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Interaction of Dodecyl Sulfate with Native and Modified β-Lactoglobulin*

Thomas S. Seibles

ABSTRACT: β-Lactoglobulin polymorphs A, B, and C (mol wt 36,000) interact with dodecyl sulfate yielding complexes containing 2 moles of dodecyl sulfate/mole of protein. Neither S-carboxymethylation, alkylation of tryptophan residues, nor limited hydrolysis of β-lactoglobulin with carboxypeptidase A affected the dodecyl sulfate complexing ability. However, low levels of photooxidation in the presence of methylene blue, which destroys histidine, primarily, does abolish the ability to complex.

The results indicate that the binding site conformation is critically influenced by a histidine residue located in the interior of each polypeptide chain.

A number of studies have been made concerning the capacity of β-lactoglobulin to bind a variety of organic compounds. Lundgren (1945) and Bull (1946) correlated the binding of anionic detergents with the sum of the positive groups in β-lactoglobulin. Klotz and Urquhart (1949) found that the protein displayed only weak or slight affinity for anionic methyl orange. McMeekin *et al.* (1949) succeeded in isolating a crystalline complex of bovine β-lactoglobulin and dodecyl sulfate. Hill and Briggs (1956) were able to demonstrate different stages of β-lactoglobulin–*n*-octylbenzene-*p*-sulfonate interaction by equilibrium dialysis and electrophoresis.

The early investigations were carried out using pooled bovine β-lactoglobulin. Recently, however, four different genetic forms of β-lactoglobulin have been detected (Aschaffenburg and Drewry, 1955; Bell, 1962; Grosclaude *et al.*, 1966). These have been designated as β-lactoglobulins A, B, C, and D. With the discovery and availability of the β-lactoglobulin polymorphs, there has developed an interest in reexamining the binding characteristics of these related proteins. The interaction of these proteins with dodecyl sulfate might detect structural differences among closely related molecular species. The alkane binding characteristics of β-lactoglobulins A and B have been studied by Wishnia and Pinder (1966), while Ray and Chatterjee (1967) investigated conformational changes and binding characteristics of bovine β-lactoglobulins A and B, and also goat and buffalo β-lactoglobulins using methyl orange, dodecyl sulfate, and dodecylpyridinium bromide.

This report examines the binding characteristics of bovine β-lactoglobulins A, B, C, and also β-lactoglobulin from pooled milks (AB). I used dodecyl [^{35}S]sulfate in order to study more precisely the quantitative aspects of the protein–detergent interaction. This is an improvement on the method of McMeekin *et al.* (1949) where the binding was measured by gravimetric sulfur determinations. This report also includes studies undertaken to identify those amino acids of the polypeptide chain which are involved in the rather specific binding of the detergent molecule. These studies include photooxidation and other chemical modifications of the protein molecule prior to interaction with dodecyl [^{35}S]sulfate.

Materials and Methods

Bovine β-lactoglobulins A, B, and C and carboxypeptidase A modified β-lactoglobulin B were kindly supplied by Dr. E. B. Kalan. Crystalline sodium dodecyl [^{35}S]sulfate (specific activity 1.87 mCi/mmole) was obtained from New England Nuclear Corp.[1]

β-Lactoglobulin–dodecyl [^{35}S]sulfate derivatives were prepared by a method similar to that of McMeekin *et al.* (1949). Typically, 4.2 ml of 0.2 M dodecyl [^{35}S]sulfate was added to 50 ml of a 1% solution of β-lactoglobulin at pH 4.2, giving a 6:1 detergent to protein molar ratio in the reaction mixture. After standing overnight, the crystalline protein–dodecyl sulfate complex was separated from the mother liquor by centrifugation and recrystallized by dialysis from 0.1 M sodium chloride until a constant specific activity was obtained.

A 6:1 detergent to protein molar ratio was also used in preparing the dodecyl [^{35}S]sulfate derivative of the chemically modified β-lactoglobulins. In these instances the binding experiments were carried out at pH 6.6 owing to the differences

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[1] Mention of products or companies does not constitute an endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

TABLE I: Stoichiometric Combination of Dodecyl Sulfate with Polymorphic Forms of β-Lactoglobulin.

Sample	Protein (μmole × 10^{-3})	Act.[a] (av cpm)	Dodecyl Sulfate[b]/ (μmole × 10^{-3})	μmoles of Dodecyl Sulfate/μmole of β-Lactoglobulin
A (1)[c]	5.05	16,739	11.6	2.3
A (2)	3.48	11,320	7.85	2.3
B (1)	3.48	11,093	7.70	2.2
B (2)	4.06	13,611	9.40	2.3
C (1)	3.29	9,711	6.74	2.0
C (2)	2.83	9,329	6.47	2.3
AB (1)	2.74	7,198	5.00	1.8
AB (2)	2.46	7,986	5.45	2.2

[a] Standard error = 317 cpm. [b] Based on standard curve measurements on pure sodium dodecyl [^{35}S]sulfate. [c] Duplicate analytical samples. The letter designations represent the different polymorphic forms of β-lactoglobulin.

in solubility. Subsequently, these samples were subjected to repeated dialysis against water until a constant specific activity was achieved.

Dodecyl [^{35}S]sulfate bound by the various β-lactoglobulins was assayed by the liquid scintillation counting technique (Raben and Bloembergen, 1951). Aqueous samples containing 40 and 80 μg of protein, respectively, were transferred to glass scintillation vials and evaporated to dryness. To dissolve the protein, 200 μl of 88% formic acid (Toennies, 1942) was added to each vial followed by 10 ml of a 1:2 (v/v) mixture of 2-ethoxyethanol and toluene containing 40 mg of 2,5-diphenyloxazole and 50 mg of *p*-bis[(2,5-phenyloxazolyl)]-benzene as scintillator. The radioactivity of the sample, suitable blanks, and reference standards was measured in a Nuclear-Chicago 722–723 liquid scintillation spectrometer.

The study of the binding sites was begun by specific chemical modification of β-lactoglobulin isolated from pooled milks, containing β-lactoglobulin A and β-lactoglobulin B in approximately equal amounts. Reductive alkylation of the protein disulfide and sulfhydryl groups was carried out using iodoacetate in the presence of mercaptoethanol according to the method of Crestfield *et al.* (1963). Tryptophan residues were alkylated using 2-hydroxynitrobenzyl bromide (Barman and Koshland, 1967). β-Lactoglobulin was photooxidized in the presence of methylene blue following the procedure of Weil and Buchert (1951). Changes in amino acid content of the modified β-lactoglobulins were followed by acid hydrolysis and automatic ion-exchange chromatographic analysis (Piez and Morris, 1960). Tryptophan, which is destroyed by acid hydrolysis, was determined by the Spies method (Spies, 1967).

Circular dichroism measurements of the various photooxidized samples of β-lactoglobulin were carried out (after removal of methylene blue by adsorption on activated charcoal) in a Durrum-Jasco ORD/UV-5 apparatus with circular dichroism attachment. The spectra were measured from 250 to 200 nm using demountable quartz cells with a 0.1-mm plastic spacer. Base-line runs were made immediately after the sample runs. Instrument tracings are presented in the figures so that the experimental signal-to-noise ratio can be seen. The experiments were repeated to verify reproducibility of the results presented. Protein concentration in all samples was determined by the micro-Kjeldahl procedure for total nitrogen using a factor of 6.25 for calculating protein.

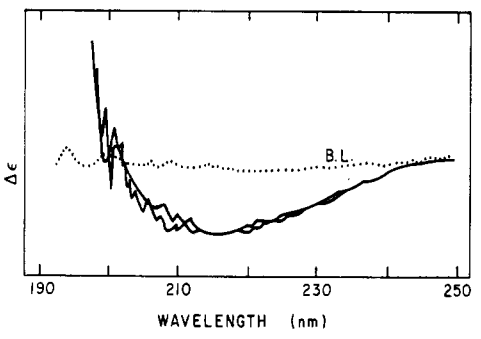

FIGURE 1: Record of far-ultraviolet circular dichroism spectrum of native β-lactoglobulin B and the β-lactoglobulin B–dodecyl sulfate complex at pH 6 in 0.1 M phosphate. Protein concentration in both cases is 7 × 10^{-5} M. B. L. is base line.

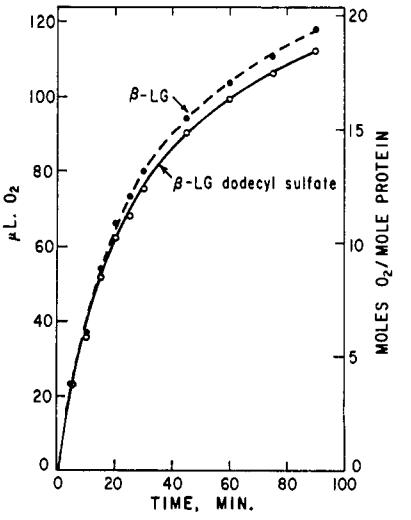

FIGURE 2: Comparison of photochemical reaction rates of β-lactoglobulin AB and β-lactoglobulin AB–dodecyl sulfate complex.

TABLE II: Dodecyl Sulfate Interaction with Modified β-Lactoglobulins.

Method	Reagent	Residues Affected	Moles of Dodecyl Sulfate Bound/Mole of Protein
S-Carboxymethylation (Crestfield *et al.*, 1963)	Iodoacetate	Half-cystine	2.2
Alkylation (Barman and Koshland, 1967)	2-Hydroxy-5-nitrobenzyl bromide	Tryptophan	2.4
Enzymic hydrolysis (Greenberg and Kalan, 1965)	Carboxypeptidase A	-His-Ile·COOH	2.1
Photooxidation (6 moles of O_2/mole) (Weil and Buchert, 1951)	Methylene blue	Histidine, tryptophan (cystine, methionine)	0

Results

As indicated in Table I, scintillation counting of the genetic variants of β-lactoglobulin studied revealed in every case the binding of 2 moles of dodecyl sulfate/mole of protein (mol wt 36,000). These results confirm the earlier findings of McMeekin *et al.* (1949), who studied the complex using β-lactoglobulin from pooled milks.

The circular dichroism spectra in the far-ultraviolet region (260 nm and below) afford information regarding the conformation of the peptide backbone of proteins (Timasheff *et al.*, 1967). Below 250 nm the spectrum of native β-lactoglobulin B is characterized by negative dichroism between 250 and 202 nm, which has an extremum at 216–218 nm and a crossover at 202–203 nm. Townend *et al.* (1967) reported that β-lactoglobulin has a positive maximum at 196 nm; however, the relatively high noise level prevented us from obtaining a reliable record below 200 nm. Figure 1 shows the record of the circular dichroism spectra of native β-lactoglobulin B and the dodecyl sulfate–β-lactoglobulin B complex from 250 to 200 nm. The tracings are identical within experimental error, indicating that the binding of 2 moles of dodecyl sulfate/mole of protein does not significantly alter the polypeptide conformation.

A comparison of the photochemical reaction rates of β-lactoglobulin and the β-lactoglobulin–dodecyl sulfate complex is illustrated in Figure 2. The reaction rates are essentially the same for both, indicating that bound dodecyl sulfate has no effect on the photooxidation of susceptible residues such as histidine. At the early stages of photooxidation the protein and the complex are equally susceptible which shows that the binding of dodecyl sulfate does not produce an increase or decrease in the number of amino acid side chains undergoing reaction.

On the other hand, if β-lactoglobulin is photooxidized beforehand, the binding capacity is sharply decreased. As can be seen in Figure 3, binding capacity is virtually eliminated after only 6 moles of O_2 uptake/mole of protein. This effect encouraged me to seek the binding sites, since it was apparent that only slight chemical modification of the protein was capable of disrupting these regions. A summary of the results of the binding of dodecyl sulfate to chemically modified β-lactoglobulin is found in Table II.

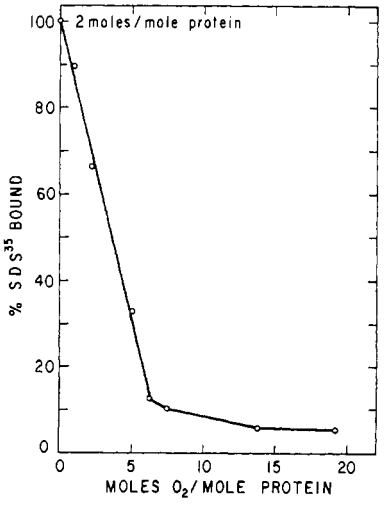

FIGURE 3: Effect of photooxidation on capacity of β-lactoglobulin AB to bind dodecyl sulfate.

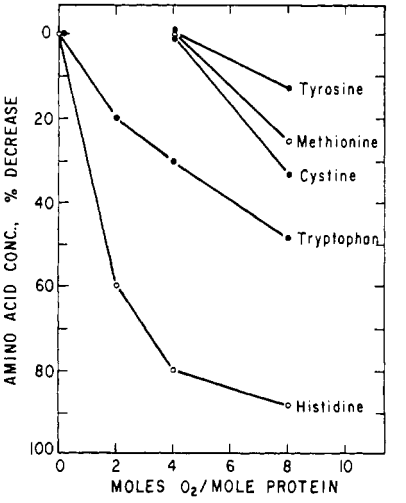

FIGURE 4: Decrease in various amino acids during photooxidation of β-lactoglobulin B.

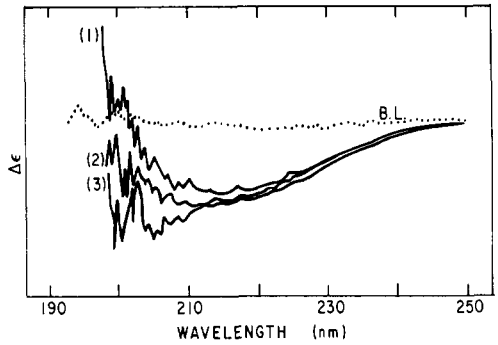

FIGURE 5: Record of the far-ultraviolet circular dichroism spectra of native β-lactoglobulin B (1) compared with β-lactoglobulin B photooxidized to 2 moles of O_2 (2), and 7.5 moles of O_2 (3) per mole of protein. Protein concentration is 7×10^{-5} M in pH 6 0.1 M phosphate buffer.

S-Carboxymethylation of the reduced protein in the presence of urea resulted in samples containing no residual cystine or cysteine. However, trace amounts of cystine were still present when urea was omitted. In each instance, amino acid analysis of the acid hydrolysate revealed the elution of a new peak at effluent volume ~96 ml. This new peak was subsequently identified as *S*-carboxymethylcystine by spiking the acid hydrolysate of the protein with the known compound. Treatment of these S-carboxymethylated β-lactoglobulin samples with sodium dodecyl [^{35}S]sulfate resulted also in the binding of 2 moles of dodecyl sulfate/mole of protein. This indicates that neither the disulfide groups nor the two free sulfhydryls of β-lactoglobulin are necessary for the binding of dodecyl sulfate. Moreover, it appears that the native conformation of the protein is not critical for binding. However, in contrast, to the native protein, the S-carboxymethylated derivative is no longer crystallizable either in the presence or absence of dodecyl sulfate.

The reagent 2-hydroxy-5-nitrobenzyl bromide has been shown to be highly selective for tryptophan (Barman and Koshland, 1967). Alkylation of β-lactoglobulin with this reagent in the presence of 6 M urea resulted in incorporation of four 2-hydroxy-5-nitrobenzyl groups per mole of protein. Four is the number of tryptophans reported by Spies (Spies, 1967) and all react under these conditions. However, in the absence of urea only two 2-hydroxy-5-nitrobenzyl groups were incorporated indicating that two of the four tryptophans are located on or near the surface of the protein molecule while the remaining two tryptophans are interior and require a change in conformation for accessibility to the reagent. Dodecyl sulfate interaction with either of these 2-hydroxy-5-nitrobenzyl-β-lactoglobulin derivatives also resulted in the binding of 2 moles of detergent/mole of protein. These findings reveal that neither alkylation of specific tryptophan residues with 2-hydroxy-5-nitrobenzyl bromide nor any changes in conformation produced as a result of this reaction has effect on the binding of dodecyl sulfate.

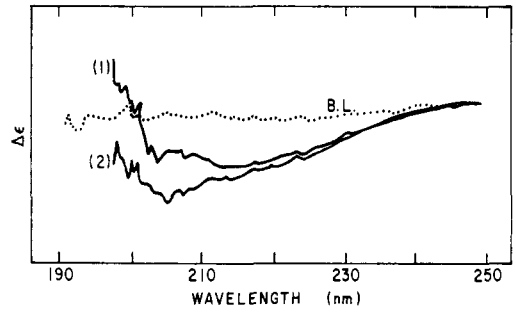

FIGURE 6: Record of the far-ultraviolet circular dichroism spectra of carboxypeptidase A modified β-lactoglobulin B (1) compared with the same protein after photooxidation to 2 moles of O_2/mole of protein (2). Protein concentration is 5×10^{-5} M in pH 6 0.1 M phosphate buffer.

Studies on β-lactoglobulin photooxidized to varying levels of O_2 uptake disclosed that the dodecyl sulfate binding was ~90% eliminated at the level of 6 moles of O_2 uptake/mole of protein. This can be seen in Figure 3. At this point, the histidine content of β-lactoglobulin had decreased ~85%, while tryptophan, cystine, methionine, and tyrosine decreased 40, 17, 13, and 6%, respectively, as shown in Figure 4.

Photooxidation also produces conformational changes in β-lactoglobulin as indicated in Figure 5. When compared with the circular dichroism spectrum of the native protein, the spectrum of β-lactoglobulin, photooxidized to the level of 2 moles of O_2 uptake/mole, shows an increase in negative dichroism at the lower wavelengths and a shift in the minimum from near 217 nm to around 210 nm. At 7.5 moles of O_2 uptake, a further negative increase is evident, the characteristic minimum near 217 nm has disappeared, and new dichroic bands at 205 and 200 nm are apparently visible.

β-Lactoglobulins A and B contain four histidine and four tryptophan residues per mole of protein. These residues are the most susceptible to photooxidation under the experimental conditions used. The results of the binding experiments carried out with 2-hydroxy-5-nitrobenzyl-alkylated β-lactoglobulin tend to rule out the tryptophan residues as essential participants in the binding sites. Nevertheless, photooxidation of β-lactoglobulin at low levels of O_2 uptake involves histidine and tryptophan, primarily, and the question persisted as to which of these amino acid residues was responsible for the binding site conformation. In order to determine this, carboxypeptidase A modified β-lactoglobulin B was utilized. This modified protein contains only one histidine residue in each of the identical polypeptide chains of the protein, the other penultimate histidine having been eliminated along with carboxyl-terminal isoleucine by the enzymatic hydrolysis (Greenberg and Kalan, 1965). As pointed out in Table II, this derivative also binds 2 moles of dodecyl sulfate/mole of protein. However, when this derivative is photooxidized to the level of only 2 moles of O_2 uptake/mole of protein, binding of dodecyl sulfate is abolished. Amino acid analysis revealed only a trace of histidine remaining and the destruction of tryptophan was limited to less than 20%. Photooxidation also produced changes in the conformation of the protein as shown in Figure 6. The circular dichroism spectrum of the photooxidized protein shows increased negative dichroism at the lower wavelengths and a shift of the characteristic minimum from near 217 to close to 205 nm.

Discussion

The results presented here indicate that 2 moles of dodecyl sulfate are bound per mole of all genetic variants of β-lactoglobulin studied, which fact agrees with the known similarity

of the proteins (Townend *et al.*, 1967). This apparently rules out the loci of the amino acid substitutions as the sites of binding.

Stabilization of the disulfide and sulfhydryl groups by reductive alkylation did not affect the quantity of dodecyl sulfate bound. Therefore, as mentioned previously, the native conformation of the protein with intact disulfide and/or sulfhydryl groups does not appear to be important in the interaction of the protein with dodecyl sulfate. Moreover, it is interesting to note that though photooxidation prevents the binding of dodecyl sulfate, the native protein and the dodecyl sulfate complex once formed are photooxidized at the same rate and to the same extent.

β-Lactoglobulin has been characterized, qualitatively, as having 10% or less of α-helical regions, the remaining structure being about equally divided between unordered and antiparallel β structure (Townend *et al.*, 1967). However, in view of the recent work of Fasman and Potter (1967) in attempting to relate optical rotatory dispersion data of lysozyme and myoglobulin with that of poly-L-lysine, and also the findings of Craig (1968) with polypeptides, rigorous conformational analysis of proteins relying solely on circular dichroism or optical rotatory dispersion measurements is of questionable value. Nevertheless, our circular dichroism measurements of photooxidized β-lactoglobulin from 250 to 200 nm show an increase in negative dichroism and blue shift with increasing levels of O_2 uptake, indicating changed conformation. Townend *et al.* (1967) observed similar changes in the dichroic spectrum of β-lactoglobulin induced by increased pH. The negative dichroic peak between 215 and 220 nm, characteristic of the native protein, shifted to shorter wavelengths and also increased in intensity as the pH increased.

Studies conducted by Hill and Briggs (1956), and Ray and Chatterjee (1967) indicate that the binding of *n*-octylbenzene-*p*-sulfonate and dodecyl sulfate, respectively, by β-lactoglobulin is dependent upon the concentration of detergent in the medium. They concluded that at low detergent concentration, one or two molecules of detergent are bound tightly, whereupon the protein unfolds to expose many other weaker sites. Our experiments were confined to the complex formed at low dodecyl sulfate concentration and our results are in general agreement with respect to the stoichiometry of the β-lactoglobulin–dodecyl sulfate complex formed under these conditions. Also, our circular dichroism studies on the dialyzed complex failed to disclose any change in protein conformation occurring as a result of complex formation. Therefore, the unfolding of the protein to expose additional binding sites must be concentration dependent and reversible as suggested by Hill and Briggs (1956).

The loss of binding produced by photooxidation is accompanied by changes in the protein conformation. This fact must be considered in light of the previously mentioned chemical modification experiments which, with the exception of the carboxypeptidase A modified protein, also produced some change in protein conformation. Therefore, the suggestion is that the binding regions for dodecyl sulfate are small and discrete, not affected by changes in certain other areas of the protein molecule. On the other hand, we observed that photooxidation of one interior histidine (as distinguished from the penultimate histidine) in each of the polypeptide chains produces a gross change in conformation accompanied by a loss in binding capability. Therefore, we submit that the polypeptide conformation necessary for the binding of dodecyl sulfate by β-lactoglobulin is influenced by the interior histidine. Or, to put it another way, destruction of the interior histidine is by itself sufficient to destroy such conformations as are necessary for the binding.

Conceivably, the binding results from the combined effects of a protonated histidinyl residue and proximal hydrophobic residues. Although Frank and Braunitzer (1967) have published the partial primary structure of β-lactoglobulin, little is known about the tertiary structure of this protein. However, it is possible that the histidinyl residue might act as the positive center of the binding site, while the hydrophobic amino acid side chains serve to augment the binding of anionic dodecyl sulfate by hydrophobic interaction with the alkyl portion of the detergent molecule.

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The Role of Iron in the Reaction between Rabbit Transferrin and Reticulocytes*

E. Baker and E. H. Morgan

ABSTRACT: The effect of the degree of iron saturation of transferrin on the kinetics of the interaction with reticulocytes was studied using pure rabbit transferrin and rabbit reticulocytes. The increase in the association rate constant measured with iron-saturated transferrin compared with iron-free transferrin was not statistically significant. The dissociation rate constant for the reaction between reticulocytes and transferrin which was low in iron content was larger than that for iron-saturated transferrin. More iron-containing than iron-free transferrin was bound to the reticulocytes at equilibrium. This difference was largely due to the iron-containing molecules having a longer mean residence time on the reticulocyte. The efficiency of the iron transfer process was 86%, indicating that a proportion of the iron-containing transferrin molecules taken up by reticulocytes was refluxed without donating all their iron to the cells.

Each molecule of the plasma iron-binding protein transferrin is capable of binding two molecules of ferric iron (Laurell and Ingelman, 1947; Surgenor *et al.*, 1949). A specific property of the transferrin molecule is its ability to donate this bound iron to hemoglobin-synthesizing cells (Laurell, 1947; Paoletti and Durand, 1958; Jandl *et al.*, 1959) and to iron-storing cells (Mazur *et al.*, 1960). The interaction between transferrin and cells has been studied in detail using reticulocytes (Jandl and Katz, 1963; Morgan and Laurell, 1963; Morgan, 1964; Kornfeld, 1968; Baker and Morgan, 1969). Contradictory evidence has been published on the conditions of exchange of iron between the transferrin–iron complex and reticulocytes. For example, Jandl and Katz (1963) presented evidence that the rate of iron transfer was dependent upon the degree of iron saturation of the transferrin and that the reticulocyte had a greater affinity for iron-containing than iron-free transferrin. In contrast, Morgan and Laurell (1963) found that iron transfer was dependent only upon iron concentration and that iron-free and iron-containing transferrin molecules had equal affinity for the cell binding sites.

This investigation was undertaken primarily to evaluate the importance of the iron moiety in the reaction between transferrin and reticulocytes. A comparison was made of the kinetics of the association and dissociation reactions between rabbit reticulocytes and rabbit transferrin which was either iron saturated, low in iron, or iron free. An estimate of the efficiency of the iron-donating process from bound transferrin molecules to reticulocytes has been obtained.

* From the Department of Physiology, The University of Western Australia, Nedlands, Western Australia. *Received March 14, 1969.* This work was supported by a grant from the Australian Research Grants Committee and from the National Institutes of Health (5 RO5 TW-00212-02).

Methods

Unless specifically noted the preparative, analytical, and experimental procedures performed in this study were carried out as described previously (Baker and Morgan, 1969).

Removal of Iron from Transferrin. Two methods were used to remove iron from transferrin. In the first the pH of the transferrin solution was lowered to 4–5 using citric acid, and the resulting iron–citrate complex was removed by passage through a column of Amberlite-IRA 410 (A. R. Grade) prepared in the chloride form (Bothwell *et al.*, 1959). The pH was immediately readjusted to 7.5 using dilute NaOH. The iron content of transferrin treated in this way was within the range of 5–17% saturation of its iron binding capacity.

The second method of iron removal was used to render transferrin completely iron free by dialysis against a solution containing 0.01 M EDTA in 0.3 M sodium acetate (pH 5.5). The EDTA–iron complex was removed by exhaustive dialysis of the transferrin solution against deionized water and Hanks and Wallace's physiological salt solution (Hanks and Wallace, 1949) for 48 hr at 4°.

Isotope Labeling. ^{59}Fe was obtained from the Radiochemical Centre, Amersham, England, in the form of ferric chloride dissolved in 0.1 N HCl. Iron-free transferrin dissolved in Hank's solution was labeled with ^{59}Fe by incubation with the isotope solution for 30 min at 37°.

Results

Uptake of Iron-Saturated and Low-Iron Transferrin. Transferrin uptake by reticulocytes was measured in duplicate systems which differed only in the degree of iron saturation of the transferrin. As shown in Figure 1, more of the iron-rich transferrin (99% iron saturated) was bound at equilibrium than low-iron transferrin (12% iron saturated). In five similar ex-