5, 1:10, and 1:20, it was found that clear resolution of antigen-antibody immunoprecipitation peaks resulted using a 1:20 dilution. This was determined by means of antigen-antibody crossed immunoelectrophoretic procedures.

Figure 8. Radiolabeling in the Ouchterlony Double Diffusion Test. The horse serum sample was labeled with iron-59 and placed in well S and the transferrin isolate was also labeled with iron-59 and placed in well T. The antibody was contained in well A, and immunoprecipitation was allowed to occur over a 24 hour period. The gel was subsequently incubated with x-ray film for 36 hours at 4°. The dark lines indicated the presence of transferrin binding the iron-59 in both the isolate and the whole horse serum.

Antigen-Antibody Crossed Immunoelectrophoresis of Horse Serum Samples Using Non-Adsorbed Antibody Preparation

In the early stages of this work, the antibody was used in a non-adsorbed state. As a result, staining of the crossed electrophoresis agarose gels revealed multiple peaks which were all thought to be transferrin components in both quarter horses and thoroughbreds (Figure 9).

Not only were multiple peaks visible in these gels, but also some aberrations appeared which were suspected to be lipids (Figure 10). Subsequent procedures were initiated in an attempt to eliminate the lipids from the horse serum samples and thus eliminate the aberrations from the stained gels.

Delipidization of Horse Serum Samples

Initially the serum was recentrifuged at 30,000 x g for 30 minutes and then used in the antigen-antibody crossed electrophoresis. However, upon staining the gels, the same aberrations appeared as did before.

Lipid extraction procedures failed to remove the aberrations in the immunoprecipitation peaks.

Staining with Phosphotunstate and Manganese Chloride

This stain was used on some of the gels which had aberrations, in an attempt to stain the immunoprecipitation peaks with a minimal emphasis on the abberations. After using this stain, it was apparent that the immunoprecipitation peaks could be visualized, but not very clearly, and that the aberrations were also still visible.

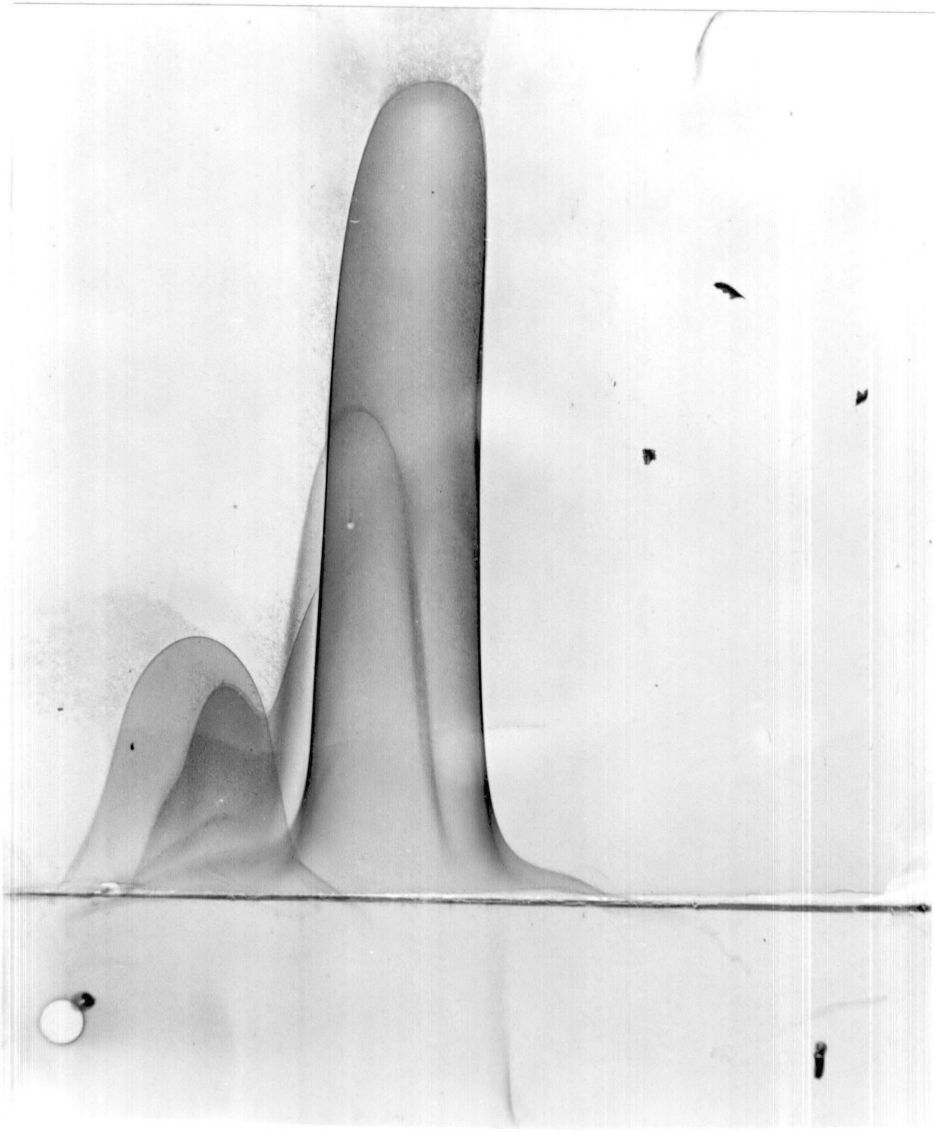

Figure 9. Multiple Immunoprecipitation Peaks in Horse Serum Samples. Following antigen-antibody crossed electrophoresis and staining of the 1% agarose gels with Crocein Scarlet, multiple immunoprecipitation peaks were noted in both quarter horse and thoroughbred serum samples. This particular gel was representative of a thoroughbred serum sample. These multiple peaks were thought to represent transferrin components.

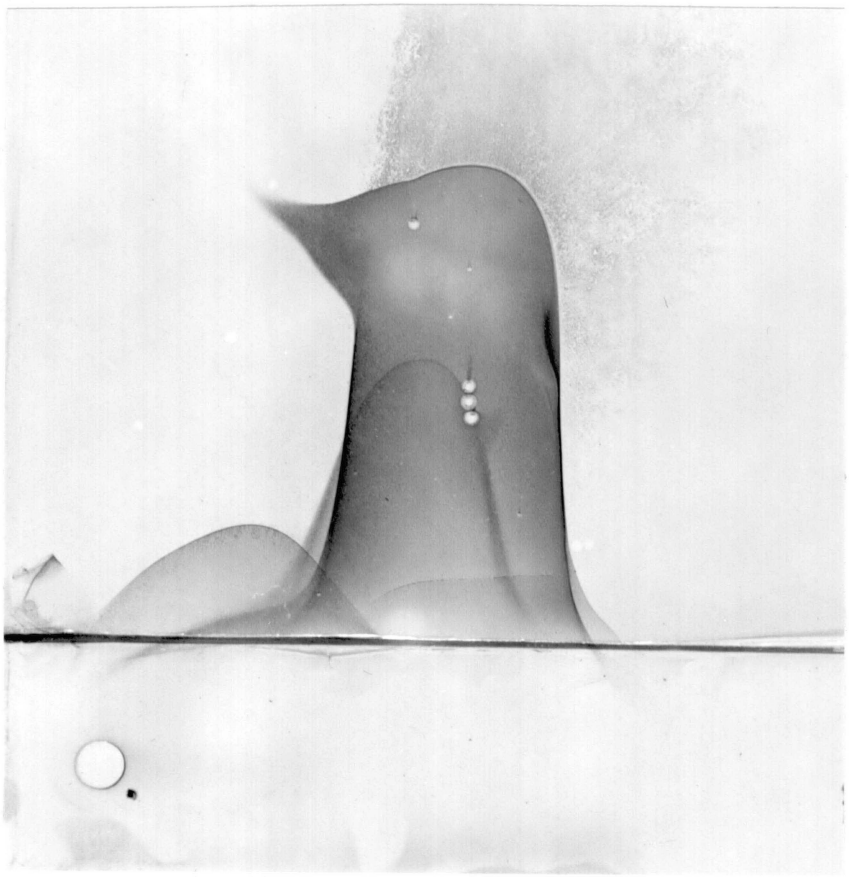

Figure 10. Aberrations in Antigen-Antibody Crossed Electrophoresis of Horse Serum Samples. Following antigen-antibody crossed electrophoresis of both quarter horse and thoroughbred serum samples, and staining of the gels were Crocein Scarlet, positively charged aberrations were noted. This particular gel represented a quarter horse serum sample and the positively charged aberration was found on the smallest of the peaks. These aberrations were suspected to be lipid contaminants in the sera.

Antigen-Antibody Crossed Electrophoresis of Horse Serum Samples Using Adsorbed Antibody Preparation

Antibody was adsorbed with both albumin and IgG and then used again in a 1:20 dilution in barbital buffer.

Upon staining the agarose gels, only 2 peaks were visible in both the quarter horse and thoroughbred samples (Figures 11-A and 11-B) rather than the multiple peaks seen before, using the non-adsorbed antibody preparation.

Antigen-Antibody Crossed Electrophoresis of Purified Horse Transferrin

The purified horse transferrin preparation was subjected to crossed electrophoresis using the adsorbed antibody in a 1:20 dilution with barbital buffer.

Upon staining the agarose gel, 2 peaks were visible (Figure 12).

Radiolabeling of Pure Horse Transferrin and Horse Serum Samples in Antigen-Antibody Crossed Electrophoresis

The horse serum samples and the pure transferrin preparation were both labeled with iron-59 in citrate and then subjected to crossed electrophoresis.

Once the gels were stained and then exposed to x-ray film for 36 hours, only a single peak contained iron-59. This peak was the smaller of the 2 peaks seen in the electrophoresis of the horse serum samples,

and the one with the slower relative mobility of the two. The smaller peak seen in the electrophoresis of the pure horse transferrin also contained the radiolabeled iron.

This peak was determined to be the transferrin components in the serum of both quarter horses and thoroughbreds (Figure 13).

Further Antigen-Antibody Electrophoresis of Horse Serum Samples

In an attempt to separate the single transferrin peak which bound the iron-59 in the radiolabeling procedure, into several components, the radiolabeled serum samples were electrophoresed for a longer period of time on an agarose gel strip measuring 12 centimeters in length, rather than the usual 8 centimeter length. The time of electrophoresis was increased from 4 hours to 9 hours for the first phase.

Following electrophoresis, the gels were stained with Crocein Scarlet and then exposed to x-ray film for 36 hours.

It was found that no further separation of the transferrin peak could be achieved by increasing the length of the agarose gel strip or the time span for electrophoresis. The smallest of the peaks, once again, bound the iron-59, but did not separate into other components (Figures 14-A and 14-B).

There are three peaks due to the fact that when this procedure was carried out, it was done using the non-adsorbed antibody preparation.

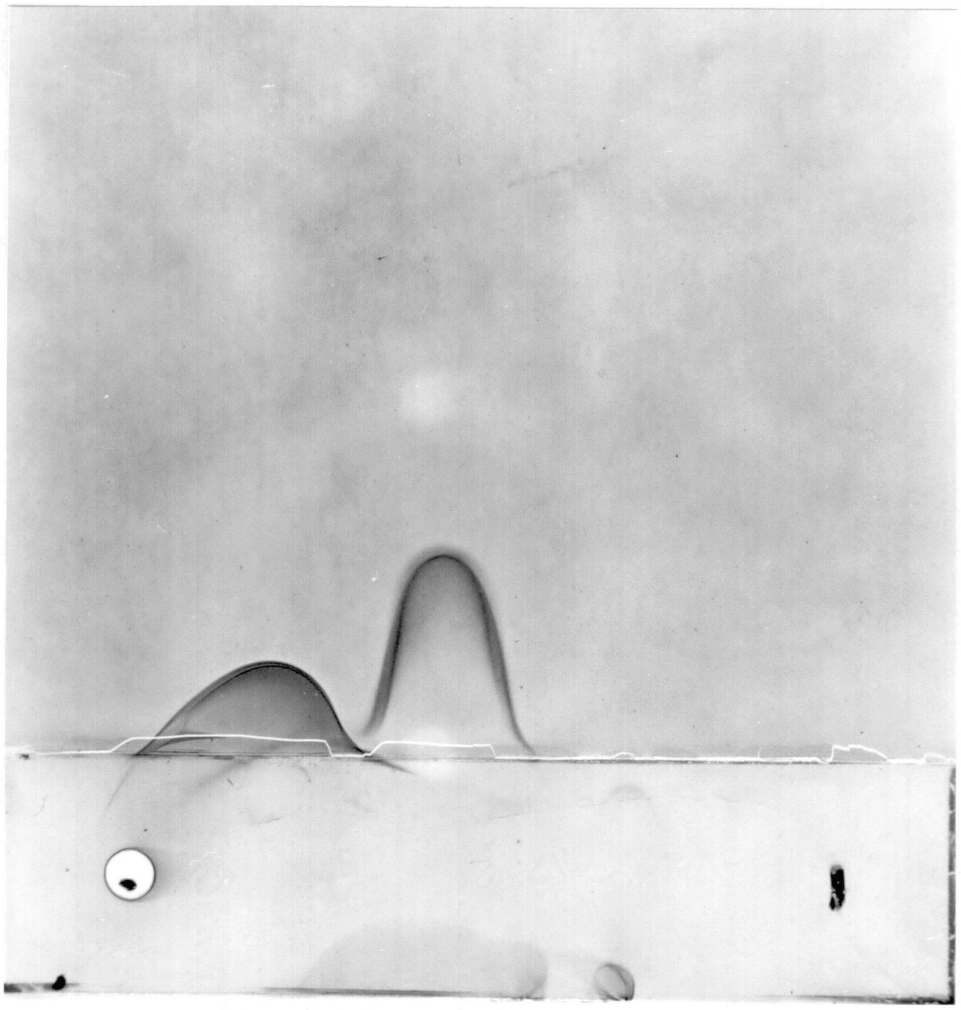

Figure 11. Antigen-Antibody Crossed Electrophoresis of Horse Serum Samples Using Adsorbed Antibody Preparation. The antibody was adsorbed with both horse IgG and albumin and then used in a 1:20 dilution in 1% agarose and barbital buffer. Horse serum samples were subjected to antigen-antibody crossed electrophoresis using the adsorbed antibody preparation. Following staining with Crocein Scarlet, only two immunoprecipitation peaks were visible in both quarter horse and thoroughbred samples, rather than the multiple peaks seen using the nonadsorbed antibody preparation (see figure 9). This particular gel represented a thoroughbred horse serum sample.

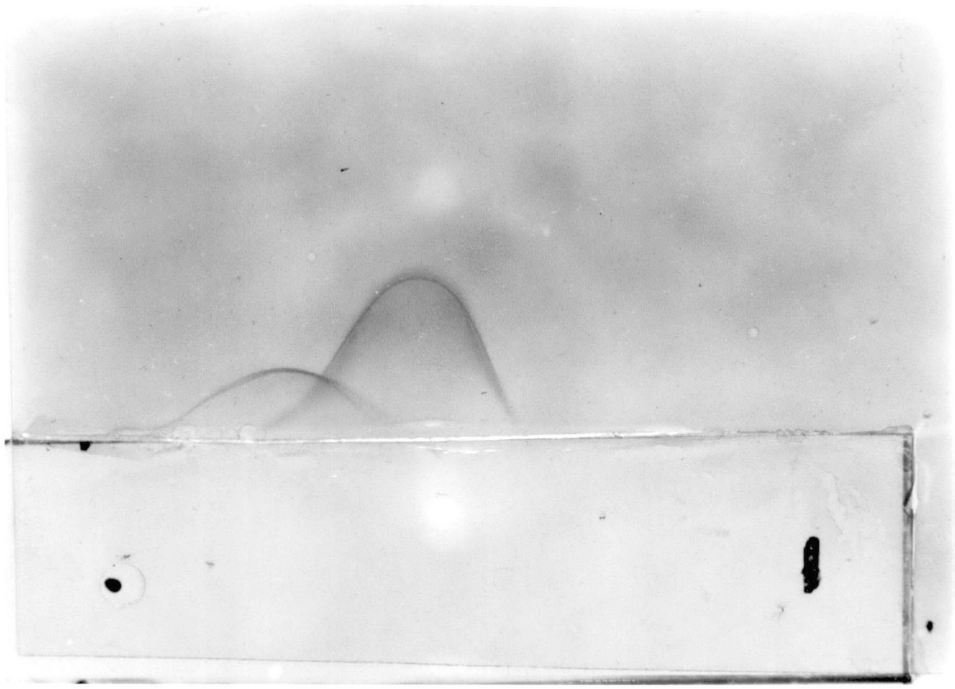

Figure 12. Antigen-Antibody Crossed Electrophoresis of the Transferrin Isolate Using Adsorbed Antibody. Following antigen-antibody crossed electrophoresis of the transferrin isolate using the adsorbed antibody preparation, two immunoprecipitation peaks were revealed using Crocein Scarlet stain.

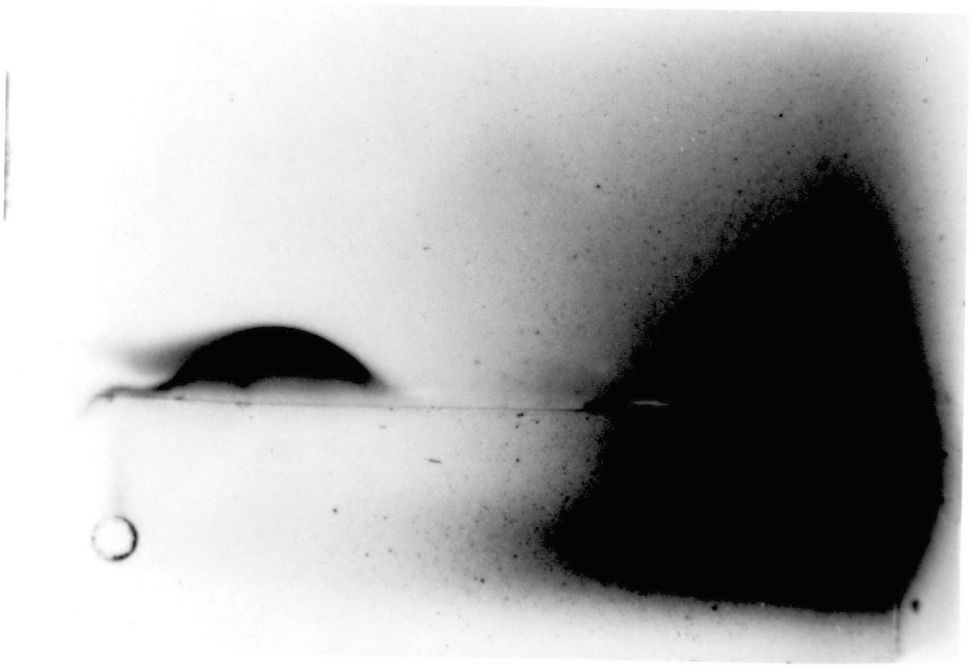

Figure 13. Radiolabeling of the Transferrin Isolate. The transferrin isolate was labeled with iron-59 and subjected to antigen-antibody crossed electrophoresis using the adsorbed antibody preparation. The 1% agarose gel was then incubated with x-ray film at 4° for 36 hours. The results indicated that the smaller of the two peaks visible in the Crocein Scarlet stained gel (see figure 12) bound the iron-59 and this smaller peak was identified as transferrin.

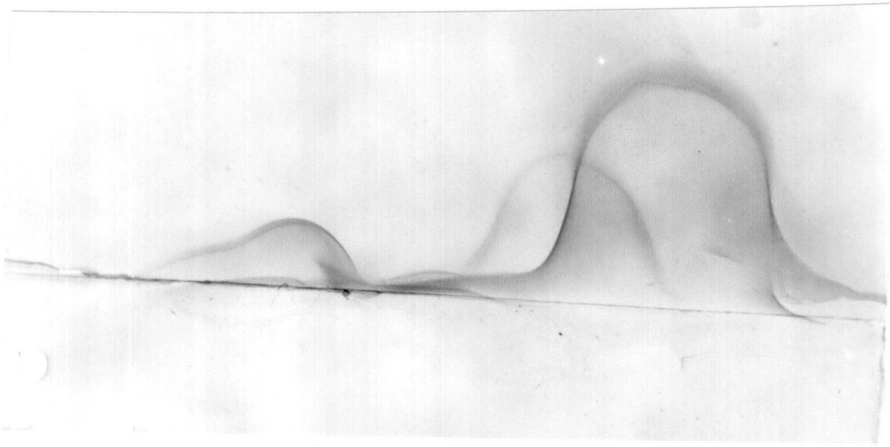

Figure 14-A. Extended Antigen-Antibody Crossed Electrophoresis of Horse Serum Sample. A quarter horse serum sample was electrophoresed for 9 hours on a 12 cm long gel strip at 70 volts during the first phase of crossed electrophoresis, rather than 4 hours on an 8 cm long gel strip in an attempt to futher separate the protein components. The presence of 3 peaks indicated the use of the non-adsorbed antibody preparation. The gel was stained with Crocien Scarlet.

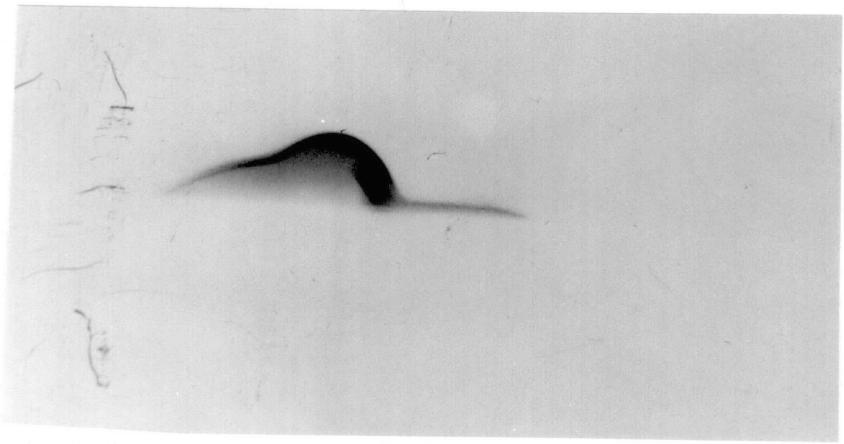

Figure 14-B. Radiolabeling of the Horse Serum Sample. The quarter horse serum sample seen in Figure 14-A was labeled with iron-59 and subjected to the usual procedure of antigen-antibody crossed electrophoresis. The gel was then incubated with x-ray film for 36 hours at 4°C, and although 3 peaks were visible in the Crocien Scarlet stained gel in Figure 14-A, only one peak (the smallest of the three) bound the iron-59 and was subsequently identified as transferrin.

Statistical Analysis of Relative Mobility Values

The following statistical parameters were calculated: The mean ($\bar{X}$), sample variance (S^2), sample standard deviation (S), variance from sample ($S\bar{X}^2$), standard deviation of sample ($S\bar{X}$), and coefficient of variability (CV).

These parameters were calculated for three sets of relative mobility values in order to determine whether each set of values could be placed in a single, specific population.

Electing to use a 95% degree of comparability, the coefficients of variability for each set of values were as follows: Population I, 2.33%, population II, 4.8%, and population III, 4.2% (Table I). These values indicated that each set of relative mobility values was not significantly different and was within the 95% degree of comparability range within their own group. Thus, three populations were defined for relative mobility values (See Table I).

Using the other calculated parameters, it was possible to perform a student t-test involving two sample means to show significant variability between each of these three populations. The calculated variation between populations I and II was -5.62, between I and III, -8.54, and between II and III, -8.29 (Table II). These values indicated significant variation between populations as they exceeded any of the values acceptable for a 95% of comparability as determined by using a t-table from Steele and Torrie (80).

Once the three populations of relative mobilities were established, the number of quarter horses and thoroughbreds having any of these three ranges of relative mobilities were categorized or classified into populations.

Population II was the most frequently observed range of relative mobility values in both the quarter horses and thoroughbreds. Eight out of 15 of the thoroughbreds tested fell into this population and 10 out of the 15 quarter horses also were in this population. Five of the thoroughbreds were found to have relative mobilities in the population I range, whereas only 2 of the quarter horses fell into this range. Two of the thoroughbreds were found to fall into the Population III range and 3 of the quarter horses were in this population range.

TABLE I

STATISTICAL ANALYSIS OF CALCULATED RELATIVE MOBILITIES OF SINGLE TRANSFERRIN PEAKS

Relative Mobility Value Range	Population	N	X	$\bar{X}$	S	S^2	$S\bar{X}$	$S\bar{X}^2$	CV
0.500-0.594	I	7	0.553	3.87	0.0129	0.00016	0.0151	0.00022	2.33%
0.617-0.694	II	18	0.687	12.38	0.01362	0.001	0.01290	0.00016	4.84%
0.708-0.757	III	5	0.727	3.63	0.03160	0.001	0.03693	0.00136	4.20%

Each coefficient of variability for each population was within the 95% degree of comparability. The statistical parameters in this table and methods for calculations are from Steele and Torrie (80).

Degree of Comparability = 95%
$\bar{X}$ = The Mean
S^2 = Sample Variance
S = Sample Standard Deviation
$S\bar{X}^2$ = Variance from Sample
$S\bar{X}$ = Standard Deviation of Sample
CV = Coefficient of Variability

TABLE II

THE STUDENT t-TEST COMPARISON OF TWO POPULATIONS INVOLVING TWO SAMPLE MEANS

T-Value	Populations Compared
-5.62	I to II
-8.54	I to III
-8.29	II to III

T values varied significantly from the t-table using a 95% degree of comparability. The table used was from Steele and Torrie (80).

TABLE III

FREQUENCY OF RELATIVE MOBILITY VALUES IN QUARTER HORSES AND THOUROUGHBREDS

Breed	Total Number Observed	Population	RM Range	Number Observed in a Population
TB	15	I	0.500-0.594	5
QH	15	I		2
TB	15	II	0.617-0.694	8
QH	15	II		10
TB	15	III	0.708-0.757	2
QH	15	III		3

TB = Thoroughbred

QH = Quarter Horse

RM = Relative Mobility

CHAPTER V

DISCUSSION

The literature suggests that the separation of transferrin into patterns of differing electrophoretic mobilities is a reliable method by which both animal and human sera can be typed and subsequently classified. However, there is no indication that any measurement or quantitation of these electrophoretic mobilities has been carried out. Instead, differences between transferrin variants have been classified by visual inspection of different positions of migration.

Ek's work (67) used starch block electrophoresis and antigen-antibody crossed electrophoresis as methods of separating and classifying transferrin variants in horse sera. He concluded that these methods provided a reliable and accurate means by which transferrin variants could be separated and classified.

The purpose of our study was to quantify and statistically categorize relative mobilities of transferrin in horse sera using methods described by Ek. Although Ek was able to identify six different phenotypes of transferrin in horses, he used Braend and Stormont's earliest work (65) as a reference standard by which these phenotypes were classified and no numerical measurement of the relative mobilities was established either in his work or in the pioneer work done by Braend and Stormont. Other investigators such as Gahne (61), Scott (70), and Arellano (72) to cite only a few, also refer back to Braend and

Stormont's original work (65) as a reference standard for typing serum transferrin in horses.

In the initial part of our study the stained gels revealed multiple immunoprecipitation peaks from which statistically valid categories of relative mobility measurements could be described. These results confirmed Ek's work, and six established categories demonstrated both homozygous and heterozygous patterns of transferrin variants. However, once radiolabeling procedures were carried out, only one of the multiple immunoprecipitation peaks bound iron-59 and was subsequently identified as transferrin, while the other peaks represented contaminants in the antigen isolate and subsequently in the antibody preparation.

The antibody was found to be reactive with both horse IgG and albumin in an Ouchterlony double diffusion test (75), indicating that the ammonium sulphate precipitation of the sheep sera was not effective in removing the IgG components and also indicating that the elutions from the G-200 molecular exclusion column which were to contain pure transferrin also contained albumin. Herbert et al. (76) reports that ammonium sulphate precipitation of IgG components from sera simply decreases the concentration of these components in sequential steps, but does not entirely remove them from the sera. This may explain the reactivity between the antibody and horse IgG.

It can be seen in standard technique books that both albumin and transferrin appear together in the large peak obtained from the gel filtration chromatography on the G-200 column and it is apparent that due to their close association, it was not possible to separate them using this particular exclusion column, although Makimura et al. (19) report that pure transferrin was to be found in the first half of the large second peak eluted from this column.

The anion DEAE column separates proteins according to charge and impurities contaminating the isolate may be of such similar charge that they were eluted along with the transferrin component. Hemopexin has been reported as a major contaminant in the purification of transferrin but the studies of separation of these two protein components have been some what contradictory. Efremov (48) reported that by treating both hemopexin and transferrin with neuraminidase prior to ion exchange chromatography on DEAE Sephadex, transferrin will remain behind in the column and hemopexin will pass through completely. However, Stratil (55) noted that once the hemopexin was treated with neuraminidase and its sialic acid residues were removed, its electrophoretic mobility was greatly decreased in starch gel electrophoresis as was that of transferrin after treatment with neuraminidase.

The question arises as to whether hemopexin might also appear as a contaminant in the elution from a molecular weight exclusion column. It has a reported molecular weight of 57,000 daltons (30) and it is also a beta-1 glycoprotein as is transferrin, although its role is to bind heme rather than iron. However, its molecular weight is close to that of both horse albumins and transferrin. Horse albumins have reported a molecular weight range of anywhere from 65,000 to 72,000 daltons, and horse transferrin has a reported molecular weight estimated at 70,000 daltons (67).

Once the antibody was adsorbed with both IgG and albumin and used in repeated antigen-antibody crossed electrophoresis, only two immunoprecipitation peaks were observed after staining the agarose gels, and the smaller of the two peaks, with the slowest electrophoretic mobility bound the iron-59 in radiolabeling procedures. The transferrin

isolate was electrophoresed again using the adsorbed antibody preparation and following radiolabeling revealed the same results. This peak was then identified as transferrin but the question was posed as to what protein contaminant was represented by the second peak.

The literature reports that not only is hemopexin a possible contaminant in transferrin isolation but haptoglobins and ceruloplasm are as well (28, 32). The haptoglobins and ceruloplasm apparently have such similar charge density and molecular weight as transferrin that they have been found on either side of the transferrin bands in both starch gel electrophoresis and polyacrylamide gel electrophoresis (32).

In the final analysis, we were unable to make a definitive identification of this second immunoprecipitation peak. However, once the smaller peak was identified as transferrin, an attempt was made to further separate this single component into multiple components to reproduce Ek's work. Increasing the time of electrophoresis in the first phase and the length of migration path in the agarose gel failed to achieve further separation of this single transferrin component. It is entirely possible that the antigen used in Ek's study may not have been pure. Ek did not institute radiolabeling procedures in antigen-antibody crossed electrophoresis in order to determine that all the multiple immunoprecipitation peaks he observed represented transferrin components.

Although we now had only a single component to analyze, statistical evaluations could be made. It was possible to establish three distinct and statistically valid categories of relative mobility values for the single transferrin immunoprecipitation peak in the 30 horse serum samples. Over half of the thoroughbred horses tested and over half of

the quarter horse tested had relative mobility values which fell into the category II range (0.617-0.694). A possible explanation for this might be that the quarter horses originated from a cross breeding of thoroughbreds with other breeds of horses brought over to America by Spanish explorers in the 17th and 18th centuries (87). Considering the history of the quarter horse evolution in this country, it is possible to attribute similar relative mobility values between this breed and the thoroughbred to a homogeneous genetic pool established over the centuries.

Can transferrin be adequately identified and separated using polyacrylamide gel electrophoresis? Our work revealed that although there were multiple bands present in the region where transferrin has been reported to appear in this electrophoretic medium, and although the transferrin isolate showed multiple bands, radiolabeling both the serum samples and the isolate was not successful. It was not possible to load the sample wells with greater than 5 microliters using a 1:5 dilution of serum to sample buffer without decreasing resolution and thus, there was not enough concentration of transferrin present to bind iron-59. Ideally, polyacrylamide gel electrophoresis as a method for separation of protein components is a most sensitive means for identification of these components and our concern was to make a definitive molecular weight determination on any band(s) which bound radiolabeled iron. An alternative approach might be to use tube polyacrylamide gels rather than slab gels, wherein the resolution is still achieved with greater sample concentration and then radiolabeling and prolonged incubation with the x-ray film might indicate iron binding. If this is possible, then the band(s) which bound the radiolabeled iron could be identified

and measured on stained tube gels, and subsequently solubulized and used to immunize either rabbits or sheep with confidence that a pure antigen would be produced.

An alternative to purification of the antigen using anion exchange chromatography is suggested by Hatton et al. (47) who reports that an efficient method of purifying bovine serum transferrin is to use cation exchange chromatography, CM-50 Sephadex, where albumin passes through the column but transferrin is allowed to bind to the column matrix and then can later be eluted by increasing ionic strength of the eluant.

We were unable to duplicate or confirm Ek's work in terms of using antigen-antibody crossed electrophoresis as a means of separating transferrin components. However, this method allows one to establish valid statistical categories of relative mobilities of a single transferrin component. Since we were dealing with only a single component, it was not possible to confirm the work done by Braend and Stormont (65) where their standard for classifying transferrin variants involved multiple transferrin components. We were, however, able to classify transferrin as to three basic types according to relative mobility values, the most rapid type falling into category III (0.708-0.757), the next in category II (0.617-0.694) and the slowest type in category I (0.500-0.594). One could designate these types with letters as is the convention of classifying transferrin variants in sera and simply refer to the fastest one as type D, the middle one as F and the slowest one as R. The most common type of transferrin variant found in horses has been reported throughout the literature as type F, and our study confirms that the greatest number of horses has this particular relative mobility value range. Since Braend and Stormont's work

reported six basic phenotypes of transferrin in terms of heterozygous and homozygous patterns, it is evident that they were dealing with multiple transferrin components. We were not able to make any genetic analysis using only a single component, but using antigen-antibody crossed electrophoresis appears to be a valid method of accurately measuring relative mobilities and statistically categorizing these values.

CHAPTER VI

SUMMARY AND CONCLUSIONS

A review of the literature revealed discrepancies concerning the position of serum proteins and of transferrin in electrophoresis procedures. It was evident that there was a need for establishing a more accurate and definative method for classifying transferrin variants.

This study has accomplished definative measurements of transferrin electrophoretic mobilities in two breeds of horses in Oklahoma: the thoroughbred and the quarter horse.

Using antigen-antibody crossed electrophoresis is a reliable and accurate method to make quantitative determinations of electrophoretic mobilities of transferrin in horse sera. The concept of classifying transferrin according to measured electrophoretic mobilities is valid and can be accomplished using antigen-antibody crossed electrophoresis.

Three types of transferrin were observed in both the quarter horses and thoroughbreds of Oklahoma. Over half of the 15 thoroughbreds tested had relative mobility values occurring in the category II range and over half of the quarter horses also had relative mobility values in this category range. Significantly fewer numbers of thoroughbreds and quarter horses had relative mobility values in either category I or III. Since quarter horses have been bred with thoroughbreds over the centuries since the 1700's, it is apparent that there are genetic similarities between the two breeds in terms of relative mobility values for transferrin.

The SDS polyacrylamide Slab gel electrophoresis revealed a distinct separation of serum proteins, but until the radiolabeling of specific bands in this procedure can be accomplished, it is not possible to identify transferrin components in polyacrylamide gel electrophoresis.

At the present time, this study cannot confirm the system of classifying and typing transferrin variants in horses established by Braend and Stormont in 1964 (65).

It was not possible to separate transferrin into more than one component using the methods of antigen-antibody crossed electrophoresis as described by Ek in 1981 (67). However, this does not preclude the fact that using this particular method may still be a viable means of separating transferrin variants if some of the method's parameters were changed and experimented with further. Perhaps changing the buffer strength or pH, or the percent agarose used in the gel medium, the time of electrophoresis and the length of the path of migration, would all contribute to separating transferrin variants using this method. This should be persued and may still prove to be an accurate and relatively simple method for classifying transferrin variants in horses.

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