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STUDIES ON IRON AND PATHOGENICITY IN *Neisseria meningitidis*

by

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Thesis submitted to the School of Graduates Studies as partial fulfillment of the requirements for the degree of Ph.D. in Biology.

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This submission is dedicated to my wife, Brenda and to my children, Trina and Daryl, in appreciation for the time and attention which was not received.

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ABSTRACT

Treatment of C_{57} black mice by the intraperitoneal route with large quantities of iron renders these animals susceptible to infection with *Neisseria meningitidis*. Within 16 h of intraperitoneal injection of low doses of meningococcal cells (10^2 cells per mouse), the number of viable bacterial cells in the blood increases more than a million-fold. Death usually follows within 48 h. This experimental model of infection provides a system for the classification of virulence of the meningococcal strains and for the study of aspects of immunity to this micro-organism. Mouse virulence of various strains correlated well with respective human virulence. The LD_{50} for mice of case strains (blood or C.S.F isolates) was reduced by 6 logs in the presence of iron (i.e. from 10^8 to 10^2 cells per mouse); whereas a carrier strain maintained a high LD_{50} even in the presence of iron (i.e. $> 10^8$ cells/mouse)..

Large molecular weight iron compounds such as iron dextrin (Ferrigen) or iron dextran (Imferon) were unable to enhance infection to the same extent as the lower molecular weight compounds iron sorbitol citrate (Jectofer) or ferric and ferrous salts.

Pre-treatment of mice with iron at different times up to 24 h before bacterial injection enhanced infection as well as did concommittant iron injection.

The requirement for injected iron could not be alleviated by growing the bacterium in or on media containing levels of iron comparable to those used in the treatment of the mice. When injected with iron, the meningococcal cells were lethal. However, once removed from the iron and injected on their own similiar cells were not lethal. The cells did not alter their character

nor store sufficient iron to lower the LD_{50} when grown in iron containing media.

Iron overload occurs following injection of the concentrations of iron required to enhance infection as was seen in livers and spleens of the mice. Although in excess of one-quarter of the normal mouse total body iron was injected hematological parameters were not significantly altered and toxicity was minimal.

Analysis of mouse sera at various times following iron treatment for iron content and % Fe saturation of the transferrin suggest that the amount of iron in the bloodstream at the time of bacterial challenge is not a major factor in determining the course of infection. The data argue against any protective *in vivo*[1] effect for mouse transferrin.

Active immunization of the mice with a meningococcal protein antigen abolished the enhancement of infection in the presence of iron, resulting in protection of the mice. The data suggest that iron might preferentially interfere with host mechanisms which operate in the early phase of microbial invasion, before the immune response of the animal has been actively developed.

The *in vivo* studies suggest that the main effect of iron treatment is upon defences of the local and reticulo-endothelial systems. Iron affects mouse resistance in such a manner that only bacteria possessing a selective character have the capacity to take advantage of this alteration.

Examination of the growth of virulent and avirulent strains "in vitro" both in liquid and agar revealed that increased iron in the media produced no difference in growth rate, yield, cellular or colonial morphology. Analysis of growth culture filtrates and sediments showed no significant difference in removal or uptake of iron by the various strains. Decreasing the

[1] *in vivo* - in the living.

availability of iron by using iron-poor media did not produce preferrential growth of the virulent strain. Iron-limited growth was obtained by addition of synthetic iron chelating agents such as Desferal to the media. Both virulent and avirulent strains grew equally well under such iron-limiting conditions. The ability to use iron *in vitro*[2] did not correlate with the ability to establish infection in mice.

Based upon examination of culture filtrates by a series of tests, meningococcal strains did not appear to secrete detectable iron sequestering agents (siderophores) into the media. Concentrates from up to 4 l of growth media did not yield catechol or hydroxamate type siderophores. A cellular extraction process with ethyl acetate failed to provide evidence of attached siderophores. Hence, production of iron chelators is not a characteristic upon which the difference in reaction of meningococci in infectivity may be based.

Mouse sera, like other mammalian sera (including human) stimulated the growth on agar of both virulent and avirulent strains. Such data indicate that exogenous iron in the mouse model of infection is not needed to overcome a defense activity of mouse serum.

A series of *in vitro* studies demonstrate that iron may affect the growth of meningococci in opposite directions depending upon the presence of different proteins or substances from various body fluids or tissues. Various strains grew in the presence of purified human transferrin containing different levels of iron. With certain lots of apo-transferrin, iron limited growth occurred. The avirulent strain showed a peculiar pattern of inhibition with apo-transferrin; inhibition increased rather than decreased when iron was initially added.

[2] *in vitro*- in glass.

Little difference existed in the capacity of either strain type to remove radioactive iron from transferrin. However, the amount of iron removed from transferrin and retained by whole cultures was least for the avirulent strain. The data suggest that spent medium or some growth by-product interferes with binding of transferrin-iron by the cells, especially avirulent cells.

Certain lots of human lactoferrin exerted selective inhibition. The highly virulent serogroup A strain grew in the presence of a concentration of lactoferrin 4 fold higher than the concentration which inhibited the avirulent strain. Inhibition was not simply due to withholding iron since inhibition was still maintained in the presence of levels of iron which well exceeded the iron-binding capacity of the protein.

Despite the lack of inhibition by certain lots of transferrin or lactoferrin, all lots of conalbumin (egg-white protein) repressed growth. Growth of the avirulent strain was inhibited more than the virulent strain.

Commercial preparations of ferritin (horse-spleen) were found to contain cadmium as a contaminant. It was necessary to remove cadmium since the levels found in the preparations were growth inhibitory. Cadmium inhibition was also iron reversible and was greatest for the avirulent strain. Reduction with ascorbate eliminated cadmium. Certain lots of reduced ferritin showed the peculiar type of inhibition which increased upon addition of iron.

Mouse liver saline extracts also expressed similar inhibition. The highly virulent serogroup A strain was resistant. The data suggest that increased virulence for mice may be due in part to elimination by exogenous iron of a bactericidal action of liver tissue.

Ascorbate in conjunction with increasing iron also caused increased growth repression. The order of susceptibility of serogroups was C > Y >

B > A. The formation of hydrogen peroxide due to oxidation of ascorbate was not an explanation for the mechanism of inhibition. Increasing iron decreased and subsequently eliminated inhibition by hydrogen peroxide. The virulent serogroup A strain which possesses a higher catalase content than the other serogroup strains possessed a three-fold higher resistance to hydrogen peroxide.

In total, the *in vitro* studies demonstrate that iron may affect growth in opposite directions depending upon the presence of other factors. Iron alone stimulates growth. However, when in combination with ascorbate, liver extracts, transferrin or reduced ferritin, iron represses growth of certain strains. On the other hand, iron may contribute to growth by reversing inhibition due to hydrogen peroxide or lactoferrin. The *in vitro* experiments show that caution must be applied when one evaluates the effects of iron during *in vivo* infections in mice or men. Iron may be playing conflicting roles during infection with the overall effect being observed.

GENERAL INTRODUCTION

I. THE ENIGMA OF THE MENINGOCOCCUS

Epidemic cerebrospinal fever was first described in 1805 by Vieusseux in Geneva, Switzerland (Vieusseux, 1805). In 1887, the meningococcus bacterium was first identified by Weichselbaum in the spinal fluid of six patients with meningitis (Weichselbaum, 1887). The bacterium, *Neisseria meningitidis*, belongs to the family Neisseriaceae which derived its name from the German bacteriologist Neisser who in 1879 discovered another important species: *N. gonorrhoeae* (Branham, 1956). The term meningitis, meaning inflammation of the membranes (meninges) covering the brain and spinal cord, is drawn from pathology. The meningococcus remains the major cause of epidemics of meningitis.

Examination by light microscopy of stained smears from purulent spinal fluid shows the bacteria to be small gram-negative cocci, each cell characteristically flattened where it is in contact with its mate (hence diplococcal). In the laboratory, the microbe grows as an aerobe, reluctantly facultative with optimum growth temperature at 35 - 37°C and a requirement for increased CO_2.

Although nearly a century has passed since the meningococcus was first identified, it still remains much of an enigma (Little-John, 1976). Many unanswered questions arise in the minds of those who make even a superficial study of the habits of this bacterium.

The infection caused by this micro-organism may appear as meningitis in one patient or as a virulent septicemia with minor effect on the meninges in another. In fact, acute meningococcal disease may occur in four clinical forms based on patient condition upon admission and the clinical course (Salmon, 1970).

Transient bacteremia. The patient is admitted to hospital with an upper respiratory tract infection. The condition is self-limited, with the patient improving in a few days without specific treatment. Blood cultures show presence of *Neisseria meningitidis*.

Acute meningococcemia. The patient arrives at hospital with a sudden onset of symptoms including fever, nausea and a diffuse petechial rash. There may be a short history of pharyngitis or upper respiratory tract symptoms. Disseminated intravascular coagulation may lead to shock, adrenal hemorrhage and renal cortical necrosis.

Meningitis. In the meningitic form of the disease, the patient has fever, headache and stiff neck. Usually there are no petechiae. The spinal fluid is purulent and blood cultures may be positive.

Meningoencephalitis. The patient presents in a coma with deep tendon superficial abdominal and cremasteric reflexes absent. The spinal fluid is purulent and clinical meningitis is present.

The matter is further complicated by the fact that in many cases the organism causes no disease at all, but simply harbours itself in the host's nasopharynx. Colonization of the mucosa of the upper respiratory tract is not of itself dangerous and is the expression of a successful commensal relationship. The factors which prevent the carrier state from developing into overt disease have not yet been characterized. Non-carriers are potentially at risk since their ability to establish the commensal relationship remains unknown.

It was the classic belief that the spreading of disease in a population was correlated with the amount of carriage. Meningococcal disease would appear if the rate of nasopharyngeal carriage exceeded 20% (Artenstein and Winter, 1974). However, many studies in the late 1970's demonstrated that no clear relationship existed between the extent of carriage in a community and

the appearance of meningococcal disease (Peltola, 1983). Variation in the incidence of the disease can no longer be ascribed to a corresponding variation in carrier rate. It is still not possible to identify the virulence of strains of meningococci cultured in carrier surveys.

Understanding is further complicated by the fact that serologically, the micro-organism is not a single entity. In 1915, Gordon and Murray proposed a serological classification of the meningococcus into four serogroups: I, II, III and IV (Gordon and Murray, 1915). Currently, strains are divided into 10 main serogroups on the basis of the antigenic specificity of their capsular polysaccharides: A, B, C, D, X, Y, Z, and Bo6, 29e and W_{135}. Most epidemic outbreaks of meningococcal disease are caused by Group A strains and small outbreaks have occurred in association with Group B, C and Y strains; the other serogroups occur less frequently. With periodic regularity, strains of a single serogroup emerge and cause an epidemic wave that spreads along a reasonably definable geographic front over several years with little leap-frogging. Hence serogroups may differ from one country to another, even from one district to the next. In Canada, in the last decade, a significant proportion of isolates from cases were serogroup A, whereas in the United States, serogroup A has only been rarely identified (Varughese and Acrnes, 1980). The 49th parallel appears to present a barrier to serogroup A strains. The same serogroup may cause overt disease in certain individuals and may simply show up as a carrier strain in others. Hence the relative virulence of a serogroup remains unknown. Overall, this epidemiological evidence would indicate that environmental conditions or host factors would be more important parameters in determining the outcome of the disease than the actual serogroup of the bacterium.

Subcapsular antigens such as outer-membrane proteins or lipo-polysaccharides have conferred upon the strains a serotype independent of capsular serogroup. Interest in this area has increased in the last decade (Ashton et al., 1980; Frasch and Peppler, 1982). Serogroup A strains are homogenous with respect to outer membrane and are not related to serotypes of other groups. Other serogroups, however, have the same serotypes. Group B and C strains have been divided into 15 to 18 different serotypes based upon immunologically distinct outer-membrane proteins (Frasch and Peppler, 1982). Of these only a few have been associated with significant levels of meningococcal disease. Serotype 2 appearance is prevalent in group B and C disease isolates (Ashton et al., 1980). However this association appears to have since declined (personal communication). It has also been indicated that expression of outer-membrane proteins (the basis for serotyping) depends upon media composition (McIntosh and Earhart, 1976). Hence the exact relevance of serotype to virulence remains to be clarified. From various sero-epidemiological studies Artenstein and Winter (1974) have concluded that "the incidence of disease may be ascribed to factors in the exposed rather than in the exposure".

II. PATHOGENICITY

The pathogenicity of a microbe is an expression of its capacity to establish disease in a host. Pathogenicity must always be defined as an expression of a specific dynamic host-parasite relationship. Characteristics of both the microbe and the host contribute to the outcome of the infection. The metabolic products of pathogenic organisms often enable them to produce disease in a susceptible host (Smith, 1960). Although peculiarities of the bacterial products are controlled by the bacterial genome, their production is phenotypically determined by the nutritional and defense conditions. Many micro-organisms infect only one particular host species, such being the case with the meningococcus which naturally infects only man.

During the infectious process, the initial requirement for a microbe is to survive on and to penetrate mucous membranes or skin. Pathogens are selective in the type of epithelia to which they attach (Savage, 1972). Most gram-negative bacteria multiply in the epithelial surface at the site of entry, producing a spreading infection in the epithelium. Local inflammation of the respiratory mucous membranes increases the permeability of the blood vessels which under certain circumstances allows bacteria to enter the circulation and subsequently cause sepsis.

At one time, it was believed that meningitis resulted from direct extension from the posterior nasopharynx. It has been generally accepted that systemic disease usually follows dissemination of the meningococcus through the blood stream from the posterior nasopharynx (Feldman, 1972).

Sanborn and Vedros (1966) provided suggestive evidence that meningococcal carriers actually experience epithelial cell invasion and are not merely superficially colonized.

The human host may contribute to maintenance of the commensal relationship by restricting the meningococcus to its site of colonization on the respiratory tract mucosa. It has been proposed that immune lysis provides the mechanism by which this process is achieved (Goldschneider et al., 1969). A model for the epidemic behaviour of the meningococcus was developed based upon virulence factors of the bacterium and susceptibility of of the host. An *in vitro* test was used to determine the ability of whole sera to kill the disease causing strain. An inverse relationship was shown between the titre of antibodies to a meningococcal group C type II strain and the level of susceptibility to disease.

However, like all proposed models, inherent discrepancies exist. Carriage and hence exposure to this strain of *N. meningitidis* was found to vary not only among different training centres but also between training companies at the same centre. These variations could not be directly correlated with variations in disease rates over time or at any given time among centres. Hence differences in disease rates could not be explained solely by the presence of a particularly virulent strain in one camp and not another. In a subsequent paper, one of the co-authors of the former study, adds that factors other than initial immune status, surface antigens of the organism and rates of transmission must operate within the recruit group setting (Artenstein and Winter, 1974).

The cyclical occurrence of epidemic meningococcal disease suggests that the disease depends for its expression on the presence of an immunologically deficient population. Since the disease is also mainly a disease of infancy and early childhood it is suggested that individuals who are susceptible to systemic disease lack humoral antibodies to the disease producing strain. However, there is no concrete evidence that humoral antibodies are the sole or even the major host defence mechanism. Recently, in a thorough review on

the mechanisms of pathogenicity of the meningococcus, DeVoe (1982) concludes that the pattern of epidemics does not correlate with the classic cycle for attack of non-immune susceptible hosts in a population.

Once the epithelial surface has been breached the micro-organism is confronted with a different micro-environment, namely the extracellular fluid of various tissues. The fate of the bacterium must be determined by the presence or absence in this fluid of nutrients and physiochemical conditions favourable for its growth as well as any antibacterial humoral factors. If the invading bacterium finds the tissue fluids a favourable medium for its growth, host resistance depends mainly on phagocytosis by macrophages and polymorphonuclear leukocytes (For excellent reviews see Mims, 1976 and Gadebusch, 1979).

It is during the decisive primary lodgement periods that the protective reactions of the host are registered against a few invading organisms. The reticulo-endothelial system (RES) serves as the first line of defence charged with prompt removal of a potentially virulent organism (Miles et al., 1976).

Most successful micro-organisms must to some extent succeed in interfering with the antimicrobial activities of the phagocyte. The encounter between phagocyte and the micro-organism is a central feature of infection and pathogenicity. The ability to grow in macrophages (resist digestion) is often a key property of successful invasive micro-organisms e.g. *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Listeria monocytogenes* and *Brucella* sp. (Mims, 1976). Extracellular replication puts a microbe at a disadvantage because it becomes exposed to all the antimicrobial forces the body can sum up. Intracellular micro-organisms must only overcome the cells' own defence mechanisms.

The intracellular association of *N. meningitidis* would be advantageous to this organism, providing the bacterium could resist degradation.

Laboratory examination of the blood or CSF of patients with meningitis demonstrated meningococci within polymorphs to such an extent that in the early years, the bacterium was actually named *Neisseria intracellulares* (Roberts, 1967). Although earlier reports suggested that meningococci resisted intracellular digestion, and that this property might determine in part their pathogenicity, recent findings demonstrate that under certain conditions, meningococci neither survive nor multiply within granulocytes. In 1967, Roberts demonstrated that various Group B laboratory strains, following ingestion by rabbit polymorphs, were rapidly killed. However, ingestion of the bacteria occurred only in the presence of type specific antibodies. A Group B laboratory strain was later shown by DeVoe et al. to be rapidly phagocytized by human peripheral blood leukocytes in the presence of 10% autologous plasma (DeVoe et al., 1973). Although most intracellular bacteria were destroyed, some bacteria remained intact within the polymorphs for the six hour duration of the experiment. The author concluded that although the leukocyte did not appear to act in any way to protect the meningococci, the intact meningocci possibly represented a portion of the population that was resistant to enzymatic attack of the leukocyte. The extent of intracellular killing of meningococci by mononuclear phagocytes remains to be defined.

A host may be resistant to a microbe if it does not provide the microbe with a suitable growth medium. A pathogenic micro-organism has the capacity to use the host environment as a growth medium. The nutritional demands of a microbe and the availability of required compounds in the tissues or organs may determine the specificity of certain microbes for specific tissues. The speed and extent of infection may be determined by the effectiveness with which microbes compete with their hosts for nutrients (Garber, 1956).

Most microbial cells need iron. Iron is one of the most versatile biocatalytic elements due to its two stable valences (i.e. Fe^{++} and Fe^{+++}) and to the wide range of oxidation reduction potential (+300 mV to -500 mV) between these two valences. Excellent reviews on the diversity of functions of iron in microbial cells have been compiled (Lankford, 1973; Byers and Arceneaux, 1977). Many enzymes contain iron. In nearly all bacteria, iron is a component of ribonucleotide reductase, an essential enzyme for DNA synthesis (Ehrenberg and Reichard, 1972; Neilands, 1977). Another major role of iron is in oxygen and electron/hydrogen movement. A deficiency of iron may greatly reduce respiratory activity.

In animals, iron is found bound to biological carriers or deposited within various cells. The effectiveness with which a microbe can remove iron from these carriers may determine its virulence. The ability of a host to deny or a bacterium to acquire such an element is "a battle of chelating agents" (Glynn, 1972).

III. IRON REGULATION IN HOST AND MICROBE

Of overriding importance in the sudy of iron in biological situations is the solubility of the metal itself. In acidic aqueous solution, ferric and ferrous ions exist as $Fe(H_2O)_6^{3+}$ and $Fe(H_2O)_6^{2+}$. The water molecules in the co-ordination sphere can be replaced by a wide range of ligands. As the pH of the solution is raised, these ions split off protons, forming hydroxy-iron species and polymers with a unique spherical geometry and a very high molecular weight of approximately 180,000 (Saltman et al., 1976). Fe^{++} ion forms ferrous hydroxide $Fe(OH)_2$ which has a solubility product of 10^{-15}M at 25°C, zero ionic strength. Thus, at physiological pH i.e. 7.0, the amount of free ferrous ion in solution is about 10^{-1}M. The solubility product for ferric hydroxide $Fe(OH)_3$ is 10^{-38}M at 25°C in the 3M $NaClO_4$, with a resulting free ferric ion concentration of 10^{-18}M at pH 7.0 (Spiro and Saltman, 1974).

In various growth media bacteria seem to have evolved multiple systems for the acquisition and subsequent transport of ferric iron (for excellent reviews see Lankford, 1973 and Byers and Arceneaux, 1977). A variety of microbes produce and release low molecular weight chelates, termed siderophores, which can solubilize ferric iron for purposes of transport. Neilands (1973) has proposed that siderochromes (siderophores) include both major classes of iron transport co-factors, i.e. the secondary hydroxamic acids and the aromatic hydroxy acids (phenolic acids). These compounds which all form oxygen ligands are among the most powerful known chelating agents of ferric ion with stability constants between 10^{29} and 10^{32}. Most naturally occurring hydroxamic acids at neutral pH bind with Fe^{+++} forming stable neutral chelates via three hydroxamate ligands per molecule, e.g. ferrichrome and deferrioxamine B (Neilands, 1952 and 1977).

Other hydroxamate compounds consist of only two hydroxamate groups per molecule with the third binding centre being provided by other O_2 containing groups e.g. Schizokinen. The two oxygen atoms of citrate provide the remaining two ligands (Mullis et al., 1971). A growth factor for *Mycobacterium paratuberculosis* termed mycobactin also belongs in this category, with the third iron-binding centre being contributed by phenolic hydroxyls (Snow, 1970).

The second major class of all-oxygen ligand compounds, are the aromatic hydroxy acids consisting of amino acid conjugates of 2,3- dihydroxybenzoic acid. Iron deficient cultures of *Escherichia coli* contain 2,3-dihydroxybenzoic acid. *E. coli* as well as *Aerobacter aerogenes* produces 2,3-dihydroxylbenzoyl serine (Brot et al., 1966). Enterochelin was isolated from *E. coli* and from *Salmonella typhimurium* (where it was called enterobactin) (O'Brien and Gibson, 1970 and Pollack and Neilands, 1970). This iron-binding co-factor is a cyclic triester of 2,3-dihydroxybenzoyl serine.

Many bacteria use not only their own endogenously produced siderophore, but also siderophores of other bacteria (Byers and Arceneaux, 1977). Some bacteria produce more than one siderochrome (Gibson and McGrath, 1969, O'Brien and Gibson, 1970 and Byers and Lankford, 1968). It is suggested that bacteria transport iron by excreting these co-factors into the medium in the deferrated state where they complex with the polymerized iron and form soluble Fe^{+++} chelates (Byers and Arceneaux, 1977). These complexes are then taken into the cell by specific transport systems involving special receptors on or within the cell (Rosenberg and Gefter, 1974).

In body fluids bacteria are also confronted with the problem of acquiring iron. They have to compete with the host iron binding proteins for the limited amount of iron available. More than 2/3rds of the host iron is found in hemoglobin and myoglobin. Almost all of the remainder is found in the storage forms ferritin and hemosiderin, located mainly within reticulo-endothelial cells of the liver and spleen. Ferritin, found in almost all types of mammalian cells, is the second most abundant iron-protein in the body (reviewed by Munro and Linden, 1978). Ferritin is composed of 24 protein sub-units each of approximately 18, 500 daltons arranged in the form of a cube (Harrison et al., 1967). A variable amount of iron is present in the centre of each molecule as a core of hydrous ferric oxide phosphate. When isolated from a tissue the protein consists of a mixture of molecules containing no iron upto fully saturated molecules containing about 4000 iron atoms, having a total iron content of over 20% by weight. The protein has ferroxidase activity, catalyzing the formation of the ferric oxide core from ferrous iron.

The daily turn-over of iron in man is approximately 30 mgs. Transferrin is the specific carrier which maintains iron in a soluble form and accommodates this traffic (reviewed by Morgan, 1974). Without transferrin, the delivery of iron to hemoglobin synthesizing sites, and its mobilization from stores would not be successfully controlled. Each transferrin molecule has two metal binding sites, consisting of a single polypeptide chain of molecular weight in the range 76,000 to 80,000. FeIII is the most tightly bound metal. For each FeIII bound, a suitable anion, preferably bicarbonate, must also be bound (Bates and Schlabach, 1975). The affinity constant for FeIII is approximately 10^{36}. Such an extraordinarily large binding constant prevents transferrin from losing its iron to the competing reaction of ferric hydroxide formation.

A major iron-binding protein found mainly in exosecretions is lactoferrin (Masson et al., 1966). This protein shares with transferrin the ability to bind reversibly 2 atoms of iron per molecule but differs from the serum protein by its antigenic properties and by its affinity for iron. Lactoferrin has a similar molecular weight and affinity constant. However, the affinity for iron is maintained by this protein at pH 4.0, at which pH transferrin releases all its iron. Polymorphonuclear leukocytes also contain this protein in their secondary granules (Masson et al., 1966c).

Whether or not an infection might develop could depend in part upon the ability of the bacterium to utilize iron bound to such proteins. Thus, pathogenic bacteria must possess a means for acquiring this iron. The bacteria can not overcome the iron deficiency in the animal body unless they create changes or produce substances which could make the iron of these complexes available to them. Direct proteolysis of the iron complex would disrupt the iron binding site. Direct interaction of the bacterial cell envelope and the iron-protein molecule could result in iron exchange. The bacteria might produce siderophores to compete and remove the iron from the iron-proteins. It is also possible that iron-starvation of the bacterium could be alleviated by a localized area of inflammation, a condition in which pH is lowered and the iron-binding protein transferrin, if present, releases its iron (Bullen, 1981).

IV. IRON AND PATHOGENICITY

A growing body of evidence suggests that hypoferremia benefits or helps a host by making it less susceptible to microbial attack (Weinberg, 1978). This conclusion is based largely on animal experiments whereby artificial induction of hyperferremia (by injection of iron compounds) increases the ease of establishing a microbial infection. Support is also provided by *in vitro* experiments in which a variety of micro-organisms grow more efficiently in the presence of fluids containing iron-binding agents when additional iron is included (Bullen et al., 1974b). Despite such evidence however, the crucial role that iron exerts in the establishment of microbial infection in a host remains controversial (Weinberg, 1977 and Stockman, 1981).

A. HOST IRON RESPONSE DURING INFECTION

Acute and chronic infectious diseases cause changes in iron metabolism in the mammalian host (Weinberg, 1978). A reduction in the quantity of iron in serum has been observed in humans and other mammals that have a variety of infectious diseases. Tuberculous patients become hypoferremic (Locke et al., 1932). In man, following exposure to an aerosol of virulent *Francisella tularensis*, a decrease in serum iron concentration occurs (Pekarek et al., 1969).

During acute infections, changes in the storage of iron have also been observed (Weinberg, 1978). Hypoferremia is achieved by an unknown mechanism that suppresses return of the metal from the R.E. system and accelerates movement of the plasma iron into hepatic storage sites. This hypoferremia in

infected persons is not reversed by the parenter administration of exogenous iron (Heilmeyer and Wohler, 1961 and Beisel, 1976). The metal accumulates in the liver and spleen rather than restoring the plasma iron level to its normal state. Hypoferremia appears to be a relatively rapid host response and thus may serve a yet undetermined role in host resistance to infection.

Weinberg (1978) has stated that perhaps all vertebrates make considerable adjustments during infection that have the effect of depriving invading microorganisms of iron. The little that is known about metabolic responses to infection suggests that at least some of these responses may tend to deny the parasite the iron required for its survival.

Increased synthesis of iron-binding proteins occurs in episodes of microbial attacks. The transferrin level in the blood of mice undergoing experimental infection with *Listeria monocytogenes* increased (Sword, 1966a). Inflammatory synovial fluid of arthritic patients contained increased levels of lactoferrin (Bennett et al., 1973). In nine patients with bacterial meningitis or pneumonia, plasma lactoferrin increased substantially within two days of onset (Hansen et al., 1976). The concentration of secreted lactoferrin was increased thirty fold above normal in the bovine mammary gland within ninety hours of the onset of experimental or natural mastitis induced by *E. coli* (Harmon et al., 1976).

Weinberg (1974) has listed a series of hyperferremic conditions in humans during which susceptibility to bacterial and fungal pathogens is increased. It is suggested that increased availability of iron enhances infection. However, other defects of the immune or inflammatory system also occur in these conditions. Such may predispose the host to bacterial invasion. It is also suggested that iron overload (by parenteral injection) enhances infection in humans (Barry and Reeve, 1977; Becroft et al., 1977). Other observations, however, indicate the opposite; iron

supplementation decreases morbidity in bacterial infections (Weinberg, 1977). Iron administration in normal neonates does not increase the risk of infection (Stockman, 1981). Polymorphs have a reduced bactericidal capacity in iron deficiency (Chandra, 1973).

Hence, as Sussman (1974) indicates, a paradox exists in the part iron plays in infection. While hyperferremia appears to favour infection, so does hypoferremia. Under conditions of normal host iron metabolism, infection can also take place. Hence, the part that iron plays in the host-iron parasite relationship requires clarification.

B. ENHANCEMENT OF EXPERIMENTAL INFECTION BY IRON

Further support for the involvement of iron in bacterial infections is provided by enhancement of infection in animals stressed with exogenous iron. Parenteral administration of iron compounds to experimental animals, reduces the size of the inoculum required to produce disease or death and permits a greater amount of multiplication of microorganisms in invaded tissues. Avirulent strains of *Pasteurella pestis* killed mice only when their *in vivo* growth was supported by adequate iron (Jackson and Burrows, 1956). *E. coli* infection in mice, rats or guinea pigs has been enhanced by hemoglobin, iron sorbitol citrate or iron salt (Bornside et al., 1968, Bullen et al., 1968b and Fletcher, 1971). Iron increased the virulence of *Klebsiella* and *Salmonella typhimurium*, but neither *Salmonella typhi* nor *Pseudomonas aeruginosa* for guinea pigs and mice (Martin et al., 1963 and Chandlee and Fukui, 1965). The LD_{50} of a virulent strain of *Listeria monocytogenes* for mice was reduced a hundred-fold by pretreatment of mice with iron salt. Iron salt also markedly enhanced the mouse virulence of *Vibrio cholerae* strains (Joo and Csizer, 1973).

In certain experimental infections, iron treatment abolished passive immunity. If iron was given within six hours of the start of infection with *Clostridium welchi*, the passive protection afforded to guinea pigs by specific antiserum was overcome (Bullen et al., 1967). Injection of iron compounds up to four hours following challenge with *Pasteurella septica* abolished the protection that specific antiserum offered to mice (Bullen et al., 1968a). These studies suggested that iron was somehow interfering with the defense mechanisms of the host. However, iron did not always abolish immunity. With experimental *Listeria monocytogenes* infection iron had no adverse effect upon actively immunized animals (Sword, 1966b). For *Vibrio cholerae*, iron did not abolish active or passive immunity (Joo and Csizer, 1973).

These studies have indicated that the effect of iron varies greatly with different bacteria and different hosts. Some authors propose that the added iron promotes development of microbial infection by becoming available for microbial utilization. The virulence-enhancing phenomenon could be explained simply as a growth stimulating effect of iron upon bacteria which are unable to neutralize tissue bound iron. It has been proposed that iron assists establishment of infection in the host by eliminating the bacteriostatic effects of iron-binding proteins such as transferrin and lactoferrin which are present in serum and various secretions (Bullen, et al., 1974b).

However, such experiments should be interpreted with caution. The routes of injection are not comparable with those in natural infections. The injection of Fe^{2+} or sufficient Fe^{3+} to saturate serum transferrin is a grossly unbiological procedure and it is not possible completely to exclude toxic effects. A site of toxicity could be the reticulo-endothelial system which would tend to enhance infection in a non-specific manner.

C. EFFECT OF IRON ON THE GROWTH OF MICROBES IN BODY FLUIDS AND TISSUES

In vitro studies with mammalian sera and body fluids support the concept that iron is somehow involved in the infectious process. The bacteriostatic and bactericidal effect of sera and body fluids can be removed by the addition of iron compounds. It is proposed that iron negates the microbicidal effects of the iron-binding proteins contained within the sera or fluids. Schade and Caroline (1944) first demonstrated that the inhibition of the growth of various bacteria by raw egg could be reversed by iron. These authors subsequently showed that the iron-binding protein transferrin contained in serum also inhibited bacteria. The inhibition by serum could be neutralized by adding enough iron to saturate the transferrin contained within. Different mammalian sera increased in capacity to support growth of *Mycobacterium tuberculosis* as the levels of iron saturation of the transferrins increased (Kochan, 1969a). It was proposed that the anti-bacterial action of serum was simply due to interference with microbial iron supply by transferrin alone or in conjunction with other serum components (Bullen and Rogers, 1969; Rogers 1967 and 1970).

Other studies, however, indicate that the explanation of the antibacterial action of serum or anti-serum based upon interference with iron supply was not sufficient. The abolition of the anti-bacterial effect of horse serum on *Pasteurella* species with iron compounds was a complex process (Griffiths, 1971). Fletcher (1971) suggested that iron interferred with the process of bacterial killing normally mediated by natural antibody and complement. Iron might bind serum proteins to the surface of the bacterium (Tandl, 1957).

It is important to evaluate with caution suggestions concerning iron and microbial infection which are derived from experiments involving the growth of microorganisms in serum. These types of experiments compare in a quasi-physiological manner the conditions of established septicemia. Growth in serum is an inadequate model of infections initiated in tissues and not the blood stream. It may be a significant model of events in bacteremia and septicemia.

The inhibition of various bacteria by milk has been attributed to lactoferrin (Cheesman and Williams, 1964, Masson et al., 1966a and Oram and Reiter, 1968). Since addition of iron to the growth media reversed inhibition, it was suggested that the effects of lactoferrin were due to the complexing of required iron. On a comparative basis lactoferrin was a more effective inhibitor than transferrin (Masson et al., 1966a). Specific antibody augmented lactoferrin inhibition (Bullen et al., 1972, Reiter et al., 1975 and Griffiths and Humphreys, 1977).

Certain evidence indicated that the effects of lactoferrin were probably not due to the binding of available iron in the media, but rather to a direct bactericidal effect due to binding of the protein at the cell surface (Bishop et al., 1976 and Arnold et al., 1977). Saturation of the protein with iron would alter its configuration and hence alter its binding to the bacterial cell surface. As a result, a reduction in inhibition would follow.

Iron compounds also abolished the bactericidal action of rabbit polymorphonuclear leukocytes for *Staphylococcus aureus* in a semi-synthetic medium (Gladstone and Walton, 1971). The growth of staphylococci inside the polymorphs was stimulated. The presence of iron or mycobactin in tissue culture media is known also to stimulate the luxuriant intra-cellular growth of tubercle bacilli in peritoneal macrophages (Kochan, 1977b). The

restricted rate of growth of *P. aeruginosa* by peritoneal fluid is restored to normal if iron is added (Bullen et al., 1974a).

D. SIDEROPHORES AND VIRULENCE

Stimulation of the growth of bacteria in sera by the addition of iron chelating compounds which had been produced by the respective bacteria in iron deficient media suggested that secretion of this type of compound by a pathogen in a host might enhance infection. Two-three dihydroxybenzoyl serine isolated from low iron cultures of *S. typhimurium* eliminated the bacteriostatic property of human sera for this bacterium and stimulated the growth of small inocula (Wilkins and Lankford, 1970). Enterochelin reversed the bacteriostatic action of horse serum for *E. coli* (Rogers, 1973). Catechols from *Klebsiella* reversed the bacteriostasis of horse serum for *E. coli* and *Klebsiella* species (Khimji and Miles, 1978).

Miles and Khimji's (1975) experiments cast doubt on the theory that the rate of siderophore production by a bacterium could be used as a measure of its ability to invade animal tissue and cause infectious disease. Chelator production was similiar for randomly selected avirulent and virulent strains of *Klebsiella* and *E. coli* and for the smooth LPS forms and their avirulent rough LPS mutants in strains of *E. coli*, *Salmonella* and *Shigella*.

The microbiostasis exerted by proteins in serum milk and egg white was more effective in agar plates for avirulent than virulent *E. coli* strains (Kochan et al., 1977a). This microbiostasis could be neutralized by the addition of enterochelin or iron compounds. These authors thought that the ability of small inocula of virulent cells to grow in serum indicated that disease producing bacteria differ from avirulent cells in the possession of a more efficient mechanism for acquiring iron. Their demonstration that

avirulent bacteria did not survive in spent mammalian sera in which virulent bacteria had grown suggested that the ability of virulent bacteria to grow in serum could not be attributed to the production of extracellular siderophores. Supportive evidence for this was provided by Kvach et al. (1977).

Exogenous microbial iron chelators have also promoted experimental infection *in vivo* (Rogers, 1973 and Jones et al., 1977). Such studies suggest that the adequate production of iron chelators *in vivo* may be necessary for the virulence of certain micro-organisms.

However, evidence concerning the role of siderophores in determining the virulence of pathogenic micro-organisms remains controversial. Catechols from the strains of both high and low virulence for guinea pigs enhanced the skin infectivity of most of the *Klebsiella* strains tested (Khimji and Miles, 1978). Catechols were not virulence factors in the proper sense of being peculiar to the virulent forms of a species since the avirulent forms also synthesized catechol.

V. IRON AND NEISSERIA

Prior to 1975, the only connection between iron and pathogenic *Neisseria* was provided by the studies of Kellogg et al., (1963). This work demonstrated that virulence was genetically linked to clonal variation. Most of the clones in primary isolates from acute gonorrhea of males were of a type 1 designation; 10% were designated types 2 and 3. Type 1 produced infections in human volunteers whereas type 4 did not. Types 1 and 2 were classified eventually as virulent and types 3 and 4 as avirulent. Increasing the percentage of ferric nitrate in the agar medium caused all four clone types to become granular in appearance and stimulated their growth rates. This was most pronounced with the virulent types 1 and 2 which became three times the size obtained without ferric nitrate.

In order to obtain adequate information on the factors involved in the virulence of a microorganism, it is better to study it *in vivo* (Smith, 1960). However, man is the sole natural host of the meningococcus and the disease may be extremely severe and rapidly resulting in death. Hence it is virtually impossible to accumulate data upon the infectious process in the natural host. The remaining alternative was to establish an *in vivo* system from which extrapolations might be made to the natural infective system in man. Various experimental models of infection have been attempted; however, the system which appeared to offer potential to better understanding the host-pathogen relationship was the mouse model of the mucin-enhanced meningococcal infection originally established by Miller (1933).

A fatal infection could be produced in mice by the intraperitoneal injection of relatively few organisms when mucin was included; without mucin a progressive infection was not obtained. In this system the bacteria

grew from small inocula, to overwhelming numbers of bacteria in the blood resulting in death within 72 hours, due mainly to septicemia.

Since mucin is a mixture of components which have not been chemically characterized, controlled administration of a standardized system did not occur. Although the infection-promoting action of mucin is of special biological interest and importance since mucin is physiological constituent of the secretions of surface tissues which so often constitute avenues of microbial infection, the factors responsible for enhancing virulence have not yet been adequately defined.

The present author undertook to study the presence of iron in the mucin suspensions employed in our laboratory and to investigate the application of iron in the mouse model of meningococcal infection. It was hoped that the results of such a search could result in the development of a standardized challenge system involving iron compounds.

Evidence that the injection of iron compounds could lead to the progressive and fatal growth of an otherwise non-lethal dose of meningococci in mice was first reported by Calver et al., (1975 and 1976). The results suggested that host iron metabolism may play a part in the onset of meningococcal infection.

Since that time, the body of knowledge concerning iron and *Neisseria* has increased significantly. The majority of these reports will be presented and discussed where applicable.

It is the aim of this thesis to report and evaluate *in vitro* studies involving iron and *Neisseria meningitidis* in conjunction with a further examination of the mouse meningococcal infection model in order to elucidate mechanisms by which iron might assist in the establishment of meningococcal infection in man its natural host.

Some of the results of this thesis have already been published. Material from Chapters 1, 2 and 3 appear in Calver et al., 1978, and 1979a. Other observations in Chapter 3 appear in Calver et al., 1979b. Since this work has been described by others, it will be referred to in the text in its published form.

CHAPTER 1 ENHANCEMENT OF THE PATHOGENICITY FOR MICE OF NEISSERIA MENINGITIDIS BY IRON

INTRODUCTION

Previous research in our laboratory had demonstrated that iron compounds could act as a replacement for mucin in the establishment of meningococcal infection in mice (Calver et al. 1976). This animal model may provide a system in which the virulence of strains of meningococci may be differentiated. This infection system may also serve for studying immunity to this micro-organism. A study of the effect of immunization with a meningococcal protein antigen upon the mouse iron model of infection was undertaken.

A closer examination of this mouse model may increase our understanding of the role iron might play in the pathogenicity of this microbe. Is the effect of iron upon the bacterium or upon host resistance, or a combination of both? Is the requirement for iron absolute or can it be circumvented by growth of the bacterium in or on iron media? If exogenous iron is interacting with host defense, where and how does this occur? Is the iron interaction at the level of the iron binding protein transferrin in such a manner that in vivo bacterial growth is due to supply of iron or does iron affect the host so that the bacterium takes advantage of such alteration and establishes infection? Insight into such questions may provide us with some clue as to how infection in humans might proceed.

MATERIALS AND METHODS

Iron Compounds. Iron salts used were $FeSO_4$ (British Drug Houses, Poole, England), $FeCl_3$ (Fisher Scientific Co., Pittsburgh, Pa.), $Fe(NO)_3 9H_2O$ (J.T. Backer Chemical Co., Phillipsburgh, J.J.). Chelated iron consisted of iron sorbitol citrate (Jectofer - 5000 molecular weight, 90% Fe^{3+} and 10% Fe^{2+}, Astra, Mississauga, Ontario, Canada), iron dextran (Imposil 200 - 180,000 molecular weight, Fe^{3+}, Fisons, Don Mills, Ontario, Canada) and iron dextrin (Ferrigen - 230,000 molecular weight, Fe^{3+}, Astra).

Hog Gastric Mucin. Granular type, 1701-W was supplied by Wilson Laboratories, Chicago, Illinois.

NCDM. Commercial *Neisseria* chemically defined medium (NCDM) was provided by Grand Island Biological Co., New York, USA and prepared according to the manufacturer's specifications. The iron contained in this medium was in the form of ferric salts (i.e. ferric chloride and ferric nitrate) at a concentration of 600 ug Fe/100 ml (Kenny et al. 1967).

Transferrin. Purified human protein (Fe content maximum 20 ug/g) was obtained from Behringwerke, West Germany.

Animals. Male Health Protection Branch (H.P.B.) black mice (a strain derived from two C_{57} sources) weighing 14 - 17 g were used in the experiments. All mice were fed the same diet of Purina mouse chow throughout the study.

Bacteria. Cells of the different serogroups of *N. meningitidis* used in this study were from the cultures regularly maintained at the Laboratory Centre for Disease Control. The strains used and their corresponding serogroups were 604-A, 608-B, 2241-C, and Slaterus Y. The strains were isolated from the following sources: blood (A and C), urethra (B) and nasopharynx (Y).

NCDM was added to the contents of freeze-dried culture ampoules. The resulting suspensions were spread on the surface of Columbia Blood Agar (CBA) plates supplemented with 5% sheep's blood, incubated and grown as previously described (Kenny et al. 1967). A second CBA plate was streaked from the growth obtained after 24h incubation. The layer of growth obtained after 24h incubation of this plate (37°C) was removed with NCDM. Subsequent dilutions were prepared in NCDM. Bacteria in all injection suspensions were counted by plating appropriate dilutions on CBA and determining viable count.

Iron Analysis. All glassware was washed in 6N HCl and rinsed thoroughly with deionized water. The iron content of injection preparations and mouse sera was determined according to the method of Caraway (1963), employing sulfonated bathophenanthroline with spectrophotometric examination at 533 nm, Bausch and Lomb Spectronic 505. For comparative purposes, iron content was also analyzed by atomic absorption spectroscopy (Perkin Elmer model 306) at wavelength 248.3 nm (0.2 mm slit) using the flame mode. The values obtained were calculated from a standard curve of $FeCl_3$ in dilute HCl (Iron reference solution - Fisher Scientific).

Iron Treatment and Injection of Bacteria. Solutions of $FeSO_4$ in physiological saline were prepared at Fe^{2+} concentrations ranging from 200 to 900 ug/ml. Mice were injected intra-peritoneally (i.p.) with different volumes of iron salt solutions (0.2 ml to 1.0 ml). A second i.p. injection of the bacteria in NCDM was given 1, 2 or 24 h later. The injection volume of the bacterial suspension was either 0.5 ml or 1.0 ml. Mice were also injected i.p. with 0.2 ml volumes of $FeSO_4$ in saline for 3 consecutive days prior to bacterial injection. $FeCl_2$, $FeCl_3$ and $Fe(NO_3)_3$ were tested in a similar manner.

The chelated iron compounds were procurred as sterile solutions adjusted to physiological pH. One milliliter of a suspension consisting of 0.5 ml of bacterial dilution and 0.5 ml of these iron compounds diluted in saline to an iron cation injection concentration of 125 ug/ml to 500 ug/ml was administered i.p.

Challenge with hog-gastric mucin and NCDM was performed as previously described (Jennings et al. 1972). Groups of mice were injected i.p. as controls with hog-gastric mucin, NCDM or the various iron solutions. All mortalities were recorded within 72 h of injection of the bacteria. LD_{50}'s were estimated by probits.

Growth of *N. meningitidis in vivo*. *Neisseria meningitidis* serogroup A, strain 604, suspended in Jectofer (final concentration 500 ug/ml) and in mucin, was injected into the mice intraperitoneally at 2.4×10^2 cells/ml. In vivo growth of *N. meningitidis* was followed for 48 h. Mouse blood (0.1 ml) was appropriately diluted in NCDM and plated on CBA. These plates were incubated at 37°C and examined 24 h later for the presence of meningococci. Mean values were recorded for groups of five mice. Appropriate control mice injected with mucin or Jectofer were also studied.

Iron Absorption in Mice. Mice in groups of five were injected i.p. with ferrous sulfate and two different concentrations of Jectofer (i.e. one which enhanced meningococcal infection in mice, and one which did not). Blood was removed by cardiac puncture at various times over a two day period, pooled and analyzed for iron content (Carawáy, 1963). A similar procedure was applied to mice which had been injected with mucin.

Mice were examined for residual iron in the peritoneal cavity by a saline-wash technique in which 3 ml of saline was injected into the cavity, the area gently massaged and as much fluid as possible withdrawn.

Iron Toxicity in Mice. Groups of 25 mice were injected i.p. with 0.5 ml of ferrous sulfate or Jectofer in saline at concentrations which resulted in 450, 225 or 110 ug Fe per mouse. Group weight determinations were recorded just prior to injection and the 3rd and 7th day following injection. All deaths were recorded.

In a further experiment, mice which had received 1.0 ml of iron as Jectofer at 500 ug/ml were individually examined for changes in hematologie parameters during a 20 hour period following injection.

Growth of Bacteria in Iron-containing Media. Iron as Jectofer was added to NCDM at the following concentrations: 500, 250, 125, 62.5 and 31.3 ug/ml. Serogroup A and C strains were grown in these media for a period of 9 hours with shaking at 37°C. The seed culture for these growth suspensions was a 10 hour culture previously grown in NCDM which had been inoculated from the overnight growth of the bacteria on CBA plates. Dilutions of the respective growth suspensions were made in growth media containing the corresponding level of iron as Jectofer. Certain of the cell preparations

were filtered through 0.45 um Millipore filters, washed with 10 times their volume in saline by refiltering and aseptically resuspended in iron-containing or iron poor medium as required. All cell suspensions were injected i.p. into mice in a volume of 1.0 ml. Deaths were recorded and viable counts of all injection suspensions were determined as previously described.

An alternative technique in which the growth suspensions were not filtered or centrifuged but were simply diluted in media with or without Jectofer (500 ug/ml) prior to challenge was also used. Cells grown in Jectofer 500 ug/ml media for 11 h were diluted in both media and injected into the mice. For comparison, cells grown in NCDM for 11 h were diluted also in both media and injected.

In separate experiments, aliquots of the growth cultures were removed at intervals following addition of the inoculum and bacteria killed as required by the addition of formaldehyde (1%). Samples were centrifuged at 3000 rpm (I.E.C. refrigerated centrifuge), washed in H_2O, and respun and resuspended in NCDM. Optical densities were recorded at 500 nm with a Bausch and Lomb Spectronic photometer.

Cell sediments were washed twice with saline and protein determined by the method of Lowry et al. (1951). Standard curves were prepared with bovine serum albumin in 0.1N NaOH.

Other cell sediments were washed in saline and resuspended in and analyzed for iron content according to the method of Caraway (1963) employing sulfonated bathophenanthroline.

Immunization of Mice. A protein vaccine extracted from the serogroup Y, Slaterus strain, according to a procedure of Jennings et al. 1972, was dissolved in citrate buffer and injected i.p. into mice in a volume of 1.0 ml at a concentration of 20 to 40 ug/ml protein. Seven days following vaccination, the mice were injected with serogroup A suspended in mucin or injected 2 h after iron salt.

RESULTS

CLASSIFICATION OF VIRULENT AND AVIRULENT SEROGROUP STRAINS BY MOUSE CHALLENGE

Table 1 compares the LD_{50} values for the four major serogroups of *Neisseria meningitidis*. The LD_{50} values of these strains for C_{57} HPB black mice, when injected in growth medium (NCDM) are fairly high. However, a progressive and fatal infection (mortality within 72 h) caused by otherwise non-lethal doses of serogroups A, B and C strains was produced in these mice following injection of ferrous sulphate or during concomittant injection with iron sorbitol citrate. (Table 2) Reduction of LD_{50} by $FeSO_4$ to levels at least comparable to those obtained in the mucin challenge system was achieved. With serogroup A, B and C strains, the LD_{50} was decreased by approximately a million fold. On the other hand, the serogroup Y strain remained relatively unresponsive, still having a high LD_{50} even in the presence of iron or mucin. Based upon this capacity for establishment of meningococcal infection in mice, the serogroup A, B and C strains were classified "virulent" and the serogroup Y strain was classified "avirulent".

For a more accurate comparison with mucin, an injection system with chelated iron as Jectofer was devised (Jectofer has a pH of 7.2 - 7.9). With this compound, as with mucin (Table 1) the bacteria could be simultaneously injected.

With serogroups A and B, an increasing % mortality was found to be proportional to the increasing concentration of iron provided by the Jectofer (Table 2). As shown, the serogroup C strain gave a response similar to the A and B serogroups when injected with the higher iron dose. Once again the serogroup Y strain showed a reduced response to growth enhancement in mice in

Table 1

N. meningitidis mouse challenge

Injection Medium	Serogroup LD_{50} (cells/ml)			
	A	B	C	Y
NCDM	10^8	10^8	10^8	10^9
Gastric mucin[a]	10^2	10^2	10^2	10^8
Iron[b]	10^2	10^2	10^2	10^8

[a]Hog gastric mucin 5%
[b]$FeSO_4$ at Fe concn of 450 µg/mouse.

Table 2

Effect of Jectofer upon Challenge with *N. meningitidis*[a]

Serogroup	Injected cells/ml	% Mortality[b] Fe concn, µg/ml 500	250	125	Mucin 5%
A	2.2×10^2	80	20	0	90
	2.2×10^3	90	30	0	
	2.2×10^4	100	80	20	
B	1.3×10^2	80	30	0	100
	1.3×10^3	100	50	10	
	1.3×10^4	90	90	80	
C	1.6×10^2	80			90
	1.6×10^3	100			
	1.6×10^4	100			
Y	2.2×10^6	10			60
	2.2×10^7	40			
	2.2×10^8	80			

[a]Based upon groups of 10 mice
[b]Mortality recorded 72 hr. after challenge

the presence of iron. An inoculum containing a fairly large number of cells of the serogroup Y strain was required to give greater than 50% mortality when injected with iron or mucin, i.e. 10^7 - 10^8 cells/ml. In this system, the mice suffered no significant ill effects injected with the highest level of iron, i.e. 500 ug/ml. Without iron, injection of these numbers of bacteria did not kill mice.

EFFECT OF GROWTH OF THE BACTERIA IN OR ON IRON-CONTAINING MEDIA UPON CHALLENGE

Cells grown for 8 h in liquid culture containing iron as Jectofer and injected as such or as dilutions in their respective iron media were also lethal at low dose levels. Mortality due to the cell suspensions was proportional to the iron content of the suspension.

The cells of serogroup A if removed from their growth suspensions, washed and filtered were not lethal at the same low dose levels when resuspended and injected in normal or low iron media (Table 3). Filtering and washing did not affect viability on CBA plates.

In separate experiments, serogroup A cells were grown for 11 h in media containing 500 ugFe/ml as Jectofer or in media without iron. Each growth suspension was diluted for challenge in both media with or without 500 ugFe/ml as Jectofer. The injection suspensions from either growth culture were lethal only when iron was included. Hence growth of the bacteria in high iron media did not alter the capacity of the bacteria to be more infective than usual in lower iron media.

Studies of the *in vitro* growth of the serogroup A strain were also carried out with commercial NCDM containing a series of iron levels similar to those used in the *in vivo* enhancement studies. Growth in liquid media

Table 3

Effect of Removal of Cells from Growth Media Upon Challenge

Serogroup cell/ml	Iron[a] added to media µg/ml	% Mortality Treatment: Nil	Filtered[b] in 62.5µg/ml	Filtered[b] in NCDM
A 9.6×10^4	500	80	0	0
1.0×10^4	250	80	0	0
6.8×10^4	125	30	0	0

[a] NCDM media containing added iron in which cells were grown for 8 hr
[b] Growth suspensions were filtered (0.45 µ Millipore) and diluted in media containing a lower level of iron and injected

was not altered in any significant manner by additional iron in the media (Fig. 1). Increase in optical density (500 nm) of washed cell suspensions was also similiar for the different growth media. No significant difference in viable counts was evident throughout the growth period. Analyses of the same washed cell suspensions as those upon which protein was determined showed no consistent increase in iron in the cell sediments grown in the various iron containing media.

Following growth on Columbia agar plates containing 400 ug Fe/ml (as Jectofer), the A strain was virulent only when injected in the presence of iron. Thus growth of this bacterium on higher iron media does not increase its capacity for growth in mice. Without adequate iron, the bacterium remains non-lethal for mice.

GROWTH OF *N. meningitidis* IN MICE

Growth of *N. meningitidis*, serogroup A, in H.P.B. black mice is demonstrated in Table 4. After the i.p. injection of 2.4×10^2 cells in Jectofer, increasing numbers of cells of *N. meningitidis* were isolated from the blood of the mice at intervals over the next 32 h. A comparison with the mucin challenge is shown. A rapid increase in viable count to a level of 10^9 cells/ml occurred within 16 h. Death usually resulted in 48 h at a bacterial concentration of $10^9 - 10^{10}$ cells/ml. Under the influence of iron sorbitol citrate a progressive and fatal infection was produced in mice by the injection of relatively few organisms.

Fig. 1

Growth of the serogroup A strain in commercial N.C.D.M. containing iron as Jectofer (ug/ml). Growth samples were removed at intervals, washed twice with saline, resuspended in 0.1N NaOH and assayed for protein by the method of Lowry et al. (1951). Cells were grown in one liter shake flasks containing 500 ml of media. Inoculum was 5 ml of 10^9 cfu/ml culture removed from overnight growth on a C.B.A.B. plate. The following concentrations of iron as Jectofer were added to commercial NCDM: 500 (··——··), 100 (——), 15 (- - - -) and 0 ug/ml (———). Viable count after 7 h of growth was 1.2, 1.1, 1.0 or 1.1 x 10^8 cfu/ml for media containing 500, 100, 15 or 0 ug Fe/ml respectively.

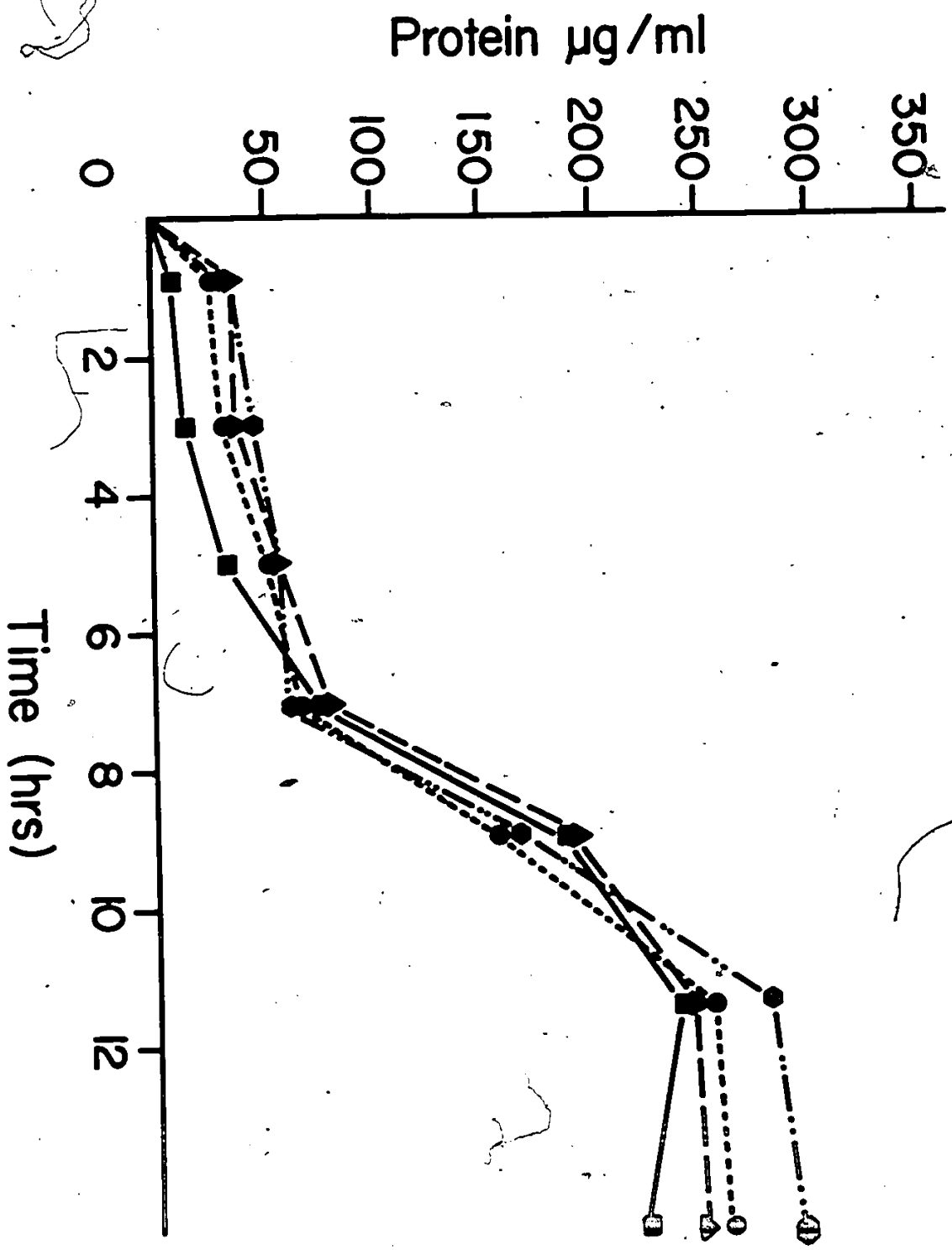

Protein µg/ml
0
50
100
150
200
250
300
350
Time (hrs)
2
4
6
8
10
12

Table 4

Growth of *N. meningitidis* serogroup A in blood of C57 mice[a]

Suspension	Time (h)	*N. meningitidis* viable count/ml blood[b]
Jectofer 500 µg/ml	0	0
	6	3.2×10^5
	16	1.2×10^9
	32	3.0×10^9
Mucin	0	0
	6	4.6×10^5
	16	2.7×10^9
	32	7.7×10^9

[a] An amount of 2.4×10^2 cells/ml injected i.p. at time 0h.
[b] A mean of blood samples from five animals; serum samples taken at 16 and 27 h following injection of bacteria in N.C.D.M. media alone were negative for viable count.

EFFECT OF OXIDATION STATE AND MOLECULAR SIZE OF IRON COMPOUND UPON CHALLENGE

The oxidation state of the iron being administered did not significantly affect the outcome of the experiment (Table 5A). With injection of lower cell inocula ferric salts were less efficient than ferrous salts at enhancing mortality. At higher cell concentrations, mortalities were comparable.

The size of the iron compound drastically affected challenge (Table 5B). When injecting different iron compounds with the same preparation of the virulent A strain no enhancement of mortality occurred with Imferon (180,000 daltons) or Ferrigen (230,000 daltons). The lower molecular weight compounds Jectofer and ferrous sulphate demonstrated a greater capacity for enhancing lethality of the bacteria.

INFLUENCE OF IRON UPON MOUSE WEIGHT GAIN AND HEMATOLOGY

In the previous experiments, injection of iron compounds alone produced an occassional death. A more extensive examination of toxicity due to iron alone was conducted. A single death occurred in the study shown in Table 6. This occurred in the group receiving the highest concentration of iron (450 ug) as ferrous sulfate. Mice in every group were seen to increase in weight within the one week study period. However, toxicity was evident at the higher iron concentrations for both iron salt and chelated iron (Jectofer); the weight gain of mice treated with these levels of iron was approximately one-half of that for untreated mice during the same time period. None of the mice suffered any visible ill effects. Weight gain in mice treated with the lowest level of iron as Jectofer was greater than that of the control mice. Weight gain was examined over 7 days according to the standard time interval established by the World Health Organization and other affiliate groups

Table 5

Comparative effect of various iron compounds upon challenge with *N. meningitidis* serogroup A

A.	% Mortality [a.]			
	Fe^{++}		Fe^{+++}	
Inoculum cells/ml	$FeSO_4$ [b.]	$FeCl_2$	$Fe(NO_3)_3$	$FeCl_3$
3×10^2	60	90	40	30
3×10^3	70	100	70	60
3×10^4	80	70	80	70

B.	$FeSO_4$ [c.]	Jectofer	Ferrigen	Imferon
1.0×10^2	70	60	10	0
1.0×10^3	80	70	10	0
1.0×10^4	100	80	0	0

a. Based upon groups of 10 mice; without iron no mortalities occurred following injection of these doses of bacteria.

b. Iron concentrations were 400 ug/mouse; mortality for iron compounds alone was nil.

c. Iron concentration was 500 ug/mouse.

Table 6

Influence of iron upon mouse weight gain

Fe injected µg/mouse		Group weight[a] (gm) Day 0	Day 3	Day 7	Group Increment (gm)	Individual Increment (gm)
$FeSO_4$	450	433	414[b]	460[b]	45[c]	1.9
	225	425	437	465	40	1.6
	110	423	443	477	54	2.3
Jectofer	450	398[d]	424	440	42	1.7
	225	397	425	460	63	2.6
	110	370	426	492	122	5.1
Saline	-	398	452	499	101	4.2

[a]Groups of 25 except where indicated
[b]One mouse died on day 2
[c]Final weight minus initial weight of 24 mice
[d]Group of 24 mice

(Code of Federal Regulations, USA 620.6), for evaluating the toxicity in mice of biological products. In the short term i.e. within 72 h iron was not toxic by the weight gain test since the average weight per mouse at this time was no less than the average weight per mouse immediately preceeding injection.

Table 7 demonstrates that in a further study, the hematological parameters of mice varied little from normal at either 4 or 20 hours after injection with 500 ug of iron (as Jectofer).

In general, these studies demonstrate that injection of iron as iron-sorbitol-citrate or ferrous sulfate into C57 HPB black mice at 27 mg/kgm causes no significant toxicity. Jectofer caused no deaths. One mouse out of 24 (i.e. 4%) died following ferrous sulfate. These data support the multitude of control studies conducted throughout this project which showed only the occassional death in mice treated with the above level of iron.

SERUM IRON ANALYSES FOLLOWING I.P. INJECTION OF IRON COMPOUNDS

This experiment was carried out to help understand the movement of iron in normal, uninfected mice.

A detailed and comparative examination of the analysis of iron in serum during the first 24 h following i.p. injection of ferrous sulfate and two different concentrations of Jectofer is presented in Table 8. For a 24 h period following injection an elevated serum iron level was provided by all doses. More iron entered the circulation from the peritoneal cavity during the first 6 1/2 h when iron sulfate was injected than when an equivalent amount of Fe as Jectofer was injected.

Total iron-binding capacity of normal sera was found to be approximately 250-300 ug/100 ml (results not shown). Hence during the first 6 1/2 h,

Table 7

Influence of iron[a] upon mouse hematology

Time Post Iron (h)	Mouse #	Hematocrit %	Hemoglobin gm %	RBC 10^6/mmc	Leukocyte 10^3/mmc
	1	35	12.8	6.0	8.9
4	2	38	13.1	6.2	6.3
	3	43.5	15.1	6.8	3.8
	5	37.5	13.1	5.9	7.0
20	6	38	13.5	6.0	5.4
	7	42.5	14.5	6.6	12.8
Controls[b]		41.6	13.2	6.6	10.9

[a]Iron as Jectofer at 500 µg/mouse
[b]Normal mouse mean value of three mice

Table 8

Serum iron after i.p. injection of iron compounds

Time (h) Post Injection	Serum Iron[a] µg/100 ml			
	Jectofer 500[b]	Jectofer 50[b]	$FeSO_4$[c]	Normal
2	1690	454	2074	72
4½	1360	450	1800	
6½	680	332	755	
24	400	223	288	80

[a]Bathophenanthroline assay-blood from 5 mice combined

[b]Concentration Fe injected µg/mouse

[c]500 µg/mouse injected

adequate iron to saturate the existing mouse transferrin was found in the circulation when any of the three doses of iron were injected. Even the Jectofer (50 ug Fe) dose provided enough iron to exceed saturation of the mouse serum transferrin. However, this lower level of Jectofer did not have the capacity to enhance meningococcal infection in mice and subsequently reduce the LD_{50} of the bacterium.

EFFECT OF TIME OF IRON ADMINISTRATION UPON CHALLENGE

Prior injection of iron salts resulted in enhancement of meningococcal infection in mice (Table 9). Consecutive i.p. administration of iron salt for three days prior to bacterial challenge resulted in an increased mortality proportional to the increasing amount of iron injected. A single dose of iron salt injected 24 h before the bacteria also significantly increased the lethality of the bacteria. The simplest and most efficient system for enhancement consisted of injection of bacterial cells in NCDM medium 2 h after injection of $FeSO_4$ in saline. Concomittant administration of iron in chelated form is also very effective in the enhancement of infection (Table 2). Iron given up to 4 h post bacteria also was capable of enhancing challenge (results not shown). Thus the time of administration is not a deciding determinant factor in meningococcal challenge. Since the mice are killed during challenge in which concomittant iron is given or in which iron is not introduced until 4 1/2 h following the bacteria, it appears that the prior disturbance of host iron metabolism by pre-treatment is not an absolute requirement for increasing the lethality of this bacterium.

Table 9 .

Effect of Time of Iron Administration Upon Challenge

Serogroup A cells/ml	Time of Iron Before Challenge	% Mortality Fe^{++} injected per mouse[a]			
		0	80	120	160
4.4×10^2	3 consecutive[b] days				7
4.4×10^3				0	13
4.4×10^4			0	7	13
4.4×10^5		0	13	27	53
		0	200	400	600
2.0×10^2	24 h[c]		0	60	70
2.0×10^3			0	60	80
2.0×10^4		0	10	60	90
					450
2.0×10^2	2 h[d]				78
2.0×10^3					78
2.0×10^4		0			100

a. $FeSO_4$ in saline.
b. Each mouse received this concentration of iron in 0.2 ml volume for 3 consecutive days prior to 1.0 ml of the bacterial suspension.
c. Iron injected in 1.0 ml volumes prior to 1.0 ml bacterial suspension.
d. Iron injected in .5 ml volume prior to .5 ml bacterial suspension.

EFFECT OF VACCINATION UPON CHALLENGE

Vaccination with a protein antigen reduced the effect of iron upon challenge. A comparison of bactericidal agents (Table 10) indicated that formaldehyde treated cells provide a more effective vaccine. Both types of vaccines however proved to efficiently protect mice during challenge in the presence of iron. Heat killing of the cells to be extracted, may have destroyed an active component of the vaccine.

Table 10

Effect of protein vaccine
upon meningococcal challenge

Bactericidal agent	Serogroup A # cells injected[b]	% Mortality[a] Vaccine[c] (µgs/ml) 0	20
Formaldehyde	5.0×10^2	100	20
	5.0×10^3	90	14
	5.0×10^4	80	26
Heat	3.8×10^2	80	27
	3.8×10^3	74	60
	3.8×10^4	87	60

[a]Based upon groups of 15 mice
[b]Challenge with iron salt as $FeSO_4$ at 450 µgs per mouse
[c]Vaccination i.p. 7 days prior to challenge

DISCUSSION

The lack of a satisfactory animal model for the experimental study of meningococcal meningitis has impeded evaluation of the host-pathogen relationship and complicated the understanding of immunity to the systemic disease. The chick embryo has been used in a variety of in vivo studies with *Neisseria*. Twelve day old chick embryos are susceptible to intravenous injection of *Neisseria meningitidis*. The neutralization potential of hyper-immune meningococcal antisera was explored with this animal model (Ueda et al. 1969 and 1971). Differences in virulence of gonococcal colony types were reproduced in 11 day-old chick embryos inoculated intravenously (Bumgarner and Finkelstein, 1973). The use of the chick embryo model was extended to examine the potential role of iron in the virulence of gonococci (Payne and Finkelstein, 1972). However, iron proved to be rather toxic to the embryos. The strains of *N. meningitidis* used proved to be equally lethal in the presence or absence of exogenous iron.

The chick embryo model of infection possessed other serious limitations. The age of the embryo has a profound effect upon the susceptibility to bacteria. Embryos less than 10 days old are easily killed by low inocula regardless of inoculation route, whereas embryos 14 days or older are resistant to challenge. Hence, such a model could not be used for the study of potential immunogenicity of meningococcal vaccines.

In addition, the presence in chick embryos (of susceptible age) of an active phagocytic function especially involving polymorphonuclear leukocytes remains questionable. Hence early defense mechanisms against meningococci due to phagocytosis and any interaction with iron could not be studied with this model.

Intra-occular injection in rabbits may be an effective tool for studying cellular events which occur in defense against *N. meningitidis*. Vitreous tissue (rabbit) supported survival and limited multiplication of this bacterium although septicemia was not detected (Pribnow et al. 1971)

Despite these previous studies, mucin enhanced meningococcal infection (originally established in 1933 by Miller) remained the model of choice for studies of virulence and immunity. The mouse possesses all defense components including active phagocytic and immune systems. The mouse also has an adequate life span with which the protective effect of various potential vaccine preparations as well as the efficacy of antibiotics can be tested. In the presence of mucin, low inocula when injected i.p. grow progressively to large numbers in both the blood and the cerebrospinal fluid eventually causing death due to septicemia. However, the factors in mucin responsible for enhancing infection were not adequately defined and the effects of mucin upon the immune response were unknown.

Enhancement of meningococcal infection in mice under the influence of exogenous iron, supports previous findings with other bacteria that parenteral administration of iron compounds to experimental animals reduces the size of the inoculu required to produce disease or death, and permits a greater amount of multiplication of microorganisms in the blood and/or invaded tissues.

Research in different laboratories has since supported our original findings (Calver et al. 1976) that iron could be used as a replacement for mucin in the establishment of meningococcal infection in mice. Bannatyne et al. (1977) examined the protective effect of polymyxin B sulfate in experimental menigococcal infection in mice using our challenge procedure

with Jectofer. The anti-endotoxic activity of polymyxin B upon mice challenged with *Serratia marscescens* and *Proteus rettgeri* was also studied (Bannatyne and Cheung, 1979 and 1981).

Addition of iron to substandard lots of 5% gastric mucin resulted in an enhancement of the virulence of typhoid strain TY-2, suspended in the mucin preparations. Ford and Hayhoe (1976) demonstrated that ferric ammonium citrate was an effective alternative to hog gastric mucin as a virulence enhancing agent in the cholera vaccine potency assay.

Our studies here demonstrate that the mouse infection model (with iron) provides a system with the potential for studying immunity to the meningococcus and for classifying the virulence of the different strains.

In the presence of iron, a fatal infection is produced in mice by relatively few cells of certain serogroups of meningococci. Strains of serogroups such as A, B and C which reacted in this manner were designated "virulent". However, not all serogroups demonstrated equal enhancement in the presence of iron. The serogroup Y, Slaterus strain remained relatively unresponsive, still having a high LD_{50} even in the presence of iron (Calver et al. 1979). Hence, based upon this lack of capacity for establishment of meningoccocal infection in mice, the serogroup Y strain is classified "avirulent". This designation of virulence in mice compares favourably with the virulence of these strains in humans: serogroup A, B and C strains were "case" strains whereas the serogroup Y strain was a "carrier" strain. Iron-enhanced infection in mice has also been used by Holbein et al. (1979) to classify meningococcal strains according to their virulence. All of nine isolates from clinical disease proved to be virulent whereas only 30 per cent of isolates from carriers were virulent. Strains of *P. aeruginosa* and *N. gonoroheae* have not demonstrated the ability to establish infection in our

mouse-challenge system (personal communication, Dr. B. Dienna, L.C.D.C., Ottawa).

Bacterial cells when grown and injected in iron media were lethal only when injected with iron. Excess iron in the media did not produce bacterial growth enhancement nor lead to an increase in the amount of iron bound to the growing cells. Hence it does not appear that the virulent strain when grown in the presence of excess iron alters its character or stores sufficient iron to lower its LD_{50}.

Is the difference in virulence of meningococcal strains due to a difference in ability to acquire some of this iron *in vivo* or is iron treatment somehow altering resistance of mice to certain strains? Reflection upon the amount of iron that must be injected in order to establish infection may provide some insight into this problem. Sorbie (1974) has examined the total iron content of similiar mice by an ashing technique. Mice weighing a few grams more than ours, fed a normal diet, had a total iron of 800 $\pm$ 50 ug. Hence, 250 to 500 ug of Fe, the concentration required to reduce the LD_{50} is rather large in comparison to the mouses' iron level. Under normal conditions injection of such an excess of iron would be considered grossly unbiological, akin to hitting the mouse with a sledge-hammer. This fact on its own tends to favour interaction of iron with host defense mechanisms as its main role.

Previous examination of mouse livers and spleens has shown that iron overload does occur when 500 ug Fe is injected (Calver et al. 1976). However, the hematological investigation conducted in the current study suggests that the blood parameters were not significantly altered for the first 20 h following iron injection. No deaths occurred in our mice with 30 mg Fe/kgm mouse body weight (corresponding to 450 ug of Fe per mouse). supplied as sorbitol citrate. The results of this study are in accordance

with many previous reports on the toxicity of iron in mice or other animals. In a series of investigations conducted by Bannatyne et al. (1977, 1979 and 1980) according to our mouse model with Jectofer, the authors report that no obvious ill effects occurred in any of the hundreds of Swiss mice injected solely with iron. A study of the acute toxicity of Jectofer in male albino mice by Svard (1961) demonstrated an LD_{50} by the i.p. route of 50.3 $\pm$ 2 mg Fe/kgm. However, Holbein et al. (1979) have reported an LD_{50} of 25 mg Fe/kgm in similiar C_{57} black mice following i.p. injection. The reasons for these differences are unknown.

Mouse serum iron analysis following i.p. iron injection shows that absorption of iron as the chelate (Jectofer) or the salt ($FeSO_4$) from the peritoneal cavity into the blood stream is rapid. Two hours following injection of 500 ug/mouse (Jectofer or $FeSO_4$) the level of iron was five-fold in excess of the serum transferrin iron-binding capacity; and excess was maintained 24 h. Lower iron doses (i.e. 50 ug) also produced an elevated iron level equal to or greater than the iron-binding capacity of the serum during this period. However, the lower iron dose did not decrease the lethal dose of bacteria as did the high doses. These data support the idea that serum iron and levels of transferrin saturation are not the only factors in determining host resistance.

The total blood volume of these mice is 2 -3 ml; thus the maximum amount of iron in the blood at any one time is 40 - 60 ug. Little iron would be lost through excretion (after 24 h approximately 20% of the dose - Lindvall and Andersson, 1961). Since each mouse initially received 500 ug of iron, it would appear that by 6 1/2 h after injection, most of the iron is in storage, either in the cells of the reticuloendothelial system (polymorpho nuclear leukocytes and local macrophages) or other body tissues or fluids.

Since previous studies with iron-dextran have shown that this type of iron is absorbed into the bloodstream by way of the lymphatics, it appears that most of the "missing iron" may be in the lymphatic system and/or deposited at the injection site, or in other tissues (Svard and Lindvall, 1961). It is possible in both our study and Holbein's that the "missing iron" is taken up by local or circulating polymorphs or macrophages and that iron may exert its influence upon the defensive action of these cells, subsequently enhancing infection.

Holbein (1981) has also shown that injection of human transferrin containing iron increases meningococcal lethality for mice. The author suggests once again that serum iron is most important. However, the study does not prove that the bacteria actually use such iron to grow, but proposes such since iron-free transferrin did not enhance lethality. However, the data show that iron-free transferrin did not inhibit infection but rather stimulated infection above normal. The possibility that iron-free or iron-containing human transferrin is taken up by the macrophages or polymorphs and inhibits their defensive activity is not ruled out.

The minimal in vitro iron requirement for growth of certain meningococci has been found to be approximately 50 ng Fe per ml medium (Archibald and DeVoe, 1978). At equivalent iron concentrations supplied by iron salts, iron sorbitol citrate (Jectofer), iron dextrin (Ferrigen) or iron dextran (Imferon) meningococcal growth on agar is stimulated to the same extent (Calver et al. 1979). Imferon agar has been proposed as an improved medium for the isolation of pathogenic *Neisseria* (Payne and Finkelstein, 1977). Archibald and DeVoe (1980) have demonstrated that Imferon iron becomes readily available for growth when in the presence of pyrophosphate or sub-physiological concentrations of citrate. If one presumes that *in vitro* findings may be extrapolated to *in vivo* then injection of 450 ug of iron as

any of the previous compounds ought to provide enough iron in excess of the mouse transferrin binding capacity (i.e. 6 - 10 ug Fe) to stimulate growth. However, *in vivo* the large molecular weight compounds, Imferon and Ferrigen were unable to enhance infection to the same extent as the lower molecular weight compounds.

Imferon was absorbed as rapidly into the bloodstream as iron salt or Jectofer (Holbein, 1980). Hence difference in absorption from the peritoneal cavity into the bloodstream does not appear to provide a basis for the difference in ability of the iron compounds to enhance infection. A difference in cellular uptake and processing by polymorphs or macrophages is an alternative explanation for such observations.

The natural resistance of mice to *Neisseria meningitidis* is similar to the natural resistance of guinea pigs to *E.coli* as described by Bullen (Bullen et al. 1968). Upon injection of iron, the normal ability of the host to suppress growth is lost. Active immunization of our mice with a protein antigen extracted from the avirulent strain abolished the enhancement of challenge with iron. It would thus appear from these studies that iron might interfere preferentially with host mechanisms which operate in the early phase of microbial invasion, before an immune response of the animal has developed. In the pre-antibody or pre-recognition state, the first line of defense lies in the normal components of serum and/or tissues which would be detrimental to bacterial growth, in conjunction with polymorphonuclear leukocytes and macrophages which are required to initially process foreign particulate matter to the antigen for presentation to the lymphoid tissue.

In summary, the data of our study indicate that iron treatment of mice is needed to establish lethal infection. Iron appears to be interacting with a defense mechanism of the host. Treatment of the mice with iron is

affecting host resistance in such a manner that only bacteria possessing a selective character have the capacity to take advantage of this alteration.

It is possible that this resistance may be due to iron-binding proteins or factors which starve the bacterium for iron. To investigate this further, in vitro comparisons are to be made of the iron requirements and binding ability of virulent and avirulent cells (Chapter 2). The ability of virulent and avirulent cells to compete with transferrin and other iron-binding proteins of the mouse and human will also be studied in a controllable in vitro environment (Chapter 3).

CHAPTER 2 IRON LIMITED *IN VITRO* GROWTH AND SEARCH FOR MENINGOCOCCAL SIDEROPHORE

INTRODUCTION

It has been suggested that exogenous iron enhances bacterial infection and mortality in animals by stimulating bacterial growth (Bullen et al. 1974). If this also occurs in mice infected with meningococci, then virulence differences might be due to differences in ability of strains to use iron for growth, or to store iron. Can iron-limited growth be produced *in vitro*? If so, does the avirulent strain require more iron?

Since various iron compounds had different *in vivo* effects on meningococcal infection it seemed important to carry out *in vitro* studies of the influence of these iron compounds upon meningococcal growth.

Many different micro-organisms can synthesize and secrete iron-binding compounds when grown in chemically defined iron-deficient medium. Iron-binding agents have been isolated from both whole bacterial cultures and culture filtrates (Table 11).

Phenolate siderophore production influences *E. coli* infection (Rogers, 1973). Two serologically different strains varying in ability to produce siderophores differed in their virulence for mice. Rogers concluded that siderophores were a virulence factor for *E. coli*, since the virulent strain produced more siderophore.

The following studies were undertaken to investigate the relation between virulence for mice and siderophore production, *in vitro*, by different serological strains of *N. meningitidis*.

Table 11

SIDEROPHORE ISOLATIONS

Investigator	Bacterium	Product	Source	Method	Yield
Brot et al. (1966)	*E. coli* K_{12}	2,3-dihydroxybenzoyl -serine	culture supernatant	$FeSO_4$ saturation; DEAE-cellulose, 1M $(NH_4)_2CO_3$ as eluant; lyophilization	75 mg/L
Byers (1974)	*B. megaterium*	schizokinen	culture supernatant	charcoal adsorption; $FeSO_4$ to eluate; acidified to pH 4; pet-ether-acetone extraction; H_2O and pH to 11; Dowex-1 1M NaCl as eluant	90 mg/L
Macham and Ratledge (1975)	*M. smegmatis*	exochelin	culture supernatant	$FeCl_3$, pH to 7; excess iron filtered off; pH to 3.5; Zerolit 225 SRC_{10} resin; NH_3 as eluant	
Young (1976)	*E. coli* AN311	enterochelin	culture supernatant	$FeSO_4$; DEAE-cellulose, 2M NH_4Cl as eluant; pH to 3.5; extracted with ethyl acetate; dried	70 mg/L
O'Brien (1970)	*E. coli*	enterochelin	culture supernatant	20 fold concentration by lyophilization residue into acid pH 1; ethyl acetate extraction; dried; DEAE-cellulose; 2M NH_4Cl as eluant	15 mg/L
Pollack (1970)	*S. typhimurium* LT 2	enterobactin	culture supernatant	20 fold concentration by lyophilization residue into acid pH 2; ether extraction dried	6 mg/L
Wilkins (1970)	*S. typhimurium*	2,3-dihydroxybenzoyl serine	whole culture	$FeCl_3$; DEAE-sephadex; filtered with .01 N HCl; fractions pooled and chromatographed	20 mg/L
Rogers (1977)	*E. coli* K_{12} *E. coli* O_{111}	enterochelin enterochelin	whole culture	EDDA medium; pH to 6.5; extraction with ethyl acetate; concentrate 24 fold; mix with ferric nitrilotriacetic; aqueous acidified and ethyl acetate extracted	21 mg/L

MATERIALS AND METHODS

Catechol. Resublimed pyrocatechin was obtained from Fisher Scientific Co., New Jersey, U.S.A. Solutions were prepared in saline and the pH adjusted to 7.0 with 1N NaOH.

Desferal. The iron chelating agent deferoxamine mesylate was supplied by CIBA Laboratories, Horsham, England.

E.D.D.A. Practical grade ethylenediamine-di-orthohydroxy phenylacetic acid (EDDA) was supplied by Sigma. Solutions were prepared by dissolving the EDDA in 5N NaOH and adjusting the pH to 7.0 with concentrated HCl.

Ferric nitrilotriacetate. A 1.0 mM ferric nitrilotriacetate solution was prepared by mixing 48.2 mg ferric ammonium sulphate (Fisher Scientific Co.) with 50 ml distilled H_2O containing 21.02 mg nitrilotriacetic acid (Eastman Kodak Co., NY, USA); the volume was made up to 100 ml with water after the pH was adjusted to 7.4 with 1N NaOH.

All other iron compounds were prepared and used as described earlier (Chapter 1).

Media: *Neisseria* chemically defined medium (NCDM) without iron was prepared using analytical grade reagents according to the manufacturer's formulations and specifications. Such medium was labelled "iron-poor NCDM." This medium was sterilized according to the manufacturer's specifications and adjusted to pH 7.2 with 5% $NaHCO_3$. As required, sterile solutions of iron salts or Jectofer were added to the sterile media prior to pH adjustment.

Tetramethylammonium hydroxide: (TMAH) The base was obtained from Eastman Kodak Co., as a 25% solution of tetramethylammonium-hydroxide in H_2O.

Agar media. The iron donating activity of various agents was tested in an iron-poor agar medium consisting of 15 g of agar per 1.0 litre of *Neisseria* chemically defined medium (NCDM), iron-poor formula (ingredient

contaminant level only). Iron was added to this agar after autoclaving and adjustment of the temperature to 50°C, as required.

Diffusion plate technique. Pour plates 10 cm in diameter were made with 20 ml of iron-poor NCDM agar. The agar depth was approximately 3.5 mm. Wells (9 mmm in diameter) were made in agar medium with a suction apparatus. Each well was filled with 0.15 ml of a test substance which was sterilized, if required, by Millipore filtration.

The test materials were allowed to diffuse at room temperature for a period of 24 hr. before 0.1 ml of a seed inoculum was distributed evenly over the surface of the agar. Plates were incubated at 37°C in a 5% CO_2 atmosphere and examined periodically over the next 48 hr.

Inocula for spreading on the agar-diffusion plates or liquid cultures were prepared by suspending overnight growth at 37°C from a plate of Columbia blood agar, supplemented with 5% sheep's blood in NCDM without iron, to a level at which a 1:2 dilution gave a 5% transmission at 530 nm (Spectronic photometer; Bausch and Lomb Inc., Rochester, NY). All inocula were counted by plating on Columbia blood agar with blood and determining viable counts. In some experiments inocula were used to prepare 8 - 10 hr cultures in non-iron NCDM medium, which were subsequently used to inoculate large shake flasks. Inocula of the different serogroups were adjusted to equal opacity using a Bausch and Lomb Spectronic photometer.

Growth in liquid media. Bacterial cells were grown in 2 litre shake flasks containing 1 litre of medium (commercial NCDM or iron-poor NCDM with or without added iron) for 32 h at 37°C, shaken at 100 rpm in an incubator shaker (New Brunswick Scientific).

Aliquots of the growth culture were removed at intervals and were killed as required by the addition of formaldehyde (1%). Samples were centrifuged at 3000 rpm (I.E.C. refrigerated centrifuge) and the cell supernatants were

removed. Cell sediments and supernatants were further processed and analyzed for iron and/or protein. The pH of killed culture suspensions and/or filtrates was recorded with a Corning Digital 110 pH meter.

Smears were prepared and Gram stains examined periodically throughout the course of the growth experiment. At various time intervals Columbia Blood Agar plates were streaked and examined for colonial morphology.

Growth in agar and liquid media containing iron chelating agents. Pour plates and agar-diffusion plates were prepared and spread with bacteria as previously described. Desferal and catechol solutions were prepared in saline, their pH adjusted to neutrality and filter sterilized. EDDA was dissolved in 5N NaOH and adjusted to pH 7.0 with concentrated HCl prior to filtration. Desferal, EDDA and catechol at various concentrations were deposited in wells of agar plates containing different iron concentrations as Jectofer and tested following diffusion.

Serogroup A and Y growth filtrates from iron-poor NCDM cultures were concentrated 5 times by lyophilization. These filtrates were deposited in centre wells of iron-poor agar and iron-agar containing Desferal and examined for growth enhancement.

Growth of A, B, C, and Y strains was tested in iron-poor NCDM containing various levels of Desferal. The extent of growth in these solutions was recorded by turbidity and viable count. Iron as Jectofer ranging from 0.31 to 10 mg/ml was added to iron-poor NCDM containing an inhibitory concentration of Desferal and tested for effect upon growth of the serogroup A and Y strains. In these experiments 0.25 ml of inoculum, prepared as described above (10^9 cfu/ml), was added to 5.0 ml of liquid medium.

Protein analysis. Cell sediments were washed twice with saline and resuspended in 0.1 N NaOH (volume equal to original suspension).

After approximately 2 hr the protein content was determined according to the procedure of Lowry et al. (1951), using bovine serum albumin as standard.

Iron analysis. Cell sediments were washed twice in 0.1 M phosphate buffer (pH 7.2) rather than H_2O to minimize dissolution and removal by the washing process of any iron that may have been sedimented. In some experiments cells were washed twice in 0.1 m citrate buffer, pH 5.0. The washed sediments were resuspended in distilled water to this original volume. One ml of the washed cell suspension was mixed with 1.0 ml of TMAH (25% in distilled H_2O) in a 5 ml glass stoppered volumetric flask. After 2 hrat 70°C the samples were filled to volume with 15 drops of concentrated HCl added from a Pasteur pipette. (Gross and Parkinson, 1974). One ml of each acidified sample was examined for iron content according to the method of Caraway (1963) employing sulfonated bathophenanthroline with spectrophotometric examination at 533 nm (Bausch and Lomb Spectronic 505), using $FeCl_3$ in dilute HCl as a standard (Iron Reference Solution - Fisher Scientific), similarily digested in TMAH. The digests were also analyzed for iron content by atomic absorption spectroscopy (Perkin Elmer model 306) at wavelength 248.3 nm (0.2 mm slit) using the flame mode.

Growth supernatants after centrifugation were filtered through Millipore 0.45 u membranes in order to remove any cellular debris. The iron content of aliquots of the supernatants was determined by the sulfonated bathophenanthroline assay and/or atomic absorption. The pH of the supernatants was adjusted to pH 6.8 to 7.0 with 0.1 NaOH or 0.1 M phosphate (pH 7.2). Following filtration of the pH adjusted supernatants (Millipore .45 u filter), the iron contents were determined and the values compared with those of the unadjusted supernatants.

Ion-exchange chromatography. Diethylaminoethyl-cellulose Type 20 (Schleicher and Schuell Co., NH, USA) was the ion-exchange resin used for the isolation of iron-binding agents. The resin was washed with 0.5 N NaOH, then with distilled H_2O. A Pharmacia (Upsala, Sweden) glass column (2.5 x 100)

was packed with washed resin, to a height of 90 cm. Prior to applying culture filtrate to the column, the packed resin was washed with 0.01 sodium phosphate buffer pH 7.0. The elution buffer was 2 M NH_4Cl. Column effluent was collected with a L.K.B. fraction collector and the fractions monitored separately at OD_{310} and OD_{280} on a Bausch and Lomb Spectronic 505.

Ultrafiltration. Amicon ultrafiltration pressure cells (Amicon Corp., USA) were used for concentration of culture filtrates and extracts. Standard stirred cells, model #'s 12, 52 and 401-S (respective processing volumes of 10, 50 and 400 ml) were fitted as required with Diaflo membranes (Amicon Corp.) of the UM or PM type. Up to 4 litre volumes of culture filtrate were passed through a 401-S cell containing a PM - 30 membrane and the ultrafiltrate was subsequently processed and examined for iron-binding agent. Model 12 and Model 52 cells with UM - 05 or PM (10) membranes were used to determine the ability of culture filtrates or extracts to remove iron from Ferrigen, transferrin or plasma.

Extraction of batch culture filtrates. Serogroup A or C strains were grown in iron-poor NCDM for 8 hr. (1 litre in 2 litre flasks). Inocula for these cultures consisted of 10 hr. cultures grown in iron-poor NCDM. Merthiolate was added to 1% and the culture centrifuged 4 hr. later 3000 rpm, 20 min.). The cells were discarded and the supernatant filtered through a 0.45 u Millipore membrane. As required, the supernatant was filtered through a PM-10 membrane (Amicon). Iron as $FeCl_3$ was subsequently added to the growth filtrate to a concentration of 10 mg/L and after 4 hr. at room temperature the pH was adjusted to 7.0. The resulting solution was filtered through a Seitz ST-3 filter pad contained in a Sartorius positive pressure filter unit.

Up to 4 litres of filtrate at a time was put on a DEAE-cellulose column. The column was washed with 0.01 M sodium phospate buffer pH 7.0.

Elution buffer was 2M NH_4Cl, pH 7.0 ; absorption of all fractions (from wash and elution buffer) were measured at OD_{310}. Fractions which gave peak absorption were pooled, adjusted to pH 1.0 with H_2SO_4 and extracted 3 times each with 0.5 volumes of ethyl acetate. The ethyl acetate extracts were combined and concentrated by flask evaporation. Benzene was added to assist precipitation of a residue which was taken to dryness. The procedure was repeated for cultures grown 10 hr. in iron-poor NCDM and in commercial NCDM (600 ug Fe/100 ml).

Extraction of cells and filtrates at intervals during growth of single shake culture. Serogroup A, B, C and Y cells were grown separately in iron-poor NCDM. A 2 ml volume of seed inoculum containing 10^9 cfu/ml was added to 1 litre of medium in 2 litre flasks. Five samples each of 10 ml were removed at 0, 2, 4, 6, 8 and 10 hr. during culture growth. Formaldehyde was added to each to a concentration of 1%. The remainder of the culture was grown for a total 24 hr. before addition of formaldehyde. Within 4 hr. of killing, the cultures were centrifuged (15,000 rpm, 10 min, on a Beckman J-6B), the supernatant removed and treated separately.

The cell sediment from 40 ml of culture was resuspended in 10 ml H_2O and mixed with 10 ml of ethyl acetate. Following vortexing and ultrasonication, the ethyl acetate layer was separated from the aqueous layer. The aqueous layer was re-extracted with a further 10 ml of ethyl acetate. The ethyl acetate layers were combined and taken to dryness (gentle heat and nitrogen). When required for use, the dried residues were reconstituted in 4 ml of 50% EtOH/H_2O. In total, the residues were concentrated 10 fold from the original medium level.

Following filtration (Millipore filter 0.45 u), the culture supernatants were freeze-dried and resuspended to one-tenth the original volume with H_2O.

Analysis for catechol. Analysis for the catechol content of cell extracts and culture filtrates was done by the method of Arnow (1937).

Analysis for hydroxamate. Analysis of the hydroxamate content of cell extracts and culture filtrates was done by a modification of the procedure of Hestrin (1949). Distilled H_2O replaced .2M hydroxylamine in both blank and sample tests. Acetyl hydroxamate (Calbiochem, LaJolla, California) was used for the standard curve. Solutions of Desferal were also analyzed.

Iron Binding Tests: Culture filtrates and cell extracts were examined for the capacity to maintain iron in solution at neutral pH. A 0.5 ml aliquot of test sample was mixed with 0.5 ml of $FeCl_3$ (10 mg/L) in dilute HCl, and let react at 37°C overnite. The pH was recorded and adjusted to 7.0 with 0.1 M phosphate buffer (sodium, pH 7.2). The solution was filtered through a Millipore membrane (.45 or .25 u), and the iron content was assayed by bathophenanthroline and/or atomic absorption.

Whole growth cultures were tested for the presence of iron binding agents by the method of Rogers et.al., (1977). Twenty ml of culture was mixed with 20 ml of ethyl acetate and vigorously vortexed. The ethyl acetate layer was removed and shaken with 5 ml of aqueous ferric nitrilotriacetic acid (100 mg/L). The ferric nitrilotriacetic phase was removed and OD_{480} recorded.

The dried residues processed from various batch growth filtrates were dissolved in ethyl acetate at concentrations up to 1 mg/ml (dry weight) and shaken with ferric nitrilotriacetic acid (100 mg/L) according to the method of Rogers et.al., (1977) and examined for an increase in absorbance at OD_{480}. Aliquots (0.5 ml) of residues in 10% $EtOH/H_2O$ (1 mg/ml) were mixed with 0.5 ml of $FeCl_3$ (2 mg/L) in dilute acid and absorbance at OD_{480} recorded.

The capacity of the dried residues to interfere with the assay of solution iron by bathophenanthroline was also examined. Aliquots (0.5 ml) of the residues in saline (1 mg/ml) were mixed with 0.5 ml of $FeCl_3$ in dilute HCl (2 mg/L). Desferal in saline (360 ug/ml) was likewise added to the iron standard. The iron contents of the solutions were assayed by bathophenanthroline.

Dried residue (1.5 mg) was added to 10 ml human plasma and let react with shaking. The plasma was subsequently ultrafiltered through a PM-10 membrane; the ultrafiltrate was collected and the retentate which had been concentrated 5 fold was resuspended back to its original volume by the addition of 8 ml of iron free saline or distilled H_2O. Retentates, ultrafiltrates and normal plasmas were analyzed for iron content by the bathophenanthroline assay.

RESULTS

EFFECT OF IRON UPON GROWTH IN LIQUID DEFINED MEDIA

Batches of commercial NCDM varied 10 - 15% in iron content from flask to flask. Filtration of media at pH 7.2 through 0.45u Millipore filters removed all recordable iron. Commercial NCDM contains 600 ug Fe/100 ml as ferric salts. It thus appeared that at neutral pH this iron exists in a polymeric insoluble form (possibly iron hydroxide or iron phosphate) removable by filtration. Hence, NCDM as supplied and prepared according to the manufacturer was not adequate for studies of the solubilization and/or co-sedimentation of iron by growing cells.

NCDM containing no added iron-salts was prepared according to the manufacturer's formulation. Repeated analyses of this iron-poor NCDM done at different times during the entire study indicated that iron contamination by other medium components was always below the level of detection by the bathophenanthroline test (i.e. < 5 ug/100 ml). Atomic absorption also indicated less than 5 ug/100 ml. The contaminant level of iron could have been lessened by absorption of this medium with iron binding agents such as hydroxyquinoline. However, this procedure was not carried out since it has also been shown that it is a difficult task to ensure that all of the iron-binding agent has been effectively removed from the medium (Lankford, 1973).

If ferric chloride or ferrous sulphate was added to this medium to a level of 600 ug Fe/100 ml it was once again found that following neutralization and filtration through Millipore 0.45 um filters all measurable iron was removed. However, very little iron was lost from iron-poor NCDM containing 500 ug Fe/100 ml as Jectofer, following Millipore

filtration of the neutralized medium. This medium was best to study iron binding and/or solubilization by growing meningococcal cells.

Growth of virulent and avirulent serogroup strains in iron-poor NCDM with 500 ug Fe/100 ml as Jectofer was similar in rate and yield, except for a larger yield of the virulent C strain (Fig. 2). No variation in cell or colonial morphology between strains was evident due to growth in this medium.

The concentration of iron in the culture filtrates decreased with increased growth of the cells in the medium. The decrease at 8 hr of growth was approximately equivalent for all four serogroups i.e. 110 - 140 ug/100 ml (Fig. 3). After 8 hr, a greater decrease occurred in serogroups B and C than in serogroups A and Y.

Analysis of the phosphate washed cellular sediments showed that iron was being co-sedimented with the cellular material in the later stages of growth (Fig. 4). Citrate (pH 5.0, buffer) removed 50 - 70% of the sedimented iron (unpublished observations). This suggests that the iron was probably attached to the external cell surface.

A further comparison of the growth of the A and Y strains was made in media containing half the previous level of Jectofer (i.e. 250 ug/100 ml) and in iron-poor media. Growth rate, yield and viability for each strain was found to be the same in both iron-poor and iron-containing media. In addition, no significant differences in growth parameters were evident between strains. A similiar reduction in the iron level of culture filtrates of both strains occurred during their growth (Fig. 5).

Samples of Jectofer 500 ug/ml in iron-poor NCDM which had been adjusted to various pH's corresponding to the different pH's of the growth cultures at sampling times were filtered and analyzed for iron. The values were found to

Fig. 2.

Growth of various meningococcal strains in iron poor NCDM (laboratory preparation), to which iron sorbitol citrate was added at 500 ug Fe/100 ml. Samples were removed at intervals, washed and analyzed for protein by the method of Lowry et.al., (1951). Cells were grown in one liter flasks containing 500 ml of medium. Seed inoculum was 5.0 ml of a 10 h. culture (10^9 c.f.u./ml) in iron-poor NCDM.

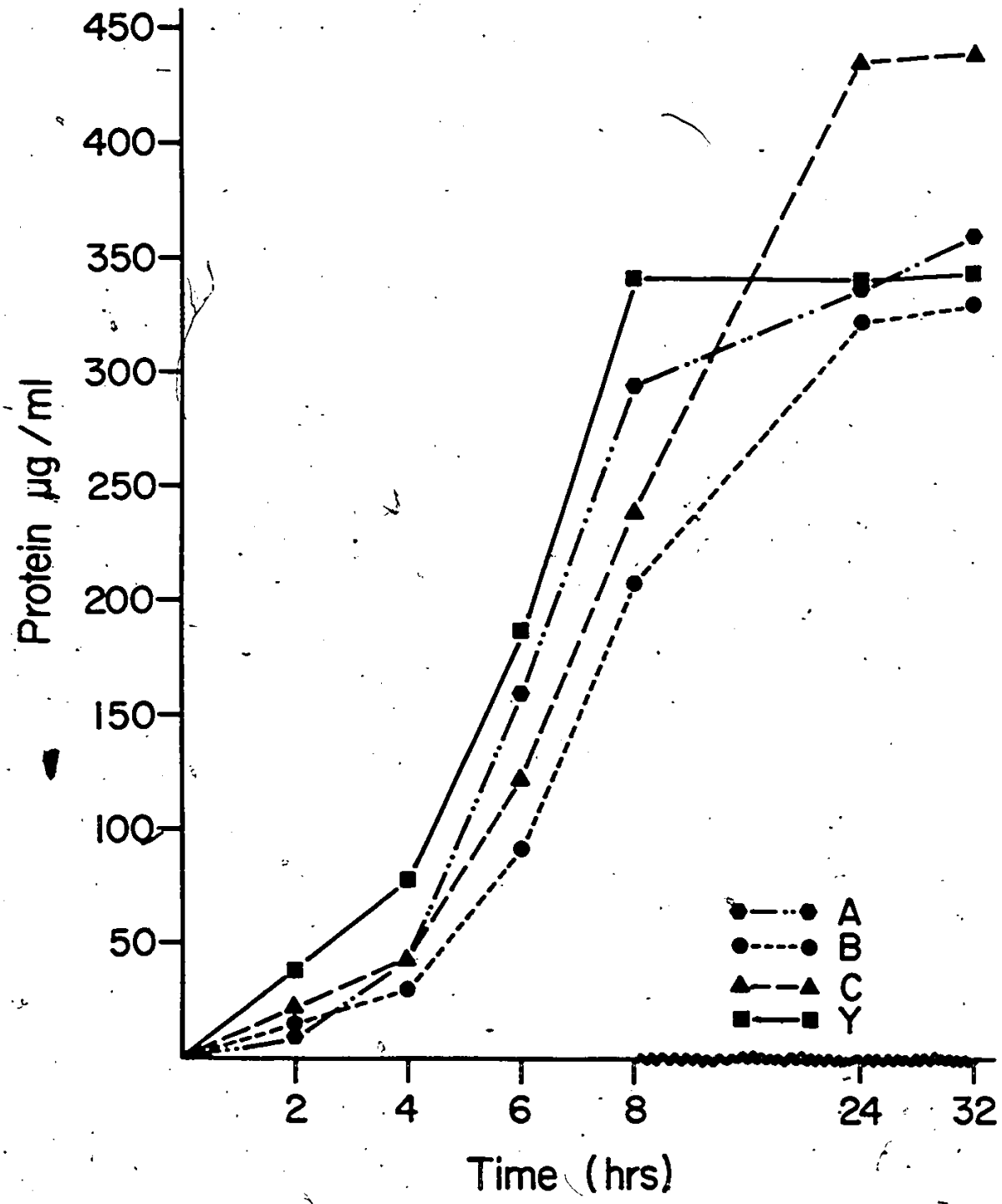

450
400
350
300
250
200
150
100
50
Protein µg/ml
2
4
6
8
24
32
Time (hrs)
A
B
C
Y

Fig. 3.

Analysis of meningococcal culture filtrates for total iron. Cultures grown in iron-poor NCDM to which iron sorbitol citrate was added were centrifuged (3000 r.p.m., I.E.C. centrifuge), superatant removed and filtered through 0.45 u Millipore and analyzed for total iron content by the method of Caraway (1963). The cultures were the same as those previously analyzed for protein.

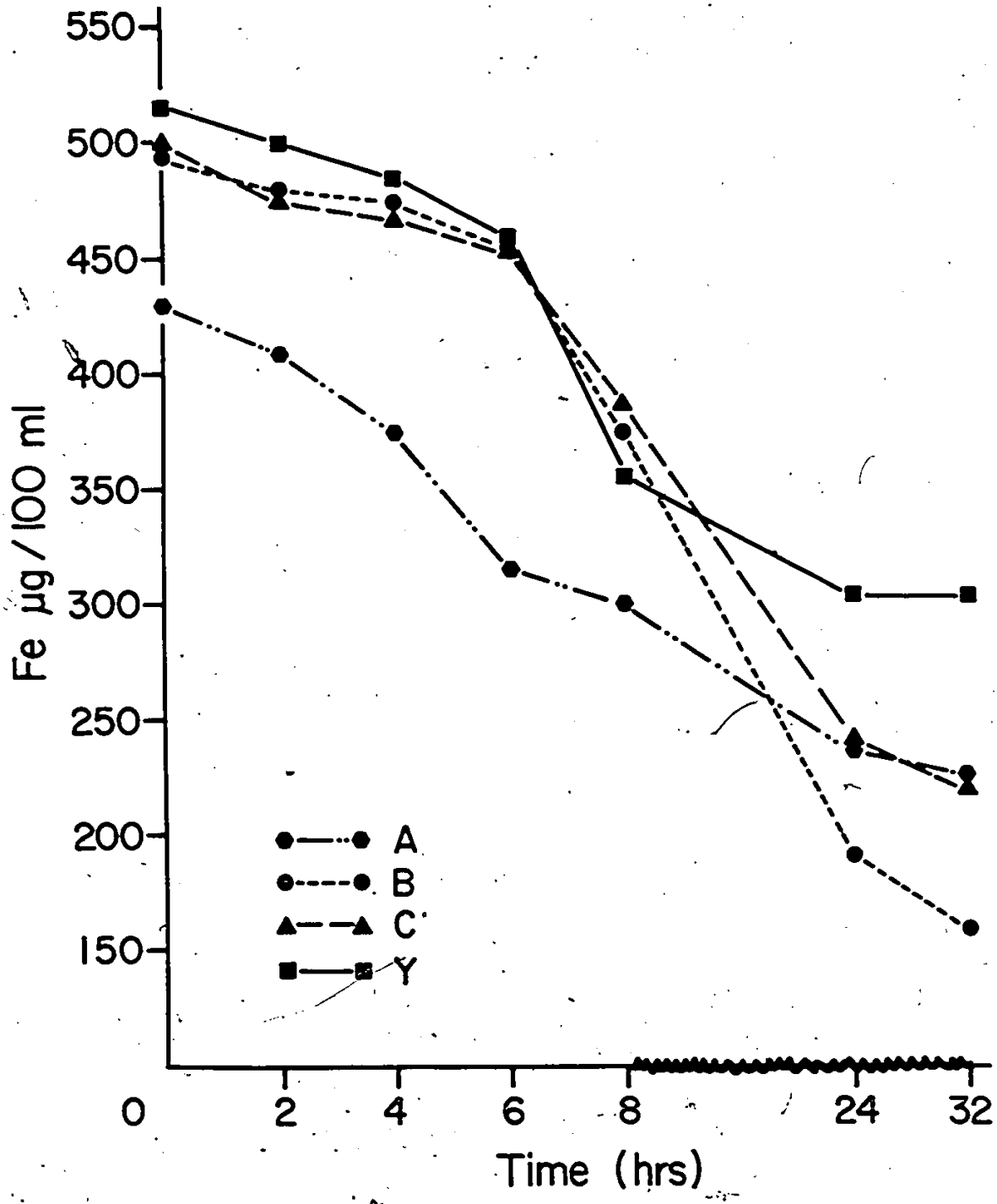

550
500
450
400
350
300
250
200
150
Fe µg / 100 ml
A
B
C
Y
0
2
4
6
8
24
32
Time (hrs)

Fig. 4.

Analyses of culture sediments for total iron content. Cultures were centrifuged, cell sediments were washed twice in phosphate buffer (0.1 M pH 7.2) and resuspended in H_2O. Aliquots were digested with 25% T.M.A.H. in H_2O (Gross and Parkinson, 1974), pH adjusted to 7.0 with HCl and the total iron determined with sulfonated bathophenanthroline (Caraway, 1963). The cultures were the same as those previously analyzed for protein.

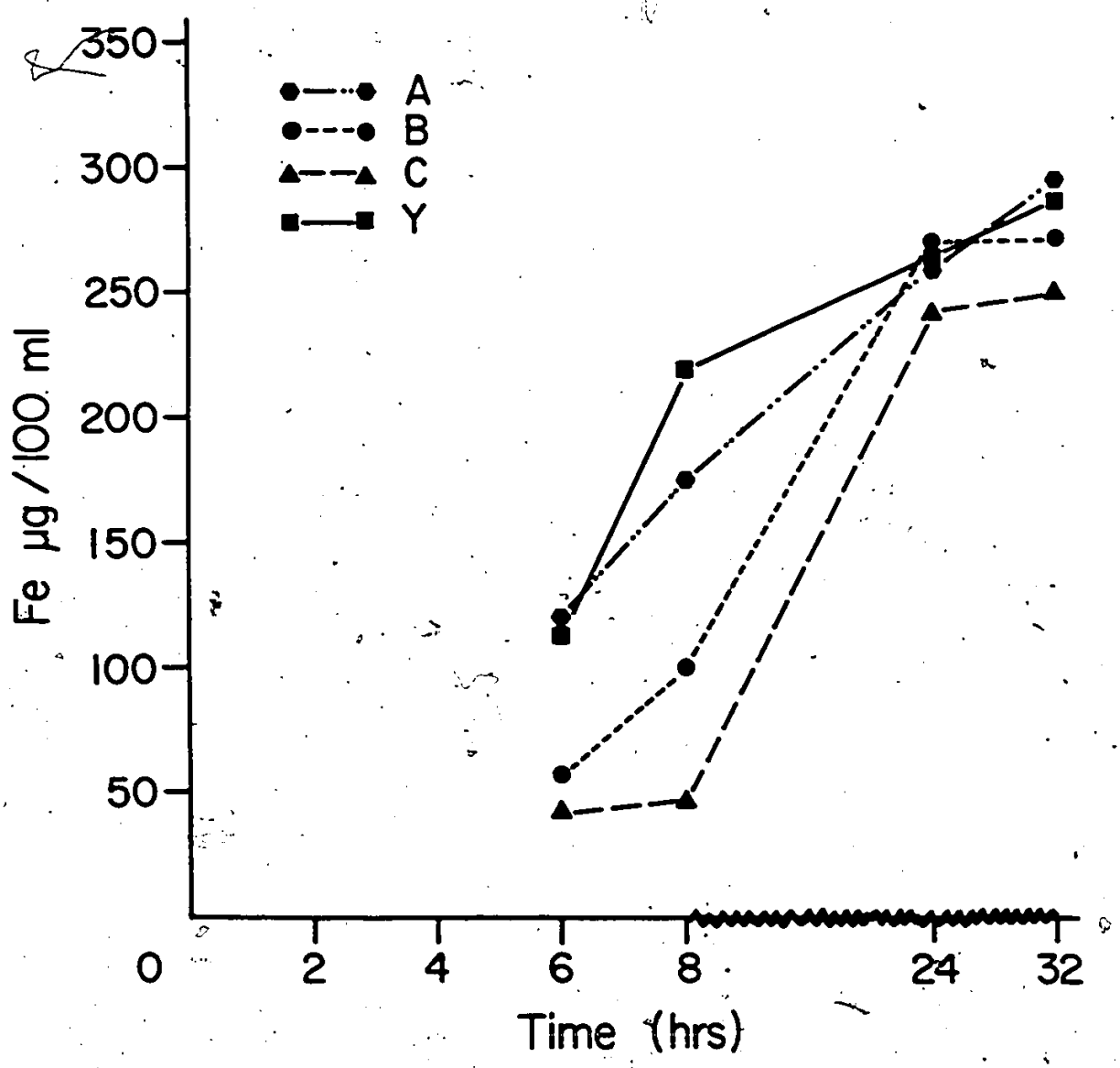
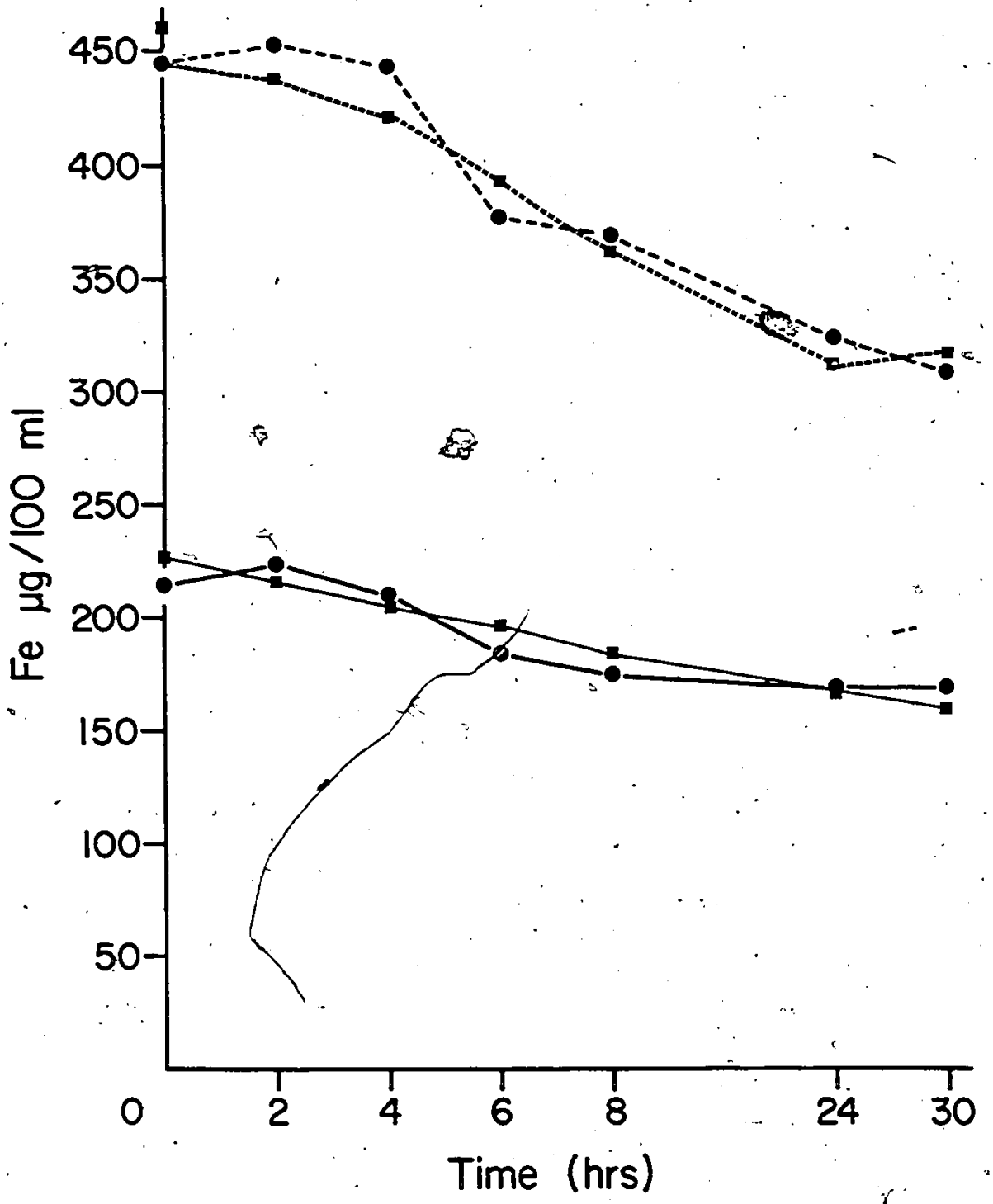

A
B
C
Y
350
300
250
200
150
100
50
Fe µg/100 ml
0
2
4
6
8
24
32
Time (hrs)

Fig. 5.

Analyses of the serogroup A and Y culture filtrates grown in iron-poor NCDM with added Jectofer: A - 500 ug Fe/100 ml (■ - - - ■), Y - 500 ug Fe/100 ml (● - - - ●); A - 250 ug Fe/100 ml (■——■), Y - 250 ug Fe/100 ml (●——●). The filtrates were assayed for total iron (Fe^{+++} and Fe^{++}) by the method of Caraway.


450
400
350
300
250
200
150
100
50
Fe µg/100 ml
0
2
4
6
8
24
30
Time (hrs)

all lie within experimental error of each other. Hence the pH of the culture filtrate as altered by growth of the bacterium did not exert any significant effect upon reduction in total iron.

No significant difference was seen in the removal or uptake of iron by the virulent or avirulent strain. Decreasing the availability of iron to the extent of using iron-poor media did not produce preferrential growth of the virulent strain. In fact, growth of the avirulent strain was slightly favoured. Further, using this media as such, iron-limited growth was not achieved.

However, addition of the iron-chelating agent Desferal to iron-poor NCDM provided a medium in which iron limited growth of the various strains could be achieved (Table 12). Growth reduction was proportional to the concentration of Desferal added to the medium. Growth of all the strains was completely eliminated at concentrations of Desferal in excess of 1.5 mg per ml of media. No correlation existed between virulence and susceptibility to Desferal. It is shown in Table 13 that the decreased bacterial growth was due to iron-limitation. In the presence of 10 mg/ml Desferal, increased growth of the virulent and avirulent strains occurred at iron levels which exceeded .63 mg/ml. Since both strains grow equally well under iron-limiting conditions, the ability to use iron *in vitro* does not appear to correlate with the ability to establish infection in mice.

EFFECT OF IRON UPON GROWTH ON AGAR PLATES

Agar plates provided better opportunity to examine the effect of iron upon lower seed inocula than did liquid cultures.

Iron supported growth of both virulent and avirulent strains. Limited growth occurred on iron-poor NCDM agar when 0.1 ml of bacterial suspension at

Table 12

Effect of Desferal upon the Growth of *N. meningitidis* in NCDM*

Desferal mg/ml	Growth Time Hr.	Optical Density 530 nm Serogroup[a] A	B	C	Y
0	0	.175	.130	.185	.185
0	20	.380	.395	.595	.430
.25	20	.315	.265	.210	.460
.50	20	.310	.200	.200	.350
1.0	20	.155	.130	.175	.230
1.5	20	.210	.135	.185	.205

*NCDM iron-poor

[a]Concentration was 10^9 cfu/ml of inoculum

Table 13

Effect of Iron on the Inhibition of Growth of *N. meningitidis* in NCDM* with Desferal[a]

Iron mg/ml	Growth Time hr.	Protein µg/ml Serogroup A	Serogroup Y
0	0	154	126
0	24	93	95
.32	24	118	112
.63	24	261	185
1.25	24	297	282
2.50	24	298	252

*Iron-poor NCDM

[a]Concentration of Desferal 10 mg/ml

10^5 colony forming units per ml (measured on CBA) was used as inoculum (Fig. $6a_1$). The same inoculum grew to a much greater extent on the same agar when it was supplemented with 500 ug/100 ml $FeSO_4$.

Significant growth occurred mainly around the wells in the areas where the various iron solutions had diffused (Fig. 6b and c). As the concentration of iron deposited in the wells was increased, the meningococcal growth zone which resulted was further enlarged (Fig. $6b_1$, $6b_2$ and $6b_3$). Generally, iron caused an extensive stimulation of growth for both virulent and avirulent strains (Table 14).

The extent of growth promotion in the presence of a high iron level (i.e. 1 mg/ml) for the virulent and avirulent strain was approximately the same (Fig. $6c_1$ and c_2). Growth enhancement appeared to be similar whether iron was supplied as $FeSO_4$ and $FeCl_3$ or chelated iron agents such as Jectofer, Imferon and Ferrigen. A variation in colony size as found by Payne and Finkelstein (1977) when using alternate media was not apparent in these studies.

Meningococcal growth enhancement in the presence of iron compounds is much more apparent in agar plates than in liquid media. Agar plate media containing 50 mg Fe/100 ml iron sorbitol citrate provides the same level of iron as the highest concentration examined previously in liquid media. In this agar-plate system, at this iron level, greater than a 1000 fold increase in yield (total amount of growth) over that from iron-poor agar is seen to occur for both strains with seed inocula of 10^5 and 10^6 cfu/ml. However, in liquid media this level of iron had no significant effect upon increase in total growth over iron-poor media.

Iron-limited growth in the presence of iron-chelating agents was very apparent again upon plates. Strong growth inhibition occurred with Desferal, EDDA and catechol. Zones of complete inhibition were evident around the

Fig. 6.

Effect of iron compounds upon the growth of *N. meningitidis*. Growth of the A strain at 10^5 CFU/ml upon NCDM iron-poor agar without added iron (a_1) and containing 500 ug of iron as $FeSO_4$ per 100 ml (a_2). Comparison of the growth of the A strain at 10^5 CFU/ml upon NCDM iron-poor agar after diffusion of iron as Jectofer from the center well at 0.5 mg/ml (b_1), 1 mg/ml (b_2), and 5 mg/ml (b_3). Comparison of the growth of the A strain (c_1) and the Y strain (c_2) at 10^5 CFU/ml upon NCDM iron-poor agar after diffusion of iron as Imferon from the center well (1 mg/ml).

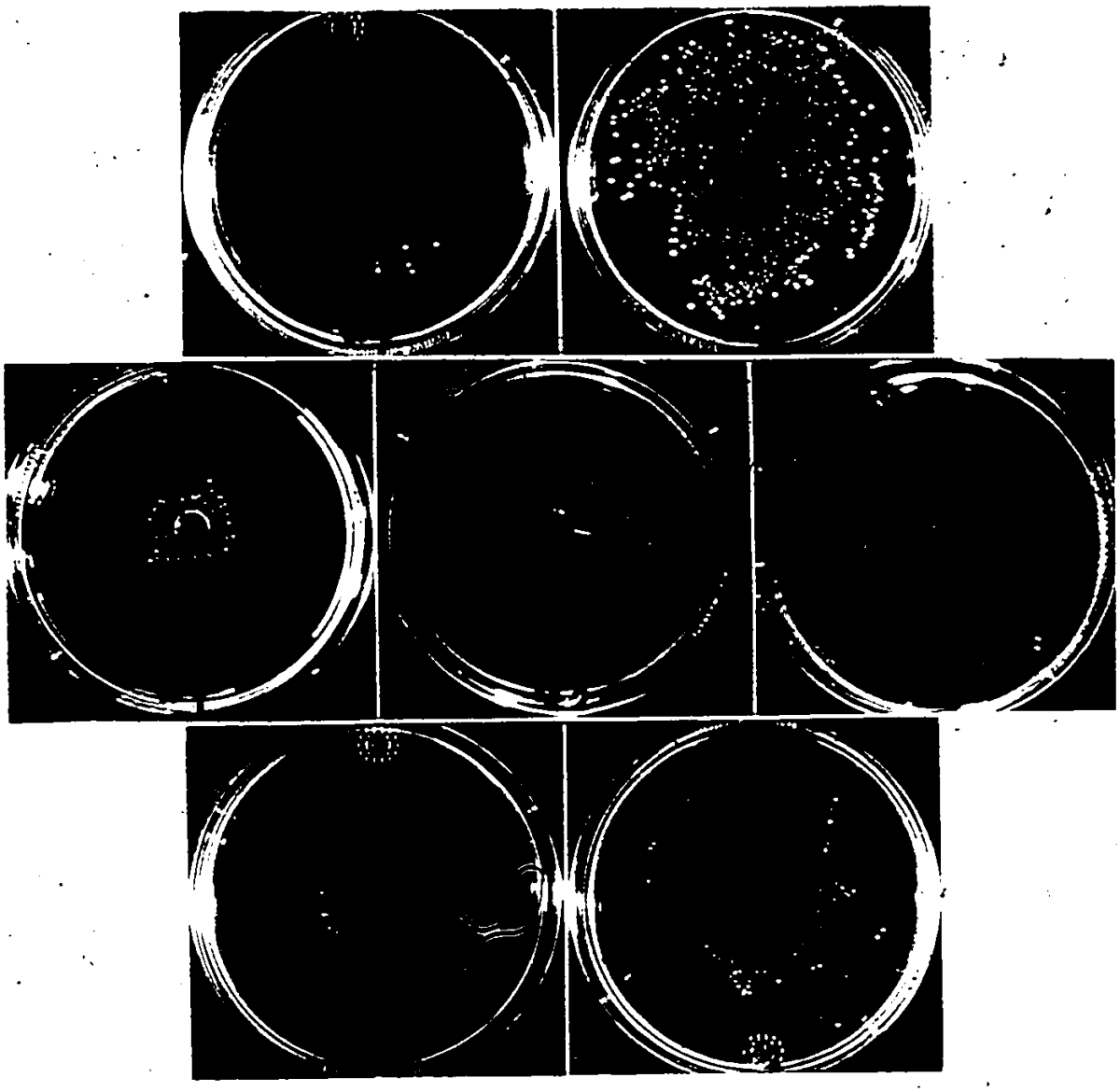


Table 14

Growth of *N. meningitidis* on Agar containing Iron Sorbitol Citrate

Iron Added	Growth c.f.u./plate					
	Serogroup A			Serogroup Y		
	Inoculum*					
a. µg/100 ml	8.0×10^5	8.0×10^6		8.5×10^5	8.5×10^6	
0	36	SEMI		NIL	20	
25	1000	CON.		NIL	1000	
50	SEMI	CON.		20	SEMI	
100	CON.	CON.		30	CON.	
200	CON.	CON.		400	CON.	
500	CON.	CON.		600	CON.	
b. mg/100 ml	6.0×10^4	10^5	10^6	6.5×10^4	10^5	10^6
0	10	35	480	~10	10	10
25	500	SEMI	CON.	410	SEMI	SEMI
50	1000	CON.	CON.	600	SEMI	CON.
100	SEMI	CON.	CON.	600	SEMI	CON.
200	CON.	CON.	CON.	420	CON.	CON.
500	CON.	CON.	CON.	408	CON.	CON.

SEMI = SEMICONFLUENT; colonies are too numerous to count - yet plate not totally covered with a layer of growth

CON. = CONFLUENT; plate totally covered with layer of growth

* Inoculum represents the cfu/ml with 0.1 ml spread on each plate.

Table 15

Effect of EDDA and Catechol upon the Growth of *N. meningitidis*[a]

EDDA (mg/ml)	Zone of Inhibition (mm)	
	Serogroup A[b]	Serogroup Y[b]
0.6	N.I.[c]	N.I.[c]
1.3	28	30
2.5	42	43
5.0	52	52
10.0	60	60

Catechol mg/ml	Zone of Inhibition (mm)	
	Serogroup A[b]	Serogroup Y[b]
.13	N.I.[c]	N.I.[c]
.25	33	30
.50	40	38
1.0	50	50
2.0	58	55

[a]Agar contained 500 µg of iron as Jectofer per 100 ml

[b]Concentration was 2.5×10^8 cells per ml of inoculum (0.1 ml applied)

[c]N.I. represents no inhibition, growth up to the well

diffusion wells even when fairly high inoculum levels were spread on the surface of the agar (Fig. 7).

As the concentration of chelating agent increased, the zones of inhibition were increased (Fig. 7 and Table 15). The extent of inhibition in the presence of these compounds appeared to be in the order catechol > EDDA > Desferal. Both the virulent and the avirulent strain were affected to the same degree by each compound.

Growth inhibition by these agents was reversible by iron (Fig. 8). Increasing the concentration of iron in the agar decreased the zone of inhibition. Eventually by adding adequate iron to the iron-poor NCDM, these zones could be totally eliminated.

The ability to use iron for growth in the presence of iron-chelating agents proved once again to be similiar for the virulent and the avirulent strains. Hence these additional *in vitro* studies strengthen the suggestion that the capacity to acquire iron may not be a virulence factor.

SEARCH FOR A MENINGOCOCCAL SIDEROPHORE

Large volumes (i.e. up to 4 litres each) of 8 and 11 hr. culture filtrates of serogroup A and C strains respectively grown in iron-poor NCDM were extracted and concentrated by a method similar to that of Young (1976). The yields of dried material obtained from different batches varied from 4 to 20 mg per litre.

The residues were dissolved in ethyl acetate (1 mg/20 ml). Ferric nitrilotriacetic acid as prepared by Rogers et.al., (1977), in a volume of 5 ml (1mM) was mixed with these ethyl acetate suspensions and OD_{490} examined. If iron-binding agents were present an increase in absorbance ought to occur. None of the ethyl acetate solutions containing the residues gave an

Fig. 7.

Inhibition of the growth of the serogroup A strain at 10^8 c.f.u./ml upon iron-poor NCDM agar containing 500 ug/100 ml iron as Jectofer, following diffusion of iron chelating agents from the center well: desferal at 10 mg/ml (a) and 1 mg/ml (b); EDDA at 5 mg/ml (c) and 2.5 mg/ml (d).

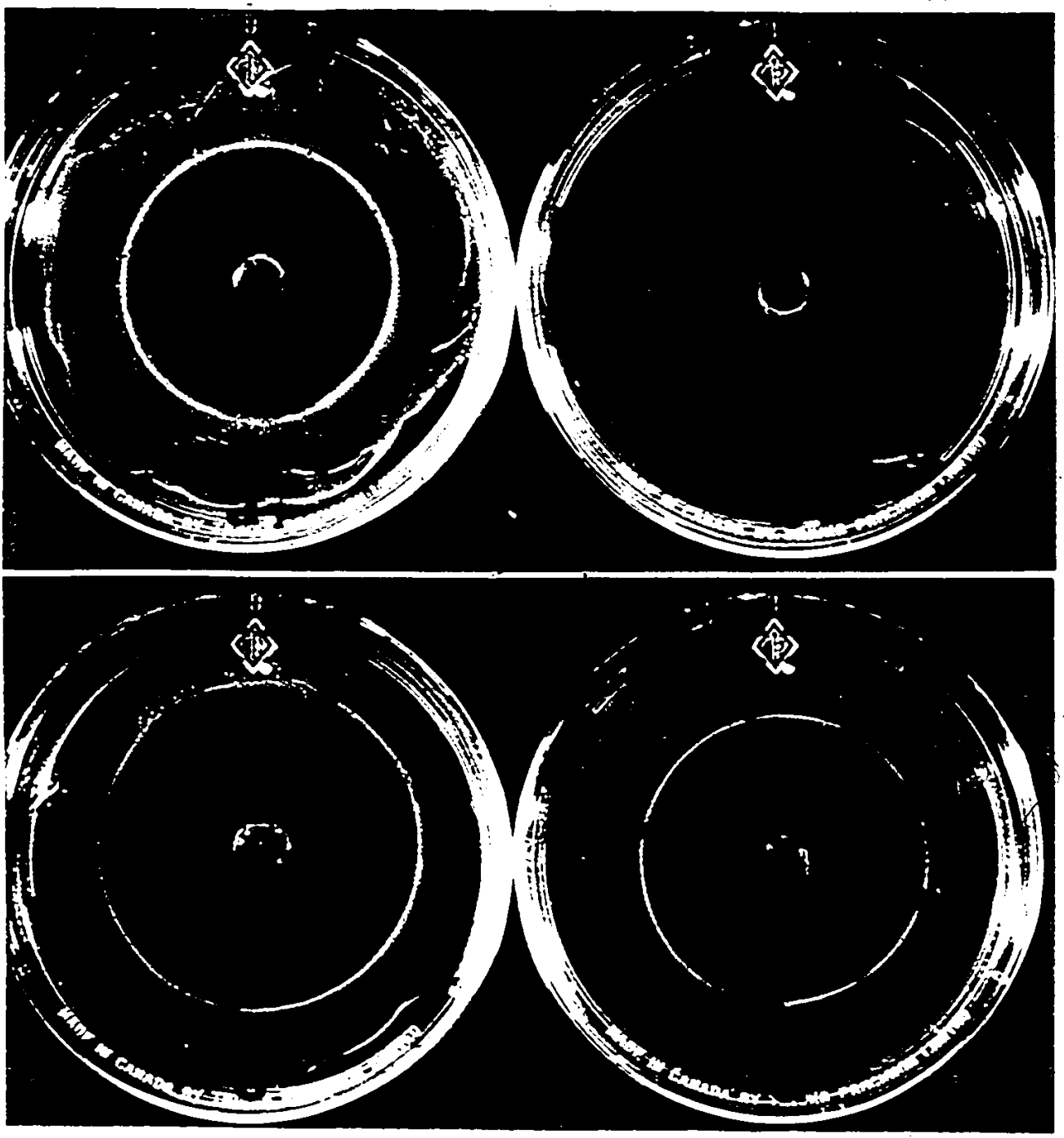
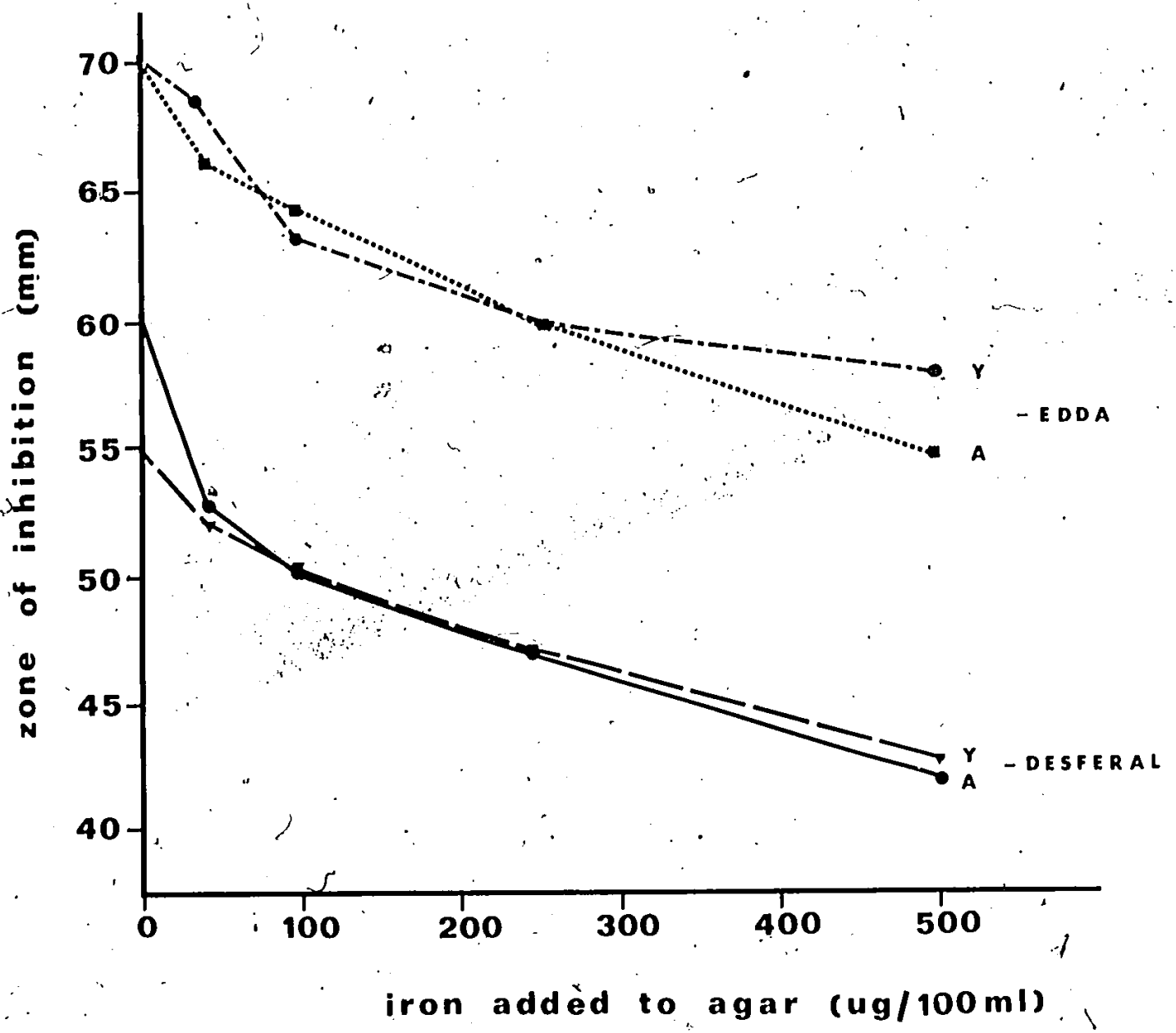


Fig. 8.

Reversal of the growth inhibition of desferal and EDDA by iron. Increasing levels of iron as Jectofer were added to the iron-poor NCDM agar prior to pouring the plates. Seed inocula of serogroup A and Y strains were applied, 0.1 ml of 10^8 c.f.u./ml following diffusion of the chelating agent, desferal 5 mg/ml or EDDA 5 mg/ml.


70
65
60
55
50
45
40
zone of inhibition (mm)
0
100
200
300
400
500
iron added to agar (ug/100ml)
Y
A
- EDDA
Y
A
- DESFERAL

absorbance value larger than the ethyl acetate solution containing ferric-nitrilotriacetic acid alone. Residues (1 mg dry wt./ml) suspended in 10% EtOH/H_2O did not give an increase in absorbance at OD_{490} upon addition 200 ug/100 ml ferric chloride. Increases in absorbance at this wavelength are known to occur with transferrin and siderophores which complex with iron and emit a pink colour (Rogers, 1973).

Catechol or hydroxamate-type compounds were not found in 2 mg/ml solutions of the various residues in 10% EtOH/H_2O or H_2O (as measured by the methods of Arnow, 1937 and Hestrin, 1949).

The presence of the extracted residue had no effect on the amount of iron measured by bathophenanthroline. Under similar conditions, the hydroxamate Desferal was found to interfere with the measurement of iron by bathophenanthroline. The extracted residues could also not remove iron from the transferrin contained in human plasma. When they were suspended in plasma at concentration of 500 ug to 2.5 mg/ml they did not subsequently remove iron from the plasma transferrin and cause it to pass through a membrane of 30,000 molecular weight cut-off. However, addition of Desferal to the plasma resulted in a loss of the amount of iron remaining after filtration.

Since attempts to isolate iron-binding agents from large volumes of culture filtrate did not prove successful, an alternative process was undertaken. To eliminate the possibility that iron-binding agents may have been lost at some stage during processing, such as filtration, concentration or chromatography, culture filtrates were concentrated by lyophilization and directly examined for the presence of iron-chelating agents and iron-binding activity. At the same time, cell concentrates were directly extracted with ehtyl acetate and likewise examined. By these techniques, an in-depth

analysis of various stages during the growth cycle of representative strains of all four serogroups (A, B, C, and Y) was conducted.

Both normal and concentrated culture filtrates of strains A, B, C and Y sampled at 0, 2, 4, 6, 8, 10 and 24 hrs proved to be below the level of hydroxamate detection i.e. 10 ug/ml of hydroxamate. In addition to the use of acetyl hydroxamate as standard in the test, Desferal at various concentrations was also examined. The lowest level of Desferal recordable by this test (Hestrin, 1949) was 10 ug/ml. Ethyl acetate cell extracts were also negative for hydroxamate.

The lower level of catechol detection by the Arnow (1939) method was 0.5 ug/ml. Analyses of concentrated culture filtrates and ethyl acetate cell indicated that the content of iron-binding agents was below this level of detection.

Ethyl acetate extracts were not capable of retaining iron in solution when the pH was adjusted to neutrality and the solution was filtered. The only concentrated culture filtrate to consistently retain an increased quantity of iron in solution (over control media) were the stationary phase samples taken at 24 h. However, no significant difference in iron solubilization by the culture filtrates of any of the four serogroup strains was seen to exist.

Hence, by the tests performed, none of the strains used in this study appeared to produce detectable iron-binding agents (siderophores) at any of the times examined during their growth cycles. It did not appear that these strains secreted iron sequestering agents into the media. Use of an ethyl acetate procedure devised to extract low molecular weight chelating agents which might be attached to the surface of the cells, did not reveal siderophore in cells of *Neisseria meningitidis*.

DISCUSSION

If the difference in virulence to mice between different strains was due to iron supply i.e. the ability of bacteria to use iron for increased growth, then one would expect that such differences might also be seen in a more controllable *in vitro* system. However, in liquid media, growth rate and yield were independent of iron concentration for all strains tested. Decreasing the availability of iron to the extent of using iron-poor media did not produce preferrential growth of the virulent strain. In addition, iron limited growth of the strain could not be obtained using iron-poor NCDM.

Addition of the iron-chelating agent Desferal to iron-poor NCDM provided a medium in which iron-limited growth of the strains could be achieved. No correlation existed between virulence and susceptibility to Desferal.

A growth promoting effect for iron became much more evident when an agar-plate system was used instead of liquid media. The defined agar plate technique, involving use of whole plates with added iron supplements or diffusion plates with deposited iron test materials, demonstrated that iron supported growth of both virulent and avirulent meningococcal types. It was also possible with the agar-plate system to show iron limitation in terms of total cell yield (Calver, et al., 1976). Low seed inocula i.e. 10^4 cfu/plate grew to a much greater extent on agar supplemented with iron. The number of colony forming units increased in proportion to the amount of iron included in the medium (up to a certain limit). Perhaps one of the main reasons that the agar-plate system can demonstrate growth promotion by iron is that the growth of this bacterium on agar can be obtained with lower seed inocula than that required for liquid media. It is also possible that the agar could bind any contaminant iron and that iron must be added in order to obtain growth.

In the agar-plate system iron-reversible growth inhibition with the synthetic chelating agents, catechol, EDDA and Desferal occurred to the same extent with both virulent and avirulent serogroups. We showed previously that Desferal lowered virulence of *N. meningitidis* when injected into mice together with mucin which served as a source of iron (Calver, et al., 1976). The increased LD_{50} for mice in the presence of Desferal may have been related to the direct inhibitory effect of this chelating agent as shown in our *in vitro* studies.

Iron limited growth upon agar containing low molecular weight chelating agents such as EDDA has also been examined by other laboratories (Miles and Khimji, 1975 and Archibald and DeVoe, 1980). Archibald and DeVoe examined the capability of various chelates and iron compounds to reverse the inhibitory effect of EDDA upon growth of meningococci. Desferal containing iron (1:3 molar ratio Fe^{+++}/Desferal) did not support [redacted] growth of meningococci on EDDA-iron-poor agar.

The ability to use iron for growth in the presence of iron-chelating agents in our study proved once again to be comparable for the virulent and avirulent strains. If iron limitation occurs in the host as a defense mechanism which indirectly determines the virulence of meningococcal strains these studies further suggest that selective interaction of the host iron-binding agents would be required; i.e., the activity of the agents would have to be such that iron is withheld from only the avirulent strain.

Attempts to understand iron status during growth of the meningococci in terms of iron solubilization and deposition were difficult to conduct with our media containing ferric or ferrous salts since the tendency to form insoluble precipitates occurred in an erratic fashion. Analyses for the iron content of commercial NCDM and/or media prepared in our Laboratory containing

added amounts of iron salts, gave values for samples from single flasks in a range of 500 to 600 ug/100 ml. Hence, the deviation between samples from the same flask could be 15 to 20%. This inconsistency appeared to be due to the formation upon neutralization of insoluble iron precipitates by the iron salts in the media. Analyses of the same solutions for iron content following filtration through Millipore 0.45 u filters proved that the iron existed in an insoluble form. Filtration removed all analyzable iron.

In the biological pH range ferric salts are known to rapidly undergo hypolytic polymerization to complexes of high molecular weight, some of which may remain soluble or may precipitate (Spiro, et al., 1966), as occurred in our media with the iron salts.

Medium with iron-sorbitol citrate (Jectofer) was better for studying iron movement during meningococcal growth. In such medium soluble, non-filtrable iron consistently remained in solution. With ferric or ferrous salts pH affects iron solubility. During growth of meningococci pH drops to 4 - 5. Such low pH increases the solubility of ferric hydrate precipitates and decreases hydrolytic polymerization. The solubility of iron as Jectofer was not affected by the range of pH found during meningococcal growth.

When meningococci were grown in the presence of iron-sorbitol citrate, a progressive decrease in total amount of soluble iron occurred to the same extent with virulent and avirulent strains. Both strains demonstrated a potential for iron binding during growth.

Sphaerotili are often classed as "iron precipitating bacteria" because the sheaths which surround these filamentous organisms can become encrusted with insoluble oxides or hydroxides of iron from their environment. A pattern of iron deposition similar to that which occurred during the growth of meningococci in our studies was also seen to occur with this

micro-organism. *Sphaerotilus* exhibited a characteristic temporal pattern of iron deposition which was delayed until the later portion of the exponential phase or the onset of the stationary growth phase (Rogers, 1976). The growth rate was also shown to be independent of the medium iron concentration over a range 0.02 to 4.0 mM. There was no evidence which indicated a relationship between the iron concentration and the final cell protein yield. Rogers speculated that the iron deposition system might include a portion of the protein polysaccharide complex comprising the sheath material of this organism.

Concentration of potential iron-binding agents from large volumes of iron-poor culture filtrates of virulent meningococcal strains was attempted by a method similar to that used by Young (1976), for the isolation of enterochelin from *E. coli*. Such a procedure is designed so that the addition of excess iron (Fe^{+++}) to the filtrate would allow any free siderophore which had been secreted into the medium to accumulate as the more stable ferric enterochelin complex. Such negatively charged complexes behave as anions and may be isolated by ion-exchange chromatography. Once the iron has been removed from such complexes by acidification, it becomes possible to isolate the chelate in an iron-free form by extraction into ethyl acetate.

Although a variety of other laboratories, using a similar type of procedure have isolated iron-binding agents from different bacteria, here this procedure failed to produce a residue which contained detectable catechol or hydroxamate, even when up to 4 litres of filtrate were combined and chromatographed. Suspension of meningococcal residue at concentrations up to 2 mg/ml contained no catechol or hydroxamate. The absence of

iron-binding activity of these residues provided additional support for a lack of catechol and hydroxamate and further contra-indicated the presence of other types of low molecular weight iron-chelates.

Since it seemed possible that failure to find iron-binding agents by the batch culture technique may have been due to loss of such agents during processing (i.e. at filtration, chromatography or extraction) an alternate procedure of direct assay of concentrated filtrates (10 fold by lyophilization) was undertaken. Various laboratories using such a direct technique had previously been successful in the isolation of siderophores from different bacteria (Pollack and Neilands, 1970 and O'Brien and Gibson, 1970). However, once again no evidence was found for the presence of iron-binding agents in the growth filtrates of meningococci.

It is known that certain iron-binding agents such as enterochelin are transported into the cell as the ferric complex and that due to the high stability of the complex, the enterochelin molecule must be hydrolyzed before the iron can be released for general cell metabolism (Bryce et al., 1971 and O'Brien, et.al., 1971). Hence, during a search for potential iron-binding agents, one must be aware that a certain percentage of the binding agent even the hydroxamate, will be found either attached to cell surface receptors or within the cell itself. Indeed, in the case of enterochelin a certain amount will be decomposed intracellularly for purposes of iron release.

To ensure that our original attempt to isolate iron-binding compounds had not failed due to the time at which the samples were taken (i.e. 8 hr) further investigations were conducted on samples taken throughout the whole growth cycle. Bulk growth samples processed in our studies (i.e. 8 hr) represented early to mid-log phase growth. Examination of the literature demonstrates that the time during the growth cycle at which growth is sampled

and processed in attempts to isolate iron-binding material varies not only with different organisms but also within the same species (Brott, et.al., 1966, O'Brien and Gibson, 1970, Rogers, 1973, Wilkins and Lankford, 1970 and Pollack and Neilands, 1970). However, even with meningococcal filtrates prepared in this manner, catechol and hydroxamate could not be detected.

Since it is possible that low molecular weight chelating agents might exist attached to the meningococcal cell surface, the cells were extracted directly with ethyl acetate. However, since this failed to indicate the presence of catechols, hydroxamates and iron-binding agent in general in cells of the four different serogroups, it would appear that such small molecular weight chelating agents are not produced or that they are so strongly attached to the cell surface that the extraction procedure was unable to remove them.

Although the exact relation of iron concentration in the medium to growth and to siderophore production has been reported for only a few organisms, it is generally accepted that siderophores are best produced in low iron media. The level of iron found in these media was within the range of iron content of media employed by other laboratories for purposes of isolation of iron-binding agents from a variety of micro-organisms (Brott, et.al., 1966, Young, 1969, Mochan and Ratledge, 1975 and Rogers, 1973 and 1975).

Archibald and DeVoe (1980) employing both liquid media and an agar plate system, were unable to demonstrate the secretion of soluble iron chelators by a serogroup B strain. They examined culture supernatant fluid both unconcentrated and concentrated by freeze-drying and ethyl acetate extracts of the supernatant fluid of the serogroup B strain in a variety of tests designed to indicate siderophores.

A disseminating strain of *N. gonorrhoeae* has been grown on a liquid defined medium made deficient in iron by the addition of EDDA. The filtrates obtained from different levels of iron deficient growth gave no indication by colorimetric or biological tests of siderophore production by *N. gonorrhoeae* (Norrod and Williams, 1978a and 1978b). Although the isolation and chemical identification of gonococal siderophores has not been established, evidence from one group indicates that culture filtrates of gonococci display biological activity attributed to siderophores (Payne and Finkelstein, 1975, 1978a and 1978b). In the case of *Neisseria meningitidis*, our evidence is in agreement with the evidence provided by Archibald and DeVoe (1980) and suggests also the lack of a soluble secreted siderophore in this pathogenic *Neisseria* species.

To the contrary, Yancey (1981) recently reported isolation of an hydroxamate type siderophore from meningococcal cultures. The amount of siderophore found was 100 to 1000-fold lower than the concentration of hydroxamate reported for *B. megaterium* or *A. aerogenes* (Byers, 1967 and Gibson, 1969). The isolation procedure, however, remains open to question. The bacteria were grown in and on a non-defined medium containing peptone. Very old cultures i.e. 48 hr which are well in death phase and subject to extensive lysis were used. Large quantities of spent culture media (i.e. 10 L) were required to produce detectable ug quantities of this siderophore. Although the compound appears to be hydroxamic in nature, the chemical structure is not yet known.

Since large quantities of meningococcal culture were required to produce even 1 ug of siderophore, the relative importance of this compound during infection is debatable. With other bacteria, injection of large amounts of

siderophore i.e. 50 to 300 ug per mouse is required to promote infection (Kochan, 1978 and Yancey, 1979). Also, these siderophores were not as effective as iron in promoting infection.

In general, evidence indicating the importance of siderophores as an infection determinant is minimal. Two strains of *E. coli*, one virulent and the other avirulent for mice have been examined for phenolate siderophores (Rogers, 1973). In the presence of some iron in a chemically defined medium, the virulent strain produced substantial amounts of iron chelators, whereas the avirulent strain produced little. Both strains, however, produced chelators well in media containing EDDA. Miles and Khimji (1975), however, when employing their agar plate system found that chelator production was equal with randomly selected avirulent and virulent strains of *Klebsiella* and *E. coli*.

As an alternative to using 2 different serotypes which might differ qualitatively or quantitatively in other virulence factors unrelated to siderophores, Yancey et al. (1979) compared the virulence of a *Salmonella typhimurium* mutant incapable of enterochelin production with that of the wild-type strain. Injection of desferri-enterochelin with the mutant (at 300 ug/mouse) reduced the LD_{50} of the mutant strain for mice to a level comparable to that of the wild-type strain. Without enterochelin, the LD_{50} of the mutant was 2 to 3 log units higher than the wild-type strain. The concentration of enterochelin was rather high in that approximately 2500 times that required for growth of the strain in serum (20 nM) was used in the injection.

Winkelman (1979) proposed that surface iron polymers in conjunction with hydroxy acids provide iron supply in sideramine-free fungi. When a sideramine-free mutant of *Neurospora crassa* was grown in the presence of iron added as $FeCl_3$ to the medium, the amount of iron accumulated by the cells was

extraordinarily high and exceeded that observed with the sideramine-producing wild-type strain. The iron accummulation by the mutant was not saturable, indicating an uncontrolled uptake process. In the presence of media containing 500 ug/100 ml $FeCl_3$, approximately 20 ug Fe/mg dry weight was accumulated. Washing with an iron-chelating agent removed iron, indicating it had been iron bound to the surface. As with our results a decrease of pH did not prevent iron hydroxide polymer absorption to the cell surface. During growth of the fungus, despite a rapid acidification during the first day, iron polymer adsorption to the mycelia of the sideramine-free mutant was observed.

Iron hydroxide polymer formation on the cell surface is completely prevented in sideramine producing strains of *Neurospora crassa*. It was suggested that sideramine-free fungi first adsorb iron as a hydroxide polymer on the cell surface and that it is gradually solubilized by excreting hydroxy acids. Thus a high local concentration of iron chelated by hydroxy acids provides sideramine-free fungi with a continuous iron supply (Winkelman, 1979).

Hence, the capacity of *Neisseria meningitidis* like other organisms to store iron in excess of immediate needs, may afford it an advantage in the event of sudden decrease in environmental iron supply. Cells grown with an excess of iron could store sufficient reserves of iron to permit several cell divisions at a normal rate in an iron-deficient medium before dilution of iron reserves in progeny cells fall growth limiting concentrations. For purposes of satisfying specific requirements of iron enzymes and/or structural components, *Neisseria meningitidis*, as an alternative to actively sequestering iron by organic chelating agents such as exochelins, may concentrate the iron from the environment onto its surface. Whatever the physicochemical mechanism involved, this iron-binding mechanism might serve

as an additional means whereby an iron reservoir could be provided for cells, for its subsequent active use under conditions of iron deficiency.

In conclusion, our *in vitro* studies suggest that production of iron chelators is not a characteristic upon which the difference in reaction of meningococcal strains in infectivity may be based. Archibald and DeVoe (1980) were also unable to demonstrate secretion of soluble iron chelators by various strains of meningococci. However, the possibility remains that siderophores could be produced in vivo under conditions of extreme stress due to iron-limitation by the iron-binding proteins of the host.

CHAPTER 3 EFFECTS OF IRON-BINDING AGENTS FROM VARIOUS TISSUE AND BODY FLUIDS UPON GROWTH OF N. MENINGITIDIS

INTRODUCTION

Ample evidence has been accumulating that the iron-binding proteins of the host, either by themselves or in conjunction with specific antibodies, are important in resistance to bacteria. The virulence of a micro-organism may be linked to its capacity to acquire iron from such protein. The response of virulent and avirulent strains to growth upon agar containing various iron-binding agents from animal body fluids and tissues may reflect upon the mechanism of reaction of the bacteria with such agents during infection in mice and man.

MATERIALS AND METHODS

The chemicals used for these experiments and their suppliers were:

L-Ascorbic Acid. Analytical reagent grade, B.D.H. Chemicals, Toronto, Ontario.

Sodium Ascorbate. Hoffmann-LaRoche Ltd., Montreal, Quebec.

Dehydro-ascorbic Acid. ICN Nutritional Biochemicals, Cleveland, Ohio.

Folic Acid. Nutritional Biochemicals Corp., Cleveland, Ohio.

Free Radical Scavengers:

Mannitol - Difco Laboratories, Detroit, Michigan.

Sodium Benzoate - Fisher Scientific Co., New Jersey.

Sodium Thiosulfate - J.T. Baker Co., Phillipsburg, N.J.

Hydrogen Peroxide. A 3% solution in H_2O, Fisher Scientific Co., N.J.

Cadmium Sulphate. Certified grade, Fisher Scientific Co., Pittsburgh, Pa.

Reducing Agents . L-cysteine HCl and glutathione (reduced) Nutritional Biochemicals Corporation, Cleveland, Ohio.

Antisera. Rabbit antisera to human transferrin, lactoferrin and ferritin were supplied by Behringwerke A.G., Germany. Antisera to horse spleen ferritin were prepared in New Zealand white rabbits by injections of solutions of ferritin in Freund's incomplete adjuvant (50 mg ferritin/ml) into the hind limb. Following weekly injections of 1.0, 2.0 and 2.0 ml, the rabbits were exsanguinated if test bleeds were positive.

Conalbumin. Type I chicken egg white (0.015% Fe) was obtained from Sigma Chemical Co., St. Louis, Mo. Conalbumin iron complex from chicken egg white type II was also used. Solutions of these proteins were prepared in 0.15 M NaCl.

Ferritin. Horse spleen ferritins supplied by Calbiochem, La Jolla, Calif. (type 341475), and Sigma (type I) were purchased as sterile solutions in 0.15M NaCl. To remove residual cadmium, the ferritins were reduced by dialysis against 0.2M L-ascorbic acid (BDH Chemicals, Canada) for 24 to 48 hr, then the ascorbic acid was removed by dialysis against distilled water. Before sterilization by membrane filtration (Millipore Corp., Bedford, Mass.), the pH of the samples was adjusted to 7.2 with NaOH. Only samples which gave a cadmium level of < 0.003% by weight as recorded by atomic absorption were employed in the growth studies. Dialysis against ascorbic acid or distilled water or both was repeated if required to reduce the cadmium level below 0.003%. The reduced ferritins had an iron content of 0.01% and a residual ascorbic acid content of < 0.01%. The unreduced ferritins had an iron content of approximately 16 to 20% and a cadmium level of approximately 0.15 to 0.20%.

Iron binding of the various lots of ferritin was determined according to Stefani et al., (1976). Ferritin (1 mg/ml) and ferrous ammonium sulphate (18 mg/ml) in 200 mM imidazole buffer pH 7.8 containing 0.05 M KIO_3 and 0.2 M $Na_2S_2O_3$ H_2O were mixed in a 9:1 ratio. Samples were periodically removed and the reaction stopped by dilution in 0.01M sodium citrate (1:10). OD_{420} nm was subsequently measured with a Bausch and Lomb Spectronic 505 spectrophotometer.

Lactoferrin. Human colostrum lactoferrins were supplied by Calbiochem La Jolla Calif. (type 427259) and Sigma (type L4881). Solutions of these proteins were prepared in saline, and the pH adjusted to 7.0 with NaOH prior to sterilization by membrane filtration (millipore 0.45 u pores).

Transferrin. Purified human protein (Fe content maximum, 20 ug/g) was produced from Behringwerke, West Germany. Solutions were prepared in 0.15 M

sodium chloride or 0.15 M NaCl – 0.024 M $NaHCO_3$. Dialysis as required was carried out against the latter solution for a 24 hr period.

To prepare transferrin fully and half saturated with iron, transferrin was reacted with different levels of ferric nitrilotriacetic acid. (Bates and Schlabach, 1973). Unbound iron was removed from the iron-complexed transferrin by chromatography on Sephadex G-20. The transferrin-iron fractions were pooled and subsequently concentrated back to the original volume (Amicon pressure filter with 30,000 molecular weight cut-off membrane).

Sera and plasma. Sera from humans, goats, rabbits and C_{57} male and female HPB black mice were collected, clarified and sterilized by membrane filtration. Human plasma was donated by the Red Cross, Ottawa.

Media. The iron binding activity of various agents was tested in iron-poor and iron containing agar medium prepared as previously described. Alternative agar media were prepared by the addition of ferritin, transferrin, goat whey or serum (mammalian, rabbit or human).

Diffusion plate technique. Conalbumin, transferrin, ferritin, cadmium sulfate, plasma, various sera and tissue extracts were examined for antibacterial effect by the technique previously described in which the test materials diffused from filled agar wells (9 mm diamater). Lactoferrin and, in certain cases, transferrin was tested by a disc diffusion technique. Twenty ul of each protein (at various concentrations) was deposited onto a 6 mm BBL differentiation disc placed upon the agar surface. Following diffusion, prior to seed application the disc was removed.

For studying the effect of ascorbate, hydrogen peroxide and similiar compounds, two wells (3 mm each) were made per agar plate with a gel puncher (LKB-Products AB, Sweden). Each well was filled with 30 ul of test substance which was allowed to diffuse at room temperature for a period of 18 hr before

0.1 ml of a seed inoculum was distributed evenly over the surface of the agar. Alternatively 30 ul volumes of iron salt solutions were added to the wells and let diffuse 2 h before sodium ascorbate solutions were added.

Electrofocussing. An L.K.B. 2117 multiphor was used for flatbed electrofocusing of ferritins upon LKB ampholine polyacrylamide gel plates of pH range 3.5 to 9.0. Anode and cathode solutions were 1M H_3PO_4 and 1M NaOH respectively. The gels were focused for 1.5 hr using an ISCO Model 494 electrophoresis power supply (constant 30 watts), fixed and stained for protein (0.1% Coomassie Blue) or for iron (1% potassium ferrocyanide). Stained bands were quantitated by a Beckman microzone densitometer model R110.

Iron-binding interference by culture filtrates. Ferric nitrilotriacetate 1.1 mM, pH 7.5 was prepared according to Rogers (1973). Human transferrin was added to Tris - HCl - Tris Base buffer, pH 7.5 (2.5 mg per ml). Concentrated culture filtrates of serogroup A, B, C and Y strains were prepared as previously described. Fifty ul of ferric nitrilotriacetate were added to a mixture of 0.8 ml transferrin in buffer with 0.2 ml of either iron-poor NCDM (pH 7.2), distilled H_2O or culture filtrate. The pH of the final reaction mixture was adjusted to approximately 7.3 with 40 ul of 1N NaOH prior to measuring the increase in absorbance due to iron binding to transferrin at 470 nm with a B & L Spectronic 505. Absorbance values at 470 nm due to concentrated culture filtrates alone were used as blank values.

Iron removal from transferrin and lactoferrin. Radioactive transferrin and lactoferrin were prepared by the following procedure. Ferrous ammonium sulfate (4.721 mg) was added to a solution of nitrilotriacetic acid (2.0054 mg per 5 ml H_2O) and the volume adjusted to 9.9 ml with distilled H_2O. One-tenth ml of 10 mCi per mg per ml $Fe^{55}Cl_3$ (New England Nuclear) was mixed with this solution, the pH of which was subsequently adjusted to 7.8 with

NaOH. A 0.6 ml volume of this Fe^{55} solution was reacted for 1 hr at room temperature with 100 ml volumes respectively of transferrin and lactoferrin. In order to remove unbound Fe^{55} these radioactive protein solutions were exhaustively dialyzed against Tris-buffer until the buffer was negative for Fe^{55}.

The ability of killed meningococcal cells to remove iron from labelled transferrin or lactoferrin was studied by incubating 0.5 ml of whole cell cultures or washed cell suspensions (3 x in formalized H_2O) for 2 hr at 37°C with equal volumes of Fe^{55}-transferrin or Fe^{55}-lactoferrin. The reaction mixtures were subsequently filtered through millipore (0.22 or 0.45 u pore size) membranes and the unbound Fe^{55} in the filtrate was measured. One-tenth ml aliquots of the filtered transferrin solutions were mixed with 5 ml of Biofluor (Emulsifying cocktail, New England Nuclear) and Fe^{55} was counted with a Beckman LSC 9000 (liquid scintillation counter), using a program with automatic quench compensation.

Tissue extraction. Extracts of mouse liver and spleen tissue were prepared by a modification of a procedure used in the isolation of tissue ferritin (Linder and Munro, 1972). Livers and spleens from 200 male C_{57} HPB black mice were separately combined, homogenized in a minimal volume of saline and stored at -22°C until further processing. Once thawed, twice the volume of physiological saline was added, and the suspensions were ultrasonicated for 20 min., heated for 20 min. at 75°C, cooled and centrifuged (3000 rpm, 30 min.). The supernatant was removed and the sediment re-extracted twice more with double volumes of 0.15 M NaCl. Cellular debris was discarded. Ammonium sulfate was added to the combined supernatants to 60% saturation. After 24 hr at 4°C the precipitate was centrifuged (10,000 rpm, 15 min.), and resuspended in H_2O. Any precipitate remaining following exhaustive against saline dialysis was removed by

centrifugation and re-extracted with H_2O after adjustment of the pH to 7.0. Clear extracts were combined with the dialyzed supernatant and concentrated 10 fold by an Amicon 52 pressure cell containing an ultrafiltration membrane with a molecular weight cut-off of 30,000. The retentate was sterilized prior to subsequent testing by Millipore filtration (0.45 u pore size). Alternatively, extracts were also prepared by acidification of the dialysed supernatant to pH 4.0 with HCl prior to concentration. The resultant precipitate was collected, resuspended in H_2O and concentrated further as required.

Extracts of a female human liver were prepared by similar techniques.

RESULTS

EFFECT OF VARIOUS MAMMALIAN SERA AND THE IRON-BINDING PROTEINS TRANSFERRIN, CONALBUMIN AND LACTOFERRIN UPON MENINGOCOCCAL GROWTH

Human, rabbit, goat and mouse (C_{57} HPB male, black) sera enhanced meningococcal growth on agar diffusion plates in the same manner as iron compounds (see Chapter 2). With low seed inocula, major growth occurred only around the agar well in the area in which the sera had diffused. Growth enhancement was similar for all serogroup strains tested (i.e. A, B, C and Y). Hyperimmune rabbit sera, on the other hand, exerted growth inhibition which was not reversible by inactivation (56°C, 30 min.) nor the presence of iron. Agar plates prepared by mixing 1 part human plasma with 3 parts iron-poor NCDM agar also stimulated bacterial growth.

Since mouse sera which contained transferrin (30-40% saturated with iron) did not inhibit meningococcal growth, transferrin of lower iron content was examined for potential to limit growth. Since an adequate supply of mouse transferrin was not readily available the effect of human transferrin was studied. The protein used was very near in form to apo-transferrin since it contained at most enough iron to provide only 1.6% saturation.

In our first experiments in which .006 M bicarbonate was included in the iron-poor agar medium no inhibition was evident even with transferrin levels up to 50 or 100 mg/ml.

It was thought possible that chelating agents in the commercial preparations of transferrin might have interfered with its inhibitory activity. Accordingly, samples which had been extensively dialyzed against 0.15 M saline with 0.024 M bicarbonate were also examined. These samples at

50 mg/ml failed to inhibit growth. We thus initially concluded and reported (G.A. Calver, C.P. Kenny, and D.J. Kushner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B109, p. 31) that transferrin did not effectively compete with our strains of *N. meningitidis* for iron.

In a further experiment, transferrin was reacted with ferric nitrilotriacetate to saturate the protein with iron. With increasing saturation of transferrin, an increased growth enhancement resulted, similar to that found with iron compounds.

However, with certain lots, patterns of inhibition emerged at high transferrin concentrations when 0.01 M bicarbonate was deposited directly with the protein in the center well (Table 16). A lot to lot variation in growth repression occurred. Some lots remained non-inhibitory despite added bicarbonate. Inhibition of the virulent strain occurred on iron-poor agar. As the iron level incorporated into the medium was increased, the inhibition of the virulent strain was reversed (Table 16). A surprising phenomenon presented itself with the avirulent strain. On iron-poor agar the avirulent strain was not inhibited (Table 16). However, upon increasing the concentration of iron, the avirulent strain began to show inhibition which increased to a higher degree and then declined. This observation was certainly not in keeping with the suggestion that the inhibitory activity of this protein was due to deprivation of iron required for growth of the bacterium.

Comparative studies were carried out with other proteins of similiar structure and size and isolated from other sources, ie. conalbumin (ovotransferrin) and lactoferrin (lactotransferrin). Substantial growth limitation of higher seed inocula (ie. 10^8 c.f.u./ml) was readily obtained with conalbumin at 3 fold lower concentrations than with transferrin (Fig. 9 and Table 16). The repression of growth was iron reversible, the extent

Fig. 9

Effect of conalbumin (30 mg/ml) upon the growth of *N. meningitidis* serogroup A strain (A_1, A_2, and A_3) and the serogroup Y strain (B_1, B_2, and B_3). The following concentrations of Jectofer were contained within the wells: no iron added (A_1 and B_1); Jectofer at 100 ug/100 ml (A_2 and B_2); and Jectofer at 500 ug/100 ml (A_3 and B_3). Inoculum levels of the bacteria were 2.0×10^8 cfu/ml.

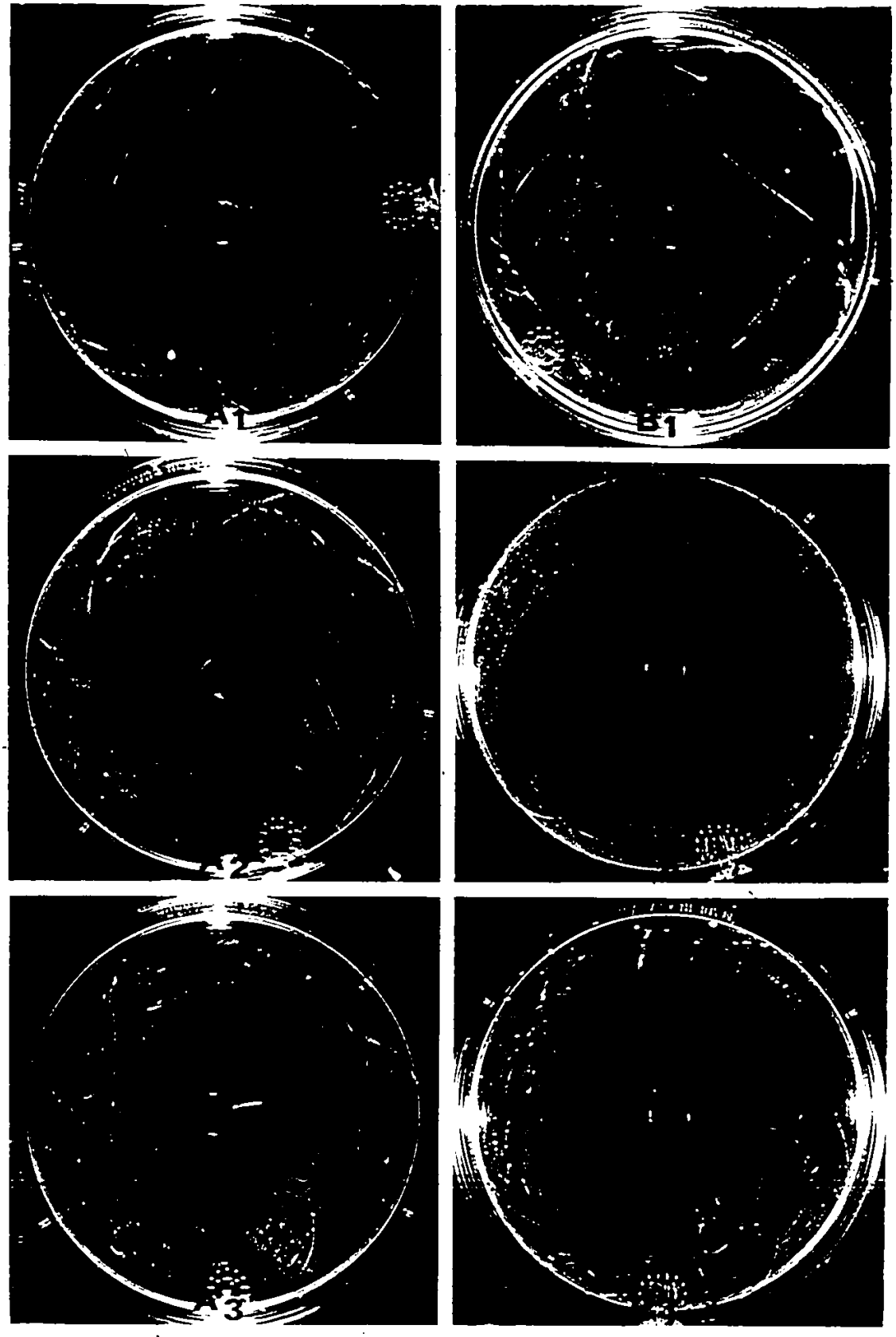

A1
B1
2
3

Table 16

Effect of Conalbumin and Transferrin upon growth of *N. meningitidis*

Iron binding protein	Serogroup[a]	Zone of Inhibition[b] 0	50	100	200	500	2000	10000
Transferrin (100 mg/ml)[c]	A	18	17	17	17	-	-	-
	Y	-	-	18	18	21	19	
Conalbumin (30 mg/ml)	A	22	20	17	15	-	-	-
	Y	23	21	19	17	12	-	-

[a]Seed inoculum for both serogroups was 10^8 cfu/ml.
[b]Each value represents millimeters at the indicated concentration (µg/100 ml) of iron incorporated into media.
[c]Transferrin in saline containing 0.01M bicarbonate.

-= No inhibition

of repression being slightly greater for the avirulent strain. All lots of conalbumin tested were inhibitory. Iron-complexed conalbumin at levels up to 50 mg/ml was non-inhibitory.

A selective inhibition occurred in the presence of human lactoferrin. Growth of the avirulent serogroup Y strain was inhibited. Growth of the virulent A strain was not affected by this protein (Table 17). Yet, susceptibility did not correlate with virulence since the serogroup B and C strains were also unable to grow in the presence of this protein. Very large concentrations of iron well in excess of the binding capacity of the protein were necessary to reverse this inhibition. Hence, it would appear that iron deprivation by this protein is not the mechanism by which limitation of growth occurs. Not all lots of lactoferrin were inhibitory.

EFFECT OF CELLS AND CELL FILTRATES UPON BINDING OF IRON TO TRANSFERRIN

Upon addition of iron to transferrin in a buffer solution, transferrin iron complexes are formed which exhibit a maximum absorbance at 470 nm. The colour emitted at this maximum absorbance is a salmon-pink colour. Culture filtrates of gonococci have been shown to interfere with the binding of iron to transferrin and subsequently to result in a reduction of the absorbance at 470 nm (Norrod and Williams, 1978).

Without concentration, our meningococcal growth filtrates had no effect upon absorbance of the transferrin-iron complex. However, significant interference in absorbance occurred with concentrated filtrates sampled later in the growth cycle, at 8, 10 or 24 hr (Table 18). No correlation exists between virulence for mice and the capacity to interfere in this test with the binding of iron by human transferrin. In fact, the greatest decrease in

Table 17

Effect of Lactoferrin upon the growth of *N. meningitidis*

Lactoferrin mg/ml	Zone of Inhibition[a] mm			
	Serogroup			
	A	B	C	Y
5	nil	nil	nil	nil
10	nil	nil	nil	10*
20	nil	12	14	15
40	nil	16	14	16

Iron added to Agar mg/100 ml	Zone of Inhibition mm[b]			
	Serogroup			
	A	B	C	Y
0	Nil	14	15	18
.5	Nil	12	11	15
25	Nil	10	11	13
200	Nil	Nil	10*	10

[a] Agar contained 10 mg of iron as Jectofer per 100 ml.

[b] Lactoferrin concentration 12.5 mg/ml.

* Inhibition zone with growth inside.

Table 18

Effect of Meningococcal culture filtrate[a] upon Absorbance of Transferrin-iron at 470 nm

Time hr	% Decrease[c] in absorbance at 470 nm			
	Serogroup			
	A	B	C	Y
0	(.082)[b]	(.082)	(.082)	(.081)
8		(.064) 21	(.080)	
10	(.035) 57			(.044) 46
24	(.036) 56	(.052) 37	(.059) 28	(.023) 72

[a]Concentrated culture filtrates (10x).
[b]Average absorbance of three different samples.
[c]% decrease from 0 hr value.

absorbance occurred with the concentrated culture filtrate of the avirulent serogroup Y strain sampled at 24 hr.

A comparison of the binding of radioactive transferrin iron (Fe^{55}) by whole cultures and corresponding washed cells is shown in Table 19. As growth proceeded, a progressive decrease of the Fe^{55} radioactivity occurred in the filtrates from mixtures of transferrin-Fe^{55} and washed cell preparations of the A or Y strain. By 24 hr, washed cells of both strains had removed approximately 50% of the radioactive iron. However, whole cultures removed less iron from the filtrates than the corresponding washed cell preparation. This was most evident at 24 hr. Cultures of strain A removed only about half as much Fe as did the washed cells, and culture of the avirulent strain removed only 1/5 as much. These results suggest that spent culture medium of avirulent cells solubilizes iron to a greater degree than spent media from virulent cells.

Removal by the cells of transferrin protein was also examined. Filtrates of the washed cell suspensions in buffer contained no protein. Filtrates from mixtures of washed cell preparations and transferrin contained as much protein as the transferrin solution. The washed cells bound little transferrin (Table 20). Although the washed cells bound less than 10% of the available transferrin, 40 to 50% of the iron was retained by the cells (Table 20). The results indicate that for most part, the cells were removing and binding the transferrin iron without retaining the transferrin.

EFFECT OF FERRITIN UPON GROWTH

Initial experiments demonstrated that ferritin produced substantial zones of growth repression even in the presence of fairly high seed inocula. The avirulent strain did not grow as well as the virulent A, B and C strains

Table 19

Binding of Transferrin[a] Iron (Fe^{55}) by *N. meningitidis* Whole Cultures and Washed Cells

Serogroup	Time Hr.	Protein µg/ml	Filtrate[c] Fe		
			Fe^{55} cpm	Fe µg/ml	%Fe Decrease[d]
1. Washed cells[b]					
	0[e]	4	38,978	3.19	3.5
	2	5	38,429	3.13	4.9
A	4	30	25,355	2.06	37.2
	6	88	22,891	1.86	43.3
	8	130	26,194	2.13	35.2
	24	220	18,863	1.53	53.3
	0	8	37,111	3.02	7.1
	2	7	38,207	3.11	5.4
Y	4	10	30,553	2.49	24.4
	6	45	22,454	1.83	44.4
	8	115	22,494	1.83	44.3
	24	210	20,018	1.63	50.4
2. Whole culture					
	0	0	40,592	3.31	
	2	10	40,032	3.26	.9
A	4	0	39,845	3.25	1.4
	6	40	33,364	2.72	17.4
	8	80	25,687	2.09	36.4
	24	190	30,306	2.47	25.0
	0	0	39,340	3.21	2.6
	2	0	38,343	3.12	5.1
Y	4	10	39,456	3.21	2.3
	6	30	35,994	2.93	10.9
	8	70	29,178	2.38	27.8
	24	180	36,381	2.96	9.9

[a]Transferrin in buffer at 2.5 mg/ml containing 3.3 µg/ml of bound iron.
[b]The cells from the corresponding whole cultures were spun down and washed (1% formalinized H_2O) and resuspended to the original volume; total protein contents of these suspensions were determined.
[c]Equal volumes (0.5ml) of washed cell suspension or whole culture were mixed with 0.5 ml of radioactive Fe^{55} - transferrin and filtered through a Millipore (0.45mm) filter after a period of time of reaction and mixing. The Fe^{55} of the filtrates was counted with a Beckman LSC9000.
[d]Transferrin - Fe^{55} buffer-medium control gave 40,393 c.p.m.; decrease from this value was recorded.
[e]Sample take after seed added, prior to incubation.

Table 20

Comparison of the Binding of Iron and the Binding of Transferrin by *N. meningitidis* washed cells[a]

Serogroup	% Decrease in Filtrate[b]	
	Transferrin	
	Fe^{55}	Protein
A	41.4	9.1
B	50.4	6.9
C	54.6	0
Y	41.1	2.3

[a]Cells sampled after 24 hr of growth.
[b]Equal volumes (0.5 ml) of washed cell suspensions were mixed with 0.5 ml of radioactive Fe^{55} - transferrin and filtered through millipore (0.45 filter) after a period of time of reaction and mixing. The Fe^{55} of the filtrates was counted with a Beckman LSC 9000. Protein of the filtrates was determined by method of Lowry et al (1951).

in the presence of horse spleen ferritin containing 20% iron. Inhibition by ferritin was reversed by iron. The avirulent strain did not respond to enhancement with iron as effectively as the virulent strain. However, not all commercial ferritin preparations were inhibitory (Calver et al., 1979).

The various lots of ferritin were examined for iron-binding activity by the procedure of Stefani et al. (1976) to determine whether variation in inhibitory activity was due to this factor. One might expect that the active inhibitory ferritins would possess a higher rate of Fe incorporation. However, the rate of incorporation and the total amount of iron incorporated at saturation was quite similar for all the ferritins examined.

Since the sub-unit composition of a ferritin determines its overall structure (Munro and Linder, 1978), a variation in type of sub-unit could affect binding and/or selective iron release during interaction with bacterial cells. The different lots of ferritin were compared by microzone electrophoresis and electrofocusing. No difference between preparations was evident by electrophoresis on cellulose acetate. However, the higher resolution obtained with flat-bed electrofocusing in polyacrylamide gel provided evidence that a heterogeneity did exist in these preparations (Fig. 10).

Since the commercial horse spleen ferritin had been isolated by a technique involving precipitation with cadmium sulfate, the cadmium contaminant level was evaluated. Analysis by atomic absorption revealed up to 600 fold higher cadmium values than reported by the manufacturer ie. 0.6% instead of .001%. Equivalent cadmium concentrations were found to be inhibitory in our plate system. A selective repression of the growth of the avirulent strain occurred. This inhibition also proved to be iron reversible (Table 21). The inhibitory activity of the ferritins correlated with their cadmium content (Results not shown).

Fig. 10

Electrofocusing of horse spleen ferritins. Various horse spleen ferritins were electrofocused on PAG plates, as previously described: 1 - Sigma 38 C, 2 - Calbiochem 702425, 3 - Sigma 117 C, 4 - Calbiochem 701170 and 5 - Calbiochem 703062. The gel was first stained for iron using 1% potassium ferrocyanide and the bands were quantitated for iron using a Beckman Microzone Densitometer model R 110 (●—●). Subsequently, the same iron stained gel was restained for protein using 0.1% Coomassie Blue and then quantitated for protein by the Beckman densitometer (■——■).

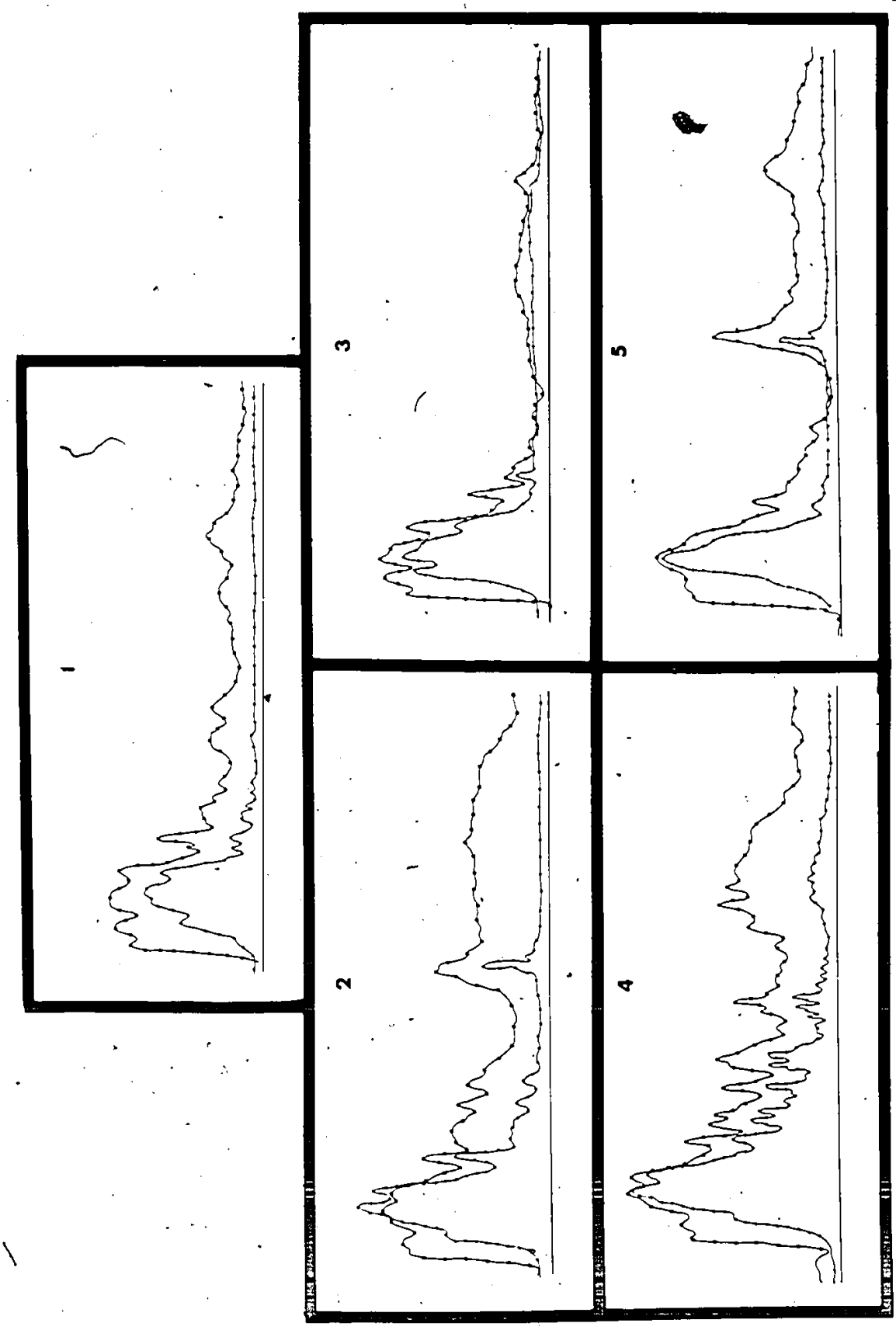

1
2
3
4
5

Table 21

Influence of Iron upon the Growth Inhibition of Cadmium

Iron[a] Mixed With Cadmium (c.w.) mg/ml	Zone of Inhibition (mm) Cadmium 50 µg/ml[b]	
	A	Y
0	26	32
5	24	28
10	21[c]	27
20	Nil	Nil

[a] Iron as Jectofer.

[b] Seed inocula for both serogroups was 10^8 c.f.u./ml.

[c] Well diameter only (9 mm).

Hence, in order to examine the effects of the protein itself, the cadmium had to be removed. Reduction with 0.2M ascorbic acid (Stefani et al. 1976), decreased cadmium to < .003%, a level well below the minimal inhibitory concentration of 5 ug/ml. Apart from having a low cadmium content, reduced ferritins had an iron content of < 0.01% and a residual ascorbic acid content of 0.005%. Reduced ferritin focused (by protein staining) in the same manner on polyacrylamide gel as normal ferritin.

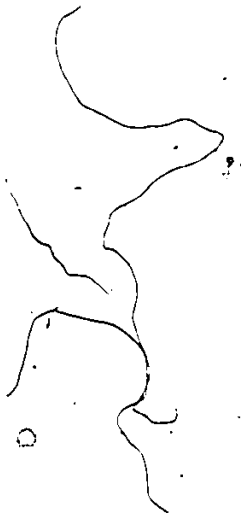
Once again, the phenomenon of inhibition on iron agar in the presence of this protein occurred. On iron-poor agar growth inhibition was not evident. However, as iron was incorporated into the media, the inhibitory effect of reduced ferritin became evident (Fig. 11). Both virulent and avirulent strains showed this same phenomenon, although the virulent strain was inhibited to a greater degree at low iron levels, whereas the avirulent strain was more susceptible to higher iron levels. Only two of seven different lots in the reduced state demonstrated inhibition at 30 - 50mg/ml protein.

EFFECT OF MOUSE LIVER AND SPLEEN EXTRACTS UPON GROWTH

The livers and spleens were surgically removed from C_{57} black mice and the iron-binding proteins extracted by a procedure similar to that of Linder and Munro, 1972). Crude liver extracts (containing ferritin and a variety of other proteins) were examined for inhibitory activity. Although growth of the virulent A strain was not affected, growth of the avirulent Y strain was greatly limited by liver extracts (Fig. 12). With certain preparations, the phenomenon of increasing inhibition on agar containing increased iron was once more apparent (Fig. 12). Growth of the serogroups B and C strains was limited to a lesser extent. Inhibition of the B and C strains was abolished by lower levels of iron than that required for the Y strain.

Fig. 11

Comparison of the effect of reduced horse spleen ferritin (Sigma Lot #38C81001, 30 mg/ml) upon the serogroup A strain (A_1, A_2, A_3 and A_4) upon the serogroup Y strain (B_1, B_2, B_3 and B_4). The agar plates contained the following concentration of iron: A_1 and B_1 - no iron; A_2 and B_2 - Jectofer 10 mg/100 ml iron; A_3 and B_3 - Jectofer 50 mg/100 ml iron; A_4 and B_4 - Jectofer 100 mg/100 ml iron. Bacterial cell inocula were 2.0×10^6 for serogroup A and 1.0×10^6 for serogroup Y.



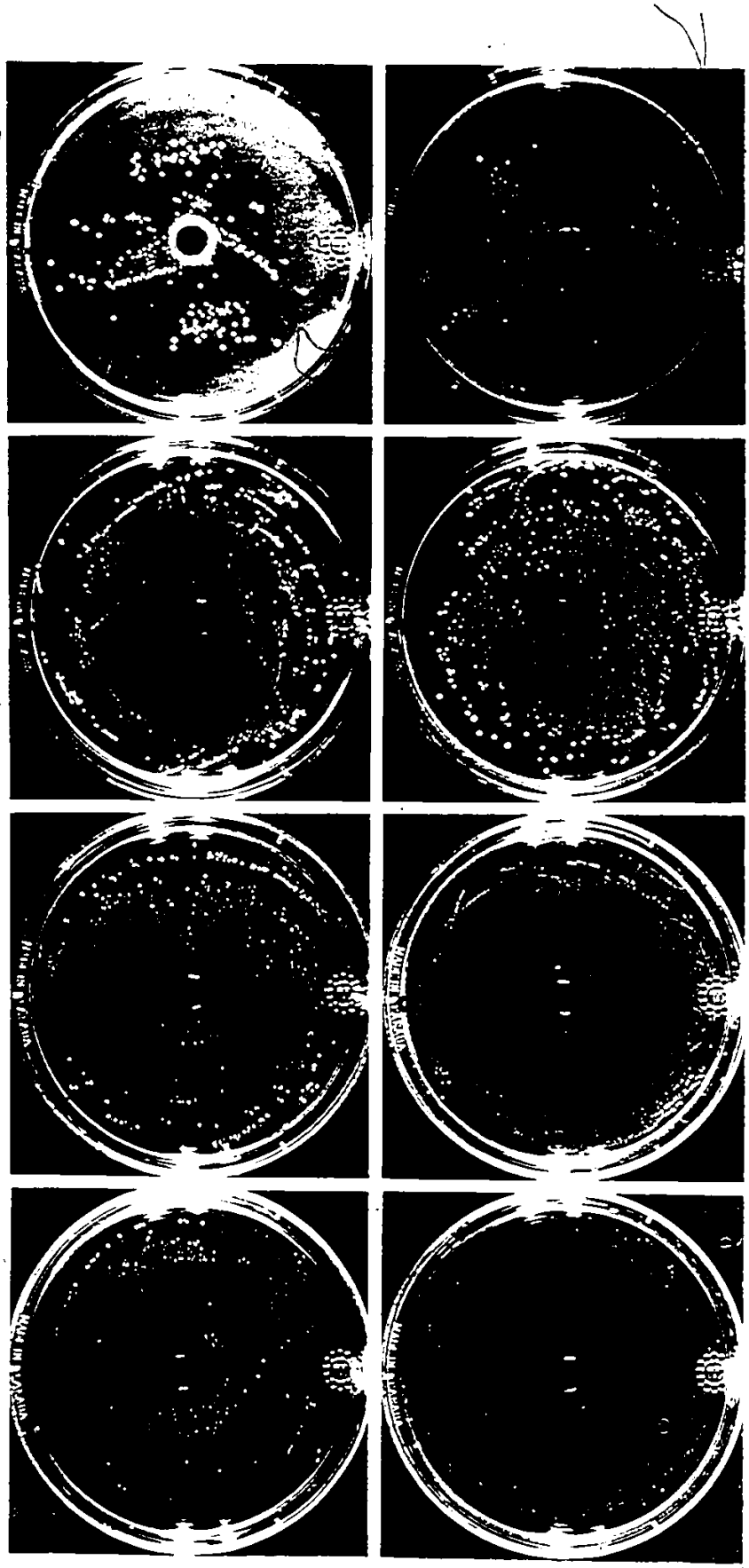
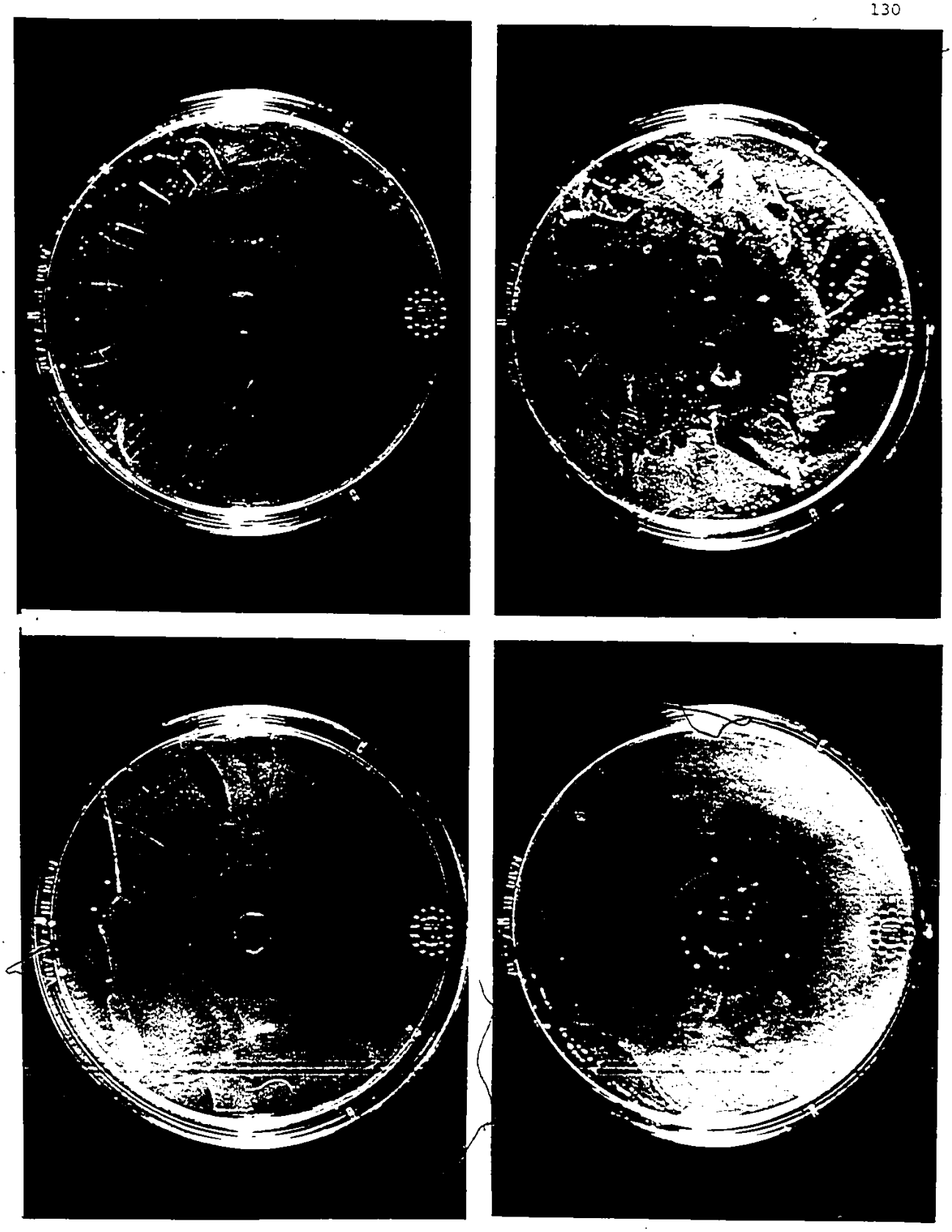


Fig. 12

Effect of mouse liver extract (M - 79) upon growth of the avirulent serogroup Y strain. The seed inoculum was applied at 1.0×10^8 cfu/ml. The agar contained increasing concentrations of iron as Jectofer: A_1 - no iron, A_2 - 1 mg/100 ml, A_3 - 5 mg/100 ml and A_4 - 25 mg/100 ml.



Ferritins from different animal species have been shown to be cross-reactive in immuno-diffusion in agar (Munro and Linder, 1978). Precipitin bands to anti-horse spleen ferritin and to anti-human ferritin (Behringwerke, Germany) were found in most liver extract preparations. However, the inhibitory activity of the different extracts did not correlate with precipitin activity.

Mouse spleen extracts were not inhibitory for any of the meningococcal strains tested.

Extracts prepared in a similar manner from human liver also limited growth of the avirulent strains. With the application of lower seed inocula, the human liver extracts caused zones of reduced growth even of the virulent A strain. Once again, this inhibition was removed by the addition of iron to the agar. In the presence of human liver extract, growth of the serogroup B strain was limited to the same magnitude as the serogroup Y strain, whereas the C strain was only slightly inhibited.

EFFECT OF IRON UPON GROWTH IN THE PRESENCE OF ASCORBATE AND HYDROGEN PEROXIDE

The puzzling phenomena which emerged in the previous studies with apotransferrin, apoferritin and the liver extracts required clarification. The observations that growth inhibition increased rather than decreased when iron was initially added to the medium was in direct contradiction with studies on other micro-organisms which suggested that an increase in % iron-saturation of similar proteins decreased their inhibitory capacity. (Bullen et al. 1974).

Since these proteins possess ferro-oxidase activity, this inhibitory behaviour might be explained on the basis of oxidation-reduction.

Ascorbic acid oxidation has an anti-microbial effect (Ericsson and Lundbeck, 1955). Hydrogen peroxide in conjunction with ascorbate has shown a bactericidal action for gram-negative bacteria (Miller, 1969). It has been suggested that free radicals may be involved in the process of inhibition (Halliwell et al., 1976). Subsequently, the chemically defined substances, ascorbate and hydrogen peroxide which are found in hosts and participate in oxidation-reduction reactions were examined for inhibitory activity in the presence of iron in the agar-plate system.

A difference in growth capacity of various serogroup strains of *N. meningitidis* occurred with sodium ascorbate. Ascorbate produced a growth inhibition which was dependent upon the concentration of iron in the media (Table 22). On iron-poor media or media containing low levels of Jectofer, inhibition was not apparent. With the levels of ascorbate in Table 25, inhibition became evident only on agar plates containing iron in excess of 10 ug/ml. However, inhibition did not continually increase as the iron concentration increased above this level. A point was reached when inhibition subsequently declined. The order of susceptibility of serogroup strains to inhibition was C > Y > B > A.

An alternative technique in which additional iron was added to the diffusion wells followed by ascorbate also produced enhancement of ascorbate inhibition (Fig. 13). Iron salts were as effective as the chelated iron, Jectofer. No difference was found between ferrous or ferric salts.

The inhibitory activity of ascorbate remained when tested in human plasma instead of phosphate buffer (pH 7.2). At comparable concentrations, dehydroascorbic acid did not produce growth repression. Folic acid also proved to be inactive. Glutathione and cysteine hydrochloride were also without effect in this system. Hence the mechanism was not simply explained on the basis of reducing power of ascorbate.

Table 22

Effect of Sodium Ascorbate upon the growth of *N. meningitidis*

Sodium ascorbate[a] mg/ml	Serogroup Strain	Zone of inhibition (mm)[b]						
		Iron ug/ml[c]						
		0	10	50	100	250	500	1000
	604- A	-[e]	-	-	-	16	13	14
200	608- B	-	-	-	19	19	20	20
	2241- C	-	18 ±[d]	23 ±	26	19	20	20
	Slaterus Y	-	-	-	25	26	22	23
	604- A	-	-	-	21	25	24	24
300	608- B	-	-	10	28	29	25	24
	2241- C	-	20 ±	25 ±	28	34	31	24
	Slaterus Y	-	15	20	34	30	31	30

a. 0.03 ml sodium ascorbate in saline deposited in centre well and let diffuse overnight.

b. Zone diameter includes 3 mm diameter (of well); mean of 4 determinations.

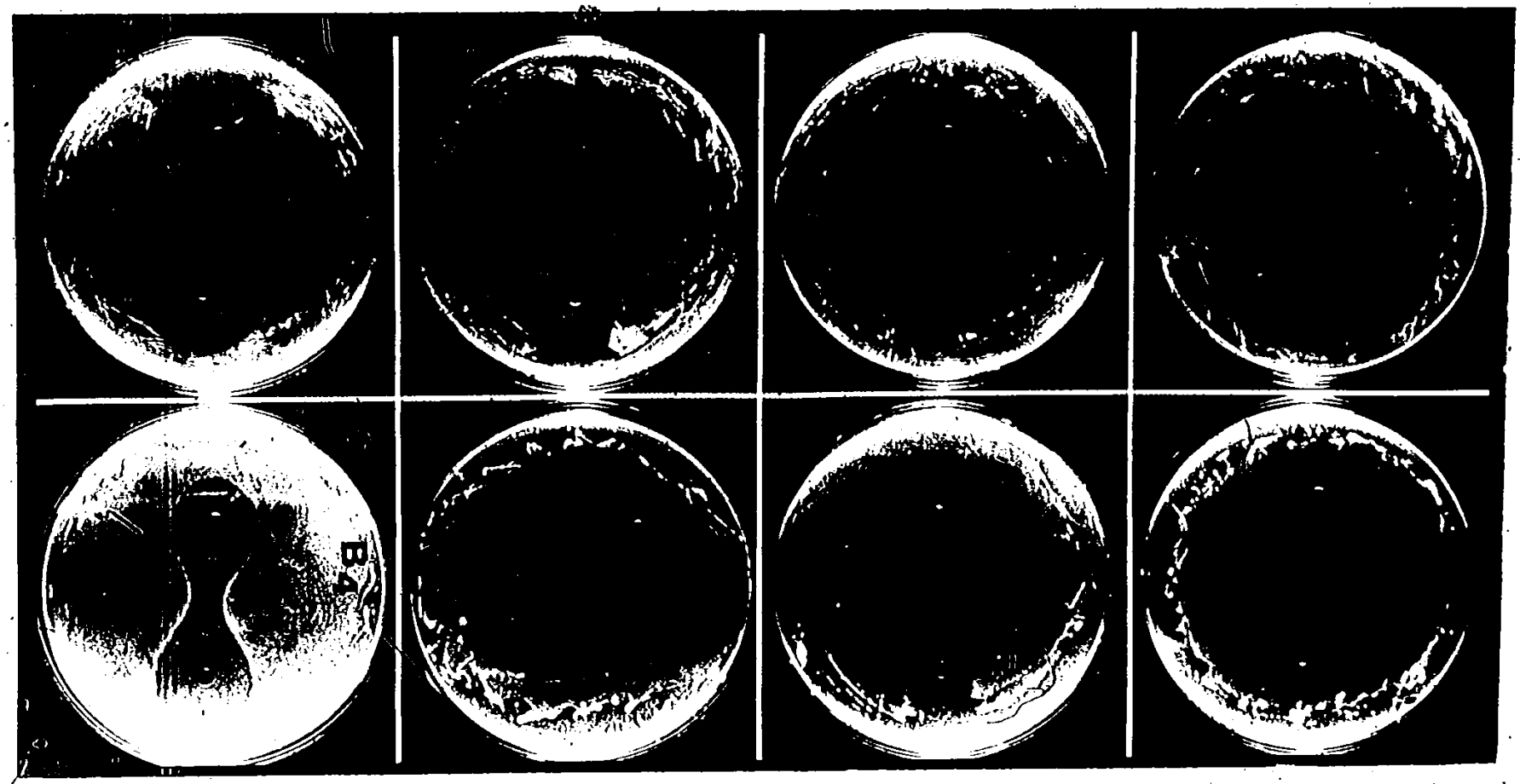
c. Iron as Jectofer added to the agar.

d. ± represents growth inside a zone; not total inhibition.

e. - represents no zone around well.

Fig. 13

Effect of sodium ascorbate upon the growth of *N. meningitidis* Slaterus Y (A_1, 2, 3 and 4) and 604-A (B_1, 2, 3 and 4). All agar plates contained iron as Jectofer at 10 ug/ml. Volumes of 0.03 ml of $FeSO_4$ in saline were added to both agar wells and let diffuse: A_1, A_3, B_1 and B_3 - saline only no iron; A_2, A_4, B_2 and B_4 - 2 mgFe/ml. (3 to 4 h later) Volumes of 0.03 ml of sodium ascorbate in saline were also added to both agar wells and let diffuse overnight before spreading bacteria: A_1, A_2, B_1 and B_2 - 200 mg/ml; A_3, A_4, B_3 and B_4 - 300 mg/ml.


B4

A series of compounds, known to scavenge hydroxyl ions in other systems (Drath and Karnovsky, 1974) were examined for capacity to reverse ascorbate inhibition. The scavengers were mixed with ascorbate in phosphate buffer (pH 7.2) and added to the diffusion wells immediately. Sodium benzoate, sodium thiosulfate, sodium metabisulfite and mannitol did not reverse ascorbate inhibition.

Measurement of pH by surface electrode showed that diffusion of sodium ascorbate produced a drop in pH of the agar medium surrounding the well on higher iron concentration plates. Diffusion of 300 mg/ml (pH 7.2) sodium ascorbate produced a pH of 6.4 in the agar around the diffusion well of 50 ug/ml iron plates. In separate experiments, for comparison purposes, similar or slightly greater drops in pH of the agar was produced using other acids. However, zones of inhibition did not occur on these plates as on comparable sodium ascorbate plates.

A variation in response of serogroup strains to growth in the presence of hydrogen peroxide was also demonstrated (Table 23 and 24). The order of strain susceptibility was 608-B > 2241-C > Slaterus Y > 604-A. The serogroup A strain possessed a four-fold greater resistance to peroxide inhibition than the other three serogroup strains (Table 26). Hydrogen peroxide was equally effective in phosphate buffered saline, pH 7.2.

The effect of iron upon peroxide inhibition was opposite to its effect on inhibition by sodium ascorbate. Iron as Jectofer directly reversed H_2O_2 growth repression (Table 24). Bacterial growth increased as the concentration of iron in the media increased. Human plasma also eliminated peroxide growth inhibition.

Table 23

Effect of Hydrogen Peroxide
on the Growth of *N. meningitidis*

Iron in agar[a] ug/ml	Serogroup Strain	Zone of Inhibition (mm)[b] H_2O_2 mg/ml[c]				
		2.5	5	10	20	30
10	604-A	-[f]	-	-	40	58
	608-B	23	38	N.G[d]	N.G	N.G
	2241-C	24	38	58	N.G	N.G
	Slaterus-Y	-	32	54	N.G	N.G
50	604-A	-	-	-	±[e]	48
	608-B	-	-	26	48	N.G
	2241-C	-	-	25	48	62
	Slaterus-Y	-	-	-	42	60

a. Iron added as Jectofer to the agar.

b. Zone diameter includes 3 mm diameter (of well); mean of 4 determinations.

c. 0.03 ml of H_2O_2 in phosphate buffered saline (pH 7.2) deposited in centre well and let diffuse overnite.

d. N.G. represents no growth.

e. ± represents less growth around well.

f. - represents no inhibition around well zone.

Table 24

Iron Reversal of the Inhibition of *N. meningitidis* by H_2O_2

H_2O_2[a] mg/ml	Serogroup Strain	Zone of Inhibition (mm)[b] Iron ug/ml[c] 0	1	5	10	25	50
5	608-B	N.G.[d]	65		50	34	-[ó]
	2241-C	63	57		46	35	-
	Slaterus-Y	65	56		45	26	-
2.5	608-B	55	44	31	25	-	-
	2241-C	51	40	34	29	-	-
	Slaterus-Y	56	43	±[e]	±	-	-

a. 0.03 ml of H_2O_2 in phosphate buffered saline (pH 7.2) deposited in centre well and let diffuse overnite.

b. Zone diameter includes 3 mm diameter (of well); mean of 4 determinations.

c. Iron added as Jectofer to the agar.

d. N.G. represents no growth.

e. ± represents less growth around well.

ó. - represents no inhibition zone around well.

DISCUSSION

Research from various laboratories suggests that hypoferremia benefits the host by making it less susceptible to microbial attacks (Weinberg, 1978). Apart from animal experiments in which artificial induction of hyperferremia (by injection of iron compounds) increases the ease of establishing a microbial infection, indirect support for this suggestion is also provided by *in vitro* experiments whereby a variety of bacteria grow more efficiently in the presence of body fluids or compounds when additional iron is included (Bullen et al., 1974b).

The bactericidal and/or bacteriostatic action of normal serum may be an important component of resistance to infection. If our mice were normally resistant to meningococcal invasion due to a bactericidal nature of the serum, one would expect that such normal sera would prevent *in vitro* growth of the bacterium. On the contrary, mouse serum like other normal mammalian sera (including human) stimulated the growth on agar of small inocula of virulent and avirulent strains. This effect was first demonstrated for this bacterium by our laboratory (Calver et al., 1978a and 1978b) and more recently by Mickelson and Sparling (1978) and Holbein (1981).

This observation with mouse sera suggests that the requirement for exogenous iron in infected mice is not based upon overcoming a defense activity of mouse serum. These results further demonstrate that virulent and avirulent meningococci are capable of growing in the presence of mouse transferrin (30 - 40% saturated with iron) contained in the serum. This observation, however, does not prove that the cells were able to grow in the presence of serum due to an ability to remove and use iron as suggested was for other bacteria (Bullen et al., 1974).

Subsequent *in vitro* tests showed that our meningococcal strains were able to grow in the presence of purified human transferrin containing various levels of iron. This ability to use transferrin iron was first demonstrated by our laboratory (Calver et al., 1979) and is also indicated in this thesis (Table 16). In certain cases iron-limited growth occurred in the presence of the apo-form of this protein. However, only certain lots of human apo-transferrin possessed an inhibitory activity at relatively high levels and this activity was dependent upon the size of the spreading inoculum and upon the presence of bicarbonate.

In our studies, since transferrin formed a pink complex in iron-containing NCDM agar, it was possible to determine the distances to which most of the protein molecules had diffused after 24 hr at room temperature. From the radius of this zone the volume in which the protein was contained could be estimated and hence the concentration of protein throughout the zone could be calculated on the assumption that it was equally distributed. For example, from a radius of diffusion of 15 mm after addition of 0.15 ml of transferrin at 100 mg/ml, it was calculated that the zone contained 6.4 mg of protein per ml.

Assuming a molecular weight for transferrin of 85,000, the maximum amount of Fe^{3+} which could be bound within the diffusion zone would be approximately 8.5 ug of Fe^{3+} per ml.

On the basis of these approximate values our virulent strain demonstrated a capacity to grow in the presence of a level of iron which provided a > 50% saturation of transferrin. Although the avirulent strain was not affected by transferrin in the presence of low levels of iron, it could not grow in the presence of transferrin 50% saturated with iron. Indeed, even with iron at a 2 to 3 fold excess of the total binding capacity

available, inhibition persisted. The normal adult range for saturation of human transferrin is 20 to 55%.

Since the concentration of transferrin in normal mammalian sera is 1.5 to 2.5 mg/ml, our results suggest that at least a 3 fold higher level of purified human apo-transferrin was required *in vitro* before limitation of growth occurred.

Although growth inhibition due to transferrin was found to be variable, from lot to lot, even in the presence of bicarbonate, such was not the situation with conalbumin. All lots of this iron-binding protein from egg-white appeared to exert selective iron-reversible inhibition. A substantial difference in the ability of the avirulent strain to grow in the presence of conalbumin was observed. The avirulent strain was inhibited to a greater degree. Since conalbumin also formed a pink complex in iron-containing agar it could be estimated that with deposition of 30 mg/ml of this protein, saturation of the iron-binding capacity would occur in agar containing 600 ug Fe as Jectofer per 100 ml of agar. When enough iron was added to the growth medium to exceed 50% saturation of the protein (i.e. 300 ug Fe/100 ml) total elimination of the growth inhibition of the virulent strain occurred.

However, total reversal for the avirulent strain was obtained at a >80% saturation. Hence, the avirulent strain demonstrated a reduced capacity to utilize available iron and subsequently eliminate growth limitation due to conalbumin.

Archibald and DeVoe (1979) also found that apotransferrin inhibition growth of meningococci. Although a variation was found to exist between trials both in the rate and the extent of zone decrease in transferrin-induced bacteriostasis with time, conalbumin induced bacteriostasis was more reproducible (Archibald and DeVoe, 1979).

In our media, without added synthetic chelating agent (EDDA), a 3- fold higher level of apotransferrin was required to demonstrate growth inhibition. However, the level of deferri-conalbumin which gave inhibition in the studies of Archibald and DeVoe was similar to the lowest level used in our studies. Examination by these authors on EDDA agar of the effect of these iron-binding proteins upon growth of four other meningococcal strains (three serogroup B and one serogroup C) of differing virulence for mice however, indicated no correlation between virulence in the mouse and the ability of a strain to compete with transferrin for Fe.

The difference in results between the two studies may be due to the use of different strains, different source and lot of the iron-binding proteins or especially that the effect alone of the iron-binding protein is being evaluated in our system, whereas in Archibald and DeVoe's system additional potent iron-binding agents such as EDDA are also present.

Recently, meningococcal isolates representing groups A, B, C, X, Y and Z were evaluated for their ability to compete with transferrin and conalbumin (Mickelson and Sparling, 1981). Only one isolate, a group A strain was inhibited by 4% Fe-saturated human transferrin on medium without added Desferral. All meningococcal strains were able to obtain the required iron for growth from 25% Fe-saturated transferrin. However, non-pathogenic commensal strains of *Neisseria* (*sicca* and *perflava sp.*) were inhibited by both 4% and 25% Fe-saturated transferrin. On the other hand, both pathogenic and non-pathogenic *Neisseria* were inhibited by 25% conalbumin(Mickelson and Sparling, 1981). Mickelson's experimental design differed significantly from that of our study. The bacteria were grown within the agar rather than spread upon the surface. The concentration of apo-transferrin used was 10 fold less.

I think that caution must be exerted when using systems which involve additional iron chelating agents such as EDDA and/or Desferal. An unknown factor in these experiments is the degree of interaction that occurs between these agents themselves and the iron-binding proteins being studied. The transfer of iron from transferrin to Desferal occurs in the presence of low molecular weight chelates (Pollack et al., 1976). Low molecular weight chelating agents are capable of forming ternary complexes with transferrin and ferric iron and can promote a rapid transfer of iron from transferrin to desferal (Pollack et al., 1976). Hence growth in the presence of transferrin containing iron may be due to decrease in the inhibition of Desferal rather than to the capacity of the cells to remove iron from transferrin.

In our studies, the amount of radioactive iron removed from transferrin and retained by whole meningococcal cultures was least for the avirulent strain. However, little difference existed in the capacity of washed cells of virulent or avirulent meningococcal strains to remove iron from transferrin. Hence, the ability of spent growth media to reduce transferrin iron retention by the cells was greatest for the avirulent meningococcal strain.

In other studies the ability of meningococcal culture filtrates to interfere with the binding of iron was also examined. Although concentrated meningococcal culture filtrates gave no indication of catechol, hydroxamate content or iron-binding capacity (refer to the previous chapter), the interference by the culture filtrates of the binding of iron with transferrin was significant.

Hence, these two separate studies support each other. An explanation for the decreased binding of iron by transferrin is that components of the spent media (filtrate) bind non-specfically to transferrin molecules and

block the iron-binding sites of transferrin. Culture filtrates of *N. gonorrhoeae* interferred with the capacity of iron-free transferrin to bind iron (Norrod and Williams, 1978). Kochan (1977) demonstrated that enterobactin isolated from spent media of other bacteria formed complexes with iron-saturated transferrin but did not remove iron from the transferrin. Carrano and Raymond (1979) have also shown that enterobactin as well as other synthetic chelates form complexes with transferrin-iron.

Hence, in our studies, it is possible that some growth by-product (i.e. of spent media) might bind to transferrin in a manner similar to enterobactin and the synthetic chelates. However, iron uptake by the cells is impaired and the effect is detrimental to growth, especially of the avirulent strain.

Archibald and DeVoe (1979) have shown that meningococcal cells were unable to obtain iron from transferrin when seperated from the protein by a dialysis membrane with a cut-off limit of 12,000 daltons. These authors suggested that direct contact of the bacteria with transferrin was required in order to remove the iron. Our results support this observation that iron is removed from transferrin by direct cellular contact. In addition, however, we have shown that spent media interferes with this process particularly so with the avirulent strain.

The results presented in Chapter 2 indicate that virulence of meningococcal strains for mice did not correlate with a difference in capacity to obtain iron from low molecular weight iron-chelating agents. Archibald and DeVoe (1980) provided additional evidence supporting this concept. The ability to obtain iron from a variety of sources was shown to be similar for 20 strains of differing isolation source and virulence for mice.

Thus, it would be expected that if iron limitation in the host was to be effective as a basis for virulence, then the host's iron binding proteins

must operate in a selective manner. Selective action could be due to a difference in recognition by or stereospecific interaction of the iron-binding proteins with the bacterium. Recently evidence has been presented that meningococci actually possess a specific surface mechanism for recognition of transferrin which is essential for removal and uptake of iron (Simonson et al., 1982). Unfortunately these authors present evidence for only a single strain which also is virulent for mice. No evidence is provided to show that avirulent strains do not express such a mechanism under comparable conditions.

In our studies as well as those of others (Archibald and DeVoe, 1979; Mickelson and Sparling, 1981) conalbumin was consistently inhibitory whereas transferrin was not. These iron binding proteins seem to act differently on the bacterium.

However, the *in vitro* evidence for suggesting that the pathogenicity of *Neisseria meningitidis* is related to an ability to remove iron from transferrin is shaky and remains controversial. On the other hand, enhancement of infection in mice can be obtained by injection of transferrin containing iron (Holbein, 1981). In addition, Holbein (1980) and Letendre and Holbein (1983) present data showing that during systemic clearance in mice of a virulent meningococcal strain, a hypoferremia occurs in which the Fe saturation of mouse transferrin is reduced to almost a zero level. Unfortunately definite evidence that a hypoferremic response does not occur during systemic clearance of avirulent strains is not presented. These authors suggest that the hypoferremic response occurs in order to limit the amount of iron available for the growth of the bacterium. However no concrete evidence is provided that the bacterium actually uses this *in.vivo* iron for growth purposes. It is possible that hypoferremia occurs for alternative reasons. Such will be discussed further on.

Human lactoferrin exerted a selective inhibitory effect upon our different meningococcal strains. The highly virulent serogroup A strain was able to grow in the presence of a concentration of lactoferrin which was 4-fold greater than the level which prevented growth of the avirulent serogroup Y strain. Resistance to lactoferrin was not a virulence characteristic per se, since the serogroup B and C strains were also partly inhibited by this protein, albeit to a lesser extent than the avirulent serogroup Y strain. However, preferential reversal of this inhibitory activity by iron occurred with the virulent strains.

If one assumes a molecular weight for lactoferrin of approximately 80,000 and that two binding sites for iron exist per molecule, then the iron-saturation level for 1 mg of the protein would be approximately 1.25 ug Fe+++. In our experiments with agar containing different levels of added iron, the amount of iron required to fully saturate the lactoferrin being deposited (i.e. 250 ug) could be estimated as .3 ug of Fe+++. Although no indication of the depth of diffusion of the protein is available, it would be expected that agar containing iron as Jectofer at .25 to 2.0 mg/ml would provide enough iron to exceed the total iron-binding capacity of the lactoferrin by 100 to 1000-fold. Thus, although the results indicate that iron-reversible growth inhibition occurs, it appears that lactoferrin exerts its inhibitory effect on the B, C and Y strains even in the presence of concentrations of iron as Jectofer which far exceed the iron-binding capacity of the protein.

It would thus appear that the mechanism by which lactoferrin exerts inhibition is not by simply withholding iron in the medium from the bacterium (i.e. iron-starvation). A direct interaction of the protein with the bacterium is suggested. Iron at high levels might interfere with this interaction by preventing attachment of the protein to the bacterium. Based

upon the amount of iron required for reversal of growth inhibition it would appear that the type of inhibition caused by lactoferrin differs from that due to transferrin or conalbumin. Our data which indicate that lactoferrin is capable of exerting a bactericidal effect independent of simple iron deprivation supports the studies of Arnold et al. (1977, 1980 and 1981) with other bacteria.

Since micro-organisms of the same species can differ in susceptibility to lactoferrin, it appears that accessibility of lactoferrin to a specific target site may account for differences in susceptibility. Increased virulence may hence be associated with changes in cell surface components which decrease lactoferrin accessibility.

An association between the response of strains of other bacteria to lactoferrin and virulence has been observed in other laboratories (Arnold et al. 1980). On the other hand, Mickelson et al. (1982) have recently demonstrated that each of 15 meningococcal strains of 6 different serogroups from different isolation sources were able to grow in the presence of 20% Fe-saturated lactoferrin. Lack of agreement with our observations could be due to use of different strains or different lots of lactoferrin. These authors seem to have used only one lot of lactoferrin. Our data showed a variability in reactivity of different lots. It is possible that different lots of lactoferrin may differ slightly in structure and hence have different interaction with the bacterial surface. Human transferrin is known to exhibit heterogeneity. Electrophoretic mobility has shown the existence of 21 variants (Bothwell et al. 1979). Our data has shown that substantial heterogeneity exists in the different commercial horse-spleen ferritins. It is possible that lactoferrin also is heterogeneic.

Due to the fact that commercial preparations of horse spleen ferritin contained cadmium, which was toxic on its own for meningococci, studies of this protein were carried out with the reduced form. On iron-poor media (5 ug/100 ml), growth of virulent and avirulent strains was enhanced by this protein. However, as the concentration of iron incorporated in the media was increased, reduced ferritin inhibited growth. At lower iron concentrations, the avirulent strain appeared more resistant to inhibition than the virulent strain, but at higher iron concentration the opposite occurred. Hence a pattern of increasing inhibition in the presence of iron similar to the inhibition which was seen in the transferrin studies also occurred with the iron-storage protein ferritin.

From a radius of diffusion of 15 mm after addition of 0.15 ml of apo-ferritin at 30 mg/ml, the concentration of this protein within the zone was found to be 4.5 mg/ml. Assuming a molecular weight for ferritin of 450,000 and that 4500 atoms at Fe^{3+} could be bound per ferritin molecule (Macara et. al 1972), the maximum amount of iron which could be bound by protein in the diffusion zone would be 2.4 mg/ml. Unlike the studies with lactoferrin, the highest level of iron used in the agar plates (i.e. 2.0 mg/ml) provided less iron than that required to saturate reduced ferritin (i.e. apoferritin).

This phenomenon of increasing inhibition in the presence of iron (rather than a decrease as would be expected) was also evident in studies with mouse liver extracts. An iron reversible activity occurred which was selective for certain types of meningococci. The increasing inhibition of growth of the avirulent serogroup Y strain which occurs in the presence of increasing iron, correlates with the inhibition patterns previously seen with human transferrin and reduced horse spleen ferritin.

This evidence suggests that the increased virulence of meningococci for mice in the presence of exogenous iron may be due to elimination of a bactericidal action of liver tissue. Such activity may be related to the content of tissue specific iron-binding proteins (Aisen, 1980). Accurate determination of the types of iron binding proteins in these extracts must await use of antisera produced against purified mouse iron binding proteins such as ferritin, lactoferrin and transferrin.

Our studies also showed that human liver extracts possessed a selective iron reversible inhibitory activity. Hence the human liver may be an important site for resistance to meningococcal infection. In the presence of iron, such resistance may be susceptible to change.

If the host iron-binding proteins were to inhibit growth of meningococci due to limitation of available iron, it would be expected that as iron was added to the medium, inhibition would be progressively eliminated. However, as seen with transferrin, reduced ferritin and mouse-liver extracts, the opposite effect occurred and growth was limited even more. Indeed in some cases, inhibition appeared with higher iron levels although none was present with lower iron levels. Enhancement of inhibition however, did not continually parallel increasing iron. Usually a level of added iron was reached, at which inhibition was reversed and subsequent growth enhancement resulted with further iron. Thus, although synthetic iron-binding agents (Chapter 2) produced an iron reversible inhibition, these host iron-binding proteins showed iron-enhanced inhibition.

The manner in which iron forms the micelle core in the ferritin protein molecule could be an important factor in determining inhibitory activity. Macara et al. (1972) showed that the rate of iron uptake by ferritin in vitro depends on the amount of iron already present in the molecule and appears to be a function of the surface area of the crystalline micelle. It is possible

that the inhibition observed in these studies depends upon the autocatalytic binding of iron by reduced ferritin. A certain level of iron is required to activate the binding process. As the ferritin iron crystal is formed, an increase in the ability of this complex to bind further iron is generated which could result in the cessation of bacterial multiplication due to iron starvation. An optimum saturation of the protein would eventually be reached, beyond which a decrease in inhibition would occur.

Increasing inhibition of meningococcal growth by transferrin may also be related to increased binding of iron by molecules to which a single atom of iron has already been added (Aisen, 1980). It has been suggested that binding of the first metal ion by transferrin strengthens binding of the second by a factor of 100 or more.

Since iron may also be involved in oxidation-reduction reactions and since ferritin and transferrin were known to possess ferro-oxidase activity (Aisen, 1980), the chemically defined oxidizing and reducing agents, H_2O_2, sodium ascorbate and ascorbic acid were examined in our system. The studies indeed proved that the phenomenon of increasing inhibition in the presence of iron did occur with sodium ascorbate. Hence the mechanism of inhibition of transferrin, reduced ferritin and mouse liver extracts could be similiar to that of ascorbate. A difference in the growth capacity of various serogroup strains of *N. meningitidis* was found when sodium ascorbate was included in the medium. This compound caused a growth inhibition which was dependent upon the iron level in the medium. Inhibition was not apparent upon agar plates containing less than 10 ug/ml of the chelated iron compound Jectofer. As the iron level in the media increased above this concentration inhibition appeared and subsequently increased to a maximum and then declined. The order of susceptibility of serogroups to inhibition was C > Y > B > A.

With certain micro-organisms, ascorbic acid alone is an effective antibacterial agent. A bacteriostatic activity has been reported for group A hemolytic *Streptococci*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (Lwoff and Morel, 1942 and Rawal, 1978). In our system, at the concentrations employed, ascorbic acid was inhibitory only in the presence of iron. Ascorbate is known to auto-oxidize if metal ions are present (Halliwell and Foyer, 1976). During this process hydrogen peroxide is produced. Ascorbate in combination with hydrogen peroxide has been found to be a potent bactericidal mixture for a variety of gram negative bacteria (Miller, 1969). It was suggested that such bacteriostatic action was due to oxidation of ascorbic acid and subsequent release of hydrogen peroxide into the medium.

The possible formation of hydrogen peroxide due to oxidation of ascorbate as a mechanism of inhibition in the meningococcal system is contradicted by the experiments with iron and hydrogen peroxide. Increasing iron decreased and subsequently eliminated inhibition by H_2O_2, whereas iron initially increased inhibition due to ascorbate.

Hydroxyl radicals are known to arise by a reaction between Fe^{++} and H_2O_2 (Haber, and Weiss, 1934). Fe^{+++} is reduced by ascorbate in a reaction involving a single electron transfer. It has been reported, however, that the interaction of Fe^{+++} and ascorbate does not produce hydroxyl radicals (Gutteridge and Wilkin, 1982). In our system with meningococci it would appear that hydroxyl radicals were not involved since free radical scavengers did not prevent inhibition.

Since both oxidized and reduced iron were equally effective at enhancing inhibition, it would be expected that reduction by ascorbate was not a necessary component of the inhibitory mechanism. However, in aerobic solution, Fe^{++} undergoes spontaneous auto-oxidation. Ascorbic acid would subsequently reduce this iron also. Since glutathione and cysteine were not

effective inhibitors when combined with iron in the meningococcal growth system, the reduction of iron per se is not the important requirement for inhibition. The main requirement for inhibition appears to be oxidation of the ascorbate itself. Ascorbic acid undergoes a reversible oxidative metabolism, first to an unstable free radical intermediate monodehydro-ascorbic acid (Eddy and Ingram, 1953). The oxidized form, however, was without meningococcal growth inhibitory activity. It is possible that the free radical form is involved. Ascorbic acid may also be converted to di-ketogulonic acid which may spontaneously oxidize to other compounds. Perhaps these compounds are involved in the inhibition of growth.

With respect to certain bacteria it is suggested that cadmium together with H_2O_2 might induce oxidative damage to cells which contain insufficient catalase to decompose all the H_2O_2 formed as a by-product of aerobic cells (Korkeala, 1980; Korkeala and Sankari, 1980).

Evidence is presented in our studies that the toxicity of cadmium for *N. meningitidis* is reversed by excess iron. In addition, the avirulent strain was more susceptible to cadmium. These studies add further support to the observations and proposals derived from experiments with hydrogen peroxide.

In vitro studies with hydrogen peroxide showed that iron reversed the inhibitory activity of H_2O_2 for meningococci. Susceptibility varied according to serogroup. The more virulent serogroup 604-A strain was more resistant. The difference may be related to catalase content. The resistant serogroup 604-A strain has recently been shown by our laboratory to possess 100 to 1000 fold more catalase activity than the other serogroup strains. In addition, the catalase content of the A strain, in contrast to the other strains appears to be iron inducible (preliminary observations).

It is possible, in our studies, that the enhancement of meningococcal growth in mice may be due in part to iron reversal of the oxidative damage to bacteria by leukocytes (Klebanoff, 1967). Kaplan et al. (1975), examining the effect of iron on leukocyte function found an inactivation of H_2O_2 by iron.

In summary, our *in vitro* studies have shown that iron may actually affect the growth of meningococci in opposing directions depending upon the presence of other factors. Iron alone stimulates meningococcal growth. However, when in combination with ascorbate, liver extracts, transferrin or reduced ferritin, iron represses growth of certain strains of meningococci. On the other hand, iron may contribute to growth of meningococci by inactivating inhibition due to H_2O_2 or lactoferrin.

A diversity in function for iron *in vitro* has been shown by others. When in conjunction with ascorbate iron produces hydrogen peroxide (Halliwell and Foyer, 1976); this could cause destruction of the meningococci to occur. On the other hand, iron decomposes H_2O_2 whether in salt form (Holbien and Weiss, 1934) or with lactoferrin (Ambruso and Johnston, 1981); this would protect the meningococcus.

The inhibitory action of ascorbate could be counteracted by the meningococcus through possession as part of its defensive armour an enzyme capable of destroying ascorbate. Perhaps, the oxidase found in association with cell wall blebs of certain strains of *N. meningitidis* (DeVoe and Gilchrist, 1976) has such a function. The inhibitory activity of H_2O_2 could be overcome through possession of adequate levels of catalase. Further investigations may clarify the involvement of catalase in the protective machinery of this bacterium.

Our *in vitro* experiments have left us with the conclusion that caution must be applied when attempting to evaluate the effects of iron during *in vivo* infection in mice. During infection, it is possible that iron may be exerting opposing effects on microbial growth, the balance of which determines the course of the infection.

CONCLUSION

The aim of this thesis was to evaluate the potential role of iron in the pathogenicity of *Neisseria meningitidis*. Pathogenicity of a microbe is an expression of its capacity to cause disease in a host. The term virulence is used with respect to the degree of pathogenicity within one species (Smith, 1972). Pathogenic bacteria have a chemical armoury which enables them to invade a host and produce disease. Although nearly a century has elapsed since *N. meningitidis* was first identified, few of the components of this pathogen's chemical armoury have been characterized.

In certain diseases it is easy to identify the weapons in the armoury when the pathogenicity is determined by a single product which is easy to produce in vitro, as with diphtheria or tetanus. However, infections in humans due to *N. meningitidis* are like the majority of infections caused by other micro-organisms whereby pathogenicity cannot be related to a single microbial product.

Very few micro-organisms can be labelled pathogenic, if pathogenic is defined to mean causing infectious disease at all times. Such is the case for the meningococcus which can be carried in the nasopharynx by a substantially high percentage of the population, without harm, and which may unexpectedly and suddenly cause an outbreak of meningococcal disease. It is an organism with a number of serogroups and yet the relative virulence of these serogroups remains unclear.

Pathogenicity and virulence cannot be applied to a microbe without reference to the host and the host's environment or special circumstances. True virulence is detectable only *in vivo* and can be influenced by changes in growth conditions due to selection of types and to phenotypic change. Genetic information which determines virulence may be expressed only under

the conditions of the test for virulence, namely during growth *in vivo*. The decisive nutritional conditions, those of the host tissues under attack are not physiological but pathological and are changing during the infection. A virulence determinant is a factor produced during infection and having biological activities directly connected with virulence.

However, bacterial behaviour during infection of its natural host is not always easily examined. Certainly with meningococcal infection in its only host, a human, detailed experimental pathology and precise biochemical determination cannot be easily accomplished. Hence the investigator in attempting to characterize true virulence determinants for *N. meningitidis* is certainly put at a major disadvantage before he starts.

Since a suitable experimental animal model of meningococcal infection which truly simulated the human infection did not exist, the only alternative for examination of this bacterium in an *in vivo* situation was study of an artificial mouse model of infection. Evidence was provided by this author in 1976 that the injection of iron compounds could lead to the progressive and fatal growth of an otherwise non-lethal dose of meningococci in mice (Calver et al., 1976). In this system bacteria grew from small inocula introduced intraperitoneally to overwhelming numbers of bacteria in the blood, resulting in death within 72 hr due mainly to septicemia.

This model of infection was examined in greater detail in the hope of providing information upon the manner in which iron was assisting in the establishment of infection. As in other experimental animal models of bacterial infection in which iron compounds are injected, the characteristic feature of the effect of iron is the stimulation of rapid bacterial multiplication. Upon injection of adequate amounts of iron, the normal ability of the host (i.e. mouse) to suppress growth of the bacterium

(i.e. *N. meningitidis*) is lost and the animal subsequently dies due to an overwhelming infection from a dose that is normally harmless.

Research from other laboratories with different bacteria would support the concept of nutritional immunity (Weinberg, 1978). It has been proposed that the iron binding proteins transferrin and lactoferrin restrict the amount of iron in the body. The microbe during its attempt to establish infection must compete with these proteins for required iron. When infection occurs in normal individuals, there is a decrease in serum iron (Heilmeyer, 1964 and Pekarek, 1969). Taken as a whole, the fall in serum iron could represent enhancement of a normal defence. Some authors have suggested that the fall in saturation of transferrin would enhance the bacteriostatic power of the plasma (Bullen, 1974b). Other authors have suggested that the iron enters the Reticuloendothelial system during infection and does so for purposes of detoxifying toxins such as endotoxin (Janof, 1960).

Evidence presented from our animal model studies would argue against a major role for mouse serum transferrin in the inhibition of meningococcal infection by the host. A study of the absorption of iron into the bloodstream of C57 normal mice following intra-peritoneal injection of the different dose levels of iron salts or chelated iron revealed that adequate iron to saturate the existing serum transferrin was found in circulation even with a dose of chelated iron which was unable to reduce the LD_{50} of the bacterium. The data indicates that the main effect of the injection of large doses of iron which enhance meningococcal lethality is to increase the levels of iron in storage either in cells of the Reticuloendothelial system or other body tissues or fluids.

The treatment of mice is affecting host resistance in such a manner that only bacteria possessing a selective character have the capacity to take advantage of this alteration. A fatal infection was produced in mice by

relatively few cells of only certain serogroup strains (Calver, 1978). Based upon this capacity to establish meningococcal infection in mice in the presence of iron, serogroups of *N. meningitidis* could be characterized as virulent or avirulent.

Virulence was not due to difference between strains in capacity to grow in vitro in the presence of iron. Growth of both strain types was equally limited by synthetic iron chelating agents such as desferal. Iron reversed this growth repression similarily for both strain types. Virulent strains did not possess an increased ability to store or take up iron from solution.

The *in vitro* production of iron chelators is not a characteristic upon which the difference in reaction of meningococcal strains in infectivity may be based. Meningococci neither secreted nor possessed cell-associated siderophores.

Mouse sera analyses showed that injection of large amounts of exogenous iron (required for infection enhancement) provided iron in the serum well in excess of the iron-binding capacity of the transferrin contained within the serum. In vitro studies with iron and growth would suggest that both virulent and avirulent strains should be able to use this iron for growth enhancement. However, the avirulent strain is not as lethal in the mouse, suggesting that local and/or Reticulo-endothelial defense mechanisms are more prominent in prevention of growth and subsequent septicemia.

In vitro the host iron-binding proteins, transferrin, lactoferrin and ferritin did not produce consistent reproducible inhibition of meningococcal growth as was produced by the synthetic iron-chelating agents. Structural heterogeneity could be responsible for lack of inhibition by certain lots of the proteins. For individual meningococcal strains, it was evident that the pattern and extent of inhibition by these proteins was not similiar but varied according to the type of protein. This response suggests there

is a direct interaction of the bacterial cell wall with the iron-binding protein, probably at the cell surface. Differences in susceptibility may be due to accessibility of the protein to specific target sites.

In vitro, virulent strains proved to be more resistant to inhibition by lactoferrin, liver extracts, ascorbate and hydrogen peroxide. These are factors which *in vivo* are associated with the local and/or reticulo-endothelial environment, especially polymorphs and macrophages. These factors would be involved in early defence systems of the host. Hence *in vitro* studies would suggest that during invasion by the meningococcus host iron binding or iron associated factors would exert their most significant preventive effects at the early stages of infection. Such effects as these, which would attempt to prevent the bacterium from becoming locally established, would be more advantageous to the host than effects that would operate once the bacterium has established itself in the circulation in septicemia.

In terms of the mouse model, a grossly unphysiological excess of exogenous iron was required to set up meningococcal infection. The *in vitro* studies suggest that such an excess of iron would be adequate to negate any inhibitory activity due to lactoferrin, transferrin, liver extracts, ferritin or H_2O_2. However, mouse virulence of the strains is due to more than resistance to iron-binding or associated factors. Some strains which were virulent for mice (eg. B and C serogroup strains) proved to have an *in vitro* susceptibility to certain iron-binding or associated factors similiar to the avirulent strain.

In mice (not pre-treated with iron), iron saturation of mouse serum transferrin declines when a meningococcal strain injected i.p. attempts to establish itself in the blood (Holbein, 1980). In normal humans the serum iron declines when a bacterial infection occurs (Pekarek et al., 1969).

Some believe that this is caused by a decreased release of iron from the reticulo-endothelial system, rather than a shift from the transferrin itself (Stockman, 1981). It has been proposed that the hypoferremic mice response occurs in order to starve the invading bacterium of serum iron (Weinberg, 1978). However, our *in vitro* studies offer an alternative explanation for movement of iron out of serum.

During infection of mammalian hosts a localization of ascorbic acid at the site of infection occurs (Stackpoole, 1975). A shift of iron from the serum to the local sites where it may react with ascorbate (causing inhibition as shown by the *in vitro* studies) could activate a first line defence mechanism to protect the host against initial bacterial invasion. Iron may also concentrate at local sites and/or in reticulo-endothelial cells in order to interact with apo-ferritin or liver constituents increasing their inhibitory activity. However, a great excess of iron in the host might be detrimental, resulting in enhancement rather than inhibition.

In vivo systems are dynamic. During infection, it is possible that iron may exert conflicting roles. Our *in vitro* experiments indicated that on one hand iron may activate ascorbate inhibition of meningococcal growth whereas on the other hand iron may inactivate inhibition by hydrogen peroxide. Meningococci which possess an armoury of special enzymes such as catalase or ascorbate oxidase may selectively take advantage of the host iron environment in promotion of their virulence.

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