
CHAPTER IV



R E S U L T S

4.1. Transferrin Polymorphism

In all 1418 animals, belonging to Sahiwal, Red Sindhi and Tharparkar breeds and their crosses with Brown Swiss, Holstein and Jersey breeds of exotic animals and buffaloes of Murrah breed from the herd of National Dairy Research Institute, Karnal, were typed for the studies of transferrin polymorphisms. Tables 4.1.1 and 4.1.2 give the distribution of animals, observed according to their transferrin polymorphic groups.

Table 4.1.1: Transferrin Phenotypes Observed in Indian Breeds

Breed	TF type								Total
	AA	DD	AD	AE	DE	EE	BE	AF	
Tharparkar	42	38	96	71	64	11	3	1	326
Sahiwal	1	7	13	33	111	121	4	-	290
Red Sindhi	3	13	12	24	45	16	6	1	120
	46	58	121	128	220	148	13	2	736

Table 4.1.2 : Transferrin Phenotypes Observed in Creebs

Eight transferrin phenotypes have been found to be present in the indigenous breeds of cattle studied. However, at a later stage, the transferrin standard for differentiating the TfD allele of cattle into TfD₁ and TfD₂ alleles could be procured and this also enabled the typing of 136 crossbred animals for TfD₁ and TfD₂ alleles. Photograph 4.1.1 shows the standard transferrin phenotypes of cattle and buffaloes used for isolation and characterisation. These were from left, viz., TfAA, TfD₁D₁, TfD₁D₂ and TfEE of cattle and TfDD and TfKK of buffaloes. The sample 5th from left was standard TfAE type of cattle and similarly, the 8th sample from left was standard TfDK type of buffalo. Photographs 4.1.2 to 4.1.4 show some of the rare transferrin phenotypes observed in the population of animals studied. The homozygous TfBB of cattle in photograph 4.1.2 was a gift sample to distinguish TfEE and TfAE phenotypes observed. The screening of large number of animals in both the species became necessary to locate and confirm the homozygous TfAA, TfD₁D₁, TfD₂D₂ and TfEE types of cattle and homozygous TfDD (faster moving) and TfKK (slower moving) types of buffaloes, required for later studies of isolation and characterisation of transferrins from these types. It is evident from Table 4.1.1, Table 4.1.2 and Table 4.1.5 that only one animal each could be confirmed for TfD₁D₁ of cattle and TfDD of buffaloes.

Tables 4.1.3 to 4.1.5 present the gene frequencies observed for different Tf alleles in the population studied. Tharparkar animals had the highest frequency for Tfa allele (0.3865) followed

Table 4.1.3: Transferrin Gene Frequencies in Indian Breeds

Breeds	Tf alleles				
	A	D	E	B	F
Tharparkar	0.386	0.362	0.245	0.005	0.002
Sahiwal	0.083	0.238	0.672	0.007	-
Red Sindhi	0.179	0.346	0.446	0.025	0.004

Table 4.1.4: Transferrin Gene Frequencies in Crossbreds

Breeds	Tf alleles				
	A	D	E	B	F
Crossbreds without D_1D_2	0.151	0.533	0.313	0.004	-
Crossbreds with D_1D_2	0.128	0.037	0.426	0.375	- 0.033

Table 4.1.5: Transferrin Phenotypes and Gene Frequency in Murrah Buffaloes.

DD	DK	KK	Total	D	K
1	70	200	271	0.133	0.867

by Red Sindhi (0.1791) and the lowest was observed in Sahiwal animals (0.0827) where only one animal could be traced for being homozygous TfAA. However, Sahiwal breed showed the occurrence of TfE allele to be highest as compared to Red Sindhi and Tharparkar breeds. The gene frequency of TfD (undifferentiated TfD₁ and TfD₂) ranged between 0.25 to 0.35 in all the three indigenous breeds studied. The occurrence of TfB and TfF alleles was found to be extremely low in all the three breeds, and TfF allele was observed absent in Sahiwal animals.

Table 4.1.4 gives the gene frequencies of different transferrin alleles in the population of crossbred animals. TfA allele was observed to be low (0.12 to 0.15) in these animals. The presence of TfD₁, TfB and TfF alleles has been observed to be extremely low. However, the gene frequencies of TfD₂ and TfE alleles were found to be high. The pooled gene frequency of TfD₁ and TfD₂ (0.4628) in the crossbred animals was found to be closer to the gene frequency of TfD (0.5327) in the undifferentiated population of the crossbred animals.

In Murrah breed of buffaloes, typed for transferrin polymorphism, it was observed that only one animal, homozygous for TfDD (faster moving) was present. The studies revealed the presence of two Tf alleles in the population. The gene frequency of TfD allele was found to be lower (0.1328) than the TfK allele (0.8671).

4.2. Isolation and Purification of Transferrins

4.2.1. Isolation of Crude Transferrins

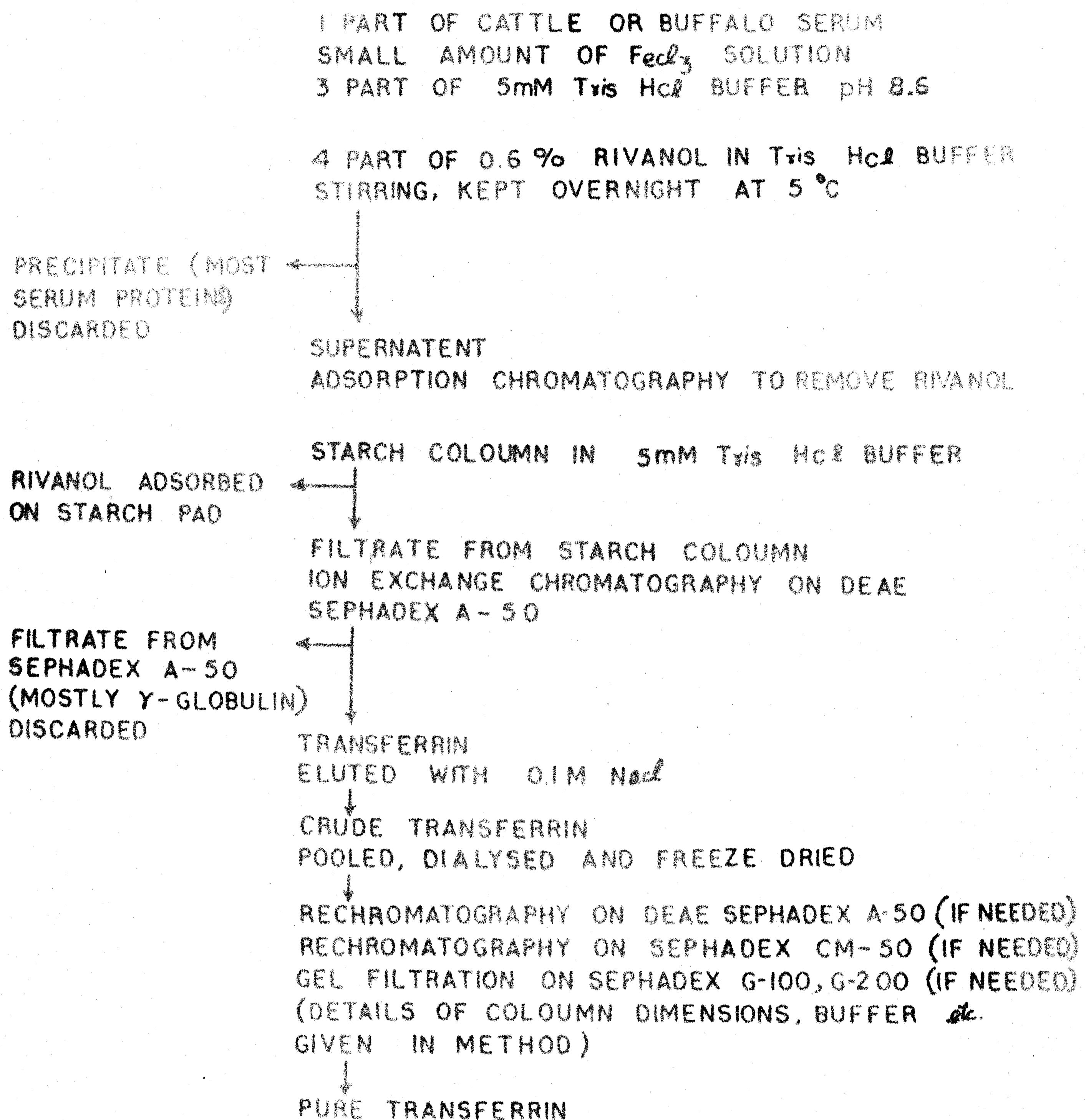
The isolation of different transferrin types from cattle and buffaloes was done according to Sutton and Karp (1965) with some

necessary modifications in the procedure. In the beginning, an effort was made to collect the bulk of crude transferrins from all the phenotypes of cattle and buffaloes, required for the study. The partly purified transferrin was further purified by the use of various procedures of gel filtration on Sephadex and ion exchange chromatography. The procedure, with the conditions used has been given as flow sheet in Fig. 4.2.1.1. The different transferrin types, prepared by this method, appeared homogeneous in starch gel electrophoresis and also by immunodiffusion and immunolectrophoresis.

The different stages of isolation of transferrin from bovine serum have been shown in the Photograph 4.2.1.1. Clear transferrin bands could be observed in all the stages. Sample number 4 from left was filtrate from DEAE Sephadex A-50 column, having no transferrin in it. Photograph 4.2.1.2 shows all the partially purified transferrin types of cattle and buffaloes viz., from left, cattle TfAA, TfD₁D₁, TfD₂D₂ and TfEE and buffalo TfDD and TfKK, respectively. During the trials for isolation of transferrin from serum, it was observed that 0.6 per cent rivanol (in Tris HCl buffer) also precipitated transferrin partly. The starch gel electrophoresis revealed the presence of transferrin in the precipitate obtained by the addition of rivanol. The second sample from left, in the photograph 4.2.1.3 shows transferrin bands.

Figures 4.2.1.2 to 4.2.1.7 show the elution patterns of different transferrin types on DEAE-Sephadex A-50 column. These elutions revealed that cattle transferrin started eluting after about 100 to

Fig. 4.2.11. SCHEME FOR ISOLATION OF BOVINE TRANSFERRIN



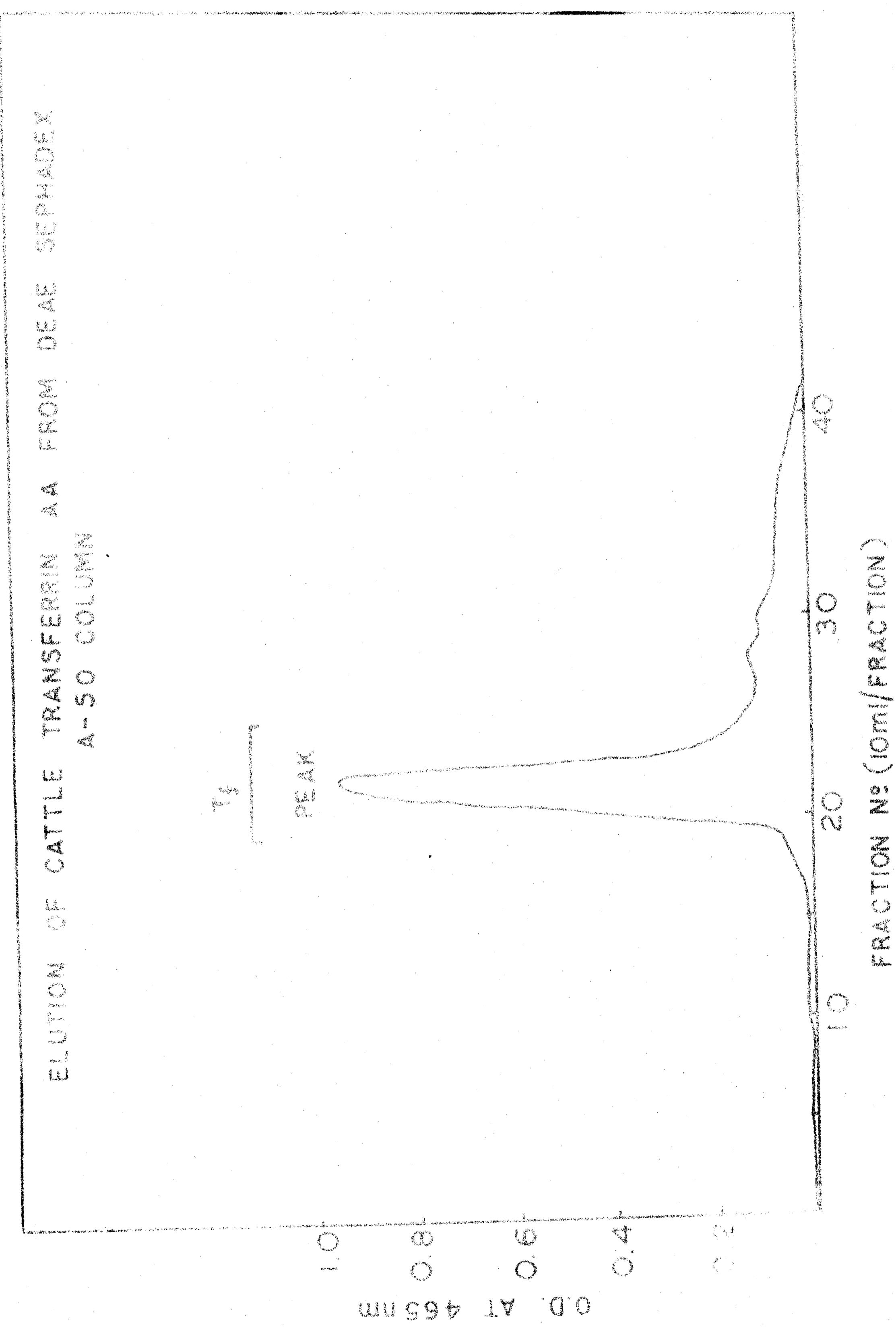


Fig. 4.2.1.2

ELUTION OF CATTLE TRANSFERRIN D₁₀ FROM DEAE
SEPHADEX A-50 COLUMN

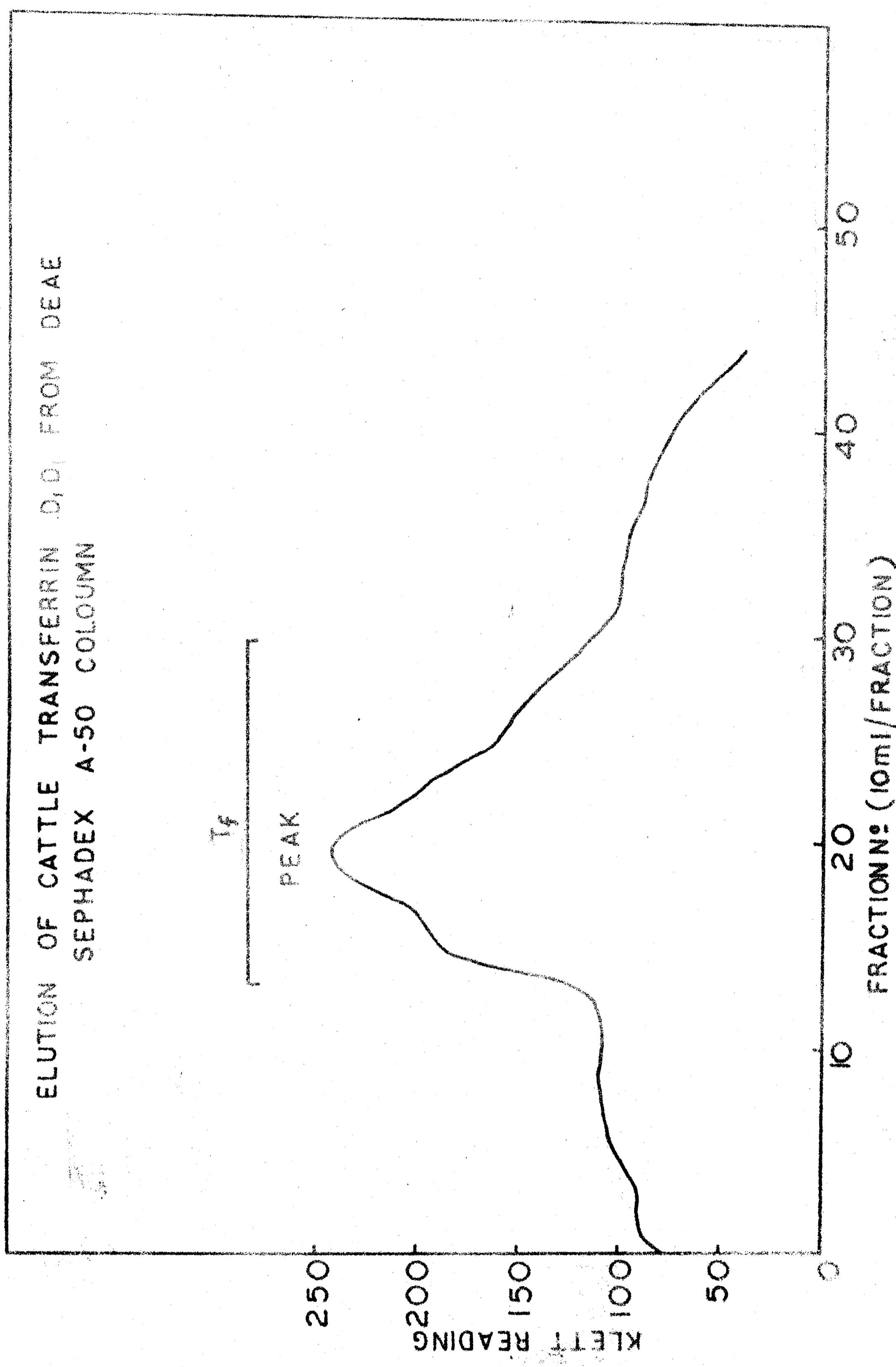


Fig. 4.2.1.3

FRACTION N° (10ml / FRACTION)

ELUTION OF CATTLE TRANSFERRIN D₂O₂ FROM DEAE
SEPHADEX A-50 COLUMN

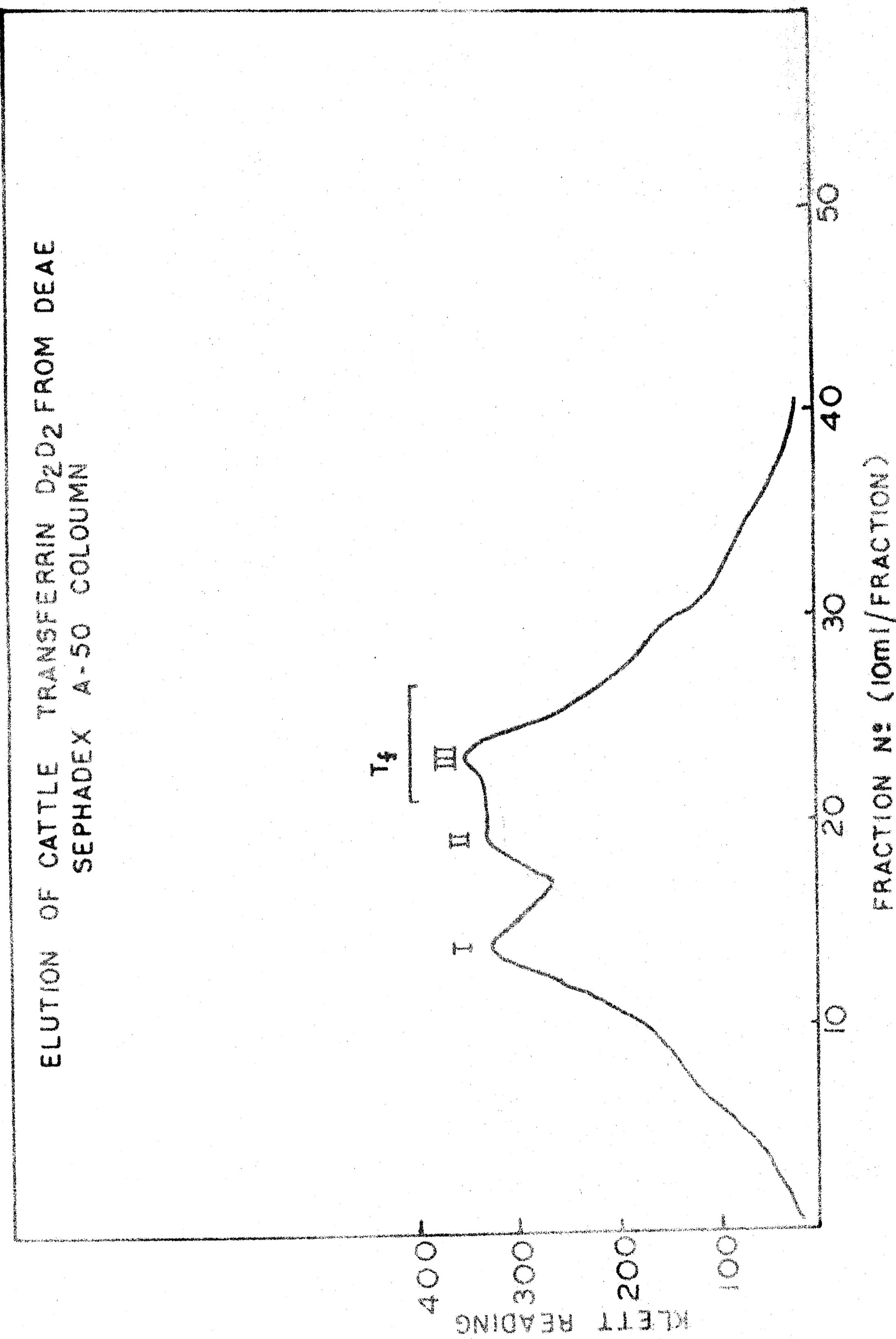


fig. 4.2.1.4

ELUTION OF CATTLE TRANSFERRIN E.E. FROM DEAE
SEPHADEX A-50 COLUMN

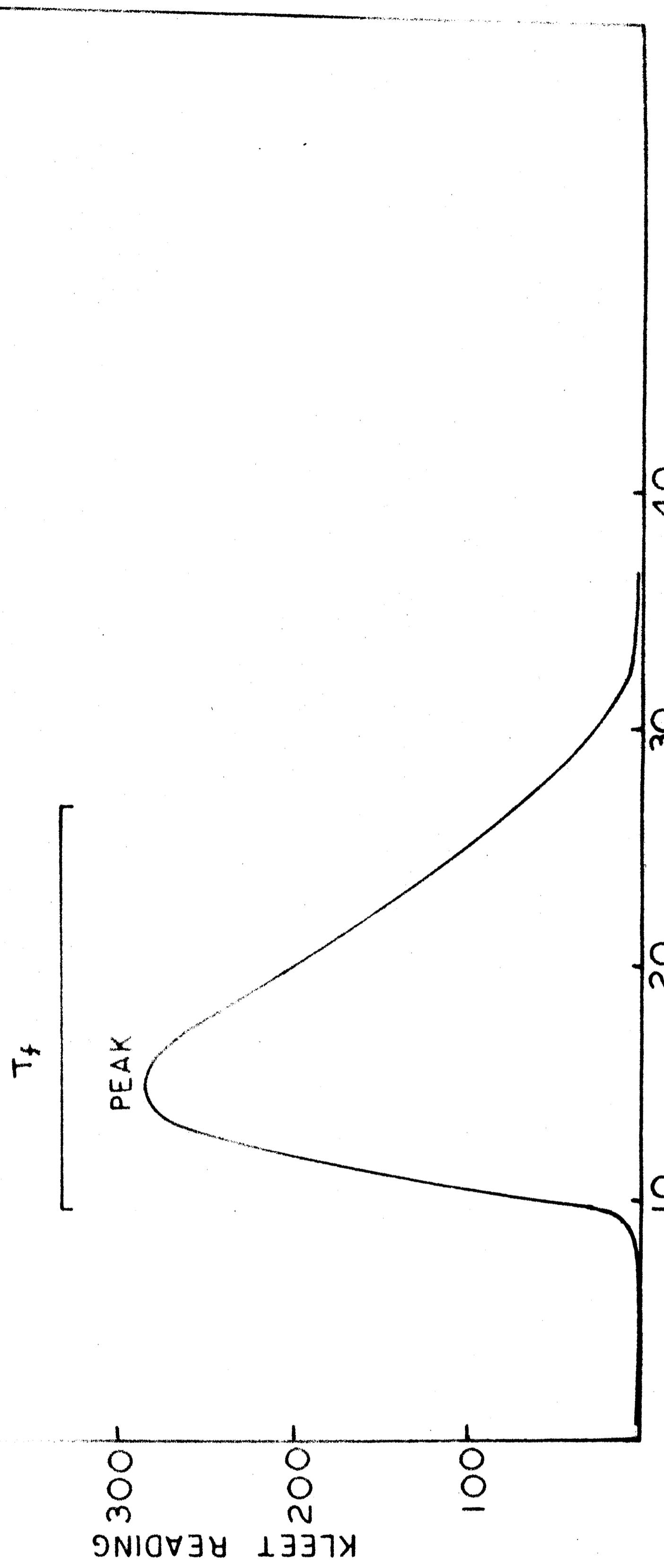
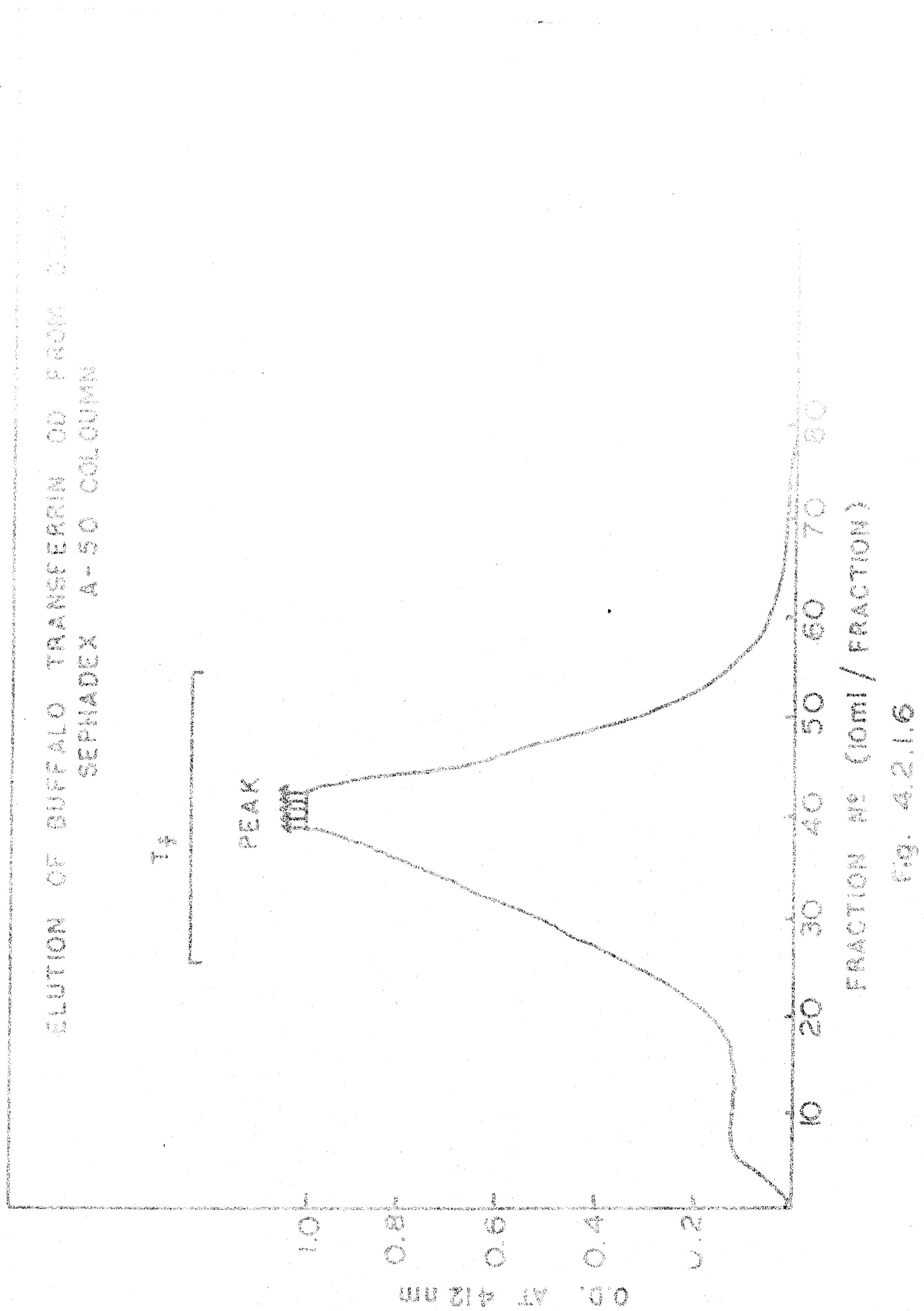


Fig. 4.2.1.5



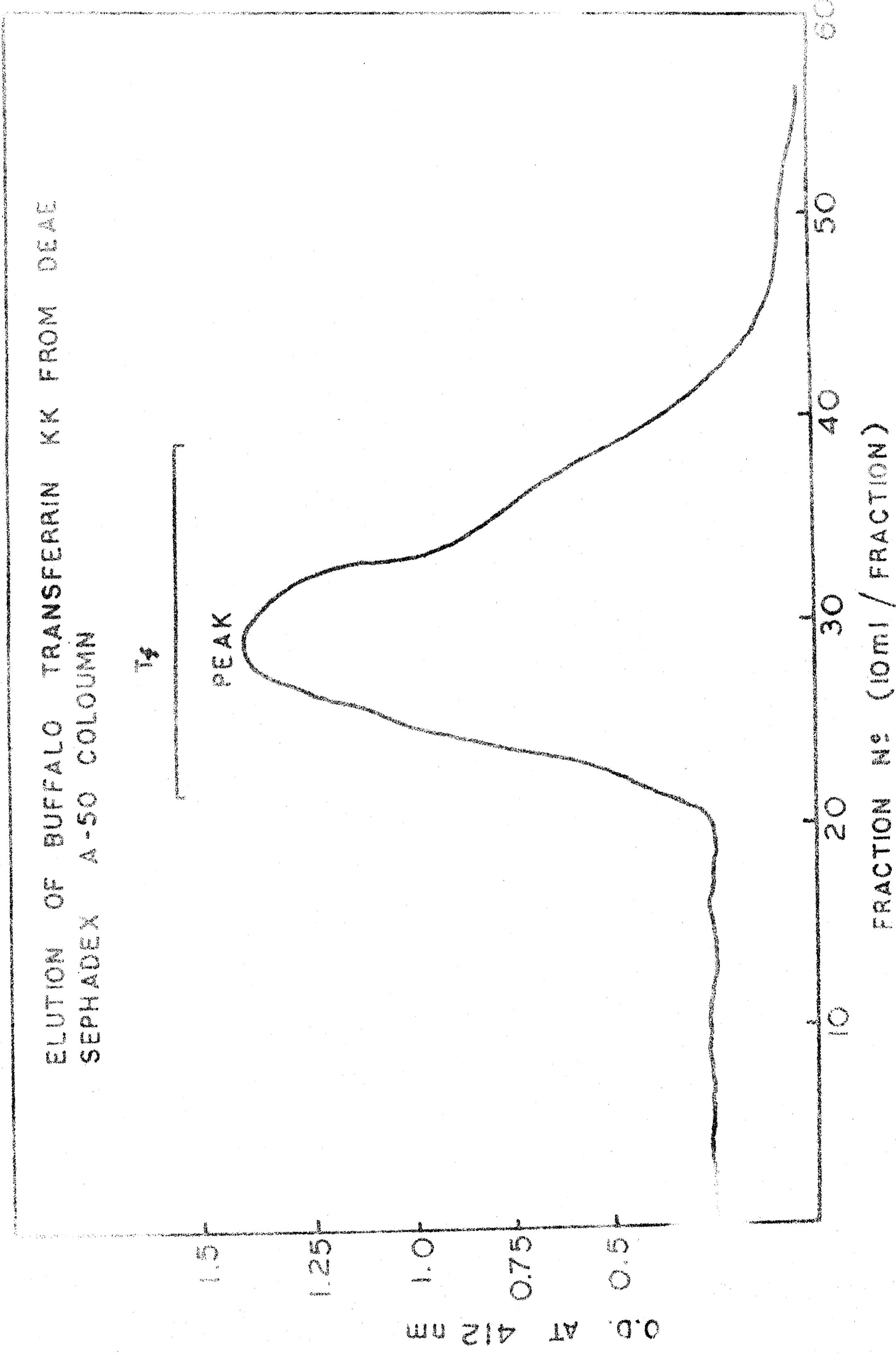


Fig. 4.2.1.7

150 ml buffer had passed through the column and it was completely eluted after 350 to 400 ml buffer had passed through the column. However, in case of buffalo transferrins, the elution started when approximately 200 ml of void volume had been collected, and was completed when about 500 ml buffer had passed through the column. The elution patterns of various transferrins showed the presence of single peak in case of both the species, except cattle transferrin D_2D_2 type where the peak was observed to be unsymmetrical and the transferrin protein was located in the third peak. The single peak of these elutions showed the presence of transferrin along with traces of other impurities, when tested by starch gel electrophoresis. Later studies on purification (Section 4.2.2) confirmed the observations made in case of cattle TfD_2D_2 , under critically controlled experimental conditions.

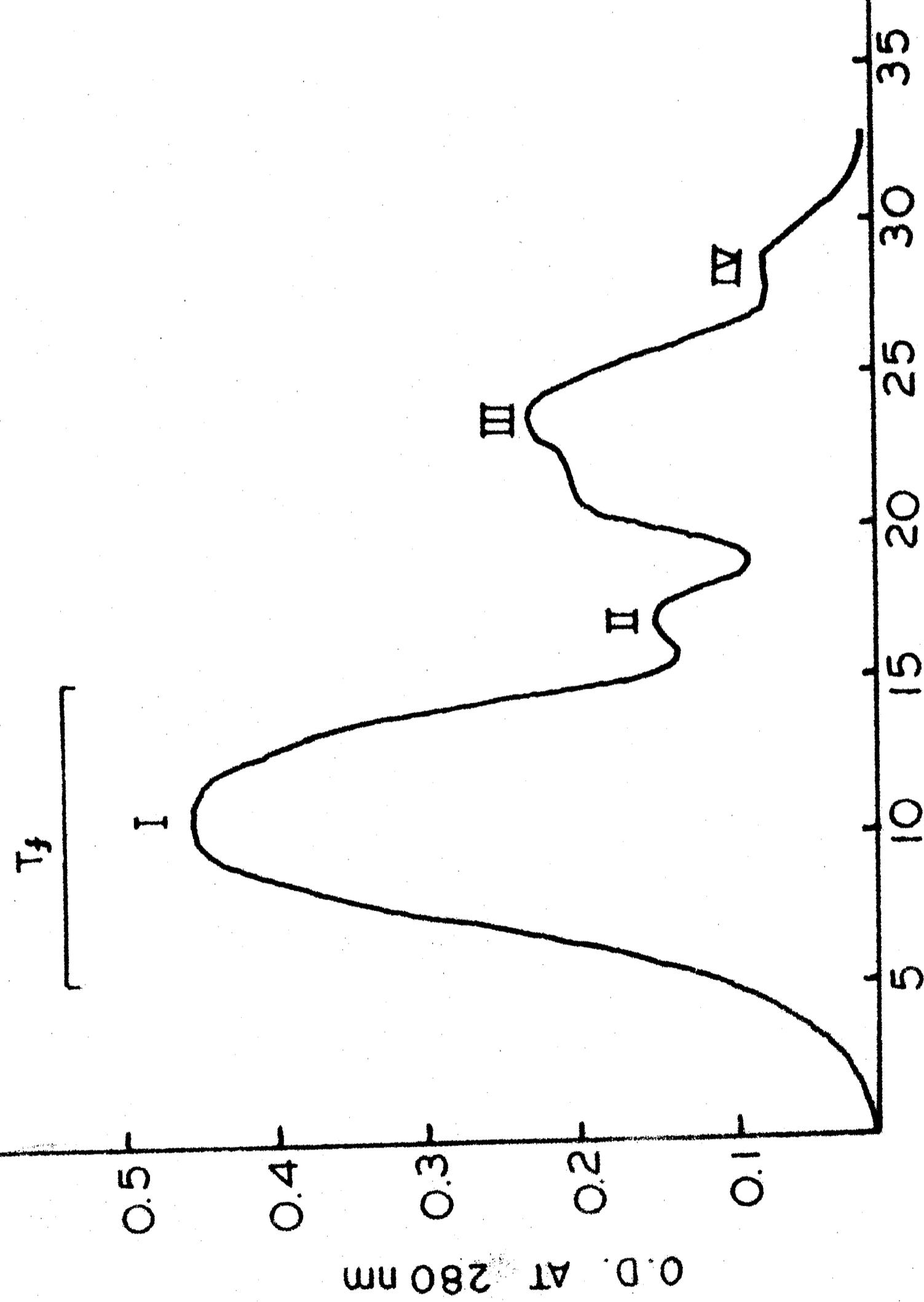
4.2.2. Purification of Transferrins

Variation in purity due to different methods of preparations, standards of purity and carbohydrate compositions necessitated preparation of electrophoretically and immunologically pure bovine transferrin samples from individual homozygotes of established genotypes. The purification of transferrin posed a difficult problem. Hence, various methodologies of gel filtration and ion exchange chromatography were used to purify the crude isolated transferrin from all phenotypes studied.

4.2.2.1. Gel Filtration on Sephadex G-100 and G-200

The preparations of crude transferrins were purified partially by the use of Sephadex gel filtration on G-100 and G-200. Fig. 4.2.2.1.1 and 4.2.2.1.2 show the elution pattern of crude transferrin on Sephadex G-100 and Sephadex G-200 respectively. It was observed in both the gel filtration experiments that the crude preparations of transferrins obtained after precipitation with rivanol, resolved into several peaks. The transferrin was, however, located in the 1st peak of these experiments. The transferrin containing peak was found to be the major peak, in case of both cattle and buffalo. The elution of crude buffalo transferrin on G-200 (Fig. 4.2.2.1.2) showed other minor peaks. However, in case of G-100 (Fig. 4.2.2.1.1) experiment with crude cattle transferrin, the other peaks were also sufficiently prominent. The elution pattern for G-100 gel filtration, experiment (cattle transferrin) revealed that the transferrin started eluting when approximately a void volume of 50 ml buffer had been collected and it was completely eluted when about 150 ml buffer had passed through the column. The complete elution of all the peaks could be achieved in this experiment when about 300 to 350 ml buffer had passed. However, when Sephadex G-200 (Buffalo transferrin) was used (Fig. 4.2.2.1.2), the elution volumes for all the peaks were observed to be almost double and the complete elution occurred after the passage of about 550 to 600 ml of buffer. It was observed, however, that the number of peaks for both the gel filtration experiments were four.

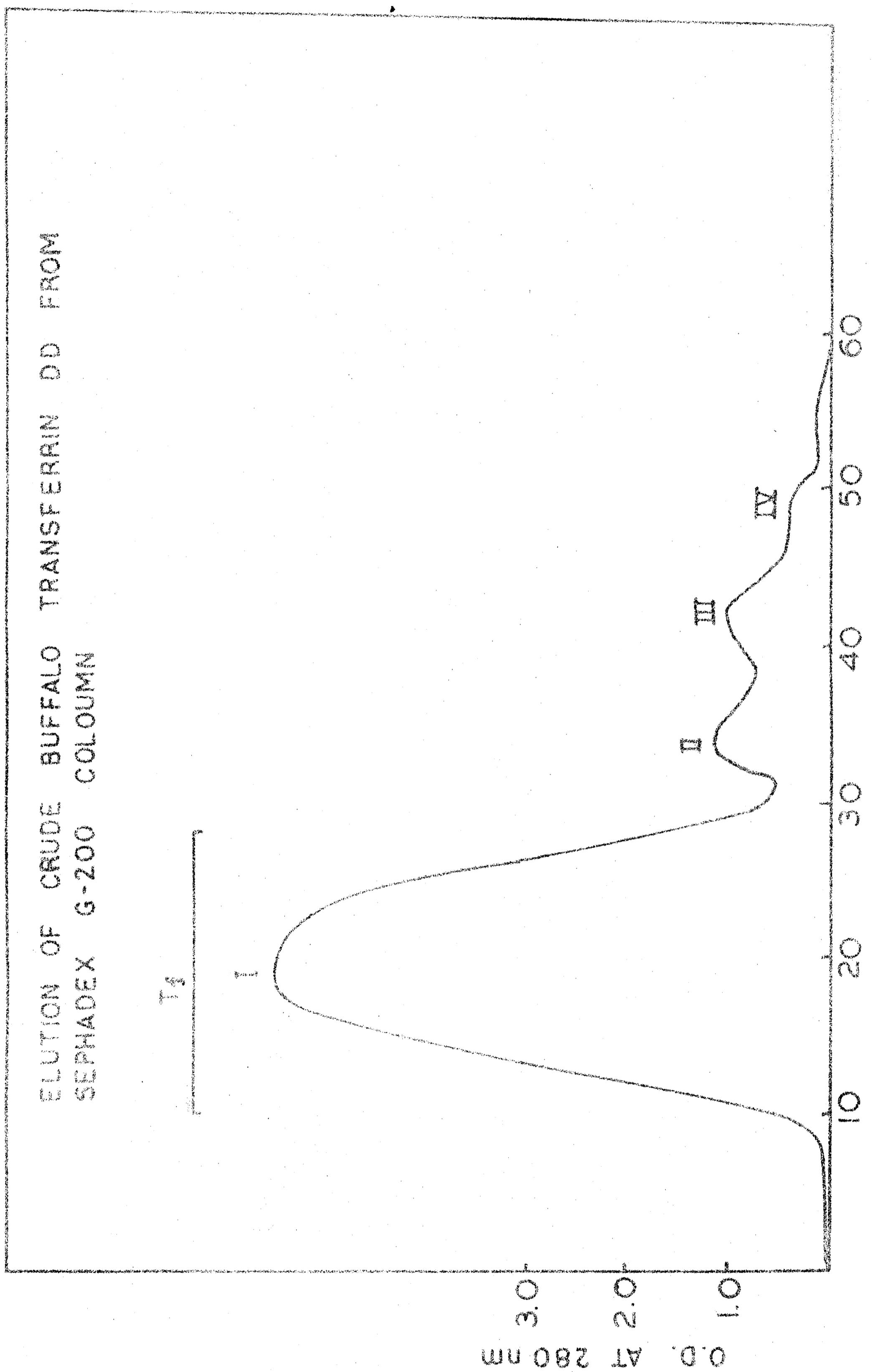
ELUTION OF CRUDE CATTLE TRANSFERRIN D₁D₁ FROM
SEPHADEX G-100 COLUMN



FRACTION N° (10ml / FRACTION)

Fig. 4.2.2.1.

ELUTION OF CRUDE BUFFALO TRANSFERRIN DD FROM
SEPHADEX G-200 COLUMN



FRACTION № (10 ml / FRACTION)

Fig. 4.2.2.1.2

when tested by starch gel electrophoresis, the transferrin was found absent in the other minor peaks. The peak containing transferrin also revealed the presence of traces of proteins of unidentified nature.

4.2.2.2. Rechromatography on DEAE Sephadex A-50

Rechromatography of crude cattle transferrin on DEAE Sephadex A-50 revealed the presence of three peaks and the transferrin was located in the third peak. Figure 4.2.2.2.1 shows the elution curve for these experiments, wherein conditions were maintained identical, as detailed in the isolation of crude transferrin. The isolation of cattle transferrin from the serum loaded with ^{59}Fe citrate radicisotope showed similar elution pattern from DEAE Sephadex A-50 column (Fig. 4.2.2.2.2). It was observed that the \times -counts per minute (C.P.M.) were highest in the 3rd peak region for the elution curve at 280 nm, and almost negligible in the 1st and 2nd peak regions. The Photograph 4.2.2.2.1 of the starch gel electrophoresis for the 1st and 2nd peaks depicts the absence of transferrin in the 1st peak in case of cattle TfAA and TfD₁D₁, respectively, and 1st and 2nd peaks in case of cattle TfEE type. The pooled fractions of these peaks having no transferrins were reddish brown in colour. Thus it is conclusively demonstrated that the serum contained another reddish brown coloured protein which eluted very close to transferrin on DEAE Sephadex A-50 column. In fact, sometimes it was practically impossible to separate these two proteins, as the elution with 0.1 M sodium chloride gave only one peak. The nature of the other protein has not been characterised.

RECHROMATOGRAPHY OF CRUDE CATTLE TRANSFERRIN
ON DEAE SEPHADEX A-50 COLUMN

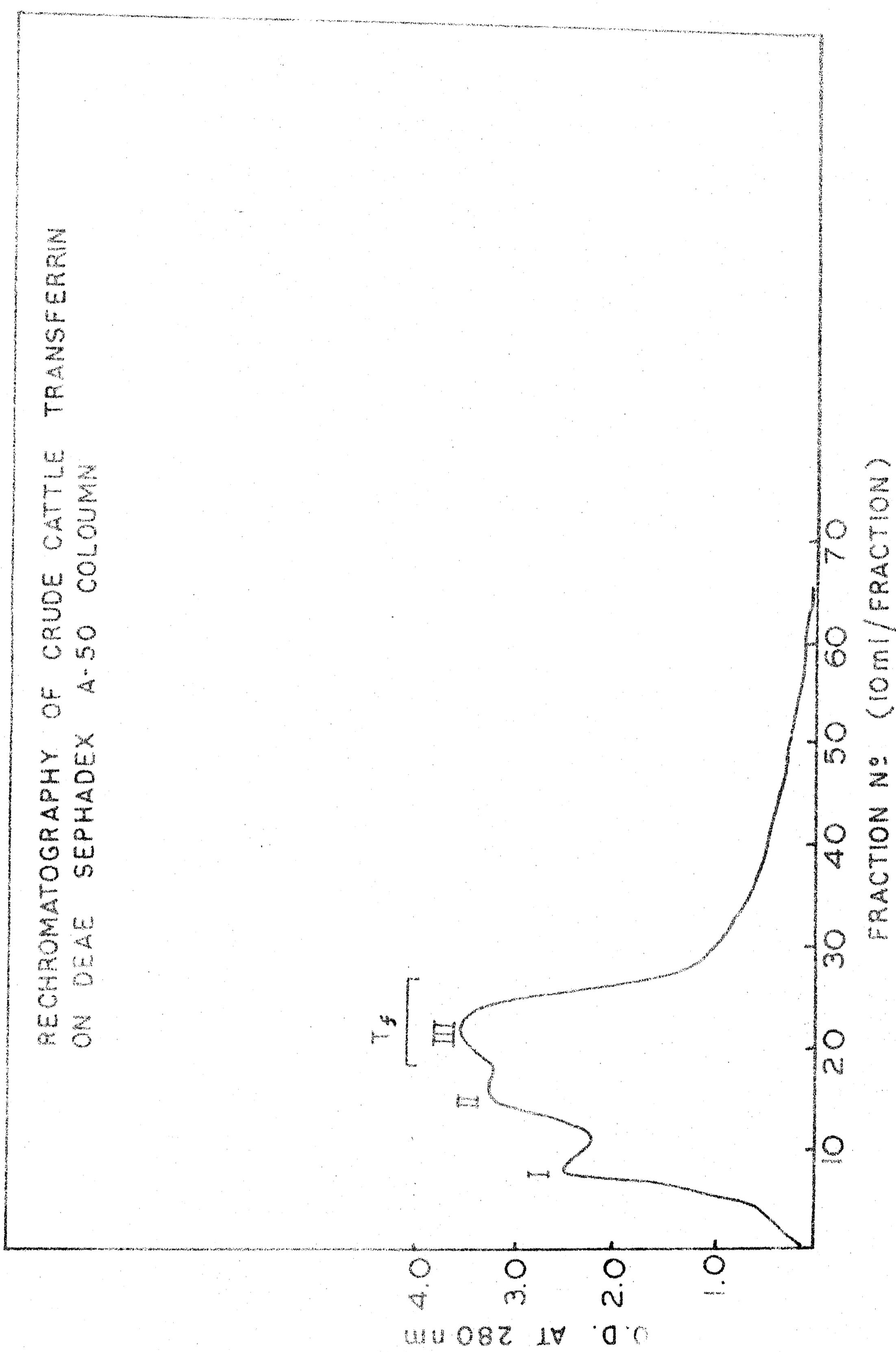
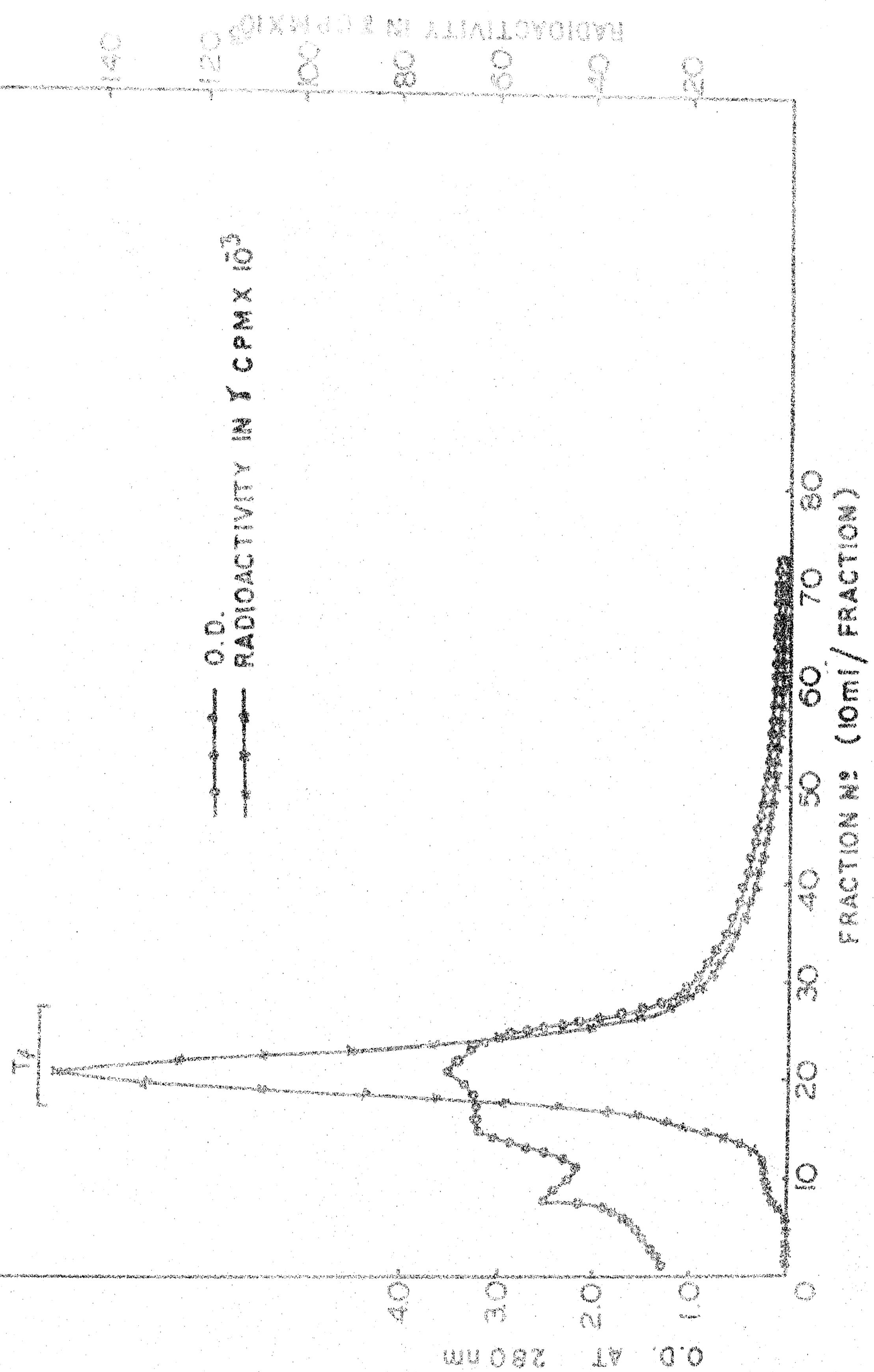


Fig. 4.2.2.1

ELUTION OF CATTLE TRANSFERRIN AA LOADED WITH Fe^{59}
THROUGH SEPARADEX A-50 COLUMN



4.2.2.3. Use of sodium Chloride Molarity Gradient Elution on DEAE Sephadex A-50

Figures 4.2.2.3.1 to 4.2.2.3.3 show the elution patterns of crude bovine transferrins when a sodium chloride molarity gradient was applied on DEAE Sephadex A-50 column. The use of sodium chloride molarity gradient gave several distinct peaks, which did not differentiate when transferrin was eluted with single molarity eluent. In case of cattle transferrin AA type (Fig. 4.2.2.3.1) three major and one minor peak could be traced in the eluted fractions. The intensity of the 4th peak was, however, very low. The 1st peak could be eluted almost immediately, when the gradient was applied. All the peaks were eluted when approximately 500 ml eluent had passed through the column. Similar observations were recorded with buffalo transferrin KK where the limit molarity of the eluent was identical (0.1 M) to the cattle TfAA experiment (Fig. 4.2.2.3.3). However, when the molarity gradient of eluent was maintained 0.01 M to 0.2 M, as in case of the buffalo transferrin DD type (Fig. 4.2.2.3.2), it was observed that the number of peaks increased to five which had sharper intensity than those obtained in earlier two experiments. The total volume of the eluent required for the elution of all the peaks, in this case, was found to be almost double than earlier elutions.

The transferrin purified in this manner indicated the absence of traces of impurities. However, when the material from all the peaks was pooled and tested for its purity, it showed the reappearance of the traces of impurities.

ELUTION OF CRUDE CATTLE TRANSFERRIN AA FROM DEAE
SEPHADEX A-50 COLUMN USING 0.01-0.1 M SODIUM CHLORIDE
GRADIENT

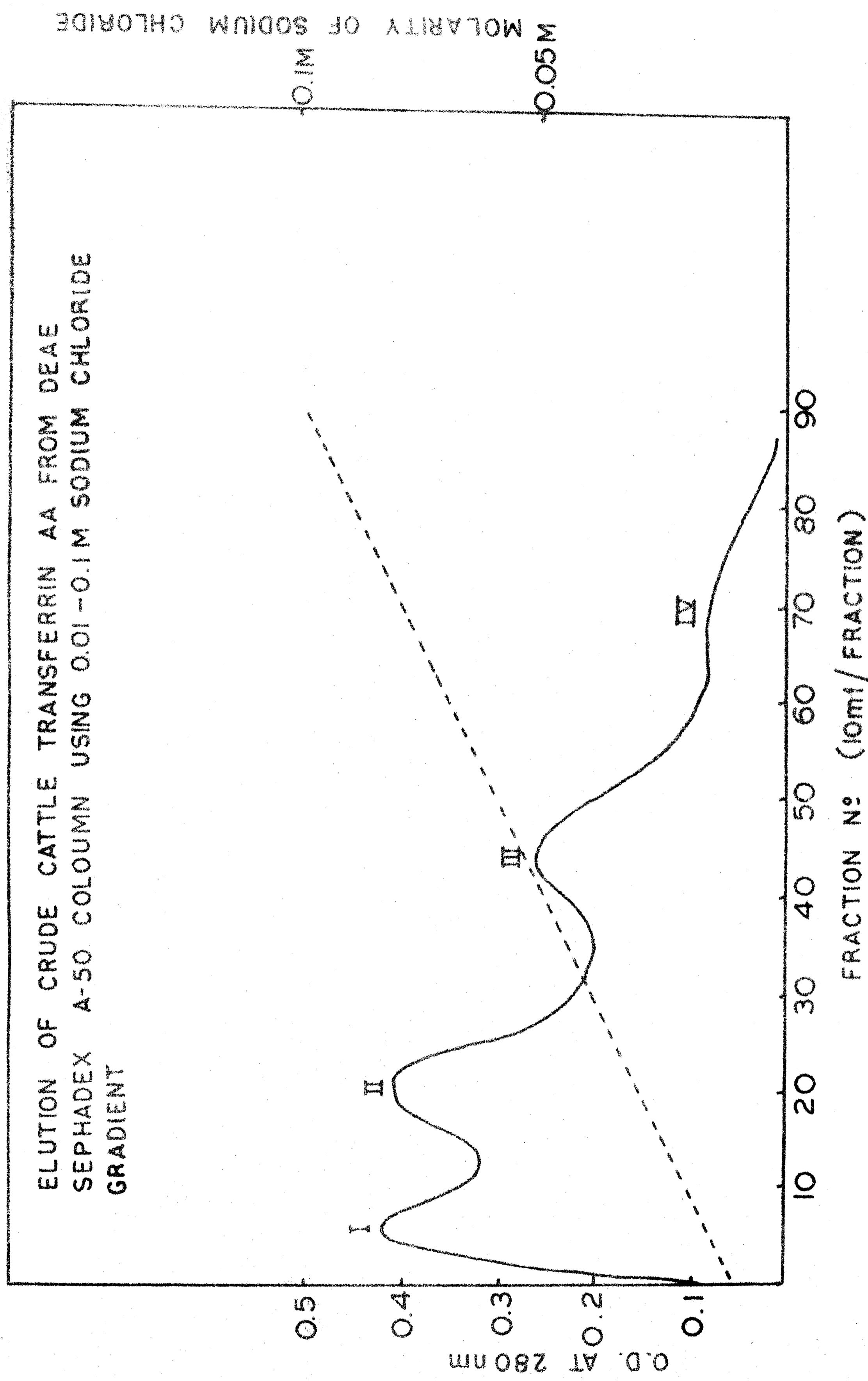


Fig. 4.2.2.3.1

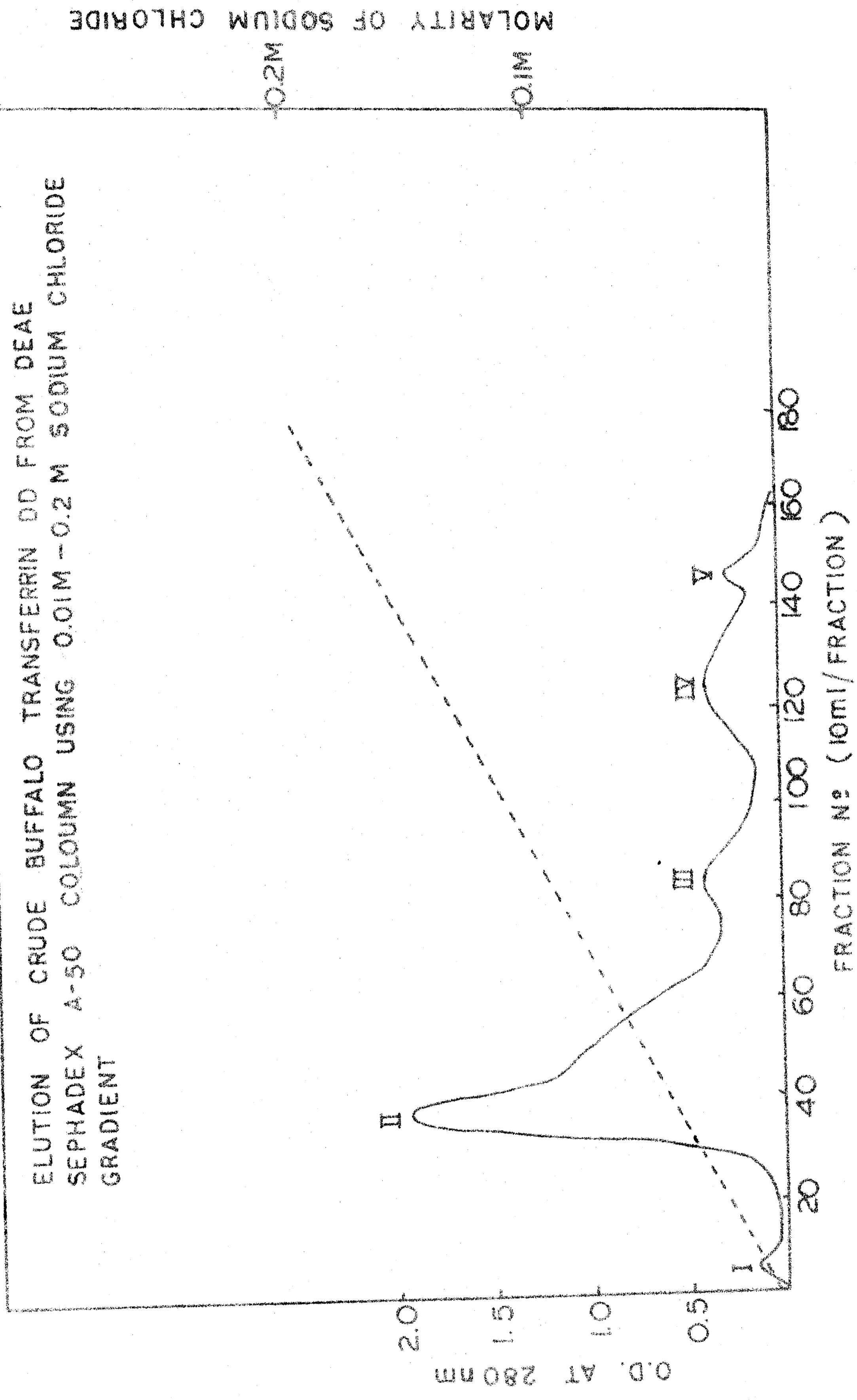


Fig. 4.2.2.3.2

ELUTION OF CRUDE BUFFALO TRANSFERRIN KK FROM DEAE
SEPHADEX A-50 COLUMN USING 0.01-0.1M SODIUM CHLORIDE
GRADIENT

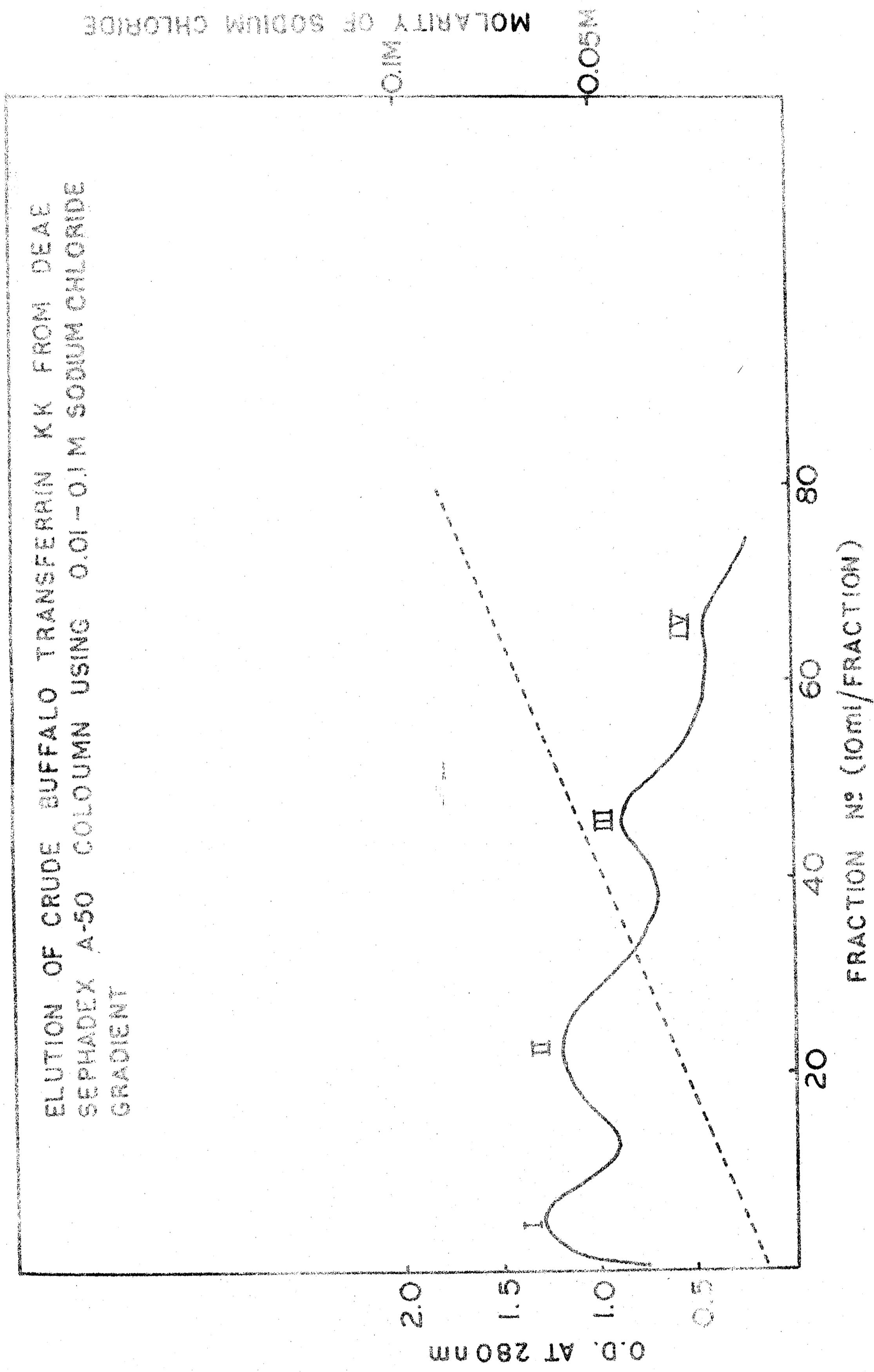


Fig. 4.2.2.3.3

4.2.2.4. Use of Tris HCl Gradient Elution on DEAE Cellulose

0.01 M to 0.3 M Tris HCl gradient was used on DEAE cellulose column for partially removing the impurity of albumin from the crude bovine transferrin. Fig. 4.2.2.4.1 shows the elution pattern of crude buffalo transferrin DD type on this column. This gradient of Tris HCl buffer resulted in the elution ^{of} five peaks and the transferrin was observed to elute in the 4th peak, when about 1.5 litre buffer had passed through the column. The other peaks were minor and not sharp. The use of DEAE cellulose was, however, found to be ineffective in completely removing the impurities from the crude transferrin. All the peaks could be eluted after about 2.5 litre buffer had passed through the column.

4.2.2.5. Chromatography on Sephadex G-50

It was observed during experimentation that anion exchangers (DEAE Sephadex A-50 and DEAE cellulose) could not remove the traces of impurities completely from the partially purified bovine transferrins. Therefore, cation exchanger Sephadex G-50 was tried at lower pH of 5.9 with sodium citrate molarity (0.01-0.1M) gradient. Figures 4.2.2.5.1 and 4.2.2.5.2 show the elution patterns of crude cattle TfD₂D₂ and buffalo TfDD type from Sephadex G-50 columns, respectively. In both the cases the transferrin was located in the first peak which was also the major peak of the elution pattern. The peaks started eluting almost immediately on the application of gradient. The

ELUTION OF CRUDE BUFFALO TRANSFERRIN DD FROM DEAE
CELLULOSE COLUMN USING 0.01-0.3 M Tris HCl GRADIENT

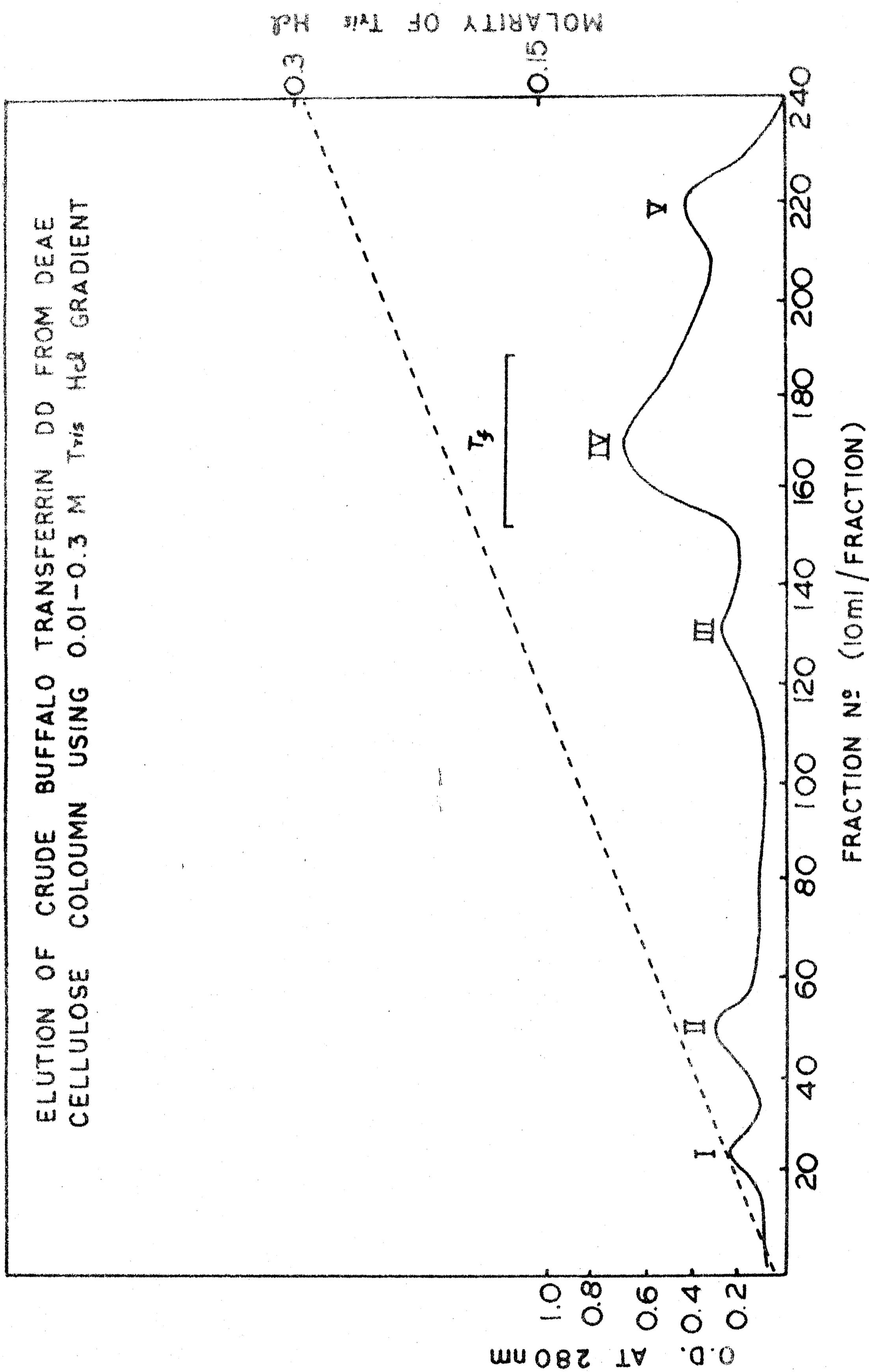


Fig. 4.2.2.4.1

ELUTION OF CRUDE CATTLE TRANSFERRIN D_2D_2 FROM
SEPHADEX CM - 50 COLUMN USING 0.01M - 0.1M SODIUM CITRATE
GRADIENT

MOLARITY OF SODIUM CITRATE

0.1M

0.05M

T_b

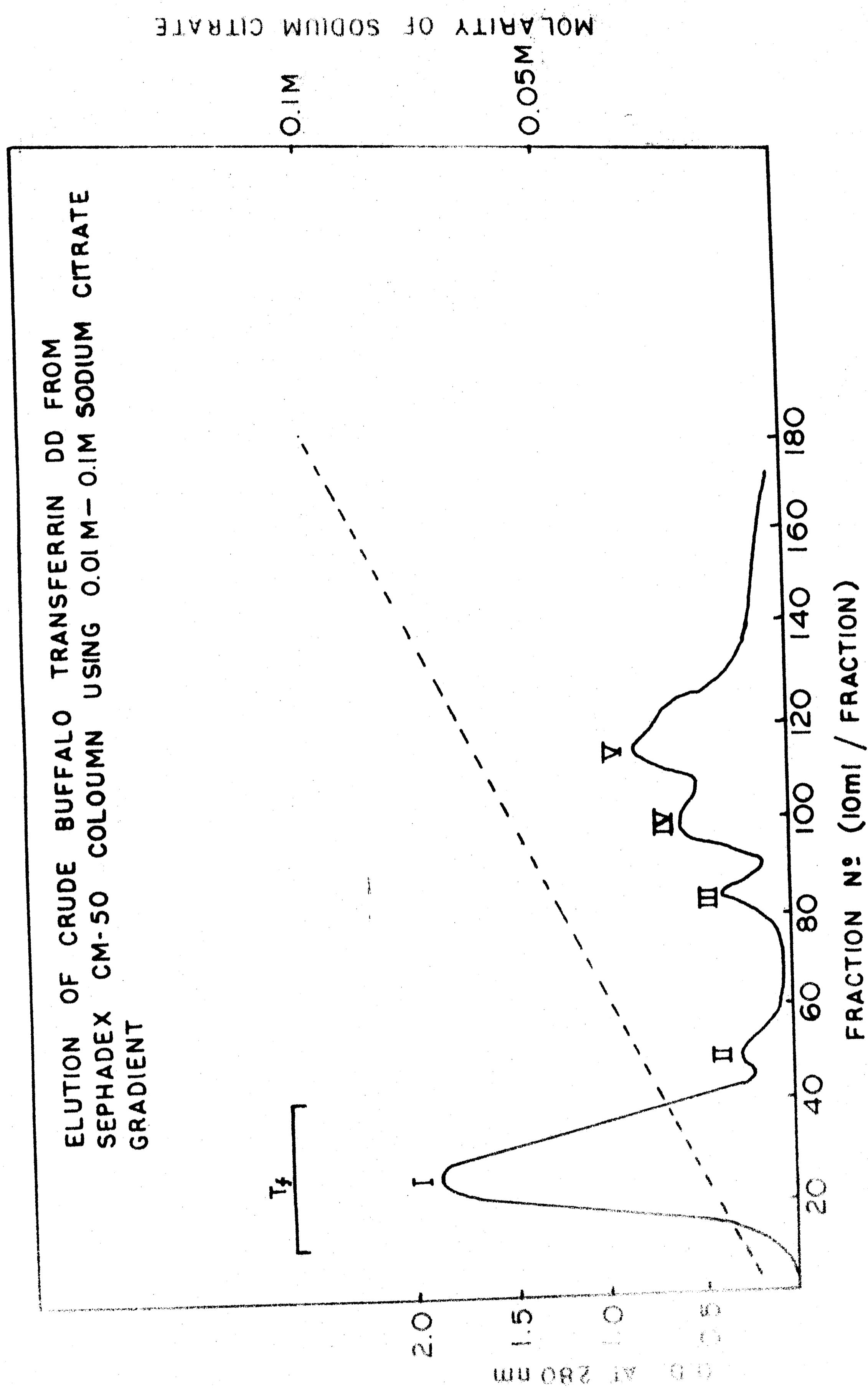
0.0
0.2
0.3
0.4
O.D. AT 280nm

20 40 60 80 100 120 140 160 180 200

FRACTION NO (10ml / FRACTION)

Fig. 4.2.2.5.1

ELUTION OF CRUDE BUFFALO TRANSFERRIN DD FROM
SEPHADEX CM-50 COLUMN USING 0.01 M - 0.1M SODIUM CITRATE
GRADIENT



total volume of the eluent required for eluting all the five peaks observed, in both the cases, was about 1.0 to 2.0 litres. The other peaks in case of cattle transferrin (Fig. 4.2.2.5.1) were of minor nature, however, the crude buffalo transferrin showed the presence of sufficiently large peaks of impurities (Fig. 4.2.2.5.2). The transferrin prepared by this method was iron-free and almost white in colour. It is evidently clear that the use of cation exchanger (Sephadex G-50) at a lower pH (5.9) could be effective in removing the traces of impurities, including the other coloured protein encountered in the crude transferrin preparations from cattle and buffaloes.

4.2.3. Purity of Transferrins

The purity of different transferrin types of cattle and buffaloes, isolated and purified by various procedures, was tested by starch gel electrophoresis and immunoelectrophoresis, respectively. Photograph 4.2.3.1 shows the phenotypes of the partially purified transferrins viz., from left, cattle TfAA, TfD₁D₁, TfD₂D₂ and TfEE and buffalo TfDD and TfKK on starch gel electrophoresis. It is observed that traces of impurities of albumin existed along with transferrin. However, on further purification of these preparations, they indicated the absence of any traceable impurity by starch gel electrophoresis. Photograph 4.2.3.2 shows the purified transferrins of various phenotypes.

The purity of the transferrin preparations was also tested immuno-electrophoretically by raising antisera against the pooled preparations of different bovine transferrins in rabbits. Photograph 4.2.3.3 shows the results of immuno-electrophoresis done with 6 mg/ml protein concentration of various purified bovine transferrins. The wells for antigen contained cattle TfAA, TfD₁D₁, TfD₂D₂ and TfEE and buffalo TfDD and TfKK from top to bottom, respectively. Similarly, the top three troughs were filled with anti-pooled cattle transferrin sera and the lowest trough (4th) contained anti-pooled buffalo transferrin sera. The absence of any extra arc other than for transferrin, revealed the absence of any traceable contaminating antigenic factor, and the degree of purity of the various transferrin types used for the physico-chemical and immunological characterisation from both the species.

4.3. Physico-chemical Characterisation

4.3.1. Estimation of Molecular Weight

The molecular weights of the purified transferrin types of TfAA, TfD₁D₁, TfD₂D₂ and TfEE of cattle and TfDD (faster moving) and TfKK (slower moving) of buffaloes were estimated by gel filtration on Sephadex G-200 column. The molecular weight marker proteins used included bovine γ -globulin (Mol. wt. 160,000), Bovine Serum Albumin (Mol. wt. 67,000), Human Serum Transferrin (Mol. wt. 76,000), Egg Albumin (Mol. wt. 21,500). These proteins in purified form were used

for calibrating the Sephadex G-200 column. The standard curve (Fig. 4.3.1.1) prepared from the elution volumes of the molecular weight markers was observed to be highly linear, except for the bovine γ -globulin, which remained slightly away from the main curve. This curve was used for extrapolation for calculation of the molecular weights of the different transferrin types of cattle and buffaloes.

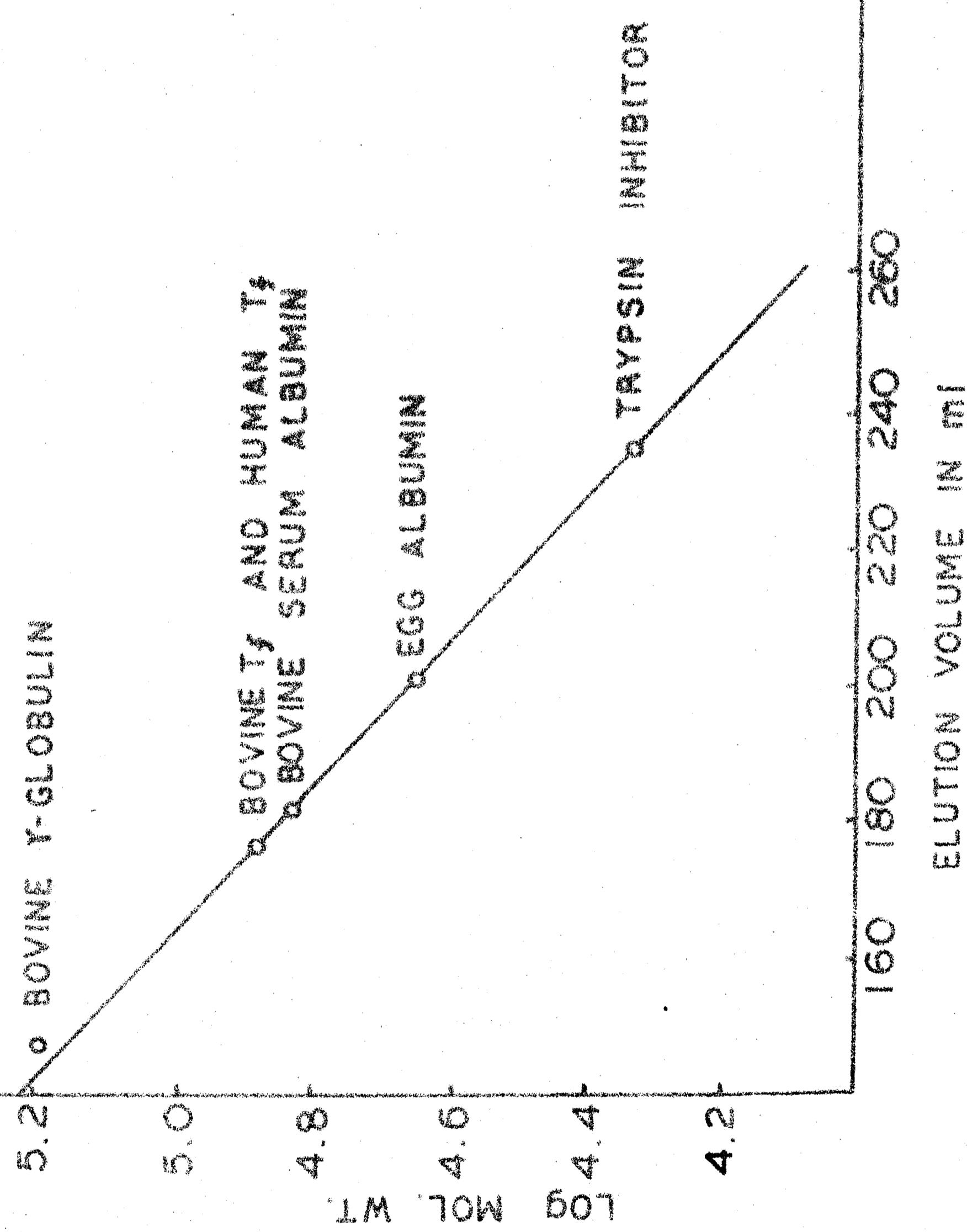
Table 4.3.1.1: Elution Volumes and Corresponding Molecular Weights of Protein Markers

S. No.	Protein	Elution volume	Mol. wt.	Log Mol.wt.
1.	Egg Albumin	200 ml	45,000	4.65
2.	Bovine Serum Albumin	182 ml	67,000	4.83
3.	Human Transferrin	176 ml	76,000	4.88
4.	Trypsin Inhibitor	234 ml	21,500	4.33
5.	Bovine γ -globulin	148 ml	160,000	5.20

Table 4.3.1.1 gives the elution volumes obtained for different protein markers used for calibration of the Sephadex G-200 column. The elution volumes of the different Tf transferrin types for both the species were found to be 176 ml. The molecular weight was estimated, therefore, to be 76,000 for all the transferrin types of cattle and buffaloes studied.

The gel filtration experiments with different purified transferrin types showed only one symmetrical peak with some traces

STANDARD CURVE FOR MOLECULAR WEIGHT BY SEPHADEX
G-200 COLUMN



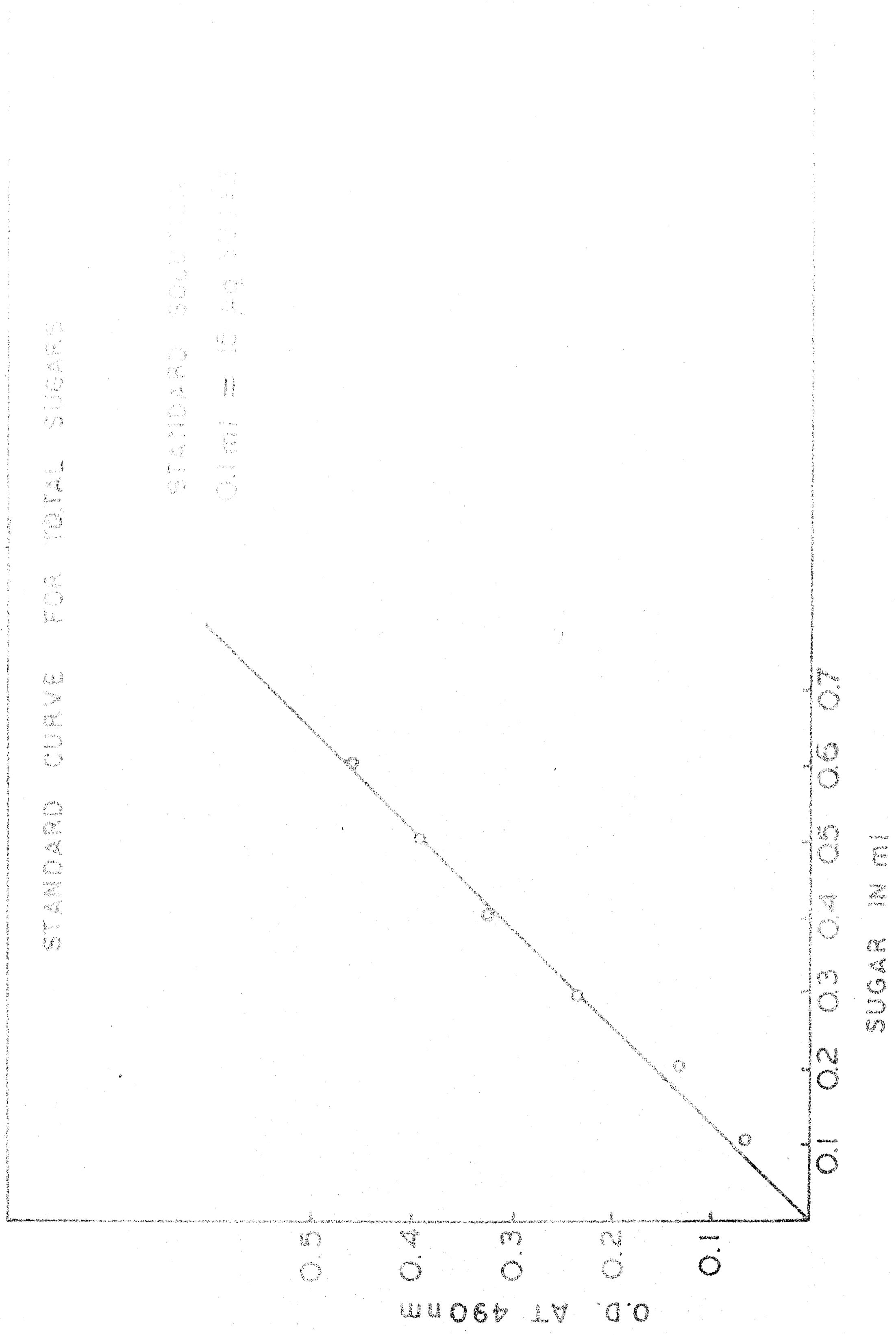
of proteins of unidentifiable nature. The separation of bands from one transferrin type could not be achieved by gel filtration on Sephadex G-200 column. It was, therefore, concluded that transferrin bands of one type and also different types, contained proteins of similar molecular size and molecular structure and conformation, since the elution volumes for all these types were identical.

4.3.2. Analysis of Hexoses, Hexosamines and Sialic Acids

The quantities of hexoses, hexosamines and sialic acids determined for the transferrin phenotypes of AA, D₁D₁, D₂D₂ and EE of cattle and DD and KK type of buffaloes have been given in Table 4.3.2.1. The results obtained were the average of triplicate analyses for each phenotype of transferrin.

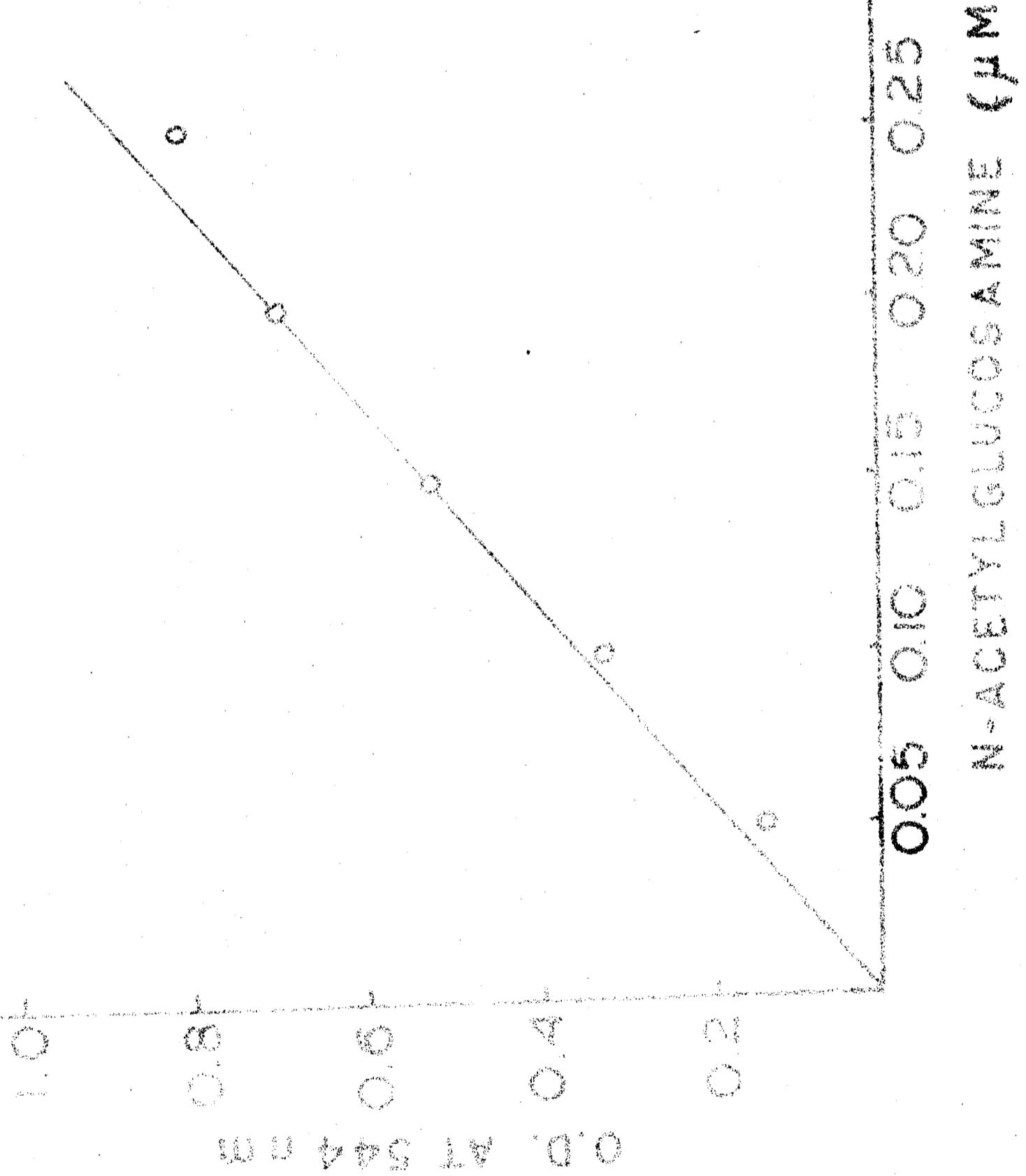
Table 4.3.2.1: Carbohydrate Analysis of Cattle and Buffalo Transferrin Phenotypes.

Tf Phenotype	Hexoses (% w/w)	Hexosamines (% w/w)	Sialic Acids (% w/w)	Total Carbo- hydrate (% w/w)
Cattle TfAA	1.25	1.01	0.74	3.00
,, TfD ₁ D ₁	1.32	0.98	0.76	3.06
,, TfD ₂ D ₂	1.35	0.95	0.73	3.03
,, TfEE	1.23	1.03	0.60	2.86
Buffalo TfDD	1.30	0.90	0.65	2.85
,, TfKK	1.28	1.04	0.75	3.07



STANDARD CURVE FOR N-ACETYLGLUCOSAMINE

STANDARD SOLUTION 0.5 μ M/ml



STANDARD CURVE FOR SIALIC ACID

STANDARD SOLUTION 32.40 µg/ml

O.D. AT 540 nm

SIALIC ACID IN ml

0.1 0.2 0.3 0.4

1.0

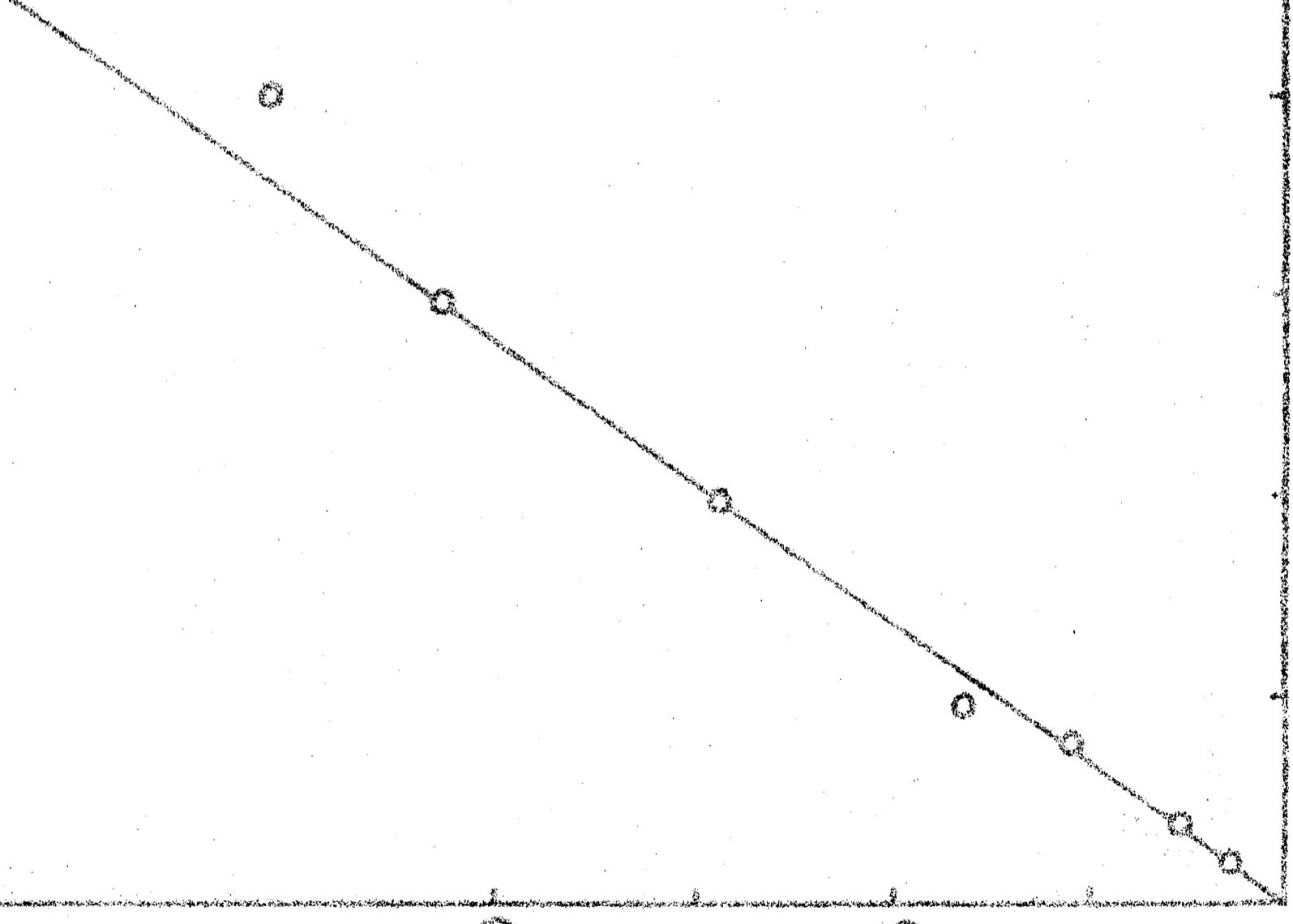
0.8

0.6

0.4

0.2

0.0



The values obtained for analysis of different sugars did not reveal any significant variation among any of the carbohydrate moieties for all the transferrin phenotypes studied. The quantities of different sugars for buffalo transferrin types were also not significantly different from those obtained for cattle phenotypes. The total hexose content ranged between 1.23 per cent to 1.35 per cent, while hexosamines and sialic acids varied between 0.90 per cent to 1.04 per cent and 0.60 per cent to 0.75 per cent, respectively, for both the species. The total carbohydrate content determined ranged from 2.85 per cent to 3.07 per cent for all the Tf phenotypes in cattle and buffaloes.

4.3.3. Radioactive Labelling with Fe⁵⁹

Transferrin AA was isolated from cattle serum after the addition of Fe⁵⁹ citrate. Fig. 4.2.2.2.2 shows the elution of transferrin from DEAE Sephadex A-50 column. The curve with round dots shows the optical density of the fractions collected, while the curve with the crosses shows the μ -C.P.M. $\times 10^{-3}$, taken on a γ -counter for 2 ml fractions. It is evidently clear from these elution curves that the fractions eluted from DEAE Sephadex A-50 column showed the presence of several peaks, including the peak which had the transferrin. It was seen that the μ -C.P.M. increased from 15th fraction onwards, while the

curve for the optical density showed that two peaks had been already eluted before the 3rd peak which contained transferrin. The transferrin, therefore, was eluted in the third peak, which was evident from the highest γ -C.P.M. of the fractions from this peak. The decline of the optical density and γ -C.P.M. was observed to be almost parallel after 25th fraction. It could, therefore, be demonstrated that the supernatant obtained after rivanol precipitation of the serum contained several fractions of other proteins along with transferrin. This could also be confirmed electrophoretically by testing these peaks. It was observed that the first peak eluted from the column did not show the presence of any transferrin, while the transferrin was present in the 2nd and 3rd peak. However, it could be observed from the elution curve (Fig. 4.2.2.2) that the γ -C.P.M. of the fractions from 10th to 25th were also sufficiently high. This could be explained due to the fact that 3rd peak started eluting before the complete elution of the 2nd peak. The nature of the protein in the 1st peak has not been characterised.

Table 4.3.3.1 gives the γ -C.P.M. for 2 ml aliquot samples obtained at different stages during the isolation of ^{59}Fe labelled transferrin. The γ -counts for the precipitate (obtained after the addition of rivanol into the serum) were taken by adding 2 ml of 0.6 per cent rivanol into 2 ml diluted sera and this was centrifuged. The supernatant was discarded and counts were taken in the precipitate thus obtained. The high counts of the precipitate of the 2nd stage as well as the electrophoretic analysis, revealed the presence of

transferrin in this (Photograph 4.2.1.3). The negligible counts in the filtrate from the DEAE Sephadex A-50 column demonstrated the absence of transferrin in this fraction.

Table 4.3.3.1: γ -C.P.M. of 2 ml Aliquots at Different Stages of Isolation of Transferrin.

S.No.	Stage of Isolation	1st γ -C.P.M.	2nd γ -C.P.M.
1.	Whole sera	183343	186367
2.	Precipitate from Rivanol	68448	68549
3.	Supernatant from Rivanol	44501	44798
4.	Filtrate from starch	33102	33520
5.	Filtrate from DEAE Sephadex A-50	27	34
6.	Transferrin peak	86385	86244

The autoradiographs were taken from the starch gel electrophoretic runs of the samples of the different stages which indicated the presence of transferrin in these fractions.

4.3.4. Preparation of apotransferrins and Iron Binding Studies

The apotransferrins of all the transferrin phenotypes of cattle and buffaloes were prepared according to Stratil and Spooner (1971). One hundred mg of transferrin of each type was dialysed against four changes of 0.25 M sodium citrate-Citric Acid buffer pH 4.0

and finally against distilled water. On dialysis, transferrins which had a characteristic reddish brown colour, were converted to yellowish white apotransferrins. These preparations of apotransferrins were used for iron binding studies. The starch gel electrophoretic resolutions of these apotransferrins showed the presence of all the bands identical to the Fe-transferrin resolutions. Also, no significant differences in the resolutions of apo and Fe-transferrin forms could be detected.

Iron binding studies were performed by the incorporation of ^{59}Fe -citrate in either the whole sera of all transferrin phenotypes of cattle and buffaloes or the apotransferrin preparations were used to estimate the iron binding capacity. Four mg of apotransferrin of each type was dissolved in one ml of gel buffer and starch gel electrophoresis was performed using these samples. Table 4.3.4.1 gives the $\gamma\text{-C.P.M.}$ for the different phenotypes in case of both apotransferrins and whole sera.

Table 4.3.4.1: $\gamma\text{-C.P.M.}$ for Various Transferrin Phenotypes of Cattle and Buffaloes

S.No.	Tf type	Apotransferrins				Whole Sera			
		Back ground count	Control zone	Net	Back ground count	Control zone	Net		
1.	TfAA	22	550	2470	1898 (min)	19	829	26705	25857
2.	TfD ₁ D ₁	18	758	12176	11400 (max)	30	868	16382	15484
3.	TfD ₂ D ₂	18	418	5730	5294	55	2494	15112	12563
4.	TfEE	19	5145	11918	6754	55	2907	10395	7433 (min)
5.	TfDD (buffalo)	19	5398	8429	3012	52	535	30337	29750 (max)
6.	TfKK (buffalo)	19	421	4564	4124	54	7702	29433	21677

The net γ -C.P.M. for the apotransferrins ranged from 1898 (minimum) to 11400 (maximum), while this range increased from 7433 (minimum) to 29750 (maximum) in case of whole sera samples loaded with ^{59}Fe . This revealed that total net γ -counts were much higher when ^{59}Fe was added to whole sera samples, as compared to the net r-counts for apotransferrins. This increase of counts for the whole sera samples was as high as three times of the average net counts for apotransferrins. The average of net γ -C.P.M. for the cattle apotransferrins was about 6,500, while for buffalo apotransferrins this was about 3,500. However, the average of net γ -C.P.M. for both cattle and buffalo whole sera of all transferrin phenotypes was approximately 15,000 and 25,000 respectively. It was observed that the counts for buffalo sera samples were higher than the cattle sera. These observations possibly indicated a remarkable variation of iron binding for different transferrin phenotypes for apotransferrins and sera samples among both the species. But, however, these observations are only preliminary and not conclusive and the contribution of other factors towards the apparent variation in iron binding could not be ignored.

Iron binding was also estimated for different bands of one single transferrin phenotype. Table 4.3.4.2 shows the γ -C.P.M. of individual bands, which have been designated from the anodic end of the resolution as I, II, III and IV respectively. The range for the net γ -counts for different transferrin types was 288 to 415.

The counts for 1st band in all the transferrin types of cattle and buffaloes were lowest and the counts increased for 2nd and 3rd bands respectively. However, the counts for the 4th band

Table 4.3.4.2: γ -G.P.M. for Individual Bands of Various Transferrin Phenotypes of Cattle and Buffaloes

S.No.	Tf type	Back ground count	Control	Total count	Net count	Band I	Band II	Band III	Band IV
1.	TfAA	21	118	436	318	68	111	135	132
2.	TfD ₁ D ₁	21	78	514	415	96	118	158	142
3.	TfD ₂ D ₂	21	61	410	349	82	97	128	103
4.	TfEE	21	123	455	332	92	125	133	105
5.	TfDD (Buffalo)	21	99	367	288	111	143	133	-
6.	TfKK (Buffalo)	21	111	443	332	114	165	126	-

(only in cattle) were found to be less than the 2nd and 3rd bands respectively, but more than the 1st band of the same transferrin phenotype. This indicated a distinctly higher iron binding capacities for the 2nd and 3rd bands in all the phenotypes of the two species. The counts for buffaloes were taken, however, for only three bands as the homozygous transferrin type of buffalo gave only three bands on starch gel resolution. In this case the net counts were found to be highest for the 2nd band, followed by the 3rd and 1st bands respectively. The average of the net counts in this experiment was much lower, as the gel was first stained and destained, before the elution of individual bands. However, all the conditions of elutions were kept identical for all the transferrin types. This could be

accomplished by running all transferrin phenotypes in a single gel simultaneously.

4.3.5. Autoradiography

The autoradiography of the different experiments using Fe⁵⁹ radioisotope was done essentially as described earlier (methodology). The autoradiographs for different incubation periods revealed that incubation of the gel having Fe⁵⁹ radioisotope for 24 hours with the X-ray plate gave the best exposition and clear bands of transferrin (autoradiograph 4.3.5.1). If the time of incubation was increased to 52 hours, it was observed that instead of clear bands, diffused zones for transferrin were obtained in accordance with their respective relative mobilities (Autoradiograph 4.3.5.2). Keeping the incubation period constant, the increase in the amount of Fe⁵⁹ to double concentration resulted in darker bands on the X-ray plate (Autoradiograph 4.3.5.3).

The autoradiograph 4.3.5.4 of the different stages of isolation, showed the presence of transferrin in all the stages. The absence of any band in the filtrate from DEAE Sephadex A-50 column, confirmed that all the transferrin got strongly adsorbed on it and did not pass into the filtrate. The autoradiograph 4.3.5.5 of the purified transferrin types indicated some trailing of Fe⁵⁹, before and after the transferrin bands.

4.3.6. Effect of Neuraminidase

Four mg of lyophilised, pure cattle transferrin AA type, which had been loaded earlier 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 incubated with 50 units of neuraminidase enzyme at 37.5° C. Samples of 0.5 ml aliquot were collected after 3, 6, 12, 24, 48 and 72 hours of incubation periods and were immediately frozen in a refrigerator. The autoradiograph 4.3.6.1 of these samples on starch gel electrophoresis, revealed a gradual decrease in mobility of transferrin bands. The complete loss of bands was not observed. All the three bands migrated slower, with the increase in incubation periods. However, the intensity of staining of the three bands varied with different periods of incubation. The decrease in mobility was not found after 72 hours of incubation. The sample incubated for 3 hours showed a quick decrease in mobility but a further decrease in mobility was less for samples incubated for longer time. The experiment, however, could not be repeated, due to the high cost of neuraminidase enzyme.

This experiment was specifically designed using Fe⁵⁹ radio-isotope so that the effect of the neuraminidase enzyme could be detected by autoradiography, without resorting to high concentrations of enzyme and protein which are normally required while using starch gel electrophoresis.

4.4. Immunological Comparisons

4.4.1. Production of Antisera

Immunological responses were investigated to further illuminate the nature of relationships among the different genetically controlled transferrin types of cattle and buffaloes. Two types of

antisera were raised by immunising the rabbits. In the first case, the sera from all the transferrin types used for study, were pooled in equal volumes and this was used to produce polyvalent antisera in rabbits. The second type of antisera was prepared against the pooled purified transferrins of all the phenotypes. Antisera were raised in two rabbits for each class. The immunisation was done subcutaneously in the footpad using transferrin in Freunds Complete adjuvant. The booster injections were given intravenously after four weeks. Photograph 4.4.1.1 shows that immunodiffusion on 1 per cent agar gel in normal saline gave the titre value of the antisera till 1/6th dilution. The antigen/antibody reaction formed distinctly well defined precipitin arcs.

Double diffusion in agar gel was performed as described by Cuchterlonny (1955) with some modifications. One per cent concentration of double ion Noble agar from Difco Laboratories was used. All the antisera, when tested against pooled cattle sera and pooled buffalo sera, showed multiple arcs between the wells for antigen and antibody. Out of eight rabbits immunised, one died during the course of immunisation while it was possible to obtain antisera from other seven rabbits. Some variation among these rabbits for antibodies production was, however, observed. Antisera from some rabbits gave very clear precipitin arcs on immunodiffusion while for others, zones of diffused precipitin formation were observed. Comparatively the specific antisera against the pure pooled transferrins gave very clear and distinct arcs (Photographs 4.4.1.2 and 4.4.1.2). The antiserum from rabbit No. 88 used for anti-pooled cattle serum production (Photograph 4.4.1.1) showed

clearly three arcs and a diffused zone near the antibody well, when it was tested against pooled cattle sera in the centre well. These could be clearly observed till 1/4th dilution, but at 1/8th dilution, only a diffused zone was observed. However, the antisera from rabbits 104 and 232, which were immunised for anti-pooled cattle transferrin, gave one arc and diffused zone. The zone gradually disappeared as the dilution of antisera was further increased. But the concentration of the arc remained uniform till 1/8th dilution. Higher dilutions of the antisera did not produce clear visible precipitin arcs.

The antisera against pooled buffalo transferrin sera and protein (Photograph 4.4.1.2) gave similar results. Antisera from rabbits 252 and 203 which were immunised for anti-pooled buffalo sera, gave multiple arcs between antigen/antibody wells. However, the antisera from rabbits 116 and 235, used for anti-pooled transferrin, showed faint arcs. Instead, diffused zones till 1/8th dilution of the antisera were observed.

4.4.2. Immunodiffusion

Photograph 4.4.2.1 shows the immunodiffusion of the partially purified cattle transferrin type AA, D₁D₁, D₂D₂ and EE against anti-pooled cattle sera and anti-pooled cattle transferrin sera. In all these cases one major precipitin arc which was most prominent and responsible for transferrin, was observed along with some very faint precipitin arcs representing the minor impurities. The arcs for

transferrin were more prominent in case of the antitransferrin protein sera than anticattle sera. The lower right centre well (Photograph 4.4.2.1) contained antibuffalo transferrin sera. The presence of arcs for all the cattle transferrin types showed that cattle transferrins had apparently cross-reacted with the antibuffalo transferrin sera.

Photograph 4.4.2.2 gives the immunodiffusion of partially purified buffalo transferrin types against the antipooled buffalo sera and antipooled buffalo transferrin sera. In this case, multiple arcs were observed for the antipooled buffalo transferrin, as was evident for the lower two wells, while there was only one arc in case of antibuffalo sera (rabbit 203). This observation is at variance with the cattle transferrin immunodiffusion results.

4.4.3. Immunolectrophoresis

The immunological nature of different cattle and buffalo transferrin types was also characterised by immunolectrophoretic experiments. Photograph 4.4.3.1 shows the immunolectrophoresis of pooled cattle sera against antipooled cattle sera in the upper trough and pooled buffalo sera against antipooled buffalo sera in the lower trough. The presence of multiple arcs in both the experiments revealed the heterogeneous nature of antibodies against those antigenic factors. The arc represented for transferrin was located in the middle of the antigen well and the antiserum trough.

The different concentrations of the purified transferrins were tested against the antisera to check the purity of the isolated proteins. It was found that the purified transferrins did not give any additional arc at 6 mg/ml concentration of the protein (Photograph 4.4.3.2), when tested against specific antitransferrin sera. This was evidently clear from the presence of a prominent arc in the gel. However, when the same experiment was repeated against the antipooled cattle sera, the intensity of the arcs was slightly decreased (Photograph 4.4.3.3).

Photograph 4.4.3.4 shows the purified cattle transferrins against pooled anticattle sera and purified buffalo transferrins against pooled antibuffalo sera. Slightly diffused arcs were observed whereas other minor arcs were absent.

Photographs 4.4.3.5 and 4.4.3.6 show the immunoelectrophoretic runs of cattle and buffalo transferrin against anticattle sera and anticattle transferrin sera, respectively. The wells for antigen viz., from top to bottom, contained TfAA, TfD₁D₁, TfD₂D₂ and TfEE of cattle and TfDD and TfKK of buffaloes, respectively. The troughs for antisera contained antipooled cattle sera (Photograph 4.4.3.5) and antipooled transferrin protein sera (Photograph 4.4.3.6) respectively. Only single arc for transferrin was observed for all these experiments. The presence of well defined arcs in the case of buffalo transferrins revealed that it clearly cross-reacted with cattle antisera.

Photographs 4.4.3.7 and 4.4.3.8 show the immunoelectrophoresis of cattle and buffalo transferrin against pooled buffalo antisera and pooled buffalo transferrin antisera. It is apparent here that along with major arcs for transferrin, some minor arcs were also present in all the cases. This demonstrated that cattle transferrin too cross-reacted with buffalo antisera. Also, the antigenic factors in cattle transferrins which could not be detected by cattle transferrin antisera, were sometimes revealed by the buffalo transferrin antisera. The presence of minor arcs in buffalo TfDD and TfKK types against buffalo transferrin antisera, indicated the traces of impurities in these proteins at higher concentrations.

The effect of heat on the immunological character of transferrin was also investigated in one experiment. Photographs (4.4.3.9 and 4.4.3.10) show the TfAA, TfD₁D₁ and TfD₂D₂ in 1, 2 and 3 antigen wells (without heating), while TfEE and buffalo TfDD and TfKK in 4, 5 and 6 antigen wells were heated. It was found that the arcs were absent in case of the transferrins which had been heated. It could be concluded, therefore, that antigenic nature of these transferrin types was lost on heating.

An immunoelectrophoretic experiment was performed, where different combinations of two and three transferrin types were pooled and charged together in the same antigen well. After the electrophoretic run the troughs were filled with pooled antitransferrin sera.

This was done specifically to locate the antigenic electrophoretic differences of these transferrin types. Photographs 4.4.3.11 and 4.4.3.12 show the results of these experiments. It was observed that one main precipitin arc appeared for all combinations of two transferrin types together at a time (Photograph 4.4.3.11) viz., from top AD_1 , AD_2 , AE , D_1D_2 , D_1E and D_2E respectively. However, some minor arcs of a lower intensity were also observed. Similarly, Photograph 4.4.3.12 shows the immunolectrophoretic run when combinations of three and four transferrin types were taken together in each antigen well. The sequence viz., from top to bottom was AD_1D_2 , AD_1E , AD_2E , D_1D_2E , AD_1D_2E and buffalo DK types respectively. Here again, one prominent arc was found in all the combinations whereas minor arcs with slightly higher intensity than that obtained in earlier experiment, were observed.