IRON PHYSIOLOGY OF NEISSERIA MENINGITIDIS

by

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Dedicated to:

Helen, Andrew, and
my mother

"Whatever pharmaka, medicines, cannot cure
iron can cure; whatever iron cannot cure
should be considered incurable."

Hippocrates

ABSTRACT

Ph.D. Frederick Archibald Microbiology

IRON PHYSIOLOGY OF NEISSERIA MENINGITIDIS

To grow the meningococcus, a simple defined medium readily made iron-limited was developed and used in both batch and continuous culture of the meningococcus. This permitted measurement of the effects of iron alone on generation times, cellular iron, respiratory efficiency and a variety of respiratory proteins. A variety of assays for siderophore production was applied to solid and liquid iron-limited cultures, all with negative results, but on a variety of iron-limited media the iron in many stable complexes such as hemoglobin, myoglobin, organic acid-iron, and polyphosphate-iron complexes was readily available. A number of meningococcal strains was tested and none could acquire iron from native ferritin, cytochrome c, insoluble ferric salts or conventional siderophores. However, if human physiological concentrations of citrate, pyrophosphate, or a number of related substances were added, many poorly available forms of iron were readily used by the meningococcus. Transferrin-bound iron was found to be available when meningococcal cells were in direct contact with the glycoprotein chelate, but not when they were separated by a dialysis membrane. Using iron-limited continuous cultures, the ability of

the cells to take up several $^{55}Fe^{+3}$ complexes was followed. The meningococcus was found to have a rapid energy-independent uptake system with saturable, heat-sensitive iron-binding sites, and in addition, a second, slower system obeying saturation kinetics and requiring both a functional respiratory chain and a trans-membrane potential.

RESUME

Ph.D. Frederick Archibald Microbiologie

LA PHYSIOLOGIE DU FER CHEZ NEISSERIA MENINGITIDIS

Les résultats obtenus au cours de cette étude sur Neisseria meningitidis ont permis de déterminer ses besoins en fer, sa possibilité d'utiliser différentes formes du fer, les effets de la privation en fer ainsi que la cinétique de l'incorporation du fer.

Un milieu défini pour le méningocoque et limité en fer a été créé pour cette recherche et utilisé tant en culture continue qu'en culture en cuvée. Ceci a permis de mesurer les effets du fer sur le temps de génération, le contenu en fer de la cellule, l'efficacité respiratoire et plusieurs protéines liées à cette chaîne repiratoire. Plusieurs tests sur la production de sidérophore ont été faits en milieu solide et liquide avec des cultures limitées en fer. Tous ces tests furent négatifs mais dans plusieurs milieux limités en fer où celui-ci forme des complexes stables tels dans l'hémoglobine, la myoglobine, l'acide organique ferrique et les complexes polyphosphatés du fer, le fer était facilement utilisable par Neisseria meningitidis. Plusieurs souches de meningocoques ont été étudiées et aucune n'a pu utiliser le fer de la ferritine, du cytochrome c, des sels ferriques insolubles ou de sidérophores

usuels. Cependant, à des concentrations physiologiques pour l'homme de citrate, de pyrophosphate ou d'autres substances similaires, plusieurs formes du fer quoique peu utilisables ont été facilement assimilées par le méningocoque. Le fer lié à la transferrine était utilisable quand les cellules du méningocoque étaient directement en contact avec un chélateur glycoprotéique mais non quand ils étaient séparés par une membrane de dialyse. La possibilité pour les cellules d'incorporer plusieurs complexes de $^{55}Fe^{+3}$ a été etudiée en culture continue limitée en fer. Deux systèmes d'incorporation du fer ont été trouvés chez le méningocoque: un qui est rapide, n'utilise pas d'énergie et possède des liaisons saturables et sensibles à la chaleur; le second est plus lent, il suit une cinétique de saturation et il requiert une chaîne respiratoire fonctionnelle ainsi qu'une difference de potentiel au niveau de la membrane.

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. An improved, simple, defined meningococcal medium capable of growing all strains tested and readily made iron-limiting was developed.

2. A low-cost dissolved oxygen monitor-controller for maintaining a constant level of dissolved O_2 in continuous culture was designed and produced.

3. The cellular iron content, distribution, and minimal and saturating iron requirements were determined in batch and continuous culture.

4. The effects of dissolved oxygen and iron, varied separately, on the levels of a variety of respiratory proteins and other cell parameters in continuous cultures of N. meningitidis were determined.

5. A variety of meningococcal strains (20) were assessed for their ability to utilize iron from or compete with a wide variety of iron compounds, complexes, and chelates. The assay substances selected provided information on how iron could be acquired in vivo and demonstrated the preferences, specificity, and affinity of the meningococcal iron-acquisition system.

6. The meningococcus was demonstrated to lack an effective siderophore, at least under the conditions used.

7. The ability of a variety of citrate-related synthetic and natural hydroxy acids and polyphosphate compounds to operate as "functional siderophores" at low concentrations and to greatly facilitate the acquisition of poorly-available forms of iron by the meningococcus was demonstrated.

8. The meningococcus can remove iron from 30% saturated transferrin *in vitro*, but only via direct cell chelate contact or via a substance with a molecular weight > 12,000 d.

9. The improved growth seen on clinical (Thayer-Martin) media with various iron supplements is due to the absence of an effective true or functional meningococcal siderophore and not a high iron requirement.

10. The iron-starved meningococcus possesses an energy-independent iron-uptake system rapidly binding $^{55}Fe^{+3}$ from a number of complexes and chelates. The binding is saturable, stable, heat-sensitive, and the initial uptake kinetics are first-order.

11. The meningococcus possesses a saturable $^{55}Fe^{+3}$-uptake system completely inhibited by blocking the respiratory chain or discharging the trans-membrane potential. The system is equally

effective with a number of ferric complexes and unaffected by competition from several other transition metals.

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GENERAL INTRODUCTION AND LITERATURE REVIEW

The experimental data presented in this thesis cover several areas of meningococcal iron physiology. In order to clarify (a) the logic behind selecting those experiments actually performed; (b) the meaning of the results obtained; and (c) the similarities and differences between the iron acquisition mechanisms of Neisseria meningitidis and those of other bacteria, a general introduction including a literature review follows. As several of the areas covered, most notably microbial acquisition of iron and the relationship between host iron and microbial pathogenesis have voluminous and rapidly expanding bodies of published data, only a brief summary can be given, with a bias towards those data felt to be most relevant to the approach and findings of the thesis work.

A. The Role of Iron

In the late 19th century iron was believed to be associated with respiration, but only in the form of hemoglobin (see Levy, 1889). When it was proposed that iron was active in cells other than erythrocytes and that it had a more universal respiratory function(MacMunn, 1886), the theory was rejected (Levy, 1889), despite good experimental evidence. However, after decades of oblivion, research, notably by David Keilin and Otto

Warburg, established the essential presence of heme iron in the respiratory activities of all aerobic cells (Warburg et al., 1933a,b; Keilin, 1933). Subsequent work showed that heme and non-heme iron compounds were employed, even by obligate anaerobes, for fundamental energy-yielding (electron- or hydrogen-transferring) reactions as well as a variety of other functions (see reviews by Postgate, 1956; Neilands, 1974a). At present it is believed that all living cells have an absolute requirement for iron, although there is some doubt about a few species of lactobacilli (Neilands, 1972). In nearly all its biological forms iron is complexed with a protein and acts as a catalyst, facilitating the transfer of electrons, oxygen, or hydrogen between two groups. So far as is known, the iron requirements of procaryotes, protists, plants and animals are much the same; 0.4 - 4.0 μM in the environment (Weinberg, 1974).

The major functional iron-containing compounds are the heme proteins such as hemoglobin, catalase, and the cytochromes, the non-heme flavoproteins including many of the major dehydrogenases, oxidases, and reductases, and the iron-sulfur proteins of the anaerobes. Not surprisingly, the unique ability of iron to facilitate this array of important biochemical reactions appears to derive from the element's fundamental physical and chemical properties. Iron has two valences readily attained in biological systems, and each valence has two arrangements of

d-shell electrons, so that a weak and a strong ligand field state exist for both the ferrous (+2) and ferric (+3) oxidation states. Iron will therefore form a great variety of complexes with organic molecules and inorganic ions and, perhaps most importantly, will have a redox potential largely dependent on iron's oxidation state and molecular environment (Neilands, 1972,1974b). The practical result of this is iron's ability to form a wide variety of complexes with redox potentials from the -500 mV found in the iron-sulfur proteins of some clostridia to the +350 mV of cytochrome oxidase (a, a_3). Therefore, while some enzymic functions may be fulfilled by proteins co-factored by several different metals, e.g. superoxide dismutases co-factored by Fe, Mn, or Cu and Zn are known, it is highly unlikely that any other metal could replace iron in its role in the classical multi-step respiratory chain.

B. Microbial Acquisition of Iron

1. The Problem

Iron is probably the earth's most abundant metal as the core is believed to consist largely of nickel-iron (Dott and Batten, 1971). On the earth's surface it ranks as the second most abundant metal behind aluminum,with a median soil iron content of 4% (Dott and Batten, 1971). However, one of the

properties making iron irreplaceable in many biochemical reactions, namely a strong tendency to form a wide variety of stable complexes also ensures that under aerobic conditions at non-acid pH, virtually all environmental iron will be bound in largely insoluble forms. In oxidizing (aerobic) environments free iron is usually in the ferric (+3) state and Fe^{+3} tends to form insoluble polynuclear complexes, such as $[Fe(OH_3)]_n$ with a β or stability product of 10^{38}; i.e. $\frac{[Fe(OH)_3]}{[Fe^{+3}]\ [(OH)^-]} \simeq 10^{38}$ (Schubert, 1963). Overall, total free iron in soil has been estimated to be 10^{-15} M (Sillen and Martell, 1964). In contrast, in environments of low redox potential, iron is usually found in the ferrous $^{(+2)}$ state which has much less tendency to form such insoluble complexes, and in oxidizing conditions at a pH of <3 ferric iron is also rather soluble. These properties are exploited by those organisms able to internalize the environment from which they take their nutrients, especially the higher animals, who usually have an acid stomach and anaerobic intestine. Since acid and anaerobic environments are not compatible with most microorganisms, the common aerobes are left with the problem of obtaining iron from highly insoluble complexes physically distant from the cell. The same problem arises for most microorganisms commensal or parasitic on animals, as animals normally have all their iron sequestered in

specific transport, storage, or functional proteins.

2. The Solution

The question of how microorganisms obtain adequate iron went unanswered and largely unasked until the discovery in 1952 of two iron-containing substances, ferrichrome and coprogen in the fungi Ustilago sphaerogena, and Pilobolus spp., respectively (Neilands, 1952; Hesseltine et al., 1953). Both substances were cyclic trimers of the hydroxylamine derivative hydroxamic acid and could specifically and tightly bind ferric iron. In the same year (1952) Lochhead et al. reported a microbial product named the "terregens factor", necessary for the growth of Arthrobacter terregens, but which could be replaced by ferrichrome and coprogen (Burton et al., 1954). At the same time it was becoming clear that the growth factor mycobactin, isolated from Mycobacterium paratuberculosis in 1949 by Francis et al. was, like the soil growth factor above, a complex iron-chelating hydroxamic acid derivative. Similar findings were obtained with other fungi and several bacilli (Garibaldi and Neilands, 1956), and by 1957 it had been proposed that microorganisms produced and released into the environment specific ferric iron chelating substances to facilitate their acquisition of adequate iron from insoluble complexes (Neilands, 1957). The large volume of work since that time has supported this hypothesis.

Up until this time all the known microbial iron chelating compounds were hydroxamic acid derivatives, but in 1958 Ito and Neilands reported the discovery in Bacillus subtilis of a a second class of ferric iron chelating compounds derived from catechol or phenolic acids with 2,3-dihydroxybenzoylglycine as the primary unit. The review by Lankford (1973) covers more recent work identifying 2,3-dihydroxybenzoate (DHB) derivatives produced by various species of Bacillus, Enterobacter, Escherichia, Salmonella, Azotobacter, Alkaligenes, Klebsiella, Micrococcus, Corynebacterium, Nocardia, Streptosporangium, and Thermoactinomyces. Since that review, Paracoccus denitrificans (Tait, 1975), Vibrio cholerae (Payne and Finkelstein, 1978b), Pseudomonas aeruginosa (Cox and Graham, 1979) and Agrobacterium tumefaciens (J.B. Neilands, pers. comm.) have been reported to produce phenolic siderophores. Recent work has also expanded the list of organisms possessing hydroxamate-type siderophores to include other strains of Enterobacter, Arthrobacter, Nocardia, Bacillus, and Streptomyces (Lankford, 1973), with the same species sometimes producing compounds of both the hydroxamate and catechol type. For example Enterobacter aerogenes can produce the catechol-type siderophore enterochelin and its DHB-serine precursors as well as aerobactin, a citrate derivative with hydroxamate functional groups (Gibson and Magrath, 1969).

Most of these iron-transporting substances are colorless

when de-ferrated but deeply colored when complexed with the ferric ion, with a λ_{max} of 400-500 nm and an ε mM > 2.0 (Lankford, 1973). Therefore, Bickel et al. (1960) first suggested the term "siderochrome" to cover the hydroxamate-type biological iron-binding compounds, a term which subsequently was expanded to cover those based on catechol. This term found general acceptance in the literature, but Lankford (1973) later suggested supplanting "siderochrome" with "siderophore" a more accurate description as most colored iron compounds are not iron transporters and all iron transporters may not be colored when ferrated. At the moment, both terms appear frequently in the literature and are identical in definition. This thesis will use the term siderophore throughout to mean those specialized secreted microbial products which are moderately specific chelators of ferric iron and presumably part of a high-affinity iron-acquisition system.

Despite their apparent differences, known siderophores are very similar in many of their physical and chemical properties. While data on all the following properties of all known siderophores are not available, much of it can be obtained from the excellent reviews by Lankford (1973) and Neilands (1973, 1974a) and references quoted therein. Molecular weights range from about 240 (rhodotorulic acid) to 1800 d. (the mycobactins), with most between 500 and 1,000 d. Many siderophores are poorly

soluble in aqueous solution, but sufficient to yield the small *in vivo* levels required. This allows most siderophores to be extracted into ethyl acetate, chloroform, or other organic solvents and concentrated. None of the known siderophores absorb visible light when deferrated, but nearly all do when ferrated, usually in the 400-450 nm region with an ε mM$\geq$2.0. In most cases maximal production of siderophores is achieved by moderate, not severe, iron privation and allowing the cells to remain in stationary phase as long as practical without cell degeneration. In addition, nearly all siderophores are based on hydroxamic acid or catechol functional groups for which there are straightforward colorimetric tests (Arnow, 1937; Csaky, 1948), and the interchangeability of siderophores allows the use of bioassay on culture extracts suspected of containing a siderophore (Burnham and Neilands, 1961; Peters and Warren, 1970; Luckey *et al.*, 1972). As all siderophores have the same function, this convenient similarity in properties is presumably the result of convergent evolution, and greatly facilitates determining the presence or absence of a conventional siderophore in a given culture.

Most siderophores, with the exception of rhodotorulic acid (Atkin and Neilands, 1968), are believed to form true chelates with ferric iron, the resulting heterocyclic rings containing 5-7 atoms of Fe, O, C, and often N (Schubert, 1963;

Lankford, 1973; Neilands, 1974a). The chelating group's dimensions are usually such that they show a far higher β (solubility or stability product) when complexed with the ferric ion than with the ferrous or any other metal cation, although in a few cases Mn^{+2}, Al^{+3} or Cr^{+3} may be competitive (Nakayama et al., 1964; Wang and Newton, 1969a,b; Byers, 1974). While various organic iron compounds bind iron through nitrogen, sulfur, and oxygen atoms, and mixtures thereof, all known siderophores bind iron through all-oxygen bonds (Neilands, 1974b). An exception is mycobactin in which both oxygen and nitrogen are coordinated to the iron, but mycobactin may not fulfill the definition of a siderophore (see Macham et al., 1975). To date, nearly all siderophores examined are synthesized by specialized branches of the amino acid biosynthetic pathways. The best documented example of this is the biosynthesis of enterochelin (enterobactin) by Escherichia coli and related enteric bacteria.

C. Some Microbial Iron-Acquisition Systems

As in many other areas of its biology, Escherichia coli is the microorganism whose iron-acquisition systems are best known at present, although the systems of several mycobacteria and bacilli have been described in some detail (Byers, 1974). E. coli and related enterics show multiple iron-acquisition systems employing most of the common features seen in those

other systems that have been examined. Also, the enteric bacteria are the only Gram-negative organisms examined in any detail and hence, theirs are the most reasonable systems to compare with the Neisseria, although recently the siderophores of Pseudomonas fluorescens (Meyer and Abdallah, 1978) and P. aeruginosa (Cox and Graham, 1979) have been isolated.

All microorganisms examined have a low-affinity iron uptake system permitting acquisition of adequate iron when it is present as hydrated ions or weakly complexed or chelated forms which are in high concentration (> 0.2 to 1.0 μg iron/ml). This system appears to employ no specific or inducible transporters, has very low substrate specificity, and does not obey saturation kinetics (Lankford, 1973; Neilands, 1974b). To date there has been no success in obtaining auxotrophs defective in this system, although auxotrophs of E. coli with defects in the enterochelin, ferrichrome, or citrate-mediated iron uptake systems are readily obtained (Rosenberg and Young, 1974; Braun et al., 1976). The low-affinity iron uptake system of E. coli has been defined in terms of its inability to remove iron from the chelate Fe^{+3} nitrilotriacetate (NTA) and the low-affinity system of the enterochelin auxotroph Salmonella typhimurium LT-2 enb-7 in terms of its inability to obtain iron bound to citrate (Luckey et al., 1972; Frost and Rosenberg, 1973). Because of the variety of phosphate, carboxylic acid, hydroxyl, and other polar or

ionized groups exposed on the bacterial cell surface, it is likely that the low-affinity uptake of iron is non-specific binding to the cell surface followed by migration of the iron into the cell, possibly with the help of a trans-membrane electrical gradient, a non-specific cation transporter, or a porin (McIntosh *et al.*, 1978).

In evaluating the importance of the low affinity iron-uptake system to microorganisms it should be remembered that even under iron-poor growth conditions when a high-affinity siderophore system is active, the organism will not benefit until the environmental siderophore concentration has reached a level at which significant numbers of (ferrated) siderophore molecules re-encounter the cell. Therefore, in relatively large bodies of circulating liquid, low concentrations of cells will initially derive little benefit from a functioning siderophore system. However, as such cell or cell metabolite dilution will presumably not affect the low-affinity iron-uptake system, and iron is only required in catalytic amounts, this system may contribute significant amounts of iron to the cell in circumstances other than high available iron or siderophore auxotrophy.

The citrate-mediated iron-acquisition system of *E. coli* uses the ton B gene product located in the outer membrane as a receptor for Fe^{+3} citrate. This system is only functional in media containing citrate, but can operate simultaneously with

the enterochelin system in wild-type cells, the two iron-uptake rates being additive (Frost and Rosenberg, 1973).

The chelate Fe^{+3}-NTA could not be taken up by this citrate system although NTA is structurally very similar to citrate (Frost and Rosenberg, 1973).

When Fe^{+3}-citrate and NTA were both present, NTA did not interfere with Fe^{+3}-citrate uptake. However, the structural similarity of the two organic acids may not be as important as the fact that NTA forms a 1:1 chelate with ferric iron while citrate, if in 20-fold excess or more over ferric iron forms a 2:1 complex (Bates et al., 1967). Interestingly, E. coli normally cannot use citrate alone as a carbon or energy source, but has a very high affinity for Fe^{+3}-(di)-citrate (Km = 0.2 μM) (Frost and Rosenberg, 1973), while the related enteric organism Salmonella typhimurium deprived of its enterochelin system will starve for iron in the presence of Fe^{+3}-citrate but can use citrate alone for a carbon and energy source (Luckey et al., 1972).

In the work with Neisseria meningitidis presented in Sections II and III of this thesis citrate was shown to act much as it does with E. coli in the absence of enterochelin or other useable conventional siderophores. The term "functional siderophore" was, therefore, adopted to include those substances like citrate which can effectively solubilize iron and transport

it to the cell, but which are not specific iron transporters and are not necessarily produced by the microorganism itself.

Another system employing citrate that has been examined in some detail is the interesting one found in Neurospora crassa in the absence of its siderophore coprogen (Winkelmann and Zahner, 1973; Winkelmann, 1979). The fungus precipitates large amounts of ferric iron as the hydroxide polynuclear complex onto its outer surface, then secretes citrate and malate which solubilize the complex and permit the iron to enter the cells. Uptake is rather slow and does not obey saturation kinetics but does appear to supply the mycelium with adequate iron. This may be a model for iron uptake in the various groups of aerobic bacteria known to accumulate insoluble iron salts, e.g. the genera Sphaerotilus, Galionella, Siderocapsa, and Magnetococcus (Bergey, 1974; Moench and Konetzka, 1978).

Citrate has also been reported to facilitate the acquisition of iron in Aerobacter aerogenes (Frost and Rosenberg, 1973), Corynebacterium diptheriae (Lankford, 1973), Microbacterium lacticum (Alexanian et al., 1972), and Micrococcus lysodeikticus (Neilands, 1957) but little data on the kinetics or characteristics of this uptake were given. In any system for the acquisition of iron there are two potentially rate-limiting steps: (a) the solubilization of insoluble iron sources distant from the cell; and (b) the removal by the cell of the solubilized iron from

the resultant soluble iron complex or chelate. Because hydroxy acids and particularly poly-carboxylic acids such as citrate and NTA are good ferric iron chelating or complexing agents and will readily solubilize many insoluble forms of iron (Schubert, 1963; Bates et al., 1967) they may allow iron-limited cells direct contact with iron. Thus, even if the transfer of iron from citrate to the cell is slow, nonspecific and obeys first-order kinetics, it may supply the cell with considerable additional iron in the absence of any E. coli-type specific iron-citrate transport system. Similar effects have been noted with other iron-solubilizing compounds, notably phosphate and simple sugars (Lankford et al., 1957; Neilands, 1957; Charnley et al., 1963; Lankford, 1973).

The acquisition of iron by the enterochelin system common to the enteric bacteria is representative of most known active transport systems in that the uptake rate is saturable (a Vmax can be determined), dependent on metabolic energy, and the concentration of substrate (ferri-enterochelin) required for a half-maximal uptake rate (Km) is quite low, about 0.1 μM (Frost and Rosenberg, 1973). Deferri-enterochelin can readily obtain ferric iron from insoluble polymers such as $[Fe(OH)_3]_n$ and most other inorganic complexes, as well as many stable organic complexes and chelates (Lankford, 1973; Rogers, 1974). The result is that, in low-iron environments with a sufficiently

restricted volume or diffusion rate to allow the accumulation of enterochelin, E. coli can obtain iron from highly insoluble forms.

The biosynthesis of enterochelin has been worked out using cell-free extracts and mutants blocked at different steps in the pathway (see Rosenberg and Young, 1974). Chorismic acid, a precursor in the biosynthesis of phenylalanine, tyrosine, and tryptophan is converted successively to iso-chorismic acid, 2,3 dihydro-2,3-dihydroxybenzoic acid, and 2,3-dihydroxybenzoate (DHB). DHB is then converted to DHB-serine (DHBS), and in an ATP-requiring process polymerized to its cyclic trimir enterochelin (Young et al., 1969a,b; Luke and Gibson, 1971; Bryce and Brot, 1972). When this siderophore binds ferric iron, the resultant chelate structure becomes very stable, and in fact the removal of ferric iron from the chelate by E. coli after uptake apparently requires hydrolysis of the enterochelin to DHB-serine by ferric enterochelin esterase (O'Brien et al., 1971; Langman et al., 1972; Cooper et al., 1978). The resulting DHB-serine is released into the medium and the iron reduced and used within the cell. All the specialized enzymes involved in the synthesis and hydrolysis of enterochelin have been shown to be induced by low iron growth conditions (see Rosenberg and Young, 1974).

In contrast to ferrienterochelin, the ferrichrome molecule is deferrated and then re-released unaltered into the

medium by E. coli, thus allowing many iron atoms to be carried by a single ferrichrome molecule (Braun et al., 1976), although the findings of Leong and Neilands (1976) suggest that deferri-ferrichrome may be retained by the cell to some extent. The ability of E. coli to effectively use ferrichrome-bound iron is an example of the ability of many microorganisms to use siderophores produced by unrelated species. This interchangeability of a range of hydroxamate and catechol based siderophores despite very different structures has been noted in the fungi, streptomycetes, bacilli, enteric bacteria and Arthrobacter species (Neilands, 1957; Burnham and Neilands, 1961; Peters and Warren, 1970; Luckey et al., 1972; Lankford, 1973). For example, Salmonella typhimurium defective in DHB synthesis was able to effectively take up iron from citrated medium when supplemented with virtually any of the known siderophores (Luckey et al., 1972). This same behavior is seen in Arthrobacter species requiring the "terregens factor" (Burnham and Neilands, 1961; Reich and Hanks, 1964), and has been used in this thesis and elsewhere to devise "bio-assays" for the presence of compatible siderophores, both in pure culture extracts (Cox and Graham, 1979; Norrod and Williams, 1978b)and in natural samples (Estep et al., 1975).

In addition to the enteric bacteria, the bacilli and the mycobacteria have had their iron uptake systems examined

in some detail. Bacillus species acquire iron much as the enterics do, producing soluble siderophores under low-iron growth conditions. Strains of B. megaterium may produce the di-hydroxamate citrate derivative schizokinen (SK), or 2,3-dihydroxybenzoyl glycine (DHBG), but so far there has been no report of a DHBG polymer analogous to enterochelin (Byers, 1974). Addition of 2,3-dihydroxybenzoate will circumvent the SK requirement of certain B. megaterium strains and suggests some versatility in accepting other siderophores (Arceneaux and Lankford, 1966). Competition by Al^{+3} or Cr^{+3} ions with Fe^{+3} for uptake on low iron medium markedly decreased the ability of B. megaterium to grow, i.e. these cations were considerably more inhibitory under iron-starved growth conditions (Arceneaux and Lankford, 1966; Davis et al., 1971). Like ferrichrome, SK appears to be re-released after uptake and deferration, although the cells appear to retain an internal pool of SK, possibly as a method of iron storage (Arceneaux et al., 1973). Bacillus subtilis also produces the phenolic DHBG (Peters and Warren, 1968, 1970) and apparently can utilize a variety of other siderophores, such as deferrioxamine, schizokinen (SK) (Downer et al., 1970), ferrichrome, and mycobactin (Peters and Warren, 1970).

Some of the mycobacteria have been shown to produce two classes of iron chelators, the exochelins and mycobactins. It has been proposed that they operate sequentially,

the exochelins ferrying medium iron to the cell surface where it is transferred to mycobactin, which carries it into the cell where it is reduced to the ferrous state and utilized (Macham et al., 1975,1977). In any case the presence of these compounds greatly increases the ability of various pathogenic and non-pathogenic mycobacteria to obtain adequate iron, especially from such important animal sources as transferrin and ferritin (Kochan, 1974; Macham et al., 1977). Interestingly, one of the major anti-tubercular drugs p-amino salicylic acid (PAS) strongly inhibits the production of mycobactin by M. smegmatis in vitro (Brown and Ratledge, 1975).

Recently, considerable data have been presented on specific transport and receptor components of the ferrichrome, enterochelin, and citrate-mediated uptake systems in the enteric bacteria. The tractability of these systems has led workers, especially in the laboratories of Braun, Rosenberg and Neilands to employ them as an aid to a general understanding of nutrient transport and cell wall structure (Braun et al., 1976; Hancock et al., 1977; Wookey and Rosenberg, 1978; Negrin and Neilands, 1978). The ton B and feu gene products, located in the outer membrane of E. coli are both required for energy-dependent ferrienterochelin uptake, the ton A and ton B gene products for the uptake of ferrichrome, and the ton b gene product alone for the uptake of iron-citrate

complexes (see Braun et al., 1976). In addition to these outer membrane components, the fep gene product, believed to be on the cell (inner) membrane is necessary for ferrienterochelin transport (Hancock et al., 1977), and inner membrane vesicles of E. coli were reported to be capable of energy-dependent transport of ferrichrome with a K_m of 0.15 - 0.25 μM (Negrin and Neilands, 1978). In addition to its iron-uptake function, the ent outer membrane site serves as a receptor for colicins B, I, and V, and the ton a site serves as a receptor for phages Ø80, T_1, and T_5, the albomycins (antibiotic analogues of ferrichrome), and colicin M (Braun et al., 1976; Wayne et al., 1976).

D. Iron and Microbial Pathogenesis

"In the contest between the establishment of a bacterial or mycotic disease and the successful suppression of the disease by animal hosts, iron is the cation whose concentration ..... at present appears to be the most important" (Weinberg, 1966). It has been recognized since the late 19th century that many animal diseases caused by microorganisms resulted from, or were only established after, a physical-chemical struggle between the host and the potential parasite. While the cellular and humoral host defenses and such parasite attributes as antiphagocytic capsules and endo- and exotoxins have long been recognized as having importance in this struggle, there was

little mention of nutritional control of the parasite by the host until recently. This is understandable both because, once dead, an animal clearly offers no nutritional limitations to the decomposers and because most organics and salts necessary for good microbial growth are readily available in body tissues and fluids, as attested to by the use of blood and tissue digests for microbiological culture media. Nevertheless, there is now considerable evidence that in certain diseases and under certain conditions, the struggle between the host iron-sequestering systems and the parasite's iron-acquisition systems is critical to the outcome of the infection.

1. Host Iron Uptake, Transport, and Scavenging

Essentially all the iron in an animal is bound in storage, sequestering, transport, or functional protein complexes. A normal adult human (70 Kg male) contains approximately 4 gm of iron, distributed as follows: hemoglobin, 2.7 gm; myoglobin, 0.14 gm; all other heme proteins, .01 gm; transferrin, .003 gm; and ferritin, 0.7-1.5 gm. Other non-heme iron proteins are negligible in iron content (Wintrobe, 1974). Of all these forms of iron, only transferrin-bound iron is normally found extracellularly in appreciable amounts. However, even this iron, present at approximately 1 μg/ml serum, if available would supply several times the total iron requirements of most of the

bacteria and fungi whose iron requirements have been quantified (see Lankford, 1973). A brief description of human iron uptake and metabolism will be given, as this represents the system that a successful pathogen must exploit.

The uptake of food iron from the jejunum and duodenum is essentially irreversible (Wheby, 1970), as animals have no known mechanism of iron excretion, the only normal loss being bleeding and epithelial and epidermal cell sloughing (Green et al., 1968). Iron is transported, both in the ferrous form and as a heme derivative, probably hemin, by the brush borders of the mucosal cells (Weintraub, 1968; Wintrobe, 1974). This iron uptake appears to be an energy-dependent, active transport process, the rate of which varies widely between individuals (Greenberger et al., 1969). The rate of iron uptake is also controlled by the rate of erythropoiesis and the amount of tissue storage iron, individuals with low levels of storage iron taking up a greater proportion of food iron (Wheby and Crosby, 1963; Wheby, 1970). Within a few hours of uptake by the gut mucosal cells, most food iron is either in the ferric iron intracellular storage (and possibly transport) protein ferritin or delivered to the serum of the adjacent capillaries where it is immediately bound by transferrin (Pinkerton, 1969; Harris, 1978; Richter, 1978).

Transferrin is the primary and nearly exclusive

transporter of iron between host cells, and between the iron storage, absorption, and functional (biosynthetic) "compartments" in different host tissues (Wintrobe, 1974). Transferrin is a serum β-globulin (M.W. = 80,000) found in nearly the same form in all vertebrates, suggesting a high evolutionary conservation (Bezkorovainy and Zschocke, 1974). It binds 2 moles of Fe^{+3} and 2 moles of HCO_3^- per mole glycoprotein, the resultant transferrin-iron complex having a stability constant of about 10^{29} (Bezkorovainy and Zschocke, 1974; Bates and Schlabach, 1975). Transferrin is synthesized by the liver parenchyma and is normally present at 2.5 mg/ml in normal sera with an iron-binding capacity of 3 μg Fe^{+3}/ml, of which only approximately 35% is saturated (Wintrobe, 1974). This large unsaturated iron-binding capacity allows transferrin to scavenge the serum for the extracellular iron generated by normal cell degradation, keeping serum free iron to $< 10^{-15}$M, a level far too low to be useful to a microorganism (Bullen et al., 1978; Weinberg, 1978). Furthermore, appreciable sudden influxes of free iron can be immediately chelated by transferrin's free iron-binding capacity. Ferrated transferrin can donate iron to growing hemopoeitic, or iron storage cells, apparently by highly specific cell-surface transfer mechanisms (Fielding and Speyer, 1974; Princiotto and Zapolski, 1978). Therefore, de-ferration of transferrin, like ferration at the intestinal mucosal cell membrane appears to

involve specific cell-surface receptors for ferrated transferrin, i.e. at no point in its travels is iron allowed to be free and extracellular (Fletcher and Huehns, 1968; Van Der Heul et al., 1978).

In addition to transferrin mammals also have a closely related glycoprotein, lactoferrin. The lactoferrin molecule has the same M.W. as transferrin, similar amino acid composition, and will reversibly bind two ferric ions and two bicarbonate ions (Bezkorovainy, 1977). Lactoferrin differs in being considerably more stable than transferrin at acid pH, binding iron down to pH 4, whereas transferrin is ineffective below pH 6.5. Lactoferrin is almost completely absent from serum, but present at moderate or high levels in external secretions and in neutrophil leukocytes, from which it is released upon leukocyte activation (Masson et al., 1966). Another of the transferrins, conalbumin (ovotransferrin), comprising 13% of chicken egg white protein, is normally entirely de-ferrated, and like lactoferrin does not seem to have an iron transport function (Williams, 1962). Both have clearly demonstrated iron-reversible bacteriostasis against a variety of bacteria (Weinberg, 1978).

Mammalian storage iron is almost exclusively intracellular (ferritin = 17 $\pm$ 7 ng/ml serum) (Koller et al., 1978), chiefly in the form of ferritin, and when excessive iron is stored, hemosiderin (Richter, 1978). Ferritin is a hollow

sphere (450,000 d.) formed of 24 protein subunits with iron stored as ferric oxy-hydroxides in the core. Up to 25% of the total weight of the molecules may be iron, which enters through 6 pores in the sphere (Richter, 1978). The major tissues of iron deposition are liver, bone marrow, spleen and reticulo-endothelial cells, and the protein has been detected in animals, plants, and fungi down to the phycomycetes. Iron in ferritin is made highly unavailable by being (a) in a highly insoluble form, and (b) tightly enclosed in the ferritin protein. In turn the protein is usually segregated from the cytoplasm in a membrane vesicle or siderosome, and the cytoplasm from the extracellular environment by the cell membrane (Richter, 1978).

Hemosiderin is a less well-described iron storage product consisting of an amorphous mass of intracellular ferritin subunits and iron oxyhydroxides. Hemosiderin becomes prominent in iron-overloaded cells and in iron storage and reticuloendothelial cells required to take up large amounts of iron in a short period (Richter, 1978). Both iron storage substances normally accomplish the objectives of keeping the inherently cytotoxic ferrous and ferric ions out of intracellular, as well as extracellular solution (Richter, 1978). However, severe iron overload results in sufficient free iron to poison the cell. If the excess absorption of iron continues, cytosiderosis

follows and the subsequent tissue death is fatal for the patient in many cases (Richter, 1978). An important application of one of the siderophores mentioned previously (deferrioxamine) is in the treatment of such hypersideremia. The synthetic sulfonate of deferrioxamine (Desferal) is injected intravenously (I.V.) daily, binds serum and tissue iron through poorly understood processes, and is excreted with bound iron in the urine (Moeschlin and Schnider, 1963; Richter, 1978). Recently, the synthetic iron chelator ethylenediamine di-o-hydroxyphenyl acetate (EDDA or EDHPA) has proven superior to Desferal in relieving iron overload in mice (Pitt et al., 1979).

2. Responses of Animal Iron Metabolism to Invading Microorganisms

If host iron sequestration limits microbial pathogens, then it would be useful to determine if the effect is passive, or if the animal host can actively respond to the pathogen, as do the classical cellular and humoral systems. Weinberg (1978), in his review of this area, has listed six general ways in which an animal's iron metabolism could respond to an infection to make iron less available to the parasite. They are as follows: (1) increased iron excretion; (2) decreased food iron absorption; (3) reduced extracellular (serum) iron; (4) prior stationing of iron sequestering molecules at potential sites of infection; (5) increased synthesis of host iron-binding proteins;

and (6) suppression of synthesis of microbial iron-binding proteins. Except for increased iron excretion, there is some evidence for all of these possible host responses in various host-parasite systems.

The most dramatic response of the host's iron acquiring system to infection is inflammatory hyposideremia in which the animal host responds to the presence of a variety of antigenic substances by a sharp decrease in the amount of serum iron (Cartwright, 1966). Deferri-lactoferrin is released by neutrophil leukocytes activated by leukocytic endogenous mediator (LEM) or any of the microbial antigens or other substances normally activating leukocytes (Van Snick et al., 1974). The released lactoferrin effectively removes iron from ferrated transferrin and binds any free iron, and then the ferrated lactoferrin is specifically taken up by monocytes and fixed cells of the reticuloendothelial system (Van Snick et al., 1974). The result is an increase in the unsaturated transferrin iron-binding capacity and a decrease in total serum iron of 50-100% within a matter of hours. Furthermore, the acidity of local infections and the stability of the Fe^{+3}-lactoferrin chelate mean that the lactoferrin released in those areas would be much more effective than the serum transferrin in binding iron. In a number of instances low serum iron levels have been related to the bacteriostatic effects of serum and to the

resistance of animals to disease (see Bullen et al., 1978; Weinberg, 1978). Such increased resistance of an animal host to a microbial pathogen due to lowered serum iron or increased iron-free binding capacity has been observed with Mycobacterium tuberculosis (Kochan, 1974), Candida albicans (Elin and Wolfe, 1974), Salmonella typhimurium (Puschmann and Ganzoni, 1977; Chandlee and Fukui, 1965), and Neisseria gonorrhoeae (Payne and Finkelstein, 1975). If a lowered serum iron level reduces the likelihood of an infection or increases the LD_{50} of a given pathogen in a particular host, then one would expect the reverse to be true also, i.e. increasing internal iron in an animal should increase its susceptibility to infection. This also appears to be so in a variety of host-parasite relationships. Some diseases shown to be markedly enhanced by iron-stressing the host are as follows: systemic candidaisis in mice (Elin and Wolfe, 1974); coliform pyelonephritis in rats (Fletcher and Goldstein, 1970); corynebacterial pyelonephritis in rats (Colenda et al., 1977); meningococcal infections in mice (Holbein et al., 1979a); gonococcal infections in chick embryos (Payne and Finkelstein, 1975); gangrene in guinea pigs (Bullen et al., 1967); malaria in rats (Murray et al., 1975); listeriosis in mice (Sword, 1966); tuberculosis in mice and guinea pigs (Kochan, 1974); staphylococcal and coliform infections in mice (Gladstone and Walton, 1970); coliform meningitis in infants (Farmer and Becroft, 1976); and

lactoferrin (Griffiths and Humphreys, 1977; Bezkorovainy, 1977; Bullen et al., 1978).

Two other possibilities for host response, an increase in host iron-binding proteins and a contraction of the extracellular iron compartment, i.e. serum iron, are both seen in inflammatory hyposideremia. Finally, there is some evidence that at least in a few cases, the host is able to reduce the production of siderophores by the production of a fever. This effect has been reported in Aeromonas hydrophila (Kluger, 1978), Salmonella typhimurium, and a fluorescent pseudomonad (Garibaldi, 1971,1972). Rogers (1974) has reported the production of horse antibodies apparently directed against iron receptor sites or siderophore release by E. coli.

Another possible effect of elevated iron levels may be on the effectiveness of phagocytosis. There is one report that iron interferes with leukocyte chemotaxis (Becroft et al., 1977) and several suggesting that additional iron reduces the bactericidal effectiveness of phagocytosis by several mechanisms, including (a) neutralization of basic lysosomal proteins (Gladstone, 1976); (b) inactivation of H_2O_2 (Kaplan et al., 1975); and (c) saturation of endogenous leukocyte lactoferrin (Bullen and Wallis, 1977; Kaplan and Basford, 1979).

Except for these effects of iron on phagocytosis, the

preceding examples of the effects of added iron on host-parasite relationships have been interpreted as nutritional effects on the parasite, but when relatively large amounts of iron (several mg/kg body weight) are given, iron may have a variety of other effects. In the case of simple salts injected or ingested: 2 n $FeCl_3$ + 6 n $H_2O \longrightarrow$ 6 n HCl + $\left[Fe(OH)_3\right]_{2n}$ which may result in some degree of shock or metabolic acidosis (Jacobs et al., 1965). The acid, particularly when in the blood or peritoneum may cause a variety of metabolic disturbances and the precipitated iron may engender reticuloendothelial blockade and cytotoxic effects, first in the reticuloendothelial cells attempting to process the iron and secondarily in the iron storage tissues (see Jacobs et al., 1965; Wintrobe, 1974; Richter, 1978; Holbein et al., 1979a,b). Holbein et al. (1979b) found that iron sorbitol citrate (Jectofer) and ferric sulfate were toxic to mice at a level only slightly greater than the level required to enhance the virulence of meningococci, but that iron dextran was efficaceous in enhancing virulence at far below toxic levels. Therefore, caution must be exerted when interpreting iron-stressed host-parasite systems if iron salts are used or if the level of iron used is not far below the level causing visible toxic effects.

The cellular and humoral immune defense systems were discovered in the late 19th century, and the complement system

shortly thereafter, but despite the pioneering observations of Schade and Caroline (1944,1946), the iron withholding and sequestering systems were not recognized as an important general host defense mechanism until very recently. As with the immune system, further research combined with an adequate understanding of this important system by the general practitioner should lead to significant improvements in medical care of infants, starvees, recipients of repeated blood transfusions, and people with defects in their iron metabolism. In particular, the recommendations for iron supplementation, especially for underweight children and the old should be reviewed (see Farmer and Becroft, 1976; Barry and Reeve, 1977).

E. Neisserial Disease

The genus Neisseria (family Neisseriaceae) is a rather closely related group of Gram-negative diplococci, including at present six species (Bergey, 1974). By DNA homology, the genus can be divided into Group I, containing N. gonorrhoeae and N. meningitidis, and Group II, containing N. flavescens, N. sicca, N. mucosa, and N. subflava (Kingsbury, 1967). All are aerobic, with occasional facultative anaerobiosis observed, non-motile, and catalase and cytochrome oxidase positive (Bergey, 1974). All members of the genus are commensals or occasionally parasites on the mucous membranes of warm-blooded animals, in

particular the membranes of the nasopharynx. With rare exceptions, the Group II organisms are nonpathogenic and inhabit the mammalian nasopharynx (Bergey, 1974). The two Group I organisms, Neisseria gonorrhoeae and N. meningitidis are the causes of gonhorrhoea and meningococcal meningitis, respectively, in man. So far as is known, man is the only natural host for these organisms, a fact which has greatly hampered the understanding of the diseases they engender (Griffiss and Artenstein, 1976). Because of their marked similarities, both the meningococcus and the gonococcus will be briefly described.

1. Gonococcal Disease

Neisseria gonorrhoeae, the only species of the genus commonly found far from the nasopharynx, normally inhabits the human genitourinary tract and is transmitted almost exclusively by sexual contact. Approximately 3% of all women carry gonococci on their genital mucosa, often chronically, but only 1% show clinical symptoms of gonorrhoea (Holmes et al., 1970; Griffiss and Artenstein, 1976). In men, a much larger proportion of those infected show clinical symptoms and it has been proposed that women be considered the carrier or natural host of the gonococcus and men the vector (Griffiss and Artenstein, 1976). Gonococci typically penetrate columnar epithelium in preference to stratified squamous epithelium of the mucous membranes of the genitourinary tract and cause an acute inflammatory response in the subepithelial tissue producing the

purulent discharge characteristic of the early stages of gonorrhoea. In the male,urethritis is the most common symptom, but if left untreated the infection may include the prostate and epididymis, followed by fibrosis and urethral stricture. In the female infection may result in inflammation of the urethra, cervix, vagina, uterus, fallopian tubes, and eventually pelvic inflammatory disease, with increasingly serious consequences (Davis et al., 1973; Griffiss and Artenstein, 1976). Occasionally, the gonococcus produces a fulminating (disseminated) bacteremia with purulent arthritis, endocarditis, and other serious conditions following the dissemination of the infection (Davis et al., 1973).

An interesting characteristic of gonococcal infections is the fact that the host develops bactericidal serum antibodies directed against antigens of the outer wall "native complex" of the gonococcus, but this does not prevent maintenance of a gonococcal infection or re-infection of individuals previously cured of the disease (Tramont et al., 1974). Since most virulent gonococci (types 1 and 2) have pili, and most avirulent strains (types 3 and 4) do not, the presence of pili has been considered a virulence factor, the pili presumably facilitating the attachment of the parasite to susceptible host mucosal cells (Punsalang and Sawyer, 1973). Other work attempting to determine how and why these fastidious and rather fragile organisms

do so well in man has reported an anti-antibody and anti-compliment gonococcal virulence factor lost upon *in vitro* culture (Ward *et al.*, 1970) and the ability of the gonococcus to resist phagocytosis and opsonizing antibodies (Bisno *et al.*, 1975; Penn *et al.*, 1977). Strains causing disseminated gonococcal infection (DGI) have been reported to differ from other gonococcal strains in several respects: (a) they are more resistant to the complement-dependent bactericidal action of normal human sera (Brooks *et al.*, 1976; Schoolnik *et al.*, 1976); (b) they are usually more sensitive to penicillin and require arginine (Schoolnik *et al.*, 1976); and (c) strains causing DGI are much more effective in obtaining nutritional iron (Payne and Finkelstein, 1978a; Payne *et al.*, 1978).

2. Meningococcal Disease

The meningococcus appears to be a normal inhabitant of the nasopharynx of 3-30% of any given human population (15% average) (Davis *et al.*, 1973; Littlejohns, 1976). This carrier state may be retained for days, months or years with no clinical manifestations. Transmission has been shown to be via aerosols generated by the nasopharynx, and in certain high-density populations, such as military recruits, the carrier rate may exceed 90%, yet incidence of overt disease is still less than 1% (Goldschneider *et al.*, 1969a; Griffiss and Artenstein,

1976). While a variety of microorganisms can cause meningitis, only the meningococcus has been found to be highly infectious and responsible for epidemics (Davis et al., 1973). Each epidemic is caused largely by a single serogroup, serogroups A, B, and C being those chiefly responsible. When overt meningococcal disease occurs, i.e. when the normally benign host-parasite relationship alters, the usual pattern is a bacteremia followed by invasion of the cerebrospinal fluid and meningitis (Davis et al., 1973). The mortality from meningococcal meningitis is approximately 85% when untreated and 15% with the chemotherapy presently used, but could be $<1\%$ if vigorous therapy followed early diagnosis (Goldschneider et al., 1969a;Davis et al., 1973).

One thousand to several thousand cases of meningococcal disease are reported every year in the U.S., but during the major epidemic of 1943-1945 there were 15-20,000 cases of meningitis per year (Aycock and Mueller, 1950). As in the gonococcus, a less common result of invasion is a systemic infection, with metastatic lesions often developing in the ears, lungs, skin, joints, spleen, liver, and adrenal glands. The lesions usually contain viable meningococii and clots restricting or preventing normal circulation. This disseminated intravascular coagulation leads to irreversible shock and is probably related to meningococcal endotoxin and possibly material egested by polymorphonuclear leukocytes which have previously engulfed

and degraded meningococci (Davis et al., 1973; DeVoe and Gilka, 1976; Griffiss and Artenstein, 1976). DeVoe and Gilchrist (1973,1975) have also shown that the meningococcus has the ability to steadily release outer membrane (endotoxic) material in the form of evaginations or "blebs" while growing. As in the case of the gonococcus, there is an observable change in the characteristics of most meningococcal strains after subculture in vitro. This takes the form of a loss of the piliation usually encountered in fresh clinical isolates (DeVoe and Gilchrist, 1975,1978).

3. Immunology of Meningococcal Infection

As a large number of people may carry the same meningococcal serotype, but only a small proportion of them contract meningococcal disease, some facet of the host's physiology would appear to be a critical determinant. On the other hand, some strains of the meningococcus (especially in serogroups X, Z and 29E) rarely cause disease while others (serogroups A, B, C) are nearly always the causative strains in overt disease. Whether or not a person contracts overt disease after becoming a carrier, he will develop bactericidal antibodies to a variety of meningococcal antigens (Goldschneider et al., 1969a,b; Frasch and Robbins, 1978a). The activity of such antibodies and their activation of the serum complement system produces antimeningococcal

bactericidal activity in human serum which appears to play an important if not decisive role in host defence (Goldschneider et al., 1969a,b). Especially in those two groups with a high incidence of meningococcal disease, military recruits and infants older than six months, there is a good correlation between lack of antimeningococcal antibodies and overt disease (Goldschneider et al., 1969a,b). The major mechanisms for acquiring natural immunity appear to be passive immunity in the case of infants, protected for about six months by maternal IgG antibodies crossing the placenta, and active immunity by the production of IgG, M, and A antibodies as a result of becoming a carrier.

As in most Gram-negative organisms, the outer wall of the meningococcus has been shown to contain a variety of protein, carbohydrate, and lipopolysaccharide (LPS) antigens, some of which have been characterized (Kasper et al., 1973; Griffiss and Artenstein, 1976; Frasch and Robbins, 1978a,b). Many meningococcal strains also appear to have an antigenic polysaccharide capsule, and together with LPS and wall protein components they form a "native complex" of cell wall components in which resides much of the antigenicity of the meningococcus (Zollinger et al., 1974). In addition, a variety of other broadly cross-reacting antigens have been noted in crude meningococcal cell extracts and may be important in inducing bactericidal host

antibodies (Kasper et al., 1973; Frasch and Chapman, 1972).

The meningococcal capsule is considered to have anti-phagocytic properties (Griffiss and Artenstein, 1976) and differences in the chemical structures of their capsular polysaccharides form the basis of the present division of meningococci into the major serogroups A, B, C, D, X, Y(Bo), Z, 29E, and 135 (Bergey, 1974; Griffiss and Artenstein, 1976). The structures of these polysaccharides have been determined, and those of serogroups A and C have been used to produce serogroup-specific anti-meningococcal vaccines of clinical value providing at least five years of protection in man (Gotschlich et al., 1969a,b,c; Brandt and Artenstein, 1975). Serogroup B meningococci produce a small capsule whose polysaccharides do not produce a satisfactory vaccine (Wyle et al., 1972) but as it is one of the major disease-producing serotypes along with A and C, recent efforts have been made with some success towards developing a vaccine against serogroup B wall proteins (Frasch and Robbins, 1978a,b; Zollinger et al., 1978). While effective serogroup-specific vaccines represent a useful advance, the fact that a person recovering from a meningococcal infection of any serogroup is subsequently immune to meningococcal infections of all serogroups, holds forth the hope of a "universal" anti-meningococcal vaccine (Littlejohns, 1976).

Antibody-mediated complement fixation has been known

for some time to have considerable importance in the anti-meningococcal effects of serum (Goldschneider et al., 1969a,b). Frasch and Robbins (1978) make the interesting observation that neutropenia did not seem to increase the sensitivity of the guinea pig to group B meningococci, while absence of a higher complement fraction did, suggesting the relative importance of the humoral and cellular defenses in meningococcal infection. However, should iron-limitation prove to be important in meningococcal disease, the guinea pig would be a poor model, having 85% iron-saturation of its transferrin, a fact shown to be of considerable importance to the survival of pathogenic mycobacteria (Kochan, 1974).

4. Treatment of Meningococcal Disease

Before the advent of chemotherapy, meningococcal antisera were used to treat meningococcal disease, but by the 1930's antisera against the Gordon type strains were ineffective against many disease strains (see Aycock and Mueller, 1950). After its introduction, sulfadiazine was found to be an excellent antimeningococcal drug for both prophylaxis and treatment of disease. However, by the mid 1960's, the spread of sulfadiazine resistance had rendered it useless, except against serogroups B and Y, and penicillin was used for both gonococcal and meningococcal disease (Artenstein, 1975). Due to the

increasing resistance of the meningococcus to penicillin and its derivatives, and for patients reacting badly to penicillin, erythromycin and chloramphenicol are often used, although the new antibiotic rifampin is proving useful in treating overt disease as well as eradicating the carrier state. Unfortunately, rifampin resistance is rising so that the drug should be reserved for acute disease (Artenstein, 1975).

The introduction of chemotherapy for neisserial infections resulted in vastly improved treatments and recovery rates, but had one unfortunate side-effect - it greatly reduced research into the basic mechanisms of meningococcal pathogenesis. Combined with the lack of a satisfactory animal model, this has left some major areas of the man-meningococcus relationship completely unknown. Some of the more important areas of ignorance are: (a) how and why the meningococcus enters the blood and cerebrospinal fluid from the nasopharynx; (b) why only a minority of persons showing no anti-meningococcal bactericidal activity and exposed to a virulent meningococcal strain contract overt disease; (c) the presence of any consistent differences in the physiology of people contracting meningococcal disease, other than low or absent anti-meningococcal activity in the serum; (d) physiological events accompanying the early stages of meningococcal infection in man; and (e) the differences between avirulent and virulent meningococcal strains.

F. Iron and Pathogenic Neisseria

When the research for this thesis was started, there was virtually nothing known about neisserial iron requirements, methods of iron acquisition, or the effects of iron limitation, except that supplemental iron enhanced both meningococcal and gonococcal growth under certain conditions. Despite the moderate levels of iron already present, growth of gonococci on GC-base (complex) media is significantly improved by the addition of hemoglobin, ferric nitrate, or iron-dextran (Kellogg et al., 1968; Payne and Finkelstein, 1977b). Iron salts and substances containing iron have been shown to have a marked effect on the virulence of gonococci for the chick embryo (Payne and Finkelstein, 1975) and on the meningococcus for the mouse (Calver et al., 1976; Holbein et al., 1979a).

In 1933 Miller reported that hog gastric mucin lowered the LD_{50} of several meningococcal strains by up to eight orders of magnitude when they were injected intraperitoneally (I.P.) into the mouse. Although this system was standardized (Branham and Pittman, 1940; Pittman, 1941) for the assessment of the antimeningococcal protective effects of the sera of human patients, the virulence-enhancing mucin varied widely in its efficacy from lot to lot. This variation was difficult to explain in terms of its presumed mode of action, that of discouraging phagocytosis and antigenic recognition by coating the

cells with viscous polysaccharides, because most preparations had roughly similar viscosities (Olitzki, 1948). Calver et al. (1976) reported that hog gastric mucin contains high but widely variable levels of iron, sometimes greater than 0.1% Fe by weight, and further that mucin could be replaced by ferrous sulfate, Imferon, a hematinic iron-dextran, or Jectofer, an iron sorbitol citrate hematinic preparation, in producing fatal meningococcal infections in mice. However, the levels of ferrous sulfate and iron sorbitol citrate employed in the study of Calver et al. (1976) were close to the levels lethal for the mouse. Recent work by Holbein et al. (1979a) using ferrous sulfate, Jectofer and Imferon also showed that the levels of ferrous sulfate and Jectofer that gave significant reductions in the LD_{50} for meningococci in the mouse model were close to fatal per se. In the case of Imferon (iron-dextran) iron, 15 mg/kg body weight ($\simeq$300 μg iron/mouse) lowered the LD_{50} of a number of meningococcal strains by six to eight orders of magnitude, yet $>$1600 mg Imferon iron/kg body weight per se was not fatal to the mice. Since the mouse model uses intraperitoneal injection for introduction of both cells and adjunct, it presumably does not assess meningococcal invasiveness well. Nevertheless, wide variations in the LD_{50} values obtained for various meningococcal strains in the mouse-iron-dextran model appear to correlate moderately well with the

virulence of their serogroups and whether they came from carriers or cases of active disease (Calver et al., 1976; Holbein et al., 1979a). In the absence of an iron-containing adjuvant, the numbers of viable meningococci required to kill the mouse approximate the numbers of heat-killed cells that are fatal (10^8-10^9) i.e. death is probably due to endotoxic effects from the injected cell material (Branham and Lillie, 1933; Olitzki, 1948; Holbein et al., 1979a). Use of the dextran fraction of Imferon per se as an adjuvant had no virulence-enhancing effect on the system (Holbein et al., 1979a). While this certainly does not prove that the dextran is of no importance in enhancing meningococcal virulence it does demonstrate that the iron is vital to the profound effect that iron dextran has on the virulence of the meningococcus for the mouse. As use of the near-fatal levels of iron salts or Jectofer can result in pathology resembling that of hypersideremia (see Richter, 1978), Holbein et al. (1979b) looked at the serum and tissue distribution of iron following i.p. injection of Imferon into mice. They reported that the iron from 15 mg Fe/kg i.p. Imferon injections appeared in the serum, with transferrin iron saturation rising from 48% to a peak of 82% at 2½ - 5 h post-injection. By 30 h post-injection transferrin saturation had returned to normal and the liver and spleen were found to contain elevated iron stores, but without any observable pathology. Therefore, the

low toxicity of iron dextran may be due at least in part to the relatively slow release of iron from the dextran complex (Holbein et al., 1979b), allowing the transferrin and the reticuloendothelial system to process the colloid without gross overload resulting in iron toxemia and blockage (Kornfeld et al., 1969; Thorén-Tollig and Jönsson, 1977).

In contrast to iron-dextran, use of the near-fatal levels of iron salts or iron sorbitol citrate required to significantly depress the LD_{50} of meningococci for the mouse or gonococci for the 11-day chick embryo may result in pathology resembling that of hypersideremia (Richter, 1978). There are, therefore, a number of possible explanations for their effect, including: (a) reticuloendothelial blockade due to particulate iron precipitates; (b) acid pH shift occurring when iron salts convert to iron hydroxides; (c) the inherent toxicity of high levels of free iron for living cells, particularly those that attempt to sequester it; and (d) the variety of effects that high iron has on phagocytosis and serum bactericidal properties (see Norrod and Williams, 1978a; Kaplan and Basford, 1979).

Within the last few years several reports have appeared presenting evidence that the ability of the gonococcus to invade and kill the chick embryo and to cause disseminated gonococcal infections in man is related to the relative abilities of different strains to obtain iron (Payne and Finkelstein,

1975,1978a; Payne et al., 1978). Strains of the virulent T_1 and T_2 gonococcal serotypes had LD_{50} values of 3-25 cells for 11-day chick embryos while strains of avirulent T_3 and T_4 serotypes had LD_{50} values of 10^5-10^6 cells. Added iron in the form of iron-dextran (500 μg/egg) reduced the LD_{50} of T_3 and T_4 strains by 1-2 orders of magnitude (Payne and Finkelstein, 1975), but the virulence of T_1 and T_2 serotypes was so high without added iron that it could not be determined if iron-dextran enhanced it, despite the authers' claims. Iron-free conalbumin was inhibitory to the growth of all strains, but when iron-saturated had no effect (Payne and Finkelstein, 1975). However, if as the authors suggest, the difference between virulent T_1 and T_2 strains and avirulent T_3 and T_4 strains is a function of differences in their ability to obtain iron in vivo, then the LD_{50} differences between the strains should disappear, but this apparently is not so according to the data presented (Payne and Finkelstein, 1975). Even if it were so, the variety of non-nutritional effects that iron can have on the host-parasite relationship (see p. 44) means that interpretation of an iron-related effect is far from straightforward. Payne et al. (1978) also examined six gonococcal strains isolated from cases of disseminated gonococcal infections and found that unlike the other gonococci examined, addition of iron-free conalbumin did not reduce their ability to acquire iron in the chick embryo.

Freshly isolated gonococci are usually resistant to the bactericidal action of serum, but upon repeated in vitro culture they become sensitive and are killed by normal human serum (Ward et al., 1970). In contrast, gonococci from disseminated infections usually remain resistant (Brooks et al., 1976; Schoolnik et al., 1976). There are two recent reports that ferric ammonium citrate, but not several other iron compounds, largely eliminated this serum bactericidal effect (Johnson et al., 1978; Norrod and Williams, 1978a). However, this is probably a good example of a non-nutritional effect, as the large levels of citrate present will probably sequester any free Ca^{+2} or Mg^{+2} in the serum. As the bactericidal effects of serum on both gonococci and meningococci have been shown to be largely complement-mediated (Goldschneider et al., 1969a; Johnson et al., 1978) and Ca^{++} is required for the proper operation of the C'1 binding step of complement fixation, citrate per se could be expected to prevent the bactericidal effects of normal sera.

There are also two reports on preliminary efforts to identify a siderophore produced by N. gonorrhoeae, one claiming success, the other failure (Payne and Finkelstein, 1978a; Norrod and Williams, 1978b). Norrod and Williams used a disseminating strain grown in a defined medium made iron-limiting with the powerful iron chelator ethylenediamine-di-(o-hydroxyphenyl) acetic acid (EDDA). Use of the Csaky test for hydroxamates,

the Arnow test for phenolics, two bioassays for the ability of the culture filtrates to enhance the growth of small inocula and to repress the antigonococcal effects of serum were all negative. On the other hand, Payne and Finkelstein (1978a), using gonococci grown on a GC base-conalbumin agar, reported growth stimulation in response to the addition of liquid culture filtrates of N. meningitidis, Vibrio cholerae, and a strain of N. gonorrhoeae isolated from a disseminated infection. Several strains of N. meningitidis were also assessed and it was reported that when grown on the GC base-conalbumin agar, the meningococci showed enhanced growth in response to liquid culture extracts of N. meningitidis, E. coli, Shigella dysenteriae, Vibrio cholerae, and Salmonella typhimurium, as well as to a sample of enterochelin. These results, being at variance with the findings presented in this thesis, are discussed further in Section II. This brings up the observation that N. gonorrhoeae shows significantly improved growth rates and extents on GC-based complex solid media already containing much iron when up to 70 μg/ml of additional iron is added as salts, iron-dextran, or hemoglobin (Kellogg et al., 1968; Payne and Finkelstein, 1977b). As the iron requirements for Gram-negative bacteria are usually ≤ 0.1 μg iron/ml (see Lankford, 1973), the need for such levels of added iron if a siderophore is produced is difficult to understand.

G. Rationale Behind the Thesis Work

From the foregoing general introduction and literature review it should be clear that: (a) in numerous host-parasite relationships microbial iron acquisition is an important factor; (b) there are fundamental questions unanswered about the man-meningococcus relationship; and (c) there is a variety of data suggesting that host iron levels are important to this relationship. Therefore, the thesis work was undertaken primarily because there was absolutely no data on the iron physiology of any of the Nesseriaceae, and secondarily in the hope of determining, or at least supplying useful information on, the role of iron in the ecology of the meningococcus.

I. *NEISSERIA MENINGITIDIS*: ITS IRON REQUIREMENTS AND THE EFFECTS OF IRON-DEFICIENCY

IA. Introduction

Despite the abundance and ubiquity of iron in nature, it is frequently not readily available to microorganisms because of its tendency to form highly insoluble complexes in aerobic soil and water and to be sequestered in specialized macromolecules in higher organisms. In recent years there has been a rapid accumulation of data on the methods by which microorganisms obtain iron from their environment, and, especially in certain members of *Bacillus*, *Mycobacterium*, and the enteric bacteria, the methods by which iron is transported into the bacterial cell (Byers, 1974; Rosenberg and Young, 1974; Macham *et al.*, 1975). Most of the microorganisms studied produce one or more specific soluble iron chelators or siderophores, when grown in iron-poor environments, and those few examined in sufficient detail appear to have energy-linked iron or ferrated siderophore uptake systems (Byers, 1974; Rosenberg and Young, 1974; Brown and Ratledge, 1975; Arceneaux and Byers, 1976; Negrin and Neilands, 1978). In the enteric bacteria, the only Gram negative group studied to any extent, interesting features of their iron uptake processes include the following: (i) specific receptor sites for siderophores synthesized by other, apparently

unrelated, microorganisms (Luckey et al., 1972); (ii) siderophore receptor sites in the outer membrane that also serve as receptor sites for colicins and phages (Braun et al., 1976); and (iii) a correlation between virulence and the ability of a bacterial strain to obtain iron in a given host (Bullen et al., 1974; Weinberg, 1978).

To date there have been no reports on the basic iron physiology of neisseriae, although a number of studies have implicated iron capture and virulence in the production of meningococcal and gonococcal disease (see p. 41-47). However, performing experiments involving the meningococcus, iron treatments and various laboratory animal models with no knowledge of basic meningococcal iron requirements and physiology one cannot hope to provide a clear picture of the importance of iron in the host-parasite relationship. Microbial iron limitation effects are usually assessed by measuring various iron-requiring functional proteins (see Neilands, 1974a). Unfortunately, most of these proteins are associated with respiratory functions and their measured activities are markedly affected by a number of inter-related factors, particularly culture growth phase and rate, carbon and energy sources, dissolved oxygen, pH, and iron concentration, if limiting. The only way to measure the effect of iron privation per se is by the use of continuous culture techniques with cells maintained under constant conditions

varying only the parameter of interest (Tempest, 1970; Light and Clegg, 1974).

The early work which I performed with the meningococcus in iron-limited batch culture showed this to be the case; not only did the parameters of interest change markedly with growth phase and dissolved oxygen, but they changed so rapidly in relation to the time required to perform the assays that the culture had significantly altered before replicates could be done. Therefore, in addition to controlling a number of important variables, continuous culture provides, at least in theory (see Tempest, 1970), cell material of unvarying characteristics for an indefinite period. In addition, a variable such as dissolved oxygen or iron could be varied and the same culture adjusted to a new equilibrium, making possible the assessment of iron or O_2 effects on a single culture instead of parallel cultures. This approach also avoided the troublesome problem of initiating meningococcal growth in iron-poor liquid media, as the iron in a high-iron continuous culture could be gradually diluted out by an influx of fresh low-iron medium, and a low-iron equilibrium (i.e. cell growth rate equals the continuous culture dilution rate) readily established.

The work presented in this section provides basic data on meningococcal iron requirements and some of the prominent effects of iron privation on the organism. The work includes

development of a simple effective defined meningococcal medium and growth of the meningococcus in iron-limited batch and continuous culture systems.

IB. Materials and Methods

Organism

The group B Neisseria meningitidis (SDIC) used was obtained from the Neisseria Repository, NAMRU, School of Public Health, University of California, Berkeley. Stock cultures were maintained in a lyophilized state, as received. Working cultures were prepared by growing a lyophilized stock culture on a plate of Mueller Hinton agar and at approximately 18 h inoculating from the plate onto each of 100 or so small slants of Mueller-Hinton agar. After 12-18 h incubation (37°C) the vials were stored at -80°C. Checks for culture purity made upon opening stock lyophilized cultures included TMPD-oxidase activity, ability of the culture to produce acid on glucose, and maltose CTA agar, gram stain, and the cultures' serogroup using sera obtained from the Neisseria Repository. Working cultures were checked again by gram stain, phase-contrast microscopy, and TMPD-oxidase activity before use. Each experiment used a fresh working culture. Meningococcus SDIC dissociates into rough and smooth colonial forms (DeVoe and Gilchrist, 1978). Only the smooth colony type was used in these studies.

Media

Cultures were grown on a complex medium, Mueller Hinton (MH; Difco), and a simple defined medium designated Neisseria

Table 1. Cellular and medium ion levels as determined by spectrophotometry.

Mueller-Hinton (M-H) broth (Difco)			Composition of Nisseria Defined Medium (NDM) (inorganic constituents)		Intracellular ions in late log-phase cells of N. meningitidis SD1C[b]
Na^{+}	140	mM	NaCl	140 mM	1040 mM[c]
$PO_4^{=}$ [a]	11.5	mM	$NaH_2PO_4 \cdot H_2O$	1 mM	-
Ca^{+2}	4.5	mM	$CaCl_2 \cdot 2H_2O$	0.5 mM	0-trace
Mg^{+2}	0.16	mM	$MgSO_4 \cdot 7H_2O$	0.2 mM	39 mM
K^{+}	1.8	mM	KCl	2.0 mM	132 mM
Fe	.0079	mM	$Fe(NH_4)_2(SO_4)_2$	as appropriate	2.6 mM
Zn^{+2}	.0084	mM	$Co^{+2}, Cu^{+2}, Zn^{+2}, Mn^{+2}$	2×10^{-8} M each[d]	-

Organic Constituents of NDM

L - glutamic acid	10 mM
D - glucose	10 mM
L - cysteine	1 mM
L - arginine	1 mM
uracil	1 mM
Trisma base	40 mM

[a] Determined by the method of Toribara and Warner (1956)

[b] Cell volume determined by assuming that all the Ca^{+2} in the pellet was interstitial (thus obtaining a value of about 30% for interstitial space in the pellet). Since pellet Ca^{+2} was only 1/3 that of the medium, true intracellular ion concentrations must be equal to or slightly greater than those given.

[c] This remarkable figure is being investigated in more detail by DeVoe *et al.*

[d] Trace metals used as sulfates or chlorides.

Cell Growth

Cultures on solid media were incubated at 37°C in a candle jar. The candle jar's atmosphere was assessed just after candle extinction by gas chromatography using a Fisher-Hamilton Model 29 gas partitioner and was found to contain about 19 mm Hg CO_2 pressure and 128 mm Hg O_2 pressure. Broth cultures were shaken (100 rpm, r = 4.5 mm) in a 37°C water bath. The atmosphere over NDM broth cultures was flushed before and during growth with sterile filtered 10% CO_2 in air. Cultures in broth media were inoculated with a 1% v/v inoculum of mid-log phase cells in the same medium. All media and materials used for cell transfers were maintained at 37°C.

For continuous cultures a model C-30 fermentor with a 335-350 ml working volume (New Brunswick Scientific Co., New Brunswick, N.J.) was modified as follows: (a) all stainless steel in contact with the culture or the medium was replaced with glass, silicone rubber (unpigmented), or polyethylene; (b) the impeller was coated with polyvinyl chloride; and (c) control of both pH and dissolved oxygen (DO) tension was provided (Figure 1). The PVC stirrer coating began to leak and degrade after repeated autoclavings, so a teflon block was machined to replace it. The block fitted

onto the glass pivot at the bottom of the culture vessel and held two standard teflon-coated stir-bars (9 x 40 mm) at right angles to the pivot. Constant culture pH was maintained by a Chemap PEC pH controller and autoclavable electrode (Ingold No. 465-35, Ingold Electrodes, Lexington, Mass.). DO was regulated by a two-position, dual-action monitor-controller, which regulated sparging gas pressure and/or impeller rpm, and employed a galvanic DO electrode (Borkowski and Johnson, 1967). Because of continuing membrane failure problems, the silver-lead galvanic probe was replaced with a modified Yellow Springs Instrument (Yellow Springs, Oh.) Model 5331 polarographic dissolved oxygen probe. Best results were obtained using a soft 1-mil polyethylene membrane (see Appendix). The culture vessel subassembly, containing 350 ml NDM, was autoclaved (121°C, 25 min), and the chemostat was assembled and adjusted to the following settings: 37 ± 0.1°C; 200-300 rpm agitation; pH 7.45 ± 0.05; and a 10% CO_2-in-air sparging rate of 85 - 115 ml/min. The culture vessel was inoculated as described above and after approximately 9 h, DO control and medium flow were started.

Equilibrium growth of SDIC ($D = \mu$, where D = dilution

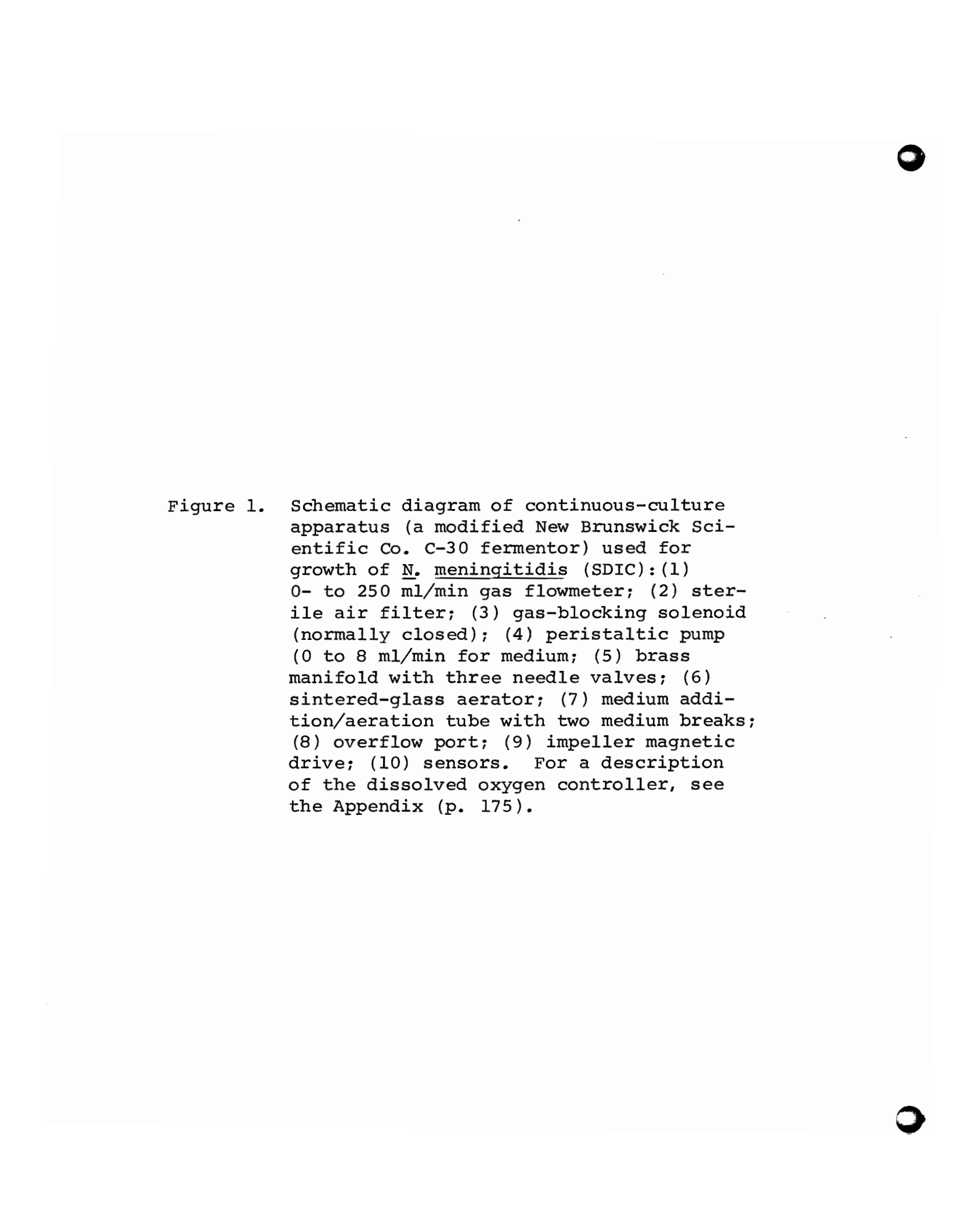
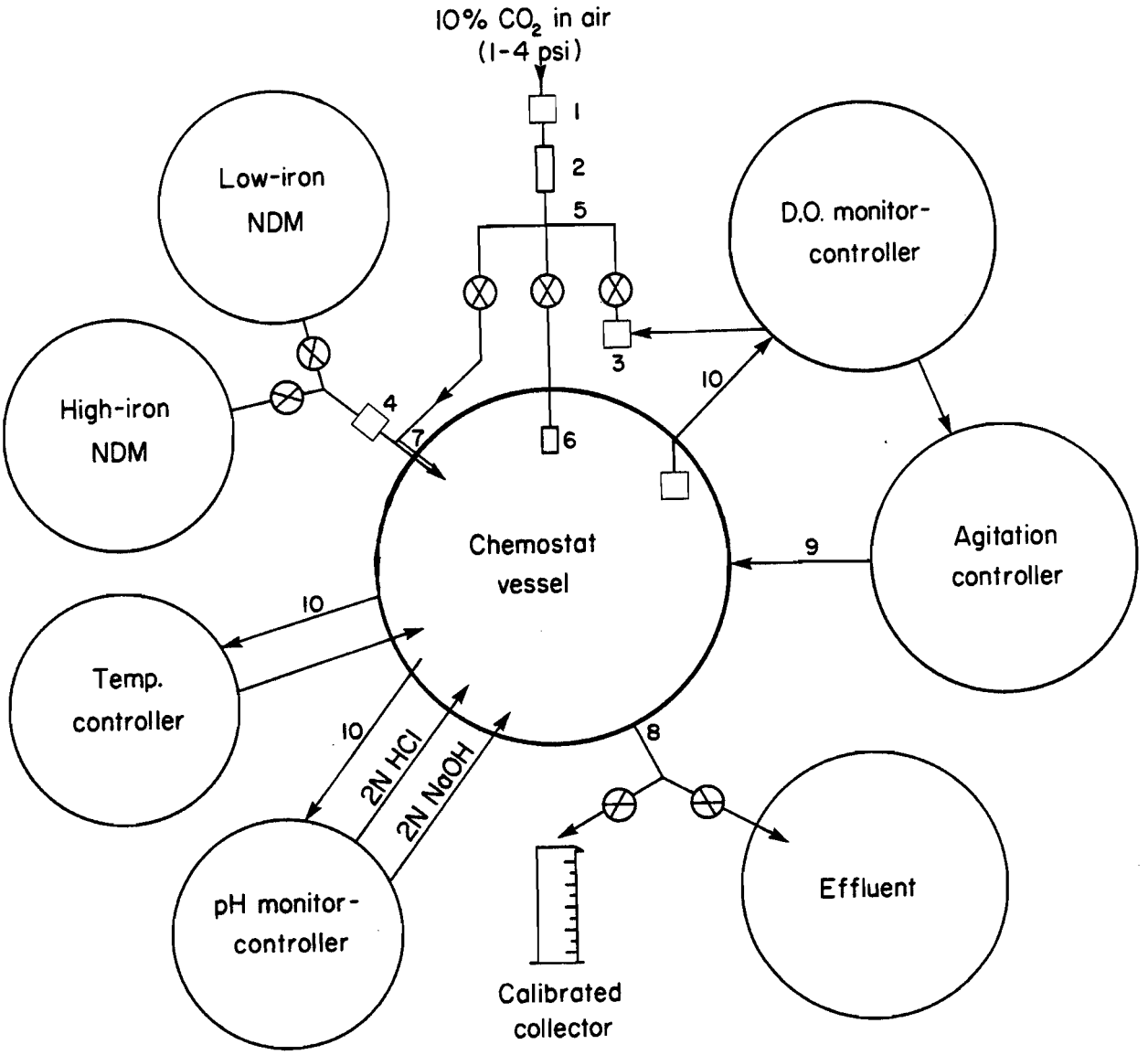


Figure 1. Schematic diagram of continuous-culture apparatus (a modified New Brunswick Scientific Co. C-30 fermentor) used for growth of N. meningitidis (SDIC): (1) 0- to 250 ml/min gas flowmeter; (2) sterile air filter; (3) gas-blocking solenoid (normally closed); (4) peristaltic pump (0 to 8 ml/min for medium; (5) brass manifold with three needle valves; (6) sintered-glass aerator; (7) medium addition/aeration tube with two medium breaks; (8) overflow port; (9) impeller magnetic drive; (10) sensors. For a description of the dissolved oxygen controller, see the Appendix (p. 175).


10% CO_2 in air
(1-4 psi)
1
2
5
Low-iron
NDM
D.O. monitor-
controller
High-iron
NDM
3
10
4
7
6
Chemostat
vessel
9
Agitation
controller
10
Temp.
controller
10
2N HCl
2N NaOH
8
pH monitor-
controller
Effluent
Calibrated
collector

rate $= \frac{\text{Flowrate}}{\text{vessel volume}}$ and μ = instantaneous growth rate) was readily maintained at a cell density of 1.5 - 2.2 x 10^9 cells per ml (OD_{600} = 0.45 - 0.65) with a fluctuation $\leq$ 2% h^{-1}. Equilibrium under iron-limited conditions was obtained by starting a flow of low-iron NDM into a high-iron NDM continuous culture. After approximately 12 h the culture stabilized, and a low-iron equilibrium was established. For stable growth it was necessary to supplement the low-iron NDM with 2 ng of iron per ml.

Vigorous cultures showed no tendency towards growth on vessel walls or foaming. Because contaminating iron from the 2 N NaOH used to control pH was a problem during iron-limited growth in the continuous cultures, the inflowing NDM was left at its natural pH of 8.3 - 8.4. The metered addition of this alkaline NDM to the culture vessel greatly reduced the amount of NaOH needed to compensate for metabolic acid production.

Iron Determinations

Initially, iron concentrations $>$200 ng/ml were determined by atomic absorption as described above, but in later work the Unicam SP-90A was replaced by a Perkin-Elmer 703 atomic absorption spectrophotometer. Concentrations $<$200 ng/ml were determined by the colorimetric method of Smith *et al.*

(1952) modified as follows: (1) all samples were adjusted to pH $\simeq$ 5.2 with HCl; (2) double-distilled hexanol (4.0 ml per 100 ml sample) was used to extract the ferroin complex and (3) absorbencies were measured on a Gilford 240 spectrophotometer at 533 nm. Iron recovery efficiency from various types of samples using this assay was evaluated with ^{55}Fe. Iron in whole cells was determined after ashing (500°C, 16 h) and dissolving the ash in 6.0 N HCl.

Quantitation of Cells

Viable cell counts were determined on MH agar plates inoculated with 10 µl of appropriate serial dilution of culture, MH broth being used as the diluent. Direct counts were performed using a Petroff-Hausser bacteria counting chamber and appropriate dilutions of the culture. The cells were counted using phase-contrast microscopy (Zeiss PM-II) and several counts averaged. Each diplococcus was counted as 2 cells. Optical densities (600 nm) of cell suspensions were measured on a Gilford 240 spectrophotometer. Salt-free cell dry weights were determined by weighing cell samples (dried to constant weight) before and after ashing (500°C, 16 h).

Batch and Continuous Culture Iron Uptake

One hour before inoculation, 3-5 x 10^4 DPM/ml $^{55}FeCl_3$

(≃9 μCi/μg) in 0.1 N HCl was added to 50 ml aliquots of NDM and MH broth in 250 ml Erlenmeyer flasks. Each flask was inoculated with 1.0 ml of 4-6 h NDM or MH broth culture, shaken (100 rpm) and samples were taken periodically and assayed for cell density, protein (Lowry et al., 1951), TMPD-oxidase activity, and the cellular ^{55}Fe content. For determination of bound ^{55}Fe, 0.2-2.0 ml aliquots of culture were passed through membrane filters (0.45 μm pore size), and the filters were washed with 5.0 ml of sterile non-radioactive medium.

^{55}Fe Determination

Liquid scintillation counting of ^{55}Fe was carried out on a Chicago Nuclear Isocap 300 counter in the SCR mode with manually set windows. Good counting efficiencies were obtained by addition of 1.0 ml of water containing 100 μg o-phenanthroline and 100 μg deferrioxamine-β-mesylate (Desferal) to 6.0 ml PCS Solubilizer (Amersham Searle, Arlington Hts., Ill.) scintillant and a 1.0 ml liquid sample or a 25 mm membrane filter.

Oxidase

Ascorbate-TMPD (N,N,N',N'-tetramethylphenylenediamine) oxidase activity was measured, after appropriate dilutions of cells in sterile growth medium, in a Rank polarographic O_2 cell (Rank Bros., Bottisham, Cambridge, England). O_2 consumption was followed after the addition of 10 μl each of 0.9 M ascorbate and

0.6 M TMPD. TMPD-oxidase activity was expressed as nmol O_2 consumed per 10^9 cells per min. Background O_2 consumption was measured after the addition of the ascorbate.

Catalase

Three ml of cell culture was placed in the O_2 cell and a few grains of sodium dodecylsulfate were added to lyse cells and stop O_2 consumption. After a flat baseline was established, 10 µl of 0.3 M H_2O_2 were added. Catalase activity was expressed as nmol of O_2 evolved per 10^9 cells per min. As the 1 mM H_2O_2 did not saturate the catalase, care had to be taken that the stock H_2O_2 was not appreciably degraded.

Cytochrome Determinations

Cytochromes b and c were determined by difference spectroscopy (Perkin-Elmer 356 Dual Beam Spectrophotometer) on medium-washed, ultrasonically disrupted cells after adding a few grains of potassium ferricyanide to one cuvette and sodium dithionite to the other. Absolute absorbances (Soret, 417 nm) were determined by comparison of aerated cell sonicates with medium blanks (Rosenberger and Kogut, 1958).

Protein was estimated using the method of Lowry et al. (1951) using bovine serum albumin standards.

Dehydrogenase Assays

Cells were grown under high and low iron equilibrium conditions in the continuous culture (D.O. = 110 ± 10 nmol/ml), and the same conditions used to obtain the high D.O. data in Table 2. Under each equilibrium 100 ml of cells were collected, centrifuged for 10 min (2500 x g), resuspended in sterile low-iron NDM and sonicated as for the cytochrome spectra. The sonicates were placed in an ice bath and used as soon as possible for the three assays. The assays were all performed at 37°C on the same sonicates and the results read using the Gilford 240 spectrophotometer. Glucose-6-phosphate dehydrogenase was quantified by the method of Malamy and Horecker (1964) using 100 µl of cell sonicate in a 1-ml assay volume. NADH dehydrogenase activity was also determined in a 1 ml assay system, containing 100 µl sonicated cells, 400 mM NADH, and 50 mM Trisma buffer, pH 7.6 with the oxidation of NADH followed at 340 nm. Succinate dehydrogenase was assayed by the method of Kasahara and Anraku, (1974), using a 3.0 ml reaction volume and 100 µl of cell sonicate. The background rate of color change was determined in the presence of the phenazine methyl sulfonate (PMS) and dichloroindophenol (DCIP) reagents used.

Chemicals

All salts and glucose were reagent grade (Fisher Scientific

Co., Fairlawn, N.J.). Other organic compounds were from Sigma Chemical Co. (St. Louis, Mo.), except for TMPD from Eastman (Rochester, N.Y.) and Desferal, a gift from CIBA, Montreal.

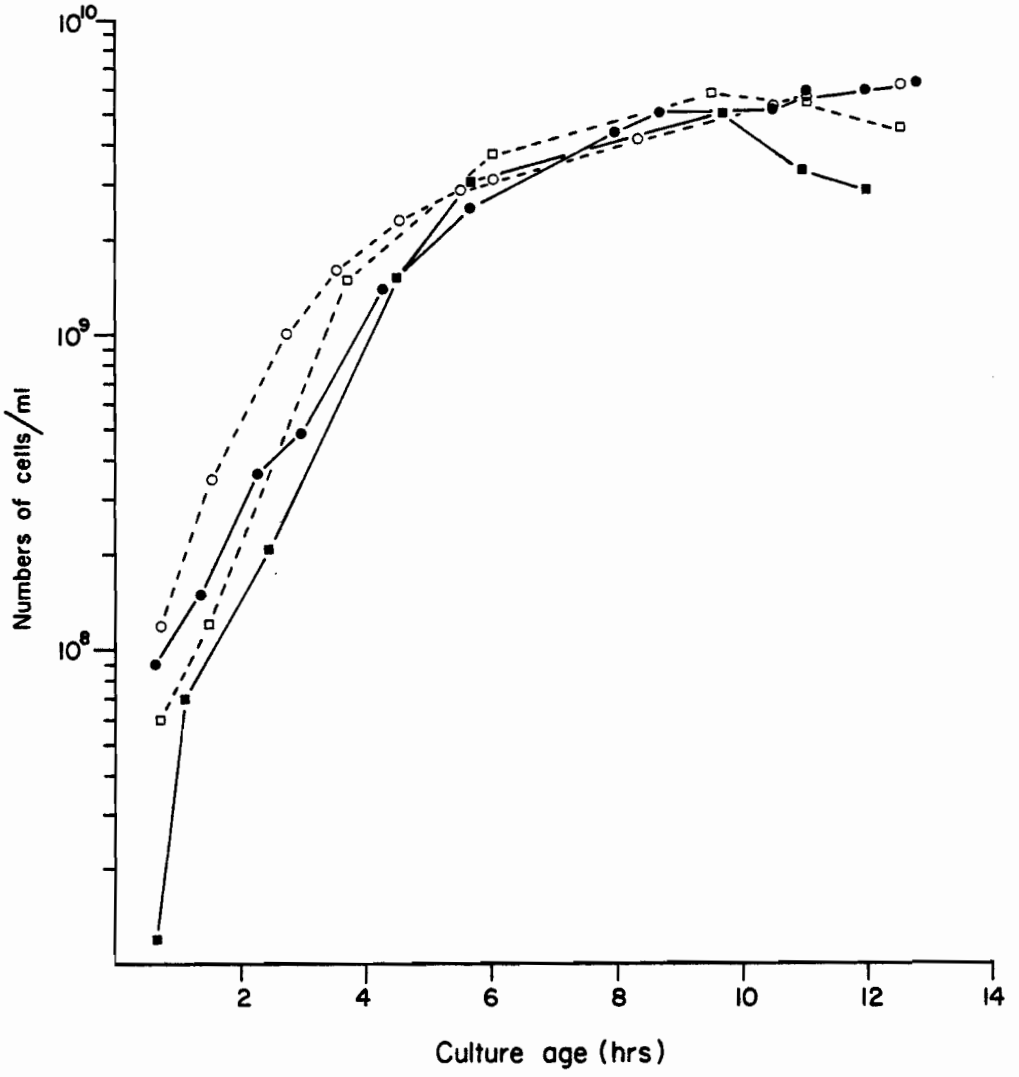
IC. Results

Batch Culture in Defined and Complex Media

The NDM and MH media were first compared for their ability to support growth of *N. meningitidis* (SDIC) in batch broth cultures. The growth, measured by either viable or direct counts, was similar (Figure 2) in both media with generation times of approximately 45 min. Moreover, the close correspondence between direct and viable counts from mid- through late-log phase in each of the media indicated that direct counting was valid as a routine measure of viable cells.

To determine the concentration at which iron became limiting for growth, NDM was adjusted to cover the range from no added iron (4-6 mg Fe/ml) to 2500 ng Fe/ml (Figure 3). The effect of this on growth was measured as the change in minimum generation time measured in the linear portion of exponential growth. The growth rate decrease became pronounced at 50 ng/ml, and below 20 ng Fe/ml not only were generation times drastically increased, but growth itself was difficult to initiate. The extent of growth, i.e., the maximum yield of cells, was found to be a poor criterion for quantitating iron limitation. Although iron at concentrations between 20 and 50 ng Fe/ml lengthened generation times, starting concentrations in the medium of $\prec$10 ng Fe/ml were sufficient to permit an increase in the

Figure 2. Growth of *N. meningitidis* (SDIC) in batch broth cultures (37°C, shaking) under 10% CO_2 in air. MH broth: direct counts (O); viable counts (□). NDM broth: direct counts (●) viable counts (■).


10^{10}
10^{9}
10^{8}
Numbers of cells/ml
2
4
6
8
10
12
14
Culture age (hrs)

population from 2 x 10^7 to 4 x 10^9 cells/ml. While Figure 3 shows that >50 ng/ml iron only slightly reduces generation time, Figure 4 shows that cells in a mid- to late-log batch culture will take up over 100 ng iron/ml, if the iron is available. These data led to the selection of 200 ng/ml as the iron concentration in "high-iron" NDM for batch and continuous culture work.

In high iron NDM, early-log phase cells had the shortest generation times in batch culture, in spite of their relatively low specific iron content (Figure 4). Therefore, the higher levels of iron observed at the late-log phase of growth were not essential for maximum growth rate. Fractionation of such cells by ultrasonic disruption and centrifugation (225,000 x g, 3h) showed 92% of the cellular iron to be in the pellet.

Growth of the meningococcus in the two media was further compared with respect to changes in dissolved oxygen, pH, extracellular iron, and oxidase activity of the cells (Figure 5). The activity of TMPD-oxidase in cells grown in either of the media progressively increased during exponential growth, whereas the concentration of dissolved oxygen in the media declined rapidly. This decrease in dissolved oxygen continued in spite of the agitation of the culture, continuous flushing of the atmosphere above the culture with 90% air - 10% CO_2, and the relatively high surface to volume ratio (0.53:1) in the flasks.

Figure 3. Generation times during the exponential phase of growth in NDM broth batch cultures containing different concentrations of iron over the range 10 to 2,500 ng of Fe per ml (0.18 μM - 45 μM). Generation times (points are means of triplicate flasks) were calculated when the cell populations were 10^9 cells per ml. The flask air spaces were flushed with 10% CO_2 in air prior to inoculation.

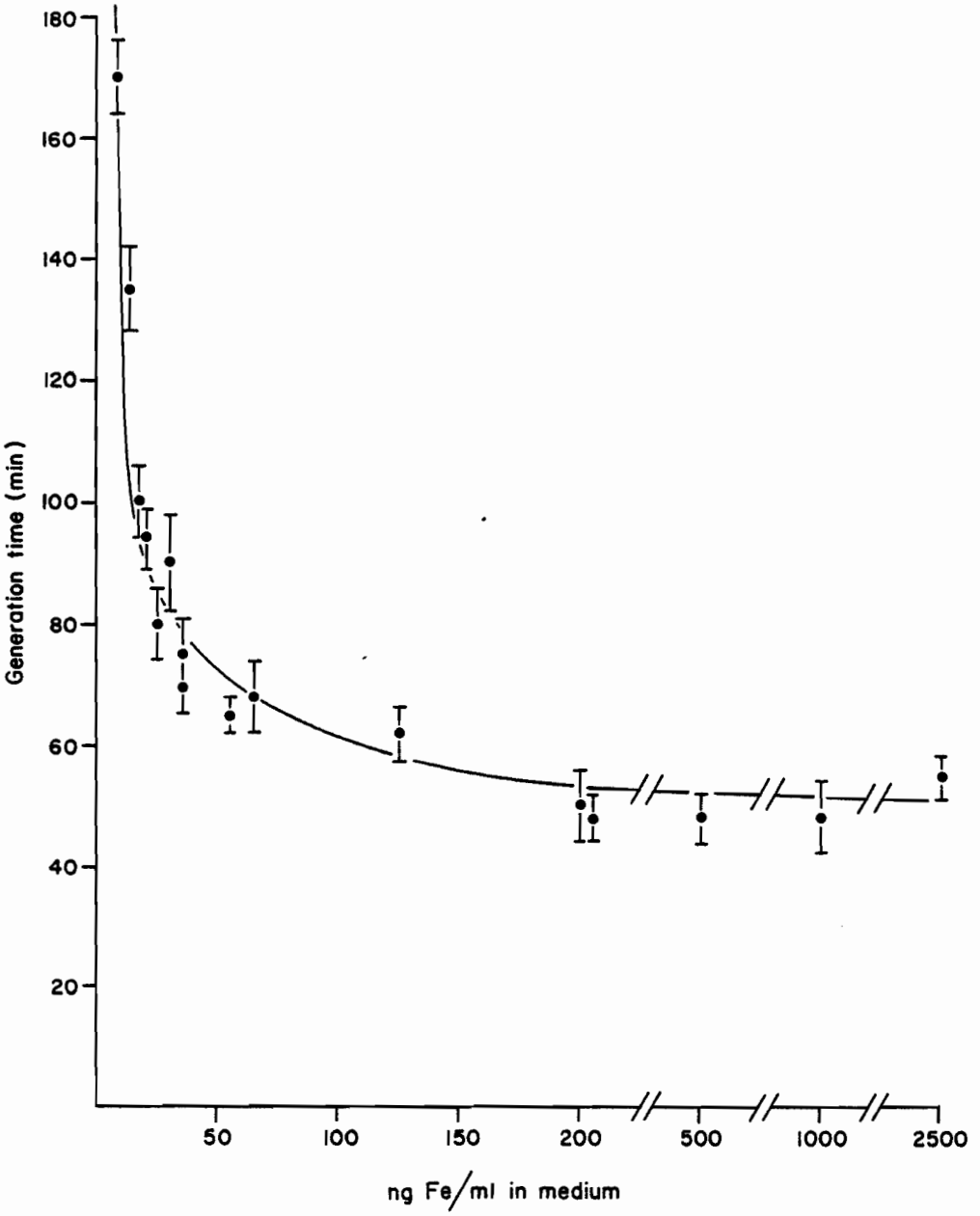

180
160
140
120
100
80
60
40
20
Generation time (min)
50
100
150
200
500
1000
2500
ng Fe/ml in medium

Figure 4. Uptake of iron (^{55}Fe) in NDM broth batch culture. Symbols: Concentration of iron in medium (●); cell counts (■); cellular iron (▲).

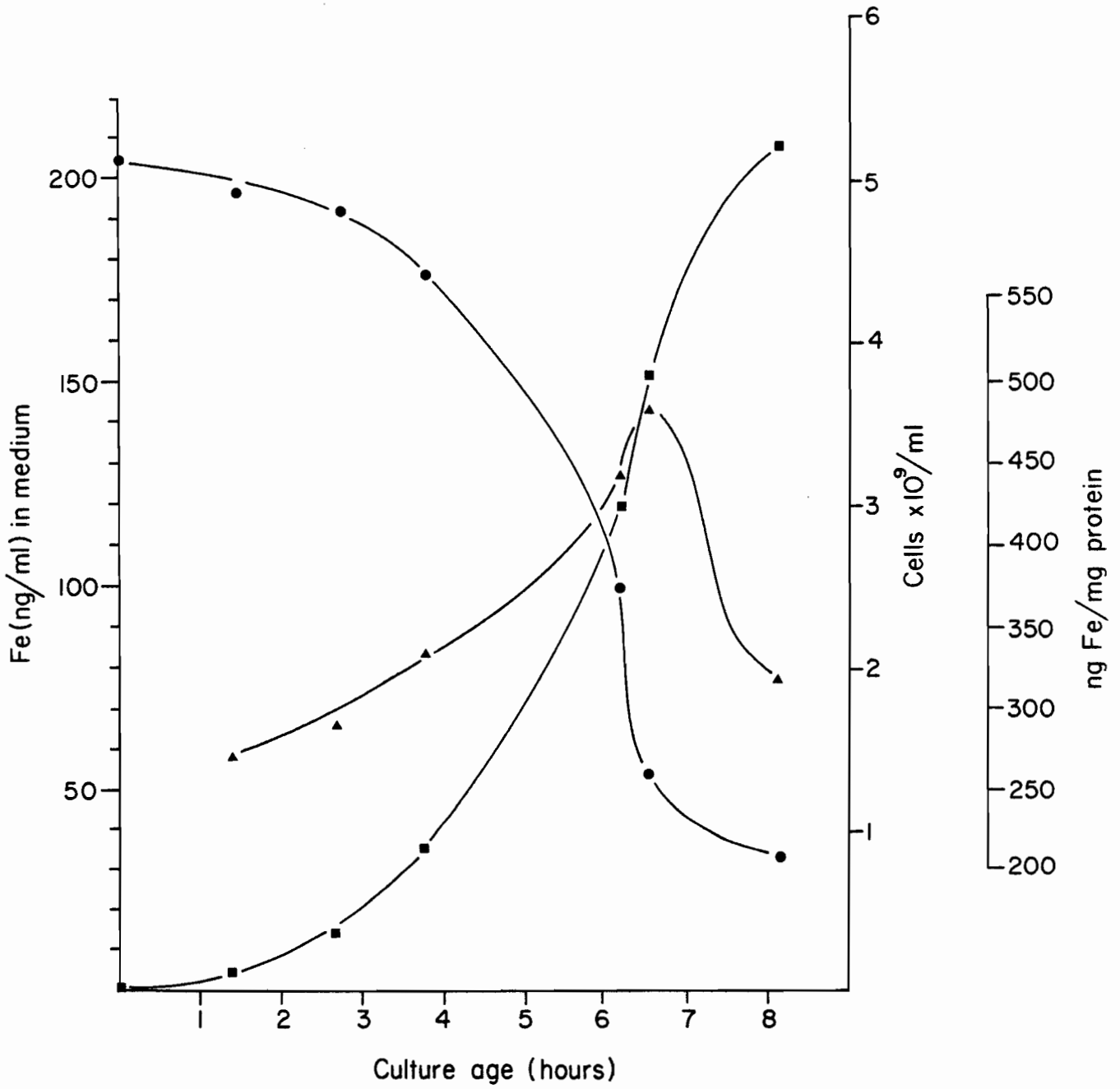
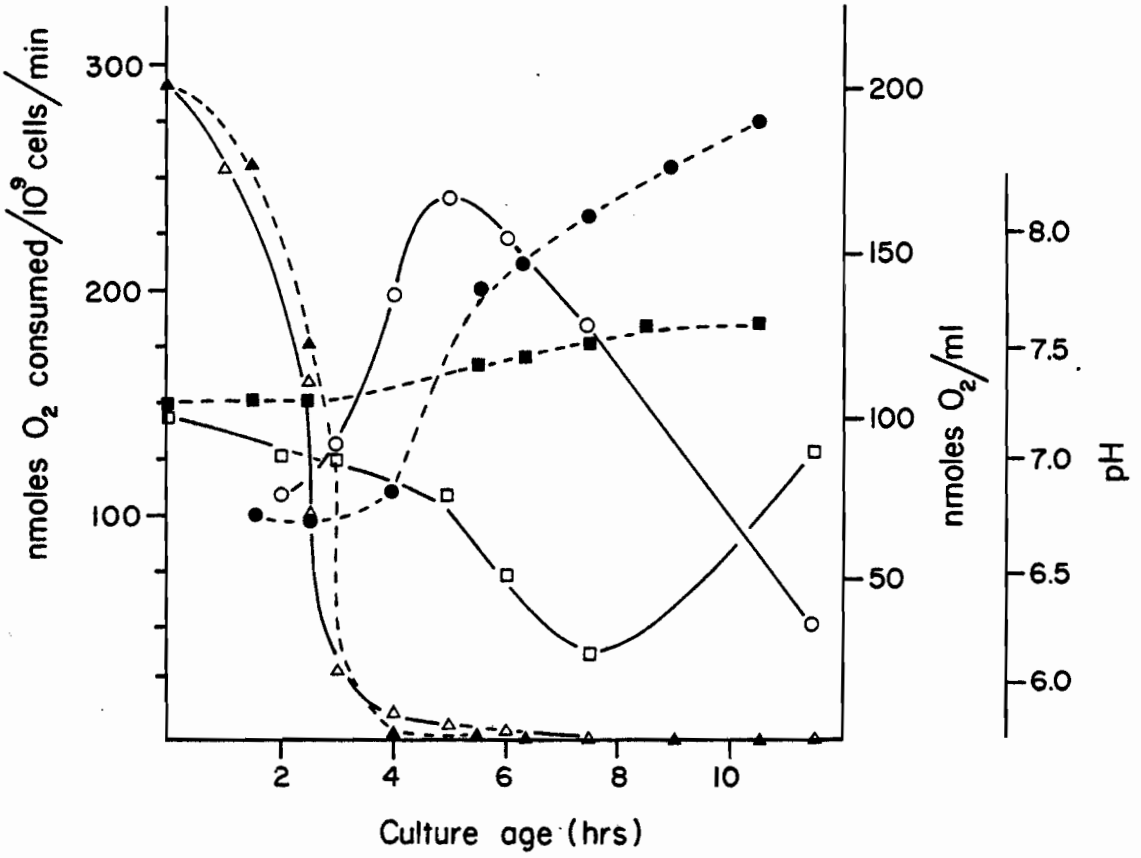

Fe (ng/ml) in medium
200
150
100
50
Cells x10⁹/ml
6
5
4
3
2
1
ng Fe/mg protein
550
500
450
400
350
300
250
200
1
2
3
4
5
6
7
8
Culture age (hours)

Figure 5. Batch cultures in MH broth (broken lines, solid symbols) and NDM broth (solid lines, open symbols). Symbols: Ambient culture dissolved O_2 (△,▲); ascorbate-TMPD oxidase activity (○,●); pH (□,■). Culture conditions as in Figure 2.


nmoles O2 consumed/10^9 cells/min
300
200
100
200
150
100
50
nmoles O2/ml
8.0
7.5
7.0
6.5
6.0
pH
2
4
6
8
10
Culture age (hrs)

An experiment using high-iron, high dissolved O_2 continuous culture gave similar specific TMPD-oxidase activities (130-140 $nMO_2/min/10^9$ cells) at equilibrium cell densities of 1.0 x 10^9, 1.9 x 10^9, and 3.0 x 10^9 cells/ml. This suggests that the observed sharp rise of TMPD-oxidase activity in batch culture (Figure 5) was caused primarily by the low dissolved oxygen in the culture. Therefore, altering either the oxygen or the iron concentration had a marked effect on the oxidase activity. Further, the interaction of other respiratory protein levels, generation times, pH, oxygen consumption and cell density with iron limitation, dictated that the culture environment be carefully controlled to quantify iron privation effects.

Continuous Culture in NDM

A number of characteristics, either directly or indirectly related to iron metabolism and cell growth, were examined during continuous culture of the meningococcus. In each experiment a single continuous culture was successively subjected to the four sets of conditions listed in Table 2. Cell division and washout rates were maintained in equilibrium at 1.5 - 2.2 x 10^9 cells/ml for at least 1 h before cells were taken for assays. Cells in chemostat cultures growing in medium containing $\leq$ 4 ng Fe/ml ceased dividing and began to wash out. This necessitated the addition of 2 ng Fe/ml to the low-iron medium.

Generation times were lengthened approximately three-fold under iron-deficient conditions (Table 2) but oxygen consumption decreased less than 50%. Therefore, 10^9 cells in the iron-poor environment required 3.1 μmol O_2 per doubling while the same culture grown in high-iron equilibrium required only 1.7 μmol O_2. Cells growing in the low-iron medium removed all detectable iron from the medium, whereas one half the total culture iron remained in the medium during growth under high-iron conditions. The cellular iron in high-iron continuous cultures was close to the iron level accumulated by cells in batch cultures (Figure 4) at the same cell density, and the values for iron per dry cell weight, after growth in the high-iron medium, were similar to those reported for other bacteria (Curran et al., 1943; Stephenson, 1949; Rouf, 1964). In these experiments, with the possible exception of catalase activity, total cellular iron changed more than any other cell parameter analyzed under conditions of iron limitation.

Growing cells in a surplus of iron (204 ng/ml) with a lowered O_2 tension (12 nmol O_2/ml) resulted in an increase in cytochromes c_{552} and b_{528} by 46% and 59%, respectively, accompanied by a corresponding increase in total heme. Also, in response to lower oxygen tension in conditions of iron excess, both catalase and TMPD-oxidase activities increased; however, as a result of iron privation, catalase activity was

TABLE 2

Changes in *N. meningitidis* SDIC grown in equilibrium chemostat cultures in both high and low concentrations of iron and dissolved oxygen.[a]

	204 ng Fe/ml medium		6 ng Fe/ml medium	
	110 ± 10 nmoles[b] O_2/ml	12 ± 5 nmoles O_2/ml	110 ± 10 nmoles[b] O_2/ml	12 ± 5 nmoles O_2/ml
Generation time (min)	55	62	164	161
Oxygen consumption (nmol $O_2/10^9$ cells/min)	30	33	19	24
TMPD-oxidase (nmoles O_2 consumed/ 10^9 cells/min)	138	197	90	125
Catalase (nmoles O_2 evolved/ 10^9 cells/min)	3.3	5.6	< 0.5	< 0.5
Cytochrome c_{552} red-oxid (O.D. x $10^{-4}/10^9$ cells/ml)	5.0	7.3	3.2	3.4
Cytochrome b_{528} red-oxid (O.D. x $10^{-4}/10^9$ cells/ml)	2.2	3.5	1.5	1.0
Heme-absolute absorbance (417 nm) (O.D. x $10^{-4}/10^9$ cells/ml)	32	46	19	19
Cell-bound iron (µg Fe/g dry wt.)	135	119	17	11
Cell salt-free dry wt (µg/10^9 cells)	86	80	71	70
Ash weights (µg/10^9 cells)	54	54	49	53
Cell protein (µg/10^9 cells)	73	75	83	85

[a] The data are from a single chemostat run but representative of those obtained from the 9 separate runs performed for this experiment.

[b] Subsequent iron uptake experiments used cells equilibrated under these conditions.

undetectable irrespective of the O_2 tension in the medium. Although the specific activity of TMPD-oxidase was somewhat lower in the low-iron medium, specific activity in this enzyme increased by 39% in response to lowered O_2 tension, irrespective of iron concentration. As 8 μM TCS (4 μM being sufficient to inhibit by 50% the rate of active iron uptake) (see Section III) did not alter the O_2 consumption rate by cells grown in high-iron, high O_2 tension continuous culture, it seems unlikely that the meningococcus has respiratory control. Therefore, the small increase in specific O_2 consumption under low O_2 tension (Table 2) may be the direct result of an increase in respiratory chain components. However, in view of the evidence that increases in O_2 consumption are smaller than the corresponding increases in cytochromes and TMPD-oxidase, when iron is not limiting, these respiratory components are probably not the rate-limiting ones.

Glucose-6-phosphate dehydrogenase activity (Table 3) was not expected to decrease directly as a result of iron privation, as this enzyme is not co-factored with iron in other organisms. However, a change in its activity could be expected if there were an overall change in glycolytic activity associated with iron privation, or if the cells shifted some of their glucose-6-phosphate from the Entner-Doudoroff and phosphoketolase pathways to the Embden-Meyerhoff pathway (Jyssum et al., 1961;

Table 3. Effect of iron limitation on the activities of 3 dehydrogenases in sonically disrupted cells of N. meningitidis SD1C grown under high and low iron equilibrium conditions.

Activity Assay	Units	204 ng Fe/ml Medium	6 ng Fe/ml Medium
glucose-6-phosphate dehydrogenase	Δ OD_{340}/min/mg cell protein	12.4 ± 1.9	11.5 ± 1.9
NADH dehydrogenase	Δ OD_{340}/min/mg cell protein	2.30 ± 0.41	1.70 ± 0.35
succinate dehydrogenase	Δ OD_{600}/min/mg cell protein	6.6 ± 0.3	2.4 ± 0.2

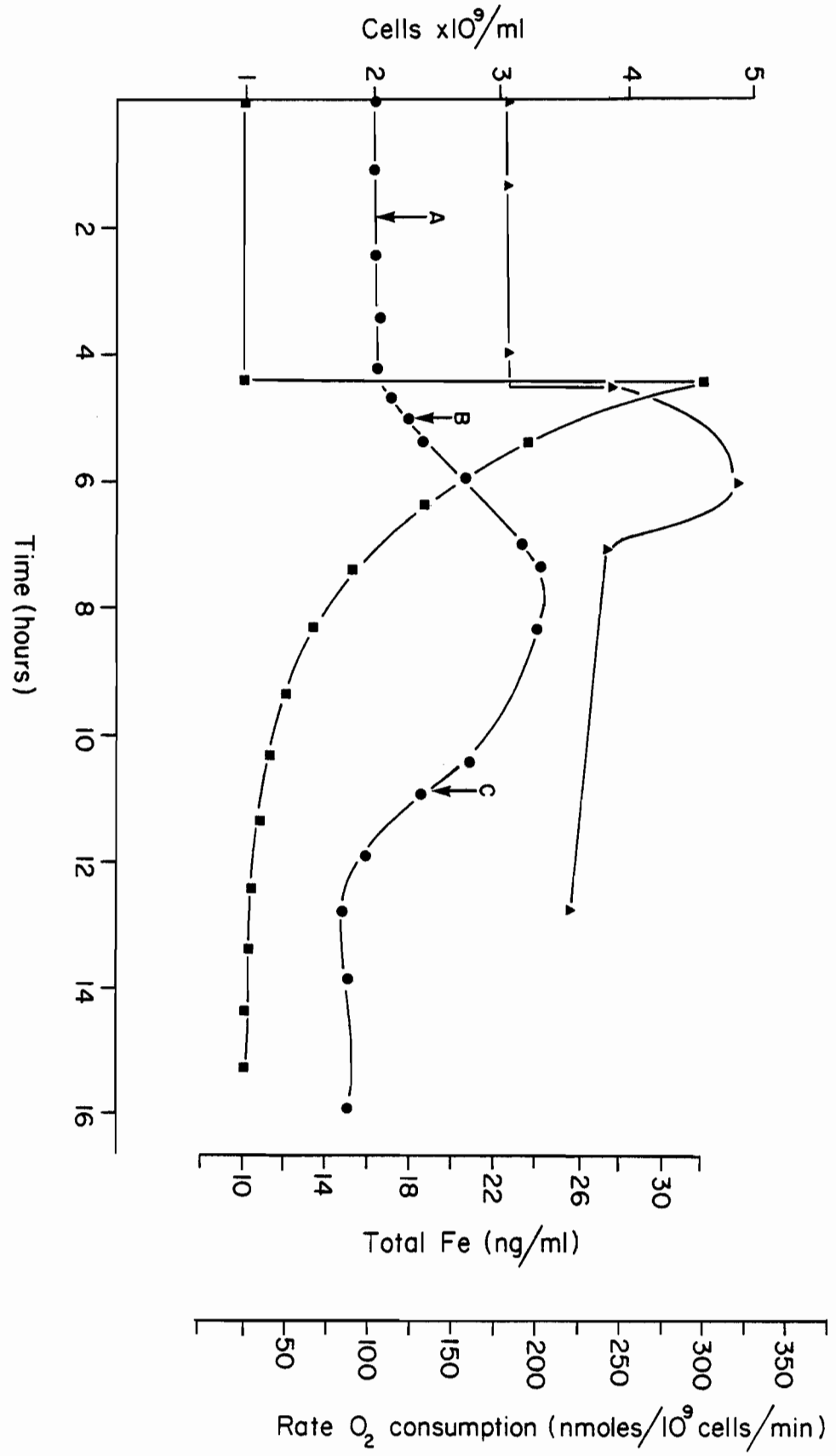
Jyssum, 1962) so that a greater proportion of the glucose-6-phosphate would be converted to fructose-6-phosphate via glucose-6-phosphate isomerase.

Both NADH and succinate dehydrogenases are presumably non-heme iron enzymes in the meningococcus, as they are in other microorganisms (Singer and Guttman, 1971; Light and Clegg, 1974), so the marked reduction in activity seen with succinate dehydrogenase and the slight reduction with NADH dehydrogenase are not surprising (Table 3), although much less than cellular non-heme iron as a whole which fell at least 5-fold (Table 2).

Response of Iron-limited Continuous Culture to an Iron Pulse

When an iron-limited continuous culture was pulsed with 22 ng/ml iron, the cellular growth rate and oxygen consumption showed marked increases within 15 min (Figure 6). Eventually, the cell density nearly doubled in response to the pulse, but as the decreasing culture iron could not sustain this population, the original equilibrium cell density re-established itself. Nearly identical results were obtained after pulsing iron-limited continuous cultures with iron (20 ng Fe/ml, final) as $FeCl_3$ or Fe^{+3}-citrate (Fe^{+3}:citrate ratio 1:3 or 1:10).

Figure 6. Effects of a pulse of ferrous ammonium sulfate (22 ng of Fe per ml, final concentration) on a continuous culture growing at equilibrium in iron-limited NDM medium. Symbols: Total culture iron (■); rate of O_2 consumption (▲); cells per milliliter (●). Generation times at points A, B and C were 115, 68, and 286 min, respectively.


Cells x10⁹/ml
1
2
3
4
5
A
B
C
Time (hours)
2
4
6
8
10
12
14
16
10
14
18
22
26
30
Total Fe (ng/ml)
50
100
150
200
250
300
350
Rate O₂ consumption (nmoles/10⁹ cells/min)

ID. Discussion

The iron concentrations required for "maximal growth" of a number of Gram-negative bacteria (see Lankford, 1973), including *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Alcaligenes faecalis* and *Pseudomonas aeruginosa*, are in the range of 20-80 ng iron per ml. Comparison of these published values is somewhat tenuous as the values will vary greatly, even in a single strain, depending on the complexity of the medium, the growth rate limiting factor, and the population density. A more meaningful measure of the cellular requirement for iron is that derived from the better defined environment of an equilibrium continuous culture.

In the work presented here, the iron required for continued viability and slow cell division of meningococcus SDIC, in continuous culture was 11-17 μg iron per gram dry weight or 2.2 to 3.3 x 10^{-9} ng per cell. This iron value is slightly less than one-tenth of that accumulated by cells grown in high iron concentration (204 ng iron per ml).

The cellular iron of an organism can be classified as that present in heme compounds, e.g. cytochromes, oxidases, catalase, or non-heme compounds which include a wide variety of storage, transport and catalytic molecules. Non-heme iron has generally been considered to form the large majority of

cellular iron, an opinion supported by the direct measurements by Kim and Bragg (1971) in E. coli (93% non-heme), and by Kurup and Brodie (1967) in Mycobacterium phlei (85% non-heme). These findings correlate well with those from the meningococcus in which total heme (417 nm), as well as the amount of cytochromes b and c, decreased less than 50% in response to iron privation, whereas total cellular iron was decreased approximately 10-fold. Further, using the 417 nm Soret absorbance and an E_mM of 120 to estimate total heme present, even iron-starved meningococci have a majority of their iron present as non-heme iron.

The very high proportion of bacterial iron found in non-heme forms and the wide variations in total cellular iron content with which the meningococcus can survive are in interesting contrast to animals. In man, about 70% of total body iron is in heme compounds, and there is only a small range through which total body iron can vary without severe anemia or hypersideremia (Wintrobe, 1974). A reasonable explanation for the high level of non-essential non-heme iron in the meningococcus would be that it was in storage forms. This would be a practical arrangement for organisms which have a high growth rate and frequently have difficulty obtaining iron.

The KCN-sensitive catalase of the meningococcus, presumably a heme protein, decreased at least eight-fold in response to iron privation. However, like the heme absorbance estimate,

the substrate turnover rates of 3.5×10^6 to 6.0×10^7/mol/sec, determined for other catalases (Chance and Herbert, 1950; Deisseroth and Dounce, 1970) suggest that the proportion of cellular iron present as catalase iron is extremely small, and thus would not account for the observed decrease in total cell iron. The difference between the non-heme iron and catalase activity levels, on the one hand, and cytochrome and TMPD-oxidase activity levels, on the other, indicates the iron priorities of the meningococcus in a low-iron environment.

Likewise, comparing the small iron privation-induced decrease in NADH dehydrogenase activity to the relatively large decrease in succinate dehydrogenase activity in the same cells may indicate the cells' iron priorities when iron becomes limiting, as well as presenting a conventional picture of NADH as the more important carrier of reducing power to the respiratory chain. An alternate explanation for the resistance of the meningococcal NADH dehydrogenase activity to iron privation may be found in Azotobacter vinelandii which, when grown under iron-limited conditions, replaced its usual NADH dehydrogenase containing four moles of iron per mole of flavin mononucleotide (FMN) with a different, but equally active NADH dehydrogenase having two moles of Fe and one to two moles of Mo per mole of FMN (Der Vartanian, 1972).

The sedimentation of 92% of the total iron in sonically

disrupted meningococci was not surprising, as one could expect a large proportion of iron-proteins to be membrane-associated; however, such sedimentation does reduce the possibility in meningococcus for a large low molecular weight iron "pool" of the type suggested by Arceneaux et al. (1973,1976) for schizokinen-Fe in Bacillus megaterium.

The stimulation of respiratory proteins by low dissolved oxygen tension that I observed has also been noted in other Gram-negative bacteria, e.g. E. coli (Moss, 1956; Wimpenny et al., 1963); Pseudomonas fluorescens (Lenhoff et al., 1956; Rosenberger and Kogut, 1958); and Enterobacter (Aerobacter) aerogenes (Moss, 1952). Under growth conditions of both batch and continuous culture, the oxygen consumption rates for the meningococcus were essentially linear in decreasing DO tensions down to < 2 nmol O_2 per ml. This is in agreement with the kinetics of oxygen consumption noted first by Gerard and Falk (1931) and subsequently by other workers (Harrison and Pirt, 1967). The K_{O_2} for the meningococcus was, therefore, 1 μM or less under all growth conditions tested. The increased TMPD-oxidase activity and cytochrome levels induced by low DO tensions would presumably make meningococcal respiration more efficient at non-saturating DO levels by increasing the number of target sites (oxidase) for oxygen.

Monod (1949) showed that the Michaelis-Menten equation

for the kinetics of enzyme-mediated reactions could be applied to microbial growth rates and continuous culture, i.e. $\mu = \mu max \frac{S}{K_s+S}$, where μ is the instantaneous growth rate, μmax the maximal growth rate, S the concentration of the growth-limiting substance necessary for a half-maximal growth rate. Therefore, if 20 ng Fe/ml decreased the generation time of the iron-limited continuous culture from 115 min to 68 min (Figure 6) and the minimum generation time (μmax) in the continuous culture with excess iron is 50-60 min (Table 2), the 50 ng Fe/ml figure for non-iron-limited meningococcal growth extrapolated from batch culture growth rates (Figure 3) would appear to apply to the continuous culture as well.

The pulsing of the iron-limited continuous culture with 22 ng/ml of iron (Figure 6) also demonstrated a sensitive system for assessing the availability of iron in any complex to the meningococcus. Since the ability of N. meningitidis to remove iron from host molecules may play a role in its virulence, the application to this assay system of a variety of relevant microbial and host iron-containing molecules was expected to prove informative (see Section II).

The work reported here has not attempted to demonstrate a link between meningococcal disease and iron metabolism, nor has it dealt with means by which meningococcus gets iron in vivo. Nevertheless, the work has provided a variety of basic

data on iron requirements, effects of iron limitation, and uptake of available iron by the meningococcus. Furthermore, this work demonstrates effective *in vitro* techniques that can be applied to determining the availability of iron from host and other iron-containing molecules.

II. METHODS OF IRON ACQUISITION AND THE AVAILABILITY OF FE IN VARIOUS COMPLEXES

IIA. Introduction

The presence of a low molecular weight siderophore (usually with either phenolic, salicylic, or hydroxamic acid functional groups), which is inducible by iron privation and promotes the efficient uptake of ferric iron from insoluble complexes, has been reported for virtually every aerobic microorganism investigated with the exception of some Arthrobacter and Cyanobacteria species (Reich and Hanks, 1964; Simpson and Neilands, 1976).

There is now considerable evidence suggesting that the ability of a variety of human pathogens to obtain iron is directly related to their ability to produce disease (Weinberg, 1978). Also, animal host iron-transport and sequestering systems have been shown to actively respond to infections by making serum iron less available to microorganisms (Weinberg, 1978). For both the gonococcus and the meningococcus there is some evidence linking iron and virulence. For example, cells of the more virulent T_1 and T_2 colonial types of N. gonorrhoeae took up iron more readily in chick embryos than the less virulent T_3 and T_4 types, and this difference appeared to be related to differences in their abilities to compete with the glycoprotein conalbumin for iron (Payne and Finkelstein, 1975).

The gonococcus has also been shown to respond to iron-limitation by the synthesis of three large proteins (76K, 86K and 97K d.) in the outer membrane (Norqvist et al., 1978). To date, there has been no siderophore identified in any member of the family Neisseriaceae, although there are two reports, one suggesting the presence of ethyl acetate-soluble "siderophores" in meningococcal and gonococcal cultures (Payne and Finkelstein, 1978a) and the other suggesting the complete absence of siderophores from gonococcal cultures (Norrod and Williams, 1978b).

Both hog gastric mucin, frequently used since the original report by Miller (1933) to establish infection in mice with low numbers of meningococci, and iron-dextran presently used for the same purpose appear to owe much or all of their infection-promoting ability to the presence of "sub-toxic" levels of iron (Calver et al., 1976; Holbein et al., 1979a,b). Not surprisingly iron privation has been shown to produce a variety of changes in the meningococcus that would presumably be detrimental to its establishment and proliferation in the host (Section I). These included the following: (a) 2-3 fold increased generation times; (b) inability to respond to low dissolved oxygen by inducing more respiratory proteins; (c) reduced efficiency of respiration; (d) no detectable catalase activity; and (e) a long and variable lag phase before growth.

Section II presents a variety of data which argue against the production by N. meningitidis of a conventional siderophore, and for the presence of an effective, specific iron-acquisition mechanism employing as functional siderophores low levels of certain organic acids and phosphate-ester compounds, some of which are normal components of host body fluids. Further, presumably by a second mechanism, the meningococcus appears to be able to effectively remove iron from transferrin but only when the cells come in direct contact with the protein. As a bacteremia occurs in most, if not all, forms of meningococcal disease, the ability of the meningococcus to remove iron from serum by either mechanism could be important.

IIB. Methods & Materials

Organisms

A range of carrier, disease, and prototype strains of N. meningitidis was obtained from various sources (Table4). Procedures for the maintenance of stock and working cultures and for routine checks of strain purity were described in Section I (DeVoe, 1976). Arthrobacter terregens (ATCC 13345) was obtained from the American Type Culture Collection, Rockville, Md., and the enterochelin auxotroph Salmonella typhimurium LT-2 enb-7 was the generous gift of Dr. J.B. Neilands. All experiments were performed with the serogroup B meningococcus SDIC, with

TABLE 4

Neisseria meningitidis strains employed

STRAIN	SOURCE[a]	SEROGROUP	REMARKS
M-136 (SDIC)	(D)	B	primary strain used in this work LD_{50}=2.0 X 10^1 CFU (DeVoe et al.,1973)
M-136 (SDIC) (R)	(D)	B	piliated rough colonial variant of SDIC (17)
M2092 (ATCC 13090)	(D)	B	LD_{50} = 1.1 x 10^6 CFU[b]
DRES 01[c]	(C)	B	LD_{50} = 6.9 x 10^1 CFU[b]
DRES 03[c]	(C)	B	LD_{50} = >10^8 CFU[b]
DRES 04[c]	(C)	C	LD_{50} = >10^8 CFU[b]
LCDC 2241[c]	(D)	C	LD_{50} = 1 x 10^0 CFU[b]
M-1011[c]	(D)	B	LD_{50} = 0.6 x 10^0 CFU[b]
C-138[f]	(C)	N/D	
C-195[f]	(C)	N/D	
SP-3424[e]	(D)	A	LD_{50} = 2.3 x 10^2 CFU[b]
SP-3428[e]	(D)	A	LD_{50} = 9.1 x 10^4 CFU[b]
SP-3453[e]	(D)	Y	
791[d]		A	*N. meningitidis* M-139
B-11[g]	(D)	B	
M-623[d]		D	*N. meningitidis* M-60
M-458[d]	(D)	B	
M-1028[d]	(D)	C	
M-405 [d]		X	*N. meningitidis* X (Slaterus)
M-407 [d]		Z	*N. meningitidis* Z (Slaterus)
M-521 [d]		29E	

a. D = strain isolated from active disease, C = strain isolated from carrier

b. LD_{50} values determined in mice by Holbein *et al.* (1979a).

c. Strains obtained from the Defense Research Establishment Suffield, Ralston, Alberta, Canada.

d. Strains received from the Neisseria Repository, NAMRU, School of Public Health, University of California, Berkeley, California.

e. Emilio Ribas Hospital, Sao Paulo, Brazil (maintained at McGill University, Montreal).

f. From Canadian Forces Base Cornwallis, Halifax, Nova Scotia.

g. Obtained through the courtesy of Dr. R.A. Finkelstein.

other strains used as noted in the text.

Media and Cell Growth

Cultures were grown on a complex medium, Mueller-Hinton (MH,Difco), or on a defined medium NDM, as previously described (Section I). Also detailed there is the preparation of low-iron NDM broth (3.5 to 6.0 ng Fe/ml), as well as its use in a continuous culture system to produce iron-limited meningococcal cultures under controlled conditions. Iron-limited continuous cultures were maintained as follows: 37°C; pH 7.4; dissolved O_2 at 60% of saturation; $pCO_2 < 50$ mm; cell density ± 2%/h at a value between 1.6 and 2.2 x 10^9 cells/ml; and generation times at a steady value between 140 and 200 min (see Section I). Congo red agar was formulated as recommended by Payne and Finkelstein (1977a). Thayer-Martin agar contained GC base, VCN antibiotic solution, 1.5% agar and 2% hemoglobin. All plates were incubated in a candle jar at 37°C (pCO_2 = 19 mm; pO_2 = 128 mmHg).

Siderophore Assays

Assays for the presence of a conventional siderophore were performed both on supernatant fluids of equilibrium continuous cultures and on supernatant fluids of continuous cultures to which fresh medium flow had been stopped so that cell growth continued for an additional 10-12 hr (final cell density 2.5 to 4.0 x 10^9 cells/ml). These cultures were centrifuged (2500 x g,

10 min), and the supernatant fluids were membrane-filtered (pore size 0.45 μm) and used immediately or after freezing (-80°C). Samples used in the seven different siderophore assays consisted of the following: (i) supernatant fluid; (ii) the supernatant fluid concentrated 20-fold by freeze-drying; and (iii) ethyl acetate extracts of the filtered supernatant fluid.

Ethyl acetate extracts were prepared by adjusting 135 ml of culture supernatant fluid to pH 1.5 with HCl and extracting into 34 ml of ethyl acetate. The ethyl acetate fraction was taken to dryness, resuspended in 1 ml of methanol, and allowed to dry on 6 mm filter paper discs (Whatman #3) for plate assays, or used directly (or diluted 1:1 with water) for difference spectra and colorimetric assays. The difference spectra (400-750 nm) of supernatant fluids or extracts, with and without the addition of several μg of iron as Fe^{+3} - citrate (1:10) or $FeCl_3$, were obtained with a Perkin-Elmer 356 Dual Beam Spectrophotometer (Chicago, III.). Absorbances in the Arnow phenolic acid (catechol) assay (Arnow, 1937) were measured at the 509 nm absorbance maximum of the dihydroxybenzoate standard. The Csaky hydroxylamine-hydroxamic acid assay was carried out with and without the sulfuric acid digestion step and measured at the 528 nm maximum of the L-glutamate-δ-monohydroxamate standard (Csaky, 1948). Cell density (600 nm) and colorimetric absorbances were determined on a Gilford 240 spectrophotometer

(Oberlin, Ohio).

Bioassays were performed with meningococcus SDIC, as well as S. typhimurium LT-2 enb-7 and A. terregens, two microorganisms that are unable to produce their own siderophores but have functional receptor sites for a wide variety of those produced by other organisms (Burnham, 1963; Luckey et al., 1972; Emery and Emery, 1973). The auxotroph S. typhimurium LT-2 enb-7 was maintained on MH agar; 6 to 8 h cultures were used to inoculate lawns onto medium E (Vogel and Bonner, 1956), and the assay was performed as described by Luckey et al. (1972) with DHB (2,3-dihydroxybenzoate) as the standard. A. terregens was maintained on Terregens Assay Medium with Desferal (Reich and Hanks, 1964), and substances to be assayed were placed on lawns of cells seeded onto Terregens Assay Medium lacking Desferal. The bioassays using SDIC were performed on the two iron-limited media exactly as were the other meningococcal plate assays.

Iron-Limited Plate Assays

A variety of substances able to form complexes or chelates with ferric iron were tested for their ability to withhold iron from the meningococcus or to facilitate iron acquisition by the meningococcus. In these experiments meningococci were grown as a lawn on a solid medium in which either total or available iron was distinctly growth-limiting. Three such media were

obtained by the following: (i) combining low-iron NDM broth and iron-extracted agar; (ii) addition of the high affinity polydentate carboxylic acid iron-chelator EDDA (ethylene diamine-di-ortho-hydroxyphenyl acetate) (Rogers, 1973) to MH or NDM agar; or (iii) extraction of 3% molten agar with NH_4OH-boiled, H_2O-washed Chelex-100 (Na^+-form) (Bio-Rad, Toronto), vacuum-filtered through sintered glass and added to double-strength, low-iron NDM medium which was then autoclaved. When saturated with iron and at >100 times the concentration used in the assay plate, EDDA itself caused no inhibition of meningococcal growth. MH and NDM agar made growth-limiting with EDDA were prepared, with final iron concentrations of 592 ng/ml (435 ng/ml in MH broth; 157 ng/ml in Difco agar) and 163 ng/ml (4-7 ng/ml in low-iron NDM broth and 157 ng/ml in Difco agar), respectively. After autoclaving, 6.44 μg EDDA (1000 ng/ml Fe^{+3}-binding capacity) per ml was added to the molten MH agar to form MHA-EDDA medium, and 1.13 μg EDDA (175 ng/ml Fe^{+3}-binding capacity) added to the molten NDM agar to form NDM-EDDA medium.

The iron-limited agar media were used to test the ability of iron-limited meningococci to respond to various compounds as follows: (a) organisms maintained on frozen slants (-80°C) were plated onto an NDM-160 (163 ngFe/ml) agar plate (37°C) and after 12-18 h subcultured onto the same medium; (b) after 8-11 h the culture was suspended in broth (3-5 x 10^8 cells/ml), and 0.1 ml

was spread evenly onto each of a series of NDM-EDDA, MHA-EDDA, or iron-extracted NDM plates; (c) test compounds were added in 5-40 µl H_2O on sterile 6 mm discs (Whatman #3) or in 4 mm wells, and the plates incubated at 37°C (candle jar pCO_2=19 mm, pO_2= 128 mmHg). All critical comparisons were performed on the same plate, and duplicate or triplicate plates run in each trial. The plate assays were scored at 10 to 12, 15 to 16, and 20 to 24 h, and the results of the earliest assessment showing sufficient lawn growth were used. Except in certain instances, e.g. those involving strains M-1011 and LCDC 2241, the 10 to 12 h plates were used, as older plates showed little or no change in growth or inhibition responses.

Plate assay results were quantified by multiplying the area of growth exhibition (mm^2) less the area of the disc or well by the intensity of growth exhibition, assigned values from +1 (faint) to +4 (heavy) or the intensity of inhibition from 0 (no response) to -3 (complete lawn inhibition). Conclusions were drawn only from repeatable differences, against a control of 1.0 µg iron as Fe^{+3}-citrate (1:10) in 5 µl H_2O. Important comparisons were made on the same plate, and only plates showing light, even lawns were scored.

In practice, the numerical values obtained for the lawn responses were reproducible and reflected visual differences (Figure 7). The obvious shortcomings of this assay included

"saturation" of the growth response by available, poorly diffusible forms of iron such as hog gastric mucin and the assumption that the arbitrary growth intensity factor was in a linear relationship with iron-availability to the test organism. Perhaps as a result of this, quantitation also seemed to be somewhat biased towards highly diffusible compounds like EDTA, DTPA or rhodotorulic acid. Since most assay plates incorporated a control of 1.0 μg iron as Fe^{+3}-citrate (1:10), the coefficient of variation (C.V.) of the growth responses to the control was determined on a large number of assays. The C.V. of growth responses between repeated assays was 20.3% and between replicate discs in the same assay 7.1%.

Transferrin Iron Uptake

The availability of transferrin-bound iron was measured as the growth response of iron-limited broth cultures of SDIC either to the addition of ^{55}Fe-transferrin (Fe-Tf) directly or within a dialysis bag. The initial pH of the culture was 7.35 to 7.40 and $[HCO_3^-]$ was 27 mM. Samples (0.3 ml) for ^{55}Fe determination were placed in 8 ml PCS (Amersham-Searle) containing 50 μg each of Desferal (CIBA-Geigy) and o-phenanthroline in 0.5 ml H_2O and were counted in an Isocap 300 Scintillation Counter (Chicago Nuclear).

Assay Substances

Suspensions of hog gastric mucin (HGM) (Wilson, Chicago) were prepared either by tyndallization or autoclaving (see Pittman, 1941). Where appropriate, HGM was dialyzed against several changes of distilled-deionized water or several changes of a solution containing 10 mM EDTA, 10 mM Tris (Trizma, Sigma; pH 7.4) followed by several changes of distilled-deionized water. Prepared HGM was stored at 4°C and before each use the sediment was resuspended. Horse spleen ferritin and apoferritin were used as received (undialyzed) or after dialysis against the 10mM EDTA-Tris solution and then water. All ferritin was filter-sterilized, stored at 4°C, and the dialyzed preparations used within a few days.

Human transferrin (Tf) (Sigma grade II) and chicken conalbumin (Con) (Sigma type I) were dissolved in 20 mM Na-citrate (at pH 5.1 and 5.2 respectively) then bound to, washed on, and deferrated by a column of CM-Sepharose CL-6B (Pharmacia) using 10-20 bed-volumes of buffer. Elution of the glycoproteins used 20 mM Na-citrate at pH 5.55 and 5.8 respectively, followed by repeated dialysis against water. Tf 30% saturated with Fe^{+3} (Fe-Tf) was prepared by dissolving purified Tf in 40 mM Tris, 2 mM $NaHCO_3$, (pH 7.4, stirring, 25°C) for 30 min with 0.42 µg $^{55}Fe^{+3}$ as Fe-citrate (1:10) /mg Tf and dialyzing repeatedly against the tris-bicarbonate (4°C). ^{55}Fe-Tf (268,000 DPM/mg)

was filter-sterilized and stored at -80°C. ^{55}Fe-Tf prepared by this method should initially have Fe^{+3} preferentially bound to the a specific site, which then may slowly be redistributed to the b site (Aisen et al., 1978).

Human hemoglobin, sperm whale myoglobin and horse heart cytochrome c were all dissolved in 150 mM NaCl, 40 mM Tris, pH 7.4 so that the 30 µl sample contained 1 µg iron. In addition, the hemoglobin and myoglobin were treated with Tris-NaCl-washed Chelex-100 (Bio-Rad) to remove any loosely bound iron. This treatment caused no change in the visible absorbances of the hemoglobin and myoglobin preparations, but could not be used with cytochrome c as the resin bound the entire protein. The commercial hematinic iron preparations were used as received. In general, assay substances were prepared so as to yield the desired amount in a volume of 5 to 10 µl of water. The proteins and HGM were exceptions in that they were applied in 20 to 40 µl quantities, nearly the maximum capacity of a 4 mm well. Most iron complexes were made up shortly before use in sterile H_2O by slowly adding acidic $FeCl_3$ to a solution of the complexing substance and adjusting the final pH. Most of the organic acids were made up in a 10:1 ratio with Fe^{+3} to enhance the solubility of the resulting Fe^{+3} complex and, at least with citrate, to have a majority of the iron solubilized in the simple, stable complex ferric dicitrate (Bates et al.,

1967). The synthetic chelators were prepared in a 3:1 molar ratio with Fe^{+3}. Nitrilotriacetate (NTA) and Fe^{+3} are known to form a simple (1:1) chelate at this ratio (Bates et al., 1967). Except for HGM and complexes which precipitated, all were filter-sterilized.

Metal Determinations

Iron and cadmium were determined by atomic absorption spectrophotometry of concentrated solutions (Perkin-Elmer model 703). Iron in viscous or insoluble materials was solubilized with 3N HCl at 121°C. Very low levels of iron were assessed by the method of Smith et al. (1952), modified as described in Section I.

Chemicals

All organic compounds, with the exception of albomycin, the siderophores, Congo red, the hematinic iron preparations and EDDA came from Sigma Chemical Co., St. Louis.

EDDA (ICN Pharmaceuticals, Plainview, N.Y.) was purified by the method of Rogers (1973). A stock solution was prepared by solution of 5 mg/ml in 0.36 N NaOH, then set to pH 8.0 with 10 N HCl and filter-sterilized.

Congo red (J.T. Baker, Phillipsburg, N.J.) was used as received.

Deferriferrichrome and albomycin were kindly supplied by

Dr. J. Coulton, and rhodotorulic acid, enterochelin (enterobactin), and ferrichrome were the generous gift of Dr. J.B. Neilands. Desferal was kindly supplied by CIBA-Geigy (Montreal). The hematinic iron preparations used were Imferon (Fisons, Don Mills, Ontario), Jectofer (Astra, Mississauga, Ontario), and Palafer (Mowatt and Moore Ltd., Montreal, Quebec).

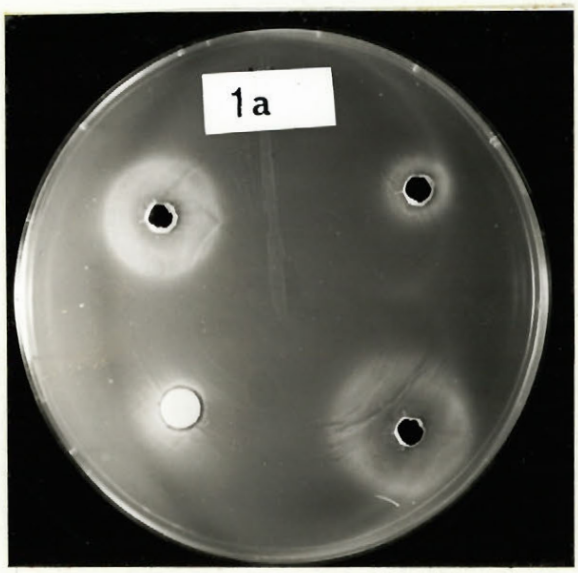
IIC. Results

When the supernatant fluids from equilibrium continuous cultures and from continuous cultures allowed to grow with the medium flow shut off for 10 to 12 h were assayed for the presence of soluble siderophores, uniformly negative results were obtained (Table 5). While no assay can prove the absence of a siderophore, one or more of the assay techniques employed in this study have been used for the initial detection of nearly all known siderophores [see references in Lankford (1973) and Neilands (1974a) as well as Tait (1975), Marcelis *et al.* (1978), Meyer and Abdallah (1978), Cox and Graham (1979) and McCullough and Merkal (1979)]. However, with most microorganisms, appreciable levels of siderophores accumulated only after the iron-deficient culture was allowed to remain in stationary phase 24 hours or more, a procedure not feasible with the meningococcus due to its rapid death and autolysis under such conditions. Nevertheless, the absence of a detectible iron difference

Figure 7. Examples of the iron-limited plate assay technique using strain SDIC grown on NDM-EDDA agar.

1a. Shows stimulation of lawn growth by 0.33 mg HGM (+180) (upper right), the same preparation plus 330 nm Na-citrate (+900) (upper left), and the same preparation plus 330 nm NTA (+1000) (lower right). NTA *per se* (lower left) although lacking Fe, apparently slightly enhances the acquisition of even the EDDA-bound iron in the medium (+100).

1b. Shows the response of a lawn of SDIC to $\simeq$ 1 mg; $FePO_4$ *per se* (lower right) (trace); with 330 nm EGTA (lower left) (+1300); and 330 nm NTA (upper right) (+2900); and pyrophosphate (upper left) (+1800).


1a

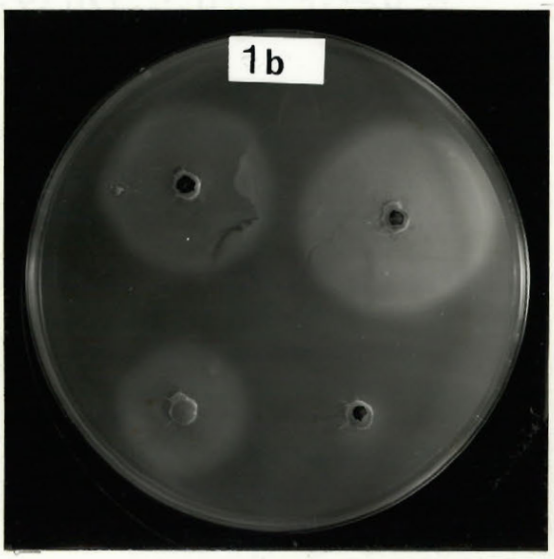

1b

TABLE 5

Siderophore assays performed using spent media from iron-limited, continuous cultures of SDIC allowed to grow 10 to 12 h after cessation of the medium flow.

Assay	Concentration of Siderophore in Culture[e]		
	Supernatant Fluid[a]	Concentrate[b]	Extract[c]
± ferric iron absorption difference spectrum (400-750 nm)	<500 nM[d]	<25 nM[d]	<3.7 nM[d]
Arnow phenolic acid assay	<1.3 µM	<65 nM	<9.6 nM
Csaky hydroxamate-hydroxylamine assay	<240 nM	<12 nM	<1.7 nM
S. typhimurium LT-2 enb-7 bioassay	<3.2 µM	<160 nM	—
A. terregens bioassay	<1.5 µM	<75 nM	—
Meningococcus SDIC bioassay (on NDM-EDDA)	no response	no response	—
Meningococcus SDIC bioassay (on iron-extracted NDM)	no response	no response	—

a From membrane-filtered culture supernatant fluid.

b From 20-fold concentration (by freeze-drying) of supernatant fluid.

c From ethyl acetate extract of culture supernatant fluid

d Assuming a ΣmM of 2.0 and an .001 ODU minimum significant response for the spectrophotometer. Most known ferrated siderophores have millimolar extinction coefficients which exceed this value (e.g. Fe^{+3} - ferrioxamine B ΣmM = 2.6 at 420 nm; Fe^{+3}-ferrichrome ΣmM = 2.5 at 440 nm; and Fe^{+3} pyoverdine ΣmM $\approx$ 20 at 402 nm) and, therefore, could be detected at correspondingly lower concentrations.

e Results are expressed as the maximum concentrations of an active siderophore that could have been present given the uniformly negative results obtained

spectrum and the inability of a supernatant fluid, untreated or concentrated, or an extract of the supernatant fluid, to stimulate growth of strain SDIC itself on an iron-deficient assay plate strongly suggests the lack of useful levels of conventional siderophore. However, these results in no way rule out the possibility of specific soluble substances capable of degrading and/or removing iron from animal iron-containing molecules such as ferritin, heme proteins, or the transferrins.

The demonstrated ability of at least some microorganisms to take up with high affinity a wide variety of ferrisiderophores produced by other microbial species was used as a basis for experiments to determine whether a conventional siderophore was produced by the meningococcus (Table 5). In the plate assays the reverse, i.e., the ability of SDIC to use the siderophores of other bacteria or fungi, was tested (Table 6). In all instances the deferrated siderophores were bacteriostatic, but in the presence of sufficient iron to saturate their binding capacity they had no adverse effect on growth or were, in fact, stimulatory. These results, together with the S. typhimurium and A. terregens bioassays (Table 5), suggest that the meningococcus neither produces its own siderophore nor uses several conventional siderophores, and cannot even successfully compete against them for the acquisition of medium iron.

The lack of a response to albomycin, an antibiotic believed to be effective only against organisms with functional ferrichrome uptake systems (Braun et al., 1976), or to potential precursors of hydroxamate-, salicylate-, or catechol-type siderophores further suggests the absence of a "conventional" iron-uptake system. As a control, the four siderophores (Table 6) were assayed against the S. typhimurium and A. terregens bioassay strains and either in the ferrated or deferrated form produced dramatic growth responses (not shown).At a dose of 0.5 μg, albomycin was highly toxic to both organisms used for the bioassay.

Many metabolic organic acids are good ferric iron complexing agents and some, notably citrate and lactate, are normally present at appreciable levels in the serum, cerebrospinal fluid, and nasopharynx. Escherchia coli and to a lesser extent some other organisms have been shown to use citrate to acquire iron (Lankford, 1973). Results in Table 7 show that the meningococcus can indeed obtain iron from a number of iron-organic acid complexes, particularly Fe^{+3}-citrate, Fe^{+3}-pyruvate, Fe^{+3}-isocitrate and Fe^{+3}-NTA. The poor growth response to the addition of some organic acids was due, at least in part, to precipitation aggravated by neutral pH and the relatively concentrated solutions used to permit the addition of required amounts of iron complex in a volume of 5 or 10 μl.

TABLE 6

Ability of some siderophores and related compounds to donate iron to or withhold iron from *N. meningitidis* SDIC growing on NDM-EDDA agar plates

Substance	Origin	Fe^{+3}:Substance (molar ratio)	Total Fe per assay (μg)	Relative Growth Response
Microbial Siderophores				
Fe^{+3}-Desferal[a]	*Streptomyces spp.*	1:3	0.5	-1100
Desferal plus Fe^{+3}-citrate (1:10)	*Streptomyces spp.*	1:0.9	0.5	+350
Fe^{+3}-Desferal plus Na-citrate (26 μg)	*Streptomyces spp.*	1:3	0.5	-1500
Fe^{+3}-ferrichrome	numerous fungi	1:1	0.35	+70
Deferriferrichrome (5 μg)	numerous fungi	—	0	-550
Fe^{+3}-rhodotorulic acid	*Rhodotorula spp.*	1:1	3.0	+1900
Deferri-rhodotorulic acid (18 μg)	" "	—	0	-1400
Fe^{+3}-enterochelin[b] (5.6 μg)	Enterobacteriaceae	1.4:1	0.66	+1000
Fe^{+3}-enterochelin[b] (5.6 μg)	Enterobacteriaceae	0.36:1	0.17	-500
Deferri-enterochelin[b] (5.6 μg)	Enterobacteriaceae	—	0	-700
Siderophore Precursors, Related Substances				
Albomycin (grisein; 0.5 μg)		—	0	0
Albomycin (0.5 μg) plus Fe^{+3}-citrate (1:10)		—	1.0	+950
2,3-dihyroxybenzoate (DHB) (16.5 μg)		—	0	0
DL-dihydroxyphenylalanine (DOPA; 20 μg)		—	0	-40
DL-dihydroxyphenylalanine plus Fe^{+3}-citrate (1:10)		—	1.0	+1400
L-glutamate monohydroxamate (20 μg)		—	0	-800
L-glutamate monohydroxamate (20 μg) plus Fe^{+3}-citrate (1:10)		—	1.0	-900
Fe^{+3}-salicylic acid		1:3	1.0	+20
Fe^{+3}-sulfosalicylic acid		1:3	1.0	+130
Fe^{+3}-salicylhydroxamate (SHAM)		1:3	1.0	+600

a. Commercial sulfonated preparation of the streptomycete siderophore deferrioxamine B.

b. A crude preparation containing 56% DHB serine and polymers thereof by the Arnow assay and having an ΣmM_{497} of 2.6 when ferrated, pure enterochelin having an ΣmM_{495} of 4.2 (O'Brien *et al.*, 1971).

TABLE 7

Ability of some organic acid-iron complexes to donate iron to *N. meningitidis* on NDM-EDDA agar plates

Substance	Fe^{+3}:substance (molar ratio)	Total Fe^{+3}(g) per assay	Relative Growth Response
Synthetic chelators[d]			
Fe^{+3}-EDDA	1:1	1.0	trace
EDDA (7 µg) alone	—	0	-1200
Fe^{+3}-ADA	1:3	1.0	+600
Fe^{+3}-DTPA	1:3	1.0	-500, +800[a]
Fe^{+3}-EDTA	1:3	1.0	+1300[a]
Fe^{+3}-EGTA	1:3	1.3	+450
Fe^{+3}-NTA	1:3	1.0	+1200
Metabolic organic acids			
Fe^{+3}-acetate	1:10	1.0	trace[b]
Fe^{+3}-cis-aconitate	1:10	1.0	+700 [c]
Fe^{+3}-citrate	1:10	1.0	+1400
Fe^{+3}-isocitrate	1:10	1.0	+950
Fe^{+3}-ketomalonate	1:3	0.5	+1100
Fe^{+3}-lactate	1:10	1.0	trace[b]
Fe^{+3}-malate	1:10	1.0	+1200[c]
Fe^{+3}-oxalate	1:10	1.0	+750
Fe^{+3}-pyruvate	1:10	1.0	+1200
Fe^{+3}-succinate	1:10	1.0	trace[b]

Footnotes

a. Large zones of light inhibition surrounded by a larger halo of light exhibition. Iron-free EDTA and DTPA caused marked inhibition unlike citrate, pyrophosphate or NTA.

b. Heavy precipitation of $[Fe(OH)_3]n$ from the stock solution (1.8 mM Fe, pH 7.0 - 7.5) before use.

c. Haze or light precipiation in stock solution within 24 h after preparation.

d. EDDA, Ethylenediamine-di-orthohydroxyphenylacetic acid, or EDHPA; ADA, N-(2-acetamido)-iminodiacetate; DTPA, diethylenetriamine pentaacetate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis (β aminoethylether)-N, N'-tretraacetic acid; NTA, nitrilotriacetate.

Because of the vigorous growth response obtained with Fe^{+3}-citrate and the structurally similar Fe^{+3}-NTA, both on the assay plates (Tables 7, 9) and in short term iron uptake experiments (Section III), a variety of related synthetic iron-complexes was assessed for their iron-donating ability (Table 7). The meningococcus was able to remove iron from all those tested, even though iron-free EDTA or DTPA has an inhibitory effect on growth. It is noteworthy that the formation constants for the Fe^{+3}-EDTA and Fe^{+3}-DTPA complexes under standard conditions are given as $10^{25.1}$ and $10^{27.4}$, respectively, and their effective constants, which take into account the side-reactions with H^+, OH^-, and Ca^{++} to be expected under physiological conditions, are reported as 10^9 and 10^{12}, respectively, at pH 7.3 (Schubert, 1963). ADA is another powerful, rather nonspecific iron-chelator whose iminodiacetate functional group forms the active portion of the metal chelating resin Chelex-100, which can efficiently extract iron from microbiological media.

These findings support the hypothesis that the meningococcus possesses an effective high-affinity iron-acquiring system with a predilection for hydroxy acid or carboxylic acid-bound iron. This hypothesis is further supported by the fact that iron bound to citrate - a weaker iron chelator than DTPA, EDTA, or ADA - is completely unavailable to S. typhimurium LT-2 enb-7 and A. terregens, organisms which do not themselves produce

TABLE 8

Ability of some hematinic and animal iron-complexing compounds to donate iron to or withold iron from *N. meningitidis* SDIC growing on NDM-EDDA agar plates

Substance	Fe:Substance molar ratio	Total Fe (µg) per assay	Relative Growth Response
Hematinic iron preparations			
Imferon (iron-dextran)	—	0.5	o-trace
Jectofer (iron sorbitol citrate)	—	0.5	+450
Palafer (iron fumarate suspension)	—	0.5	+600
Animal iron sources			
HGM[a] undialyzed	—	0.63	+270[b]
HGM[a] water-dialyzed	—	0.58	+250[b]
HGM[a] 10mM EDTA-dialyzed (<0.1 µg Fe/mg)	—	0	trace
Myoglobin (0.30 mg)	1:1	1.0	+260[b]
Cytochrome C (0.22 mg)	1:1	1.0	o-trace
Hemoglobin (0.29 mg)	4:1	1.0	+1000[b]
Ferritin, undialyzed (0.01 mg)[c]	—	2.5	+220
Ferritin, EDTA-dialyzed (0.01 mg)[c]	—	1.4	o-trace
Ferritin, EDTA-dialyzed (0.01 mg) stored[d]	—	1.4	+600
Ferritin, undialyzed (1.0 mg)	—	248	-250,+650[e]
Ferritin, undialyzed (0.50 mg)	—	124	-210,+650[e]
Ferritin, undialyzed (0.50 mg) plus 0.33 um Na-citrate	—	124	-210,+1600[e]
Apoferritin (1.0 mg)	—	0.07	-250[e]
Apoferritin (1.0 mg) plus Fe^{+3}-citrate (1:10)	—	1.0	-250,+1800[e]
Deferri-conalbumin (1.0 mg)	—	0	-400
Deferri-conalbumin (1.0 mg) plus Fe^{+3}-citrate (1:10)	1:0.7	2.0	+1800
Deferri-conalbumin (1.0 mg) plus Fe^{+3}-NTA (1:3)	1:1.4	1.0	+1000

			11 h	15 h	23 h[f]
Deferri-transferrin (1.0 mg)	—	0	-150	-70	0
Deferri-transferrin (1.0 mg) plus Fe^{+3}-citrate	1:1.4	1.0	+550	+650	+650

...../2

Page 2 of Table 8...continued

Footnotes

a. Hog gastric mucin (0.33 mg) containing 1.9 µg Fe/mg when undialyzed, and 1.8 µg Fe/mg when dialyzed against water.

b. Small zones of very heavy growth exhibition. The limited diffusion of the substance was believed to have reduced the relative growth response compared to smaller complexes with similar iron-availability to the meningococcus.

c. Dialysis reduced the Cd^{+2} content of the ferritin from 2.9 µg/mg protein to 0.13 µg/mg protein.

d. Dialyzed ferritin stored for 2 months in filter-sterilized solution at 4°C.

e. Negative values represent a sharp halo of complete inhibition around the well.

f. Only transferrin results showed significant change with time. The transferrin and conalbumin data has been published previously (Archibald and DeVoe, 1979a).

soluble siderophores. In E. coli, the low-affinity uptake system has been defined in terms of its inability to remove iron from NTA (Frost and Rosenberg, 1973), a particularly useful complex for the meningococcus.

The poly-carboxylic acid chelator EDDA, used in NDM-EDDA and MHA-EDDA to obtain iron-limited growth, has one of the highest effective ferric iron binding constants known (10^{25}) and is also the most effective substance tested for removing tissue-bound iron from the mouse (Pitt et al., 1979). It is noteworthy, therefore, that the meningococcus can grow in the presence of more EDDA iron-binding capacity than that required to bind the total medium iron. Deferri-EDDA has been shown to produce bacteriostasis in strains of Klebsiella, Escherichia, Proteus, Salmonella and other bacteria. Such growth inhibition is apparently reversible only after the production of siderophores such as enterochelin (Rogers, 1973; Miles and Khimji, 1975; Khimji and Miles, 1978).

The ability of phosphate-ester compounds to bind iron and to facilitate the removal, addition or transfer of iron to and from transferrin (Egyed, 1975; Morgan, 1977; Pollack et al., 1976; Konopka, 1978) led to the assessment of ferric pyrophosphate (1:3) as a source of Fe^{+3} for SDIC (Table 9,10). Although the structures of Fe^{+3}-pyrophosphate and Fe^{+3}-NTA chelates differ markedly, the meningococcus grow well in the presence of

TABLE 9

Siderophore-like activity of various compounds with meningococcus SDIC[a]

Potential Iron Carrier	Iron Sources		
	20 ug Ferritin (EDTA-dialyzed) (5.0 ug iron)	0.33 mg HGM[c] (0.63 ug iron)	1.0 mg $FePO_4$ (370 ug iron)
No addition	+80	+250 [+200][d]	trace
Acetate[e]	+80	+200	trace
Cis-aconitate[e]	+50	+250	+100
Citrate[e]	+330	+1050 [+900][d]	+1700
Iso-citrate[e]	+130	+300	+1000
Ketomalonate	trace	+800	+1100
Lactate	+80	+200	trace
Malate	+70	+420	+1000
Oxalate	trace	+300	+700
Pyruvate	+70	+200	+120
Succinate	+80	+200	trace
Pyrophosphate[e]	+370	+1100 [+1300][d]	+1800
Salicylate	0,-50	+240,-110[f]	-160
Sulfo-salicylate	+100	+550	+550
Salicyl-hydroxamate (SHAM)	+240,-30[f]	+700	+1200, -110[f]
Acetyl-salicylate	trace	+80	-40
ADA	+160	+600	+1200
DTPA	-800	-500	+2900, -1400[f]
EDTA[e]	-700	0	+2000
EGTA	+70	+380	+1300
NTA	+500	+900	+2900

Footnotes

a. Assays were performed on NDM-EDDA as before (Tables 6-8) and growth responses measured in the same way.

b. In all instances 0.33u mole of test compound per assay was used.

c. Hog gastric mucin (undialyzed).

d. Using 0.01 mg of iron as the hematinic Imferon

e. Na-salt of the compound was used. All others were used as the free acid.

f. Response consisted of an inner ring of inhibition, an outer ring of exhibition.

TABLE 10

Siderophore-like activity of several phosphate compounds with meningococcus SDIC[a]

Additions to 0.33 mg HGM[b]	Relative Growth Response
no addition	+200
Na^+-pyrophosphate	+1400
adenosine triphosphate	+900
adenosine diphosphate	+1000
Na^+-glycerophosphate	+200
D-glucose-6-phosphate	+200
$Na\ H_2PO_4$	+200

Footnotes

a. The assays were performed exactly as in Table 6

b. As in Table 6, 330 nm of each phosphate compound was added to wells containing 0.33 mg undialyzed hog gastric mucin.

either of these complexes.

Classically, the most popular model for meningococcal virulence has been the mouse, but disease has been produced by small numbers of organisms only when an adjunct, usually hog gastric mucin (HGM), was used. It was, therefore, important to determine whether the high iron levels in HGM, or substitutes such as iron-dextran or other hematinic preparations, were readily available to the meningococcus *in vitro*. For HGM and two hematinic preparations good iron-dependent growth responses were obtained, but the iron-dextran preparation (Imferon) did not produce an appreciable enhancement of growth (Table 8). This finding is interesting in view of the demonstration by Holbein *et al*. (1979a,b) that the presence of iron was required for iron-dextran to stimulate meningococcal infections in mice. Moreover, Payne and Finkelstein (1977b) showed improved growth on a GC base medium after the addition of 20 μg iron (as Imferon) per ml. In our experiments, HGM typically gave a small but very heavy zone of growth exhibition, the small diameter presumably due to the poor diffusion of the mucin through the agar.

Of obvious importance was the ability of the meningococcus to obtain iron from animal iron-sequestering, storage, or other functional molecules (Table 8). Results with transferrin and conalbumin show a distinct difference in that SDIC was able to overcome with time the bacteriostasis produced by human

transferrin, but not that of conalbumin, despite the strong similarity of the two glycoproteins (Aisen and Liebman, 1968b). Both unpurified and Chelex-100-extracted commercial sperm whale myoglobin and human hemoglobin (twice crystallized) gave good growth responses (Table 8) indicating that heme-bound iron was readily accessible. In contrast, a commercial preparation of cytochrome c did not stimulate growth. Some iron in an undialyzed commercial ferritin preparation appeared to be available to SDIC, but this availability was related to the presence of dialyzable forms of iron because EDTA-dialyzed fresh material containing appreciable iron had little or no effect on growth (Table 8). A well-defined zone of complete inhibition was also noted when either 0.5 or 1.0 mg ferritin samples were used. This effect was independent of ferritin-iron content, any cadmium present in the preparation, or the iron-limitation of the medium. It appeared to be a property of the concentration of the ferritin protein itself. Similar growth inhibition was not observed with hemoglobin, myoglobin, conalbumin, or transferrin, even though they were present at 3 to 13 times the molar concentration of ferritin.

In an effort to determine whether the information obtained on the acquisition of iron by meningococcal strain SDIC was also applicable to other meningococci, 20 meningococcal strains (Table 4) were surveyed using the same plate-assay technique.

As expected, all strains grew well on the simple defined NDM-160 (163 ng iron/ml) and NDM-360 (363 ng iron/ml), with the exception of strain M2092, which grew more slowly. The virulence (LD_{50}) of some strains for mice was known from the literature (Table 4). To these data were added the results from the Congo red assay for virulence (Payne and Finkelstein, 1977a) on all strains. Despite their wide variation in virulence for the mouse, all meningococcal colonies, with the exception of those of M2092, were uniformly stained with Congo red. Therefore, in contrast to the findings of Payne and Finkelstein with the gonococcus (1977a), we were unable to find any correlation between Congo red staining of colonies and the known virulence in mice of selected meningococcal strains.

This ability to bind Congo red may, according to Surgalla and Beesley (1969), demonstrate the ability of organisms to bind and deferrate hematin, a reasonable possibility with the meningococcus in view of our results obtained with hemoglobin and myoglobin. If this is the case, then the results suggest the ability to acquire heme iron does not separate virulence from avirulence among meningococcal strains. To determine whether such a correlation might exist between virulence and the ability of a strain to acquire iron from, or in the presence of, certain other compounds, all 20 strains were used in plate assays with ferritin, HGM, Imferon, Jectofer, Fe^{+3}-citrate

(1:10), Desferal, and ferriferrichrome in the amounts and by the methods used with strain SDIC, as described in Tables 6, 7 and 8. In most cases, the results (not shown) were essentially the same as those obtained with SDIC, although strains varied somewhat in their responses to 1 mg undialyzed ferritin on the NDM-EDDA media. While all strains had the sharp inner halo of ferritin "protein" inhibition, several strains (C-38, B-11, 791, M-1028, DRES 03, C-195, M-1011 and SP-3428) showed either no outer zone of growth exhibition or an enlarged inner zone of inhibition on the NDM-EDDA medium. All showed the typical response of strain SDIC to ferritin on MHA-EDDA medium. Although the results of the dialysis of ferritin suggested that most of the available iron was an artifact of initial purification or storage, this was the one iron-related assay which appeared to show distinct strain and medium-related differences. However, these differences did not appear to correlate with virulence for the mouse and did not occur on the complex medium. Also, the *in vitro* growth-inhibiting effect of ferritin protein on meningococci is of doubtful importance to extracellular *in vivo* growth as a normal (child) serum ferritin level is 17 ± 7 ng/ml (Koller *et al.*, 1978), or 4 to 5 orders of magnitude below the levels which produce inhibition *in vitro*.

Table 11 illustrates the results after comparing the ability of several strains of known virulence (mouse) to acquire

TABLE 11

Ability of four strains differing widely in their virulence for the mouse to obtain iron bound to hog gastric mucin, ferritin, or $FePO_4$ when grown on NDM-EDDA assay plates[a]

STRAIN	SUBSTANCE	TOTAL IRON PER ASSAY (μg)	RELATIVE GROWTH RESPONSE[b]
SDIC	0.01 mg ferritin (EDTA-dialyzed)	1.4	+20
LCDC-2241	" "	1.4	+10
DRES 03	" "	1.4	+50
M-1011	" "	1.4	0
SDIC	0.33 mg hog gastric mucin (undialyzed)	0.60	+180
LCDC-2241	" "	0.60	+280
DRES 03	" "	0.60	+180
M-1011	" "	0.60	+180
SDIC	$FePO_4$ suspension	≈500	0
SDIC	" " +660 nm Na-citrate	"	+1700
LCDC-2241	" "	"	+120
LCDC-2241	" " +660 nm Na-citrate	"	+2800
DRES 03	" "	"	0
DRES 03	" " +660 nm Na-citrate	"	-150[c], +2900
M-1011	" "	"	0
M-1011	" " +660 nm Na-citrate	"	+1900

a. A parallel experiment was run on MHA-EDDA agar plates and the results were essentially identical except for the hog gastric mucin plates (see text).

b. Values <100 represent a small response and showed some variation between assays.

c. Small inner halo of growth inhibition.

iron from one poorly diffusible and two poorly available forms in both complex and defined media. The highly virulent strains M-1011 and LCDC-2241 grew more slowly than the others, but the responses of all organisms to the test compounds were similar. This experiment was also performed using iron-extracted NDM and MHA-EDDA media. Iron-extracted NDM gave essentially the same responses as NDM-EDDA. The complex iron-limited medium MHA-EDDA also gave results similar to NDM-EDDA, except that the four strains showed a 1.4 to 2.0-fold greater response to HGM alone on MHA-EDDA than on NDM-EDDA. However, the addition of 660 nmol of Na-citrate to either medium caused a similar 3 to 5-fold increase in the growth response of the strains to HGM (not shown), suggesting that the better growth on MHA-EDDA was not due to siderophore production. This siderophore-like effect of Na-citrate with $FePO_4$ or HGM (Tables 9,11) was considerably greater than any other differences noted between strains or media. The addition of Na-citrate by itself had no measurable effect on cell growth in the three media. Furthermore, iron was essentially unavailable to the cells when wells were filled with large amounts of water-suspended $FePO_4$ (Table 11), whereas a particle of $FePO_4$ placed directly on the lawn gave rise to a tiny mass of heavy growth surrounding it. This finding provided additional evidence to strengthen the argument in favor of direct cell contact as a requirement for the removal of iron from

complexes and the absence of an effective diffusible siderophore.

The siderophore-like activity observed with citrate in the presence of $FePO_4$ was further investigated by testing the ability of some of the iron complexes already shown to donate iron to SDIC (Tables 6,7,8), for their ability in iron-free form to facilitate iron uptake by SDIC from insoluble forms (Table 9). Despite the small amounts used, some of the chelating and complexing agents had a dramatic effect on the ability of SDIC to obtain iron from the three iron sources. Overall, the best compounds tested were citrate, pyrophosphate, SHAM, ADA, and NTA. A few assays employing the iron-dextran preparation Imferon, with its poorly available iron (Table 8), showed that the addition of pyrophosphate or a sub-physiological concentration of citrate (330 nm) made this form of iron readily accessible to the meningococcus. This provided a plausible explanation for the apparent availability of Imferon iron to the meningococcus in the mouse model (Calver et al., 1976; Holbein et al., 1979a,b).

The reported normal range of adult human serum citrate is 100 to 135 μM (Diem and Lentner, 1970), and serum phosphoric-ester phosphates 142 to 256 μM (Helve, 1946). In adult cerebrospinal fluid the concentration of citrate is 193 and 281 μM for cisternal and lumbar fluid, respectively, and the mean total inorganic phosphate concentration was 16.1 mM, this latter

value increasing with meningeal inflammation (Friedman and Levinson, 1955).

The ability of pyrophosphate to act as a functional siderophore (Table 9) prompted the assessment of a number of other phosphate compounds (Table 10). While the number of compounds tested was relatively small, the results show that the di- and triphosphate esters had siderophore-like activity for the meningococcus, a result not unexpected in view of the metal-binding capability of phosphate-ester compounds and a variety of evidence linking phosphate esters to iron movements among biological molecules (Egyed, 1975; Morgan, 1977; Pollack et al., 1977; Konopka, 1978).

Payne and Finkelstein (1977b) reported that the growth of meningococci was enhanced on Thayer-Martin medium supplemented with iron as Imferon. As the results here and those from Section I indicate that the iron present in the Thayer-Martin medium is far in excess of that required by the meningococcus for optimal growth, it seemed reasonable that the iron in that medium might not be readily available to the bacterium. To test this line of reasoning, 350 μM NTA was added to Thayer-Martin plates and the resultant colonies measured. They were an average of 20% greater in diameter at 20 h and 34% greater in diameter at 48 h than colonies on Thayer-Martin + 20 μg/ml iron as Imferon (see Payne and Finkelstein, 1977b). This

result suggested that the apparent iron-limitation of neisserial growth on Thayer-Martin (Kellogg et al., 1968; Payne and Finkelstein, 1977b) was due to the unavailability of the large amount of iron already present, a result difficult to reconcile with the idea of the meningococcus producing an effective siderophore. In fact, previous work (Section I) has shown that near-maximal rates and extents of meningococcal growth can be obtained with 50 ng/ml total medium iron yet two components of Thayer-Martin medium, proteose peptone and hemoglobin, contribute 80-100 ngFe/ml and 70 μgFe/ml, respectively, to the final medium iron concentration. No comment can be offered on the reported stimulation of a meningococcus by enterochelin (Payne and Finkelstein, 1978a). Repeated trials in our laboratory of 0, 36%, and 140% Fe^{+3}-saturated enterochelin on the three iron-limited media gave essentially the same results reported in Table 6, i.e. enterochelin did not function as an iron chelator for the meningococcus.

While the functional siderophore effect of some polyphosphates and carboxylic acids demonstrated one effective meningococcal iron-acquisition mechanism, results with human transferrin appear to have demonstrated another, or at least that the first system is very versatile. Conalbumin (Con) and human transferrin (Tf) showed marked differences in their abilities to withhold iron and exert bacteriostasis on SDIC in the

plate assays (Table 12). While there was some variation between trials both in the rate and extent of the zone decrease in Tf-induced bacteriostasis with time, in no instance did Con-induced bacteriostasis change. Both glycoproteins exerted their bacteriostatic action by withholding iron, as evidenced by the complete reversal of growth inhibition by excess iron as Fe^{3}-citrate. With the 30% iron-saturated Tf progressive growth exhibition occurred, whereas 30% iron-saturated Con was unable to promote growth and furthermore, could still compete successfully against SDIC for medium iron. Added citrate did not appear to affect the competition for iron in the medium, but did render Tf-bound iron more readily available, as did pyrophosphate.

In light of the findings of Holbein et al. (1979a,b) that demonstrate the importance of "sub-toxic" iron supplements in producing meningococcal infection in mice, it was of interest to determine whether strains more virulent for mice would also show an increased ability to compete with Tf and Con for iron. The data in Table 13 show no correlation between virulence in the mouse and the ability of a strain to compete with Tf for iron on either the defined or complex media. All four strains were equally ineffective in reversing Con-induced bacteriostasis, and, as with SDIC (Table 12), saturating Fe^{+3}-citrate could eliminate this inhibition. These results point out not only that the behavior of SDIC is typical of other meningococci

TABLE 12

Iron-withholding or -donating ability of certain substances as measured by growth inhibition or exhibition of *N. meningitidis* SDIC on NDM-EDDA

Compound	Total Fe (µg)/assay		Growth[a] Response	
Fe^{+3}-citrate (1:10), (180 n mol)	1.0		+1200	
deferri - conalbumin (12.5 n mol)	negligible		- 400	
" " plus Na-citrate (330 n mol)	negligible		- 400	
" " plus Fe^{+3}-citrate (1:10; 180 n mol)	1.0		+ 100	
" " plus Fe^{+3}-citrate (1:10; 360 n mol)	2.0		+1800	
Fe^{+3}-conalbumin (30% saturated; 12.5 n mol)	0.4		- 400	
		11 hr.	16 hr.	24 hr.
deferri - transferrin (12.5 n mol)	negligible	-150	- 70	0
" " plus Na-citrate (330 n mol)	negligible	-170	- 20	0
" " plus Fe^{+3} citrate (180 n mol)	1.0	+550	+650	+650
deferri - transferrin (25 n mol)	negligible	-400	-350	-350
Fe^{+3}-transferrin (30% saturated; 12.5 n mol)	0.4	-150	-50/+160[b]	+230
" " plus Na-citrate (330 n mol)	0.4	-70/+100	-70/+250	+400
" " plus Na pyrophosphate (330 n mol)	0.4	+300	+450	+450

a) Negative value represents zone of growth inhibition and a positive one growth exhibition
b) (-) inner zone of growth inhibition; + outer ring of growth exhibition.

TABLE 13

Comparison of four meningococcal strains in competition with purified deferritransferrin for iron in the growth medium

Strain (Sero Group)	Medium	Reported LD_{50} in mice (ref)	Response 12 hr	Response 16 hr	Response 24 hr
SDIC (B)	NDM-EDDA	<20 (17)	-180	- 90	0
	MHA-EDDA		-140	ND	0
LCDC 2241 (C)	NDM-EDDA	1.0 (5)	-170	-110	-70
	MHA-EDDA		-160	-130	-30
DRES 03 (B)	NDM-EDDA	$>10^8$ (5)	- 90	- 30	-30
	MHA-EDDA		-150	- 70	0
M 1011 (B)	NDM-EDDA	0.6 (5)	a	-500	-30
	MHA-EDDA		a	-400	-50

a) Background lawn too light to assess inhibition at 11 hrs.

ND Not determined.

(Tables 12,13), but also that the ability to compete against Tf for iron is probably not the decisive factor of virulence distinguishing these four strains in the mouse model of meningococcal infection.

Although Con and Tf are remarkably similar in most of their physical and chemical properties (Aisen and Liebman, 1968b) rabbit reticulocytes can distinguish between Con, Tf, and lactoferrin, and will remove iron only from Tf (Zapolski and Princiotto, 1976). The observed difference in the ability of meningococcus SDIC to remove iron from or compete with the two glycoproteins for iron suggests the presence of a rather specific Tf-deferrating or degrading ability, perhaps similar to the highly specific IgA protease of meningococci (Plaut, 1978). The results are also interesting in light of the work of Payne and Finkelstein (1975) indicating that differences in virulence between strains of gonococci for the chick embryo were related to their ability to remove iron from conalbumin in the egg.

It was shown in Section I (Figure 6) that cells in iron-limited continuous culture responded rapidly to small quantities of added iron (20 ng/ml) with decreased doubling times and increased respiratory rates. Therefore, the ability of aliquots of iron-limited continuous culture to obtain iron from ^{55}Fe-Tf added directly and ^{55}Fe-Tf added in a dialysis bag (12,000 d. exclusion) could be assessed by both growth response and ^{55}Fe

uptake. The results of the dialysis bag experiments (Table 14) show that the cells were unable to move iron across the membrane even in the presence of low levels of citrate or pyrophosphate. Therefore, the production of a diffusible low molecular weight siderophore by SDIC in this system seems highly unlikely.

In contrast to the negligible response when it was separated by a dialysis membrane (Table 14), the direct addition of Fe-Tf to iron-limited culture produced rapid and vigorous growth (Figure 8), and the eventual cell mass was nearly 3-fold greater than the control lacking Fe-Tf. The average doubling times of the control and culture aliquots were 250 and 69 minutes, respectively (20 to 106 min after Fe-Tf addition), compared to the original continuous culture doubling time of 165 minutes. As can be seen in Figure 8, citrate and pyrophosphate may enhance the uptake by SDIC of iron from Fe-Tf, however are not required for the effective removal of iron from Tf by iron-starved meningococci.

The toxicity of a non-ferrous metal ion for SDIC grown on NDM with low iron over that of the same cells grown on NDM containing a higher iron concentration would presumably be the result, at least in part, of competition with iron for uptake or incorporation into functional iron-containing molecules in the cell. Plate assays with 10,000 ng samples of the cations

TABLE 14

Test of the ability of *N. meningitidis* SDIC to obtain Fe from 30% saturated ^{55}Fe-Tf isolated in a dialysis bag

Additions to low-Fe NDM broth culture aliquots [a]	^{55}Fe outside dialysis bag (DPM/ml culture) [b,c] after 30 min	after 240 min
None	194	127
Na-citrate (68 μM)[d]	121	103
" " (136 μM)[d]	136	218
" " (680 μM)	100	52
Na-pyrophosphate (313 μM)[e]	73	121

[a] Cultures all underwent <1 doubling over 4.5 hours

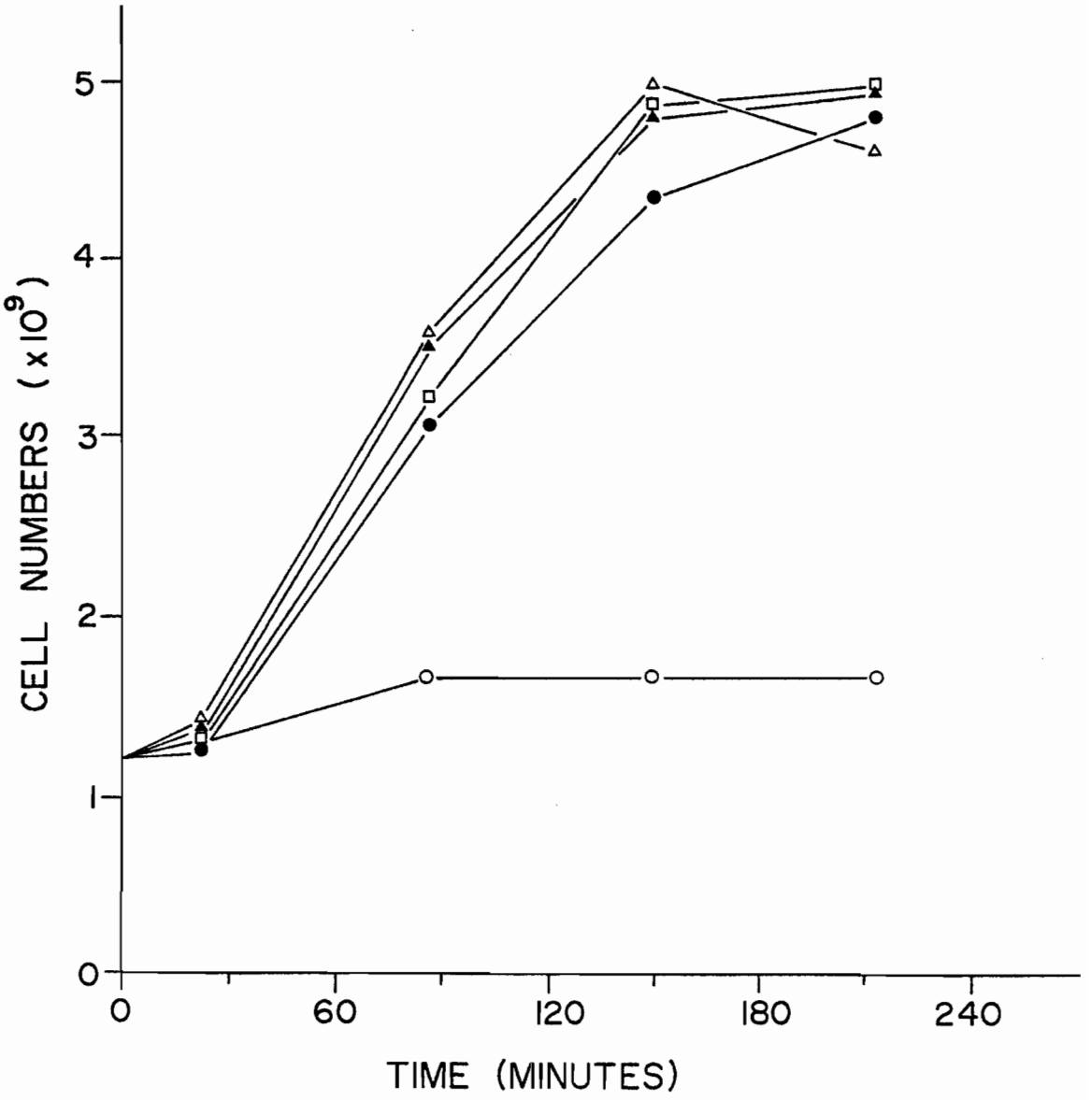
[b] ^{55}Fe-Tf in the dialysis bag was 168,800 DPM in 0.5 ml (0.63 mg protein)

[c] ^{55}Fe was so low in the samples that single channel ratio efficiencies could not be accurately calculated

[d] Values cover range of citrate concentrations in normal human serum (Diem and Lentner, 1970)

[e] Mean concentration of total inorganic phosphate in human serum (Diem and Lentner, 1970)

Figure 8. Samples taken from iron-limited continuous culture of N. meningitidis SDIC and incubated as follows: no additions Symbols: O——O (control); $^{55}Fe^{+3}$-transferrin (62 µg/ml; 0.42 µg; Fe^{+}/mg Tf) ●——●; $^{55}Fe^{+3}$-transferrin (62 µg/ml) + 100 µM Na-citrate ▲——▲; $^{55}Fe^{+3}$-transferrin (62 µg/ml) + 500 µm; Na-citrate △——△ ; $^{55}Fe^{+3}$-transferrin (62 µg/ml) + 200 µM; Na-pyrophosphate □——□.


CELL NUMBERS (x10⁹)
0
1
2
3
4
5
0
60
120
180
240
TIME (MINUTES)

Cd^{+2}, Co^{+2}, Cr^{+3}, Cu^{+2}, Mn^{+2}, Ni^{+2}, and Zn^{+3}, as their soluble Cl^- or SO_4 = salts, were carried out using SDIC lawns on NDM agar with three levels of available iron: (i) extracted-agar NDM with growth-limiting iron levels; (ii) NDM-160 (163 ng iron/ml); and (iii) NDM-360 (363 ng/ml iron). Of these cations, only Ni^{+2} was slightly more toxic on extracted-agar NDM and NDM-160 than on NDM-360, this despite the very high concentrations of metal ions near the assay discs compared to iron levels in the medium. This apparently high specificity of iron uptake and incorporation was supported by short-term uptake competition experiments using Mn^{+2}, Co^{+2}, and Ni^{+2} (Section III) and is in contrast to several reports of metal cation effects on iron uptake in siderophore-producing bacteria (Nakayama et al., 1964; Wang and Newton, 1969a,b; Arceneaux and Lankford, 1971; Davis et al., 1971). The specificity of chelators such as citrate, DTPA, EDTA or ADA for Fe^{+3} over other transition metal cations is not as great as for secreted specific siderophores such as ferrioxamine or ferrichrome, but the demonstrated high selectivity of the actual iron-uptake process in SDIC would presumably make the lower iron-selectivity of organic acids or phosphate-ester compounds functioning as siderophores of less importance.

IID. Discussion

This section of the thesis presents evidence that in

in vitro, on both defined and complex media, *N. meningitidis* did not produce siderophores detectable by any of the methods usually employed. In particular, the apparent absence from culture supernatant fluids of a siderophore able to stimulate growth of the same meningococcal strain when iron-starved is strong evidence for the lack of an effective soluble siderophore. Regrettably there is no practical way to rule out the possibility of a low molecular weight siderophore dependent for its induction on a particular chemical or physical parameter absent from the *in vitro* systems used. The results presented here are at variance with those of Payne and Finkelstein (1978a). These workers reported that both enterochelin and the supernatant fluids from iron-starved cultures of meningococci and other Gram-negative bacteria stimulated the growth of meningococci on a conalbumin-GC base agar.

Although somewhat unclear, it appears that the liquid media in which Payne and Finkelstein (1978a) grew the meningococci and the other Gram-negative bacteria (and whose supernatant fluids stimulated the growth of the meningococcus on GC base-conalbumin agar), were de-ferrated using the rather labile ion-exchange resin Chelex-100. If this resin was not treated with 3N NH_4OH at 80°C and thoroughly rinsed immediately prior to use (Bio-Rad, 1976) an appreciable level of free iminodiacetate would enter the treated media. As iminodiacetate is a

good iron chelator and similar in structure to the effective meningococcal "functional" siderophores ADA and NTA, stimulation of meningococcal growth by a supernatant fluid from this medium would not be surprising. It has been shown here that the addition of NTA to Thayer-Martin medium gives growth superior to the same medium supplemented with Imferon. This is further evidence for the absence of siderophore production under these in vitro conditions.

The similarity of the iron-acquiring abilities observed with 20 meningococcal strains in the plate assays using the three iron-limited media suggests the lack of an effective soluble siderophore was not the unique property of a single strain or of the culture medium used. Further evidence obtained in the plate assays for the absence of a conventional siderophore includes the following: (a) the absence of time-dependent overgrowth of zones of bacteriostasis around compounds which sequester iron, with the exception of transferrin; (b) the heavy, small-diameter growth in response to the poorly diffusible but readily available iron in HGM; (c) the marked improvement of iron availability from a variety of compounds in the presence of siderophore-like substances, e.g. citrate or pyrophosphate; (d) the inability of meningococci to compete with or use iron from four common siderophores; (e) the inability of meningococci to obtain iron from transferrin in a dialysis bag; and (f) the

inability of possible siderophore precursors or related compounds, with the exception of salicyl-hydroxamate, to facilitate iron uptake. This siderophore incompatibility is perhaps not surprising as the genus Neisseria is restricted to the mucous membranes, a habitat isolated from the gut or soil niches occupied by most organisms with well-characterized siderophores. The data argue only for the absence of a siderophore able to make iron available from the various forms chosen for use; however, the possibility of a substance capable of removing iron from specific host complexes, in particular the transferrins or heme proteins, remains open. Despite this apparent lack of a siderophore, the meningococcus was shown to display rapid, high-affinity ferric iron uptake from a variety of stable chelates and complexes (Section III).

This report of citrate as part of an iron-acquisition system is not unique, as facilitation of iron-uptake by citrate has been reported in E. coli (Frost and Rosenberg, 1973), Corynebacterium diphteriae (Lankford, 1973), Neurospora crassa (Winkelman, 1979), Microbacterium lacticum (Alexanian et al., 1972), and Micrococcus lysodeikticus (Neilands, 1957). However, except for E. coli, little is known of the mechanisms involved. In most reports, the use of iron-citrate was given only a casual comment. It therefore seems likely that a directed screening of microorganisms would yield still more examples of the ability

to use citrate-bound iron. There has also been some speculation on citrate being a primordial iron transporter (Simpson and Neilands, 1976), as it is unspecialized and forms the basic structure of the schizokinen-like hydroxamate chelators found in such diverse genera as Anabaena (Simpson and Neilands, 1976), Bacillus, Arthrobacter, and Enterobacter (Lankford, 1973). The use of carboxylic acid and phosphate-ester compounds, already present in body fluids, as functional siderophores, or the direct deferration of circulating transferrin would confer obvious advantages on an invading pathogen over a mechanism requiring the generation of usable concentrations of a secreted siderophore. Such an advantage would hold within a limited area of host tissue, but would be especially useful in the circulating body fluids. Furthermore, in more severe forms of meningococcal disease, there is a significant amount of host tissue degradation which would make iron readily available from the intracellular ferritin and heme proteins released, provided that at least the physiological levels of citrate or phosphate esters were present.

This leaves open the question of why small amounts of iron have such a marked effect on the virulence of meningococci for the mouse. Unfortunately, there is little data on the levels of iron, organic acids, phosphate esters, transferrins, ferritin and heme proteins in human or animal sera and cerebrospinal fluids before and during meningococcemia. Until such data are

available, as well as those on the effect of added iron on the interaction of the parasite with cellular and humoral defense systems, the relevance of the functional siderophore mediated meningococcal iron acquisition system described here to the actual disease state cannot be accurately evaluated.

Considering the results of Table 14 and Figure 8, it appears that under the experimental conditions employed, iron specifically bound to Tf at the normal level of physiological saturation is readily available to the meningococcus through direct contact with the cells. Alternatively, one could speculate that a high molecular weight siderophore or "transferrinase" acts as a vehicle for the specific removal of iron from Fe-Tf. There are no data available that would support such speculation.

Caution must be used in extrapolating these results to iron acquisition in meningococcemia. Assuming that the mechanisms of inflammatory hyposideremia proposed by Van Snick et al. (1974) function during infection, the degree of iron saturation of serum transferrin will decrease, and the ability of the organism to remove iron from lactoferrin may become important. Furthermore, the survival of meningococci in the serum must depend primarily on their successfully resisting or avoiding the phagocyte, antibody, and complement antimicrobial defenses and only secondarily on obtaining adequate nutrition to multiply.

Nevertheless, the work of Holbein et al. (1979a,b) indicates that meningococcal strains virulent for the mouse are able to survive and multiply in the blood and that the enormous virulence-enhancing effect of 15 mg iron-dextran/kg appears to be primarily nutritional. Holbein et al. (1979b) also showed that the elevated serum iron resulting from the iron-dextran treatment is apparently transferrin-bound, raising that protein's iron saturation from 48% to a peak of 82%. However, as Figure 8 shows in the in vitro work presented here, the meningococci very readily deferrated 30% iron-saturated human Tf. The apparently increased difficulty in obtaining in vivo Tf-bound iron could be due to: (a) antibody or other serum interference with the transferrin iron-acquiring system; (b) specificity of the system for human transferrin; or (c) inadequate induction of the system in the time that the meningococcal cells had been in the mouse. Possibilities (a) and (c) apply equally well to the "functional siderophore" system. The third possibility is also interesting if, like the gonococcus (see Norqvist et al., 1978), the meningococcus induces several high M.W. outer membrane proteins in response to iron-privation. These would presumably be fully induced in the iron-starved continuous culture cells used here, but not in the cells used by Holbein et al. (1978a) to inoculate their mice.

III. CHARACTERISTICS OF RAPID IRON UPTAKE BY THE MENINGOCOCCUS

IIIA. Introduction

It was established by the work described in Section I that a mid-log phase culture of Neisseria meningitidis required approximately 50 ng of iron/ml (0.9 μM) for a growth rate not greatly limited by iron. When cells were growing at much less than this iron concentration, the presentation of ferrous or ferric iron to them was followed by its rapid uptake and use (Figure 6). Work on a variety of other microbial iron uptake systems has shown that pulsing iron-starved cells with ^{55}Fe or ^{59}Fe-labeled iron compounds can yield considerable information about the kinetics and nature of the system involved. In the iron-limited continuous culture system described in Section I, the equilibrium conditions were ideal for determining the effect of iron pulses. The equilibrium cultures, where μ (instanteous growth rate) = D (culture dilution rate) could supply unlimited small aliquots of homogeneous cell material for iron-uptake comparisons and replicates, obviating parallel culture and sampling time differences inevitable with a batch culture approach, and allowing much data to be obtained from a single culture. Initially, aliquots of the low-iron continuous culture were pulsed with $^{55}FeCl_3$ because the iron would be presented to

the cells as hydrated ions, $Fe(OH)_3$ monomers, or weak complexes with medium or metabolite compounds which would simulate the uptake of "free" ferric iron *in vivo*. The iron concentrations used, up to 3.7 μM (final concentration) were far below the 200 μM "mononuclear wall" (Schubert, 1963), the concentration at which $[Fe(OH)_3]_n$ begins to form, and thus there were no precipitation or filter retention problems with $^{55}FeCl_3$ during the 10-40 min time course of individual assays.

One of the more important findings of the work presented in Section II is that human physiological concentrations of citrate greatly enhance the uptake of iron from a number of forms poorly available to the meningococcus. The initial findings using $FeCl_3$ pulses were therefore followed by uptake studies using Fe^{+3}-citrate (1:10), $^{55}Fe^{+3}$-NTA, and other ferric complexes. Because of its tendency to rapidly convert to ferric complexes, ferrous iron was not used here or elsewhere in the thesis work. In addition to following the association of the various forms of ferric iron with iron-starved cells, the effects of heat, cold, substrate concentration, various metabolic poisons and cation competition on meningococcal iron uptake were assessed, with results that shed considerable light on the nature of an iron-acquisition system.

IIIB. Methods and Materials

Organism

The group B *N. meningitidis* (SDIC) (smooth) and procedures for maintenance of stock and working cultures and for routine checks for strain purity were performed as described in Section I (see DeVoe, 1976).

Media

Cultures were grown on a simple defined medium, designated Neisseria Defined Medium(NDM), either as a broth or as a solid medium after the addition of 1.5% agar. The composition and production of NDM, both iron-sufficient (204 ng Fe/ml) and iron-poor (3.5-6.0 ng Fe/ml), have been described in Section I.

Cell Growth

The apparatus for the continuous culture of SDIC and the methods employed to obtain an iron-starved equilibrium culture were as previously described (Section I), except that no high-iron or low dissolved oxygen equilibrium growth conditions were established. Parameters controlled in the culture vessel included dissolved oxygen at 60% ± 2% of saturation (see Appendix), pH = 7.4 ± 0.05, 37°C, and cell density and generation times at steady values between 1.6 and 2.2 x 10^9 cells/ml and 140 and 200 min, respectively. In all short-term iron-pulse experiments

6 ml aliquots of the continuous culture were used immediately after removal from the culture vessel. All materials and reagents for the uptake studies were at 37°C, except the filtration apparatus and scintillation vials and reagents which were at room temperature.

Iron Uptake

The ability of SDIC cells to take up ferric iron was measured by their binding of iron from various compounds pulsed into an aliquot of iron-starved equilibrium continuous culture. The following format was used in nearly all the iron uptake studies presented in this report. Six ml of the continuous culture were transferred into a pre-warmed 50 ml flask, incubated with shaking at 37°C for 2 min and a pulse of 95 ng $^{55}Fe^{+3}$/ml (1.7 μM final concentration) of the iron-complex to be assessed was added. In the pulse experiments employing varying concentrations of $^{55}FeCl_3$, the final medium iron level varied from 4.4 ng iron/ml (0.08 μM) to 204 ng iron/ml (3.6 μM) immediately post-pulse. At timed intervals after this iron pulse, 0.3 ml culture samples were removed, deposited onto moist membrane filters (0.45 μ pores), under 15" Hg vacuum, washed with 5.0 ml of sterile low-iron NDM, placed in scintillation vials and counted for ^{55}Fe as described below.

In experiments employing KCN, TCS (tetrachlorosalicylanilide), valinomycin, NaN_3, or antimycin A, these substances

were added in 10 µl of CH_3OH after 2 min incubation of the 6 ml culture aliquot alone in the 50 ml flask and 2 min before the addition of the $^{55}Fe^{+3}$-pulse. Culture aliquots to be heat-treated were removed from the continuous culture, placed in 16 x 150 mm tubes, and the tubes placed in a water bath at the appropriate temperature. Five min after the tube contents had reached the bath temperature, they were placed in 50 ml flasks, incubated with shaking at 37°C for 2 min and treated as above.

The data points in the Figures and Tables are the averages of duplicate samplings, and all data in a given Figure or Table for this section is from a series of 6 ml aliquots from a single continuous culture, taken as close together in time as possible. All assays were performed on culture samples of at least 2 independent equilibrium continuous cultures, and cultures were checked continuously for contamination or pleomorphism. Short-term assay controls showed 1.0% ^{55}Fe retention by a membrane filter (0.45 µ pore size) after 10 min incubation of any of the ^{55}Fe preparations used in sterile, low-iron NDM, pH 7.4.

^{55}Fe Determination

Liquid scintillation counting of ^{55}Fe was carried out on a Chicago-Nuclear Isocap 300 counter in the SCR mode with manually set windows. Good counting efficiencies were obtained by

the addition of 0.5 ml of water containing 50 μg of O-phenanthroline and 50 μg of deferrioxamine-β-mesylate (Desferal) to 8 ml of PCS scintillant (Amersham-Searle) and 0.3 ml liquid sample or a 25 mm membrane filter. Earlier work (Section I) used 6 ml PCS and 1 ml of water with 100 μg each of Desferal and O-phenanthroline, but an improvement in counting efficiency (to 35-40%) plus faster clearing of the filters prompted the use of the above modifications.

Reagents

The iron complexes were prepared by dissolving appropriate amounts of the chelating or complexing agent in water, adding 1.0 mM $FeCl_3$ (final concentration), and adjusting the pH to 7 to 8. Pulsing 10 μl of this solution into a 6 ml culture aliquot yielded a final iron concentration of 1.7 μM (95 ng/ml).

Because ferric iron and citrate at close to equimolar concentrations tend to form $[Fe(OH)_3]_n$ particles (mw = 2×10^5d) coated with citrate (Bates et al., 1967), a 1:10 ratio was used for iron to citrate or iso-citrate. In this way the low concentration of iron in the culture aliquots would be in the form of a stable ferric dicitrate complex (Bates et al., 1967). In contrast, NTA forms a stable chelate with ferric iron in equimolar (1:1) concentrations, so a 3:1 ratio was used. The 1.7 μM acidified $FeCl_3$ pulsed into the culture aliquots was far below

the 200 μM "mononuclear wall" of $[Fe(OH)_3]_n$ precipitate formation (Schubert, 1963) and < 1% of the ^{55}Fe was retained by the ultra filters (0.45 μm pores) in cell-free controls during the period of the longest pulse experiments.

Just prior to use, valinomycin, antimycin a, and tetrachlorosalicylanilide (TCS) were dissolved in CH_3OH. The amount of methanol added to the culture aliquot (10 μl/6 ml) was previously shown to have no measurable effect on culture iron uptake. Due to its tendency to decompose and be lost as HCN gas, KCN was also made up only when required. KCN was dissolved in 100 mM or 300 mM Tris, adjusted to pH 7.5-8.0 and kept on ice until the addition of 10 μl to a culture aliquot. The ^{55}Fe was obtained from New England Nuclear (Boston, Mass.) as $^{55}FeCl_3$ (28 mc/mg) and was added to the ^{56}Fe complexes or chelates so that the final activity would be 4 to 6 x 10^4 DPM/ml and the final total iron would be 95 ng/ml (1.7 μM) when 10 μl of the ^{55}Fe-labeled reagent was added to a 6-ml culture aliquot. The $^{55}FeCl_3$ stock solution was used at pH = 1.5. To rule out any effects of residual medium iron on ^{55}Fe uptake the supernatant fluid from aliquots of iron-starved continuous cultures was assayed for iron content. By the colorimetric iron assay (Section I) which showed good recovery of ^{55}Fe from culture supernatant, the supernatant contained < 1 ng iron/ml.

All salts and glucose were reagent grade (Fisher

Scientific Co., Fairlawn, N.J.). Desferal was a gift of CIBA, Montreal and deferriferrichrome was obtained through the courtesy of Dr. J. Coulton. All other organic compounds were from Sigma Chemical Co. (St. Louis, Mo.).

IIIC. Results

When various concentrations of $^{55}FeCl_3$ were injected into aliquots of iron-starved culture removed from a continuous culture of meningococcus SDIC, a bi-phasic uptake curve was obtained (Figure 9). These two stages of iron uptake by the meningococcus were initially, a rapid stage covering the first 30 sec, followed in the three higher concentrations (0.96, 1.7, and 3.6 μM iron), by a secondary, slower rate, second stage. The second, slower stage was short-lived (0.5 to 1 min) in the two lowest concentrations (0.08 and 0.25 μM iron), due to the near exhaustion of extracellular iron during the initial fast stage. Cells pulsed with 0.96 μM iron took up the metal at a constant rate similar to that for the higher iron concentrations from 0.5 to 5 min, after which depletion of the iron pulse brought the rate close to zero.

To determine the need for metabolic energy for either or both stages of uptake, cells were tested for their ability to take up $^{55}Fe\ Cl_3$ iron (1.7 μM) in the presence of the bacteriostatic detergent tetrachlorosalicylanilide (TCS), KCN, and at

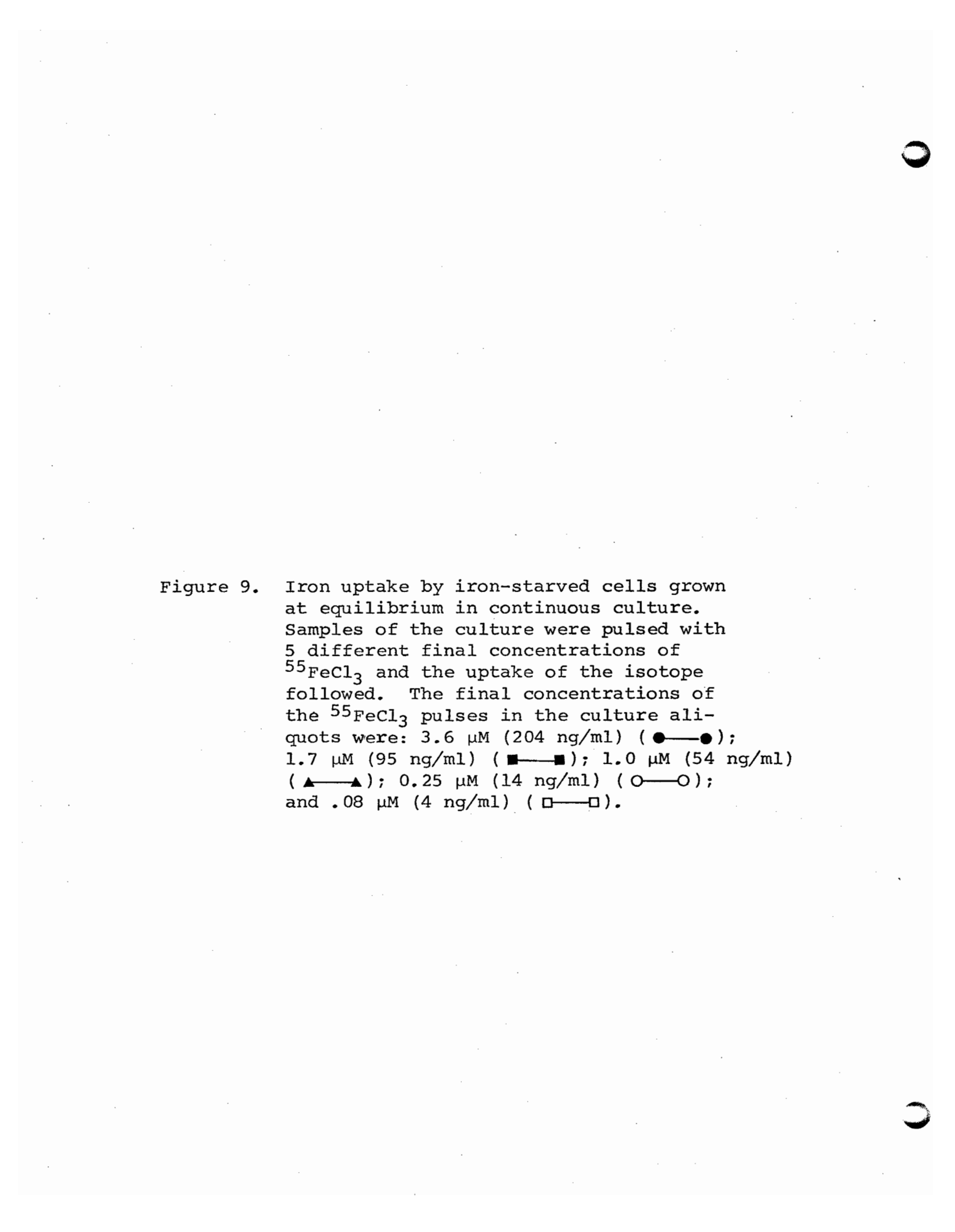


Figure 9. Iron uptake by iron-starved cells grown at equilibrium in continuous culture. Samples of the culture were pulsed with 5 different final concentrations of $^{55}FeCl_3$ and the uptake of the isotope followed. The final concentrations of the $^{55}FeCl_3$ pulses in the culture aliquots were: 3.6 μM (204 ng/ml) (●——●); 1.7 μM (95 ng/ml) (■——■); 1.0 μM (54 ng/ml) (▲——▲); 0.25 μM (14 ng/ml) (○——○); and .08 μM (4 ng/ml) (□——□).

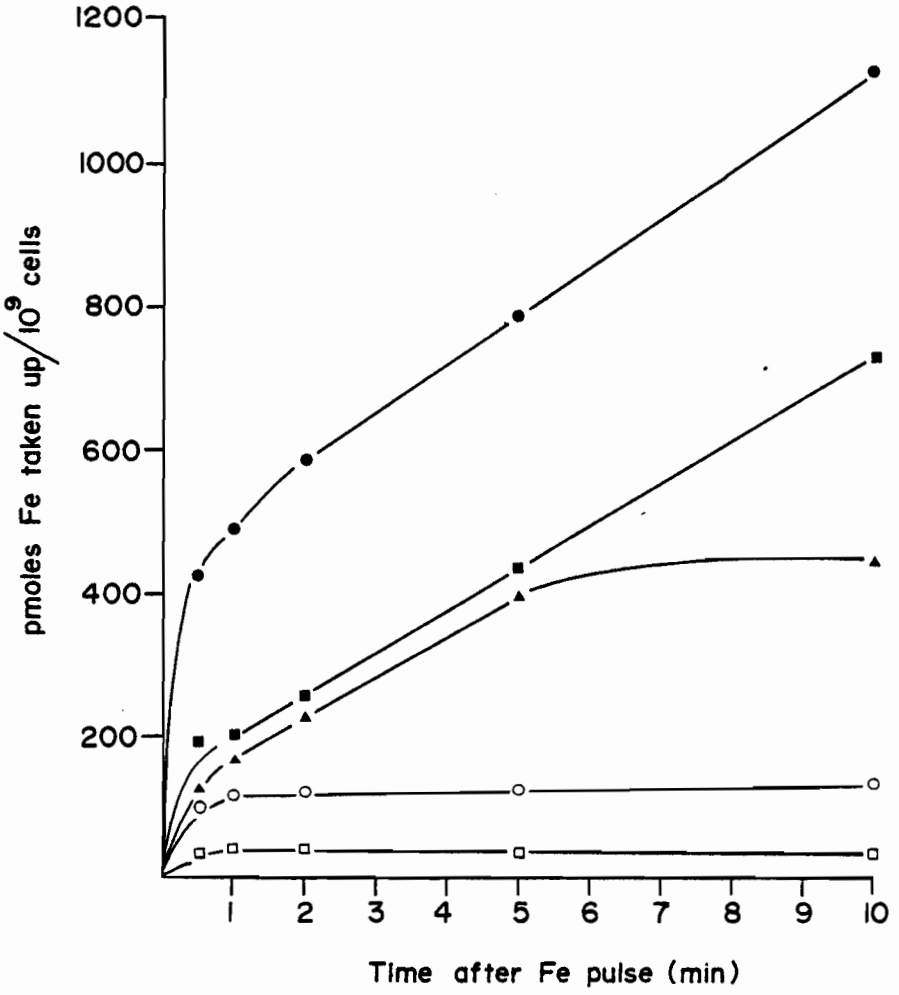
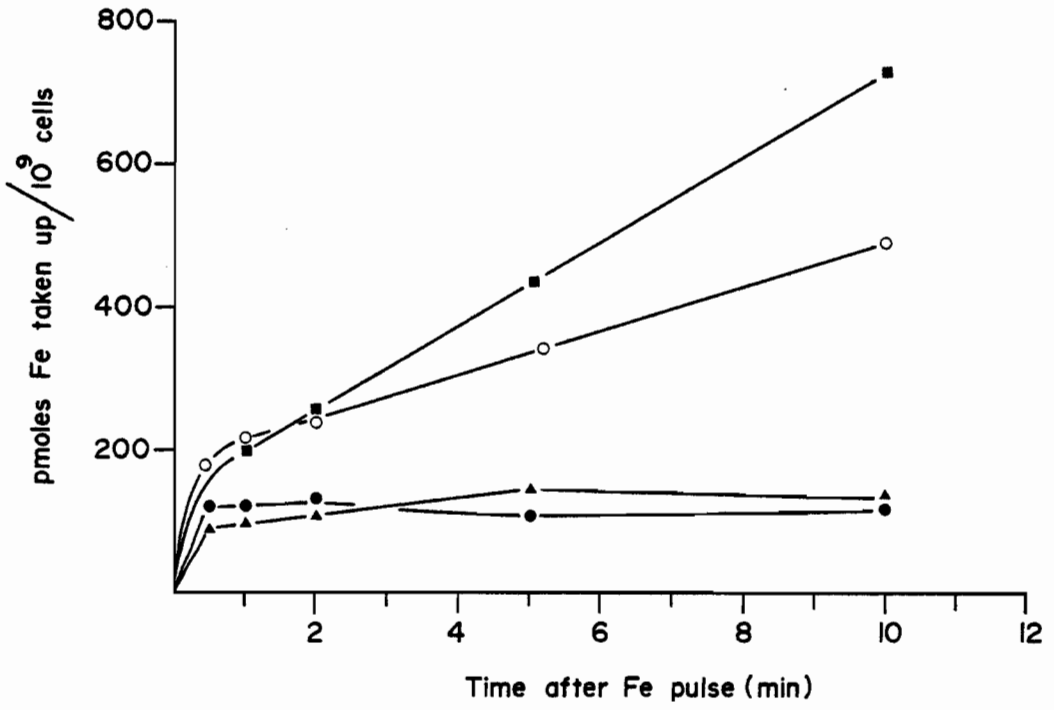

1200
1000
800
600
400
200
pmoles Fe taken up/10^9 cells
1
2
3
4
5
6
7
8
9
10
Time after Fe pulse (min)

Figure 10. Inhibition of active iron uptake by KCN (0.5 mM), TCS (4 μM), and low temperature (6°C). Cells were grown as described for Fig. 7. KCN and TCS were added 2 min prior to the iron pulse (95 ng of Fe per ml, final concentration). For the low temperature (6°C), 6 ml of culture was transferred to a precooled 50-ml flask followed by incubation (100 rpm) in an ice bath (6°C) 4 min prior to the addition of iron. Rates shown were calculated during linear uptake (2 to 10 min). Symbols: $FeCl_3$ *per se* (■——■); TCS (○——○); KCN (●——●); 6° C (▲——▲).


800
600
400
200
pmoles Fe taken up/10^9 cells
2
4
6
8
10
12
Time after Fe pulse (min)

6°C. In the presence of KCN (Figure 10) and at 6°C, the second, slow stage of uptake was completely blocked, while 4 μM TCS reduced the rate of uptake by 50%. However, greater than 60% of the iron uptake occurred during the initial fast stage in the presence of the inhibitors or at 6°C. A similar pattern was observed over a range of iron concentrations in the presence of KCN (Figure 11). Since KCN is a potent poison for respiration, and TCS has been shown to affect energy-dependent translocation in the membrane (Hamilton, 1968; Harold and Baarda, 1968), I have designated the slow stage of iron uptake inhibited by these agents as the energy-dependent system and the initial rapid stage the energy-independent system. The rate of iron uptake during the energy-independent stage, i.e. by KCN-poisoned cells, was directly proportional to the concentration of iron introduced into the medium (Figure 12), whereas iron uptake by unpoisoned cells (Figure 9) showed saturation when ambient medium iron was >0.25 μM, the uptake rate remaining steady at about 6 ng iron per 10^9 cells per min.

When these KCN-poisoned, ^{55}Fe-pulsed cells were placed in non-radioactive KCN-free sterile NDM, containing 3.6 μM iron, the initial rate of cellular iron exchanged with that in the medium was 2.8% per min for cells previously pulsed with 3.6 μM iron and 3.9% per min for cells previously pulsed with 0.08 μM iron. Therefore, the iron bound by the energy-independent

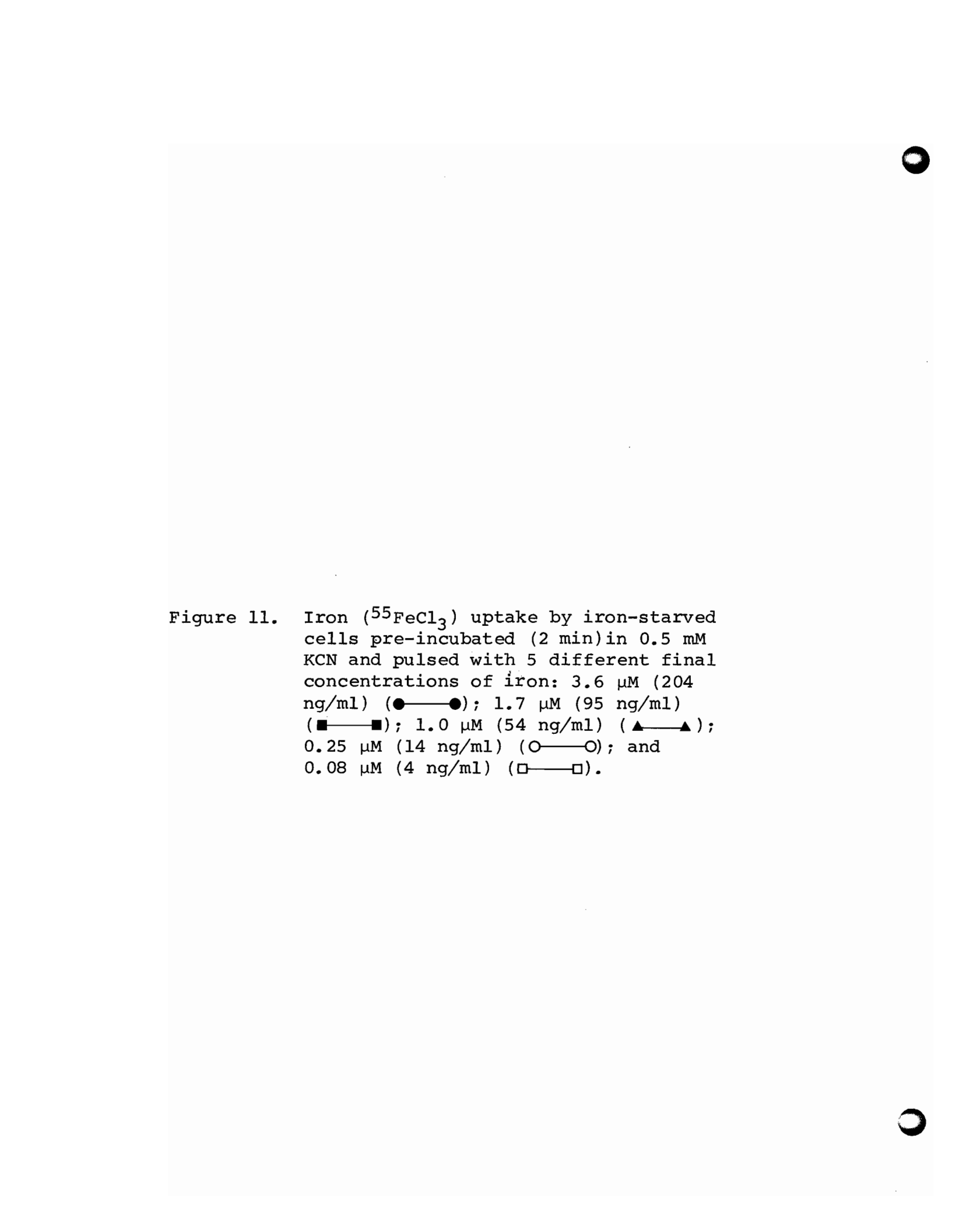
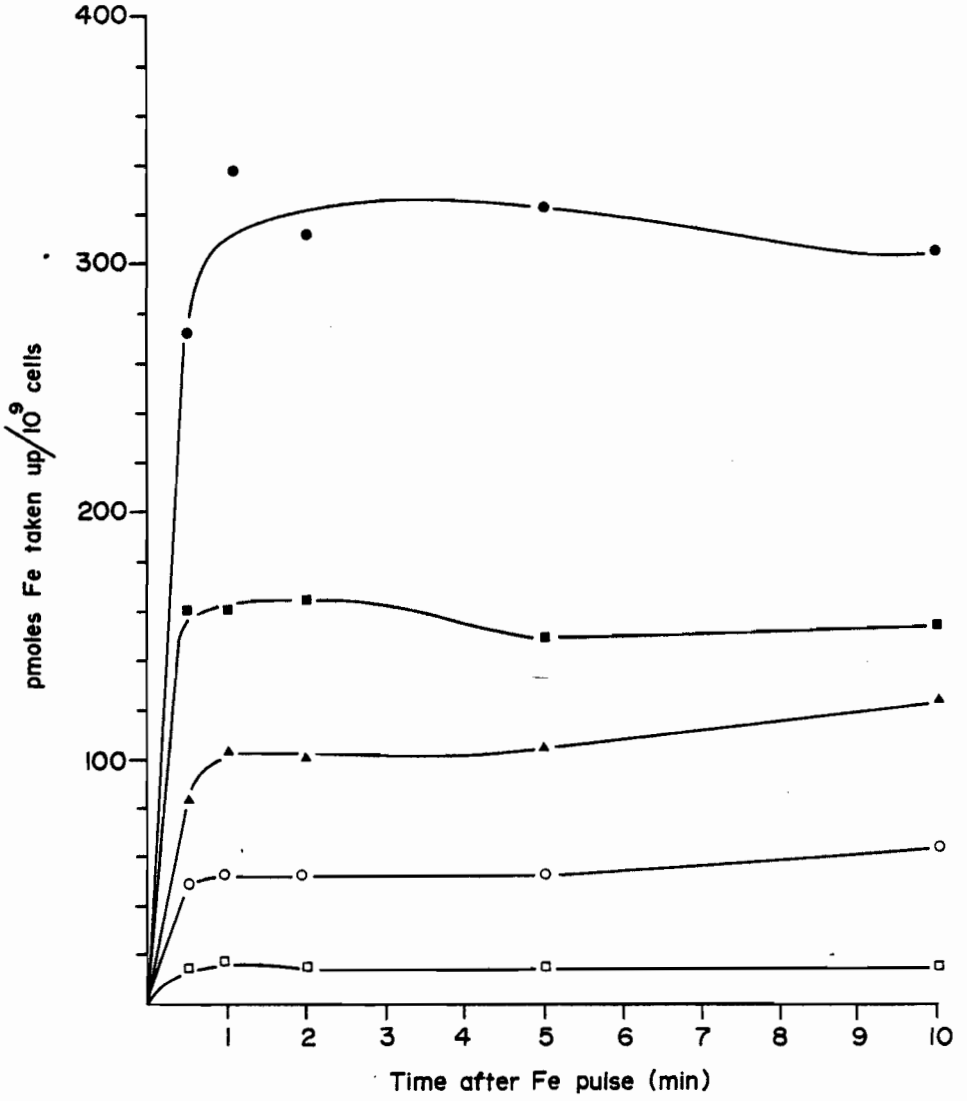


Figure 11. Iron ($^{55}FeCl_3$) uptake by iron-starved cells pre-incubated (2 min)in 0.5 mM KCN and pulsed with 5 different final concentrations of iron: 3.6 μM (204 ng/ml) (●——●); 1.7 μM (95 ng/ml) (■——■); 1.0 μM (54 ng/ml) (▲——▲); 0.25 μM (14 ng/ml) (○——○); and 0.08 μM (4 ng/ml) (□——□).


400
300
200
100
pmoles Fe taken up/10^9 cells
1
2
3
4
5
6
7
8
9
10
Time after Fe pulse (min)

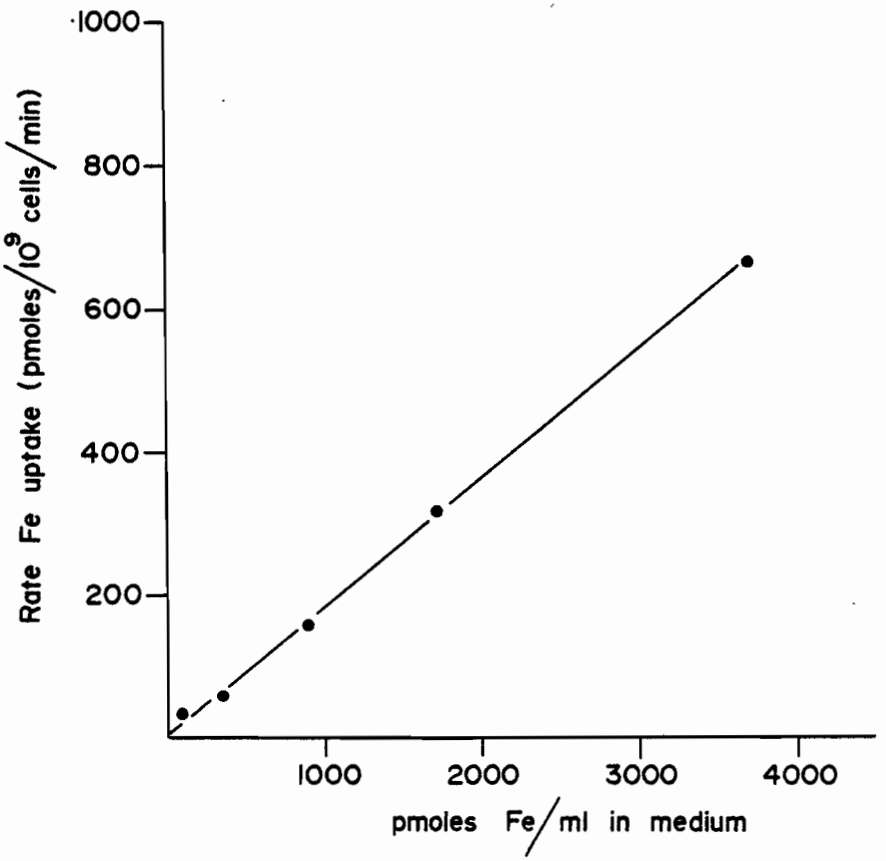
system formed a relatively stable association within or on the cells.

The energy-dependent rate of iron uptake by cells growing in a high-iron, equilibrium condition was 18-20 pmol per 10^9 cells per min, as shown by both ^{55}Fe and colorimetric iron assays. In comparison, iron-starved cells pulsed with 3.6 μM iron took up iron in the energy-dependent stage (2-10 min post-pulse) at 70 pmol/10^9 cells/min, a rate nearly four times that of cells grown in high iron. In addition to the rate of uptake, the extent of uptake was assessed by incubating iron-starved cells with 3.6 μM iron for up to 20 min. While cells grown in high-iron, high-O_2 equilibrium conditions took up 2.1 μM (54%) of the 3.6 μM iron in the medium, iron starved cells took up 2.2 μM (56%) of the 3.6 μM iron in 10 min and 2.3 μM (63%) in 20 min. It is not clear from the data whether the greatly increased, energy-dependent rate of uptake and slightly increased capacity to accumulate iron were effects of cellular mechanisms induced by iron privation, or enhanced activity of pre-existing ones.

In all iron pulse experiments, the maximum measured uptake of ^{55}Fe was only about 90% of the total added, which the controls suggested was due to fluorescence loss by quenching in the packed cells on the membrane filters.

When the complexes Fe-citrate (1:10), Fe-isocitrate

Figure 12. Initial rates (during first 30 s) of energy-independent uptake in the presence of KCN (0.5 mM) over a range of iron concentrations (4.4 to 204 ng Fe per ml) (.08 - 3.6 μM). Conditions were as described for Figure 11.


1000
800
600
400
200
Rate Fe uptake (pmoles/10^9 cells/min)
1000
2000
3000
4000
pmoles Fe/ml in medium

(1:10), Fe-nitrilotriacetate (Fe-NTA, 1:3) and $FeCl_3$ were injected into aliquots of iron-starved culture, all forms of ferric iron had nearly identical rates of saturable, energy-dependent uptake (Figures 13,14). In contrast, the rate of uptake over the first 30 ~~sec~~ ~~post~~ ~~pulse~~, the energy-independent uptake, was consistently faster from $FeCl_3$ than from Fe^{+3}-citrate, Fe^{+3}-isocitrate, or Fe^{+3}-NTA. The range of specific uptake rates for the saturable energy-dependent, Fe^{+3}-citrate-derived iron uptake from different continuous cultures run under identical conditions was 45 to 65 pmol per 10^9 cells per min after the first minute. For Fe^{+3}-EDTA (1:3) the specific rate was 0.1 to 0.2 pmol per 10^9 cells per min. This Fe^{+3}-EDTA (1:3) complex had previously been shown to promote good growth responses in an iron-limited plate assay (Section II). Therefore, the relatively slow iron uptake rate shown in Figure 13 was apparently adequate to allow near-normal growth on agar plates. On the other hand, the failure (Figure 13) of SDIC to take up a detectable amount of iron from Fe^{+3}-ferrichrome (1:3) agreed with the growth-response data in Section II showing that the meningococcus can neither use iron from ferrichrome nor compete with this siderophore for medium iron.

In Figure 10 the energy-dependent iron uptake was shown to be completely inhibited by 500 μM KCN and partially inhibited by 4 μM TCS, an uncoupler (Hamilton, 1968). The complete

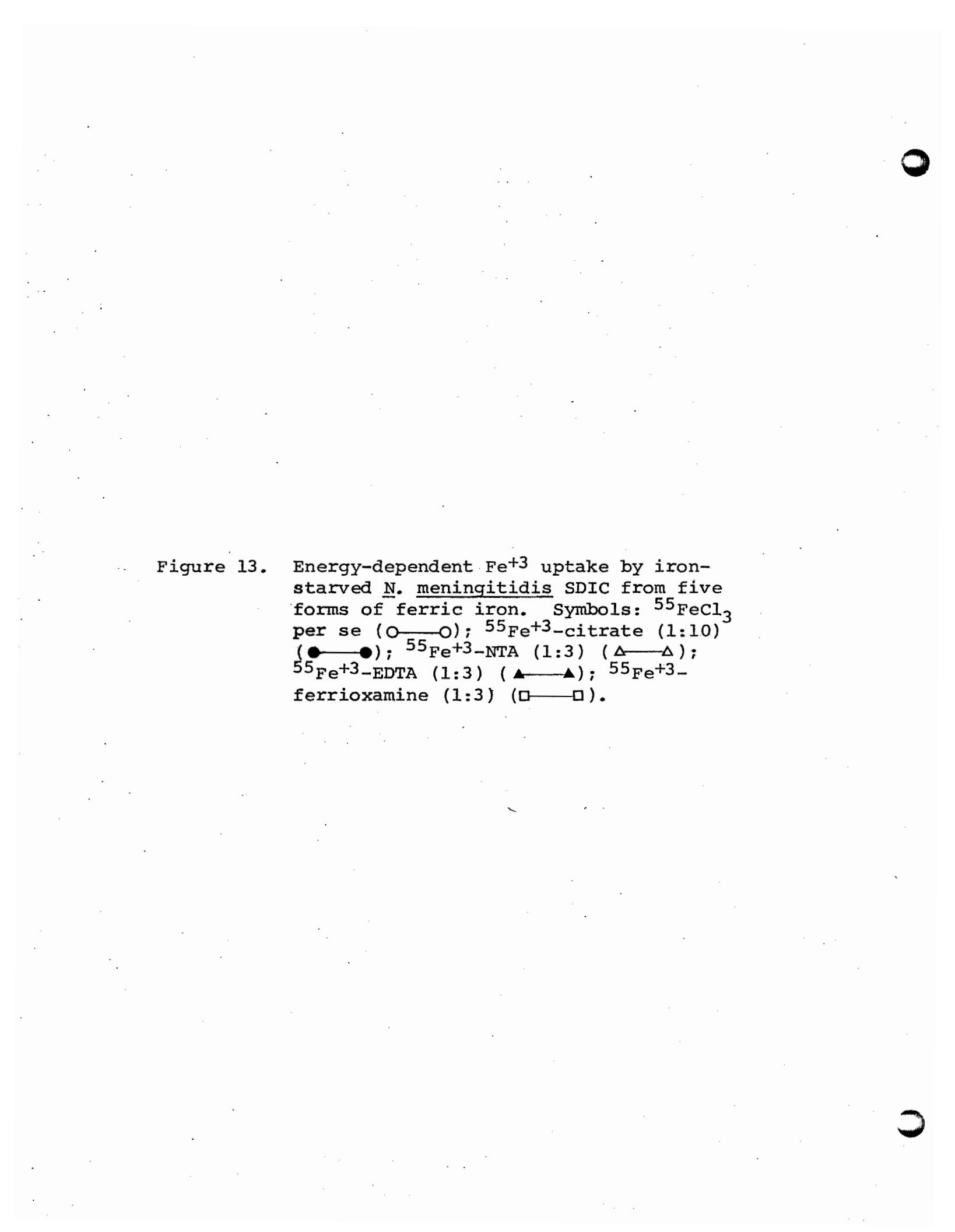


Figure 13. Energy-dependent Fe^{+3} uptake by iron-starved N. meningitidis SDIC from five forms of ferric iron. Symbols: $^{55}FeCl_3$ per se (O——O); $^{55}Fe^{+3}$-citrate (1:10) (●——●); $^{55}Fe^{+3}$-NTA (1:3) (△——△); $^{55}Fe^{+3}$-EDTA (1:3) (▲——▲); $^{55}Fe^{+3}$-ferrioxamine (1:3) (□——□).

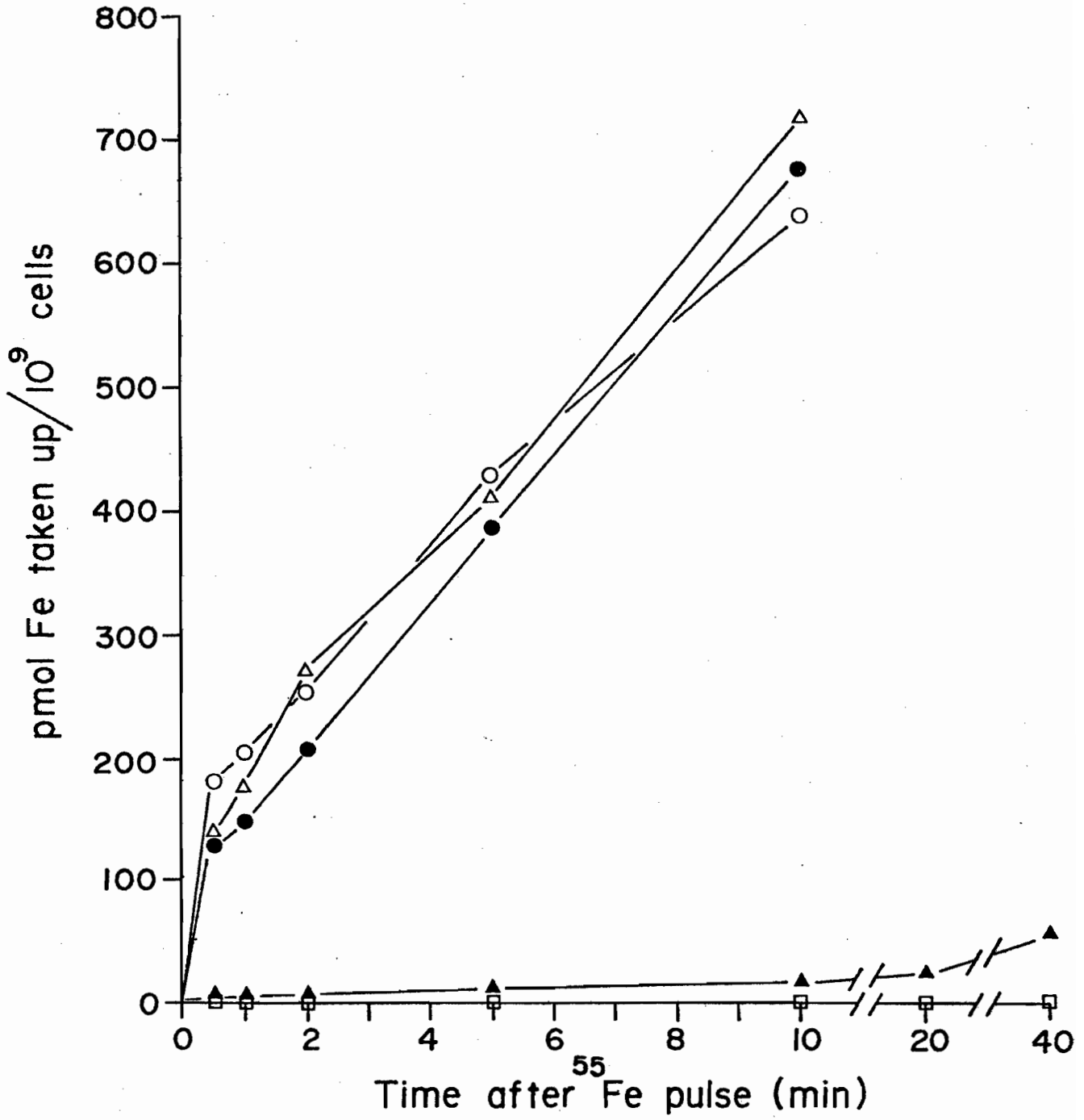
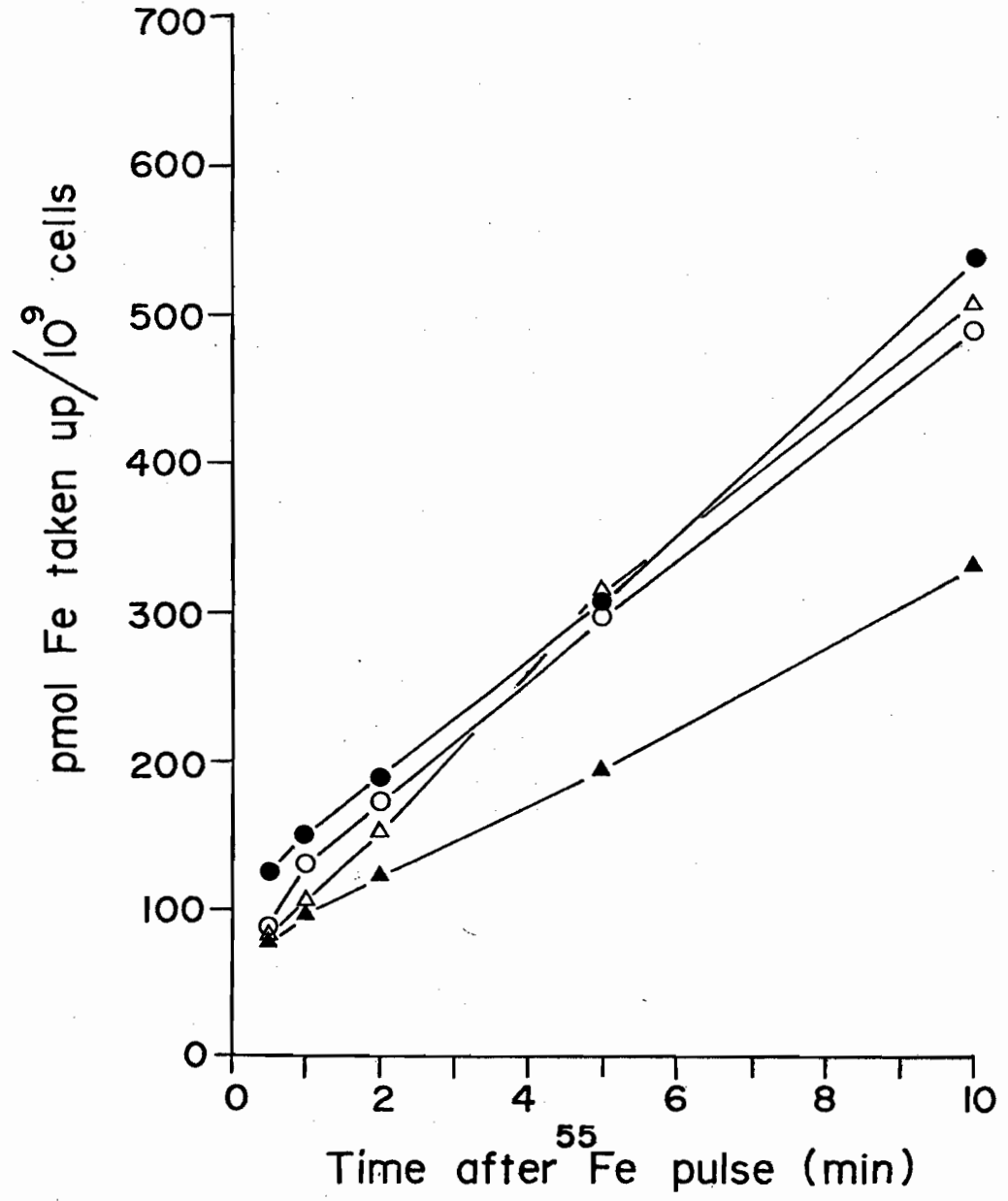

800
700
600
500
400
300
200
100
0
pmol Fe taken up/10^9 cells
0
2
4
6
8
10
20
40
Time after ^{55}Fe pulse (min)

Figure 14. Effect of the culture medium on energy-dependent iron uptake. Cells were centrifuged, resuspended and incubated at 37°C with shaking for 2 min prior to pulsing with ^{55}Fe. Symbols: $^{55}Fe^{+3}$-citrate (1:10) control (un-pelleted) (O——O); $^{55}Fe^{+3}$-citrate (1:10) pelleted (5 min, 2400x g) and resuspended in its own supernatant (Δ——Δ); $^{55}Fe^{+3}$-citrate (1:10) pelleted (5 min, 2400 x g) and resuspended in fresh iron-poor NDM (▲——▲); $^{55}Fe^{+3}$-isocitrate (1:10) un-pelleted (●——●).


700
600
500
400
300
200
100
0
pmol Fe taken up/10^9 cells
0
2
4
6
8
10
Time after ^{55}Fe pulse (min)

inhibition of the energy-dependent system by 500 μM KCN, 5 mM NaN_3, and 8 μM antimycin A (Figure 15) further demonstrated the importance of functioning respiratory chain for iron uptake, while the ability of 10 μM TCS to completely inhibit the energy-dependent-uptake showed that a trans-membrane proton gradient is also necessary. The K^+-specific ionophore valinomycin had little or no effect on iron uptake, although it had been shown at 8 μM to dissipate the internal K^+-gradient of the meningococcus SDIC (DeVoe and Archibald, unpublished). The result is in contrast to that of Negrin and Neilands (1978) who reported that valinomycin could effectively prevent ferrichrome uptake by inner membrane vesicles of E. coli.

In KCN-poisoned cells the energy-independent uptake of iron from the $FeCl_3$ pulse was compared to the iron uptake from the Fe^{+3}-citrate pulse. Uptake of iron from both sources by KCN-poisoned cells was completed within 1 min (Figures 11,15), but iron from, or as Fe^{+3}-citrate, was bound only to the extent of approximately one-half to two-thirds that of iron from $FeCl_3$ (Figures 11,14,Table 16). I have shown in Figures 11 and 12 that the amount of iron taken up from $FeCl_3$ was roughly proportional to the final concentration of iron between 0.08 and 3.6 μM in the culture aliquot. However, in the experiments presented here, the amount of iron taken up from Fe^{+3}-citrate was nearly independent of the amount added (Table 15), suggesting the

TABLE 15

Uptake of iron by KCN poisoned meningococcus SDIC from Fe^{+3}-citrate (1:10)

Final medium iron of pulse (Fe^{+3}-citrate 1:10)	Iron (pmol bound/ 10^9 cells) (5 min post-pulse)	Iron pmol bound/ 10^9 cells (10 min post-pulse)
0.85 µM	80.4	84.8
1.70 µM	87.0	81.5
3.40 µM	97.1	96.0

TABLE 16

Effect of various treatments on the binding of $^{55}Fe^{+3}$ from $^{55}Fe^{+3}$-citrate (1:10) to iron-starved, continuous culture cells of meningococcus SDIC

Pre-treatment* before pulse with $^{55}Fe^{+3}$ (95 ng/ml, 1.7 µM final)	Iron bound in 1 min/10^9 cells (pmol)	Percent total iron taken up after 1 min	Iron bound in 10 min/10^9 cells (pmol)	Percent total iron taken up after 10 min
no treatment	110	12.9	427	50.2
KCN (500 µM; 2 min)	64	7.5	62	7.3
KCN (500 µM; 2 min; after 100°C, 5 min)	12	1.4	18	2.1
45°C, 5 min	107	12.6	387	45.5
60°C, 5 min	10	1.1	18	2.1
80°C, 5 min	14	1.6	18	2.1

* All were incubated at 37° with shaking

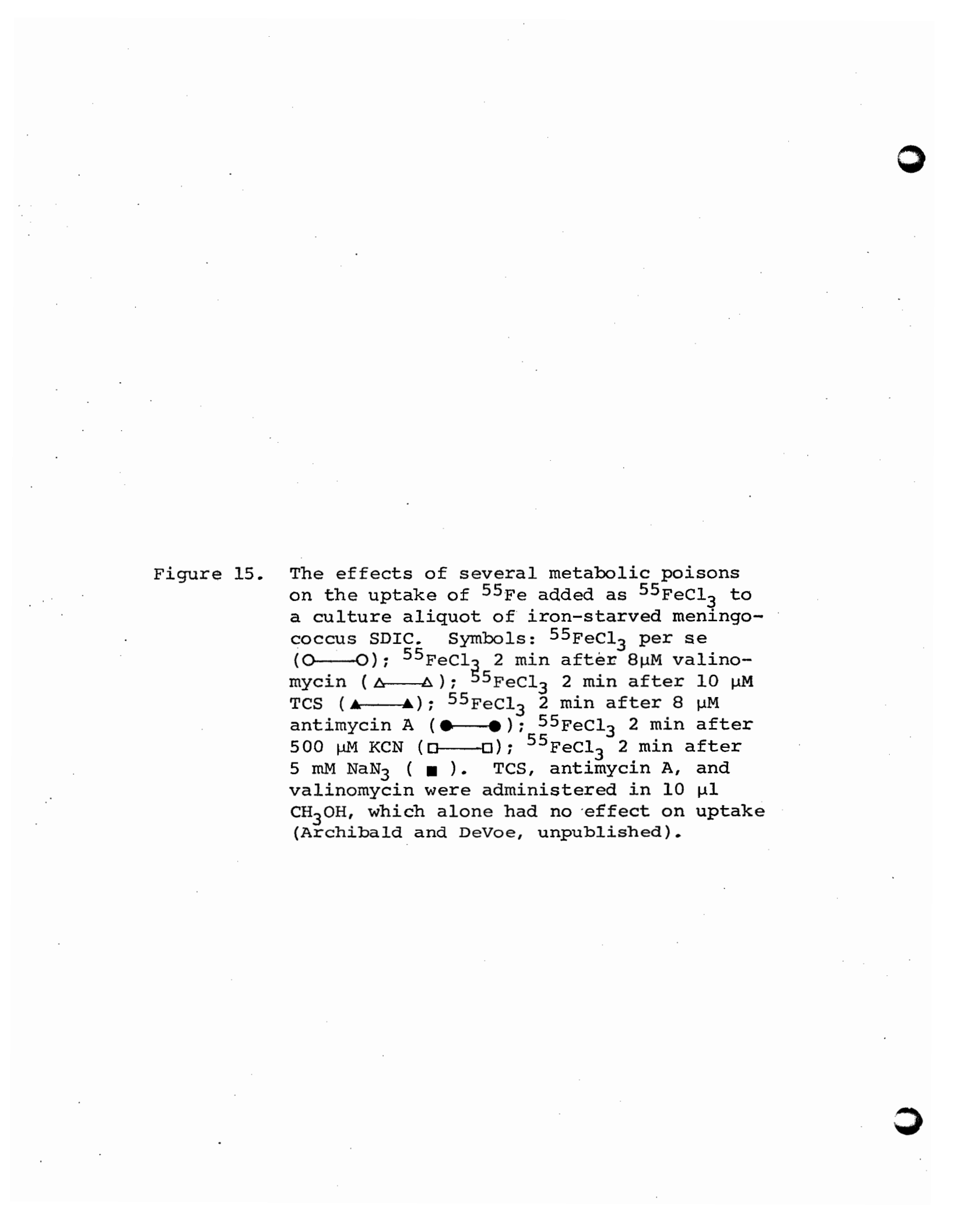
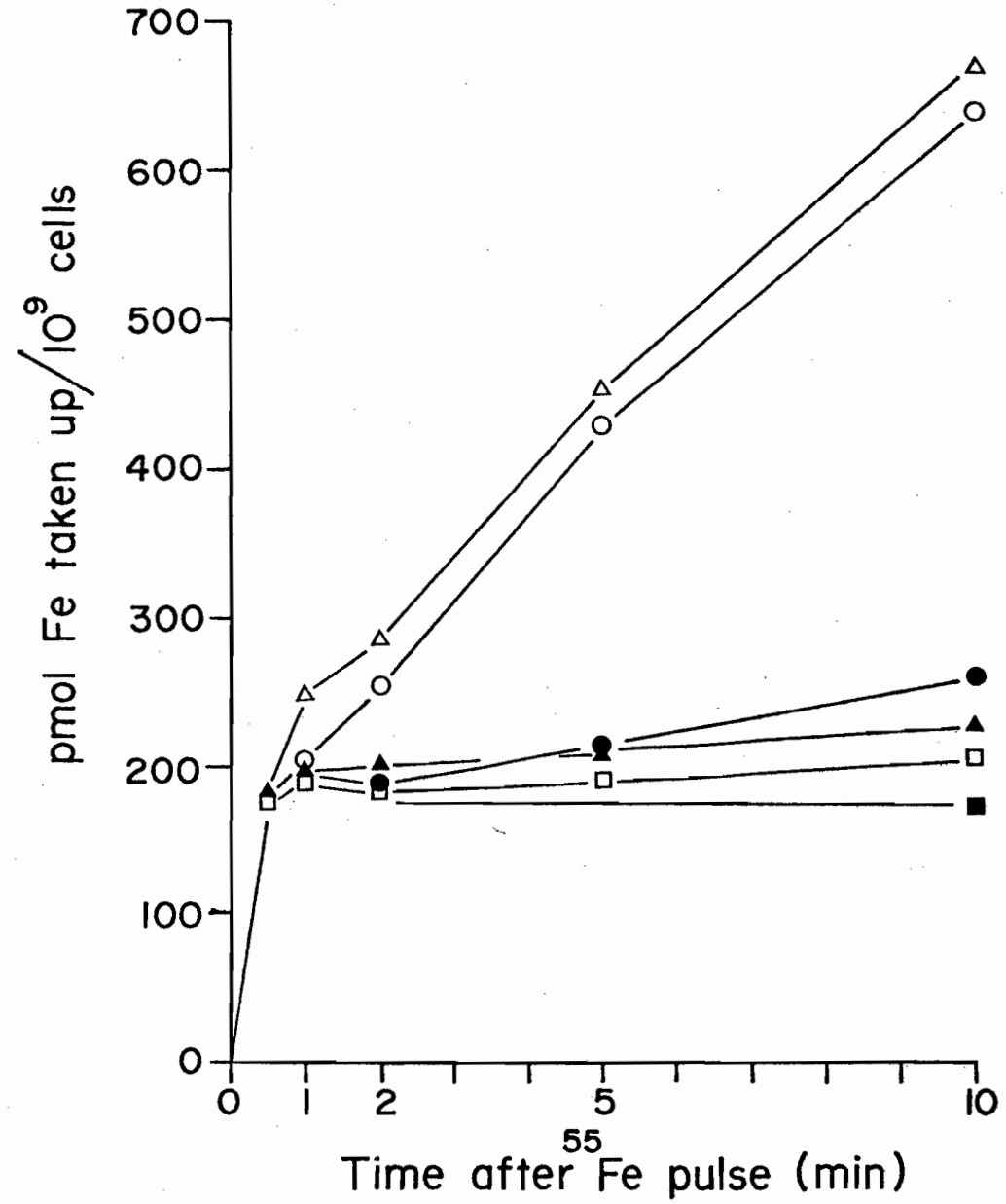


Figure 15. The effects of several metabolic poisons on the uptake of ^{55}Fe added as $^{55}FeCl_3$ to a culture aliquot of iron-starved meningococcus SDIC. Symbols: $^{55}FeCl_3$ per se (O——O); $^{55}FeCl_3$ 2 min after 8μM valinomycin (Δ——Δ); $^{55}FeCl_3$ 2 min after 10 μM TCS (▲——▲); $^{55}FeCl_3$ 2 min after 8 μM antimycin A (●——●); $^{55}FeCl_3$ 2 min after 500 μM KCN (□——□); $^{55}FeCl_3$ 2 min after 5 mM NaN_3 (■). TCS, antimycin A, and valinomycin were administered in 10 μl CH_3OH, which alone had no effect on uptake (Archibald and DeVoe, unpublished).


700
600
500
400
300
200
100
0
pmol Fe taken up/10^9 cells
0
1
2
5
10
Time after ^{55}Fe pulse (min)

presence of saturable Fe^{+3} or Fe^{+3}-citrate binding sites. To determine if such binding sites were heat-sensitive, KCN-poisoned cells were treated at 45°, 60° and 80°C for 5 min prior to iron-pulsing (Table 16). The results indicate that >80% of the iron taken up from or as Fe^{+3}-citrate was bound by sites denatured by 5 min at 60°C, but not 45°. Since both cell numbers and the quantity of iron bound by this heat-sensitive system were known it could be calculated from the data in Table 16 that there were 3.1×10^4 such sites per cell. When data from different continuous culture trials with differing doubling times and therefore different degrees of iron-limitation were compared, the range of values obtained was 3 to 5×10^4 iron atoms/cell bound by heat-denaturable sites.

The results in Section II of the thesis strongly suggested that at least under the <u>in vitro</u> conditions employed the meningococcus did not produce a low molecular weight soluble siderophore. This was tested further by resuspending cell pellets both in their own spent NDM and in fresh low iron NDM and then comparing the ability of each to take up iron from Fe^{+3}-citrate. The results (Figure 14) clearly established that no soluble substances generated and released by the cells, themselves, were required for moderately rapid and efficient energy-dependent iron uptake. However, the 35-40% reduction in the rate of energy-dependent uptake by cells in fresh NDM over cells

resuspended in spent NDM demonstrated that there was some difference between fresh and spent NDM important to obtaining a maximal rate of iron uptake. Among the possibilities, one must consider the following: (a) the presence of acidic substances known to be excreted by SDIC in low-iron NDM which may include metabolic organic acids or other adidic compounds with iron-transporting characteristics; or (b) a result of the sharp initial loss in viability which occurs when cells from an active NDM broth culture are transferred to fresh NDM (Section I, Archibald and DeVoe, unpublished). The obvious improvement in iron uptake afforded by the presence of 330 nm citrate on complex or defined solid media and reports of the exclusive production of acetate by pathogenic Neisseria (Jyssum, 1962; Morse et al., 1974) argue against the first possibility.

As the Fe^{+3}-citrate, Fe^{+3}-NTA and Fe^{+3}-isocitrate complexes that SDIC could so readily deferrate or take up were quite stable, it was of interest to determine what affinity the cellular binding sites had for Fe^{+3} from or with citrate in competition with the high-affinity siderophore Desferal (deferrioxamine B; $K_{ML} = 10^{30.6}$) (Figure 16). Although SDIC was unable to obtain measurable iron from Fe^{+3}-Desferal (1:3), most of the iron already bound to the cells with or from citrate could not be removed by iron-free Desferal in 3-fold molar excess, whether the cells were KCN-treated or not. While steric hindrance and

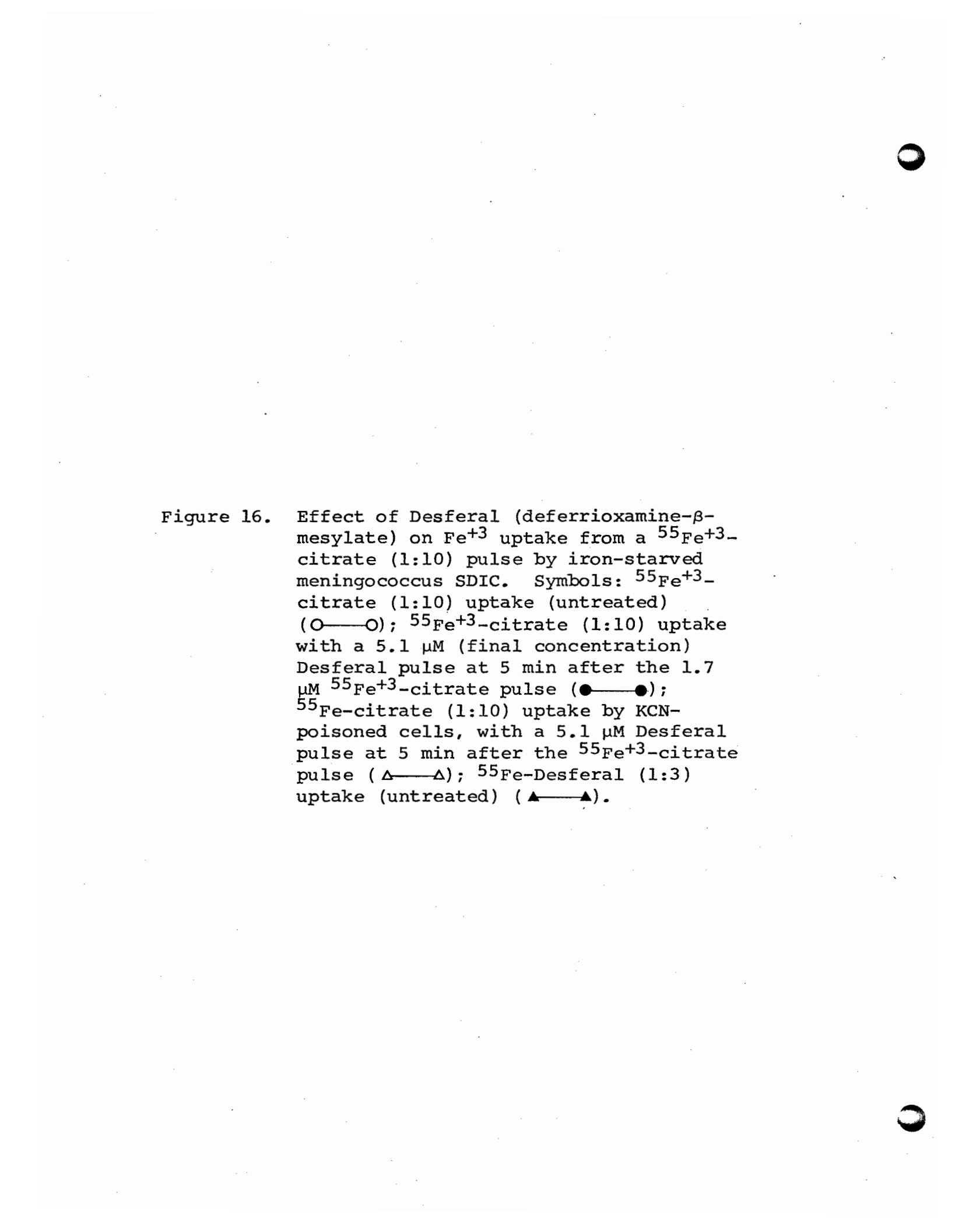
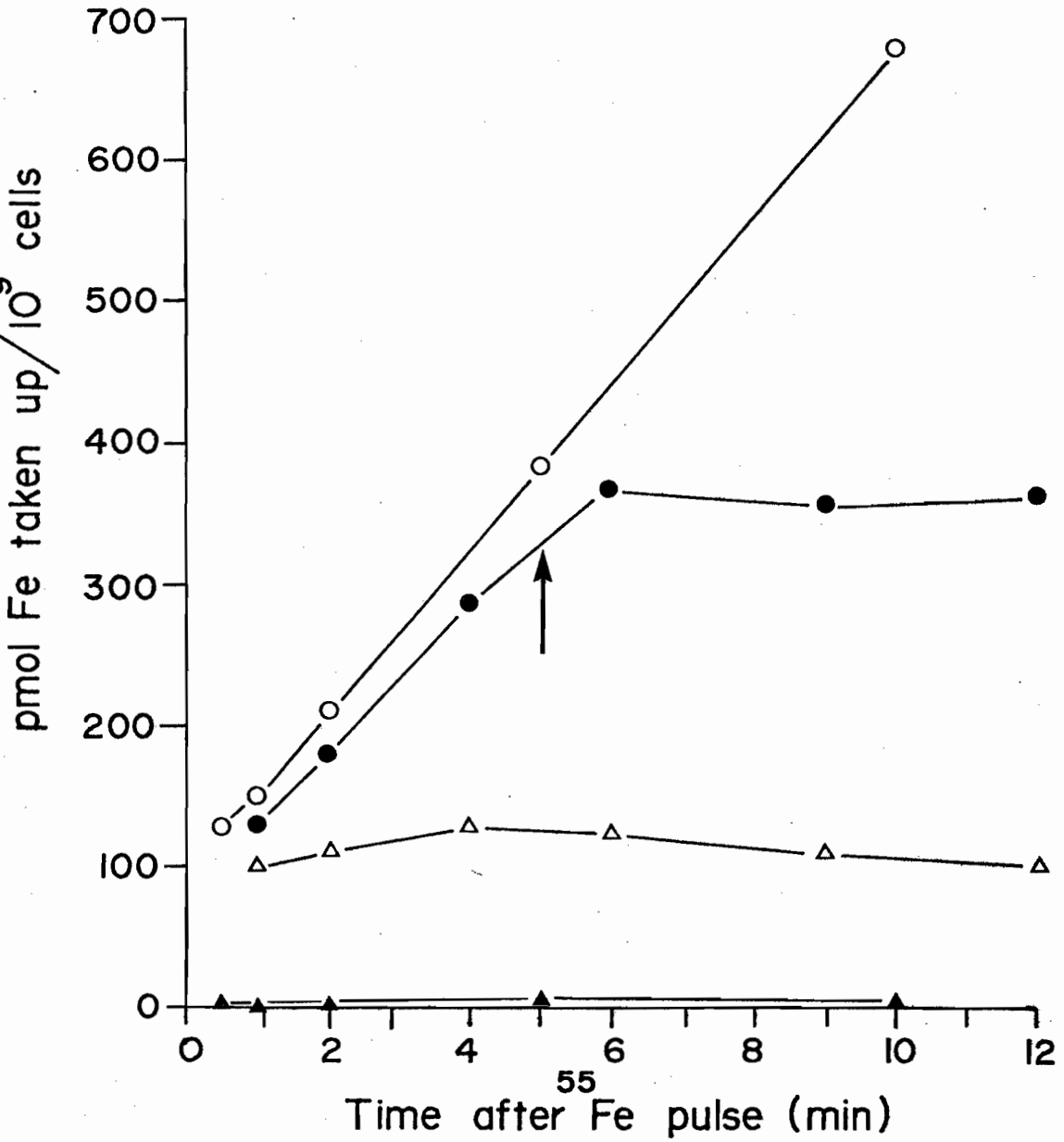


Figure 16. Effect of Desferal (deferrioxamine-β-mesylate) on Fe^{+3} uptake from a $^{55}Fe^{+3}$-citrate (1:10) pulse by iron-starved meningococcus SDIC. Symbols: $^{55}Fe^{+3}$-citrate (1:10) uptake (untreated) (O——O); $^{55}Fe^{+3}$-citrate (1:10) uptake with a 5.1 μM (final concentration) Desferal pulse at 5 min after the 1.7 μM $^{55}Fe^{+3}$-citrate pulse (●——●); ^{55}Fe-citrate (1:10) uptake by KCN-poisoned cells, with a 5.1 μM Desferal pulse at 5 min after the $^{55}Fe^{+3}$-citrate pulse (Δ——Δ); ^{55}Fe-Desferal (1:3) uptake (untreated) (▲——▲).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pmol Fe taken up/10^9 cells
0
2
4
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8
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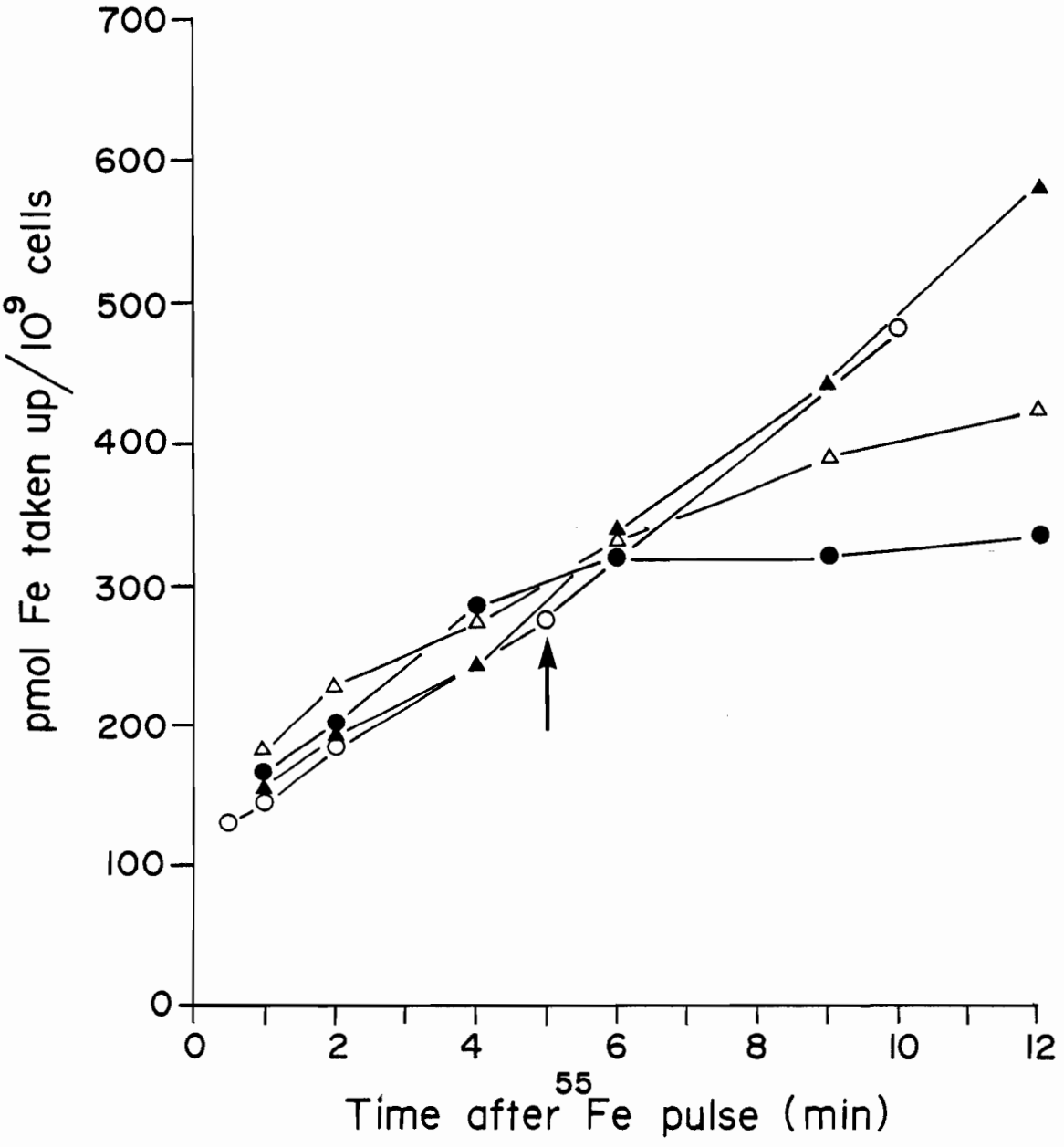
12
Time after ^{55}Fe pulse (min)

the low binding site iron exchange rate, and not just the relative stability products of the Fe^{+3}-Desferal and Fe-cell complexes may have allowed the cells to withhold this bound iron from Desferal, these results suggest, at least, that the binding sites have a very low rate of iron exchange with the environment. The energy-dependent uptake of iron was also curtailed completely by the Desferal pulse, presumably as Desferal can rapidly remove iron from Fe^{+3}-citrate in the medium and, as has been demonstrated here (Figure 16) and in Section II, the resulting Fe^{+3}-Desferal would be unavailable to the meningococcus. However, iron already bound to the cells apparently could not be removed, although the total iron bound in the first 5 min after the iron pulse, and before the Desferal pulse, was far in excess of that that could be bound by the heat-sensitive binding sites alone, i.e. binding in the presence of KCN. These results were extended by pulsing iron-starved culture aliquots with 1.7 μM $^{55}FeCl_3$ and then after 5 min with 5.1 μM deferriferrichrome, EDTA or Desferal (Figure 17). Only Desferal was completely effective in stopping energy-dependent uptake. Although iron bound to ferrichrome was inaccessible to the meningococcus in the plate assays of Section II, iron bound to the meningococcus was also apparently inaccessible to ferrichrome, and at least for a short period after the deferriferrichrome pulse, the meningococcus could compete with some success with the ferrichrome

Figure 17. Effect of iron-free Desferal, ferrichrome and EDTA on ^{55}Fe uptake from $^{55}FeCl_3$ by iron-starved meningococcus SDIC. Symbols: 1.7 mM $^{55}FeCl_3$ pulse per se (O——O); 5.1 μM EDTA pulse 5 min after initial 1.7 μM $^{55}FeCl_3$ pulse (▲——▲); 5.1 μM ferrichrome pulse 5 min after initial 1.7 μM $^{55}FeCl_3$ pulse (△——△); 5.1 μM Desferal pulse 5 min after initial 1.7 μM $^{55}FeCl_3$ pulse (●——●).


700
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100
0
pmol Fe taken up/10^9 cells
0
2
4
6
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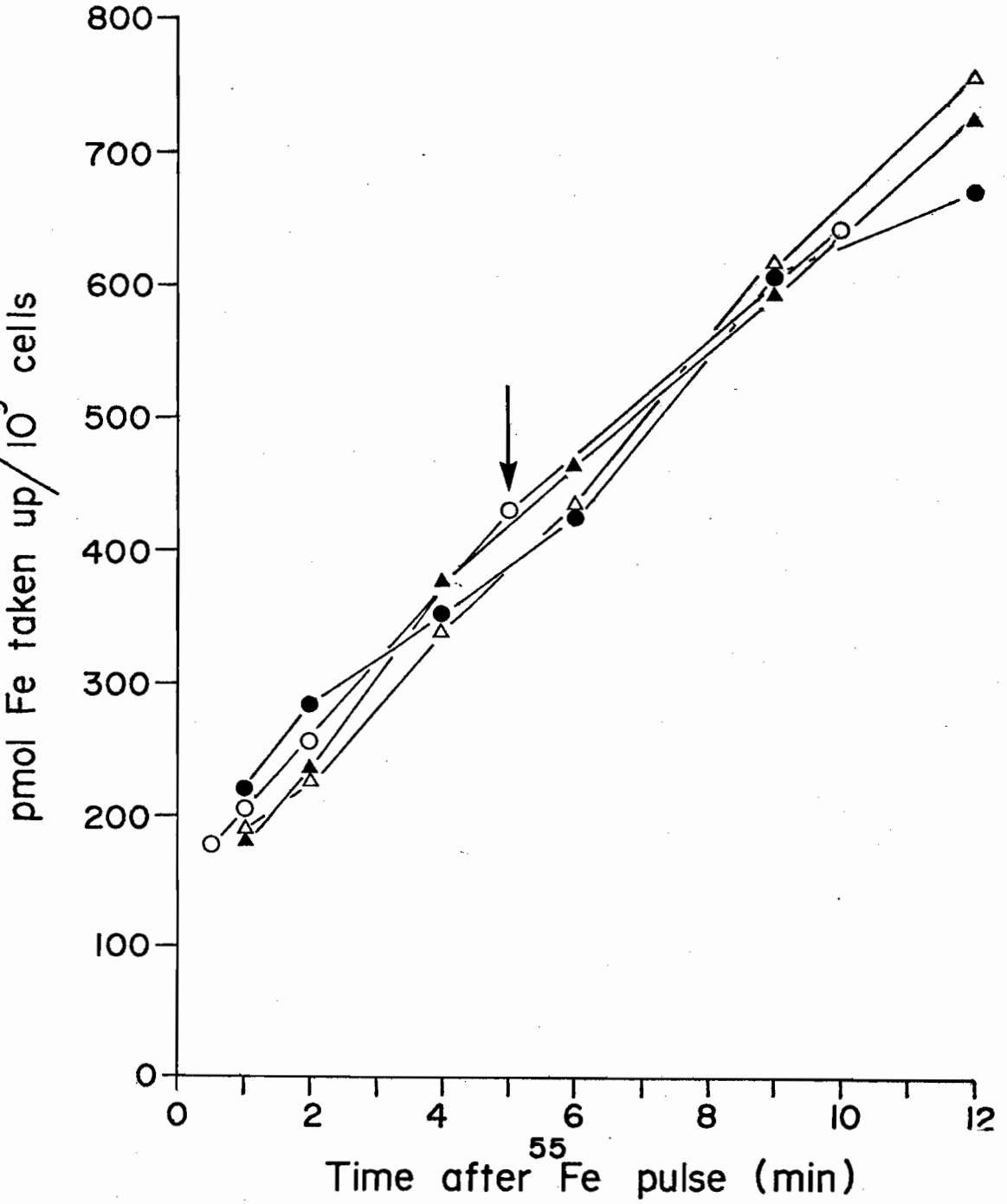
10
12
Time after ^{55}Fe pulse (min)

Figure 18. Effect of excess Mn^{+2}, Co^{+2}, and Ni^{+2} on the uptake of Fe^{+3} from $FeCl_3$ by iron-starved meningococcus SDIC. All trials were pulsed identically with 1.7 μM (final concentration) $^{55}FeCl_3$, and at 5 min post iron pulse with 5.1 μM of Ni^{+2}, Co^{+2}, or Mn^{+2}. Symbols: $^{55}FeCl_3$ per se (O——O); Ni^{+2} pulse at 5 min (Δ——Δ); Co^{+2} pulse at 5 min (▲——▲); Mn^{+2} pulse at 5 min (●——●).


800
700
600
500
400
300
200
100
0
pmol Fe taken up/ 10^9 cells
0
2
4
6
8
10
12
Time after ^{55}Fe pulse (min)

[$K_{ML} = 10^{29.1}$ (Anderegg et al., 1963)] for medium iron.

Despite the slow uptake of iron from the Fe^{+3}-EDTA (1:3) complex (Figure 13), iron continued to be taken up by the cells in the presence of the iron-free EDTA pulse (Figure 17). This suggested either interference with the binding of extracellular iron to EDTA in the culture, or the initial binding of EDTA to cells with subsequent iron-binding to the cell-associated EDTA or other sites.

In Section II the iron-limited plate assays on a variety of meningococci yielded data suggesting that cadmium and six transition-metal elements close to iron in mass did not compete with iron for uptake or insertion into functional iron-containing molecules. To support that data, the specificity of Fe^{+3} uptake was determined by pulsing cells engaged in rapid energy-dependent uptake of iron from an $FeCl_3$ pulse with 5.1 μM Co^{+2}, Ni^{+2}, or Mn^{+2}, a three-fold molar excess over the initial iron concentration. In no instance was there a significant change in the rate of iron uptake (Figure 18). Therefore, while Co^{+2} and Ni^{+2} were somewhat toxic to SDIC at millimolar concentrations (unpublished) on NDM, none appeared to exert their effects through interference with iron uptake.

IIID. Discussion

The rapid biphasic uptake of ^{55}Fe from $^{55}FeCl_3$ (Figure 9)

by meningococcus SDIC appears to demonstrate at least two different iron-binding mechanisms. The initial process (0-60 sec post-pulse) is metabolic energy-independent and shows first-order kinetics, i.e. at least within the range of the ferric iron concentrations used (0.08 - 3.7 μM), the amount taken up and the initial rate of uptake depend largely on the medium iron concentration (Figures 9,12). This iron taken up by the meningococcus within the first minute (energy-independent uptake) was maintained by the cells at high concentration relative to that in the medium. It could be due to one or more of the following mechanisms: (i) the nonspecific association of iron to cellular surface proteins or other components (see Schmidt, 1938; Gurd and Wilcox, 1956); (ii) the binding to specific cellular energy-dependent iron uptake sites without subsequent iron removal; (iii) the saturation of an internal chelator only deferrated through an energy-linked process; or (iv) a combination of these processes. In other bacteria, there have been no reports clearly demonstrating such energy-independent iron uptake, with the exception of the work of Peters and Warren (1968,1970) on Bacillus subtilis grown at low iron concentrations.

The temperature sensitivity and enzyme-like saturation kinetics, exhibited by the energy-dependent $^{55}FeCl_3$ iron uptake system, are typical of uptake systems with specific transport molecules but do not demonstrate the presence of such a system;

e.g. the iron uptake may be by nonspecific means, rate-limited by enzyme-mediated incorporation of the internal iron into functional molecules.

The complete blockage of energy-dependent iron uptake in the cyanide-treated meningococcus is to be expected in an obligate aerobe, and does not define the form of energy required for the active uptake process. However, the 50% inhibition of active iron uptake produced by the uncoupler TCS, a bacteriostatic detergent investigated for its mode of action by Hamilton (1968) and Harold and Baarda (1968), suggests that maintenance of a proton or metal-cation gradient across the membrane is important.

The iron taken up by the meningococcus in the absence of respiration (energy-independent uptake) was maintained by the cells at high concentration relative to that in the medium. This energy-independent uptake could be due to one or more mechanisms, such as the following: (i) the nonspecific association of iron to cellular surface proteins or other components (Scatchard, 1949; Gurd and Wilcox, 1956); (ii) the binding to specific cellular energy-dependent iron uptake sites without subsequent iron removal; (iii) the saturation of an internal chelator only deferrated through an energy-linked process; or (iv) a combination of these processes. Further evidence for these hypotheses is provided by the rapid uptake experiments using other forms of ferric iron.

Ferric iron bound in a citrate or NTA complex or chelate is unavailable to several bacterial strains shown to lack, through natural or induced auxotrophy, an iron-acquiring system secreting a siderophore (Reich and Hanks, 1964; Luckey et al., 1972; Frost and Rosenberg, 1973). Therefore, the uptake of ferric iron from or with its citrate, isocitrate, and NTA complexes at the same rate as from $FeCl_3$ by meningococcus SDIC strongly suggests the presence of a siderophore-free iron acquisition system considerably more effective than the "low affinity" systems used by Salmonella typhimurium (Luckey et al., 1972), E. coli (Frost and Rosenberg, 1973), and those Arthrobacter species requiring the "terregens factor" (Reich and Hanks, 1964) in the absence of usable siderophores. Thus the siderophore-free meningococcal iron-uptake system described here could be considered to be "high-affinity" both because of the ability of the system to use many stable hydroxy acid-iron complexes and because of the observed low iron concentrations required for saturating the energy-dependent uptake system and for stimulating growth.

The fact that iron pulsed into the iron-starved culture aliquots as Fe^{+3}-citrate, Fe^{+3}-NTA, Fe^{+3}-isocitrate, and $FeCl_3$ was taken up at essentially the same energy-dependent rate irrespective of their very different forms (Bates et al., 1967) suggests a low specificity for the iron-complex recognition or

binding sites on the cell. While binding of these iron chelates or complexes could occur via a number of highly specific sites followed by a common rate-limiting iron-transport mechanism analogous to the ton b gene product of E. coli (Braun et al., 1976), the presence of a specific site for the synthetic iron chelator NTA would be unlikely.

It was noted, however, that except for the phosphate-esters and salicylhydroxamate, all the low molecular weight compounds supplying iron to, or acting as, functional siderophores for meningococci in the plate assays (Section II) contained two or more carboxylic acid groups separated by two or more carbon or nitrogen atoms. In contrast to this relatively low iron-carrier specificity, both the iron-starved growth responses (Section II) and the short-term uptake competition experiments (Figure 18) indicate that the metal-cation specificity of the energy-dependent iron-uptake system of the meningococcus was quite high, unlike some other microorganisms (Nakayama et al., 1964; Wang and Newton, 1969a,b; Lankford, 1973). This would presumably be a necessity as, in contrast to most conventional siderophores, compounds such as citrate and NTA will bind with similar affinity a wide variety of di- and trivalent metal cations, and whether at the iron-complexing or internalizing step, any microorganism could be expected to need substrate selectivity in its high-affinity iron-uptake system

to minimize toxic effects from other, more abundant metals.

The combination of the absence of saturability from energy-independent $FeCl_3$ binding, the greater total amount of iron bound from $FeCl_3$ than from Fe^{+3}-citrate, and the similar rates of energy-dependent iron uptake make it tempting to speculate that iron bound from either $FeCl_3$ or Fe^{+3}-citrate occupies the same specific cellular sites, but in the case of $FeCl_3$, nonspecific ones as well. Nonspecific binding would be expected when hydrated Fe^{+3} ions or weak complexes come in contact with cellular proteins, phospholipids, and carbohydrates, and the amount bound would be dependent on the ambient concentration of iron (Schmidt, 1938; Gurd and Wilcox, 1956). It was not possible to determine by the short-term uptake experiments whether the 3 to 5 x 10^4 iron atoms bound by the heat-denaturable sites on each cell were induced by iron-starvation. Lower iron-binding by cells not starved for iron could either be due to fewer sites or a similar number of sites largely occupied by available, unlabeled medium iron. The fact that 60°C, but not 45°C, destroyed iron-uptake and binding argues against a role for high fever temperatures as a body defence for reducing the effectiveness of microbial iron acquisition, as has been proposed for several other bacterial infections. However, in all three examples of temperature sensitivity, i.e. Aeromonas hydrophila, Salmonella typhimurium, and a

fluorescent pseudomonad, the sensitive site appeared to be siderophore synthesis or release, which could not be a source of difficulty for the meningococcal system described here and elsewhere (Garibaldi,1971,1972;Greiger and Kluger, 1977.

While we did not uncover the reason for the decreased energy-dependent Fe^{+3} uptake from about 40 pmol/10^9 cells/min in washed cells resuspended in their own spent medium to 25-28 pmol/10^9 cells/min in cells resuspended in fresh low-iron NDM, it was clear that the remaining very appreciable energy-dependent uptake from the stable Fe^{+3}-dicitrate complex (Bates et al., 1967) occurred without the aid of a microbially produced true or a "functional" siderophore. It is unlikely that the weak Fe^{+3}-complexing medium components such as glutamate or glucose could fulfill this role, especially as citrate was in 10-fold molar excess over the iron.

Although it is common practice to rate the iron-acquiring or iron-retaining ability of a chelate or complex in terms of its stability product or constant (K_{ML}) (Schubert, 1963), the abilities of various chelators or complexing agents to exchange iron under in vivo conditions is often unrelated to differences in their stability constants. Generally of more importance are steric effects and the effective binding constants (K_{eff}) of the substances, which take into account side-reactions, pH and physiological concentrations (Schubert, 1963). Therefore, the

ability of SDIC to retain bound iron, even if it is exposed on the cell surface, from deferriferrichrome ($K_{ML} = 10^{29.1}$), Desferal ($K_{ML} = 10^{30.6}$), or EDTA ($K_{ML} = 10^{25.1}$), does not necessarily indicate that binding sites in or on the meningococcal cell have a greater affinity for iron than any of these compounds (Schubert, 1963; Pitt et al., 1979). It does mean that in a defined culture medium with many ions near physiological concentrations, the meningococcus, poisoned or not, is highly effective in retaining bound ferric iron, and that up to the iron-capacity of the heat-sensitive sites, apparently requires no metabolic energy for such binding.

One hypothesis to explain the data presented on iron acquisition from Fe^{+3}-citrate by iron-starved meningococci is that some or all of the energy-independent, heat-sensitive sites (Table 16) are also the iron-binding sites for the energy-dependent iron-uptake system. If this is true of all the sites, then it can be calculated, knowing the energy-dependent iron uptake rate (45 to 65 pmol per 10^9 cells) and the number of heat sensitive sites (3 to 5 x 10^4 per cell), that the average rate of uptake is slightly less than one iron atom per site per min. Although this rate appears very slow, the iron-starved continuous culture pulsed with 1.7 μM (final concentration) Fe^{+3}-citrate increased its cellular iron from approximately 30 pmol per 10^9 cells to 140 pmol per 10^9 cells in the first 30 S and

reached the level present in batch culture early log-phase cells (350 to 375 pmol per 10^9 cells) on non iron-limited NDM in 4 min. However, it should be noted that this Fe^{+3}-citrate/$FeCl_3$ uptake system may not be the only or even the major iron-uptake system present in the meningococcus. It is not known how iron is obtained from phosphate-ester iron complexes or heme and transferrin molecules, both demonstrated in Section II.

If the meningococcus bases its *in vivo* iron-acquisition on the use of environmental organic acids, phosphate esters or transferrin-type proteins as opposed to the secretion of conventional siderophores, there would be obvious advantages for such an organism growing at low cellular density on the mucous membrane surface of the nasopharynx, the serum or in cerebrospinal fluid, all of which contain usable levels of one or more of these "functional siderophores" (Diem and Lentner, 1970).

To date, the only procaryote or fungal high-affinity iron-uptake systems reported that do not employ conventional secreted siderophores are the citrate-related system of *E. coli* (Frost and Rosenberg, 1973), and the citrate-iron precipitation system in *Neurospora crassa* (Winkelmann, 1979). The saturable, stable binding of 80% of the energy-dependent Fe^{+3}-citrate uptake by the heat-sensitive binding sites on iron-starved meningococci would appear to be a useful and convenient tool for investigating binding specificity, affinity, site location and

other characteristics of this iron-acquiring system. Therefore, the iron uptake system described in this thesis may be of interest both as an example of a siderophore-free high-affinity system and as an aid in better understanding the ecology of the meningococcus.

GENERAL DISCUSSION

The data presented in this thesis, having been obtained exclusively *in vitro* must be extrapolated to the man-meningococcus relationship with considerable caution. This information does, however, allow the asking of some manageable and meaningful experimental questions concerning the role of iron in both the meningococcal carrier and disease states.

The iron physiology of *N. meningitides* is characterized both by many common and a few novel features. Most of the observed effects of iron-privation have been reported in one or more other microorganisms (see Light and Clegg, 1974), although this does not detract from the utility of demonstrating (a) the relative importance of the various iron-related functions of the meningococcus, and (b) that iron-limitation would substantially reduce the ability of the parasite to proliferate in the host. Obviously the markedly reduced growth rates under iron-limitation would have a large effect on virulence, but the lack of catalase, less efficient respiration, and the difficulty in subculturing cells under iron-poor conditions are *in vitro* effects which are probably also of importance in the host. Although the iron requirement data obtained for the meningococcus were more accurate than those obtained for most other microorganisms, the minimal levels permitting near-maximal growth

rates in log phase may be less than the level required to produce cells of maximal virulence. Because unlike carbon or energy source acquisition, iron acquisition should not be required in non-growing, respiring cells, the importance of iron in the meningococcal carrier state depends on the at present unknown growth characteristics of the meningococcus in the nasopharynx. However, to counter the relatively rapid turnover of nasopharyngeal epithelial cells and the mucous lining, it seems reasonable to assume that at least some growth of the carrier meningococcal strains must occur, and hence some environmental iron will be required. Such acquisition would presumably not be difficult via the functional siderophore system as the organic acids released by the oral and particularly dental flora bathe the nasopharynx, and saliva is reported to contain up to 100 μM citrate (Diem and Lentner, 1970). However, the picture is complicated by the lack of data on the influence of the IgA, lysozyme, and lactoferrin secreted into the human nasopharynx, and the metal-cofactored IgA protease secreted by the meningococcus. Similarly, direct extrapolation to serum of the *in vitro* results showing the accessibility of the transferrin-bound iron, and the presence in serum of useful levels of citrate and pyrophosphate would suggest that the acquisition of iron from serum offered no problems, yet the mice in the experiments of Holbein *et al.* (1979a,b) appeared to become far more

susceptible to meningococcal infection when their transferrin iron saturation was raised from 48 to 82%. This suggested both that nutritional iron acquisition is an important factor in meningococcal infections of mice and that iron was not readily accessible from the serum. Rogers (1976) has demonstrated with E. coli o111 that the concerted effort of specific antibodies and transferrin is required for bactericidal effects, and that these antibodies can prevent siderophore biosynthesis which is necessary to overcome transferrin-induced iron limitation. Nevertheless, the work reported here has presented a number of ways in which iron may be readily acquired in vitro, and if these mechanisms fail to operate in vivo, an investigation of the reasons for this failure should lead the investigator to factors of real importance in the host-parasite competition for iron. The fact that the 20 meningococcal strains examined behaved similarly in the low-iron assay plates is useful in generalizing the results of this work to all meningococcal strains, but it does not eliminate the possibility of in vivo iron-acquisition differences between strains correlated with differences in virulence.

The results of the foregoing research show a number of areas in which the work could profitably be pursued. The effects of lactoferrin on meningococcal iron acquisition should be of considerable importance, both in the carrier state, and in

meningococcal infections where inflammatory hyposideremia has presumably occurred, resulting in significant levels of the glycoprotein in serum and cerebrospinal fluid. The mechanism of transferrin deferration, whether it turns out to be a transferrinase or other specific molecule analogous to the IgA protease, or via a cell-surface site similar to the one found on the mammalian reticulocyte (see Fielding and Speyer, 1974), would also be of considerable interest. The hydroxy acid and polyphosphate mediated iron acquisition system or systems are worthy of study for two reasons: (a) the only other high-affinity iron acquisition system reported that does not require a siderophore is the citrate system of Escherichia coli; and (b) the ease with which the system could be manipulated. The apparent presence of discrete saturable binding sites that hold iron with high affinity would greatly facilitate the localization of the sites, and the use of C^{14}-labeled citrate and other iron carriers would allow site specificity and homogeneity to be evaluated, as well as determining, whether the iron complex or only the iron was transported into the cell. It may also be profitable to examine meningococcal iron uptake in a system combining physiological transferrin, citrate, and pyrophosphate levels, because of the marked ability of the two small iron complexing molecules to promote the deferration of transferrin (see Aisen and Liebman, 1968a ;Pollack et al., 1977; Okada et

al., 1978). Logically it would be reasonable and aesthetically it would be satisfying to find that the pyrophosphate, citrate and transferrin iron-acquiring systems are working in concert or are facets of the same system.

Another interesting possibility is suggested by the fact that while the present A and C polysaccharide vaccines protect only against meningococci of their respective serogroups, contraction of an overt meningococcal infection results (in survivors) in immunity to all meningococci; i.e. there appears to be one or more immunologically useful common antigens on all strains (Littlejohns, 1976). If the heat-sensitive iron-binding sites or a component of the transferrin deferrating system can be proved to be reasonably antigenic, or if anti-meningococcal sera can be shown to interfere with iron uptake, then a knowledge of meningococcal iron acquisition may be of immediate practical value. However, regardless of its importance to the mechanism of meningococcal virulence, further basic information on meningococcal iron acquisition promises to contribute some interesting data to the field of microbial iron transport.

APPENDIX

A. COST-EFFECTIVE DISSOLVED OXYGEN MONITOR-CONTROLLER FOR CONTINUOUS CULTURE

Introduction

Control of dissolved oxygen (DO) tension in continuous culture is required for many investigations and consequently the theory and construction of a number of control systems have been described (Maclennan and Pirt, 1966; Flynn and Lilly, 1967; Kilburn and Morley, 1972). Most of these designs provide control of DO tension by continuously varying culture sparge rate, oxygen content of sparging gas, or agitation speed. These devices are rather expensive, although generally less than the 2500-4000 dollars presently charged for complete commercial units.

This appendix describes a simple, flexible, dual-action two-position DO monitor-controller made with readily available components which, including valves, gas solenoid, and DO probe, cost approximately 400 dollars. This device is capable of measuring and controlling DO tension through alteration of either agitation speed, gas sparge rate, or both. It was designed to work with a New Brunswick Scientific C-30 fermentor (New Brunswick, NJ) but will interface with any continuous-culture apparatus employing low-pressure air or gas sparging and rheostat- or potentiometer-controlled agitation speed.

Materials and Methods

Monitor-Controller Design

The monitor-controller is able to achieve high stability and linearity at low cost chiefly through the use of integrated circuit operational amplifiers. The monitor input will accept either galvanic or polarographic DO probes with maximum outputs of 1-25 mV across 1000 Ω. The amplified signal from IC-1 operates the meter and recorder output and is fed to a low-gain buffer amplifier, IC-2 (Figure 19). When the resultant signal voltage from IC-2 exceeds the "preset" voltage (0-6 V) applied to the inverting input of differential amplifier IC-3, diode D, and transistor Q_1 conduct, thereby energizing relays K_1 and K_2, as indicated by light-emitting diode D_4. These relays will operate an external 115 V ac gas solenoid, switch the agitation speed between two preset values using R_7 and R_8, or perform both functions simultaneously. The values of C_1, R_7, and R_8 match the C-30 agitator control; in other fermentors R_7 and R_8 will have the same value as the original control potentiometer and C_1 may or may not be necessary.

This monitor-controller can be used in other negative feedback systems: e.g., the recorder output of a pH meter or spectrophotometer can be applied to the galvanic probe input, and a compensating pump, valve, or solenoid operated by the

controlled 115 V ac output.

Construction

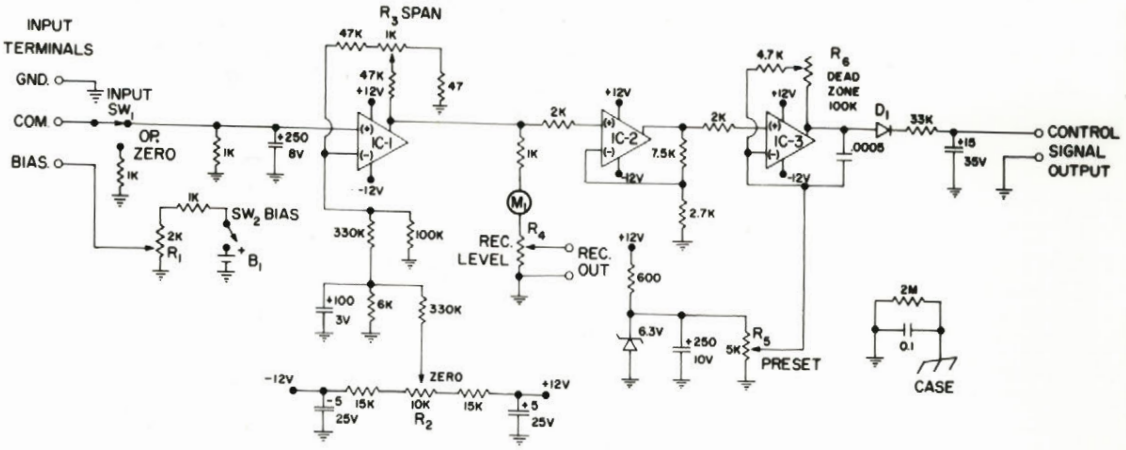
To prevent impulse-type interference from K_1 and K_2, the device was housed in two aluminum cases, one containing the ± 12 V supply and monitor circuitry, the second the + 15 V supply and the controller circuitry, as in Figure 19. Components other than transformers, relays, and controls were mounted on phenolic circuit boards and terminal strips. To connect the agitation speed control to the fermentor, the C-30 front panel potentiometer was disconnected and a cable connected from terminals S_1-S_3 of the C-30 agitator motor speed-controller to terminals T_1-T_3 on the DO monitor-controller terminal strip. When interfacing with other agitation controls, terminals T_1-T_3 would receive the wires normally going to the control potentiometer.

Operation

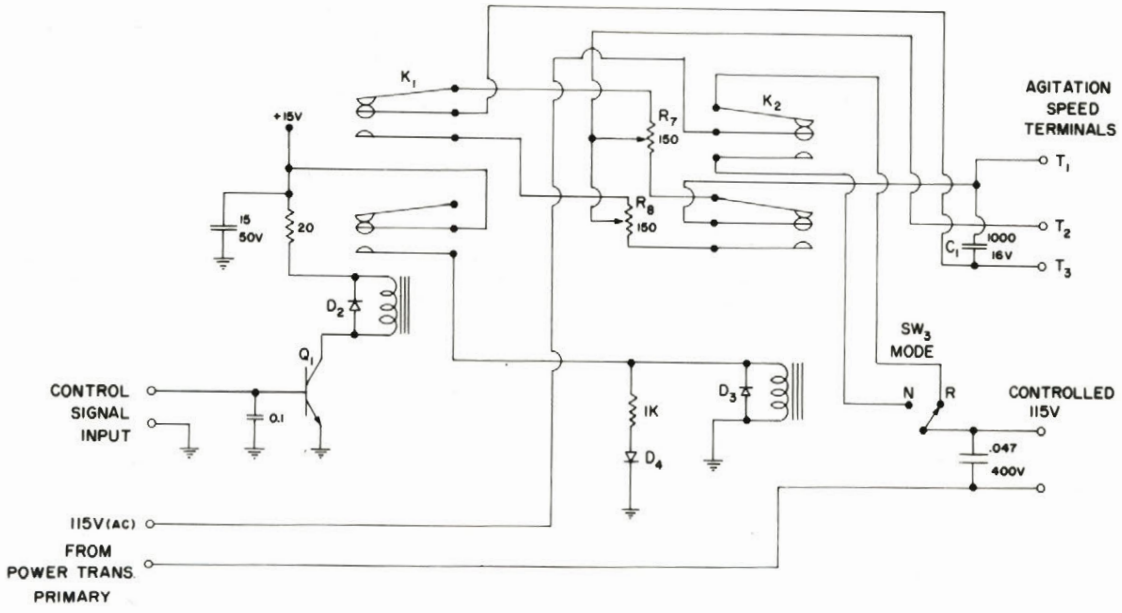
If a polarographic probe is employed, it is connected to the BIAS (+) and COM (-) terminals with the BIAS switch on and R_1 set for the proper bias, and if a galvanic probe or recorder output is employed the COM (+) and GND (-) terminals are used, with the BIAS switch off. The electrical zero is set with R_2 and the meter calibrated to the probe with R_3 using air-saturated medium at the culture temperature. SPAN control R_3 can also be

Figure 19. Schematic diagram of the dissolved oxygen (DO) monitor-controller. Power supplies were regulated, the +15 V supply using a 7815 integrated circuit and the $\pm$ 12 V supplies using zener diodes (1N-4742). Resistances are in Ω $\pm$ 10% and capacitances in μF ($\pm$ 20%). Potentiometers R_{1-8} and relay K_1 are of high quality.

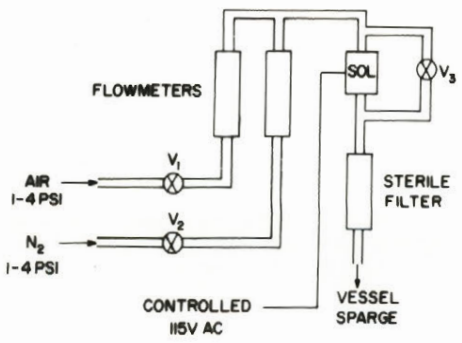
MONITOR MODULE



CONTROLLER MODULE



SPARGE CONTROL



COMPONENTS

M_1 0-1mA precision meter (10x12cm face)
K_1 Clare DPDT 975Ω No. RP 7632 G 48 or equivalent
K_2 Radio Shack DPDT 95Ω 12V, 10A, contacts
Q_1 NPN, RS 2009, or 2N2222
D_1 IN914
$D_{2,3}$ IN4003
D_4 Light emitting diode (relay indicator) XC556G
IC-1,2,3 Signetics μA 741CV operational amplifier
SW_{1-3} SPST or SPDT toggle switches
SOL Asco 120V 20watt gas solenoid No. 8262A212
V_{1-3} Nupro 4JR2R gas valves
B_1 1.5V "C" dry cell

used to expand the scale at low DO tensions, i.e., by doubling a given low meter reading, full scale represents 50% DO saturation. For operation of the monitor alone, PRESET R_5 is now turned fully clockwise.

To use the DO control circuitry, the monitor and probe are calibrated, the continuous culture established, the agitation set by the B speed control (R_7), the gas flow adjusted using the needle valves V_1-V_3 and the MODE switch to provide the desired DO tension. The MODE switch S_3 allows either normally open or normally closed solenoids to be employed, as well as manual control of gas sparging. The B speed control (R_7) is then set 2-15% higher and the A control (R_8) 2-15% lower than optimal, and the gas flow rate with the solenoid open increased 5-20%. The setting of V_3 (Figure 19) may be from zero gas flow to slightly less than adequate to maintain the desired DO tension. The PRESET (R_5) and DEAD ZONE (R_6) controls, which interact slightly, are now used to set the per cent DO at which the device will maintain the culture. This is best done by switching SW_1 to ZERO, using R_2 to place a simulated signal on the meter, and centering the dead zone on the desired preset value. The dead zone should be small, but not so small as to cause excessive activity. Control R_2 is then re-zeroed and SW_1 switched back to OPERATE.

Discussion

The precision with which the controller will maintain culture DO tension is dependent on: (a) the setting R_6; (b) DO probe condition and response time; (c) the per cent O_2 in the sparging gas; and (d) the agitator speed settings and gas flow rates. If high and low gas flow rates and high and low agitation speed settings are close together regulation is very precise, but the change in culture oxygen consumption (load change) for which the controller will automatically compensate is small. A practical compromise is a control precision of ± 1-3% at DO tensions $>$15% of saturation and a fraction thereof at lower DO tensions, this depending on scale expansion and oxygen content of the sparging gas. This DO control precision is somewhat inferior to that of proportional controllers such as the New Brunswick Scientific DO-81 (3800 dollars).

After 20 months of use in a study involving the effects of iron and DO tension on microbial respiratory proteins in Neisseria meningitidis, the monitor-controller has proven reliable, stable, and DO-tension-dependent changes in the organisms were readily quantified. In this system, sparged with air containing 10% CO_2, control precision at both 5% and 60% DO saturation was ± 2%. If the monitor scale was expanded twofold, and the air-CO_2 diluted fourfold with N_2, precision at 5% saturation was ± 1% of full scale. Drift of electrical zero and preset

points was less than 1% over 72 hr, warm up time was >5 min, and the monitor-controller was not responsive to small ac line-voltage fluctuations. The supposedly autoclavable Johnson-Borkowski type DO electrodes employed were rather unstable and subject to membrane failure and represented the chief problem in the system. Recently, improved performance has been obtained using a modified Yellow Springs Instruments (Yellow Springs, Oh.) 5331 polarographic probe. The low current output of this small probe necessitated increasing the monitor input resistor to 20,000Ω and reducing the input capacitor to 20 μF.

In addition to continuous-culture applications, the DO monitor section was used alone with polarographic probes from Yellow Springs Instruments and a polarographic oxygen cell from Rank Brothers (Bottisham, England) in assays involving oxygen consumption and evolution and gave very satisfactory results.

In conclusion, it is felt that the device meets the objectives of providing accurate and flexible DO measurement and control of small continuous cultures at a greatly reduced cost, thus rendering DO control available to many projects on limited budgets.

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