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Articles

Effect of Serum Albumin on Siderophore-Mediated Utilization of Transferrin Iron†

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ABSTRACT: The effect of serum and serum proteins on enterobactin- and aerobactin-mediated utilization of transferrin iron has been investigated. Serum was found to impede transfer of iron from iron transferrin to enterobactin and from [^{55}Fe]ferric enterobactin to cells of *Escherichia coli* BN3040 Nal^{R} *iuc*. In contrast, serum had essentially no effect on the rate of these reactions mediated by aerobactin. Three purified serum proteins, human serum albumin, bovine serum albumin, and human immunoglobulin, were comparable to human serum in their selective ability to interfere with the transfer of ^{55}Fe from [^{55}Fe]ferric enterobactin to *E. coli* BN3040 Nal^{R} *iuc*. The inhibitory effect of human serum albumin on the enterobactin-mediated transfer of iron from [^{55}Fe]transferrin was enhanced by preincubation of the protein with the siderophore. Pretreatment of the bacterial cells with human serum albumin did not affect the rate of utilization of siderophore iron. A linear, reciprocal relationship was found to hold for human albumin concentration vs. the first-order rate constant (k_{obsd}) for the velocity of iron transfer from iron transferrin to enterobactin. Binding of serum albumin to enterobactin increased the intensity of the near-ultraviolet absorption band of the siderophore and shifted it to longer wavelengths. The stoichiometry of binding to human and bovine serum albumins was established as 1:1, and the binding constant for both enterobactin and ferric enterobactin was estimated to be in the range 1×10^4–1.2×10^5 M^{-1}. These results indicate that serum albumin may act synergistically with other factors in the serum, such as transferrin, to limit iron supply and in this way restrict the growth of invading microorganisms.

Iron is probably essential for the growth of all pathogenic bacteria. Invading organisms which reach either mucosal surfaces or the circulating plasma become exposed to the iron-binding proteins lactoferrin and transferrin. These proteins, in combination with antibodies, often have powerful bacteriostatic effects in vitro and are essential for protection against many infections (Rogers et al., 1980; Bullen, 1981). Transferrin and lactotransferrin restrict the amount of ionic iron available in body fluids to 10^{-12} μM or less. This amount is insufficient for normal bacterial growth, and the ability to sequester iron, which either is insoluble or is bound to specific proteins, has been suggested as one of the factors determining the virulence of a microorganism (Weinberg, 1978).

Enteric bacteria secrete the catechol-type siderophore enterobactin, and the ferric enterobactin complex formed in the external medium is actively transported across cell membranes

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(Neilands, 1981a). Certain hospital isolates of *Escherichia coli* synthesize, in addition, a hydroxamate-type siderophore (aerobactin), the genetic determinants for which reside on the ColV plasmid (Williams, 1979; Warner et al., 1981; Stuart et al., 1982). In the clinic, *E. coli* is the most common Gram-negative bacterium isolated from the bloodstream (Young et al., 1982), and the means whereby it acquires virulence remains a challenging problem.

In a previous paper (Konopka et al., 1982), we showed that the siderophores aerobactin and enterobactin remove iron from iron transferrin at different rates depending on the composition of the medium in which the transfer occurs. In buffer solution, the transfer rate was greater for enterobactin while in serum the rate was superior when aerobactin was the ligand. The results indicate that aerobactin, despite its relatively humble affinity for Fe(III) in the siderophore series, must be endowed with special structural features or properties that enhance its ability to remove iron from transferrin in vivo. In order to determine the nature of the factor(s) in serum responsible for inhibition of iron transfer to enterobactin, we elected to investigate the effect of serum protein fractions on the siderophore-mediated utilization of transferrin iron.

Enterobactin has relatively low water solubility (O'Brien & Gibson, 1970) and contains several ester bonds which cleave to release 2,3-dihydroxy-*N*-benzoylserine and, ultimately, 2,3-dihydroxybenzoic acid (2,3-DHBA).[1] The aromatic character of enterobactin may cause it to adhere to proteins as a haptene for the synthesis of antibodies (Neilands, 1981b). Recently, it has been claimed that antibodies to enterobactin occur in normal human serum (Moore et al., 1980; Moore & Earhart, 1981). This finding led us to study the possible role of serum albumin in siderophore-mediated iron uptake in *E. coli*.

Albumin is the most abundant protein in human plasma, accounting for 60% of the total, with a normal concentation of 0.5–0.6 mM (Peters, 1975). An interesting property of albumin is its ability to bind hydrophobic ligands such as fatty acids, lysolecithin, bilirubin, tryptophan, steroids, thyroxine, drugs, and dyes. The binding of drugs to albumin affects their transport between tissues or organs and their effective concentration. Binding also protects against the toxic effects of the bound ligand, since the pharmacokinetic behavior of drugs with binding constants greater than 10^4 M^{-1} can be dependent on the binding phenomena. The fraction of the drug bound at a given instant is assumed to have no direct therapeutic effect, the albumin acting as a reservoir for the drug. A decrease in the concentration of the free drug in the blood is accompanied by a change of the drug–albumin equilibrium (Wilting et al., 1980a). Recently, more detailed studies on the structure of serum albumin and the characterization of its binding sites have been described by Brown & Shockley (1982) and Means et al. (1982). A model of albumin consisting of six paired subdomains agrees well with the ligand binding data.

Since the pharmacological effect of many aromatic, relatively insoluble compounds may be influenced by their interactions with albumin, it was of interest to known if this interaction also extends to enterobactin. Is enterobactin, because of its aromatic character, susceptible to attachment to serum albumin? In this work, we present data aimed at answering this question. Our results show that, as a consequence of the different chemical structures of the two siderophores, serum albumin binds enterobactin and not aerobactin. This specificity may account for the evolution of aerobactin over enterobactin as the siderophore commonly found in clinical isolates of *E. coli* (Neilands, 1983) and other Gram-negative pathogenic bacteria (Payne et al., 1983).

Materials and Methods

Isolation of siderophores, preparation of [^{55}Fe]transferrin and [^{55}Fe]ferric aerobactin, kinetic studies of iron removal from iron transferrin, growth media, and uptake experiments have been described previously (Konopka et al., 1982). [^{55}Fe]Ferric enterobactin was prepared according to the procedure of Hollifield & Neilands (1978). Enterobactin and ferric enterobactin were determined by use of the extinction coefficients at 318 nm (a_{mM} = 9.6) and 495 nm (a_{mM} = 5.6) for enterobactin (Neilands, 1981c) and ferric enterobactin (Anderson et al., 1976), respectively.

The protein fractions were obtained from Sigma Chemical Co. [HSA (A-8763), BSA (A-7511), and human γ-globulins (HG-11)] and were used without further purification. Albumin concentrations were based on a molecular weight of 69 000 (Brown, 1977) by using the extinction coefficients at 278–279 nm, 25 °C, pH 7.4 of a_{mM} = 36.5 for HSA and a_{mM} = 47.0 for BSA (Blauer et al., 1972).

Bacterial Strains. Two bacterial strains derived from *E. coli* K12 were used: BN3040 Nal^R (F$^-$, proC, leuB, trpE, *thi*, *entA*, *cir*) carrying the col V-K30 *iuc* plasmid (Konopka et al., 1982) and AN90 (F$^-$, *proC*, *leuB*, *trpE*, *thi*, *entD*, *cir*) (Cox et al., 1970). The ColV strain contains transport systems for both enterobactin and aerobactin but synthesizes neither siderophore; it is blocked between chorismate and 2,3-DHBA. The AN90 strain is blocked in the conversion of 2,3-DHBA to enterobactin and can utilize only the latter siderophore.

Binding Studies. Studies were performed to determine the binding constants for the interaction of enterobactin and ferric enterobactin with albumin. The binding data were determined via equilibrium dialysis at various enterobactin/albumin and ferric enterobactin/albumin ratios. Samples of enterobactin or [^{55}Fe]ferric enterobactin were placed in a dialysis bag which had a volume of 0.5 or 1 mL. The experiments were carried out at 4 °C for 24 h, which suitable control experiments showed to be sufficient time for the attainment of equilibrium. The albumin concentration was kept low to prevent dimer formation. The concentrations of enterobactin and [^{55}Fe]ferric enterobactin on either side of the dialysis membrane were determined by the Arnow (1937) reaction and measurements of radioactivity, respectively. Appropriate controls were performed to determine whether albumin penetrated the dialysis bags and to assure that the solutes used did not adhere to the dialysis membrane. The binding data were graphed by the Scatchard method (Scatchard, 1949; Steiner et al., 1966), and the binding constant was calculated from the slope of the Scatchard plot. ν/c_{free} vs. the molar ratio of bound enterobactin to albumin was analyzed by a linear least-squares computer program (HP-97, Standard Pac). ^{55}Fe radioactivity was measured with a scintillation counter, Searle Delta type 300. About 90% of the total counts of ^{55}Fe applied to the bag were recovered.

Spectral Measurements. UV and visible spectra were recorded on a Beckman Model 25 spectrophotometer at 20 °C. Solutions containing different enterobactin/albumin molar ratios were prepared, and difference spectra of albumin plus enterobactin (sample cuvette) vs. free albumin (reference cuvette) were recorded until no further changes could be seen. Variations in the enterobactin/albumin molar ratio were ad-

[1] Abbreviations: k_{obsd}, apparent first-order rate constant; 2,3-DHBA, 2,3-dihydroxybenzoic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSA, human serum albumin; BSA, bovine serum albumin.

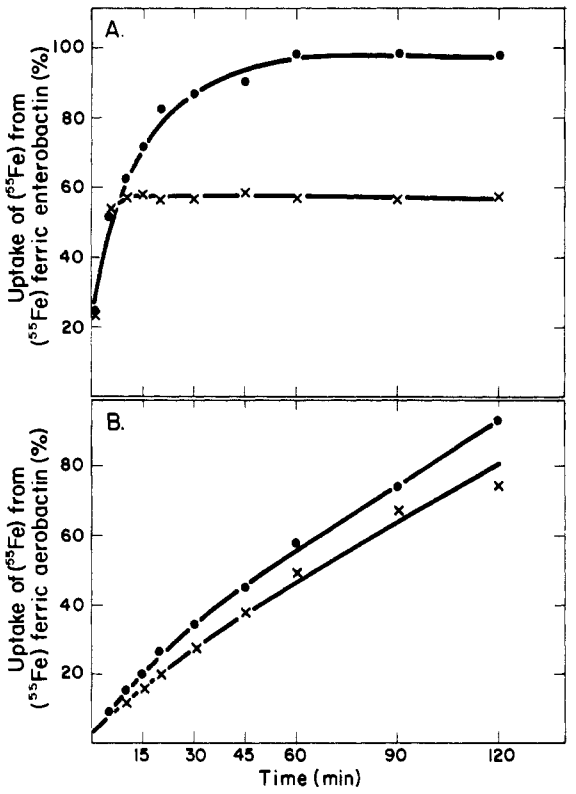

FIGURE 1: Uptake of ^{55}Fe by the BN3040 Nal^R *iuc* strain of *Escherichia coli*. Cells were shaken at 37 °C (A) with 0.9 μM [^{55}Fe]ferric enterobactin in M9 medium (●) and in human serum (×) and (B) with 0.68 μM [^{55}Fe]ferric aerobactin in M9 medium (●) and in human serum (×).

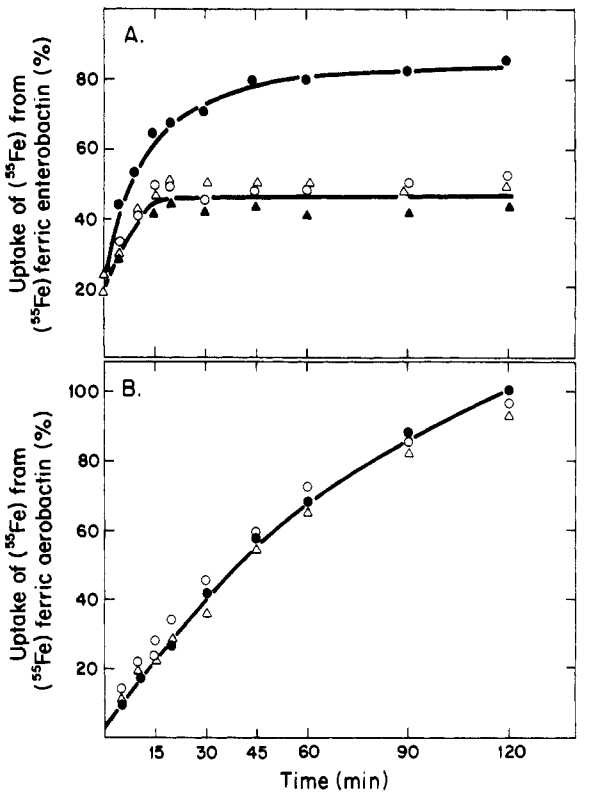

FIGURE 2: Uptake of ^{55}Fe by the BN3040 Nal^R *iuc* strain of *Escherichia coli*. Cells were shaken in M9 medium at 37 °C (A) with 0.9 μM [^{55}Fe]ferric enterobactin or (B) with 0.68 μM [^{55}Fe]ferric aerobactin: no additives (●); plus 0.64 mM HSA (○); plus 0.64 mM BSA (▲); plus human immunoglobulin (12.5 mg/mL) (△).

justed by changing the albumin concentration at constant enterobactin concentration. Possible dimerizations were neglected, and the concentration of the complex was found by means of Cramer's rule, with evaluation of the determinants by the method of pivotal condensation (Bauman, 1962). Similar experiments were performed with 2,3-DHBA substituting for enterobactin.

Results

Uptake Experiments. Repeated measurements of siderophore-mediated iron uptake from [^{55}Fe]transferrin into cells of the BN3040 Nal^R *iuc* strain confirmed that in serum the rate measured with aerobactin surpassed that found for enterobactin. Similar results were obtained by using radioactive siderophores as iron donor compounds (Figure 1). The rate of ^{55}Fe uptake from [^{55}Fe]ferric aerobactin in serum was slightly lower than that in M9 medium, and after 2 h, the velocity was about 20% inhibited. In contrast, under the same conditions, the ^{55}Fe uptake from [^{55}Fe]ferric enterobactin was reduced 50% in serum after 5 min. For investigation of the nature of the serum factor(s) responsible for inhibition, experiments were performed with serum protein fractions.

Moore et al. (1980) and Moore & Earhart (1981) demonstrated that normal human serum contains an "enterobactin-specific antibody" which inhibits the growth of enteric bacteria. The current results confirm that the antibody fraction specifically inhibits enterobactin-mediated iron uptake. Addition of human serum immunoglobulin (12.5 mg/mL) inhibited ^{55}Fe uptake from [^{55}Fe]ferric enterobactin by about 50% after 5 min (Figure 2A) but had no effect on ^{55}Fe uptake from [^{55}Fe]ferric aerobactin. Similar results were obtained with human or bovine albumin fractions (Figure 2). The similarly diminished uptake of label in the presence of all three proteins may be the consequence of a combination of binding of ferric siderophore to the proteins and to the receptor on the bacterial cell surface. The inhibition of ^{55}Fe uptake from [^{55}Fe]ferric enterobactin is not affected by sequestering albumin in a dialysis bag, suggesting that the inhibition is not a consequence of binding of albumin to the cell envelope (data not shown).

Albumin also exerts a selective inhibitory effect on enterobactin-mediated iron uptake from [^{55}Fe]transferrin. The inhibitory effect is enhanced after preincubation of enterobactin with HSA (Figure 3A). The pretreatment of bacterial cells with HSA does not interfere with iron uptake mediated by either aerobactin or enterobactin (Figure 3B). These findings suggest that the binding of both ferric enterobactin and enterobactin with albumin must occur in solution.

A similar inhibitory effect of HSA on iron uptake from [^{55}Fe]transferrin into cells of the BN3040 Nal^R *iuc* in the presence of 2,3-DHBA was observed (data not shown). The BN3040 Nal^R *iuc* strain converts 2,3-DHBA to enterobactin, which then acts as a mediator of iron uptake. Iron uptake experiments were also performed by using the AN90 strain which is blocked in the conversion of 2,3-DHBA to enterobactin. With this strain, there was essentially no stimulation of iron uptake from either $^{55}FeCl_3$ or [^{55}Fe]transferrin in the presence of 2,3-DHBA. These results are compatible with the inability of 2,3-DHBA to act as the sole iron-transporting chelate (Hancock et al., 1977) or to mobilize iron from iron transferrin (Konopka et al., 1982). Thus, no evidence was found in the uptake experiments for a direct binding of 2,3-DHBA to albumin.

Kinetics of Iron Removal from Iron Transferrin in the Presence of Albumin. The exchange of iron between transferrin and the siderophores enterobactin and aerobactin was investigated in the absence and presence of added HSA and BSA. Enterobactin is both kinetically and thermodynamically capable of removing iron from transferrin, and a possible

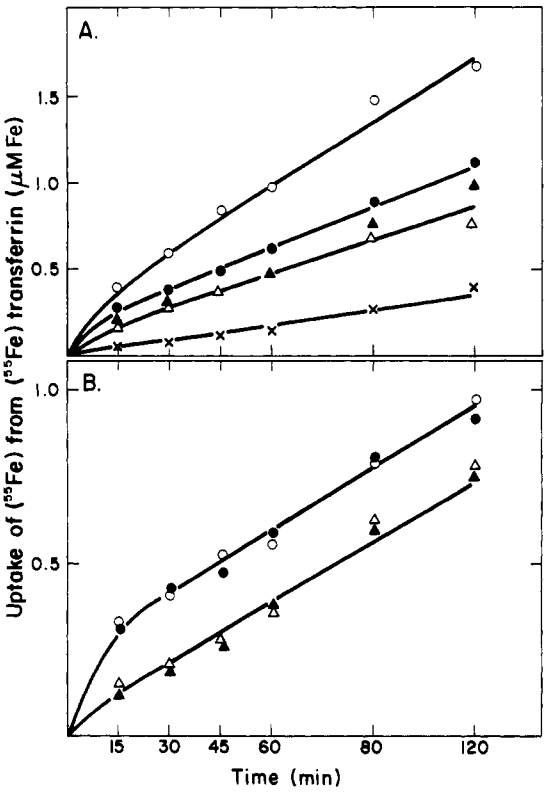

FIGURE 3: Uptake of ^{55}Fe from [^{55}Fe]transferrin by the BN3040 Nal^{R} *iuc* strain of *Escherichia coli*. (A) Cells were shaken at 37 °C with 10 μM 35% saturated [^{55}Fe]transferrin in the presence of 10 μM aerobactin in M9 medium alone (△) and plus 0.64 mM HSA (▲) and in the presence of 10 μM enterobactin in M9 medium alone (○), plus 0.64 mM HSA (●), and plus enterobactin preincubated with HSA for 30 min at 37 °C (×). (B) Cells were pretreated with 0.64 mM HSA for 30 min at 37 °C, washed 3 times, resuspended in M9 medium, and shaken at 37 °C in the presence of 10 μM aerobactin (▲) or 10 μM enterobactin (●). Control experiments in M9 medium without pretreatment with HSA: plus 10 μM aerobactin (△); plus 10 μM enterobactin (○).

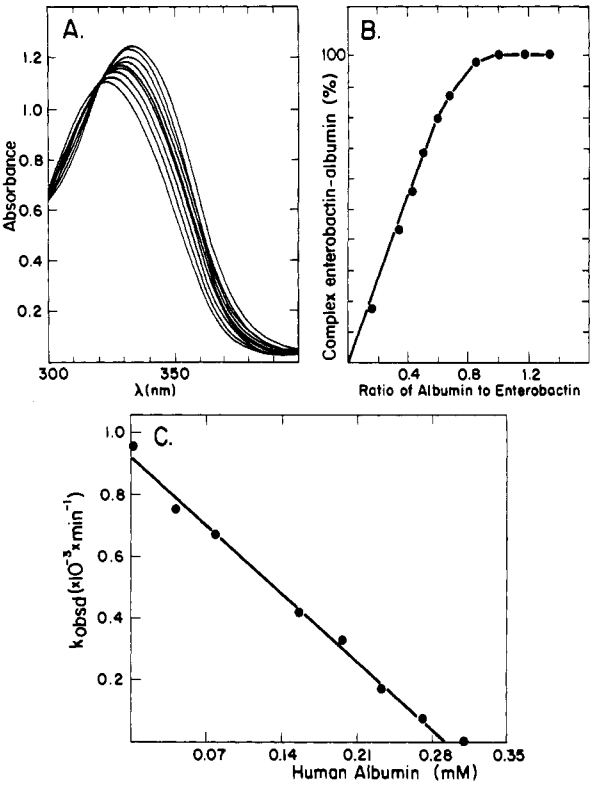

FIGURE 4: Difference spectra measurements and kinetic experiments. (A) Spectral changes associated with binding of enterobactin by BSA in 10 mM Hepes–KOH buffer, pH 7.4. The reaction mixture contained 0.12 mM enterobactin with the BSA concentration varied between 0.02 and 0.16 mM. The lowest trace corresponds to the spectrum of unbound enterobactin, and the highest curve represents the enterobactin–albumin complex. (B) Data in figure 5A plotted as a function of the molar ratio of albumin to enterobactin. (C) Relation between the first-order rate constant (k_{obsd}) and the concentration of HSA in the presence of 0.1 mM 100% saturated transferrin and 0.5 mM enterobactin.

mechanism of chelate exchange has been described by Carrano & Raymond (1979). The addition of albumin to a solution of iron transferrin and enterobactin resulted in inhibition of iron transfer. The effect of the concentration of HSA on the first-order rate constant (k_{obsd}) was tested at 0.5 mM enterobactin (Figure 4C). The calculation of k_{obsd} was based on eq. 2 from Carrano & Raymond (1979). It would seem reasonable to suppose that the observed changes of k_{obsd} reflect the binding of enterobactin with HSA and a consequent decrease in the concentration of unbound enterobactin.

The addition of albumin to a solution of iron transferrin and aerobactin does not inhibit iron transfer. The k_{obsd} was independent of albumin concentration, and the values found were 0.37×10^{-3} and 1.12×10^{-3} min^{-1} in both the absence and presence of 0.6 mM HSA at 1.0 and 5.4 mM aerobactin, respectively. The addition of 5 mM 2,3-DHBA to the solution of iron transferrin and aerobactin resulted in more efficient iron transfer from transferrin to aerobactin, and this effect was not abolished in the presence of albumin (data not shown). These direct kinetic assays confirmed the conclusions drawn from the iron uptake experiments, namely, that serum albumin enterobactin but not aerobactin.

Dialysis Experiments. Figure 5 shows typical saturation curves of binding of enterobactin by HSA and BSA as a function of enterobactin concentration and the Scatchard plots derived therefrom. The obtained data suggest the presence of one high-affinity binding site for enterobactin per mole of albumin, but the possibility that albumin also had several sites

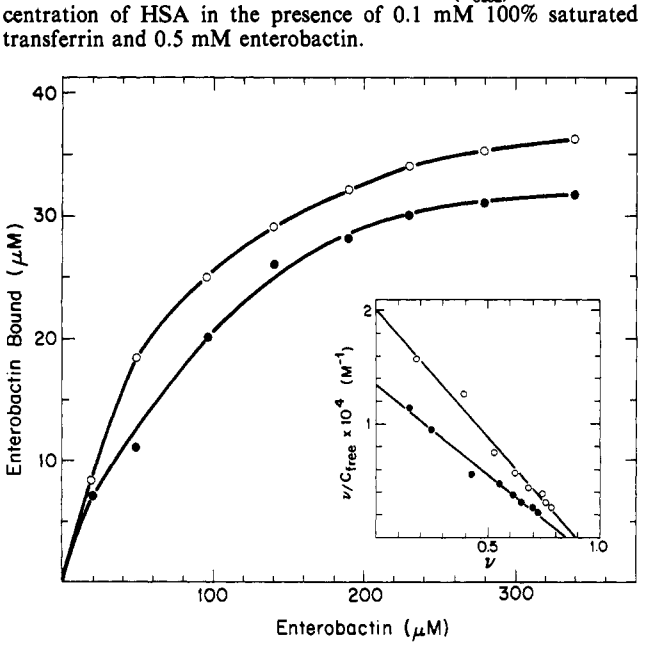

FIGURE 5: Binding of enterobactin to HSA (●) and BSA (○) in 10 mM Hepes–KOH buffer, pH 7.4, at 4 °C; [albumin] = 48 μM. Inset: Scatchard graph of the same data; ν is the molar ratio of bound enterobactin to albumin, and c_{free} is the molar concentration of free enterobactin at equilibrium.

of lower affinity for enterobactin cannot be ruled out. In Table I, the binding constants of enterobactin and [^{55}Fe]ferric enterobactin with albumin are summarized. These constants are estimated values and were determined by assuming one class of high-affinity binding sites. As can be seen, the values

Table I: Binding Constants (M^{-1}) of Enterobactin and Ferric Enterobactin to Albumin at pH 7.4, 4 °C

	enterobactin[a]		[^{55}Fe]ferric enterobactin[b]	
condition	human	bovine	human	bovine
10 mM Hepes-KOH	1.6×10^4	2.3×10^4	1.0×10^5	1.2×10^5
0.16 M NaCl	1.0×10^4	1.5×10^4	0.3×10^5	nd[c]

[a] Albumin concentration, 48 μM; enterobactin concentration, 20–340 μM. [b] Albumin concentration, 7.2 μM; [^{55}Fe]ferric enterobactin concentration, 2.0–30.0 μM. [c] nd, not determined.

obtained are not significantly different from the values for other aromatic compounds (Brown & Shockley,1982). In the presence of 0.16 M NaCl, pH 7.4, the stability constants were somewhat lower than those in 10 mM Hepes–KOH buffer. This was also observed for the binding of warfarin (Wilting et al., 1980a) and diazepam (Wilting et al., 1980b) to albumin.

Difference Spectra. Additional information on the number and nature of binding sites on albumin for enterobactin was obtained by experiments involving a spectrophotometric method based on the difference in light absorption spectra between the unbound and the bound enterobactin. As illustrated in Figure 4A, complexing of enterobactin with BSA resulting in progressive shifting of the absorption band of the siderophore in the near-UV region to a longer wavelength with increasing concentrations of albumin. The isosbestic point at 320 nm indicates the presence of two absorbing species in solution as equilibrium is approached. These are enterobactin and the complex enterobactin–albumin. Analysis of these data indicates a 1:1 complex with a mean value of 2.7×10^4 M^{-1} for the binding constant (Figure 4B). The relatively good agreement for the values of the formation constants obtained by two different methods (cf. Table I) confirms the stoichiometric binding of enterobactin by albumin. Similar results were obtained upon addition of HSA to enterobactin. In contrast to enterobactin, when HSA or BSA was added to a 2,3-DHBA solution, no shifting of the absorption band to longer wavelength occurred (data not shown). The effectiveness of 2,3-DHBA as a mediator and our failure to detect a spectra shift in the presence of albumin indicate that this compound does not bind to the high-affinity site of the serum albumin.

Discussion

The experiments described herein show that the serum protein fractions are the main factors responsible for the diminished capacity of enterobactin to complex transferrin iron when the reaction is run in serum.

Moore et al. (1980) and Moore & Earhart (1981) reported that an immunoglobulin A (IgA) isotype antibody isolated from normal human serum inhibits ferric enterobactin uptake but does not affect iron assimilation mediated by ferrichrome or citrate. There is no direct evidence regarding the origin of these antibodies, and the nature of the actual immunogenic complex remains unknown. Small molecules such as enterobactin are not normally immunogenic unless coupled to a larger carrier, and a literature reference for the generation of antibody following injection of this siderophore cannot be found.

Since serum albumin is a general binder of a variety of small molecules which may be present in the circulatory system, it seems likely that enterobactin and also ferric enterobactin fall victims of this property of the protein. Albumin, like other plasma proteins, is distributed throughout most of the extracellular fluid of the body, in both the intravascular and extravascular compartments. The passage of albumin into interstitial fluid makes this transport protein available to all cells and extends its protective effect to a number of potentially toxic compounds, among them enterobactin.

We have proposed that the reason serum albumin binds enterobactin and not aerobactin can be traced to the chemical structures of these siderophores. Enterobactin possesses an aromatic character because it is comprised of three cyclized residues of 2,3-dihydroxy-*N*-benzoyl-L-serine, while aerobactin is a conjugate of citric acid with two residues of N^{ϵ}-acetyl-N^{ϵ}-hydroxyl-L-lysine. Enterobactin coordinates ferric iron octahedrally with six oxygens from six phenolic hydroxyl groups. The electrophoretic mobility of ferric enterobactin suggests that it carries three negative charges, while enterobactin at physiological pH has no charge (Pollack & Neilands, 1970). The isoelectric point of plasma albumin is near pH 5.0, and hence the protein carries a net negative charge at biological pH. The finding that the binding constants for enterobactin are not significantly different from those for [^{55}Fe]ferric enterobactin (Table I) can eliminate ionic bonding as the only binding mechanism; this suggestion was also postulated for the complex warfarin–albumin (Wilting et al., 1980a).

Two distinct binding sites for fluorescent probes and anionic drugs on HSA (sites I and II) were characterized by the fluorescence technique. The results suggested that not only electrostatic and dipolar forces but also steric factors and nonpolar residues play a role in both strength and specificity of binding. Drugs which bind to site II (diazepam site) are all aromatic carboxylic acids, which would be largely ionized at physiological pH. The conformation of these molecules is generally extended, and the negative charge is specifically located at one end of the molecule, away from the nonpolar region. Drugs which bind to site I (warfarin site) are also all aromatic acids. These are more bulky heterocyclic molecules in which the negative charge is generally delocalized to the center of a largely nonpolar molecule (Sudlow et al., 1976).

Several lines of evidence indicated that enterobactin and ferric enterobactin bind to a high-affinity binding site, possibly the "warfarin site", on HSA and BSA with an affinity comparable to that for the binding of other aromatic compounds (Brown & Shockley, 1982). The results obtained from the kinetic studies, the dialysis experiments, and the absorption spectra measurements confirm the data from uptake experiments. The binding constant for the attachment of enterobactin to albumin and the stoichiometry of the complex were established by the independent methods of dialysis and difference spectroscopy. The data are best accounted for in terms of a single site of high affinity, possibly supplemented with additional, weaker sites.

Binding of compounds to serum albumin competes with binding to sites in tissues and organs (i.e., receptors, transport system, etc.). Larger dosages of drugs can usually be administrated to compensate for the amount expected to bind to albumin. In the case of enterobactin, a siderophore common to many enteric bacteria, binding with the serum proteins immunoglobulin and albumin decreases its effective concentration and impedes enterobactin-mediated utilization of transferrin iron. The results obtained confirm the suggestion that virulence can be dependent on the synthesis of aerobactin rather than enterobactin. Aerobactin has no detectable affinity for albumin and hence can act as a mediator of iron uptake from iron transferrin to bacteria in the presence of serum proteins. In studies of the utilization of iron in serum, our results underline the need for prior knowledge of the number and chemical types of the siderophores synthesized by the

bacterial species under investigation.

The data presented are also relevant to the design of an effective ferric ion chelating agent for the treatment of transfusion-induced siderosis. The drug of choice for this condition is Desferal (Weatherall, 1981) although a number of synthetic catechols have been prepared and tested (Carrano & Raymond, 1979; Raymond & Chung, 1983). Our results suggest that future studies should be directed to a determination of the binding constant of the ligand with albumin and the synthesis of a line of deferration drugs not highly aromatic in character and with little binding avidity for serum proteins.

Registry No. Iron, 7439-89-6; enterobactin, 28384-96-5; ferric enterobactin, 62280-34-6; aerobactin, 26198-65-2.

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