INFECTION AND IMMUNITY, Feb. 1980, p. 418-423
0019-9567/80/02-0418/06$02.00/0

Vol. 27, No. 2

Bacteriostatic Enterochelin-Specific Immunoglobulin from Normal Human Serum

DAVID G. MOORE, ROBERT J. YANCEY,† CHARLES E. LANKFORD, AND CHARLES F. EARHART*

Department of Microbiology, The University of Texas at Austin, Austin, Texas 78712

Heat-inactivated normal human serum produces iron-reversible bacteriostasis of a number of microorganisms. This inhibitory effect was abolished by adsorption of serum with ultraviolet-killed cells of species that produce the siderophore enterochelin. Bacteriostasis also was alleviated by adsorption of serum with 2,3-dihydroxy-*N*-benzoyl-L-serine, a degradation product of enterochelin, bound to the insoluble matrix AH-Sepharose 4B. The adsorption process did not add iron or enterochelin to serum, nor did it remove transferrin. The immunoglobulin fraction from normal human serum was isolated; when added to a defined medium (M199) prepared so as to mimic normal human serum, the immunoglobulin rendered the medium inhibitory to an enterochelin-defective strain of *Salmonella typhimurium*. Adsorption of this medium with AH-Sepharose 4B-2,3-dihydroxy-*N*-benzoyl-L-serine removed the inhibition. Our results indicate that enterochelin-specific immunoglobulins exist in normal human serum. These immunoglobulins may act synergistically with transferrin to effect bacteriostasis of enterochelin-producing pathogens.

Complement-inactivated normal human serum is bacteriostatic for a number of pathogenic microorganisms (20, 21), including *Mycobacterium tuberculosis* (6), *Escherichia coli* (7), and *Salmonella typhimurium* (22). One factor which contributes to this inhibitory effect is the iron-binding serum protein transferrin. Like the iron-binding proteins present in other biological fluids (e.g., lactoferrin in human milk), transferrin limits bacterial growth by sequestering essentially all of the available iron. The bacteriostatic effect of human serum can be abolished by the addition of various iron compounds which result in saturation of the two transferrin iron-binding sites. Serum bacteriostasis can also be overcome by the addition of microbial iron chelators (siderophores), which are thought to provide bacteria with iron by successfully competing with transferrin for iron (21). Thus, serum inhibition of *S. typhimurium* growth is alleviated by the addition of iron-free enterochelin (enterobactin) (24), the siderophore produced by *E. coli* and *S. typhimurium*.

As indicated above, previous work on the bacteriostatic nature of serum has concentrated on the ability of transferrin to deprive microbes of iron. In addition, Rogers (17, 18) found that serotype-specific horse antiserum strongly potentiated the bacteriostatic effect of human transferrin, an effect abolished by adding a transferrin-saturating concentration of iron or by adding an "iron-transporting catechol." Rogers has attributed this effect primarily to a specific immunoglobulin (immunoglobulin G) not found in normal serum, which he believes inhibits the bacterial synthesis of the siderophore. In this study we report that normal human serum contains enterochelin-specific immunoglobulin that apparently inhibits enterochelin-mediated iron uptake. Such antibodies probably act in concert with transferrin to impede microbial iron uptake and thereby limit the growth of invading microorganisms.

MATERIALS AND METHODS

Bacteria. The organisms used are listed in Table 1. Unless otherwise indicated, strains were from the departmental culture collection. Strains bearing the designation SHD were originally obtained from the Texas State Department of Health Resources Laboratory, Austin.

Chemicals. Aminohexyl-Sepharose 4B (AHS4B) was purchased from Pharmacia Fine Chemicals, Inc., and Affigel 701 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were obtained from Bio-Rad Laboratories. Crystalline human transferrin was purchased from Sigma Chemical Co.

Growth of bacteria. Cultures were maintained at 4°C on brain heart infusion agar (Difco Laboratories) slants. Inocula and cells for adsorption were prepared from the second of two consecutive 12-h slants that had been incubated at 37°C. Cells were harvested, washed three times by centrifugation, and diluted if

† Present address: Department of Microbiology, School of Medicine, University of Missouri-Columbia, Columbia, MO 65201.

TABLE 1. *Organisms employed and their ability to alleviate serum bacteriostasis*

Alleviation of bacteriostasis		
Complete	Partial	None
S. typhimurium SR-11 (24)[a]	*Klebsiella pneumoniae*	*S. typhimurium* Fisher (autoclaved)
S. typhimurium 96-1 Ent^{-}[b] (24)[a]	*Vibrio cholerae* Inaba	*S. typhimurium* enb-1 Ent^{-}[b] (16)[a]
S. typhimurium Fisher		*S. typhimurium* enb-7 Ent^{-}[b] (16)[a]
S. typhimurium LT-2		*E. coli* RW193 *entA*[b] (10)[a]
E. coli AN102 *fep*[b] (4)[a]		*E. coli* AN93 *entE*[b]
E. coli AB1515 (8)[a]		*Proteus mirabilis* SHD
E. coli O55:B5		*Salmonella typhi* SHD H901
E. coli O111:B4		*Serratia marcescens* SHD
E. coli O127:B5		*Bacillus megaterium* ATCC 19213
Enterobacter aerogenes SHD		*Bacillus cereus*
Citrobacter bethesda		*Staphylococcus epidermidis* SHD
A. faecalis ATCC 19018[c]		*Streptococcus faecalis*
S. flexneri type II		*Streptococcus pyogenes* ATCC 10526
S. dysenteriae type I SHD		*Corynebacterium diphtheriae* mitis SHD 387471
		Corynebacterium diphtheriae subsp. *ulcerans* SHD 1471

[a] Reference number.
[b] Relevant marker or phenotype.
[c] Shown to produce DBS (and probably enterochelin) (9).

necessary in 0.029 M phosphate buffer, pH 7.0 (PB). All growth experiments were performed with *S. typhimurium* 96-1 (24) or *S. typhimurium* Fisher.

Bacterial growth was monitored by viable counts; appropriately diluted culture samples were spread onto nutrient agar (Difco) and enumerated after 15 to 18 h of growth at 37°C.

Media. Iron-deficient solid medium consisted of MM agar supplemented to 0.1 mM with α,α'-dipyridyl (11). Enterochelin was isolated from the supernatant fluid of cells grown in medium 56 (15) supplemented with 0.5 mg of $FeSO_4$ per liter (25) and required nutrients.

TC-medium 199 (Difco; M199) was rehydrated with demineralized water, filter sterilized, and stored as a 10×-concentrated solution at 4°C until needed. This defined medium is described as an ultrafiltrate of serum (12) and was used to mimic immunoglobulin-free serum. To provide the nonspecific, bacteriostatic component of serum, we added apotransferrin (T) to a physiological concentration (30 μM). The M199-T solution was incubated at 37°C for 2 h to allow chelation of iron (18 μM) present in M199 (6): this amount of iron was equivalent to 30% saturation of the transferrin added.

Pooled serum for bacterial culture was obtained from the blood of healthy human volunteers. Within 4 h after blood collection, serum was isolated, filter sterilized, and stored at −20°C. Iron saturation levels of transferrin in the several pooled serum samples ranged from 25 to 35%. Before use, the heat-labile components of complement were inactivated by heating the serum at 56°C for 30 min. Serum cultures were incubated at 37°C in a candle jar. The inoculum represented 1% of the total volume of serum.

Glassware used in experiments involving M199 and serum was rendered iron-free as before (24).

Assays. Serum transferrin iron saturation levels were determined in all sera by the method of Williams and Conrad (23), employing the chromagens 2,4,6-tripyridyl-*s*-triazine and 2,2′,2″-tripyridyl. The presence of transferrin was determined by the colorimetric assay of Bates and Wernicke (2). Phenolate quantitation was performed by the method of Arnow (1).

Preparation of coupled ligands. 2,3-Dihydroxy-*N*-benzoyl-L-serine (DBS) was coupled to AHS4B and Affigel 701 by the carbodiimide coupling procedure recommended by Pharmacia. The conjugation efficiency was determined by testing the slurry washes for phenolates. Essentially all free amino groups on either matrix were bound with DBS.

Lipopolysaccharide (LPS) from *S. typhimurium* SR-11, isolated by trichloroacetic acid precipitation (19), was coupled to human erythrocytes or Affigel 701 as follows. LPS (100 μg/ml) was boiled for 2 h in 0.1 N NaOH to solubilize the endotoxin and degrade any enterochelin present. The pH was adjusted to neutrality, and the cooled LPS was attached to pooled human erythrocytes by the method of Nowotny (13) or to Affigel 701 by the glutaraldehyde coupling procedure of Cambiaso et al. (3).

UV irradiation of bacterial cells and serum adsorption. Cells in PB were ultraviolet (UV) irradiated with a Sylvania type A germicidal lamp to a survival level of 10^{-6}, harvested, washed three times with PB, and suspended in complement-inactivated normal human serum to a cell density of approximately 5×10^9 colony-forming units per ml. The serum-cell mixture was incubated at 37°C for 30 min in a candle jar. After adsorption, cells were removed by centrifugation, and the serum was filter sterilized.

Adsorption of serum with Affigel-DBS, Affigel-LPS, AHS4B-DBS, or erythrocytes-LPS followed a similar protocol. Serum (5 ml) was adsorbed for 1 h at 37°C in a candle jar with 1 ml of AHS4B-DBS, 1 g of Affigel-DBS or Affigel-LPS, or 1 ml of packed erythrocytes-LPS. The ligand was collected by centrifugation, and the serum was then filter sterilized.

Enterochelin and DBS. Enterochelin was isolated from the supernatant fluid of late-log-phase cultures (5 liters) of *E. coli* AN102. The method of Yancey et al. (24) was used, except the initial diethylaminoethyl cellulose step was omitted. The purity of the enterochelin preparation was determined by comparison with authentic enterochelin by one- and two-dimensional thin-layer chromatography (14, 15). Ferri-enterochelin was prepared and stored at −20°C. Aqueous desferri-enterochelin solutions were prepared as needed in 0.1 M tris(hydroxymethyl)aminometh-

ane-hydrochloride buffer (pH 6.5) containing 20% (vol/vol) ethanol (8).

The pH 2.0 and 7.0 aqueous phases resulting from the wash of the ethyl acetate concentrate were saved for the purification of DBS (15).

Isolation of immunoglobulin. Whole immunoglobulin fractions were isolated by sodium sulfate precipitation by the method of Kekwick (5).

RESULTS

Yancey et al. (24) demonstrated that 10^3 colony-forming units of *S. typhimurium* SR-11 per ml requires 24 h to reach maximal growth in complement-inactivated serum and that *S. typhimurium* 96-1, an Ent$^-$ derivative of SR-11, fails to grow at all in this medium. *S. typhimurium* Fisher, although Ent$^+$, also failed to grow in complement-inactivated serum (Fig. 1). Strain Fisher, which is known to produce less enterochelin than does strain SR-11 (21), apparently produces an insufficient amount of enterochelin to escape serum bacteriostasis.

After adsorption of heat-inactivated normal serum with certain microorganisms, *S. typhimurium* 96-1 and Fisher were able to grow (Table 1). No gram-positive organisms and only certain gram-negative organisms that are members of the family *Enterobacteriaceae* (*Alcaligenes faecalis* excepted) were able to relieve the serum bacteriostasis. The ability of UV-killed cells to alleviate growth inhibition was dependent on their ability to synthesize the siderophore enterochelin; serum which had been adsorbed with mutants bearing specific defects in enterochelin biosynthesis (*E. coli* RW193 and AN93 and *S. typhimurium* enb-7 and enb-1) did not permit growth. Serum bacteriostasis also was retained after adsorption with autoclaved Ent$^+$ cells.

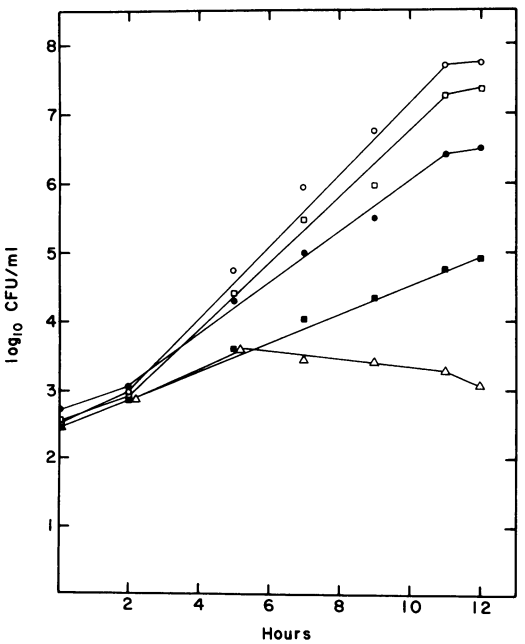

FIG. 1. *Growth of S. typhimurium Fisher in homologously adsorbed serum. The number of UV-killed cells per milliliter used for adsorption was as follows:* ○, *8 × 10^9;* □, *1.6 × 10^9;* ●, *8 × 10^8;* ■, *1.6 × 10^8;* △, *0. CFU, Colony-forming units.*

No evidence was found that the UV-killed cells released iron or enterochelin into serum during adsorption. After adsorption, the saturation level of transferrin was unaltered, and no enterochelin could be detected in serum by standard extraction and thin-layer chromatographic techniques. Thus, incubation of serum with appropriate UV-killed bacteria appeared to remove a bacteriostatic factor rather than add a growth-stimulating compound.

The growth of *S. typhimurium* Fisher in normal adsorbed serum is shown in Fig. 1. There was an initial brief lag and then approximately 8 h of exponential growth. The extent of growth and the growth rate were directly dependent on the concentration of cells with which the serum was adsorbed. Adsorption of serum with approximately 8×10^9 UV-killed cells per ml permitted maximal growth; adsorption with fewer than 10^8 UV-killed cells per ml had almost no effect. In all subsequent experiments, serum was adsorbed with approximately 5×10^9 UV-killed cells per ml.

The inability of Ent$^-$ strains to remove the serum bacteriostatic factor is shown in Fig. 2. Strain 96-1 grew normally in serum adsorbed with any of the three parental strains (*E. coli* AB1515 and *S. typhimurium* SR-11 and LT-2), and no growth was observed in unadsorbed serum. However, in serum adsorbed with Ent$^-$ cells, strain 96-1 failed to grow in all cases but one. These results implicated enterochelin biosynthetic ability with the capacity to remove the serum bacteriostatic factor. The one case in which adsorption with an Ent$^-$ strain did allow some subsequent growth of strain 96-1 was when homologously adsorbed serum was used (Fig. 2B). This suggested that strain 96-1 was "leaky" for enterochelin biosynthesis; this was confirmed when it was found that strain 96-1 cells could grow on iron-deficient solid medium. None of the other four Ent$^-$ mutants showed any growth on these plates. That strain 96-1 was leaky for enterochelin biosynthesis probably explains its ability to grow under the iron-deficient conditions present in serum.

That only Ent$^+$ cells were able to remove the serum bacteriostatic factor suggested that the inhibitor could be removed by adsorbing serum

with enterochelin-derived ligands attached to an inert matrix. DBS is a degradation product of enterochelin which, because it has a free carboxyl group, can be attached to insoluble matrices such as Affigel 701 or AHS4B. LPS (endotoxin) was also used as a ligand and coupled to both Affigel 701 and erythrocytes. Growth of strain 96-1 in serum adsorbed with these insoluble preparations is shown in Fig. 3. Adsorption in which DBS was used as a ligand completely removed serum bacteriostasis; adsorption with endotoxin was without effect. These results strongly argue that the inhibitory factor recognizes DBS and can be removed from serum because of its affinity for DBS. Other explanations for the ability of serum adsorbed with the appropriate UV-killed cells to support the growth of *S. typhimurium* 96-1 or Fisher have been eliminated. The data shown in Fig. 3 further confirm that leakage of iron or enterochelin

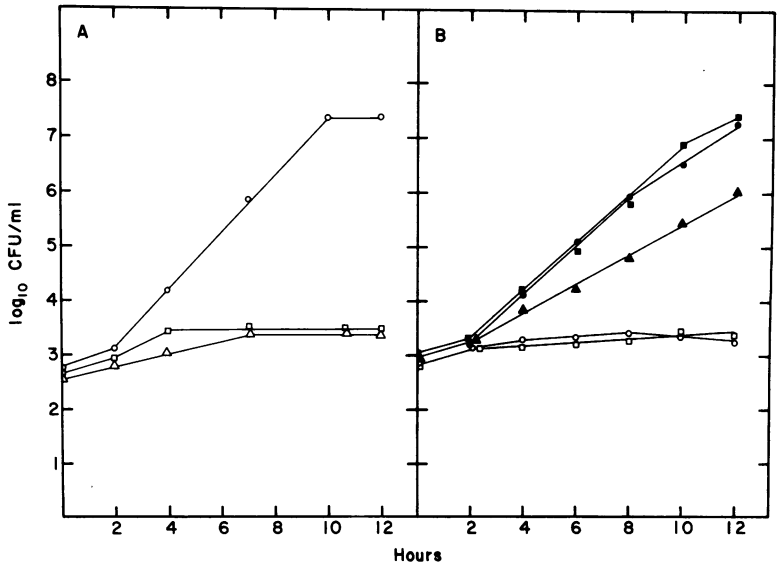

FIG. 2. *Growth of S. typhimurium 96-1 in serum adsorbed with E. coli (A) or S. typhimurium (B). (A): ○, AB1515; □, AN93; △, RW193. (B): ●, SR-11; ■, LT-2; ▲, 96-1; ○, enb-1; □, enb-7. CFU, Colony-forming units.*

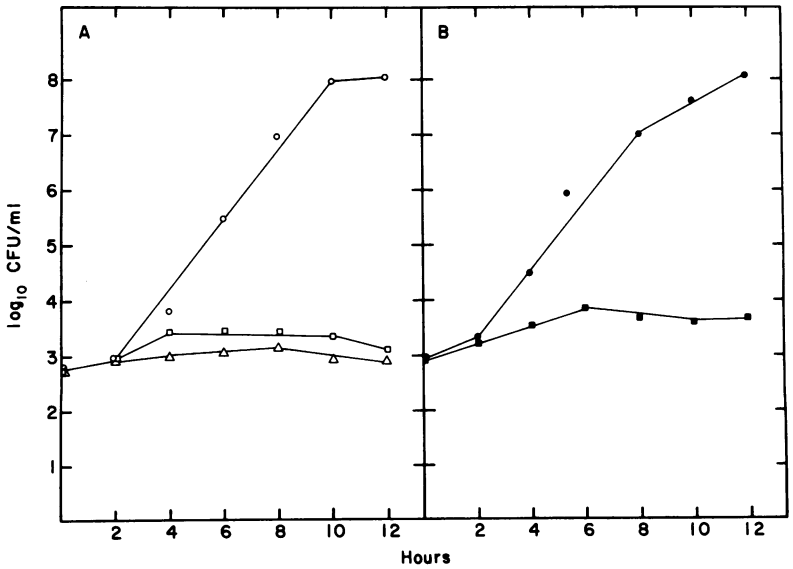

FIG. 3. *Growth of S. typhimurium 96-1 in serum adsorbed with carrier-associated DBS and LPS. (A): ○, Affigel-DBS; □, Affigel-LPS; △, erythrocytes-LPS. (B): ●, AHS4B-DBS; ■, unadsorbed. CFU, Colony-forming units.*

was not responsible. They also failed to provide evidence for the existence of bacteriostatic LPS-specific antibodies. The possibility that the abolition of serum bacteriostasis resulted from the removal of transferrin was also eliminated. The proteins bound to the AHS4B-DBS slurry were eluted with 0.5 M NaCl in 0.2 M glycine buffer, pH 2.6. After dialysis against phosphate-buffered saline, the proteins were assayed for transferrin with negative results. The possibility that the DBS coupled to the inert matrices was dissociating during adsorption was also excluded. Five milliliters of PB was incubated at 37°C for 1 h with AHS4B-DBS. The AHS4B-DBS was removed by centrifugation, and the PB was concentrated to 0.5 ml. This "adsorbed" PB, when added to serum at a final concentration of PB of 10%, did not stimulate the growth of strain 96-1.

The specificity involved in the adsorption studies suggested that a naturally occurring immunoglobulin was responsible for bacteriostasis in heat-inactivated serum. The immunoglobulin fraction was isolated from normal human serum and added in a physiological concentration to M199 supplemented with 30 μM T. This reconstituted medium (M199 + T + immunoglobulin) was assayed for bacteriostatic properties directly or adsorbed with AHS4B-DBS and then assayed for inhibitory activity. Reconstituted medium was 20-fold more bacteriostatic than medium without added immunoglobulin (M199 + T) (Fig. 4). Strain 96-1 grew equally well in M199, M199 + T, and M199 + T + immunoglobulin which had been adsorbed with AHS4B-DBS. These data showed that immunoglobulin fractions from normal human serum were capable of inhibiting the growth of strain 96-1 and that adsorption with AHS4B-DBS removed the inhibitory immunoglobulin.

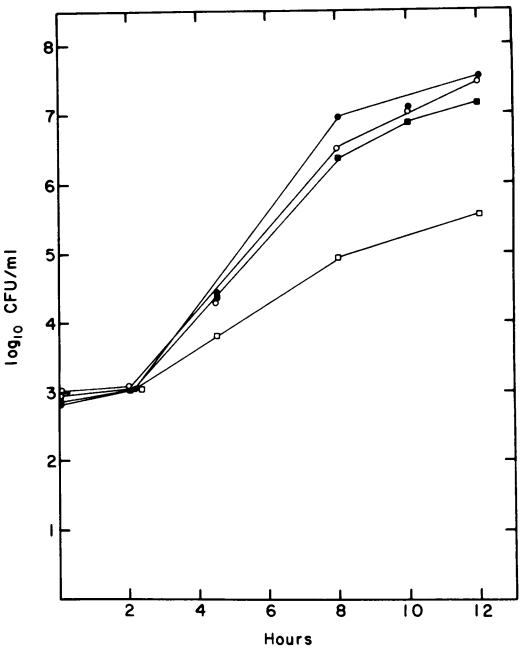

FIG. 4. *Effect of immunoglobulin and transferrin supplements on the growth of S. typhimurium 96-1 in defined media. Symbols:* □, *M199 + T + immunoglobulin;* ○, *M199 + T + immunoglobulin (AHS4B-DBS adsorbed);* ●, *M199;* ■, *M199 + T. CFU, Colony-forming units.*

DISCUSSION

Our results indicate that normal human serum contains an immunoglobulin capable of inhibiting the growth of enteric bacteria. This immunoglobulin was removed by adsorption of heat-inactivated human serum with a variety of whole-cell and antigen carrier preparations. The immunoglobulin appears to be specific for enterochelin: (i) Ent^- mutants of *E. coli* and *S. typhimurium* did not remove the inhibitory factor, and (ii) the factor could be adsorbed from serum with DBS coupled to inert matrices. Bacterial species which do not synthesize enterochelin, such as gram-positive organisms, did not remove the factor. Some of the strains used in this study have not been shown to produce enterochelin, but were capable of abolishing the bacteriostatic effect of serum. Our results suggest that organisms such as *Shigella dysenteriae* and *Shigella flexneri* synthesize enterochelin.

It is not possible at this time to answer questions regarding the origin of enterochelin-specific antibodies. They could have arisen from antigenic stimulation during a previous infection by an organism that produces enterochelin. Alternatively, such antibodies could result from the ubiquitous colonization of the human gut by *E. coli*. *E. coli* produces enterochelin as a response to iron deprivation; if immunogenic enterochelin complexes formed, subsequent antigenic stimulation of the appropriate antibody-producing cells could occur. Also, small molecules such as enterochelin are not normally immunogenic unless coupled to a larger molecule. Such a carrier molecule could be a protein involved in the excretion or uptake of enterochelin or, as Kochan et al. (7) have suggested, LPS. Research is under way to determine the nature and identity of this immunogenic complex.

The presence of an enterochelin-specific antibody in normal human serum provides an additional line of defense against enteric invaders. Upon entry into the blood, any microorganism must compete with the serum protein transferrin

for iron. To accomplish this task, the microorganisms produce siderophores. Yancey et al. (24) have shown that the production of enterochelin by *S. typhimurium* or the addition of exogenous enterochelin to normal human serum stimulates the growth of an enterochelin-defective strain and its parent in an otherwise iron-limiting environment. In addition, the production of enterochelin by *S. typhimurium* was shown to enhance the virulence of the organism in mice. Iron sequestered by transferrin would provide only a transitory defense against infection by enterochelin-producing pathogens. However, immunoglobulin specific for enterochelin could selectively inhibit this iron uptake mechanism and, by acting in concert with transferrin, iron starve invading microbes. This would be particularly true when the initial concentration of pathogens was small, so that the possibility of their synthesizing enterochelin in sufficient quantity to overcome the anti-enterochelin immunoglobulin titer would be remote.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant PCM 75-07693 (to C.F.E.) and by a grant from the University Research Institute of the University of Texas at Austin (to C.E.L.). D.G.M. is a Public Health Service predoctoral trainee.

We thank L. J. Berry for providing us with endotoxin and for reading the manuscript.

LITERATURE CITED

1. **Arnow, L. E.** 1937. Colorimetric determinations of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531-537.
2. **Bates, G. W., and J. Wernicke.** 1971. The kinetics and mechanism of iron (III) exchange between chelates and transferrin. The reaction of transferrin with iron (III) nitrilotriacetate. J. Biol. Chem. **246**:3679-3685.
3. **Cambiaso, C. L., A. Goffinet, J.-P. Vaerman, and J. F. Heremans.** 1975. Glutaraldehyde-activated aminohexyl derivative of sepharose 4B as a new versatile immunoabsorbent. Immunochemistry **12**:273-278.
4. **Cox, G. B., F. Gibson, R. K. J. Luke, N. A. Newton, I. G. O'Brien, and H. Rosenberg.** 1970. Mutations affecting iron transport in *Escherichia coli*. J. Bacteriol. **104**:219-226.
5. **Kekwick, R. A.** 1940. The multiple serum proteins in multiple myelomatosis. Biochem. J. **34**:1248-1257.
6. **Kochan, I.** 1969. Mechanism of tuberculostasis in mammalian serum. I. Role of transferrin in human serum tuberculostasis. J. Infect. Dis. **119**:11-18.
7. **Kochan, I., J. T. Kvach, and T. I. Wiles.** 1977. Virulence-associated acquisition of iron in mammalian serum by *Escherichia coli*. J. Infect. Dis. **135**:623-632.
8. **Langman, L., I. G. Young, G. E. Frost, H. Rosenberg, and F. Gibson.** 1972. Enterochelin system of iron transport in *Escherichia coli*: mutations affecting ferric-enterochelin esterase. J. Bacteriol. **112**:1142-1149.
9. **Lankford, C. E.** 1973. Bacterial assimilation of iron. Crit. Rev. Microbiol. **2**:273-331.
10. **Leong, J., and J. B. Neilands.** 1976. Mechanisms of siderophore iron transport in enteric bacteria. J. Bacteriol. **126**:823-830.
11. **McIntosh, M. A., C. L. Pickett, S. S. Chenault, and C. F. Earhart.** 1978. Suppression of iron uptake deficiency in *Escherichia coli* by loss of two outer membrane proteins. Biochem. Biophys. Res. Commun. **81**:1106-1112.
12. **Morton, H. J., J. F. Morgan, and R. C. Parker.** 1951. Nutrition of animal cells in tissue culture. V. Effect of initial treatment of cultures on their survival in a synthetic medium. J. Cell. Comp. Physiol. **38**:389-400.
13. **Nowotny, A.** 1969. Basic exercises in immunology, p. 144. Springer-Verlag, New York.
14. **O'Brien, I. G., G. B. Cox, and F. Gibson.** 1970. Biologically active compounds containing 2,3-dihydroxybenzoic acid and serine formed by *Escherichia coli*. Biochim. Biophys. Acta **201**:453-460.
15. **O'Brien, I. G., G. B. Cox, and F. Gibson.** 1971. Enterochelin hydrolysis and iron metabolism in *Escherichia coli*. Biochim. Biophys. Acta **237**:537-549.
16. **Pollack, J. R., B. N. Ames, and J. B. Neilands.** 1970. Iron transport in *Salmonella typhimurium*: mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. **104**:635-639.
17. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7**:445-456.
18. **Rogers, H. J.** 1976. Ferric iron and the antibacterial effect of horse 7S antibodies to *Escherichia coli* O111. Immunology **30**:425-433.
19. **Staub, A. M.** 1965. Bacterial lipido-proteins-polysaccharides ("O" somatic antigens). Extraction with trichloroacetic acid. Methods Carbohydr. Chem. **5**:92.
20. **Weinberg, E. D.** 1971. Roles of iron in host-parasite interactions. J. Infect. Dis. **124**:401-410.
21. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45-66.
22. **Wilkins, T. D., and C. E. Lankford.** 1970. Production by *Salmonella typhimurium* of 2,3-dihydroxybenzoylserine, and its stimulation of growth in human serum. J. Infect. Dis. **121**:129-135.
23. **Williams, H. L., and M. E. Conrad.** 1966. A one-tube method for measuring the serum iron concentration and unsaturated iron-binding capacity. J. Lab. Clin. Med. **67**:171-176.
24. **Yancey, R. J., S. A. L. Breeding, and C. E. Lankford.** 1979. Enterochelin (enterobactin): virulence factor for *Salmonella typhimurium*. Infect. Immun. **24**:174-180.
25. **Young, I. G.** 1976. Preparation of enterochelin from *Escherichia coli*. Prep. Biochem. **6**:123-131.