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Iron-Donating Properties of Transferrin†

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ABSTRACT: The transferrin molecule has two specific metal-binding sites, each of which may provide iron for the biosynthesis of hemoglobin by reticulocytes. Diferric human transferrin was shown to be a better iron donor, per iron atom, for rabbit reticulocytes, than was monoferric transferrin obtained by isoelectric focussing. The difference in binding of ^{125}I-labeled monoferric and diferric transferrin to reticulocytes may be sufficient to account for the difference in iron uptake. In contrast, diferric and monoferric rabbit transferrin both donated iron to reticulocytes at the same rate, per iron atom. In an experiment using ^{55}Fe/^{59}Fe doubly labeled transferrin, one iron binding site of human transferrin was a better iron donor than the other. In rabbit transferrin, the two sites appeared to function equivalently. Care was taken in these experiments to demonstrate that labeled iron added to dilute solutions of transferrin was indeed specifically bound to the protein. A liquid scintillation counting procedure, simpler than existing methods, was developed to quantitate ^{55}Fe and ^{59}Fe in blood.

The major physiologic role of the plasma iron-transport protein, transferrin, is the delivery of iron to hemoglobin-synthesizing immature red blood cells. This glycoprotein molecule consists of a single polypeptide chain of molecular weight near 80,000 (Greene and Feeney, 1968; Mann *et al.*, 1970) on which are disposed two specific iron-binding sites which are very similar, if not identical, by a variety of thermodynamic and spectroscopic criteria (Aasa *et al.*, 1963; Aisen *et al.*, 1966; Binford and Foster, 1974). Fletcher and Huehns (1967; Fletcher, 1969) have presented evidence which suggests that the two sites are functionally heterogeneous; one site appears to be a better iron donor for reticulocytes than the other. They also observed that iron-saturated transferrin is a better source of iron for reticulocytes than partially saturated transferrin. A similar saturation effect was observed in the delivery of iron to the liver (Fletcher, 1971) and a reverse effect was postulated to be important in iron transport to the placenta (Fletcher and Huehns, 1968). Other studies failed to confirm these findings and a myriad of conflicting reports concerning these effects now exists (Lane, 1973; Hahn, 1973, 1974; Ganzoni *et al.*, 1972; Chernelch and Brown, 1970; Lane and Finch, 1970;

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Dern *et al.*, 1963; Hosain and Finch, 1964; Zapolski *et al.*, 1974; Williams and Woodworth, 1973; Awai *et al.*, 1972).

Two major methodologic difficulties which beset studies of the physiologic properties of transferrin have been assuring that labeled iron added to the protein is indeed specifically bound, and determining the distribution of iron among protein molecules less than fully saturated with the metal. In the present studies we have taken care to establish that labeled iron is bound to the specific sites of transferrin. Further, by using monoferric transferrin obtained by isoelectric focussing, we have been able to study directly the difference between monoferric and diferric transferrin. Finally, because of variability in the behavior of transferrins from different species (Paoletti *et al.*, 1958), we have compared the properties of the two most extensively studied transferrins, rabbit and human, using rabbit reticulocytes.

Materials and Methods

All glassware was acid-rinsed and buffers were extracted with dithizone in carbon tetrachloride to remove traces of iron. Saline buffers used to wash reticulocytes were boiled to remove residual carbon tetrachloride.

Transferrin. Apotransferrin (Harris *et al.*, 1974) was prepared from human (Aisen *et al.*, 1966; Gelotte *et al.*, 1962) or rabbit (Baker *et al.*, 1968) transferrin. ^{125}I-labeled diferric transferrin, containing an average of one iodine atom per molecule, was prepared by the method of Katz (1961), using Na^{125}I in dilute NaOH from Amersham/Searle. Iron was removed and monoferric transferrin was prepared from the apoprotein as described below. In all experiments in which monoferric and diferric transferrin were compared, diferric transferrin was made from monoferric transferrin. All calculations assume a molecular weight of 80,000 for transferrins and A_{280}(1%) values of 10.9, 12.4, and 14.0 for apotransferrin, monoferric transferrin, and diferric transferrin, respectively.

Reticulocytes. Rabbit reticulocytes were obtained from adult males bled from the central artery of the ear (10–15 ml/kg of body weight) four times on alternate days prior to an experiment. The heparinized blood, containing 15–25% reticulocytes, was centrifuged at 3200*g* for 15 min at 4° and washed four times at 0° with 4 volumes of saline buffer containing 0.13 M NaCl–0.005 M KCl–0.0074 M $MgCl_2$–0.01 M Hepes[1] (pH 7.4). Most of the buffy coat was removed during these washings. The washed, packed cells, simply called reticulocytes hereafter, were maintained at 0° before use within 3 hr of the bleeding. Alternatively, blood containing 30–50% reticulocytes was obtained from rabbits 3–5 days after treatment with 1-acetyl-2-phenylhydrazine (1 ml/kg of 0.6% acetylphenylhydrazine in 0.9% NaCl administered subcutaneously for 2 days, followed by 1 ml/kg of 1.2% acetylphenylhydrazine for 2 days). Rabbits so prepared continued to exhibit 15–50% reticulocyte counts for 1–2 weeks when bled on alternate days.

Iron Nitrilotriacetate. A 1.5 mM solution of ^{59}Fe-labeled iron nitrilotriacetate was prepared by combining 0.25 ml of $^{59}FeCl_3$ (1.2 μmol of Fe, 0.5 mCi, in 0.1 M HCl, obtained from Amersham/Searle), 0.77 ml of Fe^{3+} (13.8 μmol of Fe (pH 0.5) in HCl, prepared by dissolving iron wire in 6 M HCl) and 3.0 ml of nitrilotriacetic acid (30 μmol). The pH was very carefully adjusted to 4.0 with KOH and the solution was diluted to 10 ml with water. The pH of this solution was occasionally checked to assure that it remained between 4.0 and 4.5. ^{55}Fe-labeled iron nitrilotriacetate was prepared in a similar manner, using $^{55}FeCl_3$ in 0.1 M HCl from Amersham/Searle.

Standardization of Iron Nitrilotriacetate. To a 5-ml volumetric flask was added 0.5 ml of ascorbic acid (5 g/l. in 0.1 M HCl), iron nitrilotriacetate solution containing 2–3 μg of Fe, 0.5 ml of ferrozine[2] (Hach Chemical Co., 75 mg dissolved in 25 ml of water with 1 drop of HCl), and 1 ml of 1 M NaH_2PO_4–HCl buffer (pH 2.51). The volume was brought to 5 ml and the solution was mixed and allowed to stand 45 min at 22° in the dark prior to measuring A_{562}. A blank contained all reagents except iron. Beer's law was obeyed over a wide range of concentration of either Fe^{3+} in HCl or iron nitrilotriacetate, with ϵ = 27,400 M^{-1} cm^{-1}.

Monoferric Transferrin. Human or rabbit transferrin, 45% saturated with iron, was prepared from 150 mg of apotransferrin, ^{59}Fe-labeled iron nitrilotriacetate, and 1 ml of 0.04 M K_2CO_3 in a total volume of 20 ml. The solution was incubated overnight at 4° and concentrated to 5 ml by vacuum dialysis against water in a Schleicher and Schuell collodion membrane. Isoelectric focussing was performed for 40 hr at 4° and 600 V in an LKB 440-ml column with a 1% Ampholine (pH 5–7)–sucrose gradient, using a dense H_2SO_4 anode solution and light NaOH cathode solution. Protein was applied 1/3 of the way from the bottom of the column. Fractions (3 ml) were collected at an elution rate of 0.5–1 ml/min in tubes containing 0.3 ml of 0.5 M Hepes (pH 8.1) to raise the pH of the transferrin above 7.0 as quickly as possible and thus minimize redistribution of iron among molecules. Transferrins are eluted in the order: diferric (pI ≃ 5.0) before monoferric (pI ≃ 5.3) before apoprotein (pI ≃ 5.6). Each of these species of human transferrin is further split into two components of approximate ratio 10:1, the major component being eluted first. This is the same behavior noted by Wenn and Williams (1968) for conalbumin. In the case of rabbit transferrin, we observed only monoferric and apoprotein, in contrast to the three fractions found by Van Eyk *et al.* (1969). This may be because the diferric fraction is expected near the cutoff pH of the column. Pooled fractions of the major component of monoferric transferrin were purified by dialysis against 0.05 M Hepes–0.1 M KCl (pH 7.4) and passage through a 3 × 23 cm Sephadex G-25 (medium) column equilibrated with this buffer and cleansed of iron by the passage of 10 mg of apotransferrin one void volume prior to applying the monoferric transferrin. Typical final recovery of monoferric transferrin is 70–80 mg.

Analysis of Monoferric Transferrin. Our procedure for determining iron was patterned after that of Carter (1971), modified to eliminate interference by nitrilotriacetate. To a test tube were added 0.2 ml of water, 0.2 ml of solution containing about 2 mg of transferrin, and 0.5 ml of ascorbic acid (5 g/l in 0.1 M HCl). After 10 min 0.5 ml of trichloroacetic acid (110 g/l., neutralized to pH 2.1 with KOH) was added and the solution was centrifuged. To 1 ml of supernatant was added 0.2 ml of ferrozine (75 mg/25 ml) and 0.5 ml of buffer prepared by adding acetic acid to 0.2 M sodium acetate to lower the pH to 4.0. After standing 30 min in the dark at 22°, A_{562} was measured against a blank prepared from apoprotein. Iron content was determined from a standard curve constructed using apoprotein and known quan-

[1] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

[2] Ferrozine is disodium 3-(2-pyridyl)-5,6-bis(4-phenylsulfonate)-1,2,4-triazine.

tities of iron. The composition of a protein sample was then determined from the iron content and A_{280}. Two different preparations of human transferrin were found to possess iron/transferrin ratios of 1.07 and 1.10. A single preparation of rabbit transferrin exhibited a ratio of 1.17.

Incubation Procedures. Transferrin solutions containing 1 mg of protein, the required quantity of iron nitrilotriacetate, and 5–25 μl of 0.5 M $NaHCO_3$ in about 0.15 ml were prepared a day before use and stored at 4°. Under these conditions, spectrophotometric assay at 470 nm indicated that all of the iron is bound to the specific metal-binding sites of the transferrin. The incubation medium consisted of saline buffer containing 1 mg/ml of glucose and, to minimize hemolysis, 2 mg/ml of bovine serum albumin. Incubations were performed at 37° in open flasks containing 0.15 ml of transferrin solution, 3 ml of incubation medium, and 1.5 ml of reticulocytes. This nonphysiological protein concentration (0.23 mg/ml) was chosen so that the entire quantity of iron present in solution was potentially consumable by the reticulocytes. Samples at zero time were withdrawn before the suspensions were warmed to 37°. Thereafter, 0.50-ml aliquots of cell suspension were withdrawn periodically, quenched in 10 ml of saline buffer at 0°, centrifuged at 4° for 10 min at 3200*g*, and washed twice with 10-ml portions of saline buffer. Washing a third time produced less than a 1% change in ^{59}Fe activity bound to reticulocytes, as measured to a statistical counting error of 1–2% in a well-type scintillation counter.

^{55}Fe/^{59}Fe Double Incubation Procedure. Transferrin labeled with ^{59}Fe (iron/transferrin = 1.90) was incubated with reticulocytes until about 25% of the iron had been consumed, as determined by counting the cells and supernatant of an aliquot quenched in 20 volumes of saline buffer. To the undiluted supernatant from the incubation mixture was added an amount of ^{55}Fe-labeled iron nitrilotriacetate necessary to just replace the ^{59}Fe lost, as well as 25 μl of 0.5 M $NaHCO_3$. This solution was stored at 4° overnight and then incubated at 37° for 1 hr before determining the fraction of ^{55}Fe bound to protein as described below. For future reference, we call this preincubated solution containing both ^{59}Fe and ^{55}Fe solution A. After an additional day at 4°, solution A was reincubated with fresh reticulocytes and both ^{55}Fe and ^{59}Fe uptake were monitored.

Determination of ^{55}Fe and ^{59}Fe in Blood by Liquid Scintillation Counting. To each sample of packed red cells (0.16 ml) was added 2.00 ml of distilled water and the sample was thoroughly agitated with a glass rod at 22°. Samples were further agitated at 37° for 1 hr to ensure complete hemolysis. Duplicate 0.50-ml aliquots of whole, uncentrifuged hemolysate were placed in 20-ml glass scintillation vials. Each was treated with 0.50 ml of Clorox (5.25% NaOCl) and allowed to stand at 22° for 1 hr at which time the samples were almost colorless. To reduce unreacted Clorox, 0.50 ml of freshly prepared ascorbic acid solution (120 g/l.) was then added. After an additional hour, 10 ml of Aquasol (New England Nuclear) was added under dim lighting. Samples were counted in a Beckman LS-230 liquid scintillation counter at 22° after 1 hr of dark adaption. Window settings were such that a high energy window registered essentially only ^{59}Fe and a low energy window responded mainly to ^{55}Fe. The specific activities of ^{55}Fe-labeled iron nitrilotriacetate and ^{59}Fe-labeled iron nitrilotriacetate were chosen so that the counts measured on each channel were similar. Two background samples were always prepared from a portion of reticulocytes incubated in the absence of radioactive materials. The background activity was constant at about 30 cpm on both channels after 1 hr. (In the absence of ascorbic acid, initial backgrounds were near 500,000 cpm.) Suitable standards for counting were prepared from cells incubated in the absence of radioactive material, hemolyzed, and treated with microliter quantities of ^{55}Fe-labeled iron nitrilotriacetate or ^{59}Fe-labeled iron nitrilotriacetate calculated to correspond approximately to the maximum activity expected in our samples. The averaged counts of four such samples were used as standards for determining ^{55}Fe and ^{59}Fe in each experiment. Samples were analyzed by solving the following simultaneous equations:

$$\text{low energy channel cpm} = a_1[^{55}\text{Fe}] + a_2[^{59}\text{Fe}]$$

$$\text{high energy channel cpm} = b_1[^{55}\text{Fe}] + b_2[^{59}\text{Fe}]$$

where a_1 and a_2 are the averaged standard counts produced by samples of ^{55}Fe and ^{59}Fe, respectively, on the low energy channel and b_1 and b_2 are the averaged standard counts produced by ^{55}Fe and ^{59}Fe, respectively, on the high energy channel. Background was subtracted from all counts before calculation. As a reference to define 100% of the activity present in the incubation solution, 0.50 ml of the suspension was withdrawn at the end of the experiment and pipetted directly into 2.00 ml of distilled water. Duplicate 0.50-ml aliquots of these hemolyzed samples were then prepared for scintillation counting. ^{59}Fe uptake was determined by both γ counting and simultaneously with ^{55}Fe by liquid scintillation counting. The results of these independent procedures generally agreed to within 3%.

Analysis of ^{55}Fe Binding to Transferrin. In a preliminary experiment, we found that 97% of the ^{59}Fe-labeled iron nitrilotriacetate added to ^{56}Fe-saturated transferrin (^{59}Fe/transferrin = 0.49) could be separated from the protein by gel filtration through a column of Sephadex G-25 eluted with 0.05 M Hepes–0.1 M KCl (pH 7.4). In our ^{55}Fe/^{59}Fe double incubation experiments, the ratio of ^{55}Fe to ^{59}Fe was determined for samples of solution A subjected to gel filtration and compared to the ratio found for samples not so filtered. The decrease in this ratio upon gel filtration was taken as a measure of the fraction of ^{55}Fe not bound to the transferrin.

Results

The Problem of Iron Binding to Transferrin. Many previous studies of this type utilized either ferric chloride or ferric citrate as sources of iron for transferrin. The propensity of the former to form hydrolytic polymers and to bind tightly but nonspecifically to macromolecules renders it worthless as a source of iron under physiologic conditions (Bates and Schlabach, 1973). Ferric citrate appears to be a somewhat better source of iron for transferrin, but it can be very slow in delivering its metal to the protein (Cavill, 1971). In the present work, iron nitrilotriacetate was chosen since it rapidly provides iron to the two specific metal-binding sites of transferrin and unbound iron nitrilotriacetate could be separated from the protein by gel filtration. We used this procedure to establish that about 93% of the ^{55}Fe-labeled iron nitrilotriacetate added to preincubated transferrin in dilute solution was actually bound. This is important because iron nitrilotriacetate, itself, is an efficient source of iron for hemoglobin synthesis by reticulocytes (Princiotto *et al.*, 1964; Morgan, 1971). The demonstration of iron binding in this type of experiment, and especially for *in vivo* experiments, seems mandatory.

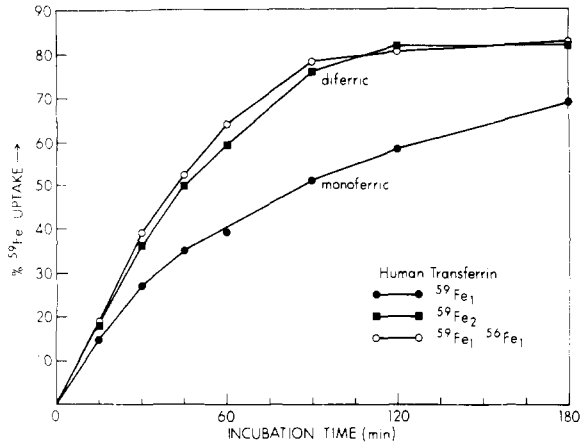

FIGURE 1: Uptake of ^{59}Fe from human transferrin. The transferrin concentration in the incubation solution was 0.22 mg/ml and the red cells contained 22% reticulocytes. 100% consumption of iron corresponds to 3.6 μg of Fe/ml of reticulocytes from diferric transferrin.

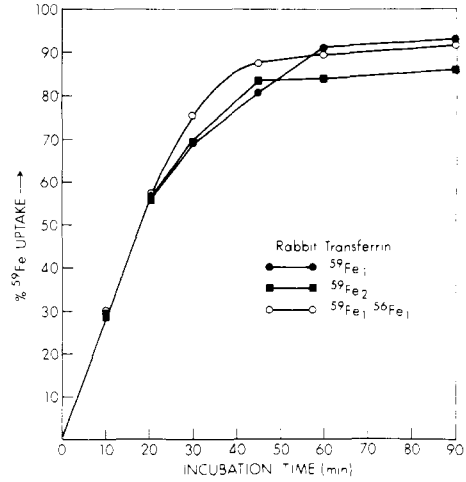

FIGURE 2: Uptake of ^{59}Fe from rabbit transferrin. The transferrin concentration in the incubation solution was 0.23 mg/ml and the red cells contained 37% reticulocytes. 100% consumption of iron corresponds to 2.5 μg of Fe/ml of reticulocytes from diferric transferrin.

Relative Effectiveness of Monoferric and Diferric Transferrins. The relative abilities of monoferric and diferric transferrin to donate iron to reticulocytes was determined by incubating three aliquots of a single, well-mixed suspension of washed reticulocytes with three kinds of transferrin: (1) monoferric ^{59}Fe-labeled transferrin; (2) monoferric ^{59}Fe-labeled transferrin brought to 97% saturation with ^{59}Fe-labeled iron nitrilotriacetate; and (3) monoferric ^{59}Fe-labeled transferrin brought to 97% saturation with ^{56}Fe-labeled iron nitrilotriacetate. For human transferrin (Figure 1) a difference between monoferric and diferric transferrin is evident. For rabbit transferrin (Figure 2) no such difference could be demonstrated.

Equal numbers of reticulocytes from different preparations exhibited a threefold variation in the rate of iron uptake, probably because the average age of the reticulocytes differs from one preparation to another, and the number of transferrin receptors decreases with maturity of the cell (Kornfeld, 1969). We consistently noted that the "slower" reticulocytes discriminated only very little between monoferric and diferric human transferrin. Reticulocytes from bled rabbits and acetylphenylhydrazine-treated rabbits gave similar results with human transferrin. Experiments with rabbit transferrin were done only with reticulocytes from acetylphenylhydrazine-treated rabbits.

In a control experiment, ^{59}Fe uptake was the same from monoferric human transferrin and monoferric human transferrin containing added nitrilotriacetate (nitrilotriacetate/Fe = 2.0), confirming that nitrilotriacetate, like several other chelating agents, had no effect on the transferrin–reticulocyte interaction (Morgan, 1971). In another experiment, the major and minor species of human monoferric transferrin obtained from isoelectric focussing were found to donate iron to reticulocytes at the same rate.

To see if the difference in iron-donating ability of monoferric and diferric human transferrin could be due to a difference in binding of each kind of transferrin to the reticulocyte, ^{125}I-labeled monoferric and diferric transferrin were incubated with reticulocytes and ^{125}I uptake was measured. Results, shown in Figure 3, indicate that diferric transferrin binds faster and to a greater extent than monoferric transferrin.

Functional Heterogeneity of the Two Sites. In order to test whether there is a functional difference between the two iron-binding sites of transferrin, 95% ^{59}Fe-saturated

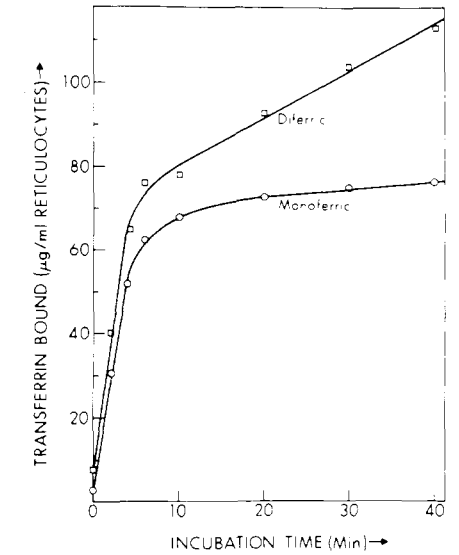

FIGURE 3: Uptake of ^{125}I-labeled monoferric and diferric human transferrin by rabbit reticulocytes. The transferrin concentration was 2.0 mg/ml and the reticulocyte count was 43%.

transferrin was incubated with reticulocytes from bled rabbits until about 25% of the iron was removed. Just enough ^{55}Fe was added to replace the ^{59}Fe lost. This doubly labeled material was then incubated with fresh reticulocytes from bled rabbits and the uptake of both isotopes was measured. Results are shown in Figures 4 and 5. The ratio of ^{59}Fe uptake to ^{55}Fe uptake was unity throughout the course of the experiment when rabbit transferrin was used (Figure 5). In contrast, ^{55}Fe was taken up more rapidly than ^{59}Fe from human transferrin, an effect most evident during the initial period of the experiment (Figure 5). It seems, then, that one site of the human protein functions as a better source of iron for rabbit reticulocytes than the other, in keeping with the findings of Fletcher and Huehns (1967).

Iron uptake was measured with whole cells, either intact or hemolyzed. In order to show that iron taken up from each iron-binding site of transferrin is equally able to penetrate the cell membrane, a suspension of hemolyzed cells was centrifuged at 6200g for 15 min and ^{55}Fe and ^{59}Fe

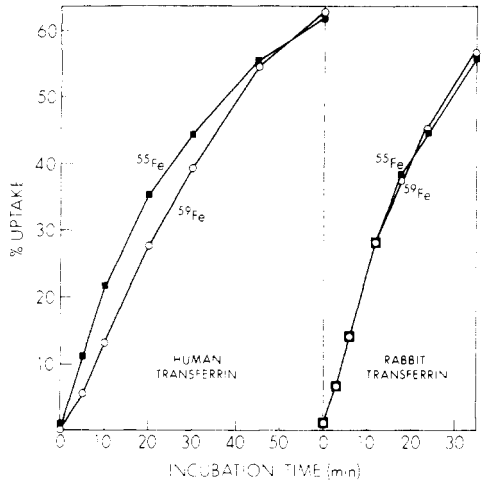

FIGURE 4: Uptake of ^{55}Fe and ^{59}Fe from human and rabbit transferrins. Human transferrin saturated to the extent of 95% with ^{59}Fe was incubated with rabbit reticulocytes until 25% of the ^{59}Fe was removed. Enough ^{55}Fe to replace the lost ^{59}Fe was added and the doubly labeled material was reincubated with red cells containing 18% reticulocytes. The transferrin concentration in each incubation mixture was 0.23 mg/ml and 100% total iron consumption corresponds to 4.9 μg of Fe/ml of reticulocytes. Conditions for the rabbit transferrin experiment were the same as described for human transferrin except that 22% of the ^{59}Fe was initially removed.

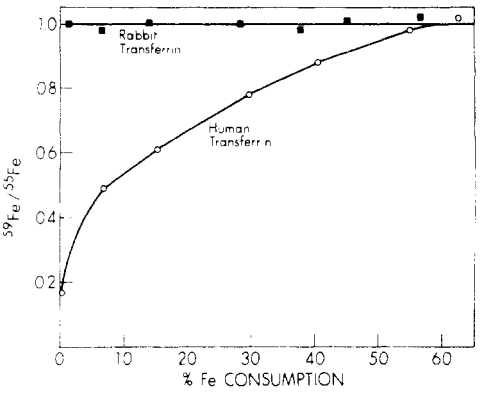

FIGURE 5: Comparison of ^{59}Fe and ^{55}Fe uptake from human and rabbit transferrin by rabbit reticulocytes. These curves are derived from the data of Figure 4.

contents of the supernatant were measured. The relative amount of each isotope was the same as that found in whole hemolysate and 80–95% of the radioactivity was present in the soluble fraction. Control experiments also showed that when preincubated transferrin was reloaded with iron, it donated iron to reticulocytes at exactly the same rate as non-preincubated transferrin. This shows that preincubation did not affect the function of the protein. In another experiment, human transferrin was brought to 20% saturation with ^{59}Fe, the pH was raised with $NaHCO_3$, and the saturation was increased to 90% with ^{56}Fe. A second sample was prepared first with 70% ^{56}Fe and brought to 90% saturation with ^{59}Fe. Both samples delivered ^{59}Fe to reticulocytes at essentially the same rate, indicating that the binding constants of the two different iron-binding sites must be very similar. Otherwise, the better iron donor site would contain a greater fraction of ^{59}Fe in one preparation than in the other, and reticulocyte ^{59}Fe uptake would be correspondingly greater. Similar results have been reported by Zapolski *et al.* (1974).

Discussion

Although the role of transferrin in the transport of iron has long been appreciated (Jandl *et al.*, 1959; Laurell and Ingelman, 1947), only recently has attention centered on a possible function of the protein in the regulation of iron metabolism (Fletcher and Huehns, 1968). Possible mechanisms for a two-sited protein like transferrin to fulfill an active regulatory role include a difference in the iron-donating properties of molecules carrying one or two iron atoms and an intrinsic functional heterogeneity of the sites. In the present studies, we have been able to examine each of these possibilities separately.

Comparison of Monoferric and Diferric Transferrin. In agreement with previous studies using human transferrin and rabbit reticulocytes (Fletcher, 1969; Lane, 1973) we find that diferric transferrin is a better iron donor than monoferric transferrin (Figure 1). This difference must be a real difference between the monoferric and diferric species since it is seen when ^{59}Fe-labeled monoferric transferrin is saturated with either ^{59}Fe or ^{56}Fe, and the results expressed as the percentage of ^{59}Fe which is taken up by the cells. If there were good and poor iron donor sites and if, for example, the monoferric species contained all of the ^{59}Fe at the poor donor site, addition of ^{56}Fe to the good site would have no effect on uptake of ^{59}Fe, contrary to what is observed.

In contrast to previous studies employing rabbit transferrin and rabbit reticulocytes (Fletcher, 1969; Lane, 1973), we find no difference between the iron-donating abilities of monoferric and diferric rabbit transferrin (Figure 2). Williams and Woodworth (1973) have found that chick embryo red blood cells do not distinguish at all between monoferric and diferric chick conalbumin (ovotransterrin),which is similar to transferrin in many of its properties (Gafni and Steinberg, 1974; Aisen and Leibman, 1968a; Tan and Woodworth, 1969; Woodworth *et al.*, 1970; Warner and Weber, 1953). Evidently species variability is of critical importance in assessing the function of transferrin.

The difference in binding of ^{125}I-labeled monoferric and diferric transferrin to reticulocytes (Figure 3) appears to be sufficient to account for the difference in iron-donating abilities of these species. This result could be anticipated from the work of Kornfeld (1969), which utilized 43% randomly saturated transferrin instead of monoferric transferrin. In contrast, the data of Baker and Morgan (1969) indicate only about a 10% difference in binding between 99 and 12% saturated rabbit transferrin. The difference in behavior between monoferric and diferric human transferrin could be related to the change in overall dimensions (and hence conformation) of the molecule known to occur when iron is bound (Bezkorovainy, 1966; Rossenue-Motreff *et al.*, 1971; Kornfeld, 1969). It would be of interest to learn if such a conformational change occurs with rabbit transferrin, as the studies just cited all deal with human transferrin.

Zapolski *et al.* (1974) have postulated that the iron atom of monoferric transferrin is either at one site capable of donation to rabbit reticulocytes or at another site incapable of such donation. Our observation that monoferric transferrin gave up 88% of its iron in one experiment suggests that this hypothesis is wrong, that iron is capable of site–site exchange during incubation, or that the two iron-binding constants of transferrin are not equal. The equality, or near equality, of the binding constants is a necessary premise for the hypothesis of Zapolski *et al.*, and agrees with all other experimental results, including those of the present work. Intermolecular iron exchange does not occur in the absence of an iron chelating agent at pH 7.4 (Aisen and Leibman,

1968b) and the potentially chelating ampholytes from isoelectric focussing have been removed by gel filtration (Vesterberg, 1973). Intramolecular site-site exchange seems unlikely since the two sites are more than 43 Å apart (Luk, 1971). Were site-site exchange important, it would not have been possible to observe a functional difference between the sites in the $^{55}Fe/^{59}Fe$ double label experiments. Hence our experiment is not consistent with the hypothesis of Zapolski *et al.*

Functional Heterogeneity of the Binding Sites. From Figures 4 and 5 it is clear that one binding site of human transferrin is better than the other as an iron donor for rabbit reticulocytes. It is also clear from Figures 4 and 5 that there is no such functional heterogeneity in rabbit transferrin. The electron paramagnetic resonance spectrum of diferric human transferrin has been interpreted by Aasa (1972) in terms of two structurally different binding sites. The spectrum of hagfish transferrin, by contrast, was consistent with only one kind of binding site. We note that the spectrum of rabbit transferrin is very similar to that of hagfish transferrin, and therefore may also indicate a single type of binding site. These observations fit nicely with the observation of a functional heterogeneity in human transferrin and a functional homogeneity in rabbit transferrin. In disagreement with such a simple correlation, however, the two binding sites of conalbumin are spectroscopically distinguishable (Aisen *et al.*, 1973; Aasa, 1972); yet they are equivalent in their ability to supply iron to chick blood cells (Williams and Woodworth, 1973).

The epr spectra of monoferric and diferric human transferrin were recorded after 54 and 32%, respectively, of the iron had been consumed by reticulocytes. No qualitative differences were noted between these spectra and that of ordinary diferric transferrin, so it would seem from these findings that the two kinds of binding sites give rise to very similar, if not identical spectra. Since the two binding sites possess similar properties (Aisen *et al.*, 1966; Aasa, *et al.*, 1963; Binford and Foster, 1974), the functional difference seems more likely to be related to an asymmetric disposition of the iron atoms in transferrin than a difference in the immediate environments of the iron atoms. For example, although the immediate ligands of each iron atom may be the same, one iron atom may be closer to the point of attachment of human transferrin to the reticulocyte and therefore more accessible to the cell. Such a proposal is consistent with all of these observations and does not require any correlation between functional heterogeneity of the sites and small differences in the immediate environments of each iron atom which might produce differences in the epr spectra.

If a functional heterogeneity of the specific sites of transferrin is important in the regulation of iron metabolism (Fletcher and Huehns, 1968), it is surprising to find that such a heterogeneity is only seen with the human and not the rabbit protein. It might be expected that such a regulatory function would be common to many species. Perhaps the rabbit red blood cell distinguishes between the iron-binding sites of transferrin at the earlier normoblast stage in its development, when the rate of hemoglobin synthesis is maximal, and this ability to differentiate between the sites diminishes as the cell matures. Alternatively, it is conceivable that the heterogeneity of binding sites which we observed is an artifact of the mixed system of human protein and rabbit reticulocytes. Clearly, the question of species variability is a critical one, and the study of human transferrin with human reticulocytes will be necessary in further assessing the physiological significance of our observations. This work is in progress.

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The α and β Subunits of *Cyanidium caldarium* Phycocyanin: Properties and Amino Acid Sequences at the Amino Terminus†

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ABSTRACT: Phycocyanin was isolated and purified from the unicellular alga, *Cyanidium caldarium.* Subunits were prepared on a Bio-Rex-70 column developed stepwise with urea solutions (pH 1.9). The α subunit eluted in 8 M urea and the β subunit eluted in 9 M urea. The α and β subunits displayed absorption maxima at 660, 354, and 277 nm in 8 M and 9 M urea. The α:β ratio of total absorbance under the 660-nm peak was 0.56 suggesting an α:β phycocyanobilin chromophore ratio of 1:2. On calibrated sodium dodecyl sulfate gels, the α subunit had an estimated molecular weight of 15,500 ± 1100 and the β subunit had an estimated molecular weight of 18,300 ± 300. Minimum molecular weights based on one histidine residue per subunit were 16,300 for the α subunit and 18,750 for the β subunit. Phycocyanin displayed a single visible absorption maximum at 625 nm and two positive circular dichroic bands at 632 and 610 nm. The α and β subunits displayed single visible absorption maxima at 618 and 600 nm and single positive circular dichroic peaks at 620 and 585 nm, respectively. Two-dimensional maps of tryptic digests of the α and β subunits revealed distinct patterns of peptides each of which was consistent with the lysine and arginine composition of these polypeptides. Maps of tryptic digests of phycocyanin contained 25 major peptides (a total of 27 lysine and arginine residues). Automated sequence analysis of separated subunits revealed a 70% homology within the first 27 residues at the amino terminus of the α and β subunits of *C. caldarium* phycocyanin.

Phycocyanin[1] is a bile pigment–protein complex which is found in the photosynthetic apparatus of the red (Rhodophyta), blue-green (Cyanophyta), and golden-brown (Cryptophyta) algae (OhEocha, 1965). This biliprotein is an accessory photosynthetic pigment which is a functional component of pigment system II (Myers, 1971). Phycocyanin occurs as a high molecular weight aggregate *in vivo,* called a "phycobilisome" (Gantt and Conti, 1966). In stained sections in the electron microscope phycobilisomes appear as granules, 350–450 Å in diameter, located on the stroma side of thylakoid membranes (Gantt and Conti, 1965). Phycocyanin from the golden-brown algae does not form high molecular weight aggregates (MacColl *et al.,* 1973) and is located within the intrathylakoid space rather than on the outer surface of thylakoid membranes (Gantt *et al.,* 1971).

The fundamental unit of phycocyanin aggregation is a monomer which has an estimated molecular weight of approximately 33,000 (Berns, 1971). The monomer can associate or dissociate into larger or smaller aggregates depending on the pH, ionic strength, and protein concentration (Hattori *et al.,* 1965; Berns, 1971). The monomer from

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[1] Abbreviations used are: phycocyanin, C-phycocyanin in which phycocyanobilin is the only chromophore; TPCK, L-tosylamido-2-phenylethyl chloromethyl ketone; dansyl, dimethylaminonaphthalenesulfonyl derivative.