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# IDENTIFICATION OF TRANSFERRIN RECEPTORS IN RETICULOCYTES

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## Summary

Experiments were performed to obtain definitive evidence for the presence of membrane receptors for transferrin on reticulocytes. Rabbit reticulocytes were incubated with $^{125}$I-labelled rabbit transferrin. The transferrin taken up by the cells was solubilized using the non-ionic detergent, Teric 12A9 (polyoxyethylene ($\bar{n}$ = 9) dodecyl alcohol). The soluble extracts of the cells were examined by gel filtration and a transferrrin-binding moiety of approximate molecular weight 275 000 was identified. This binding moiety was found only in reticulocytes, not in mature erythrocytes. The membrane component could bind only transferrin and not the other plasma proteins studied. Only transferrin could displace bound transferrin from the complex. Rabbit transferrin was bound more strongly than human transferrin. The binding of transferrin to the component was shown to be reversible and saturable. It is concluded from these studies that the transferrin binding component identified in the reticulocyte stroma is a true physiological receptor for transferrin.

## Introduction

A primary biological function of the plasma iron-binding protein, transferrin, is the donation of its iron to erythrocyte precursors in the bone marrow and to circulating reticulocytes. These cells have large iron requirements for haemoglobin synthesis.

In 1959 Jandl and co-workers [1] postulated that the acquisition of iron by reticulocytes from transferrin occurs via receptors for transferrin located on the cell membrane. Since then there have been numerous references in the literature to "transferrin receptors", not only in reticulocytes but also in other tissues [2—8]. The term "transferrin receptor" was used very loosely in these publications since definitive evidence for their existence had not been reported.

Abbreviation: Teric, polyoxyethylene ($\bar{n}$ = 9) dodecyl alcohol

Recently, transferrin-binding moieties have been demonstrated in reticulocytes by the use of extraction of cells and ghosts with various detergents [9—11]. However, it has not been established that the properties of these transferrin-binding components are those of the biologically functional transferrin receptors.

The aim of the present work was to identify the component of the rabbit reticulocyte membrane which is capable of binding transferrin, to characterize it and to establish its identity as the biological receptor for transferrin.

## Materials and Methods

Iron-59 as $FeCl_3$ of specifific activity 10—30 $\mu$Ci/g; $^{125}I$ and $^{131}I$, as NaI, carrier free; were obtained from the Radiochemical Centre, Amersham, England. Coomassie Brilliant Blue R-250 was purchased from the Sigma Chemical Co., St. Louis, M.O., U.S.A. The chromatography media, Sepharose 2B, 4B, 6B, and Sephadex G-200 superfine were supplied by Pharmacia (South Seas) Pty. Ltd., Sydney, N.S.W., Australia; Ultrogel ACA 22 and 34 were supplied by L.K.B. Stockholm, Sweden. Teric 12A9 (polyethoxylated ($\bar{n}$ = 9) lauryl alcohol) batch No. C512.31 was a generous gift from I.C.I. Ltd., Perth, Western Australia and will be referred to as Teric in the following text. Human transferrin was obtained from Behringwerke, Marburg-Lahn, Germany.

*Reticulocytes.* Reticulocytosis was induced in adult rabbits, by either phenylhydrazine treatment or haemorrhagic anaemia as described by Baker and Morgan [12]. Reticulocyte-enriched blood, referred to as "reticulocytes" in the following text, was collected from a marginal ear vein of the rabbits into heparinised test tubes. The cells were washed three times with 0.15 M NaCl at 4° C and particular care was taken to remove "buffy-coat" cells which may contain relatively high activities of proteolytic enzymes [13].

*Isolation of rabbit transferrin, albumin and γ-globulin.* Di-ferric rabbit transferrin was isolated from pooled, iron-saturated serum by four steps, viz: (a) ammonium sulphate fractionation; (b) ion-exchange chromatography on DEAE-cellulose DE-52 (Whatman); (c) gel filtration on Sephadex G-200; and (d) crystallisation, as described by Baker et al. [14]. The final product was subjected to three crystallisation cycles after which the transferrin purity was greater than 99% as determined by electrophoresis on 10% polacrylamide gels at pH 8.3 using a Hoefer electrophoresis apparatus (EF 301), the buffer systems described by Davis [15] and the Coomassie Brilliant Blue-staining method of Vesterberg [16]. Rabbit serum albumin and γ-globulin were obtained from the ion-exchange chromatography step of the transferrin isolation procedure as described previously [17].

*Isotopic labelling of proteins.* Albumin and iron-saturated transferrin were labelled with radioactive iodine using the iodine monocholoride method of McFarlane to give an average maximum of one iodine atom per protein molecule. Apotransferrin was prepared from iron-saturated transferrin and was labelled with $^{59}Fe$ as previously described [18].

*Incubation procedure.* Reticulocytes, reticulocyte ghosts, mature erythrocytes and mature erythrocyte ghosts were incubated with labelled proteins, washed with ice-cold 0.15 M NaCl and the radioactivity determined as described

by Baker and Morgan [12]. Teric extracts of these preparations were also incubated with labelled and unlabeled proteins. Details of these procedures will be given in the text.

*Extraction procedures.* Unless otherwise stated in the following text, washed cells or ghosts were extracted with 10 vol 1% Teric or five volumes 2% Teric in 15 mosM sodium phosphate buffer, pH 7.4 ("extraction solution") for 1 h at 4°C after either low speed homogenization or mixing with a glass rod. The extraction mixture was then centrifuged for 1 h at 40 000 × $g_{max}$. High speed, supernatant solutions were also prepared by centrifugation at 48 000 × $g_{max}$ for 2 h. In experiments where cell ghosts were used, cells were haemolysed at 4°C with 10—20 volumes 15 mosM phosphate buffer, pH 7.4, and centrifuged for 40 000 × $g_{max}$ for 0.5 h. The resultant pellets were washed by repeated cycles of re-suspension and centrifugation in 15 mosM phosphate buffer until they were almost haemoglobin free.

*Gel filtration.* In general, established chromatographic procedures as recommended by Pharmacia were used and all column operations were carried out at 4—6°C. Column eluants (0.1% Teric in 15 mosM phosphate buffer, pH 7.4) were pumped through a Chromatronix Absorbance Detector, model 230 (chromatronix Inc., Calif., U.S.A.). The absorption profiles at 280 nm were recorded on a 2-channel chart recorder (Linear Instrument Corp., Irvine, Calif., U.S.A.) with one channel used for fraction event marking. The eluants were collected into radioactivity counting tubes in a fixed-time mode fraction collector, and the radioactivity in the fractions was measured.

*Analytical procedures.* Reticulocyte counts were performed on blood smears stained with new methylene blue. Haematocrits were determined by the microhaematocrit method. Protein was measured by a biuret procedure using bovine serum albumin as standard [19]. Transferrin was quantitated by measurement of absorbance at 462 nm using as standard an $E^{1\%}_{462}$ value of 0.58 obtained from pure, lyophilized, iron-saturated rabbit transferrin corrected for residual water content. Lipid analysis was performed on cell extracts obtained by the procedure of Folch et al. [20] using two-dimensional thin-layer chromatography according to the method of Turner and Rouser [21]. The lipids were visualized on the thin-layer plates by the method of Vaskovsky and Kostetsky [22] and phosphorous was determined as described by Parker and Peterson [23]. Radioactivity was measured in a 3-channel, well-type scintillation detector.

## Results

*Extraction of reticulocytes with Teric 12A9*

Reticulocytes were incubated with $^{125}$I-labelled transferrin for 15 min at 37°C, haemolysed and the ghosts washed three times with 15 mosM sodium phosphate buffer, pH 7.4. Experiments were performed to investigate the effects of Teric concentration, extraction time, temperature, and ionic strength of the extraction solution of the solubilization of labelled transferrin from the ghosts.

The ghosts were extracted for 1 h at 4°C with solutions containing Teric dissolved in 15 mosM sodium phosphate buffer, pH 7.4. The Teric concentration was varied from 0.01 to 4% (w/v) and 10 volumes of the Teric solution was

used per volumes of cells from which the ghosts were prepared. The amount of $^{125}$I-labelled transferrin extracted from the ghosts increased from 16 to 91% of that present in the ghosts as the Teric concentration was raised from 0.01 to 1%. No further increase in $^{125}$I-labelled transferrin solubilization was observed at higher Teric concentrations. When 10 volumes of 1% Teric were used it was found that the percentage extraction of labelled transferrin from the ghosts was the same whether the Teric was dissolved in 15 mosM sodium phosphate 150 mM NaCl or in 15 mosM phosphate alone. Also, the degree of extraction after 1 h was the same at 37° C as at 4° C. Extraction of $^{125}$I-labelled transferrin reached maximum values after between 10 and 30 min at 4° C.

As a result of these experiments subsequent extractions of reticulocytes or ghosts with Teric were performed at 4° C for 1—4 h using either 10 volumes 1% Teric or five volumes 2% Teric dissolved in 15 mosM sodium phosphate buffer, pH 7.4.

Measurements were made of the protein and phospholipids extracted from reticulocyte ghosts by Teric. Whereas 90% or more of the $^{125}$I-labelled transferrin present in ghosts was extracted by the standard procedure only 47 ± 1.8% (mean ± S.E. of 10 determinations) of the total protein was present in the Teric extract. The results for phospholipid analysis are given in Table I. Teric extracted approx. 70% of the total phospholipids present in the reticulocytes when compared with the value for whole cells obtained by the Folch extraction method [20]. The relative distribution of the phospholipids between the various classes was similar in the Teric extract as in the total cell Folch extract.

The recovery of $^{59}$Fe and $^{125}$I in the various fractions obtained when labelled reticulocytes or their ghosts were extracted with Teric was measured in several experiments. Table II summarizes the results obtained in one experiment in which reticulocytes were first incubated with $^{59}$Fe - $^{125}$I-labelled transferrin for 15 min at either 4 or 37° C. The relative distribution of the two isotopes between the various fractions was similar when incubation was performed at 4° C.

TABLE I

PHOSPHOLIPID CLASS DISTRIBUTION IN RETICULOCYTE MEMBRANES

Rabbit blood cells (65% reticulocytes) were extracted according to the method of Folch et al. [20]. Phospholipid class distribution was determined using two-dimensional thin-layer chromatography. An identical sample of reticulocytes was haemolysed and the ghosts washed until free of haemoglobin as described in Materials and Methods. The Teric extract of this preparation was analysed for phospholipid class distribution by one-dimensional thin-layer chromatography. Attempts at two-dimensional analysis failed with this preparation, presumably because of the presence of Teric.

| Phospholipid | Intact cells (percent total P) | Ghost Teric extract (percent total P) |
|---|---|---|
| Phosphatidylcholine | 35.63 | 33.65 |
| Phosphatidylethanolamine | 23.31 | 30.26 |
| Sphingomyelin | 19.78 | 23.42 |
| Lysophosphatidylcholine | 3.03 | 9.57 |
| Phosphatidylinositol | 3.35 | |
| Phosphatidylserine | 5.61 | |
| Unknown | 9.30 | 3.10 |

TABLE II

TERIC EXTRACTION OF RETICULOCYTES

Rabbit blood cells (54% reticulocytes) were incubated for 15 min at 4 or 37°C with $^{59}Fe$-$^{125}I$-labelled rabbit transferrin (3.0 mg/ml, 50% saturated with iron). The cells were then washed and lysates and membrane ghosts were prepared as described in Materials and Methods. Samples of the intact cells and ghost preparations were extracted with Teric as described in the text. The radioactivity of each fraction is expressed as a percentage of that present in the intact washed cells.

| Incubation temperature: | Radioactivity (percent intact cells) | | | |
|---|---|---|---|---|
| | 4°C | | 37°C | |
| | $^{59}Fe$ | $^{125}I$ | $^{59}Fe$ | $^{125}I$ |
| Whole cells | 100 | 100 | 100 | 100 |
| Teric extract | 96 | 95 | 95 | 98 |
| Residue | 4 | 5 | 5 | 2 |
| Whole cells | 100 | 100 | 100 | 100 |
| Haemolysate | 22 | 28 | 68 | 10 |
| Washes | 19 | 19 | 10 | 16 |
| Ghosts | 59 | 53 | 22 | 74 |
| Teric extract | 50 | 49 | 16 | 71 |
| Residue | 9 | 4 | 6 | 3 |
| Total uptake (cpm) | 10 200 | 109 800 | 129 000 | 429 000 |

However, after incubation at 37°C a much greater proportion of the $^{59}Fe$ than of the $^{125}I$ appeared in the haemolysate and far more of the $^{125}I$ than $^{59}Fe$ was present in the Teric extract of the ghosts. Approx. 75% of the $^{125}I$ present in the whole cells was recovered in the ghosts and nearly all of that was extracted by the Teric. This high degree of retention of labelled transferrin during preparation of ghosts was found consistently whenever incubation of cells and transferrin was performed at 37°C.

*Gel filtration of Teric extracts of reticulocytes*

Reticulocytes were incubated at 37°C with $^{59}Fe$ - $^{125}I$-labelled transferrin, washed and Teric extracts were prepared from whole cells and from haemoglobin-free ghosts. The extracts were fractionated by gel filtration on Sephadex G-200 Superfine or on Ultrogel ACA 34. The pattern obtained with both gel filtration media was similar but greater resolution of the components was achieved using ACA34 than with Sephadex G-200.

As shown in Fig. 1 the absorbance profile at 280 nm of the whole cell extract fractionated on ACA 34 consisted of four peaks the major one corresponding to haemoglobin. $^{125}I$-labelled transferrin emerged as two peaks, the first just after the void volume and the second at the elution volume of native transferrin. The first peak contained approximately one-third of the total $^{125}I$ and the second peak two-thirds. Six $^{59}Fe$-containing peaks were observed, two corresponding to the two transferrin peaks, one to the heamoglobin peak, one peak before the first transferrin peak, one between the two transferrin peaks and the other a small peak, after the haemoglobin peak. The first $^{59}Fe$ peak emerged at the same elution volume as horse spleen ferritin.

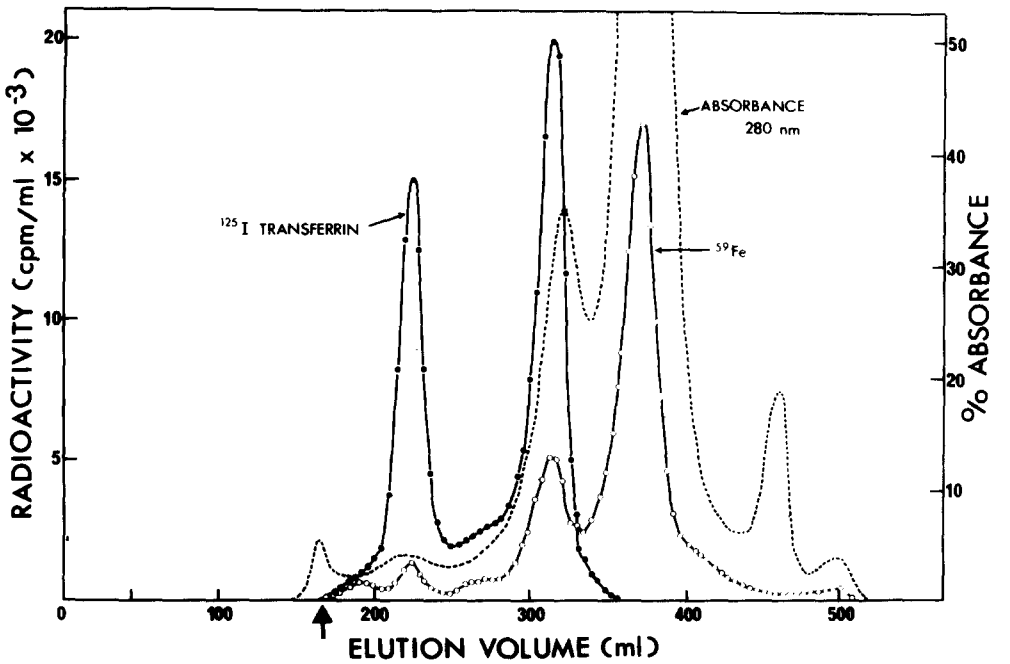


Fig. 1. Gel filtration of Teric extract of rabbit reticulocytes. A reticulocyte cell suspension (65% reticulocytes) was incubated with $^{125}$I-$^{59}$Fe-labelled rabbit transferrin for 20 min at 37°C. The cells were then washed and extracted with Teric as described in the text. A 3.0 ml aliquot of the solution obtained after centrifugation of the extract at 40 000 × *g* for 2 h was fractionated on an 85 × 2.5 cm column of Ultrogel ACA 34. The bold arrow denotes the "void volume" of the column as defined with Blue Dextran 2000.

The elution pattern obtained with the Teric extract of haemoglobin-free ghosts was similar to that obtained with the extract of whole cells, except that the protein and $^{59}$Fe peaks corresponding to haemoglobin were absent. The same gel filtration pattern was obtained with Teric extracts of ghosts after centrifugation of the extract at 40 000 × *g* for 16 h or 40 000 × *g* for 1 h.

The molecular weight of the first, larger molecular weight transferrin peak was estimated by gel filtration on Ultrogel ACA 22. The column was standardized using horse spleen ferritin ($M_r$ 430 000), catalase ($M_r$ 240 000), rabbit IgG ($M_r$ 155 000), rabbit haemoglobin ($M_r$ 68 000), and rabbit transferrin ($M_r$ 76 000). Ovalbumin ($M_r$ 45 000) and albumin (monomer $M_r$ 68 000, dimer $M_r$ 135 000) could not be used as standards because of their interaction with Teric. The value obtained for the high molecular weight transferrin peak was 350 000 ± 20 000. Hence, if this peak consists of transferrin combined with a cell component in a 1 : 1 molar ratio the binding component has an apparent molecular weight of approx. 275 000.

*Dependence of the transferrin-binding component on reticulocyte count*

The amount of transferrin in the first gel filtration peak was taken as an index of the amount of transferrin-binding macromolecule in Teric extracts of cells. Cell suspensions were prepared containing from 2 to 90% reticulocytes by mixing blood from a normal rabbit with that from a rabbit with haemolytic anaemia. The cells were incubated with labelled transferrin, extracted and frac-

tionated as described previously. The relative amount of transferrin present as the high molecular weight complex was linearly dependent on the percentage of reticulocytes present in the cell suspensions. There was no evidence for the presence of a high molecular weight transferrin complex in mature erythrocytes.

*Effect of incubation time on the amount of transferrin in the 1st gel filtration peak*

Reticulocytes were incubated with labelled transferrin for various times up to 30 min, washed and then haemolysed. Teric extracts of the resultant ghosts were subjected to gel filtration and the radioactivity in the two transferrin peaks was measured. Fig. 2 shows that the amount of transferrin fractionated as the high molecular weight complex increased with incubation time. The time dependence of formation of this complex was similar to the uptake of transferrin by intact reticulocytes, viz. an initial absorption phase (at 0 time) followed by an uptake phase to finally reach a plateau in the amount of transferrin taken up [12].

*Interaction of rabbit serum albumin and IgG and human transferrin with the transferrin-binding component.*

Reticulocytes were incubated with $^{131}$I-labelled rabbit serum albumin or IgG or human transferrin. Teric extracts of these cells were then fractionated on Sephadex G-200. In contrast with the results obtained with transferrin, both albumin and γ-globulin eluted as single peaks only. IgG emerged at the elution volume expected for a protein of 155 000 daltons. However, albumin eluted at a volume corresponding to a molecular weight considerably greater than that reported for this protein. Human transferrin was taken up by the reticulocytes and in the subsequent chromatographic analysis of the Teric extract the human transferrin behaved in a similar manner to rabbit transferrin, as described above.

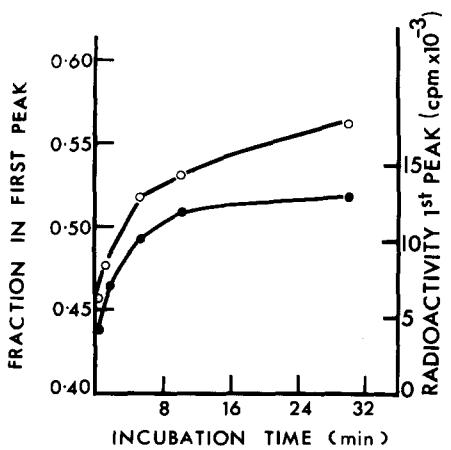


Fig. 2. Effect of incubation time on proportion of transferrin eluted in first gel filtration peak. Samples of rabbit blood cells (72% reticulocytes) were incubated at 37° C with $^{125}$I-labelled transferrin for 0—30 min. The cells were washed, haemolysed, extracted with Teric as described in the text. The extracts were fractionated by filtration on Sephadex G-200. The figure shows the appearance of radioactivity in high molecular weight transferrin peak (●———●) and the fraction of the total extract radioactivity which appeared in this peak (○———○).

In order to investigate the species specificity of the interaction between transferrin and its reticulocyte-binding component, reticulocytes were incubated with equimolar concentrations of $^{131}$I-labelled human and $^{125}$I-labelled rabbit transferrin. The radioactivity elution pattern of human and rabbit transferrin in the Teric extract prepared from these cells showed that the amount of human transferrin extracted by Teric from the the ghosts of these cells was 70% of that of rabbit transferrin. The amount of human transferrin associated with the high molecular weight peak was approx. 50% of that of rabbit transferrin.

*Binding and displacement studies*

Teric extracts of reticulocyte ghosts containing the high molecular weight $^{125}$I-labelled transferrin complex were incubated for 20 min at 37° C with unlabelled rabbit serum albumin, IgG and transferrin, and human transferrin. These solutions were then subjected to gel filtration on Sephadex G-200 (Fig. 3). The results showed that neither rabbit albumin nor IgG were capable of displacing labelled transferrin from the high molecular weight complex. However, the transferrins used did displace this transferrin. Rabbit transferrin was more effective than human transferrin in these displacement studies. Furthermore, the amount of transferrin displaced from the high molecular weight complex depended directly on the concentration of the competing, unlabelled transferrin in the incubation mixture.

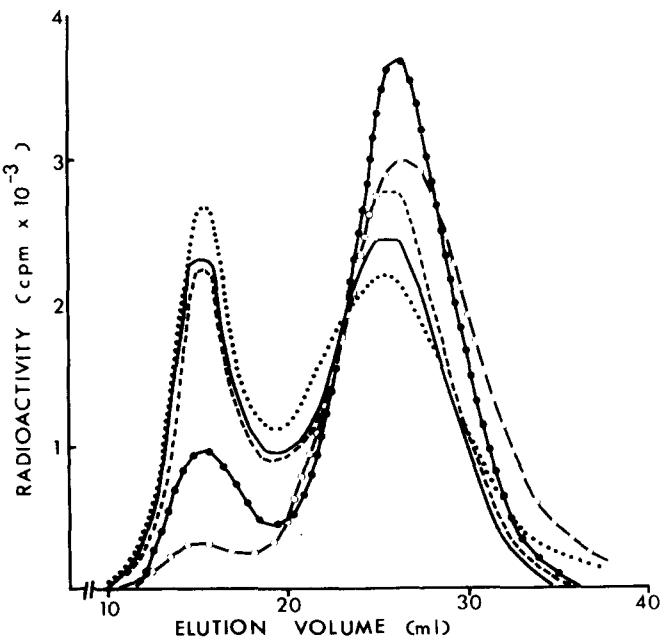


Fig. 3. Effect of rabbit transferrin, albumin and IgG on gel filtration pattern of Teric extract of reticulocytes containing $^{125}$I-labelled transferrin. Rabbit reticulocytes (65% reticulocytes) were incubated with $^{125}$I-labelled rabbit transferrin for 20 min at 37° C. Samples of a Teric extract of the ghosts prepared from these cells were incubated with unlabelled rabbit transferrin (●———●, 0.25 mg/ml; ○———○, 1.00 mg/ml), rabbit albumin (· · · · · ·, 2.50 mg/ml), rabbit IgG (- - - - - -, 2.50 mg/ml) or no other protein (———) for 20 min at 37° C. The extracts were then fractionated by gel filtration on Sephadex G-200.

The above experiments showed that the binding of transferrin to the cell membrane component was reversible and that the bound, labelled transferrin could be displaced from the complex by unlabelled transferrin. The reversibility of binding of transferrin to the complex was also supported by the following experiment. Reticulocytes were incubated with $^{125}I$ - $^{59}Fe$ labelled transferrin for 20 min at 37° C. A Teric extract was prepared from the washed ghosts and subjected to gel filtration on Sephadex G-200. The high molecular weight transferrin fractions were pooled and concentrated by vacuum dialysis against the column elution buffer (0.1% Teric in 15 mosM phosphate buffer). Rechromatography of this material again yielded the two molecular weight forms of transferrin (Fig. 4). This result shows that transferrin could be dissociated from the high molecular weight complex by gel filtration. That this dissociation was not due to any significant extent to denaturation of the binding component is indicated by the finding that this component is very stable under these experimental conditions (reported below). Furthermore, samples of the high molecular weight complex, partially depleted of labelled transferrin by rechromatography, could be made to bind additional transferrin upon incubation for 15—20 min at 37° C with labelled transferrin.

In view of these observations, it must be concluded that the binding of transferrin to the cell membrane derived component occurs in a reversible manner.

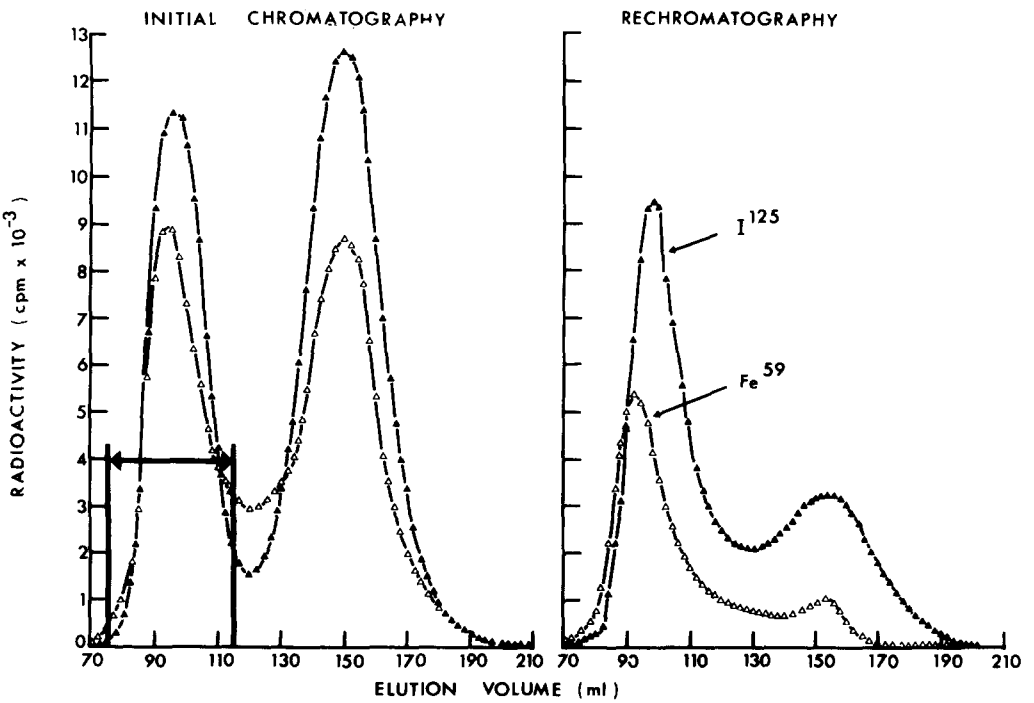


Fig. 4. Rechromatography of high molecular weight transferrin peak. A sample of rabbit reticulocytes (55% reticulocytes) was incubated with $^{125}I$-$^{59}Fe$-labelled rabbit transferrin for 15 min. The large molecular weight peak obtained by Sephadex G-200 gel filtration of a Teric extract of the ghosts from these cells (arrow, left-hand side) was concentrated by vacuum dialysis and rechromatographed on the same column of Sephadex G-200 (right-hand side).

*Effect of transferrin concentration*

Reticulocytes were incubated with increasing concentrations of labelled transferrin, washed with cold 0.15 M NaCl and transferrin uptake was measured. The cells were then haemolysed and the ghosts were washed with haemolysing solution until free from haemoglobin. The ghost pellets were extracted with Teric, centrifuged and the supernatant solution was fractionated by gel filtration. Fig. 5 shows the distribution of labelled transferrin in these fractions. Total cellular uptake of transferrin increased in a biphasic manner as the concentration of transferrin in the incubation medium was increased. This indicates that two uptake processes were operative, a non-specific one which increased linearly with increasing transferrin concentration and a specific one with a plateau at a transferrin concentration of approx. 10 mg/ml. The amount of transferrin extracted by Teric from the ghosts and the amount present in the first gel filtration peak plateaued at approximately the same transferrin concentration.

On the premise that the uptake process which reached a plateau at about 10 mg/ml represented binding of transferrin to specific receptors, it was possible to estimate the number of receptors per cell by analysing the data according to

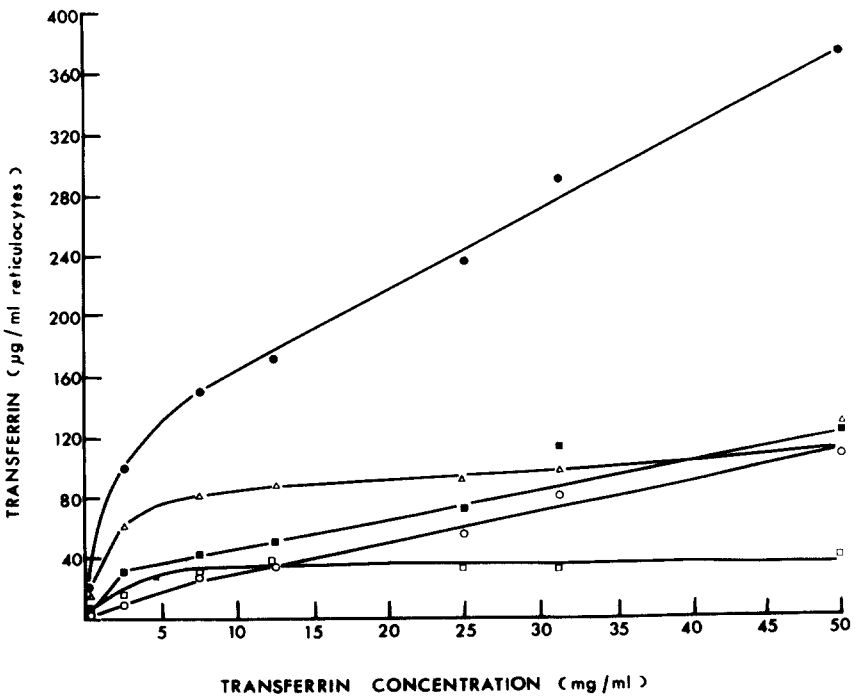


**Fig. 5. Effect of transferrin concentration on its distribution between fractions from reticulocytes. Samples of rabbit reticulocytes (83% reticulocytes) were incubated in solutions containing varying concentrations of $^{125}$I-labelled rabbit transferrin. The cells were then washed, haemolysed with 15 mosM phosphate buffer, pH 7.4 and the ghosts washed three times with same buffer. The ghosts were then extracted with 2% Teric, centrifuged at 40 000 × *g* for 2 h and the clear supernatant subjected to gel filtration on Sephadex G-200. The figure shows the concentration in the intact reticulocytes (●), the combined haemolysate plus ghosts washes (○), the supernatant (△) and residue (■) fractions of the Teric extract after centrifugation and the first transferrin-containing gel filtration peak (□).**

the method of Scatchard [24]. It was assumed that the "receptor" is univalent, i.e. one receptor molecule binding one transferrin molecule. The number of "receptors" per cell was thus estimated to be approx. 120 000 from the total cell uptake values and 90 000 from the values for the amount of transferrin extracted by Teric.

*Stability of the high molecular weight transferrin complex*

In order to investigate the stability of the high molecular weight transferrin complex, a number of experiments were carried out where various labelled and unlabelled cell fractions were stored at temperatures ranging from −20° C to 22° C before being examined by gel filtration. It was found that the tendency for complex formation between transferrin and its reticulocyte binding moiety was not diminished after storage at −20° C for at least 2 months; at 4—6° C for at least 10 days and at 20—22° C for 36 h.

## Discussion

The aim of the present work was to identify the component of the rabbit reticulocyte membrane which is capable of binding transferrin, to characterize it and to establish its identity as the biological receptor for transferrin. The binding component was extracted from the cell membrane with the non-ionic detergent Teric 12A9. This has the advantage over the more commonly used detergent, Triton X-100, in that it has negligible light absorbance at 280 nm so that proteins can readily be monitored in its presence. In addition, it has been reported to be more effective than Triton X-100 in solubilizing membrane components while retaining their biological properties [25].

Although the mechanism of interaction between Teric and cell membranes or proteins, such as those of the reticulocyte stroma and of plasma, has not been reported, a considerable body of data is available on the association of amphiphiles structurally related to Teric with membranes and proteins [25—30]. From the chromatographic data presented in the present work, it seems unlikely that transferrin bound Teric since its apparent molecular weight during gel filtration was unaltered in the presence of the detergent. Serum albumin, however, had an apparent molecular weight considerably in excess of 70 000 suggesting that albumin had bound the Teric or that it had experienced a large change in conformation from its native state in the presence of Teric.

The presence of transferrin in the high molecular weight peak observed during gel filtration (Fig. 1) indicates that transferrin is bound to a component derived from the reticulocyte stroma. The fact that this peak persisted even after the extract was centrifuged for 16 h at 40 000 × *g* is evidence that the peak represents a complex of transferrin with a solubilized component from the cell rather than with a membrane-derived particle or vesicle. Although it seems highly likely that the binding component is the reticulocyte receptor for transferrin, this has not been previously established. Before such a component is called the "transferrin receptor", there are certain prerequisites to be satisfied, [31]. These include: that the receptor should react specifically with transferrin only and not with other plama proteins; that binding of transferrin to the receptor should be reversible and saturable; that the number of receptors per

cell should be commensurate with the known biological functions and properties of transferrin; that the receptor should be present in reticulocytes but not in mature erythrocyte membranes, since mature erythrocytes do not take up transferrin [2,32]; and, finally, that cellular acquisition of iron from transferrin should be the ultimate biological event following the initial occupation of the membrane receptor by transferrin.

The results presented here show that the membrane component present in the high molecular weight transferrin peak satisfies all except the last of the above conditions. As discussed below, the results of other experiments provide strong support for the last condition [33,34]. Hence, the membrane component must be considered to contain the transferrin receptor. The component reacted with transferrin, but not with albumin or IgG (Fig. 3). The fact that it reacted with human transferrin in addition to rabbit transferrin, although not with the same affinity indicates that there are species differences in the interaction of transferrin with membrane receptors. This undoubtedly plays some part in the varied rates of iron acquisition by reticulocytes from transferrins of different species [7,35].

The reversibility of binding of transferrin to the membrane component was demonstrated by the experiment illustrated in Fig. 4, and also by the observation that unlabelled transferrin could displace $^{125}$I-labelled transferrin from the complex (Fig. 3). This reversibility probably accounts in part, at least, for the fact that less than 50% of the labelled transferrin present in the Teric extracts of reticulocytes appeared in the first transferrin peak on gel filtration. An additional explanation for this observation may be that some of the transferrin in reticulocytes is not bound to receptors but is free within intracellular endocytotic vesicles or even within the cytosol of the cells.

Evidence for saturability of the acceptor with transferrin is provided in Fig. 5. When reticulocytes were incubated with increasing concentrations of labelled transferrin the amount of transferrin present in the first gel filtration peak reached a maximum at a transferrin concentration of approx. 10 mg/ml. Thus, the binding component was saturated with transferrin under these conditions. The results of this experiment confirm an earlier observation that non-specific binding of transferrin to reticulocytes occurs in proportion to the transferrin concentration of the incubation medium once the specific binding sites are fully occupied [12] and they also strongly suggest that transferrin bound to its specific receptor is much more readily extracted with Teric than is transferrin bound at non-specific sites. This indicates that transferrin receptors are more readily released from the cell membrane than are other components capable of binding plasma proteins. Because of the reversibility of the binding of transferrin to its receptor there is a continual loss of transferrin from the receptor complex during gel filtration. Hence, an estimate of the number of transferrin binding sites or receptors based on the plateau level of transferrin in the first gel filtration peak in Fig. 5 would certainly underestimate the true number. For this reason, it seemed more likely that the amount of transferrin present in the total Teric extract when this had reached an apparent saturation level (at a transferrin concentration of approx. 10 mg/ml, Fig. 5), would give a better estimate of the number of receptors than the maximum amount of transferrin present in the first gel filtration peak. The result obtained in this manner was

90 000 sites per cell. This figure is less than the values obtained from the values for total cell uptake of transferrin (120 000 sites per cell). The reasons for this discrepancy may be that the latter estimate included some non-specifically bound transferrin, that not all the transferrin taken up by reticulocytes remains bound to receptors and that a proportion of the receptor-bound transferrin was lost in the present work during preparation and washing of the reticulocyte ghosts as suggested by the data in Table II.

The transferrin-binding component was found only in reticulocytes and not in mature red cells. Mature erythrocytes do not take up transferrin-bound iron. This is probably the result of loss of transferrin receptors during maturation of the cells, although other metabolic attributes are also lost during maturation and these, too, may be required for iron acquisition. Stronger evidence that binding of transferrin to its receptors is necessary for iron uptake by immature erythroid cells is provided by the observation that mild proteolytic digestion or incubation at 37°C with calcium chelators leads to loss of transferrin binding and iron uptake by reticulocytes [33,34]. Hence, it is most likely that iron uptake from transferrin is dependent on the presence and physiological integrity of the receptors. However, the role that the receptors play in this process is not certain. They may be required for cellular internalization of transferrin [36,37] and/or for the release of iron from the protein [11].

The chromatographic data presented in Fig. 1 resemble those of Garret et al. [9] and Speyer and Fielding [10,11] in that labelled transferrin was eluted in two peaks. These workers used Triton X-100. Certain differences should be noted between the present results and those of Speyer and Fielding. Firstly, they found that in order to obtain a "transferrin-reticulocyte binding site" ("B2") complex in chromatograms, an initial gel filtration on Sepharose 2B was obligatory before chromatography on Sephadex G-200 or Sepharose 6B. No such step was required in the present study in order to identify what would appear to be the same transferrin complex. A second difference is that Fielding and Speyer found that all of the labelled transferrin emerged as the complex with a membrane component, none being in the free form and they did not report any evidence that transferrin could dissociate from the complex.

More work is required before the structure and functions of the transferrin receptor are fully understood. Some information on its properties is already available. Thus, it appears to have a molecular weight of approx. 275 000. However, in view of the uncertainty of the amount of Teric associated with the solubilized receptor, this value must be interpreted with caution. The receptor is probably a protein since its ability to bind transferrin is lost after mild proteolytic digestion of intact reticulocytes, their ghosts or Teric extracts [33]. It is readily released from the cell membrane by non-ionic detergents and appears to be stable under such conditions since it retains its transferrin-binding properties in solutions containing such detergents even after prolonged storage.

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