SOLUBILIZATION AND CHROMATOGRAPHY OF IRON-BINDING COMPOUNDS FROM RETICULOCYTE STROMA

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SUMMARY: Rabbit reticulocytes and erythrocytes were incubated with ^{59}Fe and ^{125}I labeled transferrin. Stroma of these cells was extracted with dispersing agents including sodium dodecyl sulfate, sodium deoxycholate, N-butanol, and Triton X-100. Chromatography of extracts on Sephadex G-200 and Bio-Gel A-15m showed that in addition to transferrin the stroma contained a macromolecule which binds iron and exhibits some transferrin binding capacity. Transferrin and the macromolecule attained steady state concentrations after 5-10 min incubation. These iron-binding species were not evident in erythrocyte stroma.

Reticulocytes incorporate rapidly iron from transferrin. A cell membrane receptor for transferrin has been postulated,[1] and iron uptake has been related to energy-dependent processes.[2,3] During the initial stages of incorporation more than 50% of cellular iron may be in the stroma or membrane fraction.[2,4] To date, however, no one has elucidated the nature of stromal iron. Jandl et al.[2] attempted to extract reticulocyte stromal iron but were not successful. Thus, we report results of the extraction and partial characterization of iron compounds in the stroma of reticulocytes. In addition to transferrin, there was present in the stroma a high molecular weight component with a capacity to bind both iron and transferrin.

EXPERIMENTAL: Blood was obtained from either phenylhydrazine-treated (30-50% reticulocytes) or normal (1-3% reticulocytes) adult rabbits. Blood was collected in heparinized containers, centrifuged and washed twice with buffer [modified Ringer-Tris: NaCl, 130 mM; KCl, 3 mM; $MgCl_2$, 1 mM; $CaCl_2$, 1 mM; Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol], 19 mM; pH 7.4]. All procedures in this experiment were conducted at 4°C and all centrifugations at 1,000 x g,

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10 min, unless otherwise specified. Cells collected from normal and phenylhydrazine-treated rabbits were designated erythrocytes and reticulocytes respectively. Packed cells were suspended in buffer such that a 25-30% hematocrit was obtained. 50 ml of the suspension were incubated at 37°C with 12.5 mg ^{125}I- and ^{59}Fe-labeled transferrin. After incubation the suspension was pelleted. Packed cells were washed thrice with buffer, hemolyzed with 30 volumes of 19 mM Tris-HCl, pH 7.4, and centrifuged (erythrocytes, 20,000 x g, 40 min.) to separate stromal and cytosol fractions. After 3 washes with 10 volumes of hemolysing solution, stroma was stored at - 20°C.

For extraction of stroma, the frozen preparation was thawed and centrifuged. 50-300 µl of the compacted stroma were mixed with 1 ml of solubilizing or extracting agent at pH 7.4 and homogenized (tight fitting teflon pestle, 1,000 rpm, 10 strokes). The agents used were sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC), Triton X-100, chymotrypsin, papain, neuramidase, trichloroacetic acid (TCA), N-butanol, and chloroform-methanol. After 30 min extraction, the suspension was centrifuged 1 hr, 100,000 x g. Any material in suspension after centrifugation was considered as "solubilized." The homogenate, pellet and soluble phase were counted for ^{125}I and ^{59}Fe (Nuclear-Chicago gamma counter with radiation analyzer). Protein was determined by the method of Lowry et. al.[5] 0.3 ml aliquots of the solubilized material were chromatographed at 23°C on Sephadex G-200 and Bio-Gel A-15m columns (length, 22 cm; diameter, 0.8 cm; bed volume, 10-11 ml; flow rate, 8 ml/hr) equilibrated with buffer containing 0.1% Triton X-100. Fifty 0.25 ml fractions were collected, counted for ^{59}Fe and ^{125}I, and the optical density at 280 nm measured. Recovery of radioactivity from the columns was 80-90%.

Transferrin used in the experiment was obtained commercially (Mann Research Laboratory; assayed by supplier to be 95% pure). Non-radioactive iron on the transferrin was replaced with ^{59}Fe ($^{59}FeCl_3$, 16 Ci/g; New England Nuclear). This was accomplished by subjecting the transferrin to continuous diafiltration (Amicon Model 52 Ultrafiltration cell, UM-10 membrane) with

0.1 M ascorbic acid, pH 5.5, for 30 hr. Ascorbic acid was subsequently removed from the apotransferrin by 24 hr of continuous diafiltration with buffer. Apotransferrin thus prepared had a normal total iron-binding capacity. Measurement of total iron-binding capacity was as follows: at 23°C 50 µl of apotransferrin were added to 0.3 ml of $FeCl_3$ (10 µg iron/ml; pH 3) and 50 µl of $^{59}FeCl_3$ (0.625 µg iron). The concentration of iron was within a range that yields completely hydrolyzed products.[6] 0.02 ml of $NaHCO_3$ was immediately added and the pH adjusted to 8 to insure iron binding to transferrin. After 10 min 0.4 ml of the iron-transferrin mixture was applied to a Sephadex G-15 column (length, 22 cm; diameter, 0.8 cm; bed volume, 10-11; flow rate, 8 ml/hr; 23°C; equilibrated with buffer). Transferrin eluting in the void volume was separated from unbound iron which was retarded.

For preparing ^{59}Fe labeled transferrin, the apotransferrin solution was brought to 23°C, made 0.01 M in $NaHCO_3$, and the pH adjusted to 7-8. $^{59}FeCl_3$ was added so that the transferrin was 40% saturated with ^{59}Fe. The transferrin was subsequently labeled with ^{125}I ($Na^{125}I$, carrier-free, New England Nuclear) by the iodine monochloride method.[8] The doubly labeled transferrin was subjected to continuous diafiltration for 30 hr with buffer to remove unbound nuclides. The $^{125}I/^{59}Fe$ count ratio of the transferrin was 2.9.

RESULTS: Results of a representative experiment in which reticulocytes were incubated with doubly labeled transferrin are shown in Fig. 1. ^{59}Fe increased rapidly in the cytosol fraction. Incorporation of iron and transferrin by the stroma reached steady state values after 5 min. At 30 min the stromal $^{125}I/^{59}Fe$ count ratio was 0.7. This indicated that there was a significant amount of ^{59}Fe not bound to transferrin.

Iron containing constituents of the stroma could be extracted by SDS, DOC, triton and butanol. A direct correlation existed between the amount of protein and nuclide solubilized (Table 1). As indicated by the $^{125}I/^{59}Fe$ count ratio of the soluble phase, agents extracting less protein extracted relatively more transferrin. 0.001%, 0.01% and 0.1% chymotrypsin and papain,

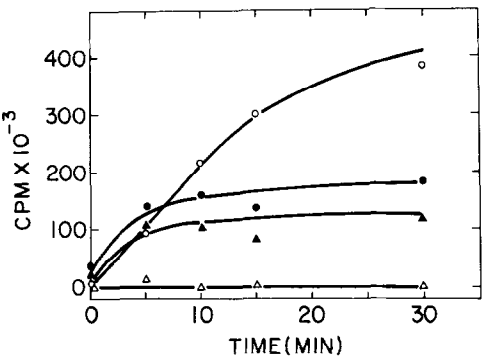


Fig. 1. The incorporation of ^{59}Fe and ^{125}I into reticulocyte stroma and cytoplasmic fractions. Reticulocyte-rich cells were incubated with doubly labeled transferrin. Incorporation of: ^{59}Fe into stroma, ●—● , cytoplasmic fraction, ○—○ ; and ^{125}I into stroma, ▲—▲ , cytoplasmic fraction, △—△ .

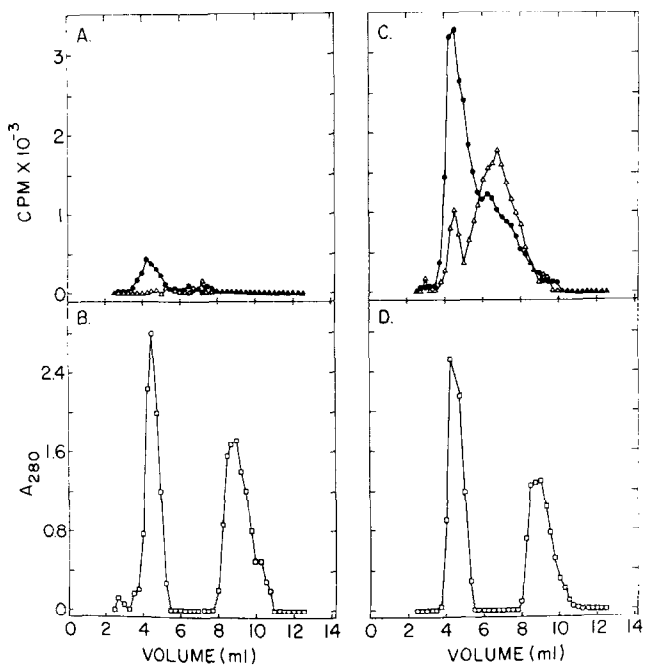
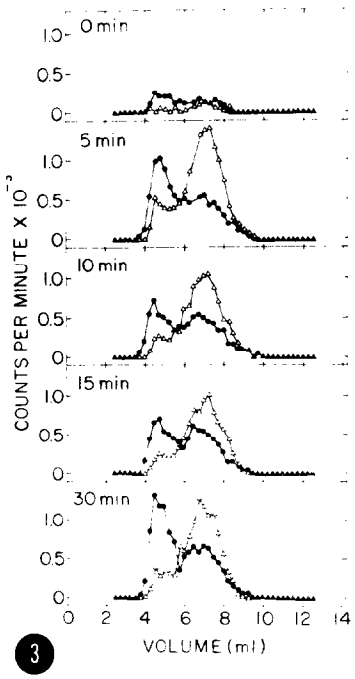


Fig. 2. Sephadex G-200 chromatography of the solubilized fraction from erythrocyte (A,B) and reticulocyte (C,D) stroma. Cells were incubated 30 min with transferrin. ●—● , ^{59}Fe cpm; △—△ , ^{125}I cpm; □—□, absorbance at 280 nm.



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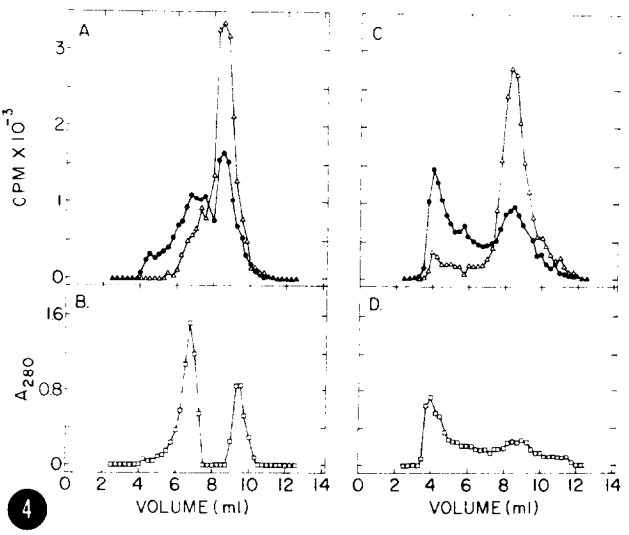

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Fig. 3. Sephadex G-200 chromatography of reticulocyte stromal extracts after 0, 5, 10, 15 and 30 minutes incubation. Stroma was extracted with 1% sodium deoxycholate. ●—● , ^{59}Fe cpm; ▲—▲ , ^{125}I cpm.

Fig. 4. Chromatography of reticulocyte stromal extract on Bio-Gel A-15m agarose gel. The column contained: (A,B) 0.1% Triton X-100, and (C,D) no detergent. Cells were incubated 30 min with transferrin. ●—● , ^{59}Fe cpm; ▲—▲ , ^{125}I cpm; □—□, absorbance at 280 nm.

0.001% and 0.01% neuramidase, 7% TCA and chloroform-methanol were relatively ineffective in extracting iron containing constituents from the stroma.

The 1% DOC extract of stroma prepared from reticulocytes and erythrocytes was subjected to chromatography on Sephadex G-200 (Fig. 2). The A_{280} elution profiles of solubilized material were essentially the same for both cell types. The ^{59}Fe and ^{125}I components extracted from reticulocyte stroma were resolved into two peaks. One component was of high molecular weight and eluted in the void volume (4.5 ml). Although this component contained some transferrin, its

TABLE I

SOLUBILIZATION OF RETICULOCYTE STROMAL IRON, TRANSFERRIN AND PROTEIN

Stroma was obtained from reticulocyte-rich cells incubated 30 min with doubly labeled transferrin. Solubilized material was contained in the aqueous phase of the N-butanol extract.

	Solubilization Agent			
	1% Sodium dodecyl sulfate	1% Sodium deoxycholate	1% Triton X-100	N-Butanol
% ^{59}Fe Extracted	56	40	30	21
% ^{125}I Extracted	55	44	35	33
Protein (mg/ml) Solubilized	6.59	4.79	3.15	0.92
$^{125}I/^{59}Fe$ Ratio	1.03	1.17	1.24	1.74

$^{125}I/^{59}Fe$ count ratio was 0.3 strongly indicating the presence of iron not bound to transferrin. The second component was retarded by the column and had a $^{125}I/^{59}Fe$ count ratio of 2.9, indicating that it was transferrin. According to the exclusion limits of the gel and the elution pattern of marker transferrin, the first component had a molecular weight of >200,000, and the second component eluted the same as marker transferrin. Erythrocyte stroma contained <10% of the radioactivity of reticulocyte stroma, and transferrin was virtually absent. However, a small amount of ^{59}Fe material was present. As shown in Fig. 3, initially reticulocyte stroma incorporated only small quantities of transferrin and iron. After 5 min incubation, however, the two nuclide-containing components reached steady state concentrations.

The DOC extract of reticulocyte stroma was chromatographed on Bio-Gel A-15m to determine the molecular weight range of the component which contained

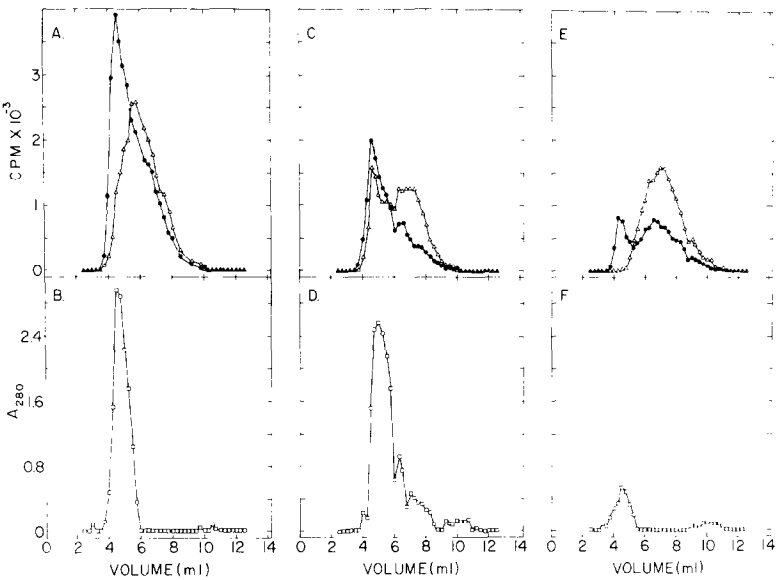

Fig. 5. Sephadex G-200 chromatography of the reticulocyte stroma extract. Solubilization with: (A,B) 1% sodium dodecyl sulfate, (C,D) 1% Triton X-100, and (E,F) N-butanol (1-3 with buffer). Cells were incubated 30 min with transferrin. ●—●, ^{59}Fe cpm; △—△, ^{125}I cpm; □—□, absorbance at 280 nm.

iron not associated with transferrin. Fig. 4 A,B shows that this component was retarded (void volume, 4-4.5 ml) and not well resolved from transferrin with its characteristically high ^{125}I/^{59}Fe count ratio. An estimate of the K_{av} of this component is 0.5-0.6, corresponding to a molecular weight of 350,000-700,000 [K_{av}=(elution volume - void volume)/ (total gel volume - void volume)]. In the absence of 0.1% solubilizing agent in the eluant, the complex reverted to a highly aggregated form (Fig. 4C,D). However, the elution volume of transferrin was independent of the presence of detergent in the eluant.

1% SDS, 1% Triton X-100 and N-butanol extracts of reticulocyte stroma were subjected to chromatography on Sephandex G-200 (Fig. 5). In contrast to DOC these agents extracted predominately high molecular weight material. N-butanol was primarily effective in transferrin solubilization and SDS did

not separate the high and low molecular weight ^{59}Fe containing components. Chromatography of Triton X-100 extract resolved partially the two components and the $^{125}I/^{59}Fe$ count ratio for the high molecular weight component was greater than that obtained with other solubilizing agents.

CONCLUSION: These experiments offer evidence that during iron incorporation by the reticulocyte, transferrin binds to the stroma and its iron becomes associated with a macromolecule of 350,000-700,000 molecular weight. The macromolecule has a greater affinity for iron than for transferrin, as indicated by its $^{125}I/^{59}Fe$ ratio of 0.25-0.50 as compared to 2.9 for transferrin. After 5 min incubation the macromolecule and transferrin in the stroma reach steady state concentrations. The pattern of iron incorporation and the solubilization characteristics of the macromolecule indicate that it is not ferritin which is highly water soluble.[8]

Of the solubilizing agents utilized, DOC was the most effective. It extracted both the macromolecule and transferrin and allowed chromatographic resolution of these components. A greater percentage of transferrin was solubilized by milder agents, indicating than transferrin was not bound as tightly to the stroma as the macromolecular component.

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