The Kinetics of the Interaction between Rabbit Transferrin and Reticulocytes*

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ABSTRACT: Kinetic characteristics of the interaction between transferrin and reticulocytes have been studied using purified iron-saturated rabbit transferrin and rabbit reticulocytes. The effects of varying transferrin concentration, reticulocyte concentration, and temperature on rate constants for the association and dissociation reactions were measured. The activation energies required for the association and dissociation reactions (10.8 and 12.0 kcal mole^{-1}, respectively) were not significantly different. The number of specific reticulocyte binding sites for transferrin was estimated to be about 300,000 sites/reticulocyte. The average equilibrium constant was 200,000 l. mole^{-1} and the average standard free-energy change for the reaction was -7.3 kcal mole^{-1}. The standard enthalpy change was negligibly small, but there was a considerable increase in entropy for the reaction. These results may be interpreted as suggesting that the reticulocyte has available a limited number of binding sites specific for transferrin and bind transferrin by several weak bonds.

The transfer of iron into cells from the plasma iron binding protein, transferrin, has been studied in detail only with reticulocytes (Walsh *et al.*, 1949; Paoletti and Durand, 1958; Jandl *et al.*, 1959; Jandl and Katz, 1963; Morgan and Laurell, 1963). The mechanism of iron uptake by these cells may involve several separate reactions. It has been shown that the initial stages of this process involve the uptake of transferrin by the cells (Jandl and Katz, 1963; Morgan and Laurell, 1963). This may take place in two steps: firstly, the formation of an initial union between protein and cell, probably by a process of physical adsorption, and secondly a firmer binding by a process which may be dependent upon the active metabolism of the cell (Morgan, 1964). Iron is then transferred to cellular receptors by a reaction which requires cellular energy, after which the transferrin is released from the cell. The precise biochemical mechanisms involved at each stage of the iron transfer process are not known.

This paper reports the results of a kinetic analysis of the interaction between transferrin and reticulocytes. The investigation was performed using rabbit reticulocytes and purified rabbit transferrin labeled with radioactive iodine. The process of transferrin uptake by, and of transferrin release from the cells were both studied.

Experimental Procedure

Materials

Reticulocytes. Reticulocytosis was induced in adult rabbits by the repeated removal of approximately 15 ml of blood/kg body weight at 2–3-day intervals by cardiac puncture or incision of the marginal ear vein.

Transferrin. Transferrin was isolated from rabbit plasma using ammonium sulfate fractionation and column chromatography on DEAE-Sephadex (Baker *et al.*, 1968). The protein was maintained in the iron-saturated form at 4° at all stages during preparation, storage and labeling with radioactive iodine.

Radioisotopes. Iodine-131 and iodine-125, carrier-free and free from reducing agents, were purchased from the Radiochemical Centre, Amersham, England.

Methods

Isotope Labeling. The pure iron-saturated transferrin was labeled with iodine-125 or iodine-131 by the iodine monochloride method (McFarlane, 1958). Nonprotein-bound iodine was removed by passage of the protein through a column of the anion-exchange resin Amberlite IRA-410, followed by dialysis against Hanks and Wallace's balanced salt solution (Hanks and Wallace, 1949).

Analytical Methods. The purity of the isolated transferrin preparation was evaluated by cellulose acetate and starch gel electrophoresis, and by examination of its absorption spectrum in the visible region. Iron concentration and total iron binding capacity were measured by a microadaptation of the method of Morgan and Carter (1960). Transferrin concentration was estimated from the total iron binding capacity assuming a molecular weight of 75,000 (Baker *et al.*, 1968) and the binding of two iron atoms per molecule, or measured using a biuret reagent (Kingsley, 1942).

The proportion of reticulocytes present in the blood samples was determined by the visual counting of one thousand cells on blood smears made after staining with new methylene blue. Hematocrits were determined

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by the microhematocrit method using heat sealed capillary tubes (Clay-Adams, Inc., New York) and a Hawksley microhematocrit centrifuge. Red blood cell counts were performed with a Model A Coulter electronic particle counter.

Radioactivity was measured in a well-type scintillation detector. When two different isotopes were present in the one sample they were differentiated using a pulse height analyzer.

Incubation Procedure. Reticulocyte-rich blood was collected in heparinized tubes and washed twice in ice-cold buffer. Hanks and Wallace's solution was used at all stages of incubation and washing of cells. No attempt was made to separate reticulocytes from mature cells as it has been shown that transferrin and iron uptake can be attributed almost entirely to reticulocytes at physiological transferrin concentrations (Paoletti and Durand, 1958; Jandl *et al.*, 1959; Jandl and Katz, 1963). The reticulocyte-rich blood will, for brevity, be referred to as reticulocytes.

To measure the uptake of transferrin by reticulocytes, a known volume of washed cells was mixed with the labeled protein and incubated with constant gentle shaking under an atmosphere of 5% CO_2 and 95% O_2. Samples were removed at various time intervals after admixture and the uptake reaction quickly terminated by cooling the cells in tubes containing ice-cold buffer. The samples were centrifuged at 4° and the supernatant was removed by suction. The cells were then washed three times in about 100 volumes of ice-cold buffer to remove unbound transferrin. Transferrin bound to the reticulocytes was estimated from the radioactivity in the washed cells and the specific activity of the incubation medium.

To measure the characteristics of the release process in which bound transferrin dissociates from the cell, the cells and protein were first incubated for 30 min at 37°. The reticulocytes were then washed free of unbound transferrin at 4°, and aliquots of the cell suspensions were pipetted into a series of tubes at 4°. Buffer at the required temperature was then added to give a hematocrit of about 35%, and the mixtures were incubated at the same temperature for different time periods of up to 3 min. The reaction was terminated in these samples by mixing them in centrifuge tubes with a large volume of ice-cold buffer, centrifuging at 4°, and measuring the radioactivity in cell and supernatant layers. The proportion of bound transferrin which had dissociated from the reticulocytes during incubation could then be calculated from the amount of radioactivity bound to the cells and that present in the supernatant solution.

Results

Uptake of Transferrin by Reticulocytes. The uptake of transferrin by reticulocytes at 37° is shown in Figure 1. The amount of transferrin bound immediately after mixing represents adsorbed transferrin (Morgan, 1964). Following this initial binding there was a progressive uptake of labeled protein, maximum values being obtained at equilibrium, approximately 30 min after admixture. The initial binding will be termed adsorption and the progressive uptake of transferrin will be called the association reaction.

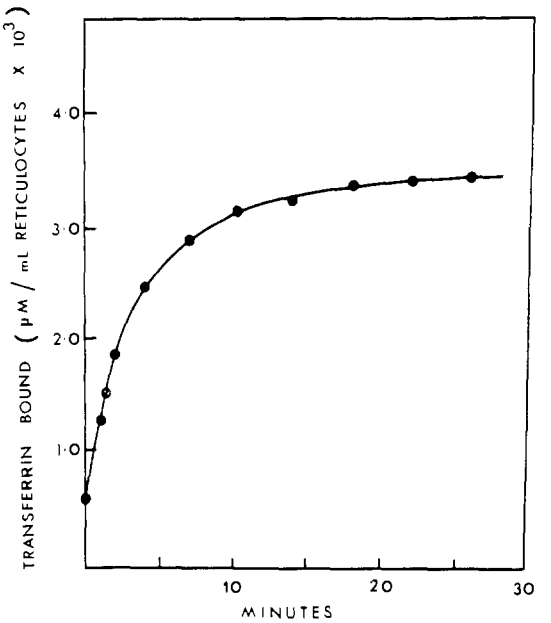

FIGURE 1: Effect of the time of incubation on the uptake of transferrin by reticulocytes at 37°. The transferrin concentration was 0.03 mM.

Determination of Specific Rate Constants. Attempts to quantitate the kinetics of the adsorption process were unsuccessful because of the extremely rapid nature of this reaction in the temperature range studied (0–40°). However, because the adsorption was not rate limiting, the kinetics of the association reaction could be measured in the type of experiment illustrated in Figure 1. The rate of the association reaction was estimated from the initial uptake of transferrin before the complication of subsequent release of bound transferrin. Samples were taken at approximately 15-sec intervals for two minutes after the admixture of cells and protein at 37°. As illustrated in Figure 2A, there was a linear relationship between the logarithm of the concentration of free transferrin and the time of incubation. The pseudo-first-order association rate constant, k_1, was evaluated from the slope of the straight line obtained.

The second-order association rate constant was evaluated in several experiments where simultaneous determinations were made of the concentration of reticulocyte binding sites and the rate of transferrin uptake in the initial stages of the reaction. The average value obtained in three experiments was 2.3×10^7 l. mole^{-1} sec^{-1}, the range being 4.1×10^6–5.0×10^7 l. mole^{-1} sec^{-1}. Because of the difficulty of making all the cell measurements on each cell population, the pseudo-first-order rate constant was used to evaluate the kinetic characteristics of the uptake reaction under different experimental conditions, employing the commonly made assumption that initial velocity is directly proportional to the specific rate constant.

The kinetics of the dissociation reaction were studied

TABLE I: Kinetic Characteristics of the Interaction between Transferrin and Reticulocytes.[a]

	First-Order Rate Constant (sec^{-1})	Activation Energy (kcal $mole^{-1}$)
Association reaction	$0.565 \times 10^{-4} \pm 0.028 \times 10^{-4}$ ($n = 8$)	10.8 ± 1.2 ($n = 4$)
Dissociation reaction	$12.1 \times 10^{-4} \pm 0.8 \times 10^{-4}$ ($n = 6$)	12.0 ± 1.1 ($n = 3$)

[a] Parameters were estimated using transferrin in the concentration range 0.04–0.09 mM and blood containing 14–20% reticulocytes. Results are presented as mean plus and minus standard deviation. The number of cell populations analyzed is given in parentheses.

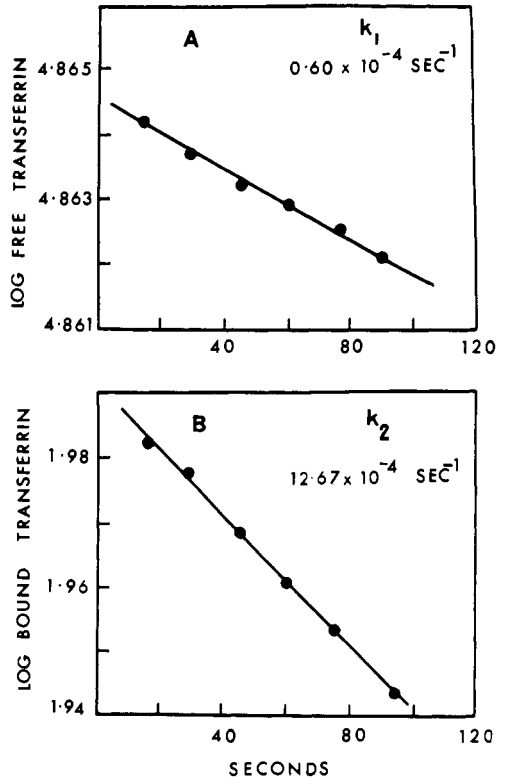

FIGURE 2: Logarithmic plots. (A) Of initial rate of decrease in free transferrin concentration (estimated as the difference between the total cpm per sample and the bound cpm per sample) for the association reaction. (B) Of the rate of decrease in the concentration of bound transferrin during the dissociation reaction. Rate constants were determined from the slope of the lines of best fit.

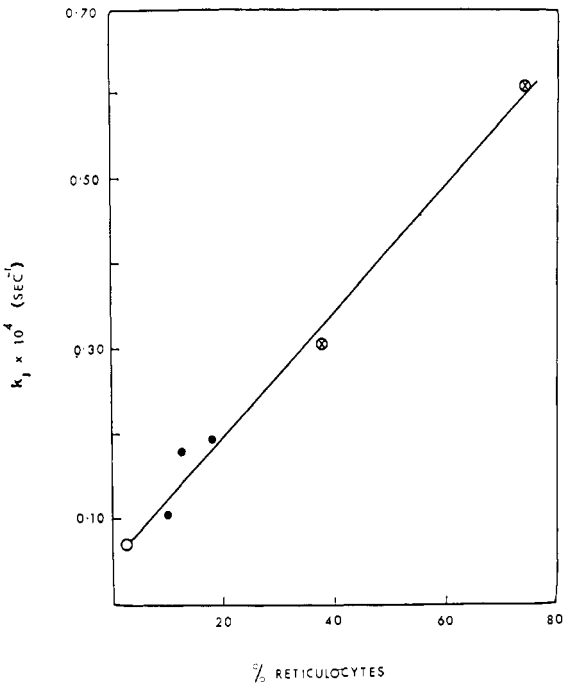

FIGURE 3: The association rate constant as a function of reticulocyte concentration. Measurements were made with 0.050 mM transferrin using reticulocytes obtained after repeated blood withdrawal (●) and with 0.065 mM transferrin using reticulocytes obtained in higher concentration from a rabbit injected daily for 5 days with 6 mg/kg of phenylhydrazine hydrochloride (⊗). Serial dilution of the reticulocyte-rich blood was made with erythrocytes from an untreated rabbit (○).

in a similar manner. The reticulocytes and transferrin were equilibrated at 37°, washed free of unbound transferrin at 4°, and reincubated in Hanks and Wallace's solution at 37°. As shown in Figure 2B the dissociation reaction followed a first-order kinetics law and the specific dissociation rate constant, k_2, was calculated from the slope of the linear relationship between the log of the bound transferrin concentration and the time of reincubation.

The kinetic constants were calculated from the raw data (presented as counts per minute of free transferrin per sample, for the association reaction; and as percentage of total transferrin which was bound to the cells, per sample, for the dissociation reaction) by a digital computer using a modified program for linear regressions. Weighted means and standard deviations were derived from the common regressions. A summary of the data obtained on the kinetics of the interaction of transferrin and several reticulocyte populations at 37° is shown in Table I.

Effect on k_1 of Alteration in Reticulocyte Concentration. The reticulocyte concentration in the incubation medium was altered while maintaining a constant packed-cell volume by serial dilution of the reticulocytes with mature red blood cells using blood containing a low proportion of reticulocytes (2.5%) obtained from a normal unbled rabbit. As shown in Figure 3, the value of the association rate constant was directly proportional to the concentration of reticulocytes in the incubation medium.

Effect on k_1 of Alteration in Transferrin Concentration. Equal volumes of cells were mixed in replicate systems

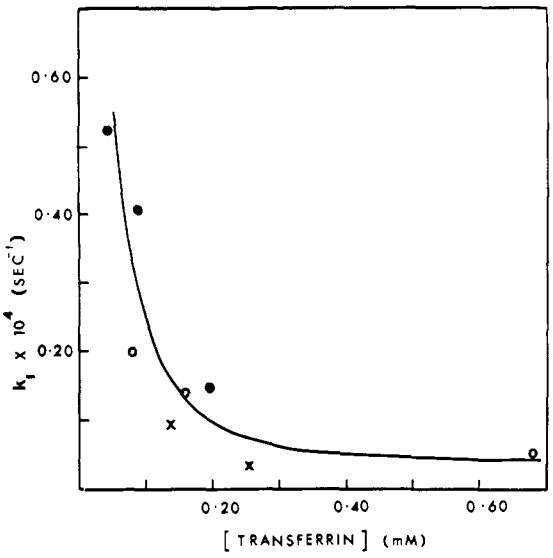

FIGURE 4: The association rate constant as a function of transferrin concentration. Measurements were made in three cell populations with reticulocyte concentrations of 20% (●), 16%, (○) and 17% (×).

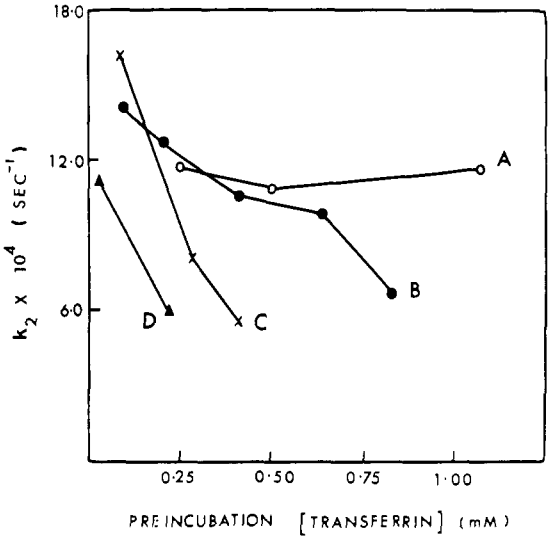

FIGURE 5: The dissociation rate constant as a function of preincubation transferrin concentration. Measurements were made on four cell populations with reticulocyte concentrations of 19% (A), 22% (B), 19% (C) and 15% (D).

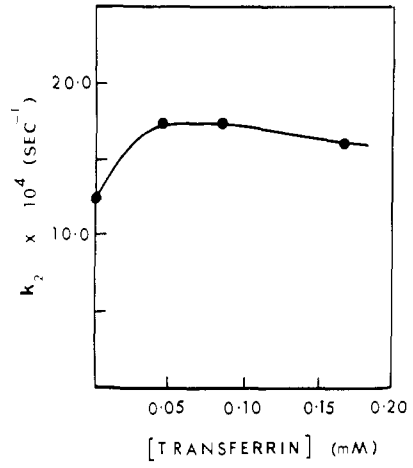

FIGURE 6: The dissociation rate constant as a function of the concentration of unlabeled transferrin in the reincubation medium. The reticulocyte concentration was 22%.

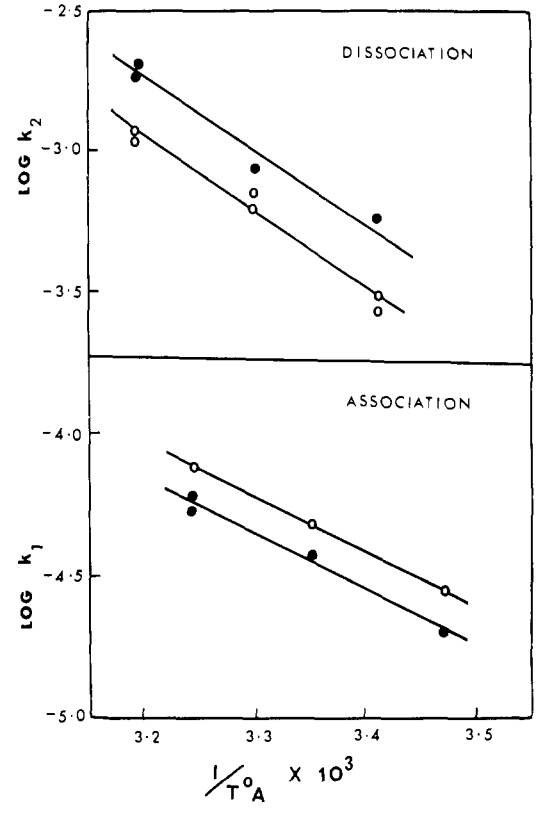

FIGURE 7: Arrhenius plots showing the effect of temperature on the rate constant for the association reaction and the dissociation reaction. The results are shown for measurements on four cell populations.

which differed only in transferrin concentration. The value of the specific association rate constant varied inversely with the concentration of transferrin in the incubation medium (Figure 4). The rate constant is maximal at low transferrin concentrations similar to those found in blood plasma.

Effect on k_2 of Alteration in Preincubation Transferrin Concentration. Equal volumes of reticulocytes were equilibrated in replicate systems which differed only in transferrin concentration. The cells were then washed and the specific dissociation rate constant was measured during incubation in an unlabeled medium. In one experiment the dissociation rate constant was not significantly altered when the preincubation transferrin concentration was varied between 0.25 and 1.0 mM (Figure 5, curve A). In three other experiments, using different cell populations, the dissociation rate constant was found to be inversely proportional to the transferrin concentration in the preincubation medium, which was varied between 0.02 and 0.80 mM (Figure 5, curves B–D).

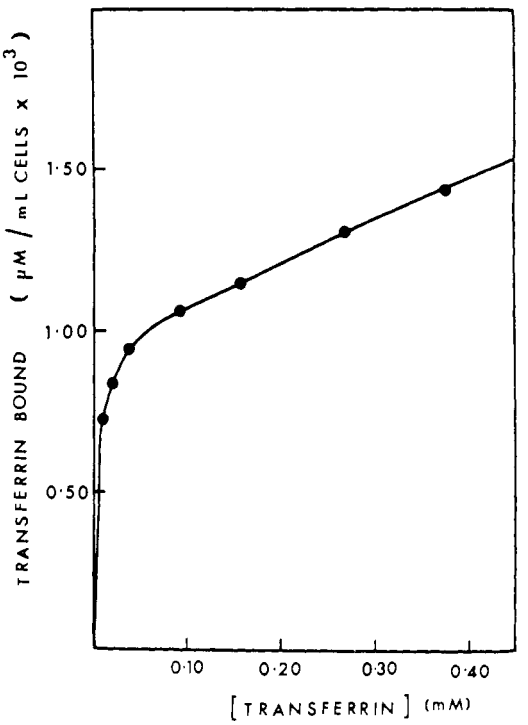

FIGURE 8: The uptake of transferrin at equilibrium as a function of initial transferrin concentration. Bound transferrin was calculated as micromoles of transferrin bound per milliliter of erythrocytes at 37°.

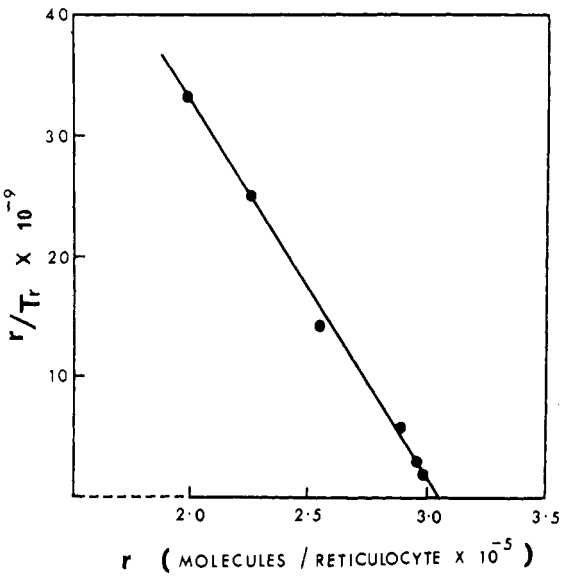

FIGURE 9: Transferrin binding by reticulocytes plotted according to the Scatchard eq 1. Extrapolation to the abcissa gives *n*, the number of specific transferrin binding sites per reticulocyte.

TABLE II: Transferrin Binding Characteristics of Rabbit Reticulocytes Measured at 37°.

n (no. of binding sites/reticulocyte)	*K* (l. mole^{-1})	$\Delta F°$ (kcal mole^{-1})
200,000	125,000	−7.24
200,000	164,000	−7.40
267,000	75,000	−6.92
270,000	49,000	−6.64
344,000	300,000	−7.70
495,000	590,000	−8.19
560,000	143,000	−7.30
Mean 334,000	207,000	−7.34

Effect on k_2 of Alteration in Transferrin Concentration in the Reincubation Medium. The rate of dissociation of transferrin from reticulocytes was measured in the presence of unlabeled transferrin in the concentration range 0–0.2 mM. The specific dissociation rate constant was slightly increased in the presence of unlabeled transferrin in the reincubation medium, as shown in Figure 6.

Determination of Activation Energies. The association and dissociation reactions were markedly temperature dependent. The rate constants were measured at temperatures varying from 15 to 40°. For the association reaction the cells and incubation mixture were equilibrated at the appropriate temperature before admixture. The dissociation reaction was started by diluting the transferrin–reticulocyte complex with buffer preincubated at the required temperature. The energies of activation of the association and dissociation reactions were estimated from the Arrhenius relationship between the log of the rate constant and the reciprocal of the absolute temperature. The curves appeared linear between 15 and 40° (Figure 7) and the activation energies were estimated from the line of best fit for each experiment. A summary of the results is included in Table I. The activation energies for the association and dissociation reactions were not significantly different.

Estimation of Number of Reticulocyte Binding Sites for Transferrin. To further quantitate the nature of the transferrin–reticulocyte interaction, the number of transferrin binding sites on the reticulocyte membrane was estimated from the maximum number of transferrin molecules which combine with a reticulocyte. The uptake reaction between transferrin and reticulocytes is reversible, so that at equilibrium the amounts of bound and free transferrin are given by the law of mass action. The amount of transferrin which would be taken up if all the transferrin sites were saturated could therefore be estimated using Scatchard's (1949) method of analysis of the law of mass action. The assumption was made that the transferrin molecule and the binding site are univalent in this reaction.

Samples containing equal volumes of reticulocytes were incubated to equilibrium at 37° with different concentrations of transferrin. The cells were then washed in ice-cold buffer, and the amount of transferrin taken up by each cell sample was measured. Figure 8 shows the relationship obtained between transferrin concentration and uptake at 37°. Transferrin uptake showed two distinct phases: an initial steep increase in transferrin bound with increase in transferrin con-

TABLE III: Thermodynamic Constants for the Reaction between Transferrin and Reticulocytes.

Temp (°C)	n (no. of binding sites/reticulocyte)	K (l. mole^{-1})	$\Delta F°$ (kcal mole^{-1})	$\Delta H°$ (kcal mole^{-1})	$\Delta S°$ (cal deg mole^{-1})
37	495,000 ± 24,000[a]	590,000 ± 70,000	−8.19 ± 0.69	+1.5	+31
24	360,000 ± 22,000	513,000 ± 68,000	−7.76 ± 0.66		
6	190,000 ± 21,000	460,000 ± 64,000	−7.23 ± 0.61		

[a] Standard error.

centration in the physiological range of about 0–0.05 mM transferrin, and a second more gradual increase in uptake with further increase in transferrin concentration. The latter phase was linear up to at least 0.7 mM transferrin. This type of binding curve is characteristic of an interaction involving two sorts of binding sites (Scatchard *et al.*, 1950).

The specificity of the two phases of transferrin uptake was investigated by comparing transferrin and albumin uptake at 37 and 4° with varying reticulocyte concentrations. A relationship similar to the second phase of transferrin uptake at 37° was obtained for transferrin and albumin uptake at 0° and this phase of uptake was not specific for reticulocytes. These results suggested that while the first phase represented a specific temperature dependent binding reaction between transferrin molecules and specific transferrin receptor sites on the reticulocyte membrane, the second phase represented a nonspecific adsorptive process, of a similar nature to that observed for albumin and γ-globulin on liver and lung (King, 1968) and red cells (Boursnell *et al.*, 1953; Pirofsky *et al.*, 1962; Grob *et al.*, 1967).

To estimate the number of specific transferrin binding sites on the reticulocyte membrane, a correction was made for nonspecific adsorption by extrapolation of the second phase back to the ordinate and subtraction of this component from the total uptake per cell sample. The corrected curve represented specific transferrin binding by reticulocytes. The number of molecules of transferrin bound per reticulocyte at each transferrin concentration was then estimated using the red cell count and the percentage of reticulocytes present. The equilibrium binding data were plotted according to the Scatchard equation

$$\frac{r}{(\mathrm{Tr})} = K(n - r) \qquad (1)$$

where r = number of transferrin molecules bound per reticulocyte; n = maximum number of molecules which can be bound per cell, which represents the number of reticulocyte binding sites; (Tr) = molar concentration of free transferrin; and K = association or equilibrium constant in l. mole^{-1}.

Figure 9 shows the Scatchard plot obtained in a typical experiment. After correction for adsorption, a linear plot was obtained, and extrapolation to the abscissa gave an estimate of n. The number of specific transferrin binding sites per reticulocyte was in the range of 200,000–560,000 in seven different cell populations. A summary of the results obtained is given in Table II. At an average physiological transferrin concentration of 0.04 mM, the specific transferrin binding sites are about 60% saturated with transferrin.

Determination of Equilibrium Constants. The equilibrium constant for the reaction between transferrin and reticulocytes was calculated from the slope of the Scatchard plot of $r/(\mathrm{Tr})$ against r, or by substitution of individual values of n, r, and (Tr) in eq 1. Plotting $r/(\mathrm{Tr})$ against r gives a straight line only if the reticulocyte binding sites are homogeneous with respect to K. The linear plots obtained indicate the homogeneity of the binding sites in individual cell populations. The variation of K between cell populations was considerable, however, the range in seven different cell populations being from 49,000 to 590,000. A summary of the results obtained is included in Table II. The equilibrium constant derived from the ratio of the mean second-order association rate constant and the mean dissociation rate constant was 10^9, differing by at least 10^3 from the values derived from the Scatchard equation.

Estimation of Thermodynamic Parameters. The standard free-energy change for the association reaction between transferrin and reticulocytes was estimated from the relationship

$$\Delta F° = -RT \ln K \qquad (2)$$

using experimentally determined values of K. The results from six experiments are included in Table II.

The equilibrium constant was estimated from the slope of Scatchard plots derived from the measurements of transferrin uptake at equilibrium at 6, 24, and 37°. The range of transferrin concentrations used was 0.004–0.500 mM. The standard enthalpy change for the reaction was derived using the van't Hoff relationship. The standard entropy change was estimated using the relationship

$$\Delta F° = \Delta H° - T\Delta S° \qquad (3)$$

The results from one cell population are summarized in Table III. The free-energy change for the reaction was about −8 kcal mole^{-1}, and the enthalpy change was positive by about 1 kcal mole^{-1}. The standard

entropy change was thus approximately 30 cal deg^{-1} $mole^{-1}$, suggesting an entropy driven reaction. The estimate of the number of specific binding sites per reticulocyte obtained by extrapolation of the Scatchard plots was markedly temperature dependent, varying from 190,000 sites/cell at 6° to 495,000 sites/cell at 37°.

Discussion

The reaction between transferrin and reticulocytes is generally assumed to involve specific receptor sites on the cell membrane. The data presented in this paper provide information on the kinetics and thermodynamics of this binding reaction, and on the number and nature of the binding sites.

The activation energy for the association reaction between transferrin and reticulocyte binding sites was estimated to be approximately 11 kcal $mole^{-1}$. This low level of activation energy approaches the value obtained in a diffusion-controlled reaction, and suggests that the reaction between transferrin and reticulocyte binding sites involves a weak type of binding, possibly due to a combination of weak forces such as hydrophobic or hydrogen bonds or van der Waal forces. The possibility of a covalent or ionic bonding cannot be discarded on the grounds of a low activation energy alone. When considered in conjunction with the relatively low change in standard free energy and enthalpy for the reaction, however, it seems more likely that a weaker type of bonding is involved. The mean activation energies for the association and dissociation reactions were not significantly different. However, the results may reflect the limits of accuracy of the method rather than true identity of the activation energies of the two processes.

The thermodynamic measurements showed that the change in standard free energy for the transferrin reticulocyte binding reaction was about -7 kcal $mole^{-1}$. This indicates that the reaction is spontaneous, but it is unlikely that the change in free energy is the driving force. This level of change in free energy is very much lower than that generally observed for a reaction involving the formation or scission of covalent or ionic bonds (Hughes-Jones, 1963). The negligible change in enthalpy for the reaction, and the considerable increase in entropy suggests an entropy-driven reaction. A negative change in entropy could be anticipated because of an increase in order in the system with binding of transferrin to cell sites. The discrepancy between the expected entropy change and the gain in entropy obtained in this system may be ascribed to the release of bound water molecules on complex formation (Haurowitz *et al.*, 1951), although reorientation of weak bonds such as hydrogen bonds within the transferrin molecule or binding site may be a contributing factor. These results suggest that the reaction is entropy driven and the small negative change in free energy, negligible enthalpy change, and increase in entropy together suggest that hydrophobic bonding may be involved in the reaction. In interpreting the results obtained, the assumptions have been made that the binding involves a single bimolecular interaction between transferrin and reticulocyte binding sites and that transferrin and reticulocytes are univalent with respect to their interaction. This may be an oversimplification. However, there is no evidence contrary to these assumptions and treatment of the data in this manner helps in the analysis of the reaction mechanisms.

There are at least two types of interaction between transferrin and reticulocytes, the adsorption reaction and the association reaction. It is also possible that the association reaction takes place in two or more steps, as described for several antigen–antibody reactions (Talmage, 1960; Lafferty and Oertelis, 1961). These factors may contribute to the wide range observed in the second-order association rate constant, apart from the intrinsic variability between individual reticulocyte populations and the experimental error incurred in the estimation of binding site number and rate of transferrin uptake. Likewise, the discrepancy between the value of the equilibrium constant calculated from the ratio of the second-order association rate constant and the dissociation rate constant and that determined from the slope of the Scatchard plot may be similarly explained.

The number of transferrin binding sites on each reticulocyte, n, was estimated in individual cell populations using Scatchard's derivation of the law of mass action. The results showed that transferrin reacted with two types of binding sites on the reticulocyte. A concentration-dependent uptake of transferrin occurred at a number of nonspecific adsorptive sites. These sites had a low affinity for transferrin and were heterogeneous with respect to this binding affinity (as calculated using Karush's derivation of the law of mass action, 1962). At a physiological temperature and transferrin concentration, transferrin was bound primarily by specific transferrin binding sites which, within each cell sample, were homogeneous with respect to their affinity for transferrin. It is clear from Table II, however, that there was a considerable range of variation in n and in K, the equilibrium constant, between different cell populations. This variation may reflect differences in the average cell age of these populations. As the reticulocyte matures it loses the ability to bind transferrin and take up iron for hemoglobin synthesis (Paoletti and Durand, 1958; Jandl *et al.*, 1959; Jandl and Katz, 1963). This loss in transferrin binding ability, whether as a result of alteration in membrane configuration, cell metabolism, or a combination of factors, should be reflected in n and in K, a measure of the affinity of these sites for transferrin.

At physiological transferrin concentrations the specific binding sites were about 60% saturated with transferrin, and almost completely saturated at concentrations greater than 0.1 mM. An inverse relationship was observed between k_1 and transferrin concentration. Provided the concentration of reticulocyte binding sites was kept constant, it would be anticipated for a bimolecular reaction, that the pseudo-first-order association rate constant would be independent of transferrin concentration. The rate of reaction between transferrin and the nonspecific sites was almost instantaneous, however, and the observed inverse relationship

between association rate constant and transferrin concentration may be due to blockage of the specific binding sites by nonspecific adsorption, reducing their effective concentration and hence lowering the estimated value of k_1. Negative interaction between occupied and unoccupied sites could also be a factor at high transferrin concentrations.

In three experiments an inverse relationship was also observed between the amount of transferrin bound and the rate constant for the dissociation reaction. In one experiment (Figure 5, curve A), variation in the preincubation transferrin concentration between 0.25 and 1.0 mM had no significant effect on k_2. The reason for this discrepancy is not known. An inverse relationship between the amount of transferrin bound and the dissociation rate constant could be due to a positive interaction between occupied sites, or to a difference in the location of the specific and nonspecific sites, the adsorbed transferrin blocking the release of transferrin from the specific binding sites which could be located deeper within the membrane. Alternatively, the specifically bound transferrin may be released at the end of a specific reaction sequence which depends on the concentration of some cellular component. With very large amounts of transferrin bound, the rate of release of the transferrin would be controlled by the concentration of this component. It was observed, however, that the dissociation rate constant was slightly increased in the presence of unlabeled transferrin in the reincubation medium. This would suggest that a part at least of the attached transferrin is more loosely bound and available for competitive exchange.

In several ways the interaction between transferrin and reticulocytes resembles antigen–antibody reactions. The concentration of binding sites, the second-order association rate constant, the equilibrium constants and thermodynamic values obtained for the transferrin–reticulocyte binding site reaction are similar to those obtained for some antigen-antibody reactions (Hughes-Jones, 1963; Talmage and Cann, 1961; Economidou *et al.*, 1967). In the case of antigen–antibody reactions, the relatively stable union is considered to involve van der Waal forces, electrostatic interactions and hydrogen bonds (Hughes-Jones, 1963). It seems possible that the specificity in both the antigen–antibody reaction and the transferrin–reticulocyte interaction may be in the complementary spatial configuration of the reactive atoms or atomic groups.

It cannot be concluded that the interaction between transferrin and reticulocytes necessarily occurs only at the outermost layer of the cell membrane. The present results could also be explained by the passage of transferrin molecules into or even through the cell membrane, with the iron-donating reaction occurring within the cell. A proposed mechanism of iron transfer into the cell (Mazur *et al.*, 1960) is through a reducing system involving ascorbic acid and ATP, to which the cell membrane is impermeable. Chelator studies have provided evidence that iron does not exist in the free ionized state at any stage in its transfer from transferrin into the cell (Jandl *et al.*, 1959). It therefore seems likely that the transferrin would deposit its iron well within the surface of the membrane. An inverse relationship was observed between the association and dissociation rate constants and the initial transferrin concentration in the medium. At low levels of transferrin the main restriction on the uptake of transferrin by the cell would be the diffusion process. As the concentration of transferrin is increased, the uptake could be restricted by competition for carrier sites, or possibly by a limitation in the number of carrier molecules if the process was one of facilitated diffusion. These observations could also be explained by the existence of a limited concentration of some energy source or enzyme activity which was involved in the binding of transferrin or uptake of iron by the cell. Depletion of this energy source or saturation of enzyme sites could produce the observed reduction in the association constant with increasing transferrin concentration. The number of effective specific transferrin binding sites on the reticulocyte varied with temperature. This variation may be due to a temperature dependent configurational change in the reticulocyte surface which renders more specific binding sites accessible at higher temperatures. Alternatively it may indicate the involvement of a supply of cellular energy needed to maintain the specific transferrin binding sites in an active form.

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Metabolism of Menadione-6,7-^{3}H in the Rat*

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ABSTRACT: Menadione-6,7-^{3}H was prepared from tetrasodium 2-methyl-1,4-naphthoquinol-6,7-^{3}H diphosphate with specific activities to 44 Ci/mmole. Distribution of radioactivity in rats 18 hr after administration of a physiological dose (10 μg) of the tritiated vitamin to deficient rats showed low levels in liver, heart, kidney, carcass, and viscera while 78–83% of the administered tritium was recovered in the urine during this period. The lipophilic metabolite of menadione, menaquinone-4, was found in all tissues examined. The identity of this compound was supported by chromatographic comparison with authentic material on adsorption and partition columns and on thin-layer plates in the presence and absence of silver nitrate. The concentration of menaquinone-4 ranged from 2.4 to 48.4 pmoles per g depending upon the tissue studied. The administration of Dicumarol® in amounts sufficient to inhibit coagulation response lowered these values to 0.8–8.2 pmoles/g.

A study of vitamin K under conditions which relate physiological response to the vitamin with its metabolic fate has been undertaken as an approach to elucidating the function of this fat-soluble vitamin. A metabolic study under these conditions requires labeled vitamin with a specific activity higher than previously attained. The work of Andrews *et al.* (1962) resulted in highly radioactive vitamin K in the form of tetrasodium 2-methyl-1,4-naphthoquinol-6,7-^{3}H diphosphate.[1] Although originally used as a cancer chemotherapeutic, this compound is easily converted to menadione-6,7-^{3}H with specific activities much higher than the labeled preparations presently available (Lee *et al.*, 1953; Martius and Esser, 1958; Billeter and Martius, 1960).

The preparation of menadione-6,7-^{3}H, its purification and the results of some metabolic studies are the subject of this report.

Materials and Methods[2]

Preparation of Menadione-6,7-^{3}H. Tetrasodium 2-methyl-1,4-naphthoquinol-6,7-^{3}H diphosphate was obtained in saline solution and used immediately or stored at 4°. New samples were sufficiently pure for direct conversion into menadione; however radiodecomposition of the diphosphate during storage is a major problem (Evans, 1966). Older samples were purified by the paper chromatographic system of Andrews *et al.* (1962): saturated ammonium sulfate–1 M sodium acetate–2-propanol (75:25:2, v/v) (R_F = 0.60).

Menadione-6,7-^{3}H was prepared from the diphosphate by acid hydrolysis in the presence of ceric sulfate according to Andrews *et al.* (1962). Up to 10 ml of solution of the radioactive diphosphate was mixed with 10 ml of hot 10% ceric sulfate in 20% H_2SO_4, allowed to stand for 2 min and washed into a separatory funnel with cold water. The resulting mixture was extracted with ether, washed with water, dried with anhydrous sodium sulfate, and evaporated without heat under a stream of nitrogen or at partially reduced pressure in a

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[1] Different forms of vitamin K are designated according to the recommendation of the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem. 241*, 2989, 1966). Menadione is 2-methyl-1,4-naphthoquinone; phylloquinone is 2-methyl-3-phytyl-1,4-naphthoquinone; and menaquinone-4 (abbreviated MK-4) is 2-methyl-3-geranylgeranyl-1,4-naphthoquinone.

[2] Nuclear-Chicago Corp., Des Plaines, Ill.