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Iron in *Neisseria meningitidis*: Minimum Requirements, Effects of Limitation, and Characteristics of Uptake

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A simple defined medium (neisseria defined medium) was devised that does not require iron extraction to produce iron-limited growth of *Neisseria meningitidis* (SDIC). Comparison of this medium to Mueller-Hinton broth and agar showed nearly identical growth rates and yields. The defined medium was used in batch cultures to determine the disappearance of iron from the medium and its uptake by cells. To avoid a number of problems inherent in batch culture, continuous culture, in which iron and dissolved oxygen were varied independently, was used. Most of the cellular iron was found to be nonheme and associated with the particulate fraction in sonically disrupted cells. Nonheme and catalase-heme iron were reduced by iron starvation far more than cytochromes *b* and *c* and *N,N,N',N'*-tetramethylphenylenediamine-oxidase. The respiration rate and efficiency also decreased under iron limitation, whereas generation times increased. The iron-starved meningococcus took up iron by an energy-independent system operating in the first minute after an iron pulse and a slower energy-dependent system inhibited by respiratory poisons and an uncoupler. The energy-dependent system showed saturation kinetics and was stimulated nearly fourfold by iron privation. In addition, to determine the availability to the meningococcus of the iron in selected compounds, a sensitive assay was devised in which an iron-limited continuous culture was pulsed with the iron-containing compound.

Iron appears to be an essential metal for all organisms with the possible exception of some lactobacilli (35). 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 (7, 30, 40). 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 (1, 5, 7, 34, 40). 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 (29); (ii) siderophore receptor sites in the outer membrane that also serve as receptor sites for colicins and phages (4); and (iii) a correlation between virulence and the ability of a bacterial strain to obtain iron in a given host (6, 47).

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. For example, growth of *Neisseria gonorrhoeae* and *Neisseria meningitidis* was stimulated on Thayer-Martin medium after the addition of an iron-dextran or ferric ions, despite the high level of iron already present in the medium in complex form (23, 38). Hog gastric mucin, frequently used since the original report by Miller (31) to establish meningococcal infections in animals, may owe at least part of its infection-promoting effect to its high concentration of iron. Calver et al. (8) were able to infect mice with low numbers of meningococci by substituting various forms of iron for mucin, and the addition of the powerful ferric iron chelator, deferrioxamine-β-mesylate, increased the 50% lethal dose of meningococci in mice.

Cells of the more virulent T_1 and T_2 colonial types of *N. gonorrhoeae* appeared to take up

iron more efficiently in chicken embryos than the less virulent T_3 and T_4 cells (36). Also, in these experiments the iron-binding capacity of the protein conalbumin appeared to increase the 50% lethal dose of T_1 but not T_3 cells, and, conversely, added iron decreased the 50% lethal dose of T_3 and T_4 cells to approximately that of T_1 and T_2. In two meningococcal strains, the ability of the colonies to retain the dye congo red correlated with their ability to obtain iron and establish a fatal infection in the chicken embryo (37).

Although such findings must be interpreted with caution, especially those involving injection of proportionally large amounts of iron compounds or metal chelators into animals, there does appear to be evidence for the enhancement of pathogenic neisserial infections by iron and some correlation between bacterial iron uptake efficiencies and virulence. The work presented here provides basic data on iron requirements and uptake characteristics, as well as some effects of iron privation on the meningococcus. The work includes the growth of cells in continuous culture on a defined medium in which the iron concentration was readily made growth limiting.

MATERIALS AND METHODS

Organism. The group B *N. meningitidis* (SDIC) used was obtained from the Neisseria Repository, NAMRU, School of Public Health, University of California, Berkeley. Procedures for maintenance of stock and working cultures and for routine checks for strain purity were previously described (14). Meningococcus SDIC dissociates into rough and smooth colonial forms (16). 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 defined medium (NDM). Each was used as a broth or as a solid medium after the addition of 1.5% agar. The organic composition of NDM (Table 1) was partially derived from earlier meningococcal media (9, 17, 22), whereas the principal cation levels were set after determining ion concentrations in MH broth with a Perkin-Elmer SP-90A atomic absorption spectrophotometer. Exceptions were Ca^{2+} and PO_4^{2-} concentrations, reduced to prevent precipitation and reduce iron contamination, and NH_4^+, which was not measured in MH medium. The atomic absorption spectrophotometer was used in the emission mode for Na^+, K^+, and Ca^{2+} and in the absorption mode for Mg^{2+} and Fe. NDM was sterilized by autoclaving (121°C) after all components, including glucose, had been added. The inclusion of glucose was necessary to keep medium iron completely solubilized (see 11), and because it was necessary to have only the quantity and not the form of iron vary between high- and low-iron NDM. To obtain the 3.5- to 6.0-ng/ml residual iron level in NDM, double-distilled water, 6 N HCl-cleaned glassware, and screening of concentrated medium components by atomic absorption were required. Some batches of salts, uracil, and all cysteine-HCl tested proved to have unacceptably high iron levels.

TABLE 1. *Composition of NDM*[a]

Component	Final concn (mM)
L-Glutamic acid	10
D-Glucose	10
L-Cysteine	1
Uracil	1
L-Arginine	1
Trisma base	40
NaCl	140
$NaH_2PO_4 \cdot H_2O$	1
Na_2SO_4	2.3
$CaCl_2 \cdot 2H_2O$	0.5
$MgSO_4 \cdot 7H_2O$	0.2
KCl	2.0
NH_4Cl	10.0
Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+} (as chlorides)	0.00002
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	(As appropriate)

[a] The medium was made with double glass-distilled water, and the pH was adjusted to 7.5 with reagent-grade HCl.

Cell growth. Cultures on solid media were incubated at 37°C in a candle jar. 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% (vol/vol) 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- to 350-ml working volume (New Brunswick Scientific Co., New Brunswick, N.J.) was modified as follows: (i) all stainless steel in contact with the culture or the medium was replaced with glass, silicone rubber (unpigmented), or polyethylene; (ii) the impeller was coated with polyvinyl chloride; and (iii) control of both pH and dissolved-oxygen (DO) tension was provided (Fig. 1). 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 revolutions per minute and used a Johnson-Borkowski-type (3) galvanic DO electrode. The culture vessel subassembly, containing 350 ml of NDM, was autoclaved (121°C, 25 min), and the chemostat was assembled and adjusted to the following settings: 37 ± 0.1°C; 200 to 300 rpm of agitation; pH 7.45 ± 0.05; and a 10% CO_2-in-air sparging rate of 85 to 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 rate = flow rate/vessel volume, and μ = instantaneous growth rate) was readily maintained at a

cell density of 1.5×10^9 to 2.2×10^9 cells per ml (optical density at 600 nm = 0.45 to 0.65) with a fluctuation <2%/h. 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 to 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. Iron concentrations >200 ng/ml were determined by atomic absorption as described above. Concentrations <200 ng/ml were determined by the colorimetric method of Smith et al. (45) modified as follows: (i) all samples were adjusted to pH ≈5.2 with HCl; (ii) double-distilled hexanol (4.0 ml/100-ml sample) was used to extract the ferroin complex; and (iii) absorbances 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 and direct counts were determined as described previously (15). 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, 3×10^4 to 5×10^4 dpm of $^{55}FeCl_3$ per ml (≈9 μCi/μg) in 0.1 N HCl was added to 50-ml samples of NDM and MH broth in 250-ml

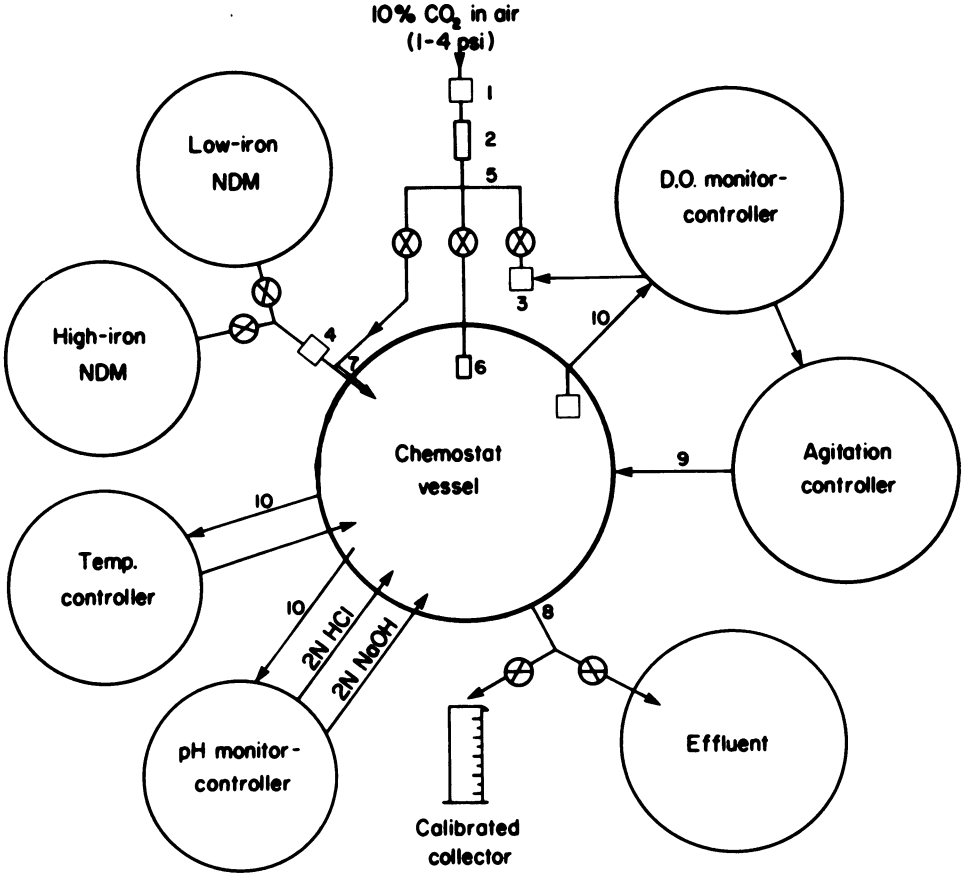

FIG. 1. *Schematic diagram of continuous-culture apparatus (a modified NBS C-30 fermentor) used for growth of N. meningitidis (SDIC): (1) 0- to 250-ml/min gas flowmeter; (2) sterile air filter; (3) gas-blocking solenoid (normally closed); (4) peristaltic pump (0 to 8 ml/min) for medium; (5) brass manifold with three needle valves; (6) sintered-glass aerator; (7) medum addition/aeration tube with two medium breaks; (8) overflow port; (9) impeller magnetic drive; (10) sensors.*

Erlenmeyer flasks. Each flask was inoculated with 1.0 ml of 4- to 6-h NDM or MH broth culture and shaken (100 rpm), and samples were taken periodically and assayed for cell density, protein (28), *N,N,N',N'*-tetramethylphenylenediamine (TMPD)-oxidase activity, and the cellular ^{55}Fe content. For determination of bound ^{55}Fe, 0.2- to 2.0-ml samples of culture were passed through membrane filters (0.45-μm pore size), and the filters were washed with 5.0 ml of sterile nonradioactive medium.

In continuous culture, the ability of cells to take up ^{55}Fe was determined on cells grown at equilibrium in high (204 ng/ml)- and low (6 to 8 ng/ml)-iron NDM. Six milliliters of the continuous culture was transferred to a prewarmed 50-ml flask, to which 4×10^4 to 6×10^4 dpm of $^{55}FeCl_3$ per ml (in different concentrations of $^{56}FeCl_3$) was added, and the flask contents were incubated with shaking at 37°C. In some experiments fresh, buffered [0.1 M tris(hydroxymethyl)-aminomethane, pH 8.3, KCN (0.5 mM final concentration), or tetrachlorosalicylanilide (TCS) in methanol (4 μM final concentration) was added 2 min prior to the addition of iron. At intervals, samples were removed, deposited onto filters, washed with 5.0 ml of sterile low-iron NDM, and counted for ^{55}Fe.

^{55}Fe determination. Liquid scintillation counting of ^{55}Fe was carried out on a Nuclear-Chicago 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 of *o*-phenanthroline and 100 μg of deferrioxamine-β-mesylate (Desferal) to 6.0 ml of PCS scintillant (Amersham Corp., Arlington Heights, Ill.) and 1.0 ml of liquid sample on a 25-mm membrane filter.

Oxidase. Ascorbate-TMPD-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 nanomoles of O_2 consumed per 10^9 cells per minute.

Catalase. A 3-ml amount of cell culture was placed in the O_2 cell, and a few grains of sodium dodecyl sulfate were added to lyse cells and stop O_2 consumption. After a flat base line was established, 10 μl of 0.3 M H_2O_2 was added. Catalase activity was expressed as nanomoles of O_2 evolved per 10^9 cells per minute.

Cytochrome determinations. Cytochromes *b* and *c* were determined by difference spectroscopy (Perkin-Elmer 351) 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 ultrasonically disrupted cells with medium blanks (41).

Protein was estimated by using the method of Lowry et al. (28), using bovine serum albumin as the standard.

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.

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 (Fig. 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 to 6 ng of Fe per ml) to 2,500 ng of Fe per ml (Fig. 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 of Fe per 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/ml lengthened generation times, starting concentrations in the medium of <10 ng of Fe per ml were sufficient to permit an increase in the population from 2×10^7 to 4×10^9 cells per ml. Whereas Fig. 3 shows that >50 ng of iron per ml only slightly reduces generation time, Fig. 4 shows that cells in a mid- to late-log batch culture will take up over 100 ng of iron per 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 (Fig. 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 $\times$ *g*, 3 h) 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 DO, pH, extracellular iron, and oxidase activity of the cells (Fig. 5). The activity of TMPD-oxidase in cells grown in either of the media progressively increased during exponential growth, whereas the concentration of DO in the media declined rapidly. This decrease in DO continued in spite of the agitation of the culture, continuous flushing of the atmosphere above the

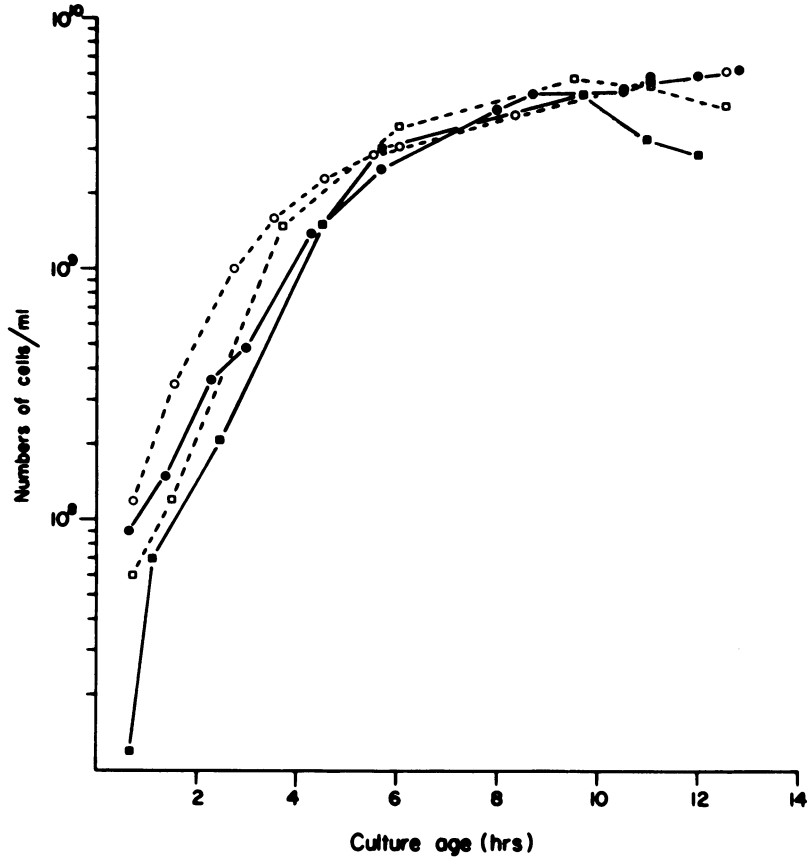

FIG. 2. *Growth of N. meningitidis (SDIC) in batch broth cultures (37°C, shaking) under 10% CO_2 in air. MH broth: direct counts (○); viable counts (□). NDM broth: direct counts (●); viable counts (■).*

culture with 90% air–10% CO_2, and the relatively high surface-to-volume ratio (0.53:1) in the flasks.

An experiment using high-iron, high-DO continuous culture gave similar specific TMPD-oxidase activities (130 to 140 nmol of O_2/min per 10^9 cells) at equilibrium cell densities of 1.0×10^9, 1.9×10^9, and 3.0×10^9 cells per ml. This suggests that the observed sharp rise of TMPD-oxidase activity in batch culture (Fig. 5) was caused primarily by the low DO in the culture. Therefore, altering either the oxygen or the iron concentration had a marked effect on the oxidase activity. Furthermore, 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 1.5×10^9 to 2.2×10^9 cells per ml for at least 1 h before cells were taken for assays. Cells in chemostat cultures growing in medium containing ≤ 4 ng of Fe per ml ceased dividing and began to wash out. This necessitated the addition of 2 ng of Fe per ml to the low-iron medium.

Generation times were lengthened approximately threefold 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 of O_2 per doubling, whereas the same culture grown in high-iron equilibrium required only 1.7 μmol of O_2. Cells growing in the low-iron medium removed all detectable iron from the medium, whereas one-half the total culture iron remained

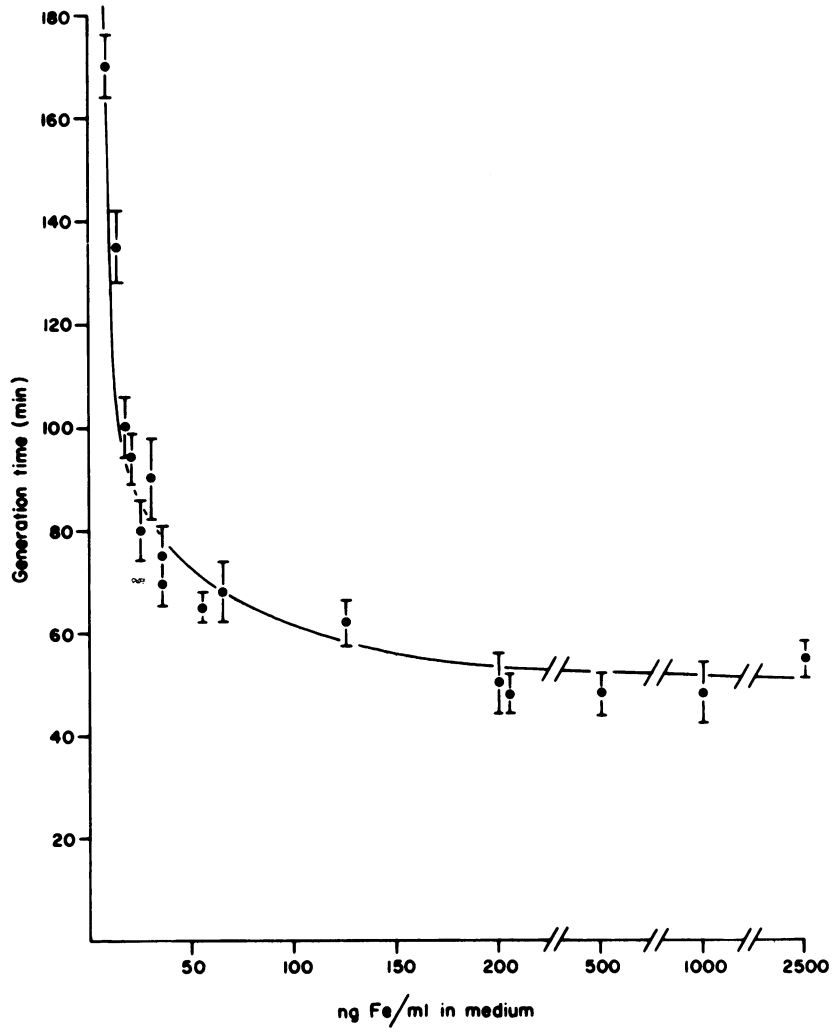

FIG. 3. *Generation times during the exponential phase of growth in NDM broth batch cultures containing different concentrations of iron over the range 10 to 2,500 ng of Fe per ml. Generation times (points are means of triplicate flasks) were calculated when the cell populations were <10^9 cells per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

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 (Fig. 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 (12, 42, 46). In our 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 of O_2 per 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 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) 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.

Response of iron-limited continuous culture to an iron pulse. When an iron-limited continuous culture was pulsed with 22 ng of Fe per ml, the cellular growth rate and oxygen consumption showed marked increases within 15 min (Fig. 6). Eventually, the cell density nearly doubled in response to the pulse, but since 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 of Fe per ml, final concentration) as $FeCl_3$ or Fe citrate (Fe^{3+}-citrate ratio, 0.35:1).

Fe uptake by the meningococcus. Iron uptake was measured in cells that had been grown at equilibrium in an iron-limited (6 to 8 ng of Fe per ml) continuous culture (110 nmol of O_2 per ml). By colorimetric iron assay, which showed good recovery of ^{55}Fe from culture supernatant fluids, the supernatant fluid of these cultures contained <1.0 ng of Fe per ml. Cells presented with as little as 4.4 ng of Fe per ml took up in excess of 80% of that iron during the first 30 s (Fig. 7). The maximum measured uptake of ^{55}Fe was only approximately 85% of the total added, which the controls suggested was due to fluorescence loss by quenching in the packed cells on the filter.

Iron uptake by the meningococcus (Fig. 7) occurred in two stages: an initial rapid stage covering the first 30 s, followed in the three higher pulse concentrations (54, 95, and 204 ng

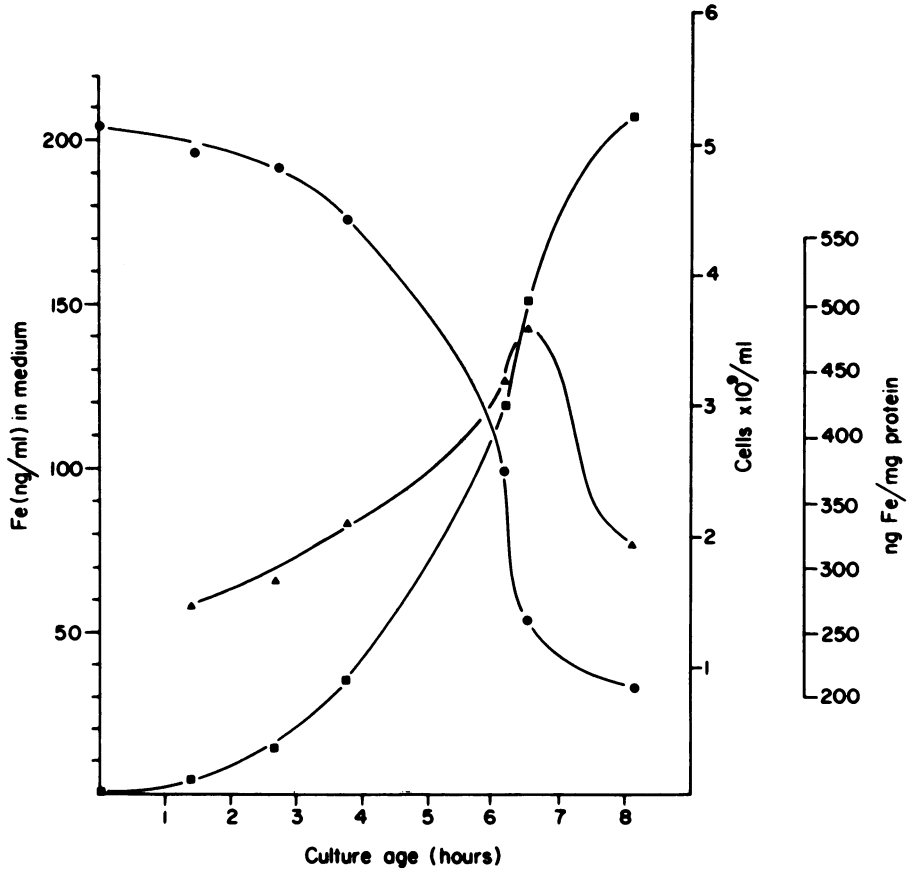

FIG. 4. *Uptake of iron (^{55}Fe) in NDM broth batch culture. Symbols: Concentration of iron in medium (●); cell counts (■); cellular iron (▲).*

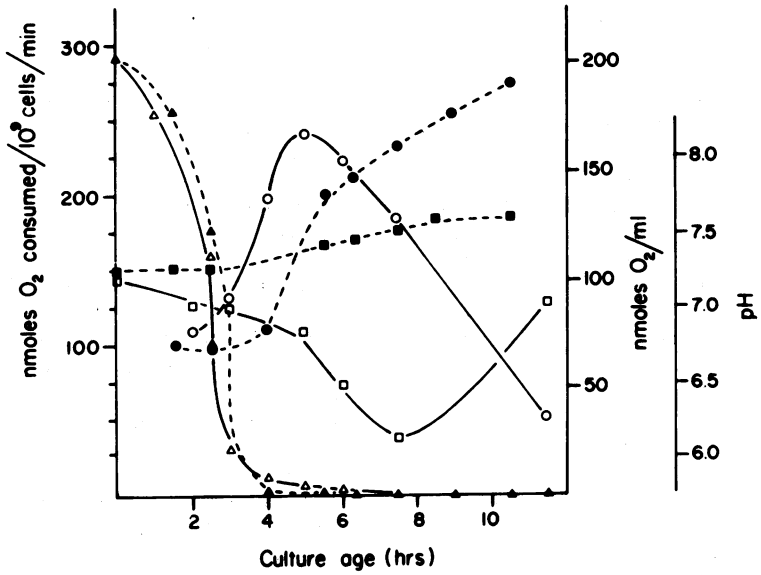

FIG. 5. *Batch cultures in MH broth (broken lines, solid symbols) and NDM broth (solid lines, open symbols). Symbols: Ambient culture dissolved O_2 (△, ▲); ascorbate-TMPD oxidase activity (○, ●); pH (□, ■). Culture conditions as in Fig. 2.*

TABLE 2. *Changes in N. meningitidis SDIC grown in equilibrium chemostat cultures in both high and low concentrations of iron and DO*[a]

Determination	204 ng of Fe/ml of medium		6 ng of Fe/ml of medium	
	110 ± 10 nmol of O_2/ml[b]	12 ± 5 nmol of O_2/ml	110 ± 10 nmol of O_2/ml[b]	12 ± 5 nmol of O_2/ml
Generation time (min)	55	62	164	161
Oxygen consumption (nmol of O_2/10^9 cells per min)	30	33	19	24
TMPD-oxidase (nmol of O_2 consumed/10^9 cells per min)	138	197	90	125
Catalase (nmol of O_2 evolved/10^9 cells per min)	3.3	5.6	<0.5	<0.5
Cytochrome c_{552} red-oxid, (OD × 10^{-4}/10^9 cells per ml)[c]	5.0	7.3	3.2	3.4
Cytochrome b_{528} red-oxid (OD × 10^{-4}/10^9 cells per ml)	2.2	3.5	1.5	1.0
Heme-absolute absorbance (417 nm), (OD × 10^{-4}/10^9 cells per ml)	32	46	19	19
Cell-bound iron (μg of 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 are representative of those obtained from the nine separate runs performed for this experiment.

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

[c] OD, Optical density.

of Fe per ml) by a secondary, slower-rate second stage. The second, slower stage was short-lived (0.5 to 1 min) in the two lowest concentrations (4.4 and 14 ng of Fe per ml), due to the near exhaustion of extracellular iron during the initial fast stage. Cells pulsed with 54 ng of Fe per ml took up iron at a constant rate similar to that for higher Fe concentration from 0.5 to 5 min, after which depletion of the iron in the medium 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 iron (95 ng of Fe per ml) in the presence of the bacteriostatic detergent TCS, in KCN, and at 6°C. In the presence of KCN (Fig. 8) and at 6°C, the second, slow stage of uptake was completely blocked, whereas 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 (Fig. 9). Since KCN is a potent poison for respiration, and TCS has been shown to affect energy-dependent translocation in the membrane (19, 20), we 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 (Fig. 10), where iron uptake by unpoisoned cells (Fig. 7) showed saturation when ambient medium iron was >14 ng/ml, the uptake rate remaining steady at about 6 ng of Fe per 10^9 per min.

When these KCN-poisoned, ^{55}Fe-pulsed cells were placed in nonradioactive KCN-free sterile NDM, containing 204 ng of Fe per ml, the initial rate of cellular iron exchanged with that in the medium was 2.8%/min for cells previously pulsed with 204 ng of Fe per ml and 3.9%/min for cells previously pulsed with 4.4 ng of Fe per ml. Therefore, the iron bound by the energy-dependent 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 29 to 31 pmol/10^9 cells per min, as shown by both ^{55}Fe and colorimetric analyses. In comparison, iron-starved cells pulsed with 204 ng of Fe per ml took up iron in the energy-dependent stage (2 to 10 min postpulse) at 113 pmol/10^9 cells per 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 204 ng of Fe per ml for up to 20 min. Whereas cells grown in high-iron, high-O_2 equilibrium conditions took up 110 ng of the 204 ng of Fe per ml in the medium, iron-starved cells took up 115 ng of the 204 ng of Fe per ml in 10 min and 128 ng/ml 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 of enhanced activity or preexisting ones.

DISCUSSION

The iron concentrations required for "maximal growth" of a number of gram-negative bac-

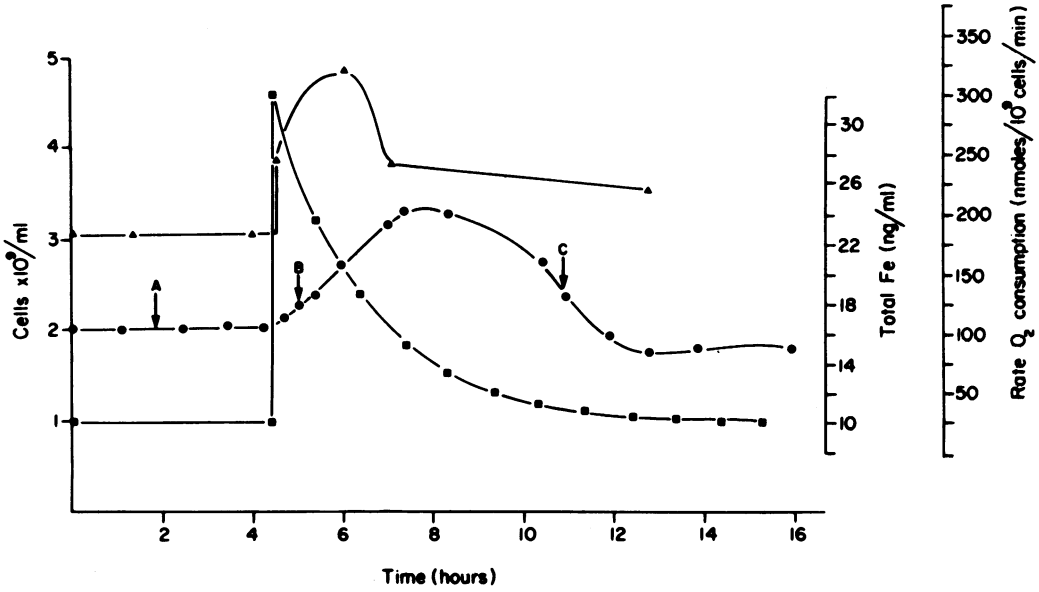

FIG. 6. *Effects of a pulse of ferrous ammonium sulfate (22 ng of Fe per ml, final concentration) on a continuous culture growing at equilibrium in iron-limited NDM medium. Symbols: Total culture iron (■); rate of O_2 consumption (▲); cells per milliliter (●). Generation times at points A, B, and C were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

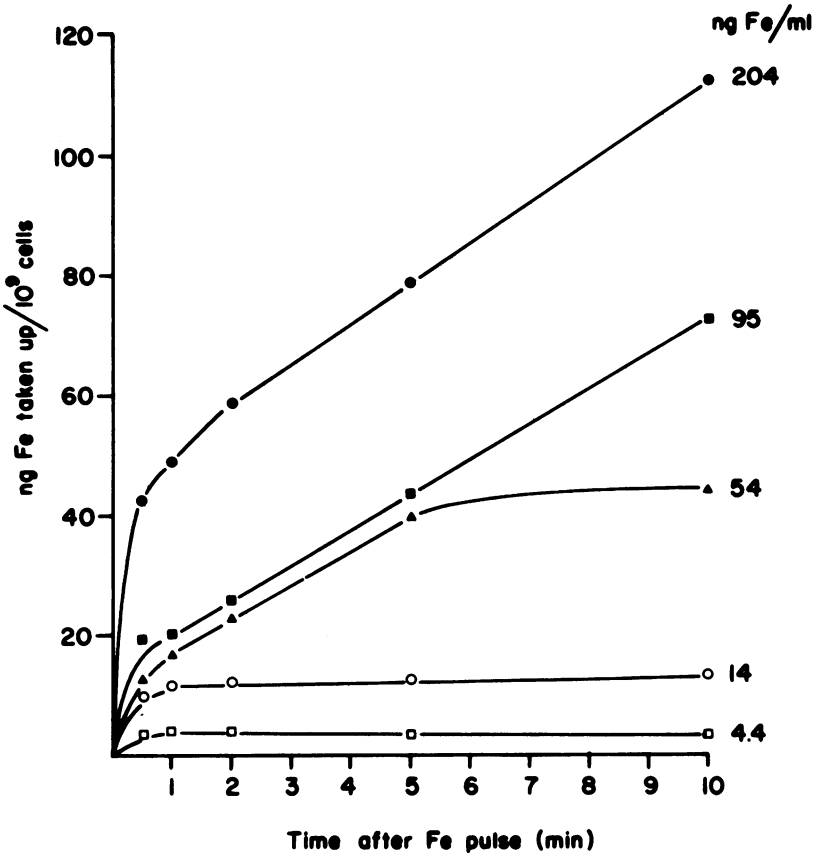

FIG. 7. *Iron uptake by cells grown at equilibrium in a low-iron (≈6.0 ng of Fe per ml, high O_2 [110 nmol of O_2 per ml]) continuous culture in NDM. Samples of the culture were pulsed with ^{55}Fe (40,000 to 60,000 dpm/ml) to give the final concentrations of iron shown.*

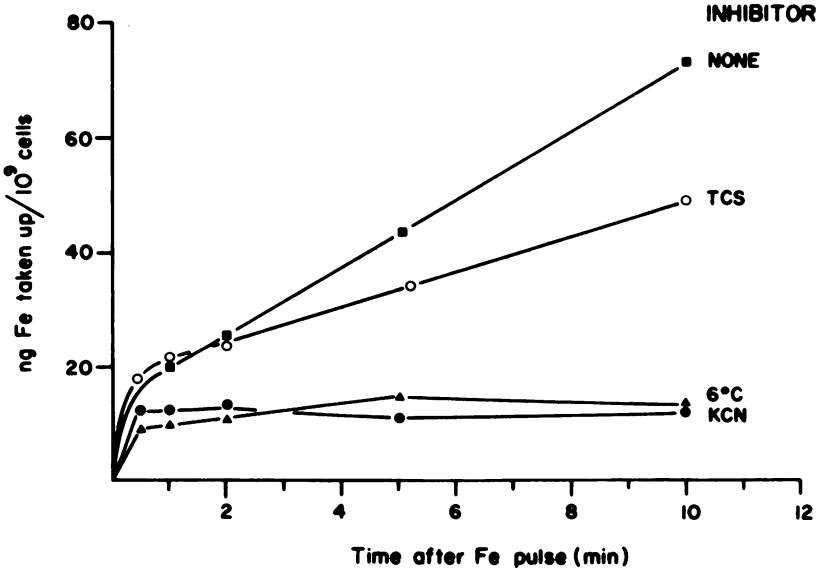

FIG. 8. *Inhibition of active iron uptake by KCN (0.5 mM), TCS (4 μM), and low temperature (6°C). Cells were grown as described for Fig. 7. KCN and TCS were added 2 min prior to the iron pulse (95 ng of Fe per ml, final concentration). For the low temperature (6°C), 6 ml of culture was transferred to 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).*

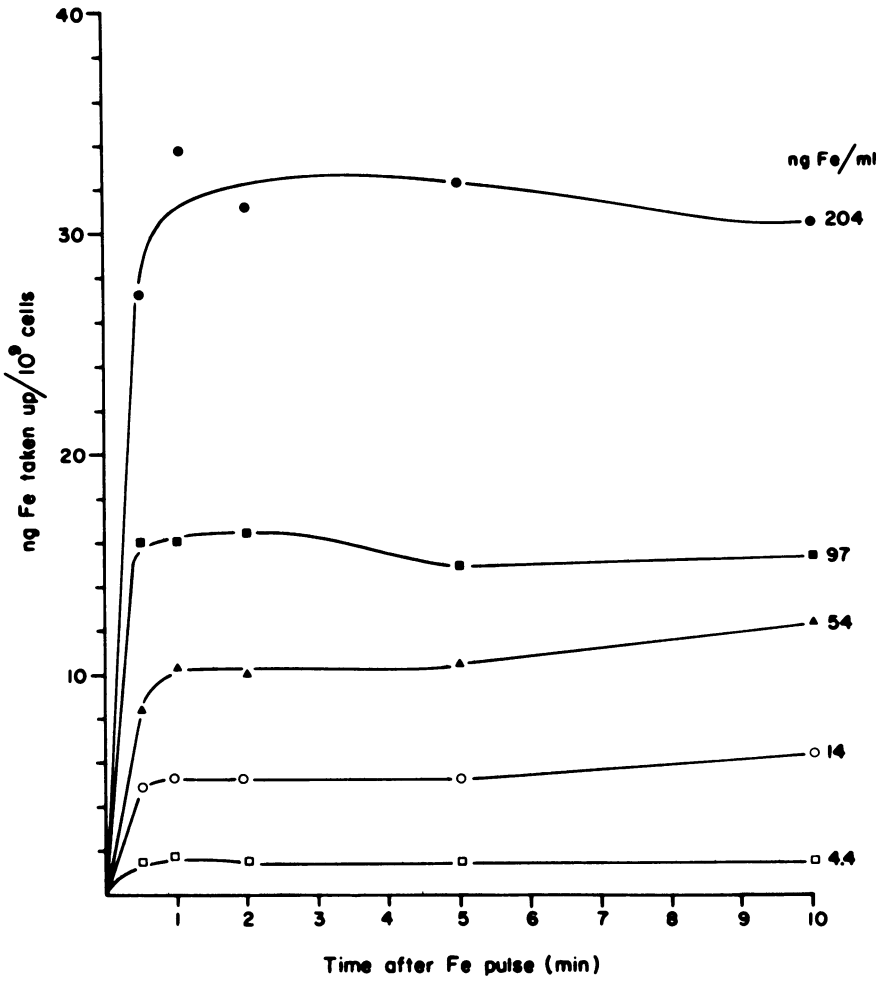

FIG. 9. *Energy-independent iron uptake by iron-starved cells in the presence of KCN (0.5 mM).*

teria (see 26), including *Enterobacter aerogenes, Escherichia coli, Salmonella typhimurium, Alcaligenes faecalis,* and *Pseudomonas aeruginosa,* are in the range of 20 to 80 ng of iron per ml. Comparison of these published values is somewhat tenuous since 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 we present here, the iron required for continued viability and slow cell division of meningococcus SDIC in continuous culture was 11 to 17 μg of iron per g (dry weight) or 2.2×10^{-9} to 3.3×10^{-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 of iron per ml).

The cellular iron of an organism can be classified as that present in heme compounds, e.g., cytochromes, oxidases, catalase, or nonheme compounds, which include a wide variety of storage, transport, and catalytic molecules. Nonheme iron has generally been considered to form the large majority of cellular iron, an opinion supported by the direct measurements by Kim and Bragg (24) in *E. coli* (3% nonheme) and by Kurup and Brodie (25) in *Mycobacterium phlei* (85% nonheme). 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. Furthermore, using the 417-nm Soret absorbance

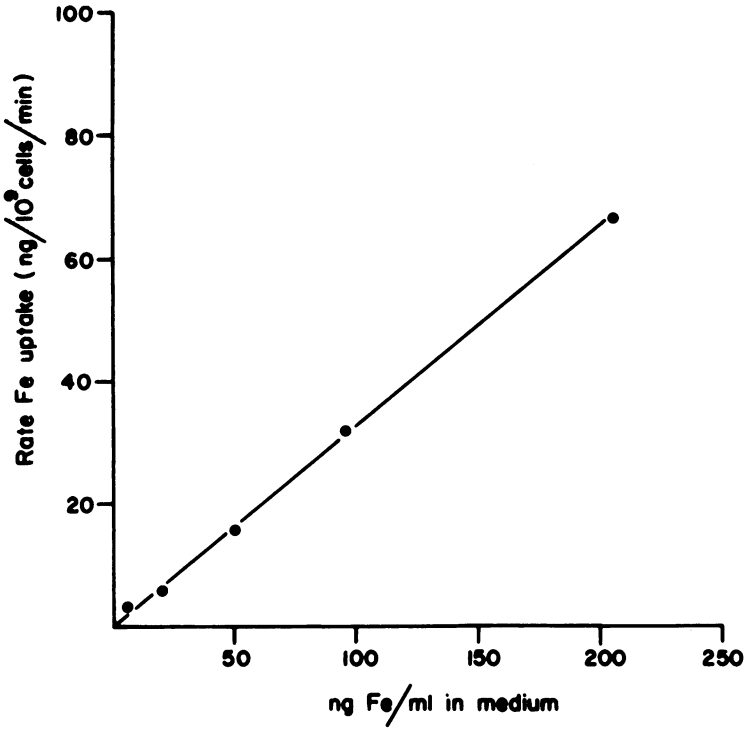

FIG. 10. *Initial rates (during first 30 s) of energy-independent uptake in the presence of KCN (0.5 mM) over a range of iron concentrations (4.4 to 204 ng of Fe per ml). Conditions were as described for Fig. 8.*

and ϵmM of 120 to estimate total heme present, even iron-starved meningococci have a majority of their iron present as nonheme iron.

The KCN-sensitive catalase of the meningococcus, presumably a heme protein, decreased at least eightfold 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 per s, determined for other catalases (10, 13), 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 nonheme 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

The sedimentation of 92% of the total iron in sonically disrupted meningococci was not surprising, since 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. (1, 2) for schizokinen-Fe in *Bacillus megaterium*.

The stimulation of respiratory proteins by low DO tension that we observed has also been noted in other gram-negative bacteria, e.g., *E. coli* (33, 48), *Pseudomonas fluorescens* (27, 41), and *Enterobacter (Aerobacter) aerogenes* (32). 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 of O_2 per ml. This is in agreement with the kinetics of oxygen consumption noted by Gerard and Falk (18) and, subsequently, by other workers (21). The apparent K_m for oxygen 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 nonsaturating DO levels by increasing the number of target sites (oxidase) for oxygen.

Pulsing of the iron-limited continuous culture (Fig. 6) 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 should prove informative.

The temperature sensitivity and enzyme-like saturation kinetics, exhibited by the energy-dependent 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 (19) and Harold and Baarda (20), 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 (see 43, 44); (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 energy-independent iron uptake, with the exception of the work of Peters and Warren (39) on *Bacillus subtilis* grown at low iron concentrations.

The work we report here has not attempted to demonstrate a link between meningococcal disease and iron metabolism, nor has it dealt with the 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 demontrates effective in vitro techniques that can be applied to determining the availability of iron from host and other iron-containing molecules.

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