
CHAPTER III

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MATERIALS AND METHODS

3.1. Animals

The animals used for the present investigations were maintained at National Dairy Research Institute, Karnal. In all 1418 animals, belonging to Sahiwal, Red Sindhi and Tharparkar breeds and their crosses with Holstein, Brown Swiss and Jersey breeds, were typed for transferrin polymorphism, to locate the homozygous transferrin types of TfAA, TfD₁D₁, TfD₂D₂ and TfEE in cattle and TfDD (faster moving) and TfKK (slower moving) type of buffaloes. Some of the standard transferrin samples used in our laboratory were compared with the transferrin standards from Blood Group Laboratory, Wageningen, Holland.

3.2. Serum Samples

The blood samples were collected from the animals which had been typed earlier for transferrin polymorphism. Approximately 300 ml blood was collected from the jugular vein in a 500 ml sterilised blood transfusion bottle with an adaptor. The bottles were left for allowing the blood to clot. At this stage necessary precaution was taken to avoid any shaking of the bottles to check the hemolysis of the clots. Blood clots were left in a refrigerator overnight for serum separation. The clear serum was separated next day morning, and the pooled sera of one transferrin type, was centrifuged to remove cells. This serum was transferred to 500 ml reagent bottles and stored in a deep freeze maintained at -15° C, till it was used for the isolation of the transferrin. For most of the isolation trials the

blood was collected from the single animal. However, occasionally the blood was also collected and pooled from two animals of same transferrin type.

3.3. Isolation of Transferrin

3.3.1. Rivanol Precipitation

The procedure followed for the isolation of transferrin was essentially as given by Sutton and Karp (1965) with necessary modifications employed to suit our working conditions. To about 300 ml serum few mg of ferric chloride was added in order to saturate the transferrin with iron thus increasing its stability at room temperature. This was diluted with 3 parts of 5 mM Tris HCl buffer, pH 8.6. To the diluted sera, an equal volume of 0.6 per cent Rivanol (2-ethoxy-6,9 dihydroxyacridinelactate) solution in 5 mM Tris HCl buffer, pH 8.6, was slowly added with stirring. A gummy precipitate appeared on the addition of Rivanol solution which quickly settled down and the clear supernatant was decanted off in a beaker. This was passed through a starch column (5.0 cm x 20.0 cm) which had been equilibrated with 5 mM Tris HCl buffer, pH 8.6. The unhydrolysed potato starch was soaked in the buffer for few hours and filled into the column. The supernatant after Rivanol precipitation, was passed through this column. Rivanol remained adsorbed at the top of column bed which could be easily observed due to its yellow colour. The clear, reddish brown

filtrate, indicating the presence of transferrin, was collected and used for ion exchange chromatography.

3.3.2. Chromatography on DEAE Sephadex A-50

The reddish brown filtrate from the starch column was a very dilute solution and this was concentrated by making use of DEAE Sephadex A-50 chromatography. A column of size 2.6 x 45 cm was filled with DEAE Sephadex A-50, ion exchanger which had been equilibrated earlier in Tris HCl buffer, pH 8.6. The DEAE Sephadex A-50 column was washed with the same buffer until the pH of the emerging buffer was 8.3 or higher. The starch filtrate was passed through this column. Transferrin remained strongly absorbed at the top of the column bed as indicated by its intense reddish brown colour. It was eluted with 0.1 M NaCl in Tris HCl buffer. Usually 10 ml fractions were collected and the readings of these fractions were taken at 280 nm and 465 nm on a Hitatchi Spectrophotometer model 100-20, or at 412 nm on Spectrocol Colorimeter. In the beginning, Klett Summerson Colorimeter was also used with green filter.

3.3.3. Dialysis

The fractions containing transferrin, were pooled and dialysed against 8 to 10 litres of distilled water which was adjusted to pH 8.0 or above by the addition of few drops of sodium hydroxide solution, to prevent the dissociation of Fe-Transferrin complex. The dialysis

was done generally at room temperature in the cellulose sacs from Arthur H. Thomas Co., using a magnetic stirrer.

3.3.4. Freeze Drying and Storage

The dialysed protein material from the peaks having transferrin, were transferred into Petri dishes and deep frozen. These were then lyophilised. The freeze dried protein was scrapped out from the Petri dishes with the help of a sharp spatula and transferred into clean glass vials which had been sterilised earlier in hot air oven. These vials containing the transferrin were properly sealed and kept in plastic boxes to avoid any absorbance of moisture. The boxes having the vials were stored in refrigerator maintained at -15°C , until further use.

3.4. Purification of Transferrin

The gel filtration and ion exchange chromatography were employed to remove traces of impurities from the crude transferrin protein obtained earlier.

3.4.1. Re-chromatography on DEAE Sephadex A-50

A column ($2.6 \times 45 \text{ cm}$) was filled with DEAE Sephadex A-50 which had been preswollen in 5 mM Tris HCl buffer, pH 8.6 for several hours. Sufficient quantity of this buffer was allowed to pass through the column till the pH of emerging buffer was the same.

Usually 300 to 500 mg of the crude transferrin sample which had been isolated earlier by Rivanol precipitation, was dissolved in 8 to 10 ml of Tris HCl buffer. This was centrifuged to remove the undissolved protein and the clear reddish brown supernatant of transferrin was used for loading, gradually on top of the column, with the help of a micropipette. Necessary precautions were taken while loading the protein, to avoid any stirring of the top layer of the gel in the column. The column was washed with few ml buffer to wash down the protein sample completely. About 100 ml of buffer was passed to remove any unadsorbed soluble proteins. Transferrin was then eluted with 0.1 M sodium chloride in Tris HCl buffer. The fractions of 10 ml were collected and the readings were taken at 280 nm on a Hitachi Spectrophotometer. All the fractions containing transferrin were pooled, dialysed and freeze dried, as detailed earlier.

3.4.2. Gel Filtration on Sephadex

Sephadexes G-100 and G-200 were used at different times for gel filtration experiments. The columns of different diameters and lengths were used in different experiments of gel filtration. The gel was swollen in 0.086 M Tris HCl - 0.2 M NaCl, pH 8.6 buffer and the technique was followed as described in the Pharmacia Fine Chemicals booklet on Gel Filtration. About 300 mg of freeze dried crude transferrin was dissolved in 5 to 8 ml of same buffer and the clear solution of protein after centrifugation, was carefully loaded on the column. The column was connected to the same buffer and the fractions

of 10 ml were collected. The fractions were monitored at 280 nm and 465 nm.

3.4.3. Chromatography on DEAE Cellulose

Several trials of DEAE cellulose chromatography were done for the removal of contaminating impurities of albumin and other trace proteins. The procedure as detailed by Regoeczi et al. (1974) was followed for this experiment. A column of size 1.6 x 30 cm was filled with preswollen and equilibrated DEAE cellulose (Sigma) in 0.01 M Tris HCl buffer, pH 8.0. Three hundred mg of freeze dried crude transferrin was loaded on the column. About 100 to 150 ml of the same buffer was allowed to pass through the column. A gradient of 0.01 M Tris HCl - 0.1 M Tris HCl pH 8.0 was connected. The mixing chamber contained 400 ml of 0.01 M Tris HCl. Ten ml fractions were collected and the readings were taken at 280 nm. The fractions of the peak showing the presence of transferrin were pooled, dialysed and freeze dried.

3.4.4. Chromatography on Sephadex G-50

Sephadex G-50 was used to remove hemopexin, a major contaminant of transferrin. The complete separation of these two proteins was observed to be virtually impossible by the use of anion exchangers. The procedure of Regoeczi et al. (1974) with some modifications, was found to be useful for this purpose. About 300 to 400 mg of the crude

protein was dissolved in few ml of 0.01 M sodium citrate buffer pH 5.9. This was dialysed at 4°C for 6 hours against the same buffer. A column of size 2.2 x 30 cm was filled with Sephadex G-50 which had been preswollen earlier in the same buffer for several hours. The protein solution was charged on the column and washed with about 200 ml of the same buffer. A gradient elution system containing (mixing chamber 600 ml 0.01 M Sodium citrate buffer pH 5.9, limit solvent 0.1 M Sodium citrate buffer, pH 5.9) was connected to the column. Ten ml fractions were collected and monitored at 280 nm. The fractions of the peak showing the presence of the transferrin were pooled, dialysed and freeze dried.

3.4.5. Isolation of Transferrin Bands (peaks)

The method as described by Stratil and Spooner (1971) was used for the isolation of different bands (peaks) of single homozygous transferrin. A column size (2.8 x 55 cm) was filled with DEAE Sephadex A-50. It was equilibrated with 0.05 M Tris HCl buffer pH 8.0. About 450 mg of freeze dried transferrin was dissolved in same buffer and charged on the column. A gradient of 0.01 M to 0.1 M Sodium chloride (mixing chamber 800 ml) was connected to the column. A flow rate of 45 to 50 ml/hour was maintained and 10 ml fractions were collected. The fractions of different peaks, representing the different bands of homozygous transferrin, were pooled, dialysed and freeze dried.

The experiments of column chromatography and dialysis of transferrin were done generally at room temperature or in cold room.

3.5. Purity of Transferrin

3.5.1. Electrophoretic - The purity of transferrin was checked electrophoretically.

Starch Gel Electrophoresis : Horizontal starch gel electrophoresis was carried out as described by Smithies (1955) with some modifications. Several buffer systems were used during the course of the study. These have been given below:

(i) Gel buffer - 0.014M Tris and 0.003MCitric acid, pH 7.3.

Electrode buffer - 0.3M Boric acid 0.1M sodium hydroxide, pH 8.7 (Poulik, 1957).

(ii) Gel buffer - 0.019 M Tris, 0.016 M Cacodylic acid, pH 7.6.

Electrode buffer - 0.3 M Boric acid, 0.1 M Sodium hydroxide, pH 8.6 (Kristjansson and Hickman, 1965).

(iii) Gel buffer - 0.043MTris, 0.012MCitric acid, 0.002MLithium hydroxide, 0.024MBoric acid, pH 7.4.

Electrode buffer - 0.092M sodium hydroxide and 10.3M Boric acid (Golderman, 1970).

(iv) Gel buffer - 0.039M Tris, 0.007MCitric Acid, pH 8.0.

Electrode 0.018M Lithium hydroxide, 0.191M Boric acid (Ashton, 1965).

(v) Gel buffer - 0.165 M Tris - 0.007 M EDTA (Ethylene diaminetetra acetic Acid) - 0.025 M Boric Acid pH 9.0.
Electrode - Same buffer in 1:1.5 dilution (Gahne et al., 1960).

However, for the routine testing of serum or for detection of transferrin in different fractions during isolation work, the buffer system of (i) Poulik (1957) was found to be very useful. Unhydrolysed starch from Patal Chest Institute, Delhi, was hydrolysed in laboratory, according to Smithies (1955) and this was used for routine starch gel electrophoresis. The electrophoresis was done at room temperature.

For transferrin typing of the animal population studied, the starch gel was made in a glass plate of size 17.5 cm x 16.0 cm, on which three layers of glass strips (size 16.0 x 0.8) were fixed with the help of an adhesive (Quick Fix). Starch gel was made of 12 to 13 per cent of the laboratory hydrolysed starch in the gel buffers given earlier. About 12 samples were charged in the gel of this size and the electrophoretic run was made at 3 to 4 m A current/cm. The gel was taken out after about 2 to 3 hours run when the borate boundary had moved about 10 to 11 cm apart from the origin. The gel was cooled for 10 to 15 minutes in a refrigerator and sliced with the help of a steel wire. The lower layer of starch gel was stained with 1 per cent Amido Black in Methanol, Water, Acetic Acid in 5:5:1 proportion for 8 to 10 minutes. The extra stain was drained out and the gel was left

for destaining in the washing solution (Methanol: Water: Acetic Acid in the proportion of 5:5:1) for about 24 hours. The transferrin phenotypes were recorded from the washed gels. Smaller glass plates of size 17.5 cm x 8.0 cm were used for occasional detection of transferrin in different isolated fractions during the purification or isolation. About 4 to 6 samples could be conveniently run on this plate.

3.5.2. Immunological Purity

3.5.2.1. Antiserum Production against Pooled Sera of all Transferrin Types

The antiserum against the pooled sera of all the transferrin types was produced in rabbits. The sera from the cattle belonging to four transferrin type viz., TfAA, TfD₁D₁, TfD₂D₂ and TfEE was pooled in equal volumes. Similarly the sera of buffaloes of TfDD and TfKK type was also pooled. These pooled sera were stored in several clean vials in a refrigerator for immunisations. The procedure as detailed in Methods in Immunology and Immunochemistry, Vol. I, for multivalent serum production, with some modifications in the immunisation schedule followed, is given below.

Two rabbits each were immunised for both anticattle serum and antibuffalo serum production. 0.5 ml of pooled sera of all Tf types was thoroughly mixed with 0.5 ml complete bacto adjuvant with the help of a shaker. One ml of this suspension was injected at 3 to 4 sites in the sole of foot intradermally in each rabbit. Similarly, 1 ml suspension of pooled buffalo sera and adjuvant was injected in

two rabbits. Exactly after one month 0.5 ml of pooled sera was injected slowly intravenously into the marginal ear vein of each rabbit. Next day 1.0 ml of pooled sera was given intravenously and on the third consecutive day 1.5 ml of pooled sera was injected in the same manner. One week after the first intravenous injection, these rabbits were bled by giving a small cut in the marginal vein of the other ear and about 5 ml of blood was collected for checking the development of antibodies. It was observed by immunodiffusion on agar plate that antibodies had developed and then the rabbits were bled in bulk after two days to harvest the antisera. In the following week only two booster injections of 1 and 2 ml pooled sera were given intravenously on the two consecutive days. Three days after the last injection, the rabbits were once again bled for harvesting the multivalent antisera. The sera thus collected were tested for their titre and pooled in glass vials which were kept frozen at -15°C , till used.

3.5.2.2. Antisera Production against Pooled Crude Transferrin Protein Types

The antiserum against the pooled crude transferrin types of both cattle and buffaloes were produced by immunising the rabbits according to the procedure given by Fourcier *et al.* (1976). A sample of 4 mg of pooled transferrin having all the transferrin types in equal amount, was dissolved in 1 ml normal saline. For a single rabbit 0.5 ml of protein solution was thoroughly mixed with 0.5 ml complete

bacto adjuvant, with the help of a shaker. One ml of this suspension was injected at 3 to 4 sites in the sole of the foot. Exactly after one month 1 ml of the suspension as prepared above was given subcutaneously in the thigh region. Next day, 1 ml of protein solution having 4 mg pooled protein per ml in normal saline, was injected intravenously into the marginal vein of the ear. The rabbit was bled for testing the development of antibodies four days after last injection. The serum was harvested the next day. The following week two booster injections were given intravenously on the consecutive days having 2 mg and 3 mg of transferrin per ml in normal saline. Again the rabbits were bled in bulk to harvest the antisera. The antiserum against pooled buffalo transferrin types was also produced in the same manner by immunising the rabbits. The antisera thus collected were checked for their titre and then pooled in glass vials. These glass vials were stored at -15°C in a refrigerator, until further use.

3.5.2.3. Immunodiffusion

Double-diffusion in agar gel was performed basically as described by Ouchterlony (1958), using some of the modifications in the technique. Glass slides of size 8.0 x 8.0 cm were used for the immuno-diffusion tests. The slides were thoroughly cleaned in alcohol: ether (1:1 mixture) and dried. These were then put on the levelled platform. 0.5 per cent Noble agar having 0.1 ml of 0.1 per cent phenol in 100 ml of agar solution was used for coating the slides with the help of a

cent-
brush. One per agar gels were prepared by taking 1 g of special agar-Noble from Difco Laboratories, U.S.A., in 100 ml normal saline, in a conical flask. This was warmed on a boiling water bath, with occasional stirring. Heating was continued till it became clear. At this stage 0.02 per cent sodium azide was added. Ten ml each of this agar solution was added to ten clean glass tubes and cotton plugs were put on the tubes. Approximately 10 ml solution was applied to make a 0.15 mm thick layer of agar gel on the 8 x 8 cm glass slide. These tubes were kept in cold, till used. At a time single tube having 10 ml solid agar gel was warmed up in a boiling water bath till the agar gel liquified. This was poured, carefully on the slide placed on the levelled platform. After the gel solidified, it was put in Petri dish having some water and transferred inside a refrigerator. After about 2 to 3 hours, punches were made by the use of an immuno-diffusion block. A single slide accommodated four such punches. Different experiments of immune-diffusion were done by charging the wells in the agar gel, with the help of a microsyringe. About 5 to 10 μ l antiserum was charged in the centre well and protein solutions of different transferrin types were charged in the other wells. The charged slides were put in a Petri dish having some water to avoid drying of the gel and this Petri dish was then placed in a refrigerator for 16 to 18 hours for diffusion. The development of the precipitin arcs was complete by 30 to 36 hours.

Staining of Gels

The slides with the developed precipitin arcs were dried by putting a moist filter paper on them. These slides were washed

thoroughly in normal saline, to remove unreacted proteins from the gels and rinsed in water to remove sodium chloride. These were stained in 1 per cent Amido Black stain having following composition for 2 to 3 minutes.

1 g Amido Black stain
 7.2 g Sodium Acetate/450 ml distilled water
 450 ml Acetic Acid.

After washing the extra stain from the slides with washing solution (5:5:1 Methanol: Water: Acetic Acid) the slides were dried. These slides were photographed.

3.5.2.4. Immunoelectrophoresis

Immunoelectrophoretic experiments were done according to the technique detailed by Scheidegger (1955). The following buffer systems were used for making 1.5 per cent agar gel on the slides of size 8 x 8 cm.

(1) Continuous Buffer System pH 8.6

Sodium barbitone	12.8 g)
Barbitone	1.66 g) per litre, pH 8.6
Calcium lactate	0.384 g)

(2) Discontinuous Buffer System

Electrode buffer

Barbitone	1.38 g)
Sodium barbitone	0.76 g) per litre, pH 8.6
Calcium lactate	0.38 g)

Gel buffer

Barbitone	1.66 g)
Sodium barbitone	10.5 g)
Calcium lactate	1.54 g)

per litre, pH 8.6

The 1.5 per cent agar gels were made in the gel buffer in the same manner as described for immunodiffusion. About 2-3 μ l of the protein solution having 1 to 6 mg protein per ml, were charged in the well and the electrophoresis was done in a small migration chamber for 70 minutes at 70 volts. After the run the agar layer from the troughs was removed with the help of the gel knife. Approximately 50 to 60 μ l of antiserum was charged per trough in the gel. The slides were put in Petri dish and kept in a refrigerator for 24 to 36 hours. The staining and photography of these was done exactly in the same manner as described earlier.

3.6. Characterisation of Transferrin Types3.6.1. Estimation of Molecular Weight

The molecular weights of transferrin types of cattle and buffaloes were estimated by gel filtration chromatography on Sephadex G-200, according to Andrews (1965). A glass column of size 2.4 x 70 cm was packed to a height of 62 cm with preswollen Sephadex G-200 in 0.1 M Tris HCl buffer having 0.5 M sodium chloride, pH 8.3. About 200 ml of the same buffer was passed to equilibrate this column. The void volume of the column was determined by passing Dextran blue-2000. This column

was standardised by passing Bovine Albumin, Egg Albumin, Human Transferrin, Bovine Globulin and Trypsin Inhibitor (Soy Bean). Ten to 15 mg of the protein samples were dissolved in 2 ml buffer and applied on the top of the Sephadex bed after the excess buffer had drained out. All the necessary precautions were taken to avoid unnecessary stirring of the bed. Five ml fractions were collected at a flow rate of 12 ml/hr. The readings were taken at 280 nm on a Hitachi Spectrophotometer and the elution volumes were calculated from the peaks. Similarly, the various transferrin types of cattle and buffaloes were passed through the calibrated column and the elution volumes were calculated. Molecular weights were estimated from the standard curve prepared from the proteins of known molecular weights.

3.6.2. Analysis of Hexoses, Hexosamines and Sialic Acids

3.6.2.1. Total Sugars

The total sugars were estimated according to the method of Dubois *et al.* (1956). Two ml of the protein sample, having sugar content approximately between 10 μ g and 75 μ g was pipetted into a clean test tube. 0.05 ml of 80 per cent redistilled phenol was added into it. Five ml of concentrated sulphuric acid was added rapidly into the liquid surface rather than the sides of the test tube. This helps in obtaining good mixing. The tubes were allowed to stand for about 10 minutes and shaken. These were placed in a water bath maintained at 25 to 30°C for 10 to 20 minutes. The yellow orange colour developed

remained stable for several hours. The readings were taken at 490 nm on a Hitatchi Spectrophotometer. Blanks were prepared by substituting distilled water for the sugar (protein) solution. A standard curve was prepared by taking authentic sample of glucose, between the range of 10 μ g to 75 μ g.

3.6.2.2. Hexosamines

Hexosamines were estimated by the method of Reissig et al. (1955). The protein was hydrolysed at a concentration of 0.2 - 0.4 per cent in 3.5 M HCl for 4 hours at 105° C. Excess of HCl from the hydrolysis was neutralised by 4 M Sodium hydroxide. To 1 ml of the sample, 0.2 ml of Potassium tetraborate solution (0.8 M, pH 9.1) was added, in a test tube. The tubes were heated on a vigorously boiling water bath for 3 minutes and cooled under the running tap water. Six ml of the p-dimethylaminobenzaldehyde reagent was added and immediately after mixing the tubes were placed in a water bath maintained at 37° C for 20 minutes. The tubes were cooled under tap water and readings were taken immediately at 585 nm. The blanks were taken for the unhydrolysed protein sample and treated in the same manner. N-acetylhexosamine concentration was determined from the standard curve prepared using a known sample of N-acetylglycosamine-HCl.

3.6.2.3. Sialic Acids

Sialic acid content of the transferrin was determined by the thiobarbituric acid assay method of Warren (1959). The protein samples

were hydrolysed in 0.05 M Sulphuric acid at 85° C for 1 hour. The standard, N-acetyl-neuraminic acid (NANA) was subjected to hydrolysis in the same manner. 0.1 ml of 0.2 M periodate solution was added to 0.2 ml of sample containing upto 0.05 μ mole of N-acetylneuramine acid. The tubes were shaken and kept at room temperature for 20 minutes. One ml of 10 per cent arsenite solution was added and the tubes were shaken until the yellow brown colour developed and disappeared. Three ml of thiobarbituric acid solution was added, the tubes were shaken and heated on a vigorously boiling water bath for 15 minutes. The tubes were removed and placed in a cold water for 5 minutes. The colour developed, was extracted by the addition of 4.3 ml of cyclohexanone. The tubes were shaken and centrifuged for 3 minutes. The clear upper cyclohexanone phase was pipetted out and the intensity of the colour was measured at 549 nm on a spectrophotometer. The sialic acid content was calculated from the standard curve using N-acetylneuraminic acid (0-20 μ g).

3.6.3. Radioactive Labelling with Fe⁵⁹

Radioactive labelling of transferrin was done with Fe⁵⁹ citrate obtained from Radiochemicals, Amersham. The isolation of transferrin AA type was done from the serum loaded with Fe⁵⁹. To 50 ml serum, 300 μ c of Fe⁵⁹ was added at the rate of 6 μ c/ml serum. The isolation of transferrin was done according to Sutton and Karp (1965) as described earlier. Ten ml fractions were collected and the readings of these fractions were taken at 280 nm. The γ -counts of

2 ml aliquot from these fractions were taken on a γ -counter. The fractions showing the high γ -counts were pooled, dialysed and freeze dried. The isolated TfAA loaded with Fe^{59} was used for other experiments.

3.6.4. Preparation of Apotransferrin

The apotransferrins of all the transferrin types of cattle and buffaloes, were prepared according to Stratil and Spooner (1971). One hundred mg of protein for each type was dissolved in 2 ml 0.25 M sodium citrate citric acid buffer pH 4.0 and to this 2 ml distilled water was added. This was transferred into a dialysis tube which was sealed from one side. The dialysis of the proteins was done against four changes of same buffer and then against distilled water. The dialysed apotransferrin types were freeze dried and used for iron binding experiments.

3.6.5. Iron Binding Capacity

The iron binding of Fe^{59} with transferrin was done according to Neethling and Osterhoff (1966). The quantitative binding of Fe^{59} with transferrin was done both by the addition of Fe^{59} to whole sera of all transferrin types and also by the addition of Fe^{59} to the apotransferrins. For the first experiment 6 μ c of Fe^{59} was added to 1 ml of serum of each transferrin type. The electrophoresis of the loaded sera was done as described earlier. After the run the gel

was sliced and the bottom slice was used for staining and an area of 7.5×5.0 cm from the zone of transferrin was cut with a blade from the upper slice. The gel for control was cut from the region just below transferrin. The slice of transferrin zone was cut into small pieces and put into small glass tubes. The counts were taken on a γ -counter for these tubes. The background counts of the equipment were taken both before and after taking actual counts for the material.

The iron binding of Fe^{59} was also measured in the same manner using the apotransferrin solutions.

The quantitative binding of Fe^{59} with individual bands of a single transferrin type was also measured by the same method. For this experiment the bands of different transferrin types were eluted with the help of a blade very carefully after destaining the gel in methanol water (7:3) to prevent the loss of radioactive iron. The areas below the slowest and above the fastest moving bands served as controls in the determination of radioactivity.

3.6.6. Autoradiography

The autoradiography of the Fe^{59} loaded sera samples was done as described below. Fifteen μ c of Fe^{59} citrate was added to each one ml transferrin serum sample. The electrophoresis was done as usual for $2\frac{1}{2}$ hours at $25 m A$. The upper slice of the sliced gel was thrown away and the lower slice was transferred to another glass plate. This gel along with the glass plate support was put inside a cellophelin

envelope and the paper was slowly adhered to the surface of the gel such that no air bubble remained. Indu X-ray film of the same size as that of the gel was put on this with the emulsion side of the film facing towards gel. Two rubber bands were put on the two ends of the gel to avoid shifting of the X-ray film from the gel. The whole assembly was put inside a black envelope which was again put in another black envelope. This was put inside a small photographic paper box and left inside a refrigerator for 16 to 18 hours. The X-ray film was developed in Kodak formula D8 developer for 3 minutes. The positive prints were taken from the autoradiographs thus obtained. All the experiments of autoradiography were done in the dark room of the photography laboratory.

3.6.7. Treatment of Transferrin with Neuraminidase

The experiment to see the effect of neuraminidase on the mobility of transferrin, was done as described by Stratil and Spooner (1971). Four mg of pure TfAA type transferrin loaded with Fe⁵⁹ was dissolved in 0.5 ml of 0.2 M acetate buffer, pH 5.4 having 0.04 M calcium chloride. This was taken in 5 ml Corning vial with a lid. About 40 to 50 units of neuraminidase enzyme (Sigma V) were dissolved in 2 to 5 ml distilled water and added to the protein. The vial containing the enzyme and protein was incubated at 37°C in an incubator. Aliquots of 0.5 ml were taken out after 3, 6, 12, 24, 48 and 72 hours and transferred to other glass vials. These were frozen and stored in a refrigerator immediately. The starch gel electrophoresis and autoradiography were performed from these samples.
