*Biochimica et Biophysica Acta*, 598 (1980) 293—304


BBA 78724

# TRANSFERRIN-BINDING AND IRON-BINDING PROTEINS OF RABBIT RETICULOCYTE PLASMA MEMBRANES

## THREE DISTINCT MOIETIES

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(Received July 9th, 1979)

*Key words: Plasma membrane; Transferrin-binding protein; Iron transport; (Rabbit erythrocyte)*

## Summary

1. Transferrin-membrane complexes and iron-binding membrane complexes were solubilized with sodium dodecyl sulfate from the plasma membranes of reticulocytes that had been incubated with ($^{59}$Fe,$^{125}$I)-labeled transferrin. Gel filtration of solubilized material demonstrated $^{125}$I-labeled transferrin complexed to two moieties, a minor component (Peak I) of apparent molecular weight 435 000 and a major component (Peak II) of apparent molecular weight 200 000. Most of the membrane $^{59}$Fe was located in Peak I.

2. Sepharose-bound anti-transferrin was used to purify the $^{125}$I-labeled transferrin-membrane complexes. The $^{59}$Fe/$^{125}$I ratio in the transferrin complex purified from Peak I was the same as in the original transferrin and thus contained membrane-bound transferrin to which the $^{59}$Fe was still attached. The $^{59}$Fe/$^{125}$I ratio in the purified Peak II transferrin complex was 0.33 times that of the original transferrin, indicating that more than 60% of its $^{59}$Fe had been delivered to the reticulocyte.

3. The purified transferrin complexes analyzed by SDS-polyacrylamide gel electrophoresis demonstrated a single band of apparent molecular weight 78 000 both by Coomassie blue stain for protein and by $^{125}$I radioactivity. The specific activity of this material was 0.27 and 0.56 times that of the original transferrin for Peak I and Peak II, respectively, indicating that transferrin in Peak I and II was bound to a membrane component with a molecular weight similar to that of transferrin.

4. The isoelectric focusing pattern of the Peak II transferrin complex showed isoelectric points of pH 6.7 and 6.2 compared to pH 5.4 for transferrin.

5. On the basis of these studies we propose that transferrin is first bound to a

membrane protein and then delivers iron to a membrane component distinct and separate from the transferrin-binding moiety. Prior to its release, transferrin markedly depleted of iron is still bound to a component in the plasma membrane.

## Introduction

The delivery of iron to and transport of iron across the reticulocyte membrane are complex processes which are still poorly understood. The early studies of Jandl and Katz [1] suggested that transferrin, the iron transport protein in plasma, binds to specific sites on the reticulocyte membrane followed by release of iron to the membrane. Subsequently, many workers have demonstrated specific binding of transferrin on the reticulocyte [2] and earlier precursors [3,4] but efforts to isolate and define transferrin and iron-binding moieties in the membrane have yielded divergent results.

In general, the approach for analysis of transferrin-binding sites on the reticulocyte has utilized solubilization of reticulocyte membranes with bound $^{125}$I-labeled transferrin by non-ionic detergents. The molecular weights of the solubilized complexes of transferrin with membrane proteins are then estimated by gel filtration [5—10].

The present studies employ the ionic detergent sodium dodecyl sulfate to solubilize ($^{59}$Fe,$^{125}$I)-labeled transferrin bound to rabbit reticulocyte plasma membranes. Subsequent gel filtration of the solubilized membrane clearly distinguishes a plasma membrane fraction with an apparent molecular weight of 435 000, which is markedly enriched for iron, and two transferrin-membrane protein complexes of apparent molecular weights 435 000 and 200 000. The transferrin-protein complexes have been purified further by immunoabsorbance with anti-transferrin antibody and characterized by polyacrylamide gel isoelectric focusing.

## Methods

*($^{59}$Fe,$^{125}$I)-labeled transferrin.* Rabbit transferrin was purified and labeled with $^{125}$I and $^{59}$Fe as described previously [11]. In brief, transferrin was isolated from rabbit plasma by chromatography on DEAE-Sephadex, equilibrated with and then eluted with 0.15 M sodium phosphate (pH 5.0), followed by gel filtration on Biogel A-1.5. The transferrin obtained by these methods was at least 95% pure as determined by: (1) electrophoresis on SDS-polyacrylamide gels with staining of proteins by Coomassie blue and (2) the ability to saturate the transferrin with iron [12]. Transferrin was labeled with $^{125}$I either by the lactoperoxidase method [13] or the iodine monochloride method [14]. Transferrin was charged to an iron saturation of 50—65% with $^{59}$Fe by the method of Bates and Schlabach [12].

*Labeling of reticulocytes with ($^{59}$Fe,$^{125}$I)-labeled transferrin.* Reticulocytes were produced in rabbits in response to phenylhydrazine hydrochloride as an initial stimulus (10 mg per kg body wt. for 3 days). The rabbits were then bled every other day from the marginal vein of the ear. After two such bleedings the

reticulocyte count was 50—70% and there was little residual effect of the phenylhydrazine on the cell membrane as judged by the ease of preparing white plasma membranes. A 10—20% suspension of rabbit reticulocytes was incubated with or without 10 mM isoniazid in phosphate-buffered saline (pH 7.4) for 15 min at 37°C. Cells were washed, ($^{59}$Fe,$^{125}$I)-labeled transferrin (5—10 $\mu$M) was added and the incubation continued for 20 min. Cells were washed three times with phosphate-buffered saline. Low transferrin concentrations were used to minimize nonspecific binding of transferrin to the reticulocyte.

*Preparation of ($^{59}$Fe,$^{125}$I)-labeled reticulocyte membranes and $^{59}$Fe-labeled mitochondria.* ($^{59}$Fe,$^{125}$I)-labeled reticulocytes were lysed with 10—20 vols. of 20 imosM (imosM, ideal milliosmolarity) Tris-HCl (pH 7.6) and membranes pelleted by centrifugation at 15 000 × *g* for 15 min. Membranes were resuspended with lysis buffer and washed until no hemoglobin was visible in the supernatant, generally five times. After each centrifugation the small hard pellet at the bottom of the tube was eliminated by suction. We have previously demonstrated minimal contamination of plasma membranes with mitochondria prepared in such a fashion [11]. Failure to remove the hard pellet results in significant contamination with mitochondria. $^{59}$Fe-labeled mitochondria were prepared by the method of Guggenheim et al. [15] after incubation of reticulocytes for 20 min. with ($^{59}$Fe,$^{125}$I)-labeled reticulocytes. Membranes were stored at −80°C and generally used within 7 days.

*Membrane solubilization and gel filtration.* The ($^{59}$Fe,$^{125}$I)-labeled membranes were diluted to approx. 1 mg protein/ml with 20 imosM Tris-HCl (pH 7.6), made up to 4 mg/ml in SDS (Bio-Rad Laboratories), and heated for 30 min at 37°C. The solubilized membranes were cooled on ice and loaded onto a Biogel A-1.5 column (1.0 × 40 cm) which was equilibrated and then eluted with 20 imosM Tris-HCl (pH 7.6) in 0.56 ml fractions. $^{59}$Fe and $^{125}$I radioactivity and absorbance at 280 nm were determined in the collected fractions. Recovery of $^{125}$I and $^{59}$Fe membrane radioactivity was greater than 90%. In trial experiments the radioactivity from $^{59}$Fe-labeled transferrin in solution with, and dialyzed against, 0.4% SDS remained non-dialyzable for periods of at least 7 days.

The amount of SDS bound to membrane constituents was quantified using $^{35}$S-labeled SDS. The radioactive SDS (New England Nuclear, spec. act. 53 Ci/M) was dissolved to a final concentration of 100 mg/ml SDS, 46 $\mu$Ci $^{35}$S/ml. Reticulocyte membranes prepared after incubation with unlabeled transferrin were solubilized with $^{35}$S-labeled SDS and subjected to gel filtration as detailed above. $^{35}$S radioactivity was determined with Aquasol II (New England Nuclear) as scintillation fluid in a Nuclear Chicago Mark II scintillation counter. No quenching was detected with up to at least 0.2 ml of aqueous sample.

The Biogel A-1.5 columns were calibrated for assessment of apparent molecular weight by elution of the following protein standards (molecular weights in parentheses): ferritin (440 000), catalase (232 000), aldolase (158 000), transferrin (78 000) and hemoglobin (62 000).

*Sepharose-bound anti-rabbit transferrin.* Goat anti-rabbit transferrin (Research Plus Labs) was purified by immunoabsorption against Sepharose-bound rabbit transferrin. The purified antibody was then attached to cyanogen bromide-activated Sepharose beads as described previously [11].

*Other analytical methods.* SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [16]. Apparent molecular weights were assigned by calibration of the gels with proteins of known molecular weight (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase) and by comparison with the proteins of rabbit erythrocyte plasma membranes using the nomenclature of Fairbanks et al. [17]. Quantitation of proteins bands was performed by densitometry at 550 nm using a Gilford gel scanner as described by Kahn and Rubin [18]. Isoelectrofocusing in polyacrylamide gels was performed in 5% acrylamide cross-linked to 0.2% *N,N'*-methylenebisacrylamide containing 0.1% *N,N,N',N'*-tetramethylenediamine, 5% glycerol, 2% ampholine pH 3.5—10 (LKB Instruments, Rockville, MD) and gelled with ammonium persulfate. The anodal and cathodal buffers were 0.05 M $H_2SO_4$ and 0.03 M NaOH, respectively. Gels were prefocused for 15 min at 1 mA/gel, the sample added at the cathodal end and focused at 1 mA/gel to a constant voltage of 400 V for 16 h. Gels were sectioned into 2-mm slices, 0.5 ml distilled $H_2O$ was added and pH and radioactivity were measured. Radioactivity was measured in a Searle 1185 double-channel automatic gamma counter. Correction was made for the cross-over of $^{59}Fe$ radioactivity into the $^{125}I$ channel.

## Results

*Distribution of $^{59}Fe$ radioactivity between plasma membrane and mitochondria*

To assay for possible contamination of plasma membrane with mitochondria the specific activity of $^{59}Fe$ was measured in plasma membrane and mitochondria prepared from reticulocytes incubated with $^{59}Fe$-labeled transferrin. In two such experiments the mean specific activity of the $^{59}Fe$ radioactivity was 13 018 cpm/mg protein in the plasma membrane and 21 600 cpm/mg protein in the mitochondria. After treatment with isoniazid the specific activities in the membrane and mitochondria were 44 353 and 30 143 cpm/mg protein, respectively. We have previously demonstrated [11] that reticulocyte plasma membranes are contaminated by less than 5% with mitochondrial proteins. Therefore, contamination with mitochondria could account for only a little of the $^{59}Fe$ in reticulocyte plasma membranes under normal conditions and the experiments with isoniazid indicate that such contamination must be very small indeed.

*Solubilization by SDS of ($^{59}Fe$,$^{125}I$)-labeled transferrin bound to reticulocyte plasma membranes*

In preliminary experiments, 2—4 mg/ml SDS in 20 imosM Tris-HCl, pH 7.6, either at room temperature or 37°C were found to solubilize all $^{59}Fe$ and $^{125}I$ radioactivity from ($^{59}Fe$,$^{125}I$)-labeled membranes at a concentration of 1—2 mg protein/ml. The $^{59}Fe$ radioactivity remained bound to a macromolecular component, as evidenced by the fact that the $^{59}Fe$ radioactivity was non-dialyzable. In contrast, solubilization of membrane radioactivity with 1% Triton X-100 yielded extremely variable results, with 55—75% of the membrane-associated $^{59}Fe$ remaining insoluble although the solubilization of transferrin bound to the membrane was greater than 90%. The SDS-solubilized membrane was next sep-

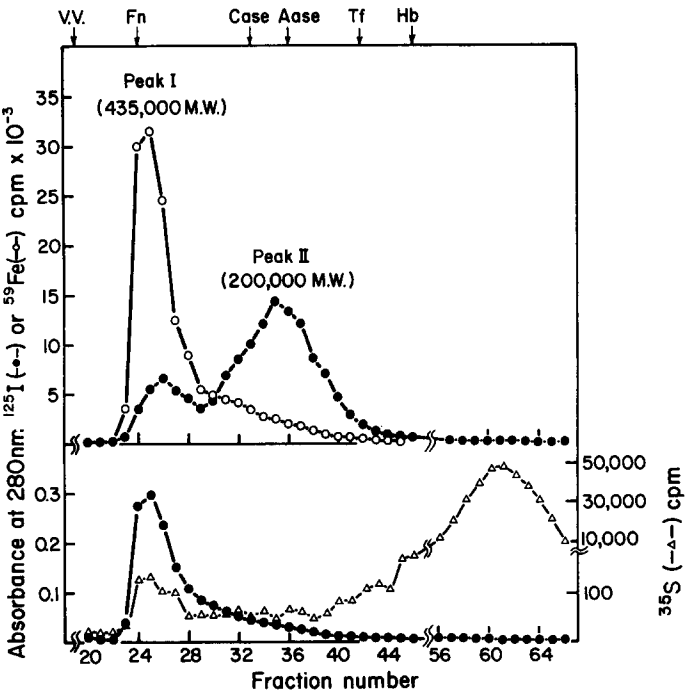


Fig. 1. Gel filtration of SDS-solubilized ($^{59}$Fe,$^{125}$I)-labeled reticulocyte plasma membranes. As described in Methods, SDS-solubilized ($^{59}$Fe,$^{125}$I)-labeled membranes were fractionated on Biogel A-1.5. The collected fractions (0.56 ml) were assayed for $^{59}$Fe (○———○) and $^{125}$I (•———•) radioactivity (upper panel) and absorbance at 280 nm (lower panel). Elution of protein standards, ferritin (Fn, 440 000), catalase (Case, 232 000), aldolase (Aase, 158 000), transferrin (Tf, 78 000) and hemoglobin (Hb, 62 000) are indicated in the figure as is the void volumn (V.V.). Reticulocyte plasma membranes prepared from reticulocytes incubated with non-radioactive $^{56}$Fe-labeled transferrin were solubilized with $^{35}$S-labeled SDS and also fractionated by gel filtration. The $^{35}$S radioactivity (△- - - - -△) in the fractions is shown in lower panel.

arated by gel filtration through Biogel A-1.5 in the absence of SDS in the buffer. As seen in Fig. 1, after solubilization at 37°C for 30 min about 70% of the membrane protein, 30% of the $^{125}$I and most of the $^{59}$Fe were found in Peak I, apparent molecular weight 435 000. The remainder of the $^{125}$I, with little associated $^{59}$Fe, was located in Peak II, apparent molecular weight 200 000. Similar patterns were observed if membranes were solubilized at room temperature and immediately filtered or if they were prepared in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (2 mM); these experiments indicate little likelihood that Peak II represents a degradation product of the larger Peak I.

The amount of protein-bound SDS in the gel filtration fractions was determined using $^{35}$S-labeled SDS to solubilize non-radioactive plasma membrane. When aliquots of these $^{35}$S-labeled SDS-solubilized membranes were subjected to gel filtration (Fig. 1), the SDS/protein ratios in Peaks I and II were 0.0022 and 0.0104 mg SDS/mg protein, respectively. The bulk of the radioactivity was separated from the protein and eluted with apparent molecular weight of about 10—20 000. In analogy to experiments with Simliki virus the low molecular weight material may correspond to mixed SDS-lipid micelles [19].

*Distribution of $^{125}$I-labeled transferrin and $^{59}$Fe in Peak I and Peak II*

The difference in distribution of $^{125}$I and $^{59}$Fe is further illustrated in Table I. In these experiments reticulocytes accumulate 2.5-fold more $^{59}$Fe relative to $^{125}$I in the plasma membrane as compared to the transferrin used in the incubation. After solubilization with SDS and gel filtration the $^{125}$I-labeled transferrin in Peak II is markedly depleted of $^{59}$Fe whilst Peak I is about 7-fold enriched for $^{59}$Fe as compared with the original transferrin. The differences in the relative distribution of $^{59}$Fe and $^{125}$I are accentuated by using labeled membranes prepared from reticulocytes in which heme synthesis has been inhibited with isoniazid. As shown in Table I the plasma membranes of these reticulocytes have accumulated 6-fold more, and Peak I 16-fold more, $^{59}$Fe relative to $^{125}$I, whilst Peak II-associated transferrin is more than 60% depleted of $^{59}$Fe. Hence, at the level of the membrane a distinction can be made between a membrane component(s) which is associated with $^{125}$I-labeled transferrin and a separate membrane component(s) to which the transferrin has delivered $^{59}$Fe.

The distribution of radioactivity was examined after shorter periods of incubation of reticulocytes with ($^{59}$Fe,$^{125}$I)-labeled transferrin. With periods of incubation of 5 min, 40% of $^{125}$I-labeled transferrin was already located in Peak II. By 10 min of incubation the pattern was the same as that shown in Fig. 1. Even at 5 min the ratios of $^{59}$Fe to $^{125}$I radioactivity in purified Peaks I and II were the same as in Table II.

*SDS-polyacrylamide gel electrophoresis of Peak I and Peak II*

Under the more vigorous dissociating conditions of incubation in 1% SDS and 100 mM 2-mercaptoethanol at 100°C for 2 min, the proteins of the two peaks were dissociated into their monomeric components and separated by SDS-polyacrylamide gel electrophoresis (Fig. 2). As anticipated, Peak I, containing the bulk of the membrane protein, presented the complex pattern of the rabbit erythrocyte plasma membrane enriched, however, for bands 1, 2 and 3. The $^{59}$Fe was dissociated from these proteins and traveled with the tracking dye. The column fractions comprising Peak II also show a complex pattern of membrane proteins (Fig. 2, c—f). The gel electrophoresis pattern changes from

TABLE I

RATIO OF $^{59}$Fe TO $^{125}$I-LABELED TRANSFERRIN RADIOACTIVITY IN THE SEPARATED MEMBRANE COMPONENTS

Reticulocytes were preincubated for 15 min with (+) or without (—) 10 mM isoniazid and then incubated for 20 min with ($^{59}$Fe, $^{125}$I)-labeled transferrin. Reticulocytes were extensively washed. Plasma membranes were prepared, solubilized with SDS, fractionated by gel filtration and the fractions comprising Peak I and II pooled separately (see Fig. 1). The ratios of $^{59}$Fe to $^{125}$I radioactivity in the membranes and in Peaks I and II were normalized to a ratio of 1.0 in the original transferrin. Presented are the means ± S.E. for four experiments (—isoniazid) and three experiments (+isoniazid).

| Sample | $^{59}$Fe/$^{125}$I radioactivity | |
|---|---|---|
| | —isoniazid | +isoniazid |
| Membrane | 2.45 ± 0.21 | 6.0 ± 0.25 |
| Peak I | 6.90 ± 1.84 | 16.6 ± 2.88 |
| Peak II | 0.43 ± 0.07 | 0.35 ± 0.03 |

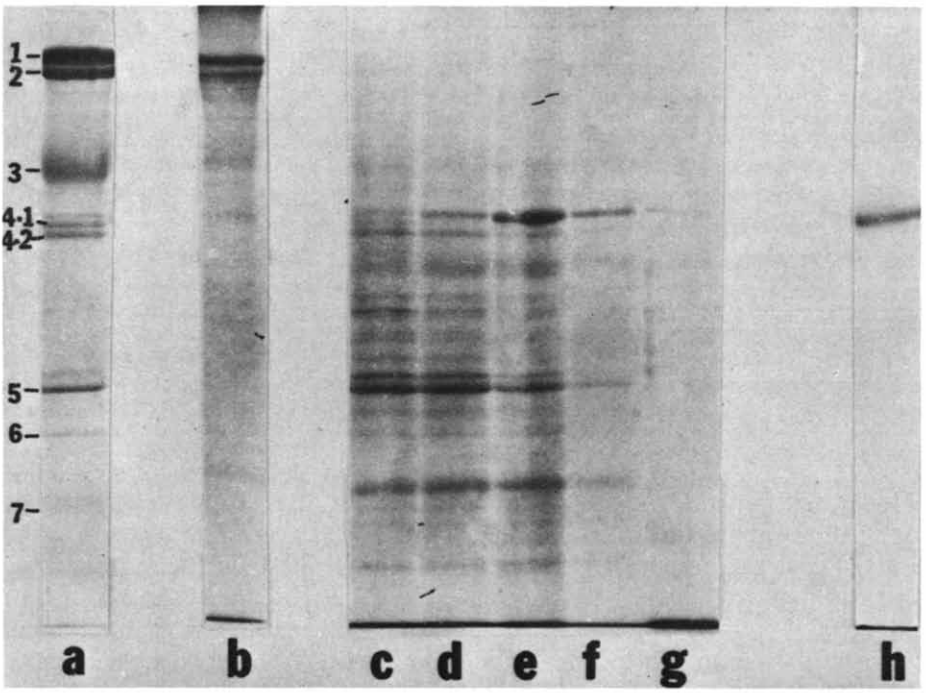


Fig. 2. SDS-polyacrylamide gel electrophoresis of Peak I and Peak II. The protein composition of fractions comprising Peaks I and II (see Fig. 1) were analyzed by SDS-polyacrylamide gel electrophoresis [17]. The proteins were detected by Coomassie blue stain, and molecular weight was estimated by comparison to the proteins of co-electrophoresed rabbit erythrocyte plasma membranes. The electrophoresed samples were: (a) Rabbit erythrocyte plasma membrane. Protein bands were enumerated according to the system of Fairbanks et al. [17]. (b) Peak I fraction 24. (c—g) Peak II fractions 31, 33, 35, 37 and 39, respectively. (h) Peak II purified by immunoadsorption. Peak II was adsorbed onto Sepharose anti-rabbit transferrin, eluted with 2 M KSCN (see Table II), dialyzed against 20 imosM Tris-HCl, pH 7.4, and then concentrated on Amicon A25 centriflow cones. The migration of pure transferrin was identical to the Coomassie blue stained material in this panel.

the more rapidly filtered fractions (i.e., those closest to peak I) to the fraction with maximal $^{125}I$ radioactivity. Fraction 31 (Fig. 2, c) is composed of many of the lower molecular weight proteins of the membrane (bands 4—7). With progression to the peak $^{125}I$ fraction, fraction 35, (Fig. 2, e), there is a relative enrichment for proteins of molecular weights 78 000, 43 000 and 29 000. The 78 000 molecular weight protein migrates with $^{125}I$-labeled transferrin and contains all the $^{125}I$ radioactivity; evaluation of the specific activity of this material (see below) suggests that the $^{125}I$-labeled transferrin is migrating with a nonradioactive protein of similar molecular weight.

*Purification of $^{125}I$-labeled transferrin-membrane complexes from Peak I and Peak II by immunoabsorbance (Table II)*

After dialysis of Peak I and II for 72 h against 0.1% Triton X-100 in 20 imosM Tris-HCl, pH 7.4, to remove SDS, the material was absorbed onto goat anti-rabbit transferrin bound to Sepharose beads. 65.4% of the $^{125}I$, but only 8.5% of the $^{59}Fe$, in Peak I was retained by the antibody. The ratio of $^{59}Fe$

TABLE II

BINDING OF PEAK I AND PEAK II TO SEPHAROSE-BOUND ANTI-TRANSFERRIN

Peak I and Peak II obtained from gel filtration of SDS-solubilized ($^{59}$Fe, $^{125}$I)-labeled membranes were dialyzed against phosphate-buffered saline-0.1% Triton X-100 and then incubated with Sepharose-bound goat anti-rabbit transferrin for 1 h at room temperature and then overnight at 4°C. The radioactive material not bound was washed from the Sepharose beads with phosphate-buffered saline. The ratio of $^{59}$Fe to $^{125}$I radioactivity in the bound fractions was normalized to a ratio of 1.0 for the original transferrin. Presented are the means ± S.E. of three experiments.

| Sample | $^{125}$I-labeled radioactivity (% of total sample) | $^{59}$Fe-bound radioactivity (% of total sample) | Ratio $^{59}$Fe/$^{125}$I |
|---|---|---|---|
| Peak I | 64.5 ± 6.80 | 8.46 ± 4.31 | 1.1 ± 0.06 |
| Peak II | 88.9 ± 4.21 | 51.5 ± 8.08 | 0.33 ± 0.10 |

radioactivity to $^{125}$I radioactivity in the retained material from Peak I was the same as in the original transferrin present in the incubation mixture whilst the non-retained material was nearly 18-fold enriched in $^{59}$Fe. Of the radioactivity in Peak II applied to Sepharose anti-transferrin, 89.9% of the $^{125}$I and 51.5% of the $^{59}$Fe initially present in the peak were bound. This material now had a $^{59}$Fe/$^{125}$I ratio of 0.33 compared to the starting transferrin. When $^{59}$Fe-labeled transferrin by itself was subjected to similar antibody binding no loss of $^{59}$Fe occurred. Therefore, though both Peak I and Peak II contain $^{125}$I-labeled transferrin, the $^{59}$Fe content in these transferrins is markedly different: transferrin in Peak I has an iron saturation similar to the transferrin in the original incubation mixture whilst transferrin in Peak II is markedly depleted of $^{59}$Fe iron. It is of note that most of the $^{59}$Fe in Peak I did not become attached to the transferrin antibody, again strongly suggesting the presence of an iron-binding moiety other than transferrin.

*Composition of purified Peak I and Peak II*

Peak II bound to anti-transferrin antibodies was eluted from the immonoabsorbant by the chaotropic agent KSCN [20]. The eluted Peak II analyzed by gel electrophoresis (Fig. 2, h) demonstrated only a single Coomassie blue-stained band containing all of the radioactivity in the region corresponding to pure transferrin. To determine whether another protein was comigrating with transferrin the specific activity of the band was determined as follows: varying amounts of $^{125}$I-labeled transferrin were electrophoresed, stained with Coomassie blue, and quantitated by densitometric scanning by the method of Kahn and Rubin [18]. A linear relationship was found between $^{125}$I radioactivity and the absorbance of the transferrin band, at least within the range of 0.5—2.0 μg transferrin protein. The specific activity ($^{125}$I radioactivity/area of the scanned peak) of purified Peak II was similarly determined and found to be 0.59 ± 0.06 (mean ± S.E. of five experiments) of that of transferrin alone; that is, Peak II had 1/0.59 or 1.7 times more protein than that contributed by $^{125}$I-labeled transferrin alone. Hence, purified Peak II represents a complex of transferrin and another membrane protein of similar apparent molecular weight or mobility on SDS-polyacrylamide gel electrophoresis. Likewise, the transferrin

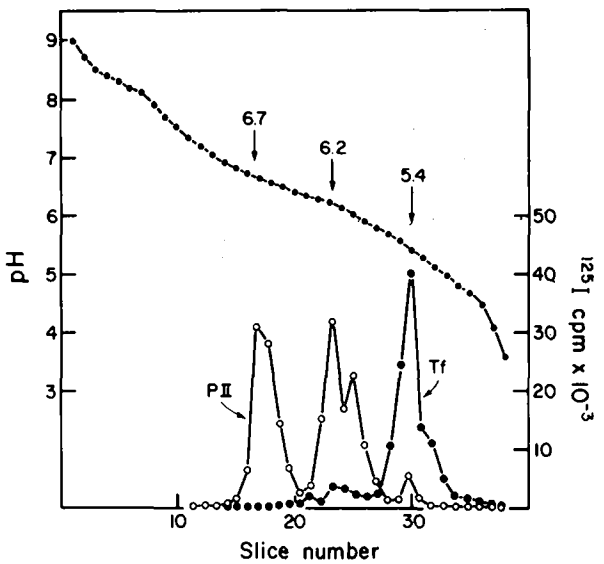


Fig. 3. Polyacrylamide gel isoelectrofocusing of purified Peak II. Peak II (○———○) purified by immunoadsorption and $^{125}$I-labeled transferrin (●———●) were subjected in parallel to isoelectrofocusing on polyacrylamide gels. The gels were sliced into 2-mm fractions and pH and radioactivity determined. The arrows indicate the pH of the peaks of radioactivity.

complex isolated from Peak I by immunoabsorption was found to have a specific activity of 0.27, implying a complex of transferrin with another protein or proteins of similar molecular weight in a ratio of 1 transferrin to 2—3 binding proteins.

*Analysis of purified Peak II by isoelectric focusing on polyacrylamide gels (Fig. 3)*

Purified Peak II was analyzed by isoelectric focusing in polyacrylamide gels in a gradient of pH 3.5—10. $^{125}$I radioactivity was detected at pH 5.4, 6.2 and 6.7. $^{125}$I-labeled transferrin focused in parallel gels was detected at pH 5.4 and 6.2 only. Pretreatment of transferrin with SDS and dialysis prior to focusing did not alter its focusing pattern. These findings suggest that transferrin is complexed to a membrane moiety (of similar molecular weight) that imparts to the complex an altered isoelectric point when compared with transferrin. An alternate hypothesis would be that the transferrin itself has been altered after interacting with the membrane. However, isoelectric focusing of the transferrin in the incubation mixture after interaction with reticulocytes was qualitatively similar to that of the original transferrin: a small amount (5—6%) of the radioactivity could now be detected at pH 6.7, perhaps representing a transferrin-membrane complex released into the incubation medium.

## Discussion

Since the initial evidence by Jandl and Katz [1] of specific binding of transferrin and release of its iron to the reticulocyte membrane, considerable effort

has been devoted to isolating the transferrin binding site and to determining whether transferrin in fact releases iron at the level of the cell membrane or enters the cell to deliver its iron. Two findings reported here give credence to the view that transferrin need not enter the reticulocyte to deliver iron: (1) the membrane of the reticulocyte accumulates $^{59}Fe$ in excess of that expected from membrane-bound transferrin (Table I), and (2) gel filtration of SDS-solubilized membranes allows a clear distinction between the Peak II $^{125}I$-labeled transferrin-membrane complex, markedly depleted of $^{59}Fe$, and $^{59}Fe$ in Peak I which is many-fold enriched over the iron-binding capacity of transferrin. If transferrin were transported into the cell to deliver iron, one would not expect to find an accumulation of $^{59}Fe$ not associated with transferrin in the membrane. These findings suggest instead that transferrin delivers $^{59}Fe$ to a membrane component and that, prior to its release from the cell, the transferrin may be found still bound to the membrane.

*The transferrin-binding proteins in Peak I and Peak II*

The primary approach to identify and isolate the transferrin binding site has been gel filtration of solubilized $^{125}I$-labeled transferrin bound to reticulocyte plasma membranes. The results of earlier studies are summarized in Table III to allow comparison with the present data. The initial studies of Garret et al. [21] demonstrated a differential solubility of membrane-bound $^{59}Fe$ and $^{125}I$-labeled transferrin. As with our studies, an ionic detergent, deoxycholate, solubilized $^{59}Fe$ bound to a macromolecule of $M_r$ 350 000—700 000: under these conditions, however, $^{125}I$-labeled transferrin was dissociated from any membrane constituent. In all subsequent studies a non-ionic detergent, either Triton X-100 or the related Teric 12A9, was used. In four cases [6—9] only a transferrin-binding moiety was detected and the transferrin was partially depleted of $^{59}Fe$ though not to the extent found in the present studies. A distinct $^{59}Fe$-binding component was not observed. However, Speyer and Fielding [5] found, in addition to a transferrin-membrane complex, $^{59}Fe$ bound to a macromolecule of approx. $M_r$ 1 000 000. In different studies the reported molecular

TABLE III

MOLECULAR WEIGHT

| Solubilization detergent | Molecular weight of transferrin-membrane protein(s) complex determined by gel filtration | Putative receptor(s) subunit molecular weight determined by SDS gel electrophoresis | Reference |
|---|---|---|---|
| 1% Deoxycholate | 350 000—700 000 | — | 21 |
| 1% Triton X-100 | 230 000 | — | 5 |
| 1% Triton X-100 | 200 000 | 120 000<br>60 000 | 7 |
| 10% Teric 12A9 | 350 000 | — | 6 |
| 1% Triton X-100 | 445 000 | 175 000<br>95 000 | 8 |
| 0.7% Triton X-100 | >200 000 | 200 000 | 24 |
| 1% Triton X-100 | 230 000 | — | 10 |
| 0.4% SDS | 200 000 | 78 000 | present studies |

weight of the transferrin complex varied from 200 000 [7] to 445 000 [8]. These estimates of molecular weight must be viewed cautiously for, as recently discussed by Hu and Aisen [22], elution of gel filtration gives an estimate of the Stokes' radius and not molecular weight and the former parameter can change with the detergent, pH and ionic strength of the elution buffer. That the molecular weight of the transferrin-membrane complexes isolated in the present study is similar to several listed in Table III may in part be coincidental, as SDS binds to hydrophilic as well as hydrophobic regions of the membrane proteins whilst Triton X-100 binds only to hydrophobic regions. Hence, different domains of the membrane sharing the transferrin binding site might be solubilized by the two types of detergent.

A striking difference between the present studies and those reported in Table III is our finding of two distinct transferrin-membrane protein complexes, distinguished both by size and iron saturation. The Peak I transferrin-membrane complex had a $^{59}Fe/^{125}I$ ratio similar to that of the original transferrin, whilst the Peak II material was markedly depleted of $^{59}Fe$. Hence, transferrin which had donated its iron was still bound to the reticulocyte. It is also unlikely that Peak II represents an endocytotic vesicle returning to the plasma membrane, since after only 5 min of incubation of reticulocytes with ($^{59}Fe$,$^{125}I$)-labeled transferrin 40% of the membrane-bound transferrin is already in Peak II. These findings suggest that ($^{59}Fe$,$^{125}I$)-labeled transferrin binds first to 2—3 membrane moieties with similar apparent molecular weight to transferrin to form Peak I. With release of $^{59}Fe$ to another membrane protein, the $^{125}I$-labeled transferrin-membrane complex may either (a) dissociate to the lower molecular weight Peak II complex of transferrin plus one binding moiety or (b) transfer the $^{125}I$-labeled transferrin to a separate membrane moiety forming Peak II before the transferrin is finally released from the reticulocyte. It appears unlikely that the Peak II transferrin complex represents a proteolytic product of the Peak I transferrin complex as both the immunogenicity of transferrin in the complexes was retained and polyacrylamide gel electrophoresis of the two complexes gave identical patterns, i.e., a single band both of $^{125}I$ radioactivity and Coomassie blue staining protein.

*The iron-binding protein (Peak I)*

The nature of the moiety to which iron is bound in Peak I has not been elucidated, since the SDS gel electrophoresis pattern shows a complex mixture of membrane proteins and under the conditions of electrophoresis the $^{59}Fe$ dissociates from these proteins and migrates with the tracking dye. The data in Table II suggest that the $^{59}Fe$ and the $^{125}I$-labeled transferrin-binding moieties of Peak I represent different entities, since Sepharose-bound anti-transferrin retains only the $^{125}I$-labeled transferrin with its complement of $^{59}Fe$. In addition, preliminary experiments have shown that (1) the isoelectric point determined by polyacrylamide gel isoelectrofocusing of Peak I $^{59}Fe$ is approx. pH 7.4—7.8: (2) $^{59}Fe$ in Peak I can be mobilized by reticulocyte cytosol in experiments analogous to those in which whole plasma membranes are the source of the $^{59}Fe$ [11,23]; (3) $^{59}Fe$ in Peak I, in contrast to $^{59}Fe$-labeled nitrilotriacetic acid, is not retained by ion-exchange chromatography on AG 11A8 beads and (4) $^{59}Fe$ in Peak I is precipitable by polyethylene glycol.

The ability to dissect three separate membrane domains — two with bound $^{125}$I-labeled transferrin of different iron saturations, the other with $^{59}$Fe — supports the hypothesis that transferrin is first bound to the reticulocyte membrane and then donates its iron to an iron acceptor membrane moiety that subsequently delivers the iron to the cytosol. Further characterization of the transferrin binding and iron-containing proteins will indicate whether these moieties are structurally related or represent separate membrane components.

## Acknowledgements

This work was supported in part by USPHS Grant AM-17148 and American Cancer Society Grant CH-51. J.G. is a recipient of Public Health Service Research Career Development Award CA 00513-01.

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