An Alternative Model for the Binding and Release of Diferric Transferrin by Reticulocytes†

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ABSTRACT: The biphasic binding of diferric transferrin to reticulocytes has been reevaluated with a series of kinetic and equilibrium studies. Identical binding progress profiles were observed for reticulocytes in the presence or absence of oxygen. The relative size of the rapid initial adsorption step could be increased to ca. 65% of the total binding by stripping the cells of endogenous transferrin or reduced to 0% by preloading the cells with nonradiolabeled diferric transferrin. Preloading the cells with ^{125}I-labeled diferric transferrin and chasing with ^{131}I-labeled diferric transferrin revealed identical rate constants for release and binding. Scatchard plots of equilibrium binding of diferric transferrin to reticulocytes showed no significant effects of anaerobiasis or 2,4-dinitrophenol on the equilibrium binding constant or the maximum number of binding sites. The potent microtubule inhibitor nocodazole had no effect on the progress curves for transferrin binding or iron uptake by reticulocytes. It was concluded that the rapid adsorption step in the binding profile represents binding to open receptors and that the slow first-order binding phase represents binding of radiolabeled transferrin to receptors already occupied by nonlabeled endogenous transferrin as this endogenous transferrin leaves the receptors. Furthermore, this first-order binding phase, unlike iron uptake, does not require the presence of active oxidative phosphorylation. These findings are consistent with a specific desorption–adsorption model for the interaction of diferric transferrin with reticulocytes.

The plasma pseudoglobulin transferrin binds inorganic iron and transports it to erythroid tissue for ultimate incorporation into hemoglobin and to various tissues, e.g., liver, for storage as ferritin. A familiar in vitro system for the study of iron transfer from diferric transferrin to cells employs reticulocytes as the iron-receiving species. Reticulocytes contain membrane receptors specific for transferrin (Williams & Woodworth, 1973; Leibman & Aisen, 1977; Aisen et al., 1978; Witt & Woodworth, 1978). The manner in which reticulocytes handle bound transferrin is presently a subject of some controversy.

In the present study the kinetics of the binding and release of two transferrins by their isologous reticulocytes have been investigated in order to ascertain the physiochemical nature of the protein–cell interaction. Most of the studies involved ovotransferrin (conalbumin) from hen egg white and chick embryo red blood cells (Williams & Woodworth, 1973), hereafter called chick reticulocytes. Other experiments involved rabbit plasma transferrin and rabbit reticulocytes, the more usual experimental preparation (Schade, 1964; Morgan & Laurell, 1963). The primary phenomenon under investigation was the well-documented kinetic profile for the binding of transferrin to reticulocytes (Figure 1), consisting of an "instantaneous" phase followed by a slow approach to equilibrium binding phase (Baker & Morgan, 1969). Morgan and co-workers have interpreted this phenomenon as involving a rapid, temperature-insensitive adsorption step, followed by a slow internalization of transferrin (Paterson & Morgan, 1980). We propose an alternative model for the interaction of transferrin with reticulocytes. Morgan has shown (Hemmaplardh & Morgan, 1974) that reticulocytes tenaciously retain some endogenously bound transferrin, carried along from the host animal at harvest. The fast adsorption step, we feel, reflects rapid binding to vacant transferrin receptor sites, whereas the slow approach to equilibrium phase reflects the filling of sites occupied by endogenous transferrin and filled with radiolabeled transferrin only as the endogenous transferrin desorbs from the membrane. Furthermore, we find this slow phase to be non energy dependent. The entire binding and release profile can be understood in terms of desorption and adsorption of transferrin by the specific membrane receptors.

Experimental Procedures

Chick Studies. The methods described by Williams & Woodworth (1973) were used without modification for the following: preparation of ovotransferrin (OTf)[1] from hen egg white, formation of Fe_2–OTf or $^{59}Fe_2$–OTf complexes, ^{125}I or ^{131}I radiolabeling of ovotransferrin, preparation of chick reticulocytes, equilibrium binding studies, and iron acquisition rate studies.

The equilibrium study of Fe_2–[^{125}I]OTf binding to reticulocytes used the following preparations. Chick reticulocytes were harvested from 14-day-old embryos, washed 3 times with EGA and once with EGAB, and held on ice until used. Cells were usually held on ice for 0.5–2 h before use in an experiment. Neither this variable time nor holding them in EGA overnight in a shallow plastic tray in the cold room appeared to compromise their ability to bind Fe_2–OTf or to take up iron from Fe_2–OTf. Aliquots of cells were transferred to stoppered tubes and incubated with shaking at 37 °C under flowing 95% air/5% CO_2 or 95% N_2/5% CO_2 or with 0.75 mM 2,4-DNP and 95% air/5% CO_2 as required for 30 min. The gas mixture was presaturated with H_2O by passing it through H_2O in a fritted gas-washing bottle. Appropriate amounts of 50 μM Fe_2–[^{125}I]OTf in EGAB were pipetted into the tubes to give a range of transferrin concentrations from 0.1 to 6 μM. Incubation was continued as above for 45 min. Duplicate 200-μL

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[1] Abbreviations: Tf, rabbit plasma transferrin; OTf, hen egg white ovotransferrin; NTA, nitrilotriacetate; EGA, Earle's balanced salts medium with 5 mM glucose and 0.5% bovine serum albumin, pH 7.4; EGAB, EGA made to 20 mM $NaHCO_3$ and equilibrated with 5% CO_2 at 37 °C; 2,4-DNP, 2,4-dinitrophenol; nocodazole, methyl [5-(2-thienylcarbonyl)-1*H*-benzimidazol-2-yl]carbamate; DEAE, diethylaminoethyl.

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samples were then taken and centrifuged through ice-cold 0.3 M sucrose to halt protein binding. The supernate was aspirated into radioactive waste, and the pelleted cells were counted in a Beckman Biogamma scintillation counter. The number of OTf molecules bound per cell was determined from the radioactivity in the pellet and the number of cells in the original sample as measured by microhematocrit and a calibration curve of hemocytometer counts vs. hematocrit. Anaerobic cells were found to have volumes 6–16% larger than aerobic cells.

Actual concentrations of Fe_2–[^{125}I]OTf at equilibrium with cells were determined by centrifuging an aliquot of each cell suspension and counting a small aliquot, e.g., 10 μL, of the supernate. Plots of Fe_2–OTf bound vs. free Fe_2–OTf were first constructed. The final limiting slope at the higher OTf concentrations was taken to represent nonsaturable binding. The slope of this line was used to correct the experimental points. The percentage of total binding ascribable to nonsaturable binding varied from 5% at 3 μM Fe_2–OTf to 8% at 10 μM Fe_2–OTf to 32% at 30 μM Fe_2–OTf under aerobic conditions. In the absence of O_2 or presence of 2,4-DNP the nonsaturable binding was less. From the corrected data, values needed for construction of Scatchard plots were calculated. The points were fitted by a linear least-squares fitting routine, which yielded estimates of the slope, intercepts, and standard deviations of these parameters.

All other binding and release studies followed a similar protocol, except that aliquots of reticulocyte suspensions from 14-day-old to 15-day-old embryos were transferred into 10–20 volumes of ice-cold EGA and centrifuged and the cells washed twice by resuspension in ice-cold EGA and centrifugation in a Beckman microfuge B. Unless otherwise specified the concentration of transferrin in the bathing medium was 10 μM.

Data from kinetic studies of diferric transferrin binding and release by reticulocytes were fitted to a first-order rate equation by the PAR biomedical computer program (Ralston, 1979), which gives estimates of the apparent first-order rate constant, k, initial and final values of the dependent variable, and their standard deviations. The lines in figures in which values of k are reported are the computer-fitted lines to the experimental points presented in these figures. Fitting these data to an exponential function is justified on the basis that any isotope-exchange process can be shown to follow an overall first-order rate process regardless of the detailed reaction mechanism (Moore & Pearson, 1981).

Results from chick reticulocytes have been expressed on a per cell basis as all cells are active in acquiring iron from ovotransferrin (Williams & Woodworth, 1973).

Rabbit Studies. Reticulocytosis was induced in young New Zealand white rabbits of either sex by bleeding from a marginal ear vein, 10–15 mL/kg of body weight, every other day for 1 week. Blood was collected into physiological saline, 0.1% in heparin as anticoagulant. At harvest the reticulocyte count was ca. 20% as determined by staining with New Methylene Blue and counting a total of at least 1000 cells. These cells were washed and handled in the same manner as the chick reticulocytes. Results of experiments with rabbit reticulocytes have been corrected for the reticulocyte count.

Rabbit transferrin (Tf) was isolated by modification of the procedure of Baker et al. (1968). The unsaturated iron binding capacity of pooled rabbit plasma was saturated with iron prior to fractionation. Transferrin-containing fractions from chromatography on DEAE-Sephadex A-50 (Pharmacia) were pooled, dialyzed vs. several changes of glass-distilled water, and concentrated under N_2 pressure to minimum volume on an Amicon ultrafilter with a PM10 membrane. The concentrate was dialyzed to equilibrium against an equal volume of 50% sucrose and introduced at the isodense region of an electrofocusing column (LKB) after prefocusing for 12 h. The linear gradient from 50 to 0% sucrose contained 1% w/v ampholine, pH range 5–7 (LKB). The column was maintained at 4 °C with a recirculating cooling bath (Precision Scientific Co.). Electrofocusing of the protein was carried out at 1000 V for at least 12 h; the current did not exceed 4 mA. When focusing was complete, the column was drained, and the effluent was monitored at 280 nm. The pH was determined on the fractions comprising the transferrin peak. The pI of rabbit diferric transferrin was found to be 5.45.

The fractions containing diferric transferrin were pooled and dialyzed against 0.2 M phosphate buffer, pH 6.0, containing 1 mM Na_2EDTA and 1 mM disodium nitrilotriacetate (NTA). Two changes of this buffer were sufficient to remove nearly all of the transferrin bound iron, and additional dialysis did not remove more. The solution was dialyzed exhaustively against glass-distilled water and then against an equal volume of 50% sucrose freed of iron with Chelex-100 (Bio-Rad) and electrofocused as above. This final focusing was necessary to remove hemopexin, which, when in the heme form, tended to cofocus with diferric transferrin. A small amount of monoferric transferrin was usually present, focusing at a pI of about 5.7. The major portion of the apotransferrin was found at pH 5.9 with a shoulder at 6.1, thought to be a genetic variant.

The transferrin was assayed for purity by discontinuous gel electrophoresis (Ornstein, 1964). Under these conditions, hemopexin, when present, could be visualized as a band moving just ahead of the transferrin and stainable by dianisidine–H_2O_2 (Woodworth & Clark, 1967). After the two isoelectric focusings, hemopexin was not detected by this method. Spectral ratios of $A_{280}/A_{465} = 21$ and $A_{465}/A_{410} = 1.4$ were found for the pure diferric rabbit transferrin. An increase in the first ratio was indicative of either contamination by proteins other than transferrin or incomplete saturation of the transferrin with iron. A decrease in the second ratio suggested either contamination by heme–hemopexin or nonspecific binding of iron to transferrin. Transferrin, purified by this method, was stored frozen in the iron-free form. As needed, aliquots were removed and iron was added. A millimolar coefficient of absorbance at 280 nm of 92.0 mM^{-1} was used to determine the concentration of apotransferrin.

On addition of iron to apotransferrin, care was taken to avoid nonspecific binding of iron to the protein. This was usually done by the inclusion of NTA in the reaction mixture. The apotransferrin solution was made 20 mM in $NaHCO_3$, a 10-fold molar excess of Na_2NTA over the protein was added, and sufficient iron was then introduced from a stock solution of 20 mM ferrous perchlorate. A 95% saturation of the sites was usually sought. If [^{59}Fe]ferric transferrin was being prepared, the radiolabeled iron was added (as carrier-free $^{59}FeCl_3$ in 0.1N HCl, New England Nuclear) to the stock iron solution to give the desired specific activity prior to addition to the protein.

Iodination of transferrin was carried out by the iodine monochloride method of McFarlane (1963). Incorporation of iodine did not exceed an average of 1 atom of iodine per protein molecule. Non-protein-bound iodine was removed by exhaustive dialysis of the protein against glass-distilled water or by gel filtration on Sephadex G-25.

All chemicals were reagent grade. Ampholytes were obtained from LKB or Bio-Rad Laboratories. Nocodazole, vinblastine, and 2,4-DNP were obtained from Sigma. Nocodazole was dissolved in dimethyl sulfoxide for addition to

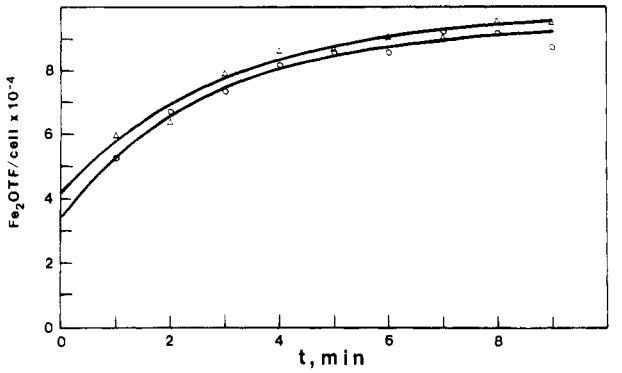

FIGURE 1: Progress curve for binding of Fe_2–[^{125}I]OTf to chick reticulocytes under 95% air/5% CO_2 (○) and under 95% N_2/5% CO_2 (△), at 37 °C. Calculated first-order rate constants (±SD) are as follows: aerobic, 0.38 (±0.05) min^{-1}; anaerobic, 0.33 (±0.09) min^{-1}. Fe_2–[^{125}I]OTf was 10 μM; cells were washed with EGA, not stripped of endogenous ovotransferrin.

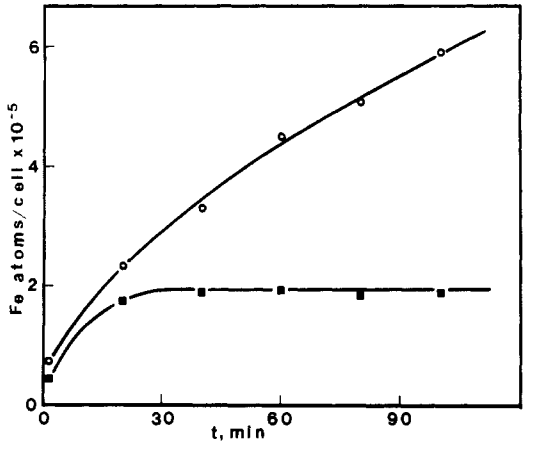

FIGURE 2: Effect of 2,4-dinitrophenol on iron uptake by chick reticulocytes from $^{59}Fe_2$–OTf: control (○) and 0.75 mM 2,4-dinitrophenol (■). Cells were preincubated with or without 2,4-DNP for 30 min at 37 °C under 95% air/5% CO_2 prior to addition of $^{59}Fe_2$–OTf to 10 μM. Cells were washed with EGA, not stripped of endogenous transferrin.

cell suspensions, and an equal volume of dimethyl sulfoxide was added to the control suspension.

Results

Aerobic and anaerobic chick reticulocytes showed comparable rates for the binding of Fe_2–OTf with only a slight difference in the total number of molecules bound (Figure 1).

Figure 2 illustrates the inhibition of iron uptake by chick reticulocytes treated with 0.75 mM 2,4-DNP, an agent that uncouples oxidative phosphorylation in mitochondria and hence significantly reduces ATP production. Note that the progress curve in the 2,4-DNP run reflects the binding of the expected number of Fe_2–OTf molecules per cell, but no net release of iron to the cell. More accurate determinations of the average number of Fe_2–OTf receptors per cell and estimates of the apparent binding constant, K, were obtained from the equilibrium binding studies, which yielded statistically similar values for aerobic and anaerobic reticulocytes and for reticulocytes inhibited by 2,4-DNP (Figure 3).

Rabbit reticulocytes showed binding progress curves for rabbit Fe_2–[^{125}I]Tf that were dependent on their pretreatment (Figure 4). Thus, relative to cells that had simply been washed in cold EGA, cells preincubated at 37 °C with 20 μM nonradiolabeled Fe_2–Tf showed a virtual absence of the fast adsorption phase and a decrease in the extent of the slow first-order approach to equilibrium of the radiolabeled protein

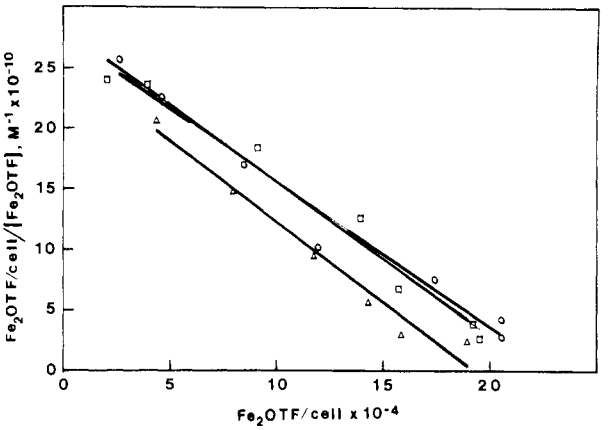

FIGURE 3: Scatchard plots of Fe_2–[^{125}I]OTf binding to chick reticulocytes under 95% air/5% CO_2 or 95% N_2/5% CO_2 at 37 °C. Calculated binding constants, K (±SD), and maximum number of binding sites, n (±SD), are as follows: aerobic (○), 1.20 (±0.09) × 10^6 M^{-1} and 2.30 (±0.11) × 10^5 sites/cell; anaerobic (△), 1.33 (±0.12) × 10^6 M^{-1} and 1.92 (±0.12) × 10^5 sites/cell; 0.75 mM 2,4-DNP (□), 1.27 (±0.09) × 10^6 M^{-1} and 2.22 (±0.10) × 10^5 sites/cell.

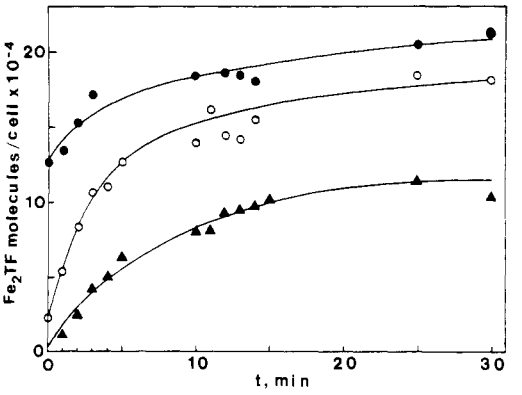

FIGURE 4: Progress curves for binding of rabbit Fe_2–[^{125}I]Tf to rabbit reticulocytes of cells stripped of endogenous Tf (●), cells preloaded with 20 μM rabbit Fe_2–Tf (▲), and control cells (○) under 95% air/5% CO_2 at 37 °C. Control cells were held on ice during pretreatment of other cells. Fe_2[^{125}I]Tf was 20 μM.

because of dilution of the radiolabeled with nonradiolabeled Fe_2–Tf. Conversely, cells stripped of their endogenous transferrin by four sequential incubations at 37 °C in fresh changes of 150 mM NaCl, 50 mM sodium phosphate, and 20 mM $NaHCO_3$, under 95% air/5% CO_2, pH 7.4, showed a marked increase in the extent of the fast adsorption phase, a decrease in the extent of the approach to equilibrium phase, and an increase in the final level of equilibrium binding of Fe_2–[^{125}I]Tf. Similar results were obtained in experiments with chick reticulocytes and Fe_2–[^{125}I]OTf (Christensen et al., 1980).

Figure 5 represents the kinetics of a dual label uptake and chase study with chick reticulocytes. On addition of Fe_2–[^{125}I]OTf the binding profile was followed for 10 min. At the end of the uptake experiment the remaining cells from the sample were diluted with 4 volumes of ice-cold EGA, centrifuged, washed once with cold EGA, and resuspended in cold EGAB, pH 7.4. To the cold suspension was added Fe_2–[^{131}I]OTf. The mixture was replaced in the 37 °C shaking incubator, and sampling was continued for 15 min. Counts on the ^{125}I channel were corrected for cross talk from the ^{131}I, and Fe_2–OTf/cell ratios were calculated for each labeled species. The lines (Figure 5) are nonlinear least-squares fits for the data presented in the figure. The values of the apparent first-order rate constant, k, for binding of Fe_2–[^{125}I]OTf, re-

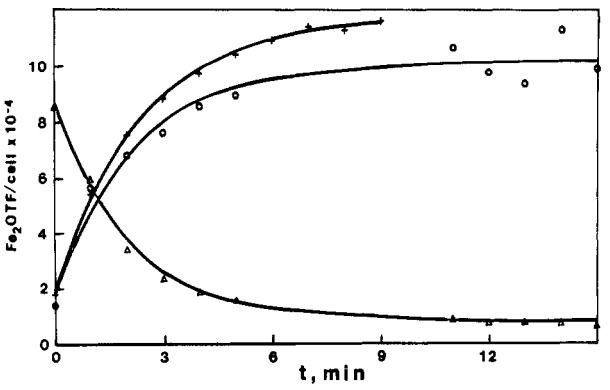

FIGURE 5: Progress curves for initial binding of Fe_2–[^{125}I]OTf to chick reticulocytes (+) and release of Fe_2–[^{125}I]OTf (Δ) with concomitant binding of Fe_2–[^{131}I]OTf (O) under 95% air/5% CO_2 at 37 °C. Calculated first-order rate constants (±SD) are as follows: Fe_2–[^{125}]IOTf binding (+), 0.42 (±0.02) min^{-1}; Fe_2–[^{125}I]OTf release (Δ), 0.49 (±0.03) min^{-1}; Fe_2–[^{131}I]OTf binding (O), 0.46 (±0.08) min^{-1}.

lease of bound Fe_2–[^{125}I]OTf, and concomitant binding of Fe_2–[^{131}]OTf are statistically similar (Figure 5).

Rates of the approach to equilibrium phase of Fe_2–[^{125}I]OTf binding to chick reticulocytes were measured as a function of Fe_2–[^{125}I]OTf concentration (1, 2, and 3 μM). No significant differences were found among the rate constants (data not shown). Thus the concentration of free Fe_2–OTf apparently has no effect on this rate process.

In binding experiments of Fe_2–[^{125}I]OTf to chick reticulocytes at 4 °C, only the fast adsorption phase of binding was observed, as reported for rabbit reticulocytes by Baker & Morgan (1969).

The effect of the highly specific microtuble inhibitor nocodazole (De Brabander et al., 1976) and less specific inhibitor vinblastine on Fe_2–OTf binding and iron uptake by chick reticulocytes is shown in Figure 6. The progress curves show no inhibition by nocodazole of either Fe_2–OTf binding or iron uptake by the cells in spite of the fact that concentrations of nocodazole up to 10^4 times greater than those found to be effective in cultured fibroblasts were used (De Brabander et al., 1976). Similar results were found in the rabbit reticulocyte system (data not shown). On the other hand vinblastine strongly inhibited iron uptake and decreased both the rate and extent of binding of Fe_2–OTf.

Discussion

From the results presented previously (Williams & Woodworth, 1973) and in this paper we find the chick reticulocyte–ovotransferrin system to be analogous to the rabbit reticulocyte–transferrin system and therefore omit the distinction between them in most of the discussion.

Aerobic and anaerobic reticulocytes, as well as those inhibited by 2,4-DNP, are capable of binding diferric transferrin with virtually the same progress profile and to nearly the same extent (Figures 1–3) and with the same association constant (Figure 3). Although 2,4-dinitrophenol effectively inhibits iron uptake by reticulocytes (Figure 2; Morgan, 1964), it has virtually no effect on the association constant and number of receptors (Figure 3) or on the progress profile for binding of diferric transferrin to reticulocytes (Figure 2; Morgan, 1964). Although we find no effect of anaerobiasis on the rate constants for binding or release of diferric transferrin by reticulocytes, iron uptake ceases under anaerobic conditions (Morgan & Baker, 1969; Williams & Woodworth, 1973). From these various pieces of evidence we conclude that although the monotonic uptake of iron by reticulocytes is dependent on

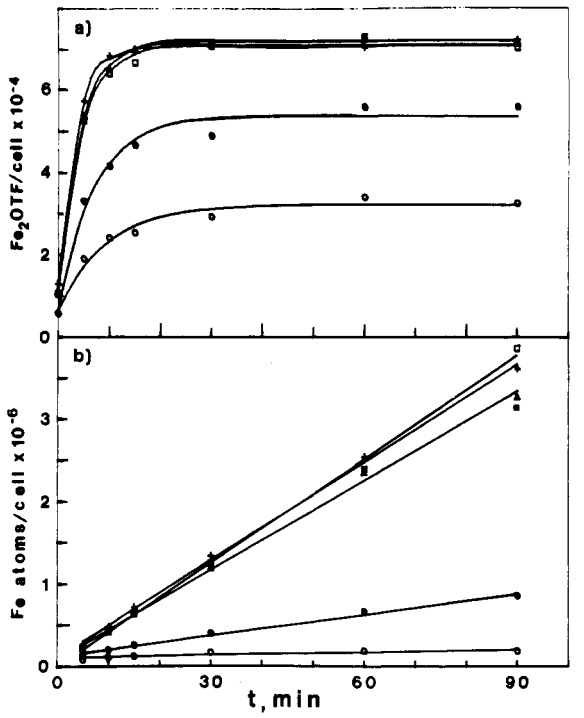

FIGURE 6: Effect of microtubule inhibitors on progress curves for (a) binding of diferric ovotransferrin and (b) uptake of iron by chick reticulocytes. All cells were preincubated with or without inhibitor for 30 min at 37 °C under 95% air/5% CO_2 prior to addition of $^{59}Fe_2$–[^{125}I]OTf. Calculated first-order rate constants (±SD) for Fe_2–OTf binding are as follows: control (+), 0.29 (±0.01) min^{-1}; 1.0 mM nocodazole (▲), 0.23 (±0.01) min^{-1}; 0.10 mM nocodazole (□), 0.23 (±0.02) min^{-1}; 1.0 mM vinblastine (O), 0.11 (±0.02) min^{-1}; 0.50 mM vinblastine (●), 0.14 (±0.02) min^{-1}. Calculated rates (±SD) for iron uptake are as follows: control (+), 4.0 (±0.1) × 10^4 Fe atoms/(cell·min); 1.0 mM nocodazole (▲), 3.6 (±0.1) × 10^4 Fe atoms/(cell·min); 0.10 mM nocodazole (□), 4.2 (±0.1) × 10^4 Fe atoms/(cell·min); 1.0 mM vinblastine (O), 0.11 (±0.03) × 10^4 Fe atoms/(cell·min); 0.50 mM vinblastine (●), 0.85 (±0.04) × 10^4 Fe atoms/(cell·min).

oxidative metabolism, the binding of diferric transferrin to these cells is not similarly dependent.

The "instantaneous" adsorption phase of binding between reticulocytes and diferric transferrin is variable in extent depending on whether the cells have been preloaded with nonradiolabeled diferric transferrin or stripped to some degree of the endogenous transferrin carried over from the animal during isolation and washing (Figure 4). The control curve for washed cells held on ice shows a high degree of carry-over of endogenous transferrin in this experiment. The role of endogenously bound transferrin in iron uptake from low molecular weight chelates by reticulocytes and the difficulty of removing this transferrin from the cells have been well documented (Hemmaplardh & Morgan, 1974). We conclude that the adsorption phase simply reflects the rapid binding of transferrin to nonfilled transferrin receptors on the reticulocyte membrane.

The slow phase proceeding to binding equilibrium is interpreted in terms of the data presented in Figures 1 and 5. The data as presented in these figures have been fitted to an exponential (first-order) function by a nonlinear least-squares program (Ralston, 1979), which estimates the initial (zero-time) and final (infinite-time) values of the dependent variable as well as the first-order rate constant. Data for the initial uptake of diferric [^{125}I]transferrin and its release with concomitant diferric [^{131}I]transferrin binding yielded first-order rate constants that are the same within experimental error; notice that these progress curves (Figure 5) show a mirror

image relationship. Because these experiments involve isotope exchange, the overall rate process is expected to be first order irrespective of the detailed molecular mechanism (Moore & Pearson, 1981). It is apparent that the rate of binding in the progressive phase is the same as, and is therefore controlled by, the rate of release of already bound transferrin. If the cells are preloaded with nonradiolabeled diferric transferrin, the entire binding profile becomes progressive; and if the endogenous transferrin is largely stripped away, the progressive phase is much reduced in extent (Figure 4). The final extent of equilibrium binding of the radiolabeled protein reflects the relative amount of nonlabeled protein added to the cells (preloading) or endogenously bound to the cells (control). We interpret these results as follows: in the progressive (approach to equilibrium) phase of binding, receptors are occupied by nonlabeled transferrin that must first leave (desorb) in order for labeled transferrin molecules to bind to these receptors. Therefore, the rate of progressive uptake is determined by the rate of desorption. The lack of effect of anaerobiasis on the desorption rate suggests that transfer of iron to the cell is not required for release of transferrin from the cell; i.e., diferric transferrin is released (see below for further elaboration).

If the progressive uptake phase represented the reaction between transferrin and open receptors, the rate constant ought to be affected by the concentration of transferrin free in solution, as assumed by Baker & Morgan (1969). An especially sensitive range should be at concentrations of free transferrin below that for half-saturation of reticulocyte receptors, e.g., below ca. 10 μM. Yet at initial concentrations of Fe_2–[^{125}I]OTf of 1, 2, and 3 μM, we found identical rate constants within experimental error. On the other hand, if the binding of labeled transferrin depends on a nonlabeled transferrin molecule vacating its receptor (a first-order process, as found here and as reported by Baker & Morgan, 1969) and refilling that receptor is very rapid relative to the rate of transferrin release, then the binding rate is not properly viewed as a second-order process but as an overall first-order process; i.e., in all cases the rate of progressive uptake (approach to equilibrium) is actually the rate of desorption of transferrin from already occupied receptors, and the filling of the vacant receptors with labeled transferrin is much faster. Our data support the following summary of reactions:

$$Fe_2\text{–Tf–cell} \xrightarrow[\text{rate limiting for (2)}]{\text{first order}} Fe_2\text{–Tf} + \text{cell} \qquad (1)$$

$$Fe_2\text{–*Tf} + \text{cell} \xrightarrow[\text{very rapid}]{\text{presumed second order}} Fe_2\text{–*Tf–cell} \qquad (2)$$

$$Fe_2\text{–Tf–cell} \xrightarrow[\text{slower than (1)}]{\text{zero order}} \text{Tf–cell–(Fe)} \qquad (3)$$

Taken together, eq 1 and 2 comprise the isotope exchange condition at chemical equilibrium.

If one takes as a reasonable value for desorption of transferrin from the cell a first-order dissociation constant of 0.35 min^{-1} (Figures 1, 5, and 6), the average lifetime for a transferrin molecule on its receptor is 2.8 min. If we take for the binding constant a value of 1.27×10^6 M^{-1} (Figure 3), then the apparent second-order binding rate constant of free diferric transferrin to unoccupied receptors can be calculated to be 0.44×10^6 min^{-1} M^{-1}. This calculation, of course, assumes that the cellular transferrin receptors can be considered to be free in solution; on the contrary, they are a component of a surface. If we take as reasonable values 2×10^5 transferrin receptors per cell (Figure 3) and an iron uptake rate of 4×10^4 atoms of Fe/(cell·min) (Figure 6) and assume that all receptors are equally effective in removing iron from diferric transferrin, then the average time required for removal of one iron by the cell is 10 min. If we further assume that the iron atoms are removed in pairs from diferric transferrin (Williams & Woodworth, 1973; Groen et al., 1982), then the average residence time for a diferric transferrin molecule to give up both irons would be 20 min. Thus a given receptor could turn over its bound transferrin 4–7 times for each actual transfer of iron to the cell. This again shows that iron removal from transferrin is not requisite for release of transferrin from its receptor and that diferric transferrin is in "prior equilibrium" with its receptors. To the extent that iron is transferred to the cell, the "equilibrium constant" calculated here takes on the nature of the reciprocal of K_m in an enzymatic reaction. Note, however, that neither anaerobiasis nor 2,4-DNP (conditions preventing iron removal from diferric transferrin by reticulocytes) affected the apparent equilibrium constant (Figure 3).

The temperature dependence of the progressive binding phase need not depend on the relative metabolic activity of the cells. Morgan (1964; Baker & Morgan, 1969) noted a 2–3-fold change in binding rate for each 10 °C change in temperature between 40 and 15 °C. Such a change is quite consistent with the common 2–3-fold change in reaction rate per 10 °C change in temperature. Also, the disappearance of progressive uptake at 4 °C might well arise because the membrane lipids are below the fluid transition temperature (Shimshick & McConnell, 1973). The temperature dependence then may well reflect the change in the desorption rate of already bound transferrin.

Although the microtubule inhibitor vinblastine has been reported to inhibit iron uptake by reticulocytes (Hemmaplardh et al., 1974), we find no effect of the more specific microtubule inhibitor, nocodazole, on either iron uptake or transferrin binding by reticulocytes, although vinblastine inhibits both effects (Figure 6). Furthermore, although nocodazole causes morphological changes in rat L-6 myocytes in culture, it shows no effect on binding of rat Fe_2–Tf or iron uptake by these cells (R. A. Fava, personal communication).

In summary, we conclude that the binding and release of transferrin by reticulocytes can be viewed as a specific adsorption–desorption process. The initial fast adsorption reflects very rapid binding to open membrane receptors, whereas the progressive approach to equilibrium is dependent on release of endogenous transferrin from occupied receptors and is not dependent on active metabolism. Endocytosis of the bound transferrin–receptor complex (Baker & Morgan, 1969; Paterson & Morgan, 1980) need not be invoked to explain the binding progress profile.

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Influence of Cholesterol on the Rotation and Self-Association of Band 3 in the Human Erythrocyte Membrane†

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ABSTRACT: The cholesterol/phospholipid mole ratio (C/P) in the human erythrocyte membrane was varied by incubating cells with liposomes. The rotational mobility of band 3 proteins was measured in these membranes by observing flash-induced transient dichroism of the triplet probe eosin maleimide. Measurements were performed with membranes in which associations of band 3 with cytoskeletal proteins were removed by mild proteolysis with trypsin. It was found that decreasing C/P resulted in a more rapid decay of the flash-induced anisotropy. The anisotropy decay curves were analyzed by curve-fitting procedures, which indicated the existence of different sized small aggregates of band 3. The changes in the decay curves with varying C/P can be explained by an effect of cholesterol on the size distribution of these aggregates. The experiments suggest a possible role of cholesterol in regulating associations between integral membrane proteins.

The effect of cholesterol on the molecular motions of the hydrocarbon chains of lipids in bilayers and cell membranes is well documented (Oldfield & Chapman, 1972; Vanderkooi et al., 1974; Cooper et al., 1978; Kawato et al., 1978). Above the gel to liquid-crystalline phase transition, lipid-soluble probes report a decrease in "fluidity" in the presence of cholesterol. From time-resolved fluorescence depolarization measurements with the probe diphenylhexatriene, Kawato et al. (1978) deduced that the effect of cholesterol (above the phase transition) is principally to change the amplitude of the chain motions rather than their rate. Exactly how such changes in chain motions might affect the mobility of membrane proteins is by no means clear. However, it was rather surprising that in a previous study, no change in band 3 rotational mobility in the human erythrocyte membrane could be detected when the cholesterol/phospholipid mole ratio (C/P)[1] was varied from 0.34 to 1.66 (Nigg & Cherry, 1979). In these experiments, band 3 was selectively labeled with the triplet probe eosin maleimide. Rotation was measured by observing flash-induced transient dichroism of the eosin probe. Other measurements of band 3 rotational mobility have been made by Austin et al. (1979) and Johnson & Garland (1981).

It is well established that band 3 can be selectively cleaved with different proteases (Steck et al., 1978). Mild proteolysis of erythrocyte ghosts with trypsin releases a 41 000-dalton cytoplasmic fragment of band 3. It has been shown that there is a concomitant enhancement of the rotational mobility of the remaining 55 000-dalton membrane-associated fragment (Nigg & Cherry, 1980). A detailed analysis of these and other experiments led to the conclusion that about 40% of band 3 molecules have a restricted mobility due to association of the cytoplasmic moiety with cytoskeletal proteins. This finding suggests a possible explanation of the earlier failure to detect changes in band 3 rotation with varying C/P. The rotation of the fraction of band 3 that associates with cytoskeletal proteins is probably determined by protein–protein interactions and hence is insensitive to changes in the composition of the lipid bilayer. Changes in the rotational mobility of the remaining band 3 with varying C/P may be difficult to detect, since resolution of the two fractions by multiexponential curve fitting of the anisotropy decay curves is not particularly accurate.

To test whether the above explanation is correct, we have investigated the effect of varying C/P on band 3 rotation in membranes treated with trypsin. Cleavage of the cytoplasmic moiety releases those band 3 molecules that are associated with cytoskeletal proteins (Nigg & Cherry, 1980). Hence, more of band 3 (strictly, the 55 000-dalton membrane-associated fragment of band 3) becomes potentially sensitive to changes in membrane fluidity, and the test for an effect due to varying C/P is correspondingly more sensitive.

Materials and Methods

Fresh human blood (O^+) was centrifuged, some of the plasma to be used for the lipid dispersions was stored at 4 °C, and the erythrocytes were washed and labeled with eosin maleimide as described by Nigg & Cherry (1979). As shown

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[1] Abbreviation: C/P, cholesterol/phospholipid mole ratio.

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