
CHAPTER V

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DISCUSSION

5.1. Transferrin Polymorphism

5.1.1. Cattle

Tables 4.1.1 and 4.1.2 show the number and phenotypes of the animals (cattle), namely, Tharparkar, Sahiwal and Red Sindhi and their crosses with Brown Swiss, Holstein-Friesian and Jersey exotic breeds typed for transferrin polymorphism. Eight transferrin phenotypes were observed in the indigenous breeds of animals. The more frequent of these were found to be TfAA, TfAD, TfAE, TfDD, TfDK and TfEE types. Out of 411 crossbred animals typed, 136 animals also could be differentiated for TfD₁ and TfD₂ alleles as the transferrin standard for these alleles could be procured at the later stage of investigation. Fourteen phenotypes were observed in this population of crossbred animals. The number of phenotypes involving the rare alleles viz., TfD₁, TfF and TfB respectively was extremely low. Only one animal each was traced and confirmed for TfD₁D₁ and TfD₁D₂ phenotypes. The present study confirmed the occurrence of TfB and TfF alleles in zebu cattle as was earlier reported by Ashton (1959) and Singh (1974). Braend and Khanna (1968) also recorded the TfB allele in the Gudali breeds of West African cattle and supported the view that zebu cattle and animals of Africa might have had a common origin. In a recent work, Prasad et al. (1978) also showed the presence of these transferrin alleles in these breeds. The presence of TfB and TfF alleles in Haryana, Kankrej, Ongole and Gir cattle has also been reported by Singh et al. (1972).

Tables 4.1.3 and 4.1.4 present the gene frequencies observed for different transferrin alleles in the cattle population studied. Tharparkar animals showed the highest frequency for TfA allele (0.3865) followed by Red Sindhi (0.1791), and the lowest was observed in the Sahiwal animals (0.0827). There seemed to be a balance between TfA and TfD alleles in Tharparkar animals, the frequencies of which were almost equal (0.3865 and 0.3619 respectively), followed by TfE allele (0.2454). However, in case of Sahiwal breed the TfE allele was observed to be highest (0.6724) followed by TfD (0.2379) and TfA (0.0827). Similar trend was also found in the Red Sindhi animals, although the differences of the gene frequencies of these alleles were less wide as compared to the Sahiwal breed. Singh (1974) also reported TfE allele highest (0.704) in Sahiwal whereas in Mariana, Kankrej and Red Sindhi, these were 0.584, 0.570 and 0.476, respectively. Similar observations have also been recorded by Prasad et al. (1978) in these breeds. A high frequency of TfE allele has been also reported by Singh et al. (1972) in other Indian breeds. The gene frequencies of TfB, TfF, TfD₁ were observed to be extremely low, as was reported earlier by these workers.

Table 4.1.4 shows the gene frequencies of transferrin alleles in the population of the crossbred animals. TfA allele was observed to be low (0.12 to 0.15) in these animals. However, the gene frequencies of TfD₂ and TfE alleles were observed to be high. The pooled gene frequency of TfD₁ and TfD₂ (0.4628) in the crossbred

population was found to be closer to the gene frequency of TfD (0.5327) in the undifferentiated population of the crossbred animals. Similar observations have been reported by Prasad et al. (1978) in the crossbred animals.

On comparing the gene frequencies of Tf alleles in Tables 4.1.3 and 4.1.4 for tracing their differences in the indigenous and crossbred animals. Some interesting differences among gene frequencies were observed. The gene frequency of TfA allele in Sahiwal animal was extremely low, while in Tharparkar animals it was observed to be quite high (0.3865). The majority of the animals of the crossbred population studied, originated from the crossing of Sahiwal with Brown Swiss exotic breed. It was observed that the gene frequency of TfA allele was between 0.13 to 0.15. This indicated that to a large extent an increase in the occurrence of this allele in the crossbreds, was due to the exotic inheritance. The gene frequency of TfA in the same range (0.137) also has been observed by Singh (1974) in the crossbred animals.

Similarly, the gene frequency of TfD allele in the indigenous breeds ranged between 0.24 to 0.36, but it was observed to be in the range of 0.45 to 0.53 in the crossbred population. Once again a rise in the gene frequency of TfD allele (pooled) could be attributed to the exotic inheritance in the crossbred animals. However, when we considered the TfE allele, the trend was found to be rather opposite in this population. An increase in the gene frequency of TfE allele in crossbreds could be largely due to the indigenous inheritance.

A nil to low frequency of TfE allele in the exotic animals has been reported by Ashton (1958), Datta et al. (1965) and Ashton (1965). The gene frequency changes of different transferrin alleles in a livestock herd might be useful in giving an idea of the possible changes in the genetic make up of that population at different intervals of times. The utility of transferrin as a genetic marker is comparatively more due to the availability of a multiple allelic series at this locus especially for understanding the structural relationships of various breeds in cattle. This is of greater interest for Indian crossbred populations which have evolved from the germ plasm of several exotic breeds which differed almost entirely in their genotypes from the indigenous Zebu animals. The work on these lines in Indian crossbred population is limited and needs to be investigated in detail before any conclusive findings could be established.

5.1.2. Buffaloes

Table 4.1.5 presents the various phenotypes and the gene frequencies of the transferrin alleles observed in the population of Murrah buffaloes, typed for the transferrin polymorphism. The nomenclature for the transferrin variants was followed as originally suggested by Braend (1965) and later used by Khanna (1969). Homozygous TfDD could be detected and confirmed only in one animal in the population studied. More than 70 per cent animals were observed to be TfKK type. Two alleles for transferrin were detected, which were

reported earlier by Khanna (1969) and Naik et al. (1969). The gene frequency of TfK allele has been observed to be very high (0.8671) as compared to TfD allele (0.1328). Khanna and Singh (1978) reported another transferrin allele TfN and five transferrin phenotypes, i.e. TfDD, TfDK, TfKK, TfDN and TfKN in the order of decreasing mobilities. They also observed that TfK allele was most frequently followed by TfD and TfN alleles. Similar observations have been reported by Basaviah et al. (1978). The present study is in agreement with the findings of the above workers. Similarly, a high frequency of the allele governing the transferrin type having mobility, probably equivalent to TfK allele was reported by Makaveev (1968) in Bulgarian buffaloes (0.84) and Indian buffaloes (0.94); Abe et al. (1969) in Formosan buffaloes (0.85); Zubareva et al. (1969) in Caucasian buffaloes (0.79); Masina et al. (1971) in Italian buffaloes (0.63); Granciu et al. (1972) in Rumanian buffaloes (0.91) and Naik et al. (1969) in Indian buffaloes (0.86). Out of the three variants viz., TfD, TfK and TfN observed in Indian water buffaloes (Khanna, 1973), the TfN was not reported earlier. This variant was observed in a low frequency in Mili and Surti breeds. These two breeds belong to different regions of India and have little similarity in their biometrical and morphological characteristics. In other species of buffaloes from other parts of the world viz., American bison (*Bison bison*), European bison (*Bison bonasus*) and African buffalo (*Syncerus caffer*) the transferrin has been shown to be monomorphic (Braend and Storment, 1964; Braend and Gasparski, 1967; Osterhoff and Young, 1970). A very high gene frequency

of one allele (TfK) in this species might be attributed to some selective advantage for this allele under natural selection. The absence of polymorphism in other species of buffaloes and a low variation in the Indian water buffaloes could be ascribed to a transitional stage of polymorphism at this particular locus. In due course it may become monomorphic as in other species of buffaloes.

5.2. Isolation and Purification of Transferrins

5.2.1. Isolation of Crude Transferrins

Crude transferrins of all phenotypes were isolated as shown in the flow sheet in Fig. 4.2.1.1 which was an outcome of different variations tried at different steps. This was ultimately found suitable for the isolation of bovine transferrin from serum. Initially an effort was made to collect the bulk of the transferrins of all types. For this purpose the methodology for preparation of crude transferrin was modified to reduce the number of steps for shortening the total period for isolation. The method of Sutton and Karp (1965) using rivanol for precipitation of majority of other serum proteins except transferrin and γ -globulin followed by subsequent removal of rivanol by adsorption on starch pad was useful in the isolation work. In few isolation trials the serum was directly precipitated with 0.6 per cent rivanol without diluting it in 1:4 proportion with 5 mM Tris HCl buffer, pH 8.6. However, it was soon discovered that dilution of the sera was essential for complete precipitation of all other proteins as the crude transferrin

isolated without dilution showed sufficient quantities of albumin (Photograph of partially purified transferrin 4.2.1.2). The addition of 0.6 per cent rivanol into serum also precipitated transferrin partially which was indicated by its presence in the precipitate (Photograph 4.2.1.3) on starch gel electrophoresis. However, other workers (Kistler et al., 1960; Stratil and Spooner, 1971, Efremov et al., 1971) did not report such observations by the use of rivanol precipitation. Various methods have been used by different workers for removing rivanol viz., adsorption on charcoal (Kistler et al., 1960), chromatography with Sephadex G-25 (Hoop and Putnam, 1967) by addition of sodium chloride (Stratil and Spooner, 1971). The passing of supernatant through the starch column was found to be simple, convenient and most effective in removing the rivanol completely. However, the starch column should be thoroughly washed with the buffer containing a bacteriostatic agent.

DEAE Sephadex A-50 has been extensively used by majority of the workers for adsorption of transferrin and removal of other soluble proteins from the supernatant obtained after rivanol precipitation. However, certain workers used ammonium sulphate precipitation (Stratil & Spooner, 1971) for removal of γ -globulin in bovines. Efremov et al. (1971) used gel filtration on Sephadex G-150 while Chen and Sutton (1967) used starch block electrophoresis for this purpose. DEAE Sephadex A-50 was found suitable in concentrating the large volume of dilute solution obtained after filtration from starch column. However, in case

of intense hemolysis of sera DEAE Sephadex A-50 also adsorbed other coloured protein which eluted along with transferrin with 0.1 M sodium chloride. The difficulty encountered as a result of contamination of other coloured protein (perhaps hemopexin, as detected by Hatton et al., 1977) was also experienced by Efremov et al. (1971). This has been depicted in Fig. 4.2.2.2.1 where transferrin was located in the third peak. The elution curve of the serum loaded with ^{59}Fe from DEAE Sephadex A-50 confirmed these observations. The separation of these two proteins was found extremely difficult by the use of single molarity eluent (0.1 M sodium chloride), as was also reported by Hatton et al. (1977). However, the fractionation did occur sometimes, as was observed in case of cattle TfD₂D₂ (Fig. 4.2.1.4) where transferrin was located in the third peak. But the nature of the protein in the 1st peak has not been characterised, except that this peak did not show any transferrin in it (Photograph 4.2.2.1). It is possible that both transferrin and the other coloured protein observed in sera got eluted with almost the same molarity of sodium chloride but they were separated in the DEAE Sephadex A-50 column due to gel filtration effect. This could be achieved simultaneously, if the column length could be maintained by keeping a slow rate of flow of the eluent. Under these critical conditions of experiments, the dark reddish brown zone at the top of the A-50 column separated into two coloured zones, on elution with 0.1 M sodium chloride. Although both zones were reddish brown in colour, transferrin resided in the last zone and eluted as 3rd peak. In other experiments where the length of DEAE Sephadex A-50 column got

reduced by shrinkage (A-50) on the application of 0.1 M sodium chloride, the opportunity for these two proteins, to be separated due to gel filtration effect, was lost. They, therefore, usually eluted as single peak. There is no report, so far, in the literature about similar observations on DEAE Sephadex A-50 for bovine transferrin isolation.

5.2.2. Purification of Transferrins

Transferrin preparations isolated from the various trials revealed impurities of different nature. It was experienced during the course of investigations that removal of all the impurities from the partially purified transferrins by the use of one methodology of purification, was not possible. The crude transferrins of various types, isolated in different batches, were purified, therefore, by several procedures of the ion exchange chromatography and gel filtration on Sephadex. Moreover, it was thought important to prepare electrophoretically and immunologically pure bovine transferrins from all the homozygous types, as there could be a possibility of variation in purity due to different methods of isolation and purification. This could also affect the results of characterisation of transferrin types viz., molecular weight, carbohydrate composition, etc.

The difficulty of obtaining haptoglobin-free transferrin from bovine serum was experienced by Efremov et al. (1971). These investigators succeeded in separating the two proteins only by incubation with neuraminidase and subsequent chromatography of the desialylated products.

on DEAE Sephadex. Under the conditions used, the contaminant passed through the ion exchanger but asialotransferrin was retained and recovered later by gradient elution. Several other workers have reported the difficulty in obtaining transferrin free of impurities in bovines viz., Hines (1965), Chen and Sutton (1967), Hatton et al. (1977).

The experiments of gel filtration of crude preparations of transferrins on Sephadex G-100 and G-200 revealed the presence of several peaks. Transferrin was located in the 1st peak which was found to be the major peak of the elution pattern (Fig. 4.2.2.1.1 and 4.2.2.1.2). This was observed for the transferrins from both the species and in case of both the grades of Sephadexes used viz., Sephadex G-100 and G-200. The starch gel electrophoresis of the 1st peak showed the presence of traces of proteins of unidentifiable nature. Hence, it was concluded from these experiments that the impurities of proteins detected along with transferrin, had a very similar molecular conformation and structure and therefore always eluted along with transferrin. The use of Sephadexes purified the crude transferrins only partially. Gel filtration as methodology of purification has been used by several workers viz., Roop and Putnam (1967) (in humans), Efremov et al. (1971) (in bovines).

DEAE Sephadex A-50 was used for the purification of transferrin as early as 1965 by Sutton and Karp, however, Stratil and Spooner (1971) applied sodium chloride molarity gradient to isolate as many as 12

subfractions from a single homozygous bovine transferrin. Various workers have used this methodology for isolating different subfractions of transferrin, from pigs (Stratil and Kubek, 1974), sheep (Spooner et al., 1975), fish (Valenta et al., 1976), bovines (Richardson et al., 1973). The experiment of Stratil and Spooner (1971) was repeated under identical conditions. However, the same number of peaks were not obtained. Fig. 4.2.2.3.1 and 4.2.2.3.2 show the elution patterns obtained by the application of sodium chloride molarity gradient on DEAE Sephadex A-50 column. It was found that only four peaks were eluted when the molarity gradient of sodium chloride was kept from 0.01 to 0.1 M, while an additional peak was observed for 0.01 to 0.2 M sodium chloride gradient. The single peak of these elutions did not reveal single band of bovine transferrin as observed by these workers. However, these experiments showed that the use of sodium chloride molarity gradient on the DEAE Sephadex A-50 had the capacity to subfractionate the transferrin, which otherwise got eluted together as single peak when done with a single molarity eluent (0.1 M sodium chloride).

The use of rechromatography for the purification of crude transferrin on DEAE Sephadex A-50 was found to be useful to a limited extent. Three peaks were observed under critically controlled conditions of experimentation and the transferrin was located in the third peak. However, this occurred rarely when single molarity eluent was used. Fig. 4.2.2.4.1 shows the elution of partially purified trans-

ferrin from the DEAE Cellulose column. Regoeczi et al. (1974) and Hatton et al. (1977) have also used DEAE Cellulose for the purification of crude transferrin. The traces of the impurities, however, could not be removed by the use of DEAE Cellulose ion exchanger.

Hatton et al. (1977) made use of cation exchanger at low pH of 5.9 for separation of transferrin from contaminating hemopexin, as earlier employed by Regoeczi et al. (1974) for the isolation of rabbit serum transferrin. The procedure as outlined by Hatton et al. (1977) was followed and the Fig. 4.2.2.5.1 and 4.2.2.5.2 show the elution of partially purified transferrin of cattle and buffalo respectively, from Sephadex G-50. The iron free transferrin was located in the 1st peak which was also the major peak. The results obtained broadly agree with the results of these workers.

5.2.3. Purity of Transferrins

The purity of the isolated and purified transferrins of all the phenotypes of cattle and buffaloes was tested by starch gel electrophoresis and immuno-electrophoresis. The starch gel electrophoresis revealed the absence of any traceable impurities in these preparations (Photograph 4.2.3.2). While testing the purity of samples by starch gel electrophoresis, it was found that the minimum concentration of the sample required was 4 to 5 mg/ml. This amount was necessary to locate the presence of the transferrin bands on the gels by protein stain. Sometimes, it was difficult to trace the nature of minor peaks by

starch gel electrophoresis. The characterisation of the major peaks having transferrin, however, could be done easily by starch gel electrophoresis. Majority of the workers (Stratil & Spooner, 1971; Efremov et al., 1971; Richardson et al., 1973) have employed this technique for testing the purity of the isolated transferrins.

However, Chen and Sutton (1967) used horizontal polyacrylamide gel electrophoresis and Hatton et al. (1977) used disc polyacrylamide gel electrophoresis for this purpose.

The purity of the purified transferrins was also tested immunolectrophoretically. It could be observed (Photograph 4.2.3.3) that these purified bovine transferrins did not show any additional arc against antitransferrin sera at 6 mg/ml concentration. Higher concentrations of the proteins were not tested, however, Hatton et al. (1977) used bovine transferrin till 2 to 3 mg/ml purity for the characterisation. The additional band detected by starch gel and immunolectrophoresis by Efremov et al. (1971) was attributed to the presence of impurities. Foucier et al. (1976) isolated several vertebrate transferrins to find the immunological relationship with the transferrin isolated from Pleurodalle waltlii. They reported the homogeneity of transferrins at a concentration of 2 mg/ml. Stratil and Spooner (1971) used starch gel electrophoresis for detecting the purity of individual fraction of bovine transferrin. They detected another weak arc along with main transferrin arc by immunolectrophoresis when the transferrin was tested at a concentration of 0.5 per cent.

homozygous transferrin. A molecular weight of 77,500 was reported by them; however, after reduction with 6 M guanidine hydrochloride, they observed a value of 62,200.

It is clear from the results of a number of workers (Feeney and Allison, 1969) that the molecular weights of iron-binding proteins range from 70,000 to 90,000. The molecular weights determined in this study have been found to be in the same range as reported by other workers. Although the molecular weights estimated by gel filtration on Sephadex are not absolutely accurate (Andrews, 1965), it is evident that the molecular weight of cattle transferrin is similar to the molecular weight of hen egg conalbumin and pig transferrin. The molecular weight of 76,000 determined for cattle and buffalo transferrin types on Sephadex G-200 seems to be more valid than the values of 103,000 (Hines, 1965) and 62,000 (Dostal and Hunter, 1970a). Hudson et al. (1973) reported the molecular weight values close to 77,000 for bovine, rabbit, equine and porcine serum transferrin, in dilute aqueous solution as well as in mercaptoethanol-reduced protein in the presence of 6 M guanidine hydrochloride. This is close to the value observed in the present investigations.

However, Efremov et al. (1971) calculated the molecular weight of bovine transferrin, from intrinsic viscosity and sedimentation coefficient to be 67,000. This value was close to the molecular weight of 68,000 reported by Charlwood (1963), and slightly lower than the values (73,200 - 76,000) found by Roberts et al. (1966), the value of

(77,000) observed by Mann et al. (1970) for human transferrin and approximately 76,000 for human, rabbit and frog transferrin by Palmour and Sutton (1971). They obtained the molecular weight of bovine transferrin as 72,4000 when calculated by substitution of the sedimentation and diffusion data into the Svedberg equation. However, Richardson et al. (1973) estimated the molecular weight of bovine transferrin as 77,500.

5.3.2. Analysis of Hexoses, Hexosamines and Sialic Acids

The analyses of different sugars for various transferrin phenotypes of cattle and buffaloes are given in the Table 4.3.2.1. The values of the carbohydrate moieties for all types did not reveal any significant variation for both the species. Also, no significant differences could be detected for all the sugars between cattle and buffaloes. The carbohydrate compositions are similar, and agree well with the analysis of bovine transferrin described by Hudson et al. (1973). The data for the sugar analysis are also in close conformity with the reports of Hatton et al. (1974, 1977) but not with the gas-liquid chromatographic analysis of Graham and Williams (1975), who found approximately two residues of sialic acid, and 13 residues of hexose and nine residues of hexosamine. The sialic acid values are smaller than those reported by Stratil and Spooner (1971) and Chen and Sutton (1967). Stratil and Spooner (1971) subfractionated bovine transferrin AA by DEAE-Sephadex and obtained transferrin peaks varying in sialic acid content from zero to five residues per 77,500 daltons. Chen and

Sutton (1967) used starch block electrophoresis to isolate three bovine transferrin bands and the sialic acid content of these ranged from 2.2 to 4.2 residues per 100,000 daltons. From the present studies and those of Efremov et al. (1971), it seems possible that these higher sialic acid values were a consequence of slight contamination with hemopexin. Hatton et al. (1977) reported that the preliminary analyses of bovine hemopexin gave values of 2.2 per cent sialic acid, 4.0 per cent hexosamine, and 4.8 per cent hexose. Evidently, it was a glycoprotein and thus small quantities of contaminating hemopexin could affect the carbohydrate analyses of transferrin. The small variation in the sugar analysis of both the species did not warrant the need for any statistical analysis.

5.3.3. Radioactive Labelling with Fe⁵⁹

Fig. 4.2.2.2 shows the elution of transferrin labelled with ⁵⁹Fe citrate from DEAE Sephadex A-50 column. The readings of optical density for the various fractions have been shown by round dots while the curve with the crosses shows the $\times 10^{-3}$ for 2 ml fractions. It could be conclusively demonstrated from this experiment that supernatant obtained after Rivanol precipitation of bovine serum, eluted into several peaks from DEAE Sephadex A-50 column under critically controlled experimental conditions. Transferrin was located in the third peak as was evident from the highest \times -counts for this peak region. Although the colour of the protein obtained from 1st peak region was reddish brown, yet it indicated the absence of transferrin

on starch gel electrophoresis. It was evidently clear that serum contained another coloured protein, which was adsorbed on DEAE Sephadex A-50 column along with transferrin. The separation of this protein (presumably Hemopexin) from transferrin was found to be difficult, as this was always eluted along with transferrin under normal experimental conditions. Similar observations have been reported by Efremov et al. (1971) who could separate hemopexin from transferrin by splitting sialic acid with neuraminidase.

It is observed from the elution curve that the γ -C.P.M. from 10th to 25th fraction were also sufficiently high. The starch gel electrophoresis showed the presence of transferrin in the 2nd peak. This could be attributed to the fact that 3rd peak started eluting before the complete elution of the 2nd peak. Or else, the 2nd peak contained another Fe^{59} binding protein. Hatton et al. (1977) reported that bovine serum transferrin isolated from grossly hemolysed sera were often contaminated with heme-hemopexin which eluted very close to the peak containing transferrin from Sephadex G-50. Although the nature of the other protein isolated in 1st peak has not been characterised, it seems there is a possibility that it could be free hemopexin as reported by several other workers viz., Efremov et al. (1971), Hatton et al. (1977).

Table 4.3.3.1 presents the γ -C.P.M. for 2 ml aliquot samples obtained at different stages during the isolation of Fe^{59} labelled transferrin. Sufficiently high γ -counts of the precipitate and further

electrophoretic analysis indicated the presence of transferrin. The precipitation of bovine serum with 0.6 per cent rivanol also precipitated partially transferrin, as has been discussed earlier (5.2.1). The presence of transferrin in this precipitate has not been investigated by Sutton and Karp (1965), Stratil and Spooner (1971). The autoradiographs revealed the transferrin in different stages through isolation. It is observed from this table that sum of the γ -counts for all the stages is approximately equal to the γ -counts for whole sera. This gives the approximate quantitative recovery of transferrin from the serum.

5.3.4. Preparation of Apotransferrins and Iron Binding Studies

Various apotransferrin types of both the species were prepared by the dialysis against sodium citrate - citric acid buffer pH 4.0. The transferrins lost their characteristic reddish brown colour and apoforms became yellowish white. The change of colour was apparently due to the loss of iron from the Fe-Transferrin complex on dialysis at the lower pH 4.0. The Fe-Transferrin complex becomes unstable at acidic pH but remains stable at alkaline pH. The resolution of various apotransferrins as revealed by starch gel electrophoresis was not different from the resolution of Fe-Transferrin type. Presumably, this could either be due to the fact that starch used for the electrophoresis must be having traces of iron which could bind with the apotransferrins and form Fe-Transferrin complex. Or possibly the preparations of different apotransferrins, could contain traces of iron. However,

since the dialysis of the transferrin with the buffer at low pH, was done for sufficiently long time, the possibility of iron having been left due to incomplete dialysis, was remote. It is, therefore, inferred that for detecting the electrophoretic differences between apo and Fe-transferrin forms, the starch should be of highest purity (even iron in traces is undesirable).

Stratil and Spooner (1971) identified three regions on starch gel electrophoresis of apotransferrins. They designated the region nearest to the origin as the transferrin without iron the intermediate region being transferrin with one atom of iron and the fastest region having transferrin with two atoms per molecule. They observed that the separation of apotransferrin depended largely on the buffer system and the concentration of the protein. The low concentrations of apo-transferrins could easily become saturated with iron from starch or buffer during electrophoresis, and the resulting pattern is identical to Fe-Transferrin pattern.

The studies of iron binding with transferrin were performed by two methods. In the first case the Fe^{59} was added into the whole sera of various transferrin type and thus Fe^{59} labelled sera was used for starch gel electrophoresis. However, in the second method the apotransferrins were incorporated with Fe^{59} radioisotope for doing the starch gel electrophoresis. Table 4.3.4.1 gives the λ -counts for the two experiments done for different phenotypes of transferrin.

On an average the net γ -counts were observed to be approximately three times higher in case of whole sera of cattle, while in case of buffaloes this increase in counts was as high as seven times.

High net γ -counts for whole sera samples as compared to the apotransferrins, have been recorded for all the transferrin phenotypes among both the species. Possibly, this might be due to the fact that either sera contained higher concentrations of transferrin in it than the apotransferrin samples or it contained some iron binding protein, other than transferrin. Perusal of available literature revealed the paucity of information on these lines of investigations.

The data indicated an astonishing variation of the net r-counts among various transferrin polymorphs for both the whole sera and the apotransferrin experiments. It should, however, be emphasised that individual samples from different animals differ in the amount of iron which might be already bound to the transferrin in the serum and, therefore, will differ in the amount of ^{59}Fe which can be bound. Neethling and Osterhoff (1966) observed a similar variation of iron-binding among some polymorphic forms of cattle transferrin. In their study, they noticed highest relative iron-binding ability (RIBA) by TfAA followed by TfDD and TfEE, being 2.9, 1.7 and 1.00, respectively. The values determined in our study were 3.48, 2.08, 1.69 and 1.00 for TfAA, TfD₁D₁, TfD₂D₂ and TfEE, respectively, which were comparable with those obtained by these workers. They, however,

failed to ascribe any reason for this variation of iron-binding among different transferrin polymorphs of cattle. In the present investigations, only homozygous forms of transferrin were used for estimating the iron-binding.

Similarly, a wide variation in the net γ -counts among both the species was observed for the experiment done with the apotransferrins, where an entirely different ranking of relative iron-binding ability was observed. Once again, it should be remembered that the apotransferrins prepared from the sera of various transferrin polymorphs might have differed in their ability to release all iron from Fe-transferrin complex. Hence, they might have also possibly differed in the level of Fe^{59} which could be bound to them. These observations, however, should be considered only preliminary and not conclusive until the repeatability of these results is made on large number of animals to establish the significance of the variation in the iron-binding with various phenotypes of transferrin. Buis (1972) failed to appreciate significant differences between the iron binding among various transferrin phenotypes by colorimetric estimation.

The binding of iron with the different bands of single transferrin phenotype was also estimated. A significant pattern of variation was observed in the iron binding of different transferrin

bands which have been designated from the anodic end of the gel. The 3rd band (in case of cattle) had the highest count followed by 2nd, 4th and 1st bands. This could be explained due to the fact that the amount of transferrin also varied in the same order as was apparent from the staining intensity of these bands. In case of buffaloes the counts were highest for 2nd band, followed by 3rd and 1st bands respectively. The variation for differences in the iron binding of different bands of bovine transferrin, has been also reported by Neethling and Osterhoff (1966).

Although variation for the net γ -counts of all the transferrin phenotypes of both cattle and buffaloes existed this was very small due to the low counts in this experiment. The subsequent destaining of the starch gel could have resulted in the loss of Fe^{59} before the elution of different bands.

5.3.5. Autoradiography

The experiments of autoradiography using Fe^{59} radioisotope revealed that the X-ray plate exposed for 24 hours gave the best results (Photograph 4.3.5.1). The increase in the time of incubation period resulted in the diffused zones for transferrin with their respective mobilities (Photograph 4.3.5.2). Similarly, the increase in the amount of concentration of Fe^{59} in the sample gave darker bands in the X-ray plate (Photograph 4.3.5.5). Both these observations can be ascribed to longer exposition to γ radiations emanating from Fe^{59} .

Autoradiography also confirmed the presence of transferrin at various stages of isolation from serum (Photograph 4.3.5.4). Autoradiography has been used by several workers viz., Giblett et al. (1959), Spooner et al. (1970) as one of the definite methodologies for locating the transferrin in the β -globulin region of the starch gel.

5.3.6. Effect of Neuraminidase

The experiment for detecting the effect of neuraminidase enzyme on the mobility of bovine transferrin was specifically designed using ^{59}Fe radioisotope so that the effect of the enzyme could be detected by autoradiography. This was done to avoid higher concentrations of enzyme and transferrin which are normally required while using starch gel electrophoresis. The autoradiograph (4.3.6.1) shows the samples from left incubated for 3, 6, 12, 24, 28 and 72 hours, respectively. Wherever necessary the position of the transferrin bands has been illustrated in the photograph. It was observed that the mobility of the sample incubated for 3 hours was quickly reduced and thereafter the proportionate reduction in the mobility was less with the further increase in incubation period. The effect of treatment with neuraminidase on the electrophoretic mobility of bovine transferrin has been studied by several investigators; Chen and Sutton, 1967; Spooner and Baxter, 1969; Stratil and Spooner, 1971; who have reported similar observations. It was observed that all the three bands moved slowly, without any loss of band due to the treatment with neuraminidase. There was no further decrease in mobility after 72 hours of incubation as observed by other workers too (Chen & Sutton, 1967; Stratil & Spooner, 1971).

The quick decrease in the mobility of transferrin the sample incubated for 3 hours with neuraminidase enzyme could be explained by the availability of the enzyme in the initial stages of the reaction. Presumably, major portion of terminal sialic acid is detached in the earlier stage of the treatment. However, no reports are available where the concentration of neuraminidase enzyme has been increased in the middle of the experiment. Also no work is cited in the literature to show whether this increase in the concentration of enzyme, further reduces the mobility of bovine transferrin bands.

5.4. Immunological Comparisons

5.4.1. Production of Antisera

The antisera against the transferrin were raised in rabbits. Two types of antisera were produced for studying the immunological differences of the various transferrin phenotypes of cattle and buffaloes. The first type of antiserum was produced by the immunisation of rabbits with pooled sera having equal proportion of all the transferrin types. These were called pooled cattle serum and pooled buffalo serum. For the second type of antiserum, the rabbits were immunised against the pooled cattle transferrin and pooled buffalo transferrin, respectively. The partially purified freeze dried transferrins of all the types were mixed in equal proportion and the pooled mixture was used for raising the antiserum specifically against the transferrin. For each class of antiserum two rabbits were immunised simultaneously. Only one rabbit

which was immunised against pooled cattle sera died during the course of immunisation. The immunisation was done subcutaneously in the footpad using Freund's complete adjuvant. The booster injections were given, however, intravenously after four weeks.

All the rabbits produced the antisera which gave the titre till 1/8th dilution. Photograph 4.4.1.1 shows the immunodiffusion on 1 per cent agar gel in normal saline for testing the titre of anti-pooled cattle sera (Set No. 1 and 4) and antipooled cattle transferrin sera (Set No. 2 and 3). The antigen-antibody reaction formed well defined precipitin arcs. Multivalent antisera gave several arcs. Specific antiserum against transferrin gave only one major arc and a diffused zone which disappeared at 1/4th and 1/8th dilutions. The titre of these antisera was tested against pooled cattle sera in the antigen well. Similarly, photograph 4.4.1.2 shows the titre of the two type of buffalo antisera. The multivalent sera showed multiple arcs in this case too (Set No. 1 and 4). The specific antisera against buffalo transferrin showed only faint arc and a diffused zone till 1/8th dilution. This was in contrast to the results obtained for specific anticattle transferrin sera, where distinct arcs were observed.

The presence of only faint arc and a diffused zone could be presumably due to several reasons, in that the buffalo transferrin had lower antigenicity as compared to cattle transferrin, or the rabbits used for antibuffalo transferrin sera did not respond very favourably to produce high concentration of antibodies. The presence of multiple arcs in case of multivalent sera showed the multiple antigenic

factors available and also antibodies against these factors. The characteristic absence of other minor arcs for the antitransferrin sera, indicated the presence of specific antibodies against transferrin in the antisera. In the absence of any work reported on buffalo transferrin it is difficult to compare the results obtained in the present study. Antiserum against cattle transferrin has been specifically raised in rabbits by several workers (Hines, 1965; Stratil & Spooner, 1971). VanEijk et al. (1972) produced antisera to study the immunological relationships of rat, rabbit, fish and human transferrins. Fourrier et al. (1976) used antiserum against transferrin of Pleurodeles waltlii to study the immunological relationship with 25 other animal transferrins from cyclostome, fish, amphibian and mammalian species.

5.4.2. Immunodiffusion

The results of immunodiffusion for cattle and buffalo transferrins have been shown in the photographs 4.4.2.1 and 4.4.2.2 respectively. All the four cattle transferrin types viz., AA, D₁D₁, D₂D₂ and EE gave one prominent arc and some very faint arcs. Apparently the prominent arcs were formed due to transferrin and the other faint arcs could be due to the traces of impurities present in the partially purified transferrin types. In the lower right set antibuffalo transferrin sera was charged in the centre well against the cattle transferrins. The presence of the arcs for all the four cattle transferrins indicated that the cattle transferrin AA, D₁D₁, D₂D₂ and EE had cross-reacted with the antibuffalo transferrin sera. However, the

intensity of crossreaction was not observed to be very strong as the arcs in this case were less prominent when compared to cattle transferrin that had reacted with anticattle transferrin sera.

So far there is no report available in literature to confirm our investigations. The results of immunodiffusion did not reveal any immunological differences among the four cattle transferrin types. Immunological comparisons made by Hines (1965) revealed that all transferrin band samples observed had one antigenic component in common. Transferrin band samples from genotypes TfAA and TfDD appeared identical. They observed that transferrin band sample from TfEE, however, failed to form one of the lines common to samples TfAA and TfDD.

The results of immunodiffusion for the buffalo transferrins have been shown in photograph 4.4.2.2. These are similar in nature to those obtained for cattle transferrins. Several arcs were formed when specific transferrin antisera was used against the partially purified buffalo transferrins DD and KK type (lower two sets). This evidently revealed the presence of traces of impurities in the partially purified buffalo transferrins. However, when these were tested against multivalent sera (upper right set), they showed only one prominent arc and other minor arcs were not present in this case. The presence of identical arcs for both TfDD and TfKK type indicated the immunological similarities of the two polymorphs of the buffalo transferrin. On comparing the results of the two types of antisera, it is inferred that the specific transferrin antisera having higher

concentrations of specific antibodies against buffalo transferrin could detect the traces of impurities which were otherwise not detected when multivalent sera was used. The nature of the impurities, however, was not characterised.

5.4.3. Immunolectrophoresis

The immunological characterisation and the purity of the finally purified transferrins from both the species was done by immuno-electrophoresis. The experiments concerning purity have been discussed in section 5.2.3. Photograph 4.4.3.1 shows the results of immunolectrophoresis for pooled cattle sera against antipooled cattle sera in the upper trough and pooled buffalo sera against antipooled buffalo sera in the lower trough. Multiple arcs were formed in both these experiments which indicated the polyvalent antibodies present in the antisera. The number of arcs in case of buffalo antiserum was, however, more than the cattle antiserum. This evidently reflected that buffalo antiserum contained broader spectrum of antibodies than the cattle antiserum. Although no specific characterisation of these arcs was done but presumably they represented various proteins of the serum.

The experiments to study the cross reaction of cattle and buffalo transferrins with anticattle and antibuffalo sera were also performed by immunolectrophoresis. Photographs 4.4.3.5 and 4.4.3.6 show the results for these experiments respectively. It was observed that the transferrins from both these species cross reacted with antisera from other species. This apparently revealed the close antigenic

relationship of transferrin molecule from cattle and buffaloes. The intensity of the arcs was, however, not so well defined, as compared to the results obtained when the cross-reaction was checked against the specific antisera against cattle transferrin and buffalo transferrin. Photographs 4.4.3.7 and 4.4.3.8 show the results for these experiments. The immunological nature of buffalo transferrin has not been reported so far in literature. Hence, much more specific and detailed studies are required before the characterisation of the protein from this species can be established.

An experiment was also performed to see the effect of heat on the antigenic nature of the various transferrins. The results have been shown in the Photographs 4.4.3.9 and 4.4.3.10. These indicated that the transferrin types which were subjected to heat treatment did not give any reaction against antisera. This can be satisfactorily explained by fact that the transferrin types which were heated lost their antigenic nature. This could be attributed to denaturation of the protein molecule and ultimately the subsequent loss of the antigenic site.

Photographs 4.4.3.11 and 4.4.3.12 show the results of experiment which was done to locate the antigenic electrophoretic differences of the various transferrin types. These were pooled in the combinations of two and three transferrin types charged together in one antigen well. These revealed that one main precipitin arc appeared for all combinations of two types together in one well (Photograph 4.4.3.11). Some minor arcs of lower intensity were also observed along with the main arc. However, the intensity of the minor

arcs increased slightly when the combinations of three and four transferrin types were charged together in one antigen well (Photograph 4.4.3.12). This could be explained by the overlapping of several less intense minor arcs when higher combinations of various transferrins were pooled together in one antigen well. This experiment thus indicated that the differences, if any, in the immunological nature of various cattle and buffalo transferrin phenotypes were of very minor nature although the presence of the small arcs did indicate these differences. However, due to paucity of information on these aspects in the literature, these observations should be considered only preliminary, before any conclusive evidence can be established.
