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Assmilation of Iron by Pathogenic *Neisseria* spp.

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Iron assimilation by *Neisseria meningitidis* and *Neisseria gonorrhoeae* has been shown to be important to their growth and virulence. Iron acquisition in vitro was studied in an agar diffusion assay employing the iron-binding protein conalbumin. The ability of various iron compounds to alter the growth-inhibitory effect of conalbumin was investigated. On an equimolar iron basis, citrate-containing iron compounds were most effective; hemin was slightly less effective; ferrous sulfate and ferrous ammonium sulfate were even less effective; and the ferric compounds, ferric nitrate, ferric chloride, and ferric dextran (Imferon), were least effective. The results suggested that, as with *Escherichia coli* and certain other bacteria, the *Neisseria* spp. may utilize a citrate-mediated iron transport system. Microbial siderophores were also tested for their ability to relieve the growth-inhibitory effect of conalbumin. Two phenolate siderophores and Desferal enhanced growth inhibition in the deferrated form, but were inactive in the ferrated form. Several trihydroxamates of the ferrichrome family and coprogen were inactive in either the deferrated or ferrated forms. Of the 12 different siderophores tested, only the dihydroxamates (schizokinen, arthrobactin, and aerobactin) were stimulatory, but then only in the ferrated forms. Apparently, even though those siderophores could be utilized as specific iron transport agents by the *Neisseria* spp., they could not compete with conalbumin for iron under these assay conditions.

Iron, a nutrient which is essential to all forms of life (20, 22), is made relatively inaccessible to parasitic microorganisms by the powerful iron-binding proteins of the body fluids of vertebrate hosts (37). These iron-binding glycoproteins of the transferrin class (1), transferrin in plasma, lactoferrin in secretions, and conalbumin, the transferrin counterpart, in egg white and chicken blood, are located at sites frequently assaulted by microbes. Numerous in vitro and in vivo studies have demonstrated that the iron-binding capability of these proteins is an important mechanism in nonspecific resistance to infection (5, 37).

To multiply in the normally iron-restricted environment of the host, microbes have developed mechanisms for iron acquisition and transport. These are dependent upon production of low-molecular-weight, iron-transporting and -solubilizing agents, known collectively as siderophores (20, 33). The characterized siderophores are of two chemical classes: hydroxamate, such as ferrichrome (10), Desferal, and schizokinen (6); and phenolate, such as enterochelin (enterobactin; 23, 32). The production of these iron-chelating agents was demonstrated to influence the outcome of infection with *Escherichia coli* (34) or *Salmonella typhimurium* (17, 19, 38). However, little is known about the role of siderophores in the virulence of other pathogens.

Payne and Finkelstein demonstrated that iron availability influences the virulence of *Neisseria gonorrhoeae* and *Neisseria meningitidis* (25, 26, 28, 30). The organic iron compounds, ferric ammonium citrate, Imferon, and Blutal, enhanced the apparent virulence of normally avirulent T3 or T4 colony types for 11-day chicken embryos (25). Treatments which decreased iron availability, such as injection of conalbumin or lipopolysaccharide, decreased apparent virulence of normally virulent T1 and T2 colony types. Whereas the exalted virulence of wild-type *N. meningitidis* for chicken embryos was not appreciably affected by manipulation of iron levels, the virulence of avirulent mutants, isolated on the basis of their reduced ability to bind Congo red dye, was markedly enhanced by iron (26). Gonococcus strains isolated from disseminated gonococcal infections more closely resemble meningococci than urogenital isolates in their ability to acquire iron (28).

In in vitro studies, Kellogg et al. (18) observed that ferrous iron, but not hemin or ferrous gluconate, influenced the colony diameter of *N. gonorrhoeae*. More recently, Payne and Finkel-

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stein (27) found that iron dextran (Imferon) and, to a lesser extent, ferric nitrate, increased the average colony size of both *N. gonorrhoeae* and *N. meningitidis*, and these authors recommended the addition of Imferon to diagnostic media for the isolation of pathogenic *Neisseria* spp. Hafiz and co-workers (13, 24) reported that ferric ammonium citrate and the iron-chelating agents, nitrilotriacetate and citrate, promoted reversion of *N. gonorrhoeae* from colony type 4 (T4) to colony type 1 (T1). This, however, was not the basis for the increased virulence of iron-supplemented T3 or T4 inocula observed previously (25) since in vivo selection of virulent colony types did not occur (25).

The in vitro iron requirement of *N. meningitidis* has been more extensively studied (2, 4, 7) than that of the gonococcus. Calver et al. (7) found that apoferritin and the iron-chelating agents Desferal and ethylenediamine-diorthohydroxyphenyl-acetate interfered with the ability of the meningococcus to acquire iron. Archibald and DeVoe (4) reported that a variety of iron-containing substances, including certain forms of chelated iron and transferrin, but not conalbumin (3), could fulfill this organism's iron requirement. Iron compounds, especially iron dextran, influenced the course of *N. meningitidis* infection in mice (8, 16), whereas Desferal, the iron-chelating agent, reduced mortality due to the bacterium (16).

The present paper compares the ability of a variety of iron compounds to promote the growth of *N. gonorrhoeae* and *N. meningitidis* in an environment rendered deficient in iron by the iron-binding protein conalbumin. The ability of a variety of microbial siderophores of exogenous origin to function in the iron metabolism of the pathogenic *Neisseria* spp. was also evaluated.

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MATERIALS AND METHODS

Bacterial strains. All strains were as previously described (28). *N. gonorrhoeae* 6555, isolated from a disseminated gonococcal infection was obtained from K. K. Holmes, University of Washington, School of Medicine, Seattle. Strain 2686, isolated at the Center for Disease Control from a genital tract infection, was provided by T. M. Buchanan, University of Washington, School of Medicine. *N. meningitidis* serogroup strains A-17, B-11, and C-11 were obtained from H. Schneider, Walter Reed Army Institute of Research, Washington, D.C. (28). Strains were maintained by lyophilization and by storage at −70°C in buffered peptone-saline broth (GC medium base formulation as per Difco Laboratories, without agar and starch) with 20% glycerol. All cultures were incubated at 36°C with 5% CO_2.

Media. Imferon agar (27) was used routinely for the growth of all strains.

The conalbumin agar medium used for assay of iron and siderophores was modified from that used previously (28). This was composed of GC medium base (Difco; lot no. 671369) with 2% defined supplement (C. E. Lankford, Bacteriol. Proc., p. 40, 1950; omitting the ferric nitrate), and 500 μg of "iron-free" conalbumin (type 1, lot no. 39C8000; Sigma Chemical Co., St. Louis, Mo.) per ml of medium. The lot of medium base and conalbumin greatly influenced the efficiency of the assay, probably due to the amount of iron contaminating either constituent. The amount of iron contaminating the lot of medium base used in these studies, as determined by the ferrozine method of Stookey (36), was theoretically sufficient to saturate 96.5% of the iron-binding capability of the conalbumin used. Conalbumin (20 mg/ml) in deionized water was sterilized by filtration through a 0.45-μm pore size membrane filter (Millipore Corp., Bedford, Mass.) and added aseptically to the molten 48°C medium.

Conalbumin agar bioassay. The conalbumin agar plate technique of Payne and Finkelstein (28) was used as a biological assay for iron and iron-chelating agents. Cultures (18 h) from Imferon agar were harvested and diluted in buffered peptone-saline to a final inoculum of 5×10^4 to 10×10^4 colony-forming units per ml of molten conalbumin agar medium (48°C). Plates (100 by 15 mm) were poured immediately, with 20 to 25 ml of seeded medium per plate. When the medium had solidified, 4.5-mm wells were cut in the plates, and 50 μl of test solution was added to the wells. After 20 and 48 h of incubation, the plates were examined, and the zones of stimulation or inhibition of growth were measured by using a stereoscopic dissecting microscope fitted with an optical reticle.

Siderophores. Enterochelin was isolated from culture supernatant fluids of *E. coli* AN 102 as previously described (38). The phenolate siderophore of *Vibrio cholerae* (29), designated herein as "vibriochelin," was crystallized from culture supernatant fluids of *V. cholerae* 3083 by the same procedure, except that the siderophore was directly extracted with ethyl acetate from the culture fluid. Desferal, the methane sulfonate of iron-free ferrioxamine B, was purchased from Ciba Pharmaceutical Co., Summit, N.J. Aerobactin was generously provided by B. R. Byers, University of Mississippi Medical Center, Jackson. All other siderophores were a gift from C. E. Lankford, University of Texas at Austin (6).

Ferric-hydroxamic acid siderophores were deferrated by the alkaline precipitation of iron (10). Phenolic acid chelators were isolated in the deferri- form (38) and saturated by the addition of ferric iron as $FeCl_3 \cdot 6H_2O$.

Iron compounds. The inorganic iron compounds used were ferric chloride, ferric nitrate, ferrous ammonium sulfate (Mallinckrodt, Inc., St. Louis, Mo.), and ferrous sulfate (Sigma). These were dissolved in mM HCl at a concentration of 54 mM iron (3 mg/ml) and sterilized by membrane filtration. The organic iron compounds tested included ferric ammonium citrate (J. T. Baker Chemical Co., Phillipsburg, N.J.; iron

concentration determined by the ferrozine method of Stookey [36]), iron sorbitol citrate (Jectofer; Astra Pharmaceutical Products, Inc., Worcester, Mass.), and iron dextran (Imferon; Merril-National Laboratories, Cincinnati, Ohio). Ferric ammonium citrate was dissolved in deionized water at a concentration of 54 mM and filter sterilized; the other two organic iron compounds were supplied as sterile solutions.

RESULTS

Conalbumin agar bioassay. The addition of iron as ferric ammonium citrate to the wells of a conalbumin agar plate supported the growth of the strains of *N. gonorrhoeae* and *N. meningitidis* tested. At 20 h of incubation, growth of the indicator strain was completely suppressed by the iron-restricting effect of conalbumin, except around the wells to which iron was added. The size of the zone was proportional to the quantity of iron compound added to each well (Fig. 1A). With no iron added, no zone of stimulation appeared around the wells (not shown in Fig. 1). After 48 h of incubation, the zones of stimulation, while still evident, became less distinct. The ability of the bacteria eventually to grow in this iron-restricted environment probably was due to production of endogenous siderophore(s) which competes with conalbumin for iron (11, 39). The ability of the bacteria eventually to overcome inhibition by conalbumin also allowed the assay to be used to detect inhibitory compounds. Those compounds produced zones of inhibition or clearing on the background lawn similar to the inhibition produced by antibiotics. Although *N. meningitidis* B-11 only is pictured in Fig. 1, the conalbumin agar assay was equally effective for studying the iron requirement of *N. gonorrhoeae*. Whenever possible, a control solution of Imferon at 15 μg of iron per well was added to the assay plates. The coefficient of variation between numerous assays was less than 10%, whereas the variation within a given assay was never more than 4.7%.

Effect of iron compounds. Since the size of the zones was directly proportional to the concentration of iron added to the wells, different iron compounds were compared for their ability to negate the growth-inhibitory effect of conalbumin for *N. gonorrhoeae* (Fig. 2). By this assay the citrate-containing iron compounds, iron sorbital citrate (Jectofer) and ferric ammonium citrate, were nearly equivalent, and they were the most effective of the iron compounds tested. Sodium citrate (pH 7.0), citric acid (pH 7.0), and *d*-sorbitol were not effective at concentrations up to 3 mg/ml (150 μg/well). Hemin, on an equimolar iron basis, was only slightly less effective than the citrate-containing compounds. The ferrous iron compounds, ferrous sulfate and ferrous ammonium sulfate, were the next most effective. The ferric iron compounds, ferric chloride, ferric nitrate and, finally, iron dextran (Imferon), were the least effective.

Although there was some strain-to-strain variation, the meningococcus responded to iron addition in the conalbumin agar assay with larger zones of exhibition than did the gonococcus. The

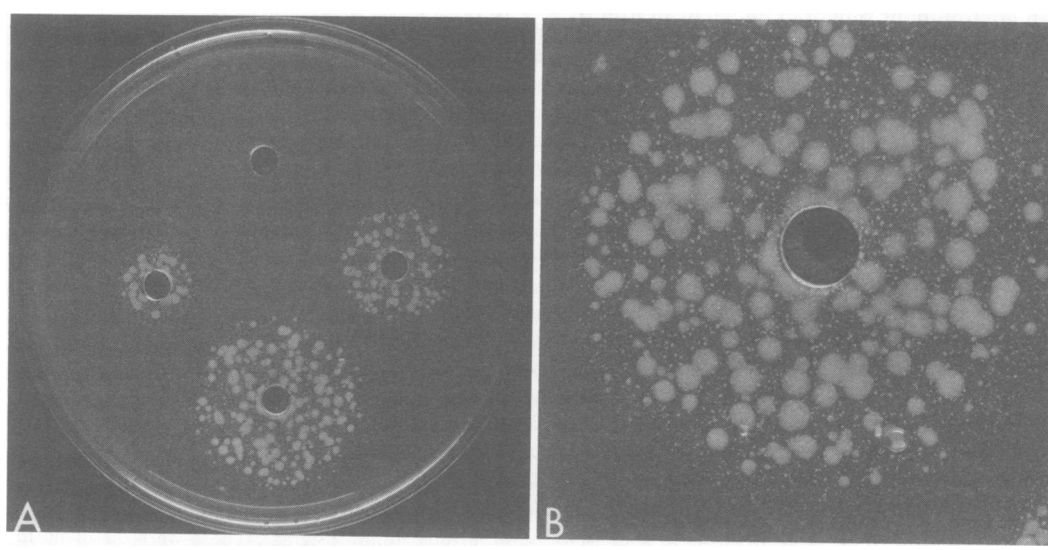

FIG. 1. *Growth of N. meningitidis strain B-11 in conalbumin agar (20 h at 36°C in 5% CO_2) in response to various concentrations of iron provided as ferric ammonium citrate. (A) Response to 150, 15, 1.5, and 0.15 μg of iron per well. (B) Higher magnification of the zone at 150 μg of iron per well. The actual diameter of the zone was 30 mm, and the wells were 4.5 mm in diameter.*

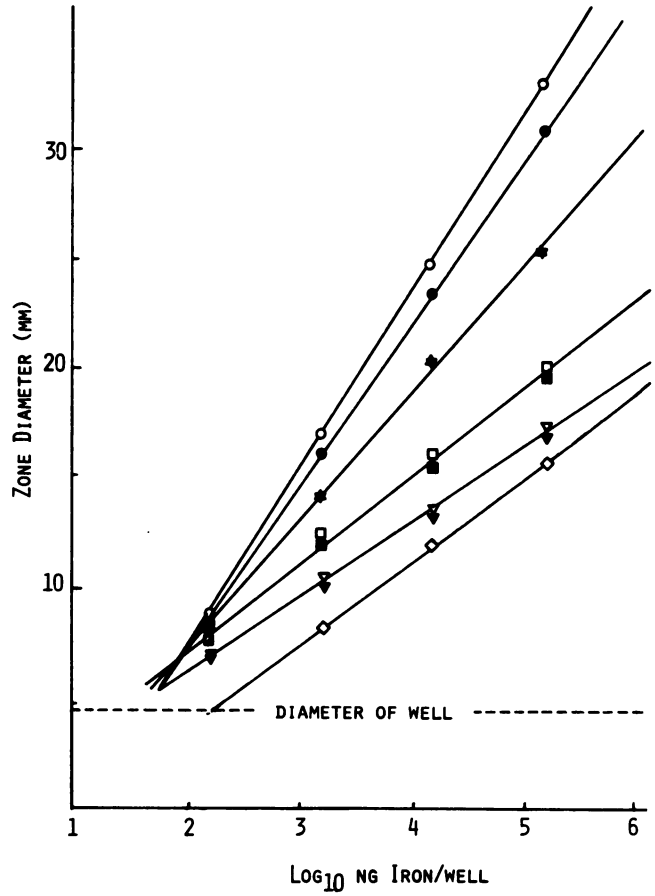

FIG. 2. *Response of N. gonorrhoeae strain 6555 to various iron compounds in the conalbumin agar assay. Iron was provided as follows (mean number of determinations in parentheses): Jectofer (3), ○; ferric ammonium citrate (5), ●; hemin (2), ★; ferrous sulfate (5), □; ferrous ammonium sulfate (3), ■; ferric chloride (4), ▽; ferric nitrate (3), ▼; and Imferon (5), ◇.*

relative effectiveness of the iron compounds, however, was identical. In general, also, the avirulent colony types (T3 and T4) of *N. gonorrhoeae* 6555 and 2686 produced slightly larger zones in response to iron than did the virulent colony types (T1 and T2).

Effect of microbial siderophores. Microbial siderophores varied in their ability to relieve the growth-inhibitory effect of conalbumin for *N. gonorrhoeae* 6555 and 2686 and *N. meningitidis* B-11 when tested in the conalbumin agar assay (Table 1). One group, including the phenolate siderophores, enterochelin and vibriochelin (29), and the secondary trihydroxamic acid, desferrioxamine B mesylate (Desferal), was found to be inhibitory in the deferrated form and had no effect in the iron-saturated form. Another group, which included the fungal hydroxamate siderophores of the structurally related ferrichrome family (33), ferrichrome, ferrichrome A, ferrichrysin, ferricrocin, and ferrirubin, was inactive in either the iron-bound or iron-free forms. This also was true for the structurally dissimilar trihydroxamate, coprogen. A third group of siderophores, represented by the dihydroxamate derivatives, schizokinen, aerobactin, and arthrobactin, was effective in overcoming the inhibitory effect of conalbumin, but only in the iron-complexed form. The deferri-compounds were not stimulatory for either the gonococcus or the meningococcus.

That the growth-enhancing effect of the dihydroxamate siderophores was due to the iron-chelate complex and not due to the presence of uncomplexed iron in these solutions is demonstrated in Fig. 3. In this experiment schizokinen was deferrated and then referrated by the addition of ferric chloride to provide an iron/siderophore ratio of 1:1 (100% saturated in Fig. 3) or 1:4 (25% saturated). When the ferri-siderophores

TABLE 1. *Effects of various siderophores on the pathogenic Neisseria spp.*

Compound tested[a]	Microbial source	Chemical class[b]	Effect[c]	
			Ferrated form[d]	Deferrated form
Enterochelin	*E. coli*	P	0	I
Vibriochelin	*V. cholerae*	P	0	I
Desferrioxamine B mesylate	*Streptomyces* spp.	3H	0	I
Ferrichrome	*Ustilago* sp.	3H	0	0
Ferrichrome A	*Ustilago* sp.	3H	0	0
Ferrichrysin	*Aspergillus* spp.	3H	0	NT
Ferricrocin	*Aspergillus* spp.	3H	0	NT
Ferrirubin	*Penicillium* spp.	3H	0	NT
Coprogen	*Penicillium* spp.	3H	0	0
Schizokinen	*B. megaterium*	2H	S	0
Arthrobactin	*Arthrobacter pascens*	2H	S	0
Aerobactin	*Aerobacter aerogenes*	2H	S	0

[a] Compounds were in 500 μM solutions, 50 μl per well.
[b] Abbreviations: P, phenolate; 3H, trihydroxamate; 2H, dihydroxamate.
[c] Abbreviations: 0, no effect (<5-mm zone diameter); I, inhibition (>20-mm zone diameter); S, stimulation (>20-mm zone diameter); NT, not tested.
[d] Ratio of siderophore/iron (as $FeCl_3$) was 1:1.

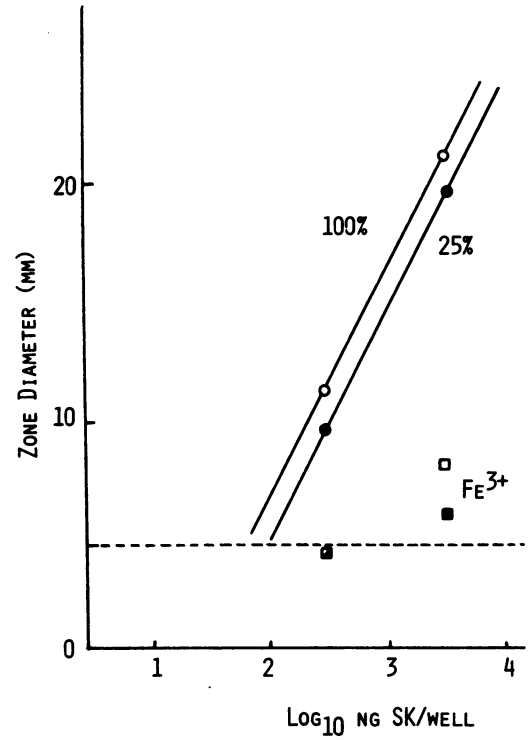

FIG. 3. *Influence of schizokinen (SK) saturation level on the growth of N. gonorrhoeae strain 2686. Schizokinen was deferrated and referrated by the addition of ferric chloride to provide an iron/siderophore ratio of 1:1 (100% saturation), ○, or 1:4 (25% saturation), ●. The square figures represent the response of N. gonorrhoeae to that amount of iron without chelator. The dotted line represents the diameter of the wells.*

were added to conalbumin agar assay plates containing *N. gonorrhoeae* strain 2686 (T3), the zones of exhibition induced by both the 100% and the 25% saturated solutions were of similar size at the two concentrations tested. The same quantity of iron used to form the ferric complex produced zones more than 10 mm in diameter smaller at the highest concentration tested. At the lower concentration of ferri-schizokinen that quantity of iron was totally ineffective.

As with the iron compounds, the meningococcus was more responsive to addition of schizokinen than was the gonococcus (Fig. 4). *N. meningitidis* strain B-11 formed larger zones in response to lower concentrations of schizokinen than did *N. gonorrhoeae* strain 6555.

DISCUSSION

A bioassay derived from one used previously by our laboratory (28) allowed partial analysis of the mechanisms of iron acquisition by the *Neisseria* spp. Conalbumin, the transferrin counterpart in egg white and chicken blood, was incorporated into GC medium base at a concentration adequate to chelate all the iron present in the medium, providing a theoretical iron saturation level of the conalbumin of 96.5%. This saturation level of conalbumin caused growth inhibition at 20 h which was overcome by the addition of various iron compounds or iron siderophores. The bacteria were able to acquire the iron necessary for their growth, presumably by endogenously produced siderophores when the incubation time was extended. Conalbumin has previously been shown to inhibit growth of *N. gonorrhoeae* (28) and *N. meningitidis* (3, 28) in agar diffusion assays. The size of the zones was proportional to the amount of iron added to the assay wells. As expected, higher concentrations of the iron-binding protein decreased the sizes of the zones, although the relative effectiveness of the compounds remained the same. The size of the surface colonies was influenced by the proximity of the colonies to the well and, thus, by iron availability. Kellogg et al. (18) and Payne and Finkelstein (27) observed a colony size variation influenced by the iron content of the medium for *N. meningitidis* or *N. gonorrhoeae* or both.

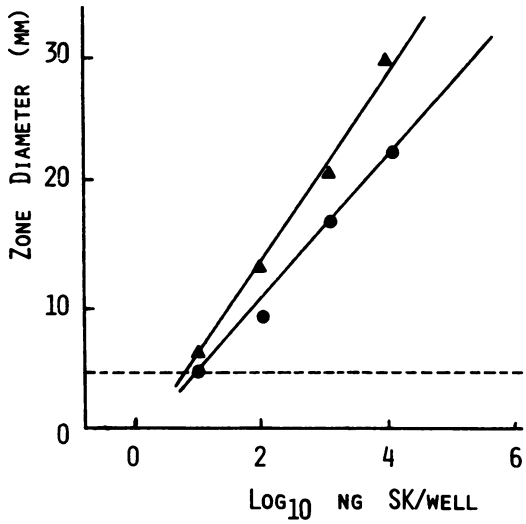

FIG. 4. *Response of N. gonorrhoeae strain 6555 (●) and N. meningitidis strain B-11 (▲) to various concentrations of schizokinen (SK). The dotted line represents the diameter of the wells.*

The greater effectiveness of citrate-containing iron complexes might suggest that both the gonococcus and meningococcus utilize a citrate-dependent iron transport system similar to that of *E. coli* (12). Archibald and DeVoe (4) observed that citrate enhanced iron utilization by *N. meningitidis*. However, it should be emphasized that the assay results with this or other agar-diffusion assays are affected significantly by the solubility and diffusibility of the iron sources. That Imferon was less effective than ferric nitrate and ferric chloride was probably due to the lower diffusibility of this iron-dextran complex, since under proper conditions, Imferon has been shown to be a highly potent iron source both in vitro (27) and in vivo (16, 25). This assay was also influenced by the ability of conalbumin to chelate the iron sources provided and to sequester the iron in the medium. The greater effectiveness of the ferrous iron compounds compared with the ferric compounds possibly was due to the inability of the iron-binding protein to bind ferrous iron until it is oxidized to the ferric state. However, the difference could also be a reflection of the greater solubility and diffusibility of ferrous iron. That hemin is readily utilized by the *Neisseria* spp. was interesting, but not unexpected, since hemoglobin-containing media have been used for years to grow pathogenic *Neisseria* spp. The meningococcus and gonococcus are not, however, as exquisitely responsive to heme compounds as was *Yersinia pestis* (31). Whether the difference in zone sizes produced by the *Neisseria* spp. in response to the iron compound, i.e., *N. meningitidis* > *N. gonorrhoeae* T3/T4 > *N. gonorrhoeae* T1/T2, was due to enhanced ability of these organisms to grow in vitro or reflected some real difference in their ability to utilize iron compounds cannot be accurately assessed by this assay.

The microbial siderophores tested fell within three groups, based upon their effectiveness for *N. gonorrhoeae* and *N. meningitidis*. One group of siderophores evidently could compete successfully with conalbumin (and endogenously produced siderophores) (39) for iron, but could not be used for iron transport by the pathogenic *Neisseria* spp. These included the phenolate siderophores, enterochelin and vibriochelin (29), and the secondary trihydroxamate, Desferal. They were inhibitory in the deferri form, but ineffective when iron saturated.

Payne and Finkelstein (28) reported that the gonococcus was unable to use enterochelin, and Archibald and DeVoe (4) found that *N. meningitidis* was also unable to use that phenolate. Our results were in agreement with those two observations, but in variance with the report of Payne and Finkelstein (28) that crude culture supernatant fluids of several enterochelin-producing enteric strains and *V. cholerae* were stimulatory for the gonococcus (after removal of an inhibitory factor by gel filtration). It is probable that those culture supernatant fluids contained factors other than enterochelin or vibriochelin, siderophore or otherwise, active in those assays. The explanation offered by Archibald and DeVoe (4), that this growth stimulation was the result of iminodiacetate present in the media as a result of Chelex-100 treatment, is untenable since the media were deferrated by conalbumin treatment (28).

Desferal has been reported to be inhibitory by virtue of its iron-binding ability for the meningococcus both in vitro (7, 28) and in vivo (16). Recently, Mickelson and Sparling utilized the ability of Desferal to sequester iron from the *Neisseria* spp., including *N. gonorrhoeae*, to develop an agar diffusion assay to study the iron metabolism of the *Neisseria* (P. A. Mickelson and P. F. Sparling, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B82, p. 30).

Another group of siderophores, all of which were trihydroxamates, could not compete with conalbumin (and endogenously produced siderophores) for iron and were not utilized for iron transport by either the gonococcus or the meningococcus. These included several trihydroxamic acids of the ferrichrome family and the fungal siderophore, coprogen. These compounds were ineffective in this assay in either the deferri- or ferrated forms. Neilands earlier reported that iron-free ferrichrome could not remove iron

from saturated conalbumin (21). It is interesting, and probably reflects differences in the assays themselves, that deferri-coprogen, which is structurally related to deferri-rhodotorulic acid (33), was not inhibitory in our hands for either *Neisseria* species, whereas Archibald and DeVoe found deferri-rhodotorulic acid inhibitory for *N. meningitidis* (4).

The third group of microbial siderophores, the structurally related dihydroxamic acids, schizokinen, arthrobactin, and aerobactin, could not compete with conalbumin (and endogenously produced siderophores) for iron, but could be utilized for iron transport; these compounds were only effective in the iron form. Their ability to serve as iron transport compounds may be related to a citrate-dependent transport mechanism since citrate forms the backbone of all of these compounds. On the other hand, a citrate-mediated transport system may simply be a byproduct of a transport system specific for these dihydroxamic acids.

The ability of a pathogen to acquire its iron from the host appears to be an important determinant of infection (37). Unless, as has been suggested (3), the pathogenic *Neisseria* spp. can acquire iron directly from the host iron-binding proteins such as transferrin, the most likely candidates for transfer of iron from the iron-binding protein to the microbe are endogenously produced (and/or exogenously produced [i.e., by other bacteria], but readily utilized) siderophores. The endogenously produced siderophores, which appear in many respects to be quite similar to the dihydroxamate type siderophores, are the subject of another report (39). Exogenously produced siderophores which can chelate iron from iron-binding proteins, but which are not utilized by the Neisseria spp. (e.g., enterochelin and Desferal), would provide an added barrier to infection. Enterochelin, which has the largest formation constant (log K_f, 52) of any iron chelator reported (15), can effectively compete with transferrin for iron (9) and may be one of the mechanisms of bacterial interference provided by enteric bacteria (35). Desferal, although a markedly less effective chelator of iron than enterochelin (9), was one of the most effective hydroxamate ligands (14) and was also inhibitory in our assays. The potential relevance of these compounds and chemical derivatives thereof in chemotherapy or prophylaxis of meningitis, gonorrhea, and other bacterial diseases, although implied, has not been evaluated.

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