
CHAPTER II

REVIEW OF LITERATURE

2.1. Introduction

Iron exists in combination with or as an integral part of a variety of molecules in mammals. Of the approximately 4.3 g of iron in an adult human being, 55 per cent is present in hemoglobin of red cells, 10 per cent occurs as part of various other tissue proteins such as cytochromes, myoglobins and catalases, 0.1 per cent is present in the combination with ferritin (Laurell, 1947). Most of the acid soluble iron in plasma is reversibly bound to a specific metal combining protein named transferrin by Holmberg and Laurell (1945) for its function of transporting iron to bone marrow and to tissue storage organs such as liver. Serum transferrin, a β -globulin which reversibly binds two moles of iron, transports this essential metal between sites of absorption (e.g. intestinal mucosal cells), sites of utilisation (e.g. bone marrow), sites of storage (e.g. liver) and sites of hemoglobin degradation. It is believed that transferrin determines the distribution of iron in body. Transferrin is also important in controlling the build-up of toxic amounts of excess iron (Morgan, 1972; Crichton, 1975; Fletcher & Huehns, 1967, 1968; Jandl & Katz, 1963; Laurell & Ingelman, 1947). This function was recognised in the earlier synonyms for transferrin i.e. siderophilin (Schade et al., 1949) and B_1 metal binding globulin (Surgenor et al., 1949).

In serum, transferrin is about 30 per cent saturated with iron, as a consequence it has the capacity to take on or release iron as required by various tissues. This is dramatically illustrated by the

fact that in the blood, iron bound to transferrin turns over approximately ten times in a 24-hour period (Morgan, 1972, Fletcher & Huehns, 1968). The physiological significance of this protein is apparent from its central role in the cyclic process whereby iron derived from the catabolism of hemoglobin and other proteins is conserved by its almost quantitative return to hematopoietic tissue. The association of the bound iron with the globulin fraction was established by Starkenstein and Harvalik (1933), and with the advent of electrophoretic techniques, iron-binding was localised to the β -globulin fraction (Surgenor *et al.*, 1949). Serum levels of transferrin are typically in the range of 200-400 mg/100 ml (Cartei and Meani, 1970).

The term transferrin applies to the class of proteins which, in addition to the serotransferrins, includes the ovotransferrins and the lactotransferrins. Ovotransferrin (Conalbumins) is found in rather large quantities in egg white. Its physiological function is not known with certainty. Because of its large affinity for iron, it is an effective antimicrobial agent which could be important in the protection of the developing chick embryo (Alderton *et al.*, 1946). In addition, ovotransferrin can donate iron to hemoglobin-synthesizing chick embryo red blood cells (Williams & Woodworth, 1973). Similar physiological function of serum transferrin in pigeons, also has been observed by Frelinger (1972).

Lactotransferrin (also known as lactoferrin and milk red protein) was originally isolated from human milk (Groves, 1960) but has since been found in various external secretions (Masson *et al.*, 1966). The role of lactoferrin is not firmly established, but like ovotransferrin, its bacteriostatic activity might be important in

depriving microorganisms of essential trace metals (Masson et al., 1966). Transferrin also binds a wide variety of divalent and trivalent transition metal ions (Teuwissen et al., 1972). Whether transferrin also plays an important physiological role in the transport of trace metal besides iron is not known. Evans & Winter (1975) reported the evidence for transport of Zn^{2+} from intestine to liver via portal blood transferrin in rat.

However, it is now believed that transferrin plays an important role in the transport of not only major elements but also several trace minerals. This critical function of transferrin, makes it a very important protein for mineral metabolism. In addition to mineral transport, transferrin may have a second function, that of bacteriostasis through the regulation of the iron available for bacterial growth (Schade & Caroline, 1946; Schade, 1961; Weinberg, 1974, 1975; Ganzoni and Puschmann, 1975; Reiter, 1976). Anderson et al. (1977) reported the stimulation of reduced lysozyme regeneration by transferrin and lactoferrin, while Hudland et al. (1977) found the specific requirement of transferrin and iron salts for the cell division of cultured cells.

2.2. Genetic Polymorphism of Transferrin in Bovines

2.2.1. Cattle

Smithies discovery in 1957 of heritable transferrin variants in human beings stimulated intensive investigations of the distribution and structure of these polymorphic proteins in various other species

(Horsfall and Smithies, 1958). As a result, transferrin polymorphism has been discovered in many species having a short or long series of multiple alleles without dominance (Lush, 1966; Bouw and Oosterlee, 1969). The various phenotypes of β -globulins in cattle were described for the first time independently by Ashton (1957 and 1958) and Smithies and Hickman (1958). Giblett *et al.* (1959) proved these β -globulins to be specific iron binding proteins of serum and were named as transferrin. Ashton (1958) and Ashton and McDougall (1958) provided the evidence for the presence of three autosomal codominant alleles, namely, TfA, TfD and TfE in cattle. Subsequently, Ashton (1959) reported TfB and TfF in zebu cattle only and these were not seen in taurine breeds. Later on, Ashton (1965) and Braend and Khanna (1968) confirmed the presence of TfB allele in the Droughtmaster cows. The presence of usual three transferrin alleles TfA, TfD and TfE were also reported by Datta and Stone (1963) in Holstein Friesian cattle. The studies of Kraay and Oosterlee (1965) in three Dutch breeds revealed the occurrence of TfA, TfD and TfE alleles.

Kristjansson and Hickman (1965) improved the method of separation of serum proteins by starch gel electrophoresis and this enabled to discover that TfD allele was in fact divisible into TfD₁ and TfD₂. The bands of TfD₁ had slightly faster mobility than TfD₂ bands. This was also confirmed by Ashton (1965). The presence of new alleles TfD₁ and TfD₂ in Holstein and Ayrshire and TfF alleles were confirmed independently, later by Kristjansson and Ashton (1965) by reciprocal testing of blood samples.

Osterhoff and VanHeerden (1965c), while testing sera of over 3200 animals representing 17 breeds of cattle, observed yet another new allele at transferrin locus. Ashton and Lampkin (1965) reported the occurrence of a new variant of transferrin in bovines of Boran breed of East Africa. This new form was observed only in one family and in heterozygous form, which they designated as TfG. Incidentally, both Osterhoff and VanHeerden (1965c) and Ashton and Lampkin (1965) had allotted the same symbol C to two different variants. The timely introduction of new symbol A_1 by Jamieson (1966) for the TfG allele as discovered by Ashton and Lampkin (1965) and symbol A_2 for the original TfA allele saved much of the confusion. Sartore and Bernoco (1966) observed a similar allele like that of Ashton and Lampkin's (1965) findings. The new allele TfH, seen in indigenous Piedmont cattle, was in fact similar to allele TfG in mobility.

Braend and Khanna (1967), while studying Norwegian Red cattle, observed one more new allele which they called TfN. They observed three different phenotypes namely TfAN, TfD₂N and TfNE. The phenotype TfAN was described earlier by Gahne (1961), however, the other two phenotypes were observed for the first time in Norwegian cattle. The new allele TfN had a lower migration rate in electrophoresis than the TfD₂ allele. Therefore TfD₂N phenotypes showed six bands with two very faint bands in front. The TfAN phenotypes also showed six bands but TfNE phenotypes appeared only five bands due to overlapping of bands. It was shown that TfN allele was in fact a new allele and was different from TfF allele.

Spooner and Baxter (1969) reported the abnormal expression of normal transferrin alleles. During the course of their investigation they observed that the two fastest strong protein bands had the same mobility as the two other slow bands. This was presumably due to the effect of a recessive epistatic gene operating at a locus distinctly different from transferrin locus.

The variation in the gene frequency of different transferrin alleles has been reported by various workers for different breeds of animals. Kraay and Oosterlee (1965) in three Dutch breeds revealed the occurrence of TfA, TfD and TfE alleles only. The TfD allele was highest in Black and White breed whereas TfA allele was predominant in Red and White and White faced breeds. In the latter breed, the TfA allele was even twice as frequent as TfD. In all the breeds, it was observed that the frequency of TfE allele was lower than 10 per cent. Kristjansson and Hickman (1965) reported the gene frequencies of Holstein herds to be as 0.62 for TfA, 0.13 for TfD₁ and 0.16 for TfD₂ and 0.09 for TfE. Similarly, for Ayrshire, the frequencies were observed to be TfA:0.29, TfD₁:0.25, TfD₂:0.21 and TfE:0.25.

The occurrence of TfE allele in taurine cattle of Europe and America from nil to very low frequency was observed by Jamieson (1965) also. The relative high frequency of this allele in zebu type was considered as an indication of climatic and ecological tolerance of these cattle by Ashton (1959).

A high frequency of TfE allele among Indian breeds has been observed by many workers. Singh (1974) reported a high TfE allele in

Red Sindhi, Sahiwal and Tharparkar breeds. However, he observed a low occurrence of TfB allele in these breeds. Similar observations were also made by Singh et al. (1972) in Haryana, Kankrej, Ongole and Gir breeds of cattle and in Red Sindhi, Tharparkar and Sahiwal breeds by Pandey (1974). Prasad et al. (1978) in a recent communication have reported similar observations in these breeds. They reported a very low frequency of TfF and TfB alleles. Singh (1974) also observed similar findings in these breeds. However, Singh et al. (1972) found the occurrence of TfF allele in Haryana, Ongole and Kankrej breeds, to be quite frequent. The occurrence of low TfF and TfB alleles has been observed in African zebu breeds, also by Osterhoff and VanHeerden (1965).

2.2.2. Buffaloes

Loupetjra (1962) is reported to have studied the polymorphism of transferrin in Siamese water buffaloes for the first time. He described three phenotypes having mobilities equivalent to TfAA, TfAB and TfBB of cattle. Naik et al. (1969) reported the transferrin polymorphism in Indian water buffaloes, and he designated three phenotypes viz., TfAA, TfAB and TfBB in order of mobilities in the Murrah buffaloes. The occurrence of TfAA type was observed to be lowest, followed by heterozygote and the highest percentage was observed of the TfBB types. Makaveev (1968, 1970) reported polymorphism in water buffaloes from Bulgaria, imported Indian water buffaloes and their crosses. He found three phenotypes controlled by two codominant alleles called as TfB and TfC. Khanna (1969) described the transferrin polymorphism in Indian water buffalo and designated three phenotypes as DD, DK and KK in

decreasing order of mobility towards the anode, which were governed by two codominant alleles. Khanna and Singh (1978) in their recent report have described three alleles TfD, TfK and TfN, in several Indian breeds of buffaloes. TfK allele has been reported most frequent while TfD and TfN were found in low frequencies. Basavaiah et al. (1977) observed five phenotypes viz., DD, KK, NN, DK and DN in Murrah buffaloes. The KK type of homozygotes accounted for more than 80 per cent of the buffaloes studied. Similar observations were made by Pandey (1974) while studying the transferrin polymorphism in Murrah breed.

Three transferrin phenotypes controlled by two codominant alleles mirrored in different nomenclatures have been reported in buffaloes from various countries. Abe et al. (1969) designated the transferrin alleles as TfA and TfD in buffaloes from Formosa, Zubareva et al. (1969) as TfA and TfB in buffaloes from Russia. Masina et al. (1971) as TfD and TfE in buffaloes from Italy and Granciu et al. (1972) as TfB and TfC in buffaloes from Rumania. Masina et al. (1971) found a few animals with an abnormal TfD phenotype in which bands stained more intensely than the bands of the common TfD. In other species of buffaloes, namely, American bison, European bison and African bison, only one type of transferrin was reported (Stormont, 1964; Braend and Gasparki, 1967; Osterhoff and Young, 1967 and Osterhoff et al., 1970).

Transferrin polymorphism in Indian water buffaloes was reported simultaneously by Naik et al. (1969) and Khanna (1969). Naik

observed the occurrence of the faster moving (AA type) to be lowest (1.94%) and the slower moving (BB type) to be highest (72.90%). The heterozygotes were about 25.16 per cent. Khanna (1969) followed the nomenclature as suggested by Brasend (1965), viz., TfD, TfK and TfN in decreasing order of mobility. TfN was not reported earlier in Indian water buffaloes. Pandey (1974) observed only three phenotypes in Murrah buffaloes viz., TfAA, TfAB and TfBB. The slower moving phenotype (TfBB) accounted for more than 70 per cent of the population. Basaviah et al. (1977) reported five phenotypes as designated by Khanna (1969). They observed TfKK homozygotes to be more than 80 per cent of the population studied. TfN allele was found to be present in the population studied. Khanna and Singh in their recent work (1978) have confirmed the five phenotypes originally designated by was the most frequent allele (range 0.74 to 0.95) among them, in Indian water buffaloes. TfK was also observed by Pandey (1974), followed by TfD and TfN. Similarly, a high frequency of the allele governing the transferrin type having mobility, probably equivalent to TfK was reported by Makaveev (1968) in Bulgarian buffaloes (0.84) and Indian buffaloes (0.94), Abe et al. (1969) in Formosan buffaloes (0.85), Zubarev et al. (1969) in Caucasian buffaloes (0.79), Naik et al. (1969) in Indian buffaloes (0.86); Masina et al. (1971) in Italian buffaloes (0.63) and Granciu et al. (1972) in Romanian buffaloes (0.91).

2.3. Isolation and Purification of Transferrin

Transferrin has been prepared in satisfactory purity by a variety of methods, including one or a combination of several methods:

alcohol fractionation, ammonium sulfate precipitation, aluminium hydroxide adsorption, gel-filtration, ion-exchange chromatography, Rivanol precipitation and starch-block electrophoresis. The isolation of transferrin was achieved for the first time by ethanol fractionation procedure (Cohn et al., 1946). Surgenor et al. (1949) further sub-fractionated this to obtain transferrin in fraction IV-7 in an estimated 76 per cent purity. Using this as a starting point, Koechlin (1952), by careful manipulation of pH, ionic strength and temperature of ethanol-water mixtures, concentrated the material to 90 to 95 per cent purity and crystallized it. Inman et al. (1961) developed a large scale purification procedure, beginning with Cohn fraction IV-4. This material yielded crystalline apotransferrins of greater than 90 per cent purity.

In other methods not beginning with alcohol fractionated plasma, either ammonium sulfate or Rivanol (2-ethoxy-6,9, diaminaacridine lactate) has commonly been used to precipitate the bulk of the proteins, followed by chromatography with CM-, TM-, or DEAE-cellulose, and gel filtration with Sephadex G-200. A simple method proposed by Hoettcher et al. (1958) and reported in detail by Kistler et al. (1960), depends on the precipitation of albumin and other proteins with Rivanol, leaving γ -globulins and transferrin in the supernatant solution. Rivanol is then removed by adsorption on charcoal (Kistler et al., 1960). Sutton and Karp (1965) used starch for adsorbing Rivanol, after precipitating other proteins with Rivanol. They used DEAE Sephadex A-50 for separating γ -globulin and transferrin. Roop (1963), Roop and Putnam (1967) combined Rivanol precipitation, ammonium sulfate precipitation, gel-filtration, and ion exchange chromatography for the

isolation of transferrin. For the purification of smaller quantities of transferrin, some very mild techniques have been developed. Starch block electrophoresis was used by Sutton and Karp (1965), and Chen and Sutton (1967) for separating individual bands of transferrin, while Parker and Bearn (1961, 1962) employed starch block electrophoresis followed by TEAE cellulose chromatography. Galotte et al. (1962) have used gel-filtration on Sephadex G-200 as the first step, with either zone electrophoresis on Sephadex G-25, or anion-exchange chromatography on DEAE Sephadex A-50 as the second. In a recent work, Wong and Regoegi (1977) isolated five subfractions from commercially available human transferrin, by using a gradient on DEAE cellulose column. Regoeczi et al. (1974) used combination of ammonium sulfate precipitation and chromatography on CM-Sephadex and DEAE cellulose to isolate rabbit transferrin. Beskrovny et al. (1963) also had employed precipitation with ammonium sulfate and chromatography on DEAE Sephadex A-50 for isolation of human transferrin.

In addition to the preceding work with human transferrin, several workers have isolated and purified various animal transferrins. Gordon and Louis (1963) prepared rat transferrin by either starch-gel electrophoresis or DEAE Sephadex chromatography. Williams (1962) used ammonium sulfate fractionation and ion exchange chromatography on CM and DEAE cellulose for the purification of chicken transferrin. Laurell and Ingelman (1947) employed a combination of ammonium sulfate and alcohol fractionation techniques to prepare swine transferrin. Regoeczi et al. (1974) used combination of ammonium sulfate precipita-

tion and chromatography on G- Sephadex and DEAE cellulose to isolate rabbit transferrin. Van Eijk and Leijnse (1968) used Rivanol precipitation, gel filtration on Sephadex G-150 and ion exchange chromatography on DEAE Sephadex A-50 to isolate transferrin from rabbit, rat and human serum. They again used the same methodology to isolate transferrin from human, rabbit, rat and fish (Van Eijk et al., 1972). The isolation of pig transferrin was done by Stratil and Kubek (1974) by using slightly modified procedure of Stratil and Spooner (1971). They precipitated immunoglobulin G by adding 2.0 M ammonium sulfate and transferrin by adding 3.0 M ammonium sulfate. This preparation was purified by using a shallow gradient on DEAE Sephadex A-50. Stratil and Spooner (1971), for separation of bovine transferrin, used the combination of Rivanol-ammonium sulfate precipitation as described by Schultze and Heremans (1966). However, further purification of transferrins and separation of individual bands was accomplished by ion exchange chromatography on DEAE Sephadex A-50. Spooner et al. (1975) used the same methodology to isolate sheep transferrin subfractions.

Sfrémov et al. (1971) used a series of four techniques for isolation and purification of bovine transferrin (a) precipitation with Rivanol (b) Chromatography of the soluble fraction on a column of Sephadex G-150 (c) Chromatography of the transferrin containing protein zone on a column of DEAE Sephadex and (d) chromatography on a column of DEAE Sephadex after transferrin was treated with neuraminidase. Valenta et al. (1976, 1977) isolated fish transferrins by a combination of gel filtration on DEAE Sephadex A-50. Hatton et al.

(1977) used sodium citrate gradient on Sephadex OM-50 at pH 5.9 to separate the contamination of hemopexin from the crude bovine transferrin preparations. There is no report to date for the isolation of transferrin from buffaloes.

2.4. Physico-chemical and Immunological Characterisation

2.4.1. Electrophoretic Identification

Because of its relatively high concentration in serum, transferrin is readily demonstrated by starch gel electrophoresis with the use of discontinuous buffer system of Poulik (1957). However, a reference serum must be used to identify transferrin variants because of the slight difference in mobility and the large number of other plasma protein components in the α and β regions. For the same reasons radioautoradiography with Fe^{59} by the method of Giblett *et al.* (1959) should be used to verify that the components are transferrin. This elegant technique visualizes only the iron binding protein and facilitates the identification. Spooner and Baxter (1969) detected the abnormal expression of normal transferrin alleles, in cattle, by the use of Fe^{59} autoradiography. They observed that the two fastest strong protein bands, apparently had the same mobility as the two slow bands. Fe^{59} was used for preparing labelled peptide maps of the major and minor components of Hens Egg Ovotransferrin by Williams and Wenn (1970).

The removal of the iron from the Fe-Transferrin complex, apparently does not change the electrophoretic mobility and banding pattern of transferrin. It was at first puzzling that serum from human

being homozygous for transferrin C gave only a single iron-binding band in starch gel electrophoresis. Since serum usually is not saturated with iron, one might have expected to find up to three transferrin bands: one representing transferrin combined with two iron atoms, another for transferrin combined with one iron atom and the third for apoprotein. The explanation for the presence of only one band is that the commercially available hydrolyzed starch contains sufficient iron to saturate the transferrin. Sass-Kortek and Vamosi (1964) and also Giblett (1968) have shown that the iron-saturated and iron free forms of transferrin can be separated by starch gel electrophoresis, provided the starch is iron free. Although they failed to find an intermediate component representing transferrin that was half saturated, Aisen et al. (1966) showed the presence of two different forms of transferrin (Fe_1Tf and Fe_2Tf) by the method of free boundary electrophoresis. Also an intermediate complex of ovotransferrin with one iron atom has been demonstrated by starch gel electrophoresis after scrupulous removal of contaminating metals from the starch powder and other reagents and glassware (Williams et al., 1970).

2.4.2. Molecular Weight

Although a molecular weight of about 90,000 was long accepted for human and animal transferrins, recent experiments in several laboratories have come to substantial agreement for a molecular weight of about 76,500. The earlier molecular weight of 90,000 was based on sedimentation-diffusion measurements, sedimentation equilibrium, light scattering, osmotic pressure and iron binding. Some of the references

are listed in the Table 2.4.2.1. Charlwood (1963) was the first to dispute this figure and gave a value of 68,000 for human, monkey, and rat transferrins and attributed the higher earlier values to aggregation.

Table 2.4.2.1: Molecular Weight of Human Transferrin

Method	M. W.	References
Iron binding capacity	90,000	Koechlin (1952)
Sedimentation diffusion	89,000	Schultze <u>et al.</u> (1957)
Sedimentation equilibrium	93,000	Bezkerovainy <u>et al.</u> (1963)
Sedimentation equilibrium	68,000	Charlwood (1963)
Iron binding capacity	90,000	Aisen <u>et al.</u> (1966).
Sedimentation diffusion	73,200	Roberts <u>et al.</u> (1966)
& Sedimentation equilibrium)	
	76,000)
Sedimentation diffusion	93,000	Bezkerovainy & Grohlich (1967)
Sedimentation velocity	77,700	Aisen <u>et al.</u> (1970)
Sedimentation equilibrium	76,600	Aisen <u>et al.</u> (1970)
Sedimentation equilibrium	76,600	Mann <u>et al.</u> (1970)
Sedimentation equilibrium	76,200	Palmour and Sutton (1971)

In the period 1966-1971, four groups of workers using the methods of sedimentation equilibrium and sedimentation velocity arrived at values close to 76,500 for human transferrin (Table 2.4.2.1) (Roberts et al., 1966; Mann et al., 1970; Aisen et al., 1970; Palmour and Sutton, 1971). No difference was observed for apotransferrin or transferrin

containing either one or two Fe atoms (Aisen *et al.*, 1970). The validity of these results was confirmed by other methods such as intrinsic viscosity, gel filtration, and electrophoresis in sodium dodecyl sulfate gels, as well as by sedimentation equilibrium measurements in the presence of denaturing agents.

With an exception of a dispute about the transferrin of hagfish, other vertebrate transferrins fall in the same range of molecular weight as human transferrin i.e. about 77,000. Greene and Feeney (1968) gave values of 77,500 and 76,000, respectively, for rabbit transferrin and ovotransferrin, based on sedimentation equilibrium in 8 M urea. Sedimentation equilibrium in the absence of urea yielded molecular weights of 74,590 for rabbit transferrin and 79,370 for that of the frog (Palmour and Sutton, 1971). The latter authors reported a somewhat high figure of 93,700 for transferrin from the turtle. All these values were confirmed by gel filtration and by SDS gel electrophoresis.

From sedimentation equilibrium studies, Hudson *et al.* (1973) reported molecular weight values close to 77,000 for bovine, rabbit, equine and porcine serum transferrins both in dilute aqueous solution and for the mercaptoethanol-reduced proteins in the presence of 6 M guanidine HCl. The fact that the molecular weight of all these transferrins is not decreased by denaturation or reduction-alkylation indicates that transferrin consists of a single polypeptide chain. Efremov *et al.* (1971) reported molecular weight of bovine transferrin to be 67,000 and 72,400 from sedimentation and viscosity and sedimentation and diffusion measurements respectively. Van Eljk *et al.* (1972)

calculated the molecular weights of human, rabbit, rat and fish by Archibald procedure, to be in the range of 81,000 to 83,000. The molecular weight of pleurodale (*Pleurodeles waltlili*) transferrin was determined by Foucier et al. (1976) to be 78,000 by SDS electrophoresis. Spooner et al. (1975) calculated the molecular weight of sheep transferrin to be 77,000 by the method of sedimentation equilibrium ultracentrifugation. Stratil and spooner (1971) reported the molecular weight of bovine transferrin to be 74,000 by gel filtration on Sephadex G-200.

An interesting dispute has arisen regarding the molecular weight of the transferrin of a primitive vertebrate, the Pacific hagfish. Palmour and Sutton (1971) by a variety of methods, obtained a molecular weight of about 44,000 and concluded that the hagfish has a primordial transferrin with a binding capacity of one atom of Fe per molecule. On this basis and on other suggestive evidence for duplicate structure in the transferrins of higher vertebrates, they postulated that a gene duplication took place early in vertebrate evolution leading to an elongated protein with some repeating sequences. Unfortunately for this argument, Aisen et al. (1970), after extensive molecular weight studies by a variety of methods, reached the contrary conclusion, namely, that hagfish transferrin has a single polypeptide chain with a molecular weight of 75,000 to 80,000 and has two similar or identical iron-binding sites thus closely resembling human transferrin.

2.4.3. Carbohydrate Composition

Transferrin is one of the few plasma glycoproteins for which the complete sequence of the oligosaccharide chains has been established

and for which biological functions have been associated with the carbohydrate moiety. The carbohydrate content of human transferrin was first reported by Heimburger et al. (1964) and since then it has been studied by a number of investigators who agree on the presence of hexose, hexosamines, and sialic acid. The hexoses were identified as mannose and galactose by Schultze et al. (1958), Montreuil et al. (1961), Jamieson (1964a) and Spik (1968). Reports of trace amounts of fucose have not been generally verified. The ratio of galactose to mannose reported by different investigators has varied; 2:1 (Schultze et al., 1958), 1:1 (Montreuil et al., 1965) and 1:2 (Jamieson, 1964a). The hexosamine consists entirely of N-acetylglucosamine (Montreuil et al., 1961; Jamieson, 1965b). The sialic acid is N-acetylneurameric acid (Montreuil et al., 1961; Jamieson, 1966a).

The significance of small differences reported by various workers is not known. The complete carbohydrate sequence was determined by Jamieson et al. (1971). They later established the presence of 4 residues of sialic acid, 4 of galactose, 8 of mannose and 8 of N-acetylglucosamine per molecule of transferrin.

Table 2.4.3.1 gives the composition of Hexoses, Hexosamine and Sialic Acid in percentages for Human serum, Bovine serum, Human milk and Bovine milk. The percentage of Hexose, Hexosamine and Sialic acid Human serum varies from 2.4 to 2.8, 2.0 to 2.03, 1.2 to 1.4 respectively (Beskorovainy et al., 1963; Jamieson, 1964a; Heimburger et al., 1964; Spik, 1968; VanEijk et al., 1972; Hatton et al., 1974; Wong & Regocczi, 1977). The variation for these carbohydrates ranged as

Table 2.4.3.1: Carbohydrate Assays of Transferrin and Lactoferrin.

Source	Hexose (%)	Hexosamine (%)	Sialic Acid (%)	References
Transferrins				
Human serum	2.55	2.03	1.40	Hatton <u>et al.</u> (1974)
	2.8	1.8	1.2	Bezkorovainy <u>et al.</u> (1963)
	2.4	2.0	1.4	Heimburger <u>et al.</u> (1964)
	2.6	2.0	1.3	Jamieson (1964a)
	2.4	2.0	1.4	Spik (1968)
	2.6	2.0	1.3	Wong & Regoeczi (1977)
	2.4	2.0	1.4	VanEijk <u>et al.</u> (1972)
Bovine serum	1.25	1.06	1.73	Hatton <u>et al.</u> (1972)
	1.34	0.90	0.60	Hatton <u>et al.</u> (1974)
	1.32	1.03	0.74	Hudson <u>et al.</u> (1973)
Chicken serum	2.8	1.4	0.35	Williams (1962)
Human Lactoferrin	3.1	2.9	1.3	Spik <u>et al.</u> (1966)
	3.7	2.0	0.9	Got <u>et al.</u> (1966)
Bovine Lactoferrin	4.5	2.2	0.3	Groves (1960).

1.25 to 1.34 per cent, 0.90 to 1.06 per cent and 0.60 to 0.74 per cent for Hexoses, Hexosamines and Sialic Acids respectively, in Bovine serum (Hudson et al., 1973; Hatton et al., 1974, 1977). The figures for chicken serum were reported closer to human serum for hexoses but the hexosamine and sialic acids were low (Williams, 1962).

The analysis for the lactoferrin from Human and Bovine milk, for carbohydrate analysis has been given in Table 2.4.3.1. The percentage of Hexoses in lactoferrin is quite high as compared to human transferrin and Bovine transferrin. However, the content of hexosamine and sialic acid have been observed quite close to human and bovine transferrins (Spik et al., 1966; Got et al., 1966; Groves, 1960).

The reports on the carbohydrate composition of different peaks or subfractions of one single transferrin type of one species are scanty. However, Wong and Negoezzi (1977) in humans and Hatton et al. (1977) in bovines reported an insignificant variation among the different subfractions of transferrin for Hexoses, Hexosamines and Sialic acids. Similar observations have been made also for sialic acid content, in bovines (Stratil & Spooner, 1971), in sheep (Spooner et al., 1975), in pig (Stratil & Kubek, 1974).

2.4.4. Effect of Neuraminidase

Variations in the carbohydrate moieties of transferrins have assumed special interest since the demonstration that sialic acid differences account for the complex patterns obtained on gel electrophoresis of many transferrins (Chen and Sutton, 1967). In addition, tissue differences in electrophoretic pattern are due to different sialic acid contents. For example, both cerebrospinal fluid and cord blood of human beings contain transferrin which differs from plasma transferrin only by the carbohydrate moieties, of which the presence or absence of sialic acid has a striking influence on charge

(Parker et al., 1963). The presence of terminal sialic acid was early recognised (Blumberg and Warren, 1961; Poulik, 1961; Parker and Bearn, 1961a). Action of neuraminidase (sialidase) alters the mobility of serum in a stepwise manner (Parker and Bearn, 1961a). As the glycosidic bonds are hydrolysed by the enzyme, an increasing but random removal of sialic acid occurs resulting in a succession of four new bands, equally spaced one from another, from a single human transferrin C type. With sufficient enzyme action all of the transferrin appears as a single band that migrates most slowly toward the anode, as if it had lost four negative charges. This indicates that all of the sialic acid residues are accessible to the enzyme.

The sialic acid content of genetic variants of human transferrin is apparently the same, and neuraminidase treatment has little effect, hence the mobility differences of the genetic variants are attributed to substitution in protein structure. Parker and Bearn (1962a) investigated the effect of neuraminidase on nine variants of human transferrin. In case the enzymatic removal of sialic acid produced stepwise patterns of four additional slower moving components whose relative intensities depended on the concentration and duration of activity of the enzyme. The transferrin which is found in rat and rabbit milk has fewer sialic acid residues than the corresponding plasma transferrin, and chicken conalbumin, whose polypeptide structure is clearly under control of the same structural gene as the plasma transferrin, also differs from the plasma transferrin in its carbohydrate portion (Williams, 1962).

The situation can be readily visualised from the studies on bovine transferrin (Chen and Sutton, 1967). Amino acid analyses of individual bands were identical to each other and to analysis of the purified complex (Chen, 1968). Treatment with neuraminidase causes the pattern to shift by two charge units. However, it appears that bovine transferrin is considerably more resistant to neuraminidase than human transferrin, and that the reaction does not go to completion readily. Instead, as shown by the treatment of isolated individual bands, several products may be formed depending on the number of potential cleavage points. Until now only that part of heterogeneity, for which sialic acid is responsible has been studied. In some species sialic acid accounts for all the heterogeneity (e.g. chicken, Williams, 1962; rabbit, Baker *et al.*, 1968; sheep, Spooner *et al.*, 1975) in some other species it is responsible only for a part of the heterogeneity (e.g. cattle, Stratil and Spooner, 1971; pig, Stratil and Kubek, 1974).

2.4.5. Interaction with Iron and other Metals

Each transferrin molecule can combine with two atoms of ferric iron in an ionic bonding in which one bicarbonate ion is taken up per iron atom (Schade *et al.*, 1949; Schade and Reinhart, 1966). The complex is stable in the pH range from 7.5 to 10 but begins to dissociate at pH 6.5 and is completely dissociated on acidification.

to pH 4 where the iron can be removed by dialysis and the transferrin loses its red colour. The mechanism of iron-binding has been studied by a variety of techniques with generally concordant results.

Holmberg and Laurell (1947) found that transferrin will bind both iron and copper, but that the affinity for iron is much greater.

Transferrin can also combine loosely with metals of the transition series and the lanthanide series including copper, manganese, cobalt, chromium, scandium, terbium, and zinc ions (Aisen et al., 1969; Ford-Hutchinson and Perkins, 1971; Charlwood, 1973; Gafni and Steinberg, 1974). Although Ca^{2+} , Cu^{2+} , Zn^{2+} and Co^{2+} and other metal ions present in plasma bind to transferrin, the binding does not appear to be of physiological importance, except possibly for Cu^{2+} (Laurell, 1960; Jones & Perkins, 1965). However, Hopkins and Schwarz (1964) have proposed that transferrin may have a biological role in the transport in plasma of the trivalent ion of chromium. They observed that Cr^{51} when given orally to rats about 70-90 per cent of the isotope bound to the β -globulin fraction. The chromium appears to compete for the metal binding site in the transferrin and also affects the binding of transferrin to reticulocytes (Karmfeld, 1969). Both metal-binding sites of transferrin form specific complexes with chromium, manganese and cobalt ions, with bicarbonate being bound also in a ratio of unity compared to the metal ion. Magnetic susceptibility measurements indicate that each of the metal ions is bound in the trivalent state (Aisen et al., 1969).

In plasma under aerobic conditions the formation of the iron-transferrin complex occurs more rapidly with ferrous than with ferric ions. Although the complex that is formed from ferrous ions with purified transferrin is not different chemically or spectrometrically from that prepared from ferric salts, there has been a conflict of opinion as to which form of iron is more efficient with respect to rate and completeness of reaction. Ross *et al.* (1963) found that ferrous salts show smooth stoichiometric reaction above pH 6.5, and the rate rises with pH and in the presence of oxidizing reagents. Ferric salts show high initial rates of complex formation at pH 5 and above but the yield is below theoretical and tends to decrease as the pH rises, probably due to competitive hydrolysis of the ferric salt.

The iron also affects other properties of transferrin, including its stability and reputedly its isoelectric point. Transferrin containing iron is much more resistant to denaturation by heat and to hydrolysis by trypsin and chymotrypsin (Azari and Feeney, 1958) and also to denaturation by urea or guanidine HCl (Bron *et al.*, 1968; Bezkorovainy and Grohlich, 1967). The increased stability which accompanies iron binding is probably the consequence of conformational changes in the molecule. The isoelectric point is reported to be pH 5.45 when the protein is fully saturated and pH 5.80 when iron-free (Keller and Pennell, 1959). Isoelectric focussing experiments by Wenn and Williams (1968) show that the iron-saturated and the apo forms of ovotransferrin have different isoelectric points.

As a result of iron binding, the net charge on the molecule is changed by -1 for each iron atom bound, with a consequent change in electrophoretic mobility (Warner and Weber, 1953) and behaviour on ion exchange chromatography (Lane, 1971, 1972). Iron binding does not produce a significant change in molecular weight (Bezkorovainy, 1966), but it does cause the molecule to become more compact and more spherical in shape (Charlwood, 1971; Rosseneau-Motreff *et al.*, 1971).

2.4.6. Comparison of Transferrin with Conalbumin and Lactoferrin

2.4.6.1. Conalbumin

Williams (1962) has shown that chicken conalbumin and chicken serum transferrin differ only in their carbohydrate content. They have identical amino acid and polypeptide composition, molecular weights and immunological properties. Their reactions with iron are indistinguishable (Feeny and Komatsu, 1966). Conalbumin is synthesized by the tubular gland cells of the oviduct magnum (Williams, 1962; Palmiter, 1972a,b), whereas serum transferrin is almost certainly synthesized by the liver in birds as in mammals.

2.4.6.2. Lactoferrin

Lactoferrin is a protein present in milk that is often confused with transferrin because of their similarity in iron-binding properties, colour and molecular weight. Despite this

resemblance, lactoferrin and transferrin from the same species generally appear to differ widely with respect to primary structure as suggested by amino acid composition and tryptic maps; they also differ in carbohydrate content and antigenic determinants. Although lactoferrin is also known as lactotransferrin, lactosiderophilin iron binding protein from milk, and the "red protein", Schultze and Heremans (1966) urge that the root name transferrin or siderophilin be abandoned and lactoferrin used instead because of the absence of structural and antigenic relationship with serum transferrin. Lactoferrin is the predominant iron-binding protein of milk and colostrum, the transferrin content of these fluids being less than 1 mg/100 ml (Schultze and Heremans, 1966). The function of lactoferrin is probably to inhibit the growth of certain microorganisms by chelating iron, thereby competing for this essential nutrient (Kirkpatrick *et al.*, 1971).

Despite their lack of structural and antigenic relationship lactoferrin and transferrin have many properties in common suggesting that they may have similar iron-combining sites (Aisen and Leibman, 1972). Human lactoferrin is saturated with iron or copper at a ratio of 0.025 μ moles of metal per milligram of protein (Masson and Heremans, 1968). On the basis of a molecular weight of 75,000 this corresponds to an uptake of two iron atoms per molecule. Copper is bound in the same molar proportion. As with transferrin, one bicarbonate ion is taken up per atom of iron or copper bound during

the formation of the coloured lactoferrin-metal complex. With iron, the absorption spectra of the two protein-metal complexes are similar for lactoferrin and transferrin. One significant difference is the much greater stability of the lactoferrin-iron complex at acidic pH (pH 2 - 4.6) where the transferrin-iron complex readily dissociates. This reflects the great difference in the strength of metal binding of the two proteins (Aisen and Leibman, 1972).

2.4.7. Immunological Characterisation

Although antisera specific for transferrin are readily prepared and are available commercially, little is known about the antigenic structure of transferrin molecule. An immunochemical difference between iron-saturated and unsaturated human transferrin has been demonstrated by Kourilsky and Barton (1968). With certain rabbit antisera but not with commercial antisera a spur appears between the precipitin lines for purified iron-unsaturated transferrin and the transferrin in iron-saturated sera from patients with hemochromatosis; also two antigenically different fractions of transferrin can be detected in normal human serum. Since these differences can be abolished by saturation with iron, it appears that a reversible immunochemical modification of transferrin occurs on iron binding that involves only part of the antigenic determinants of the molecule. This is attributed to a modification of the quaternary structure of the transferrin molecule possibly resulting from steric hindrance by the metal ions of antigenic determinants near the binding site.

Limited tryptic digestion releases two fragments from iron-unsaturated transferrin but not from saturated transferrin. Fully succinylated transferrin loses its ability to form precipitates with antiserum to the native protein and gives rise primarily to antibodies specific for the succinyl residue (Zschoke and Bezkorovainy, 1970). The carbohydrate moiety of the transferrin molecule (or at least the sialic acid portion of it) does not appear to influence the antigen-antibody reaction, for neuraminidase-treated and untreated transferrin C (human) show 100 per cent cross-reactivity (Wang et al., 1968).

It would be of great value if specific antisera capable of distinguishing genetic variants of different species transferrin could be developed to facilitate rapid screening of sera. However, no spur formation takes place when rabbit antihuman transferrin reacts in immunodiffusion with various transferrin phenotypes (Giblett, 1969). In principle, it should be possible to prepare antisera that will distinguish transferrin phenotypes by immunising human subjects of the common C phenotype with purified variants or by absorption of animal antisera prepared against individual phenotypes. However, using rabbit antisera prepared against purified variants, Wang et al. (1968) were unable to distinguish human transferrins TfC, TfD₁ and TfD₃ by sensitive technique of radioimmune inhibition of precipitation. Stratil and Spooner (1971) did not observe any antigenic differences among the various subfractions obtained after chromatography of also, Brummerstedt-Hanson (1962) failed to detect any immunological difference between Tf phenotypes in cattle homozygous cattle transferrin. This suggests that the aminoacid

sequence differences in these variants are insufficient to affect major antigenic determinants. In an extensive immune-chemical investigation, Wang et al. (1968) studied the evolution of antigenic determinants of transferrin and other serum proteins in 23 primate species when they observed a good correlation of percentage cross-reactivity of transferrin with other proteins in these species. Van Kijk et al. (1972) observed that the antibodies to the different transferrins were very specific, antihuman transferrin precipitated only with human transferrin. Foucier et al. (1976) used specific antiserum, in a study of immunological relationship of *Pleurodeles waltlili* with 25 other animal transferrins from cyclostome, fish, amphibian and mammalian species. They observed common antigenic sites only in Salamandridae family.
