MODIFIED PROCEDURES AND PRELIMINARY RESULTS IN THE ANALYSIS OF POSSIBLE IRON TRANSPORT COMPOUND SYNTHESIS BY BACTERIA

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ABSTRACT

MODIFIED PROCEDURES AND PRELIMINARY RESULTS IN THE ANALYSIS OF POSSIBLE IRON TRANSPORT COMPOUND SYNTHESIS BY BACTERIA

By

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The synthesis of phenolate and/or hydroxamate compounds during iron deficiency was investigated in Klebsiella pneumoniae, Salmonella enteriditis, Serratia marcescens, Shigella sonnei, and Pseudomonas aeruginosa.

A minimal medium and method of iron extraction described by Waring and Werkman (1942) were employed. Commercially prepared penassay broth was also deferrated (iron-extracted) by this method. A study with K. pneumoniae indicated that the deferri-penassay broth may contain more residual iron than the deferri-dextrose broth.

The original bathophenanthroline iron assay procedure, as described by Diehl and Smith (1960), was found to be unsatisfactory for the analysis of low iron concentrations in media and water. Extensive modifications of this procedure were necessary to insure adequate reduction of ferric ions. Incomplete reduction occurred unless samples were steam-heated for one hour in the presence of 0.1 ml of concentrated HCl, and L-ascorbate was substituted for hydroxylamine. Minor modifications of assays for the detection of phenolate and hydroxamate compounds were tested for their sensitivity and reliability.

Deferri-dextrose cultures of K. pneumoniae and Sal. enteriditis incubated at 25 C and 37 C synthesized phenolate compounds. At 25 C Ser. marcescens also produced phenolate compounds. At 37 C Ps. aeruginosa synthesized hydroxamate compounds during iron deficiency. In deferri-

dextrose broth cultures at 25 C and 37 C, Sh. sonnei made both phenolate and hydroxamate compounds.

In deferri-penassay broth, Pseudomonas fluorescens synthesized hydroxamate compound(s) while Shigella boydii produced both phenolate and hydroxamate compounds. Four strains of Staphylococcus aureus, two strains of Yersinia pestis, four species of Proteus, and Yersinia pseudotuberculosis grew well in deferri-penassay broth without synthesizing detectable levels of phenolate or hydroxamate compounds.

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INTRODUCTION

Trace amounts of metallic elements such as iron, manganese, zinc, copper, cobalt, and molybdenum are essential for microbial growth. The concentration ranges of these trace metals necessary for maximal microbial growth are greatly affected by a number of synthetic and natural metal-binding compounds. Secondary metabolism and differentiation occur at trace metal concentration ranges which are generally narrower than those ranges allowing growth. For bacteria, iron appears to be the trace metal most critically affecting growth, longevity, and secondary metabolism, while zinc is important in yeasts and molds. The genus Bacillus is an exception since manganese apparently is the most important factor. Manganese is also a key factor for some fungi (Weinberg, 1970; 1972).

For a number of these trace metals, evidence of their mediated transport into microbial cells has been described. Although a biological requirement for chromium has not been established, some yeast strains produce a chromium transport compound (Weinberg, 1970). Cells of Escherichia coli have been shown to accumulate manganese on the one hand by an active transport system (Silver and Kralovic, 1969) and magnesium on the other hand by a temperature dependent process inhibited by dinitrophenol and cyanide (Silver, 1969; Lusk and Kennedy, 1969). Cells of Salmonella typhimurium grown on limiting sulfur sources synthesize a protein capable of binding sulfate ions (Pardee, 1968).

Numerous microorganisms are autosequesteric; they produce small organic compounds which solubilize and transport exogenous iron into the cell (Weinberg, 1974a). These small microbial compounds have been variously termed iron transport compounds (ITC's), ionophores, sideramines,

and siderophores. The synthesis of these ITC's has been found to be inhibited by iron concentrations which are adequate for growth (Weinberg, 1974a).

The wide variety of microbial species possessing iron transport systems suggests that microorganisms often must exist where iron is not readily available. Although iron is the fourth most abundant element on earth, it is almost entirely present in forms unavailable for biological use. Due to its chemical properties, ferric iron is extremely insoluble at room temperature and at alkaline and neutral pH's. In sea water the iron present (0.5 to 5.0 ppb) is mainly in a particulate form and the free ionic concentrations are very low. Although abundant in soil and rock, iron is present almost entirely as insoluble aggregates and precipitates (Lankford, 1973). In mammals, internal conditions are extremely iron limiting because of the presence of a complex system of ligand proteins which sequester iron (Weinberg, 1974a). Generally, iron need not be added to complex synthetic media since iron contamination alone meets microbial growth requirements (Weinberg, 1971).

Evidence is accumulating that the iron transport systems of some bacteria play an important role in pathogenesis. In-depth studies of these systems have been limited to a relatively small number of bacteria. Analysis of iron metabolism in other bacteria (especially pathogens) will further clarify this biological role of bacterial iron transport systems.

The purpose of this investigation was (1) to improve and develop procedures necessary for the investigation of ITC synthesis and growth by both pathogenic and saprophytic bacteria during iron deficiency, and (2) to study the effect of iron concentration and temperature upon bacterial ITC synthesis. The test organisms were selected either because their growth patterns suggest ITC syn-

thesis, or because their capacity for ITC synthesis is undetermined. In preliminary experiments, Perry and Weinberg (1973) observed that Pseudomonas aeruginosa, Staphylococcus aureus, Shigella sonnei, Shigella alkalescens, and Proteus vulgaris as well as other bacteria competed with orthophenanthroline (OP) for iron.

REVIEW OF LITERATURE

Iron Metabolism of Microorganisms

Iron Requirements

In order to function, numerous microbial enzymes require the presence or direct incorporation of ferrous or ferric ions. The active sites of metapyrocatechase and 3,4-dihydroxyphenylacetate-2,3-oxygenase contain ferrous ions. Pyrocatechase and pryocatechuate-3,4,-oxygenase contain ferric iron at their active sites (Hayaishi, 1966). Iron deficiency in Aerobacter indologenes severely reduced the activities of catalase, peroxidase, formic hydrogenlyase, and hydrogenase (Waring and Werkman, 1944). Theodore and Schade (1965) have proposed that Staph. aureus enzymes which oxidize pyruvate, formate, acetate, succinate, malate, and citrate require iron for activity. At low pH's iron promotes increased production of penicillinase by Staph. aureus (Leitner and Cohen, 1962). Staph. aureus is unable to produce coagulase under conditions of iron deficiency (Schade et al., 1968). Rosenberg and Gefter (1969) found that E. coli cells grown under iron-deficient conditions synthesized abnormal transfer RNA for the amino acids phenylalanine, leucine, serine, tyrosine, and cysteine. There are many other examples in the literature on the effect of iron deficiency upon bacterial metabolism (see Weinberg, 1970). Of course iron is extremely important in many of the respiratory enzymes (e.g., cytochromes).

The synthesis of many bacterial toxins is also affected by iron concentrations (e.g., tetanus toxin and staphylococcal enterotoxin require moderate iron concentrations for synthesis). However, in some instances, low iron concentrations facilitate toxin production (e.g.,

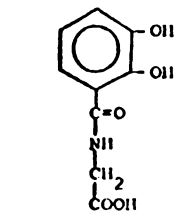
α-toxin lecithinase of Clostridium perfringens, neurotoxin of Shigella shigae, and diphtheria toxin of Corynebacterium diphtheriae) (Weinberg, 1966).

Because of its established role in bacterial metabolism, the iron concentration of the environment greatly influences the growth and longevity of many bacterial species (e.g., Perry and Weinberg, 1973; Sword, 1966; Davis et al., 1971; Garibaldi, 1970; Garibaldi, 1972).

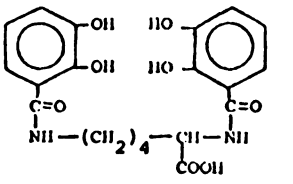
Iron Transport Compounds (ITC's)

The various ITC's fall primarily into two groups: phenolate compounds and hydroxamate compounds. The iron metabolism of bacteria has been primarily studied in Sal. typhimurium, E. coli, Enterobacter aerogenes, Bacillus subtilis, Bacillus megaterium, and the mycobacteria. The first four organisms produce phenolate compounds (Peters and Warren, 1968a; Pollack and Neilands, 1970; Young et al., 1971), whereas Ent. aerogenes and B. megaterium produce a hydroxamate compound (Davis and Byers, 1971; Gibson and Magrath, 1969). Mycobacteria synthesize a more complex ITC called mycobactin, whose chemical structure varies from strain to strain but always contains both phenolate and hydroxamate moieties (Snow, 1970).

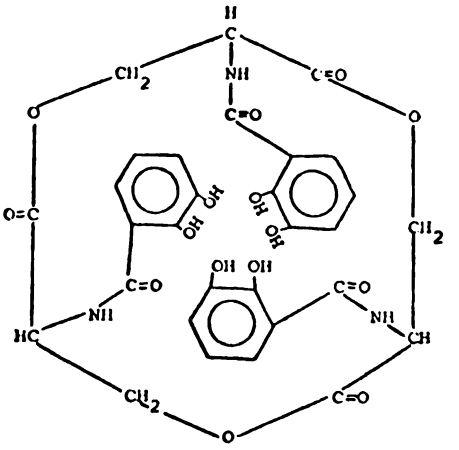
A number of microorganisms produce phenolate compounds when grown under iron-deficient conditions. B. subtilis produces 2,3-dihydroxybenzoylglycine (Peters and Warren, 1968a). Enterochelin is synthesized by E. coli, Sal. typhimurium, and Ent. aerogenes (Pollack and Neilands, 1970; Young et al., 1971). Cultures of Azotobacter vinelandii synthesize 2-N, 6-N-di-(2,3-dihydroxy-benzoyl)-L-lysine (Lankford, 1973). These structures are depicted in Fig. 1.



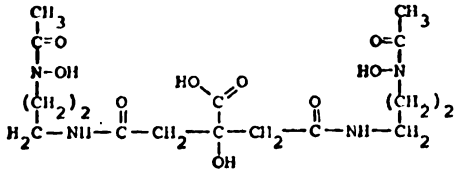
2,3-Dihydroxybenzoylglycine



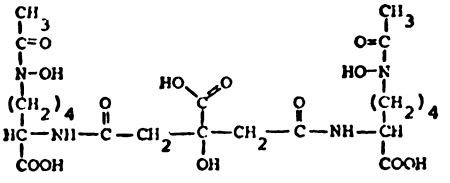
2-N,6-N Di-(2,3-Dihydroxy-benzoyl)-L-lysine



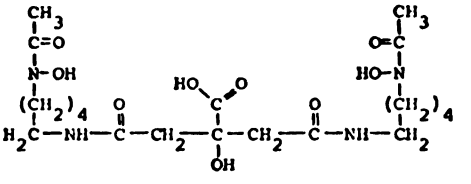
Enterochelin



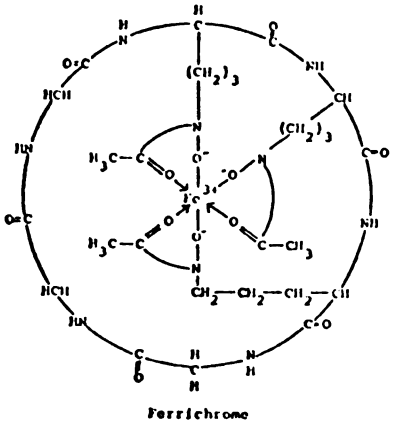
Schizokinen



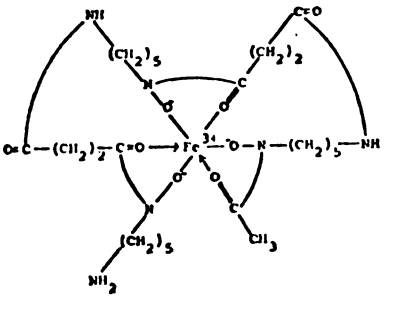
Aerobactin



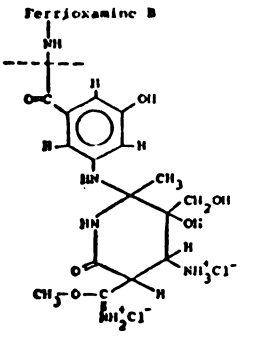
Arthrobactin
(Terregens Factor)



Ferrichrome



Ferrioxamine B



Ferrimycin A_1

Figure 1.--Structures of some iron transport compounds. Adapted from Lankford (1973).

Hydroxamate-producing microorganisms are more divergent and numerous than those producing phenolate compounds. Various species of Streptomyces produce ferrioxamines and ferrimycins (Neilands, 1967; Nüesch and Knüsel, 1967). Desferal (CIBA, Summit, New Jersey) is the commercial name for the iron-free (deferri) form of ferrioxamine B. Ferrichrome is synthesized by the smut fungus Ustilago sphaerogena (Neilands, 1957). A number of fungal species produce hydroxamate compounds (Lankford, 1973). Arthrobacter pascens synthesizes arthrobactin (also called terregans factor) (Demain and Hendlin, 1959). Schizokinen and aerobactin are synthesized respectively by B. megaterium and Ent. aerogenes (Davis and Byers, 1971; Gibson and Magrath, 1969; Lankford et al., 1966). The structures of a number of these compounds are diagramed in Fig. 1. Chemically defined species of mycobactin are shown in Fig. 2.

Halmann and Mager (1967) have partially purified a growth-initiating substance (GIS) from Pasteurella tularensis which is of low molecular weight and complexes with iron and copper ions. The requirement for GIS can be replaced only by iron salts and by high levels of certain hydroxamates. Although the role of ornithine as a precursor for GIS suggests that it may be a hydroxamate compound, assays for hydroxamate compounds yielded negative results (Halmann and Mager, 1967).

Straube and Fritsche (1973) have suggested that riboflavin serves as an iron transport compound for Candida guilliermondii. Overproduction, excretion, and later uptake of riboflavin by C. guilliermondii under conditions of iron deficiency have been noted. These observations and the observed formation of a complex between iron and riboflavin are the only data which presently implicate riboflavin in iron transport (Straube and Fritsche, 1973).

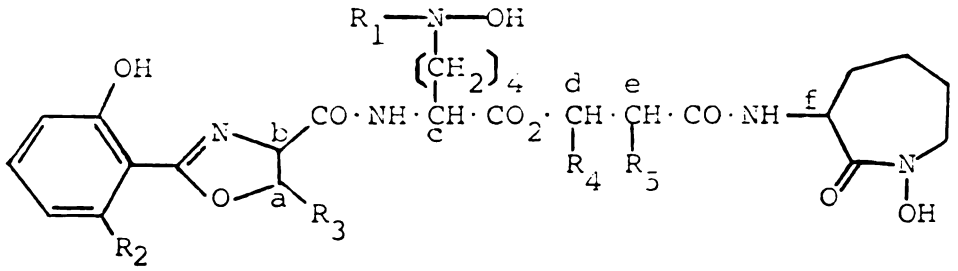


Figure 2.--Structures of chemically defined species of mycobactions.[+] The R_1 side chains are acyl groups containing the total number of carbon atoms shown below. Side chains most commonly seen are underlined. Unbranched chains are represented by n and known double bonds are indicated. Symbols a-f are asymmetric centers; (), lacks an asymmetric center; -, configuration undetermined.

Mycobactin	R_1	R_2	R_3	R_4	R_5	a	b	c	d	e	f
A*	14Δ^2	CH_3	H	CH_3	H	-	-	-	-	-	-
F	10Δ, 12, 14, 16, 18	H	CH_3	CH_3	H	-	-	-	S	()	L
H	18, 20Δ	CH_3	CH_3	CH_3	H	R	L	L	S	()	L
P	14, 16, 18, 20 cis-Δ^2n	CH_3	H	C_2H_5	CH_3	()	L	L	S	R	L
S	14, 16, 18, 20 (cis-Δ^2)*	H	H	CH_3	H	()	L	L	S	()	L
T	18, 19, 20, 21 18, 19, 20, 21Δ	H	H	CH_3	H	()	-	-	R	()	L

*Tentative structure.
+Adapted from White and Snow (1969) and Snow and White (1969).

Recently, a citrate-dependent iron transport system has been identified in E. coli K12 (Frost and Rosenberg, 1973). Induction of this system requires new protein synthesis and occurs within twenty minutes of exposure to sodium citrate. In E. coli mutants unable to utilize the enterochelin transport system, citrate has been shown to stimulate iron uptake. This citrate-dependent system appears to be less widely distributed than other iron transport systems, even among other strains of E. coli. Studies with various mutants have indicated that the iron uptake observed in the citrate system and the enterochelin system equal the sum of iron uptake observed in wild-type E. coli strains endowed with both systems. These results indicate that the citrate system is wholly independent of the enterochelin system and may operate simultaneously in wild-type strains of E. coli. In Ent. aerogenes citrate has been shown to repress 2,3-dihydroxybenzoate (DHB) biosynthesis and may therefore serve as a less energetically expensive method for the transport of soluble chelated iron from the environment into the cell. A citrate-dependent transport system has also been reported in Neurospora crassa, which produces coprogen, a hydroxamate (Frost and Rosenberg, 1973).

Mechanisms of Bacterial Iron Transport

Iron Transport by Enterochelin

Langman et al. (1972) have proposed a mechanism of iron chelation and utilization in which external iron is chelated by enterochelin and transported into the cell (see Fig. 3). The iron in the ferri-enterochelin complex cannot be utilized until it is broken down by the cell into 2,3-dihydroxybenzoylserine (DHBS) subunits. By this

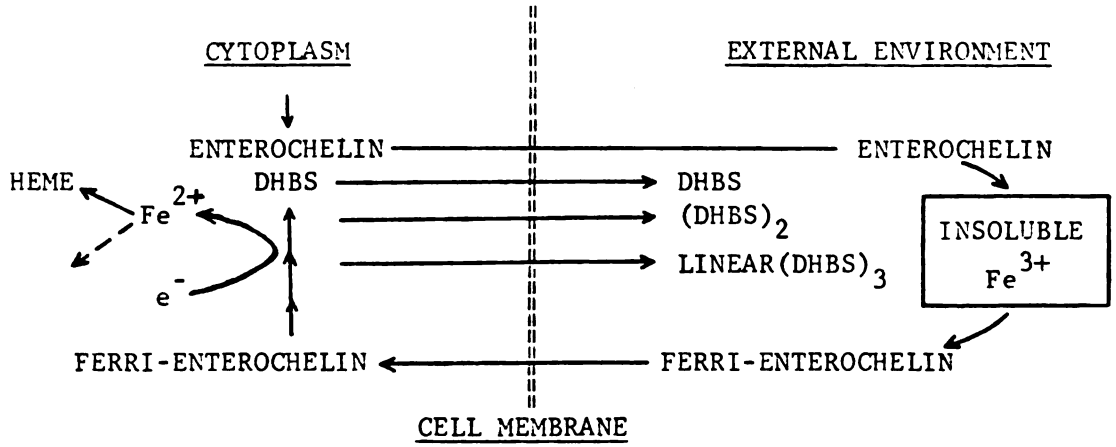


Figure 3.--A model for iron transport by enterochelin in E. coli. Adapted from Lankford (1973).

scheme, DHBS is a breakdown product of enterochelin. This proposed mechanism is supported by the observation that higher levels of DHBS are required for stimulation of growth than enterochelin which indicates that the Fe-DHBS complex alone is a poor substrate for the iron uptake system (O'Brien et al., 1971). Although this scheme has been proposed only for E. coli, it could also apply to other bacteria producing enterochelin-like compounds. Thus the 2,3-dihydroxybenzoylglycine (DHBG) compound produced by B. subtilis might be a breakdown product of a cyclic DHBG trimer. Indeed, Peters and Warren (1968b) propose a ferri-$(DHBG)_3$ chelated complex at neutral pH.

Iron Transport by Hydroxamates

From an elegant study involving labelled $^{59}Fe^{3+}$-^{3}H-schizokinen complexes, Arceneaux et al. (1973) have proposed a mechanism of iron transport by schizokinen in B. megaterium. Their results show an initial temperature-independent binding of the ferri-schizokinen complex to the outer surface of the cell membrane followed by a temperature-dependent transport of the entire complex into the cell (Arceneaux et al., 1973). Movement across the membrane is likely to involve one or more membrane-bound proteins due to the generally low solubility of hydroxamates in lipid solvents (Haydon et al., 1973). The iron is reductively released from the complex without hydrolysis of the hydroxamate. This reaction is probably catalyzed by specific membrane-bound enzymes. The deferri-schizokinen is then released into an intracellular pool where it is excreted when a critical intracellular concentration is obtained. The possible utilization of the intracellular deferri-schizokinen for internal movement of iron has also been suggested. A similar pattern has

been observed for aerobactin in Ent. aerogenes (Arceneaux et al., 1973).

There appear to be specific membrane receptors for hydroxamate transport with recognition of a specific hydroxamate based on its chemical structure. This is suggested by the finding that ferri-aerobactin does not stimulate iron uptake in B. megaterium. There is also evidence that deferri-hydroxamates compete with ferri-hydroxamates for transport across the membrane; however, the receptor has a higher affinity for the ferri-hydroxamate complex (Haydon et al., 1973).

In U. sphaerogena, Emery (1971b) identified ferrichrome as the functional iron carrier. Naturally occurring analogs of ferrichrome such as ferrichrome A which differs from ferrichrome by the replacement of two glycines in the peptide ring with serines and the replacement of acetate by β-methylglutaconate as the hydroxamate acyl groups, as well as non-iron di- and trivalent metal complexes of ferrichrome are not transported into the cell. Thus, in U. sphaerogena, it appears that the conformation of the complex near the metal ion is important for recognition and transport across the membrane (Emery, 1971b).

The specificity displayed by the hydroxamate receptor sites in B. megaterium and U. sphaerogena is not found to the same degree in all microorganisms. The arthrobactin requirement of Arthrobacter terregans can be replaced by a number of secondary hydroxamates as well as by several synthetic iron-chelating compounds (Morrison et al., 1965). Arthrobactin stimulates the growth of a number of species of Arthrobacter as well as Microbacterium lacticum (Burton, 1957; Demain and Hendlin, 1959). Hydroxamates such as ferrichrome, coprogen, propionyl ferrichrome, ferrioxamines, aspergillic acid, grisein, butyryl ferrichrome, ferrichrysin, nocardamin, and rhodotorulic acid can act as growth factors for several

species of Arthrobacter; conversely fusarinine, hadacidin, ferrichrome A, ferrichrome A trimethylester, and hexahydroferrichrome A trimethylester showed no activity (Emery, 1971a; Atkin and Neilands, 1968; Emery and Emery, 1973; Burnham and Neilands, 1961). Ferrioxamines also stimulate the growth of B. megaterium, B. subtilis, and U. sphaerogena (Emery, 1971a).

B. subtilis apparently uses ferrioxamines as ITC's even though this microorganism does not synthesize hydroxamates. This phenomenon has also been observed in mutants of Sal. typhimurium blocked in the synthesis of enterochelin (Luckey et al., 1972). The growth of these mutants is supported by the following hydroxamates: ferrichrome, ferrichrome C, ferricrocin, ferrichrysin, ferrirhodin, ferrirubin, rhodotorulic acid, dimerum acid, coprogen B, desferal, danomycin, and schizokinen. It has been suggested, therefore, that Sal. typhimurium and other microorganisms known to produce phenolate ITC's may have once possessed both phenolate and hydroxamate iron transport systems. Although the hydroxamate ITC's are no longer synthesized, the ability to transport these compounds has been retained as a scavenger system. The observation that albomycin and other hydroxamate antibiotics inhibit the growth of a number of microorganisms which do not synthesize hydroxamate ITC's also suggests that these microorganisms have retained the hydroxamate transport system (Luckey et al., 1972).

Iron Transport by Mycobactin

A more complex system for the transport of iron by mycobactins and salicylate or 6-methylsalicylate in mycobacteria has been proposed and is outline in Fig. 4. (Lankford, 1973). In Mycobacterium smegmatis, where

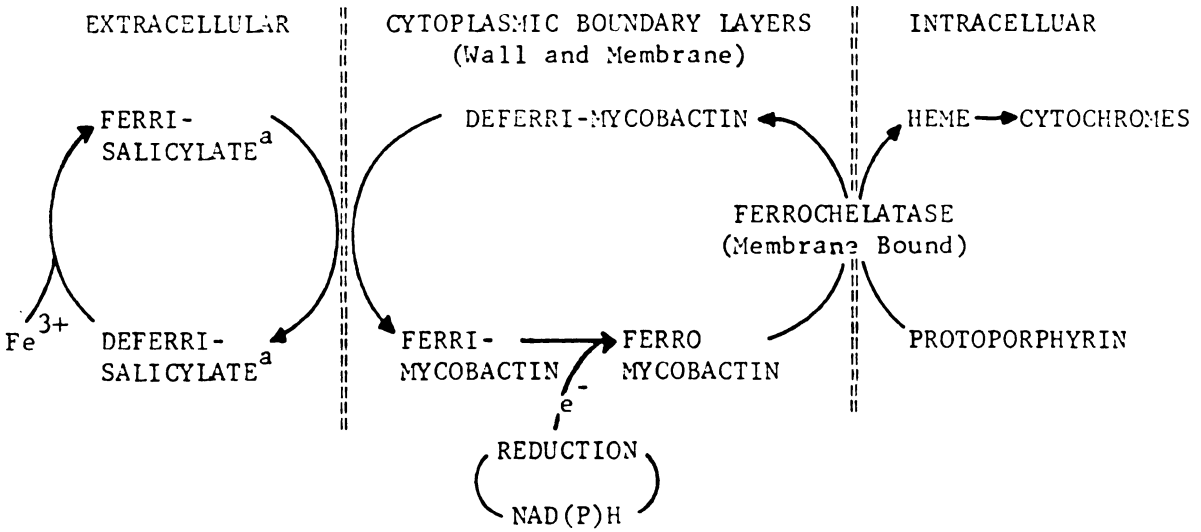


Figure 4.--A model for iron transport by mycobactin and salicylate in mycobacteria. Adapted from Lankford (1973).

[a]or 6-methylsalicylate

greater than 95% of the mycobactin present is membrane associated, salicylate chelates extracellular iron and transfers it nonenzymatically to mycobactin at the cell surface. The ferri-mycobactin complex subsequently moves with a concentration gradient to the surface of the inner membrane where the iron is reduced and removed. The mycobactin then returns to the outer membrane surface (Lankford, 1973). Enzymatic removal of iron from mycobactin may serve to prevent iron overload and to allow ferri-mycobactin to act as an iron-storage molecule (Ratledge and Marshall, 1972). Studies with mutants of M. smegmatis have shown that both mycobactin and salicylate are required for growth in deferri-media (Ratledge and Hall, 1972). Mycobactin is more firmly cell-bound when the membrane and wall contain high levels of lipids. These cells are more virulent than cells with loosely-bound mycobactin (Golden et al., 1974). These observations support the model of iron transport described above.

Metabolic Pathways for the Synthesis of Microbial ITC's

Biosynthesis of Enterochelin and Salicylate

Synthesis of phenolate ITC's, which proceeds from a branch point in aromatic amino acid biosynthesis, is depicted in Fig. 5a (Young and Gibson, 1969). Chorismate is an intermediate in the biosynthesis of tyrosine, tryptophan, and phenylalanine. Bryce and Brot (1972) have proposed the following mechanism for the series of reactions synthesizing enterochelin from DHB and serine:

1. $E_5\text{-SH} + \text{ATP} + \text{L-serine} \rightleftharpoons E_5\text{-SH-serine-AMP} + PP_i$
2. $E_5\text{-SH-serine-AMP} \rightleftharpoons E_5\text{-S-serine} + \text{AMP}$
3. $E_6 + \text{DHB} + \text{ATP} \rightleftharpoons E_6\text{-DHB-AMP} + PP_i$

4. E_5-S-serine + E_6-DHB-AMP ⟶ E_5-S-DHBS + E_6 + AMP

5. 3(E_5-S-DHBS) ⟶ enterochelin + E_5-SH

The protein components necessary for this reaction to occur are E_5, E_6, and E_7. These components have not yet been isolated and characterized. Enzymes 1 through 5 (see Fig. 5a) in this pathway are known to be repressed by high iron concentrations (Lankford, 1973).

Biosynthesis of Hydroxamates

The metabolic pathways for the synthesis of hydroxamates have not been well characterized; however, ornithine appears to be a common precursor in the synthesis of several hydroxamates. The first two steps in the synthesis of ferrichrome and rhodotorulic acid (synthesized by Rhodotorula pilimanae) are identical and involve the direct N-hydroxylation of the δ-amino group of ornithine followed by the δ-acetylation of δ-N-hydroxyornithine (see Fig. 5b) (Ong and Emery, 1972; Akers and Neilands, 1973). Ong and Emery (1972) have isolated the enzyme (δ-N-hydroxyornithine: acetyl CoA δ-N-transacetylase) from U. sphaerogena which catalyzes the second reaction in the synthesis of ferrichrome. The enzymes catalyzing the first and third steps in the biosynthesis of rhodotorulic acid are iron-repressible (Akers and Neilands, 1973).

Biosynthesis of Mycobactins

The biosynthetic pathways of mycobactins have not been well characterized. It is believed that the units obtained from hydrolysis of mycobactins are synthesized by separate pathways followed by assembly in an undetermined sequence (Tateson, 1970).

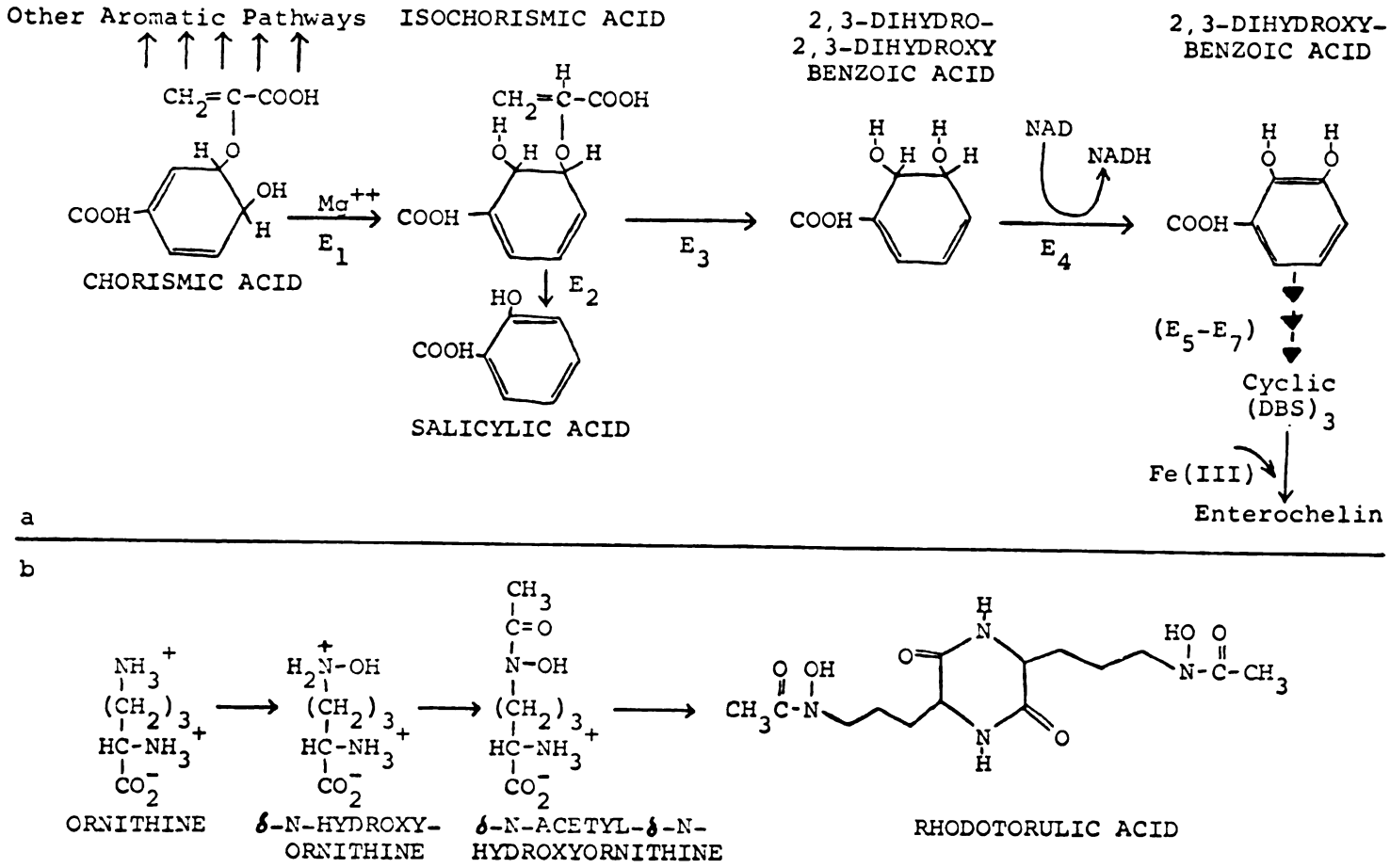


Figure 5.--Metabolic pathways for the synthesis of microbial ITC's. Symbols for Figure 5a: E_1, isochorismate synthetase; E_2, salicylate synthetase; E_3, 2,3-dihydro-2,3-dihydroxybenzoate synthetase; E_4, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase. All enzymes are iron-repressible. Adapted from Lankford (1973). Figure 5b adapted from Akers and Neilands (1973).

Mycobactins are unusual in that their two hydroxamate moieties have different acyl functions. Acylation may be the first step in the incorporation of lysine into mycobactins. Hydroxylation of the acylated-lysine compound would follow. These steps are reversed in the biosynthesis of ferrichrome (Tateson, 1970).

Either salicylate or 6-methylsalicylate is incorporated into mycobactin depending upon the type of mycobactin synthesized by the organism (Hudson and Bentley, 1970).

Conditions for the Synthesis of Microbial ITC's

It is evident that ITC's act as survival factors by sequestering iron in an environment of limited iron concentration; when iron concentrations are sufficient, these iron transport systems are necessary. Indeed, numerous studies have shown that the iron transport systems of a variety of bacteria are repressed by high iron concentrations (e.g., O'Brien *et al.*, 1971; Garibaldi, 1971; Byers *et al.*, 1967; Pollack and Neilands, 1970; Peters and Warren, 1968a; Gibson and Magrath, 1969). The structural genes for the enterochelin-iron transport system of *E. coli* are located together on the bacterial genome (Young *et al.*, 1971; Luke and Gibson, 1971), and it has been suggested that these genes form an operon which is regulated by iron (Luke and Gibson, 1971; Bryce and Brot, 1971). In most studies where various iron concentrations have been tested, concentrations of iron ranging from 10^{-5} M to 10^{-4} M have been found to inhibit synthesis of ITC's.

Temperature is a second important factor regulating the synthesis of ITC's in a fluorescent pseudomonad and strain Tm-1 of *Sal. typhimurium* (Garibaldi, 1971; 1972). The ability to synthesize ITC's at elevated temperatures

is reduced or completely lost in these organisms. Reduced synthesis of enterochelin has been observed in thirteen species of Salmonella (Garibaldi, personal communication).

Host Iron Metabolism during Infection

The concentration of free ionic iron in the human body is extremely low since most of the metal is complexed with protein ligands such as transferrin, lactoferrin, hemoglobin, and ferritin. The invading pathogen must either produce competitive iron chelating compounds or destroy the host iron ligands. The association constant of transferrin for iron is about 10^{30}. Likewise, many microbial ITC's have association constants for iron of approximately 10^{30} and the removal of iron complexed with transferrin by mycobactins and enterochelin has been demonstrated (Weinberg, 1974a). Thus it has been proposed that ITC's should be considered virulence factors (Kochan, 1973; Rogers, 1973; Weinberg, 1971).

At the onset of a bacterial infection there is a prompt reduction in the level of serum iron. This reduction is achieved by a transfer of serum iron to the liver and by blockage of intestinal absorption of iron (Weinberg, 1974a). This serum hypoferremia can be detected before the onset of any clinical symptoms and the intensity of the hypoferremia can be directly correlated with the severity of the infection. If an acute infection develops, a second lowering of serum iron occurs (Weinberg, 1971; Pekarek et al., 1969; Weinberg, 1974b).

Fever may play an important role in host defense against microbial infection since fever is invariable accompanied by serum hypoferremia. The rise in temperature may also favor the host by restricting the ability of the pathogen to synthesize its ITC's (Garibaldi, 1972;

Weinberg, 1974b). This hypothesis is supported by the *in vitro* temperature studies on various *Salmonella* species (Garibaldi, 1972; personal communication). However, fever may come into play only when the other defense mechanisms of the host have failed to halt the invasion, and should be viewed as a stop-gap measure to help contain the infection while resistance is being built up (Weinberg, 1974b).

The Effect of Iron Availability upon Virulence

Various studies have shown that the addition of small molecular weight iron compounds enhances the growth and virulence of *Klebsiella pneumoniae*, *Ps. aeruginosa*, *E. coli*, *Staphylococcus epidermidis*, *Mycobacterium fortuitum*, and *Listeria monocytogenes* in mice and rats (Martin *et al.*, 1963; Fletcher and Goldstein, 1970; Sword, 1966). Infection of iron-treated mice with a *Yersinia pestis* strain of reduced virulence greatly increases the severity of the infection (Jackson and Burrow, 1956; Brubaker *et al.*, 1965). When injected into iron-treated mice, an avirulent strain of *L. monocytogenes*, which was incapable of growth in non-treated mice, multiplied and caused some deaths (Sword, 1966). However, injection of avirulent strains of *Y. pestis* and mycobacteria into iron-treated animals does not promote infection(Kochan, 1973). Bullen *et al.* (1967; 1968) demonstrated that injection of iron compounds into experimental animals abolished the protective effects of antisera against *Yersinia septica* and *Clostridium welchii*.

In mammals, a state of hypoferremia seems to be advantageous to the host and detrimental to the invading pathogen. Animals rendered hypoferremic with endotoxin are more resistant to some bacterial infections than untreated animals (Kochan, 1973). Furthermore, the degree

of iron saturation of endogenous transferrin can be directly related to the survival of an invading pathogen. In humans, normally 30.0% of the endogenous transferrin is complexed with iron, and the serum is tuberculostatic. The addition of iron to human serum destroys tuberculostasis. This tuberculostasis can be reconstituted by the addition of quantities of transferrin which thereby lower the degree of iron saturation of transferrin to normal levels. Similarly, guinea pig serum, which contains transferrin that is 84.4% iron-saturated, supports the growth of mycobacteria (Kochan, 1973). Therefore the addition of transferrin to untreated serum should result in decreased virulence of the invading pathogen. Indeed, addition of transferrin causes growth inhibition of Staph. aureus in blood samples (McFarlane et al., 1972), and affords a slight protection to rats and mice infected with K. pneumoniae and Ps. aeruginosa (Martin et al., 1963). Likewise, animals made hypoferremic with desferal show an enhanced resistance to infection with L. monocytogenes (Sword, 1966). Desferal also inhibits the multiplication of mycobacteria in guinea pig serum (Kochan et al., 1969). However, desferal enhances the growth of mutants of Sal. typhimurium and B. megaterium and counteracts the actions of sideromycins in Staph. aureus (Arceneaux and Lankford, 1966; Luckey et al., 1972; Knusel et al., 1969). The growth of Bacillus stearothermophilus is not affected by desferal (Oram and Reiter, 1968).

The Antibiotic Activity of Sideromycins

Naturally occurring secondary hydroxamates that chelate iron have been divided into three groups; sideromycins, sideramines, and siderochromes. The sideramines are ITC's, while sideromycins, which are only synthesized by a few

species of actinomycetes, display antibiotic activity against a number of microorganisms. The biological role of siderochromes is undetermined (Nüesch and Knüsel, 1967). The antibiotic activity of sideromycins is competitively antagonized by sideramines with similar structures. This antagonism probably occurs as a result of competition for receptor sites on the cell surface (Knüsel et al., 1969).

The mechanism of action of the sideromycins has not been definitely established. While sideromycins neither disrupt hemin biosynthesis nor inactivate enzymes containing hemin, there is some evidence suggesting that carbohydrate metabolism may be affected (Nüesch and Knüsel, 1967). There is no evidence, however, that the mode of action of all sideromycins is the same. In cell-free systems (obtained from Staph. aureus SG511), albomycin had no effect on poly U-directed incorporation of phenylalanine; ferrimycin A_1 increased incorporation; while danomycin and antibiotic A22765 inhibited incorporation. Thus sideromycins should be considered a homogeneous group only in regard to their competition with sideramines for transport into the cell (Knüsel et al., 1969).

Resistance is the major problem in the application of sideromycins as antibiotics. Although many sideromycins are active against Gram-positive bacteria, certain Gram-negative organisms (e.g., Shigella dysenteriae, Salmonella typhi, and Ps. aeruginosa) are resistant to them. In addition, susceptible bacteria rapidly develop resistance to sideromycins (Nüesch and Knüsel, 1967).

MATERIALS AND METHODS

Microorganisms

The following microorganisms were examined for possible synthesis of iron transport compounds: Klebsiella pneumoniae ATCC 13883, Proteus mirabilis ATCC 9240, Proteus morganii ATCC 8019, Proteus rettgeri ATCC 9918, Proteus vulgaris ATCC 13315, Pseudomonas aeruginosa, Pseudomonas fluorescens[1], Salmonella enteriditis ATCC 13076, Serratia marcescens ATCC 13880 (a nonpigmented mutant), Shigella boydii ATCC 9207, Shigella sonnei ATCC 9290, four clinically isolated strains of Staphylococcus aureus, Yersinia pestis Kuma P^+VW^-, Yersinia pestis Kuma P^-VW^-, and Yersinia pseudotuberculosis PB 1/+.

All cultures were routinely maintined in a glycerol-phosphate storage medium at -28 C. The storage cultures were prepared by pipetting 4.0 ml of 0.033 M KH_2PO_4 onto a 24 hour sloped agar culture and mixed until the bacteria were suspended in the buffer. The suspension was then pipetted into a screw-cap tube containing 6.0 ml of sterile glycerol, mixed thoroughly, and stored at -28 C (Brubaker, personal communication).

Equipment

All growth study cultures were incubated in a New Brunswick gyrotory water bath shaker (model G76). Optical density measurements were determined on a Perkin-Elmer double beam spectrophotometer (Coleman 124). A potentiometric recorder (Sargent-Welch, model SRG) was attached to

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[1]This organism was isolated and identified as the contaminant found in a preparation of deferri-penassay broth.

the spectrophotometer for ultraviolet scanning of the prepared media. During growth studies, cell-free supernatant samples were obtained by centrifugation in an International centrifuge (model SBU). Distilled water was demineralized by passage through a Bantam demineralizer (model BD-1).

Assays for ITC's

The Arnow Assay

Phenolate compounds were routinely assayed by the procedure of Arnow (1937). This procedure has been slightly modified by the deletion of the final step (addition of 1.0 ml of water). Optical densities were determined at a wavelength of 525 nm.

The Csáky Assay

Hydroxamate ITC's may be assayed by the procedure of Csáky (1948). The modified procedure used in this study is outlined below.

(1) A volume of 1.0 ml of 1% (w/v) sulfanilamide (Sigma, St. Louis, Mo.) in 30 % (v/v) glacial acetic acid was added to 1.0 ml of cell-free supernatant in a test tube.

(2) After the addition of 0.5 ml of 6 M H_2SO_4, the sample was capped and boiled in a 100 C water bath for one to five hours.

(3) The sample was removed from the water bath and 3.0 ml of 35% (w/v) sodium acetate were added.

(4) A three to five minute reaction time was observed between the addition of 0.5 ml of 1.3% (w/v) iodine in glacial acetic acid and the

subsequent addition of 0.5 ml of the sodium arsenite reagent.

(5) The color intensity was allowed to develop for five hours after the addition of 1.0 ml of 0.3% (w/v) N-1-naphthylethylenediamine dihydrochloride (Sigma) in 30% (v/v) glacial acetic acid.

(6) The optical density was measured at 500 nm.

The sample was mixed after the addition of each reagent.

The modifications from the original procedure were as follows: (1) sulfanilamide and N-1-naphthylethylenediamine dihydrochloride were respectively substituted for sulfanilic acid and α-naphthylamine; (2) the volume of the sulfanilamide reagent added to the sample was increased from 0.5 ml to 1.0 ml; (3) the boiling time and time allowed for color development were changed; and (4) the sample was not diluted to 10.0 ml with water.

The 1.3% (w/v) iodine reagent was prepared by dissolving 3.0 g of iodine and 4.58 g of KI in 500 ml of glacial acetic acid. The sodium arsenite reagent was prepared by adding 6.0 g of As_2O_3 and 16.0 g of $NaHCO_3$ to 500 ml of water. The solution was heated to boiling with steam under a hood and continued until the compounds had dissolved and CO_2 evolution had ceased. The reaction is $As_2O_3 + 6\ NaHCO_3 \longrightarrow 6\ CO_2 + 3\ H_2O + 2\ Na_3AsO_3$ (Partington, 1933).

The $FeCl_3$-ITC Assay

This procedure may be used to detect both reported types of ITC's (Gibson and Magrath, 1969). The modified procedure consisted of the addition of 1.0 ml of cell-free supernatant to 3.0 ml of a 2% (w/v) $FeCl_3$ solution in 5 mM HCl. The minor modification made was the addition of

1.0 ml of cell-free supernatant instead of 0.1 ml.

The Bathophenanthroline Iron Assay

Several modifications of the bathophenanthroline iron assay method of Diehl and Smith (1960) were tested for their effectiveness in measuring the low iron concentrations present in culture media, water, and glassware. The modification subsequently used in all iron determinations is outlined below.

(1) Samples (100 ml) were heated with steam for one hour after the addition of 4.0 ml of 15% (w/v) L-ascorbate and 0.1 ml of concentrated HCl.

(2) After the samples had cooled to room temperature, they were transferred to separatory funnels; 5.0 ml of 10% (w/v) sodium acetate were added and the samples were vigorously shaken.

(3) Subsequently, 4.0 ml of 1 mM bathophenanthroline (Sigma) in 50% (v/v) ethanol were added, followed by vigorous shaking of the samples.

(4) After a reaction time of thirty minutes, 10.0 ml of isoamyl alcohol were added.

(5) The samples were shaken vigorously and allowed to stand overnight until the aqueous and alcohol phases had completely separated.

(6) The lower aqueous layers were drawn off and discarded.

(7) The optical densities of the alcohol layers were measured at 533 nm.

Contaminating iron was partially extracted from the L-ascorbate and sodium acetate reagents by the same procedure used in the assay itself, but the hour of steaming was omitted. The ethanol was redistilled before use as a solvent for the bathophenanthroline. No treatment of the

concentrated HCl and reagent grade isoamyl alcohol was necessary.

Preparation of In Vitro Iron-deficient Conditions

Preparation of Deferri-glassware

Contaminating iron was removed from glassware by immersion in 50% (v/v) HCl for approximately 24 hours. Traces of HCl were removed by thorough rinsing with distilled deionized water.

Preparation of Deferri-water

Distilled water used in the preparation of iron-deficient media was demineralized by passage through a demineralizing column (Bantam standard cartridge) followed by redistillation.

Preparation of Deferri-media

A liquid medium described by Waring and Werkman (1942) and a commercially prepared penassay broth (Difco, Detroit, Mi.) were used in all growth studies. The compositions of these two media are denoted in Tables 1 and 2. The medium described by Waring and Werkman (1942) was slightly modified by the addition of 10 mg of nicotinamide (Sigma) per liter and will be hereafter referred to as the dextrose broth.

Contaminating iron was extracted from all growth media by the method of Waring and Werkman (1942). In this procedure, the iron is chelated with 8-hydroxyquinoline (8-HQ) (Sigma) and the chelated complex is removed by repeated

Table 1.--Composition of dextrose broth[a]

Substance	Quantity per liter
Dextrose	20.0 g
K_2HPO_4	4.0 g
KH_2PO_4	1.0 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$ [20% (w/v) solution]	0.5 ml
Nicotinamide (10 mg/ml solution)	1.0 ml
Trace mineral solution[b]	0.1 ml

[a]Adapted from Waring and Werkman (1942).

[b]A 100 ml solution containing 44 mg of $ZnSO_4 \cdot 7H_2O$, 40 mg of $CuSO_4 \cdot 5H_2O$, 41 mg of $MnSO_4 \cdot 4H_2O$, and 42 mg of KI.

Table 2.--Composition of penassay broth[a]

Substance	Quantity per liter
Bacto-beef extrace	1.5 g
Bacto-yeast extract	1.5 g
Bacto-peptone	5.0 g
Bacto-dextrose	1.0 g
NaCl	3.5 g
K_2HPO_4	3.68 g
KH_2PO_4	1.32 g

[a]Adapted from Difco, Detroit Mi.

extractions with chloroform. Residual chloroform was removed by heating the extracted media under a hood. In order to avoid carmelization, the dextrose broth was sterilized by membrane filtration (Millipore Filter, type HA, pore size 0.45 μm, Millipore Corp., Bedford, Mass.). The media were examined for traces of chloroform and 8-HQ by ultraviolet scanning spectroscopy.

In Vitro Growth Study Procedures

Sloped nutrient agar cultures were used to inoculate 25.0 ml of the ferri-dextrose broth in an 125 ml Erlenmeyer flask. After 24 hours of incubation at 25 C, 0.2 ml of the culture was pipetted into a second 125 ml Erlenmeyer flask containing 25.0 ml of ferri-dextrose broth. This culture was incubated at 37 C on a shaker. During exponential phase, cells from 5.0 ml of the culture were harvested by centrifugation. The cell-free supernatant fluid was discarded and the cell pellet resuspended in deferri-dextrose broth at a concentration of 7.5×10^8 colony forming units (CFU)/ml. One tenth milliliter of this suspension was used as the inoculum in growth studies.

Growth studies were performed at three different iron concentrations in the deferri-dextrose broth. All experimental cultures contained 70.8 ml of the deferri-dextrose broth. A 10^{-4} M iron concentration was prepared by aseptically pipetting 4.2 ml of a sterile solution (0.1 mg Fe^{3+}/ml) into the 70.8 ml of deferri-dextrose broth. An iron concentration of approximately 10^{-7} M was prepared by aseptically adding 4.2 ml of a sterile solution (0.1 μg Fe^{3+}/ml) to the deferri-dextrose broth. The third culture contained only iron which had not been extracted from the deferri-dextrose broth and was prepared by the aseptic

addition of 4.2 ml of sterile doubly distilled deionized water to the deferri-dextrose broth.

In growth studies using the penassay broth, ferri-penassay broth and deferri-penassay were substituted for ferri-dextrose broth and deferri-dextrose broth, respectively. Although the 10^{-4} M iron and residual iron concentration cultures were prepared as previously described, a third iron-limiting culture was prepared by aseptically adding 4.2 ml of 10^{-5} M orthophenanthroline (OP) (Sigma) to 70.8 ml of the deferri-penassay broth. The final concentration of OP under this condition was 5.6×10^{-7} M.

For growth studies in deferri-dextrose broth, viable counts and ITC assays were performed at four-hour intervals over a period of 24 hours. To assure that contamination of the stock cultures had not occurred, subcultures were biochemically identified before inoculation into experimental flasks. At the end of the experiment, a sample from each flask was subcultured on a nutrient agar plate to screen for contamination. Isolated colonies from each subculture were identified biochemically.

For growth studies in deferri-penassay broth, viable counts and ITC assays were performed at regular intervals for a period of three days. Cultures were screened for contaminants as above at 24 hour intervals during the experiment.

RESULTS

Procedural Results

Assays for ITC's

The Arnow Assay

A comparison was made of the standard curves generated by use of fresh reagents, one-month old (aged) reagents, and the original procedure of Arnow (1937) with aged reagents (Fig. 6). In all cases 2,3-dihydroxybenzoate (DHB) (Aldrich Chem. Co., Milwaukee, Wis.) was used as the standard. The standard curve obtained with fresh reagents has a line equation of $y = (0.018)x - (0.00272)$, a correlation coefficient (r) of 0.9996, and a standard error of the estimate (SEE) of $\pm$ 0.011. The same reagents one month later gave the following results: (1) r = 0.9994; (2) $y = (0.01687)x + (0.00617)$; and (3) SEE = $\pm$ 0.009. The unmodified Arnow assay procedure includes the addition of 1.0 ml of water as the final step and yielded the following results: (1) $y = (0.01238)x - (0.00427)$; (2) r = 0.99 r = 0.9999; and (3) SEE = $\pm$ 0.004. Since the original assay procedure did not produce a standard curve significantly more reliable, the modified procedure was used in all subsequent growth studies. The aged reagents were used in all growth studies since a reliable standard curve was still obtained (see Fig. 6).

During the course of preliminary experiments it was noted that the color intensity of the Arnow reaction diminished with time. Accordingly, a comparison was made of the standard curves obtained with DHB at various time intervals after the addition of the last reagent. The results are reported in Table 3 and Fig. 6. Only three

Figure 6.--Arnow assay standard curves with DHB. Modified procedure standard curves with zero incubation time using fresh reagents (●) and one-month old (aged) reagents (○). Original procedure standard curve with zero incubation time using aged reagents (▲). Modified procedure standard curves using aged reagents with incubation times of zero hours (△), three hours (■), and seven hours (□).

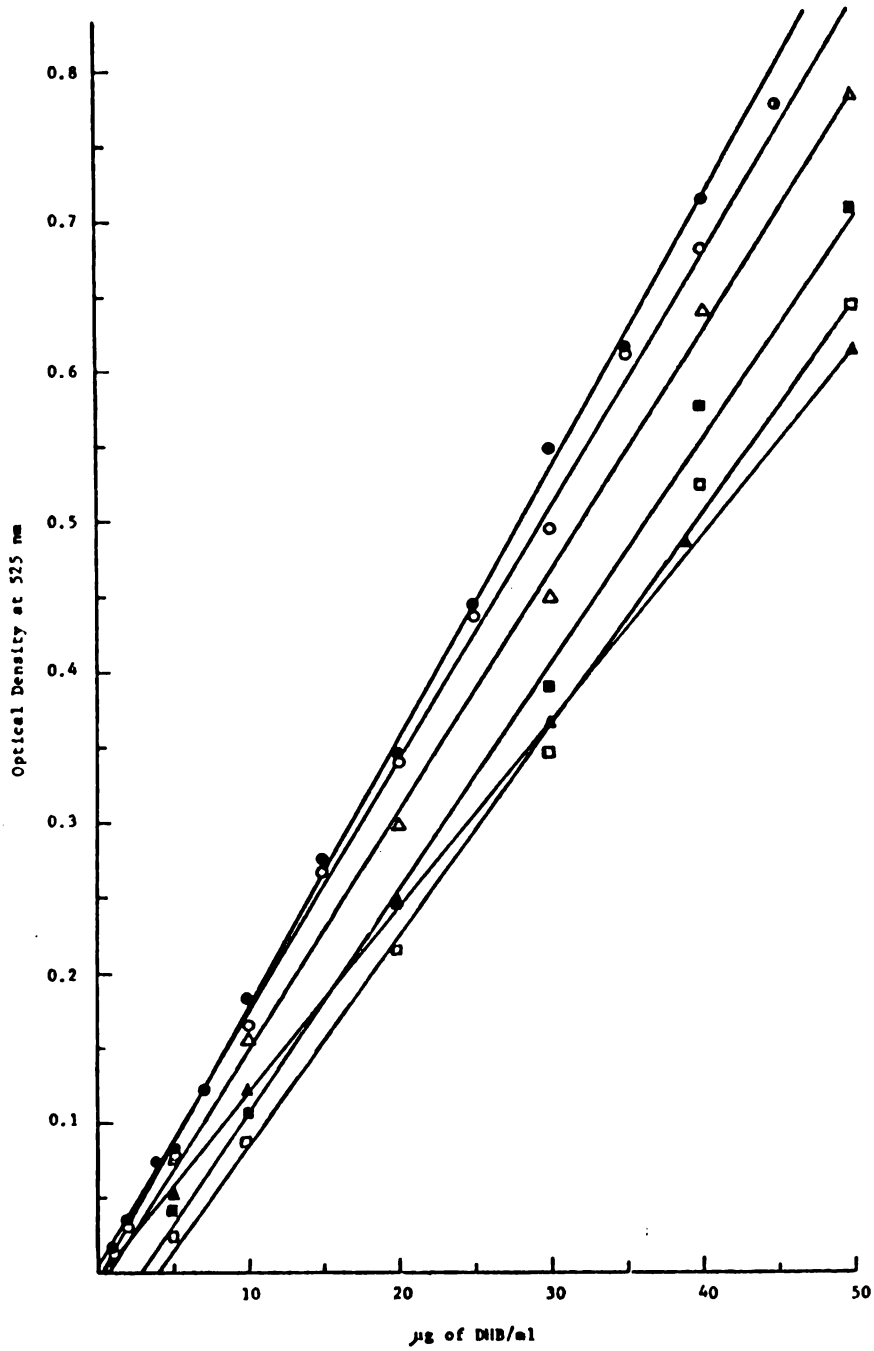

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical Density at 525 mμ
10
20
30
40
50
μg of DNB/ml

Figure 6

Table 3.--Correlation between incubation time and color intensity (Arnow assay).

Incubation time h	Statistical calculations: Line equation	r	SEE
0[a]	y = (0.01581)x - 0.00935	0.9982	± 0.015
2	y = (0.01527)x - 0.04047	0.9978	± 0.015
3[a]	y = (0.01499)x - 0.04334	0.9795	± 0.017
4	y = (0.01464)x - 0.04501	0.9980	± 0.015
5.5	y = (0.01436)x - 0.05743	0.9976	± 0.017
7[a]	y = (0.01399)x - 0.05465	0.9977	± 0.016

[a]The calculated line equations of these incubation times are diagramed in Fig. 6.

times are recorded in Fig. 6 since the differences between the two, three, and four hour time intervals and the five and one-half and seven hour intervals were small. During subsequent growth studies all Arnow assay optical densities were measured immediately after the addition of the last reagent.

The standard curves seen (Fig. 6) were calculated from equations using data points obtained from triplicate samples at each concentration. This assay appears to be an accurate quantitative method for detecting phenolate compounds.

The Csáky Assay

Desferal was used as the standard in all studies involving the determination of standard curves prepared by various conditions of the Csáky assay. The standard curves obtained with the dextrose and penassay broths appear to be fairly accurate. The standard curves were calculated from equations using data points obtained from triplicate samples at each concentration and yielded the following results in dextrose and penassay broths, respectively: (1) $y = (0.00179)x + (0.0289)$, $r = 0.9951$, SEE = $\pm$ 0.007; and (2) $y = (0.00412)x - (0.01041)$, $r = 0.998$, SEE = $\pm$ 0.015 (see Fig. 7). In the dextrose broth, an increase in color intensity is incurred by eliminating a five to six minute delay between the addition of sodium arsenite and N-1-naphthylethylenediamine dihydrochloride (see Fig. 7). This variation yielded a line equation of $y = (0.00461)x + (0.00848$, an $r = 0.9947$, and an SEE = $\pm$ 0.02. Because of the large number of samples processed during the course of subsequent growth studies, the standard curve obtained with the five to six minute delay has been used in the calculation of all hydroxamate concentrations.

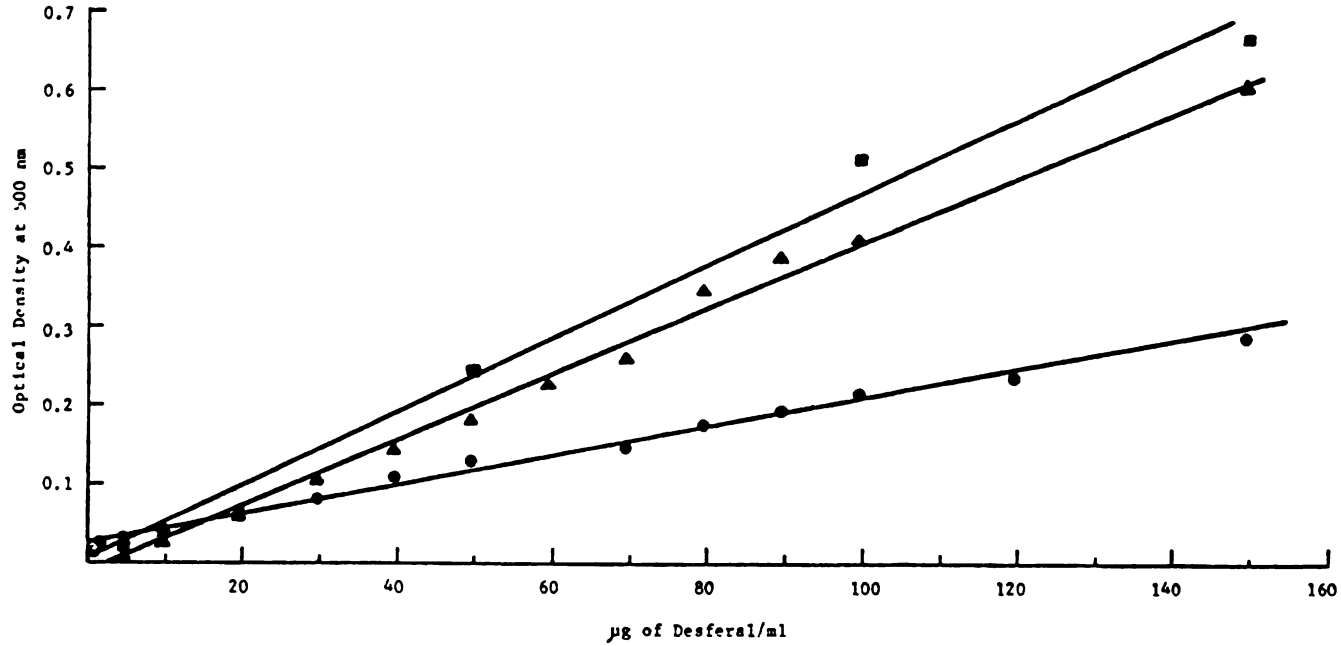


Figure 7.--Csáky assay standard curves with desferal in dextrose and penassay broths. Standard curves in dextrose broth (●), penassay broth (▲), and dextrose broth obtained by eliminating a five to six minute delay between the addition of sodium arsenite and N-1-naphthylethylenediamine dihydrochloride (■).

Optimal boiling times for release of unbound hydroxamates in penassay and dextrose broths were determined and are reported in Table 4, Fig. 8, and Fig. 9. The optimal boiling time in penassay broth was five hours, while one hour was the optimal boiling time in dextrose. These boiling times were used in the preparation of the standard curves reported in Fig. 7 and in later growth studies.

Since the color intensity of the reaction increases with time, optical density readings of the standards in the dextrose broth were taken at one-half, two and one-half, and five hour intervals after the addition of the last reagent. A significant difference in intensity was noted between the samples allowed to stand for one-half hour and those allowed to stand for two and one-half and five hours (see Fig. 10). The five hour time interval was used in the determination of standard curves as well as in later growth studies, thereby eliminating conflicts with other procedures being performed concurrently.

The Csáky assay is at best a rough estimate of the quantity of hydroxamates present. Although this assay is sensitive to small quantities of hydroxamates, the harsh treatment necessary to cleave the secondary hydroxamate and release the unbound form partially destroys the compound. The large differences in color intensity caused by small differences in the time intervals between the addition of various reagents further reduce the accuracy of this assay.

The $FeCl_3$-ITC Assay

The standard curves generated by this assay using DHB and desferal are represented in Fig. 11. DHB yielded a line equation of $y = (0.000965)x - (0.03429)$, an $r = 0.9978$, and an SEE = $\pm$ 0.013. Desferal gave a line equation of

Table 4.--Correlation between boiling time and release of unbound desferal (Csáky assay)[a].

Boiling time	Medium	Statistical calculations: Line equation	r	SEE
0.25	Dextrose	y = (0.00248)x + 0.01109	0.9957	± 0.014
0.5	Dextrose	y = (0.00209)x + 0.02137	0.9821	± 0.004
1.0	Dextrose	y = (0.00315)x + 0.00421	0.9955	± 0.012
2.0	Dextrose	y = (0.0029)x + 0.02157	0.9954	± 0.011
3.0	Penassay	y = (0.00336)x + 0.00005	0.9980	± 0.013
4.0	Penassay	y = (0.00408)x - 0.01599	0.9976	± 0.016
5.0	Penassay	y = (0.00412)x - 0.01041	0.9980	± 0.015

[a]A thirty minute time interval after the addition of the last reagent was allowed for color development. The line equations in the dextrose broth are diagramed in Fig. 8; those in penassay broth are diagramed in Fig. 9.

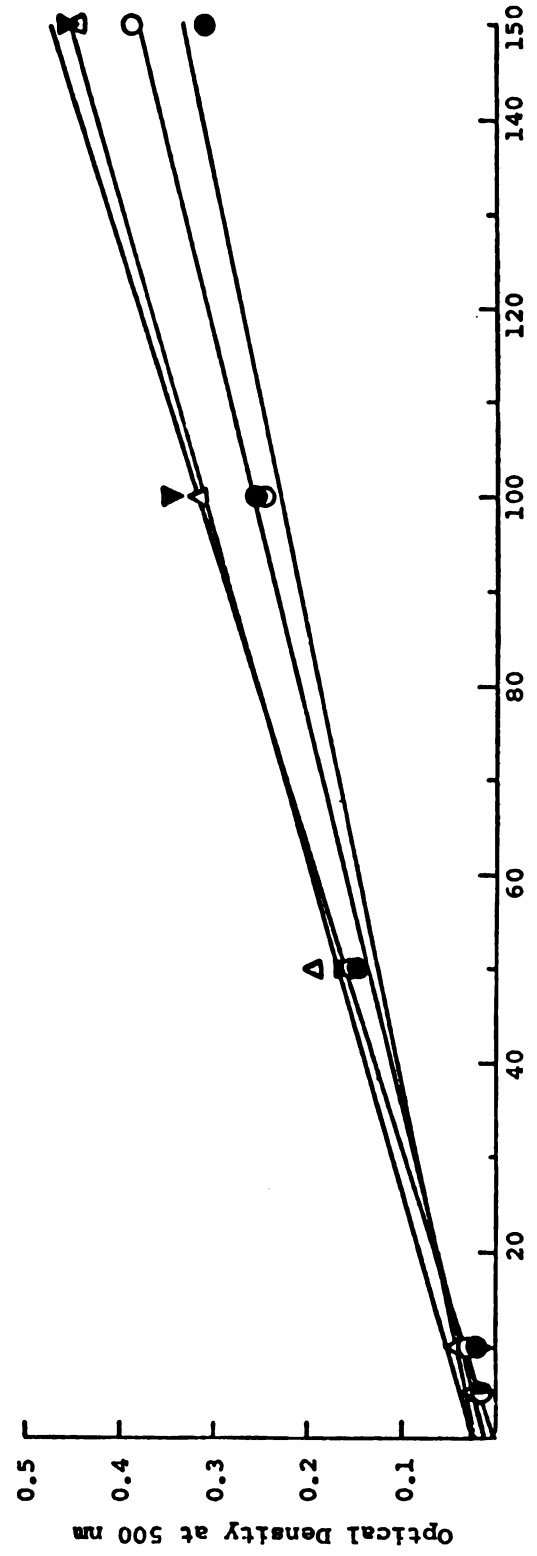


Figure 8.--Csáky assay standard curves with desferal in dextrose broth obtained at various boiling times. Symbols: -o-, 0.25 hour of boiling; -●-, 0.5 hour of boiling; -▼-, one hour of boiling; and -▲-, two hours of boiling.

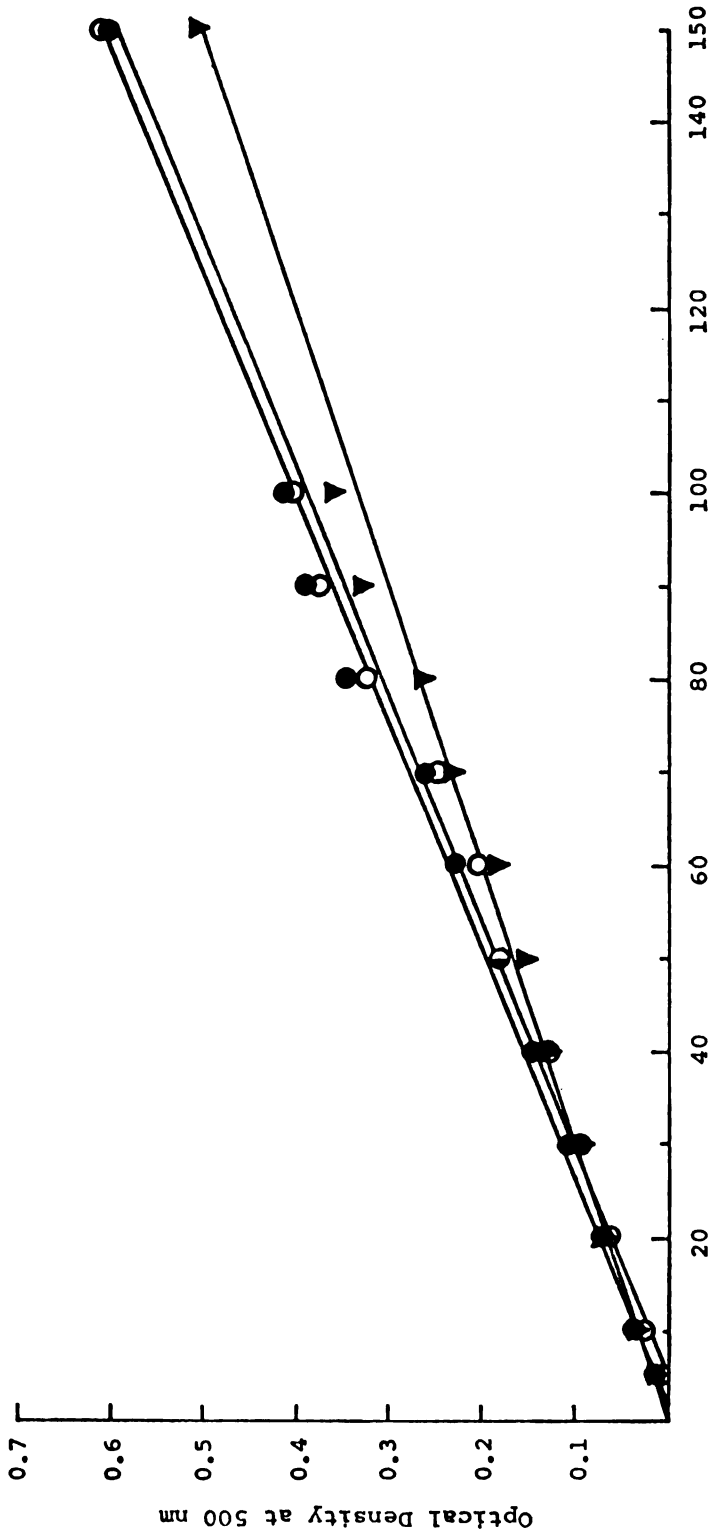


Figure 9.--Csáky assay standard curves with desferal in penassay broth obtained at various boiling times. Symbols: ▲, three hours of boiling; ○, four hours of boiling; and ●, five hours of boiling.

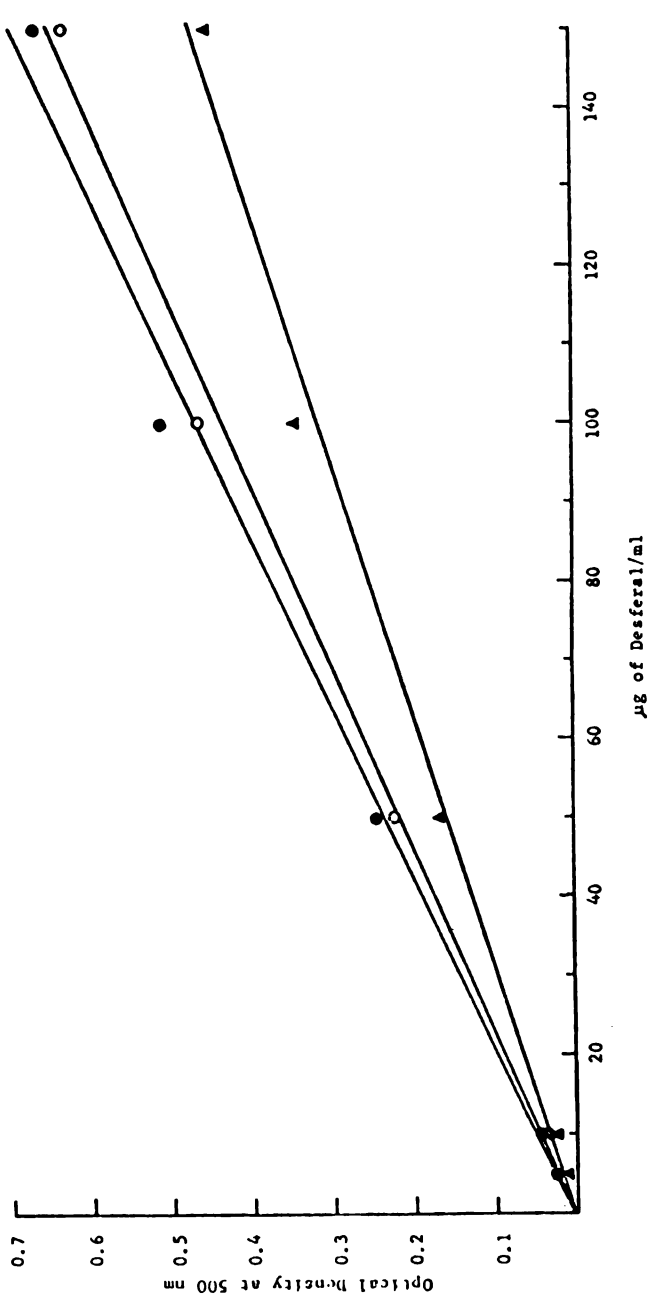


Figure 10.--Csáky assay standard curves with desferal in dextrose broth obtained at various incubation times. Symbols: ▲, thirty minutes of incubation; ○, two and one-half hours of incubation; and ●, five hours of incubation.

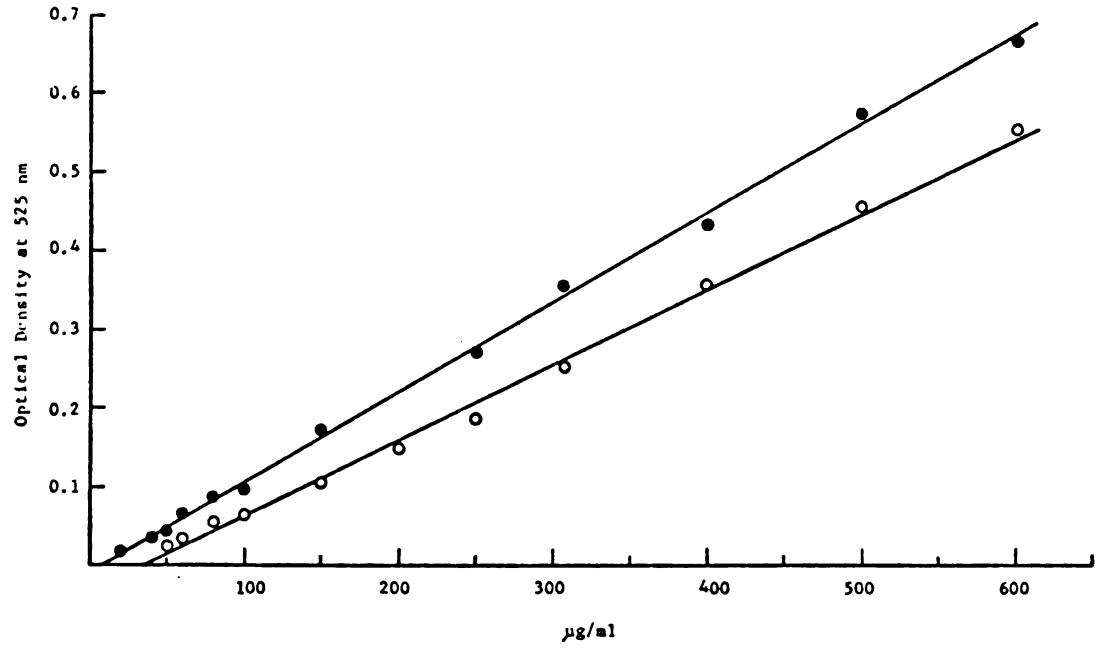


Figure 11.--$FeCl_3$-ITC assay standard curves with desferal and DHB. Symbols: -●-, desferal standard curve; and -○-, DHB standard curve.

$y = (0.001141)x - (0.008535)$, an $r = 0.9991$, and an SEE = $\pm$ 0.002.

With the standards used, this assay is at best accurate down to 20 µg/ml, and therefore has little value in the monitoring of ITC synthesis during growth studies. If enterochelin had been available for use as a standard for phenolate compounds, the accuracy might have been improved slightly due to the higher association constant of enterochelin as compared to DHB. This assay was used in preliminary studies after 24 and 48 hours of growth to show the presence of a compound or compounds which chelate iron.

The Bathophenanthroline Iron Assay

A number of modifications of the bathophenanthroline iron assay procedure of Diehl and Smith (1960) were tested. The original procedure and all modified procedures (other than that reported in the section on methods) failed to reduce iron adequately from the ferric to the ferrous state. When iron is not reduced to the ferrous state, incomplete analysis of the iron content occurs since bathophenanthroline reacts only with ferrous ions. The various concentrations of hydroxylamine did not affect iron reduction. Ascorbate reduced iron only when 0.1 ml of concentrated HCl was added and the sample was steamed for one hour. Reducing either the HCl concentration or the steaming time decreased the reduction of ferric ions. The modification reported in the section on methods was significantly more effective in reducing ferric ions. In order to assure that this reduction was occurring, standard curves were prepared using $FeCl_3$ and $FeSO_4 \cdot 7\ H_2O$ standards (Fig. 12). Analysis of these standard curves indicates that the modified procedure reduces approximately 60% of the iron present as ferric ions. Statistical

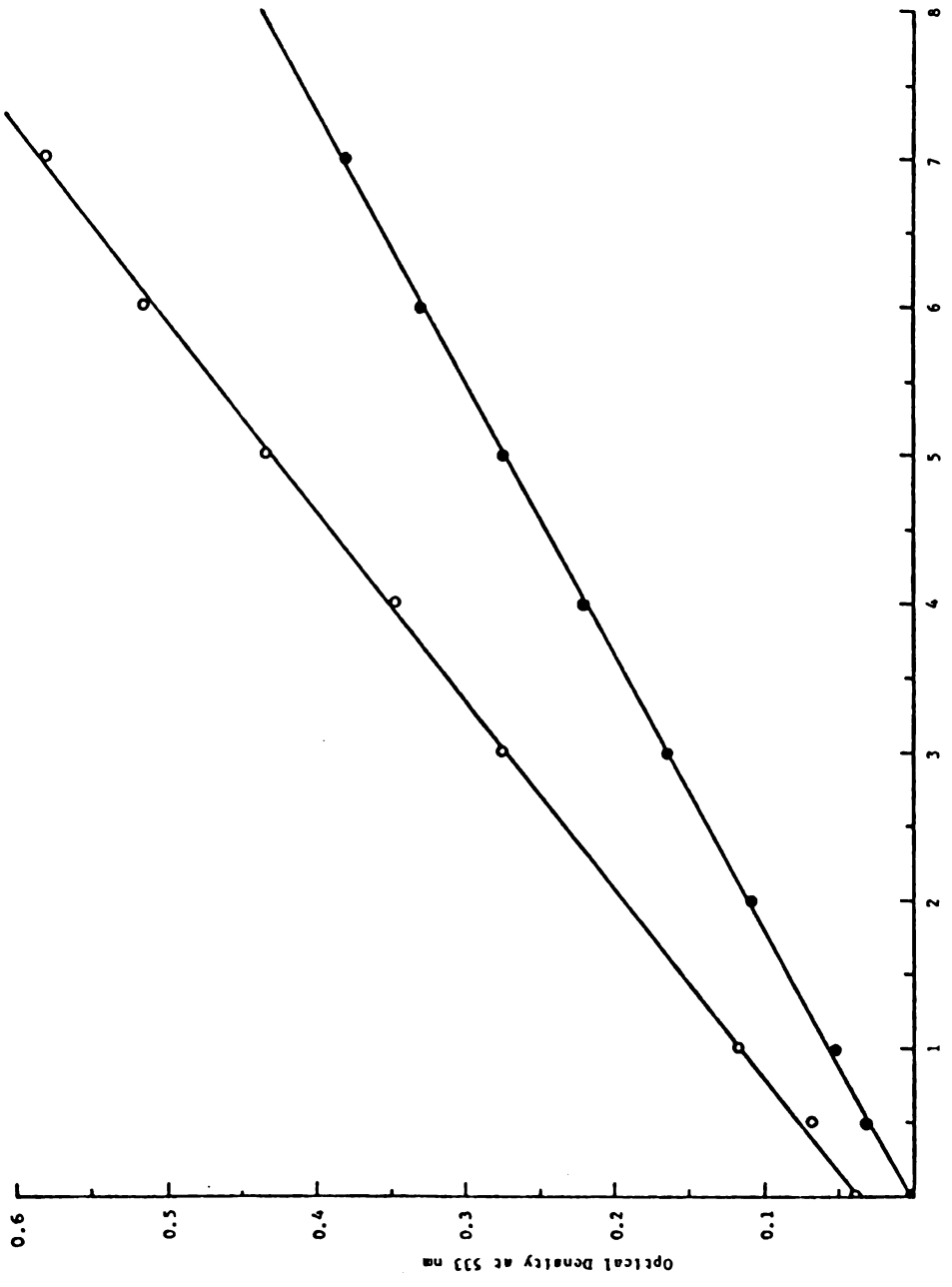


Figure 12.--Bathophenanthroline iron assay standard curves with $FeCl_3$ and $FeSO_4 \cdot 7H_2O$. Each point represents the mean of values from duplicate samples. Symbols: -●-, $FeCl_3$; and -○-, $FeSO_4 \cdot 7H_2O$.

analysis of the $FeSO_4 \cdot 7\ H_2O$ standard curve yielded a line equation of $y = (0.0795)x + (0.0315)$, an $r = 0.9997$, and an SEE = $\pm$ 0.005; whereas the $FeCl_3$ standard curve yielded a $y = (0.0545)x + (0.0009)$, an $r = 0.9998$, and an SEE = $\pm$ 0.002.

It is impossible to determine the amount of iron added to the sample from the reagents used in this assay. This introduced a maximal error of 9.33×10^{-8} M in all determinations of total iron content.

Preparation of Iron-deficient Conditions

Preparation of Deferri-water

A comparison was made of the iron content of doubly distilled deionized water and water further treated by the 8-HQ method of Waring and Werkman (1942). The doubly distilled deionized water had an iron content of not more than 1.18×10^{-7} M, whereas the 8-HQ treated water had an iron content of not more than 1.15×10^{-7} M. Since this difference is insignificant, doubly distilled deionized water was used in the preparation of all media and reagents.

Preparation of Deferri-glassware

A comparison of acid-treated flasks and flasks further treated with 8-HQ showed that differences in contaminating iron concentrations were insignificant. The iron concentrations were respectively 9.33×10^{-8} M and 1.15×10^{-7} M. An iron concentration of 1.1×10^{-6} M was detected with washed, untreated flasks. This analysis of residual iron in glassware was made by simply running iron assays in the variously treated glassware.

Preparation of Deferri-media

The deferri-media, prepared by the method of Waring and Werkman (1942) were analyzed for iron content. The deferri-dextrose broth had a residual iron concentration of 4.25 ($\pm$ 0.933) x 10^{-7} M, and the deferri-penassay broth had a residual iron concentration (as detected by the bathophenanthroline iron assay) of 4.68 ($\pm$ 0.933) x 10^{-7} M. However, it is possible that iron-protein complexes present in the deferri-penassay broth were not detected by this method. This hypothesis is supported by the observation that after 24 hours of growth at 25 C in deferri-dextrose broth, K. pneumoniae had accumulated 9.05 µg/ml of a phenolate compound. Under similar conditions in deferri-penassay broth, this organism had accumulated only 2.7 µg/ml of a phenolate compound after 24 hours of growth.

Two substances used in the iron extraction process, chloroform and 8-HQ, inhibit bacterial growth. While chloroform kills bacteria by disrupting cell membranes, the toxic effect of 8-HQ is more complex and not merely a simple metal deficiency death. The compound appears to penetrate the cell wall and membranes and to exert its toxic effect intracellularly, especially in Gram-positive organisms (Rubbo et al., 1950). Therefore, complete removal of chloroform and 8-HQ from the deferri-media must be accomplished.

All deferri-media were analyzed for traces of chloroform and 8-HQ by ultraviolet scanning spectroscopy before use in growth studies. In ferri-dextrose broth, the addition of 8-HQ produces an absorption peak at 240 nm, whereas chloroform has an absorption peak at 200 nm. The absorption spectra are shown in Fig. 13. It was noted that when the concentrated solution of deferri-dextrose broth was

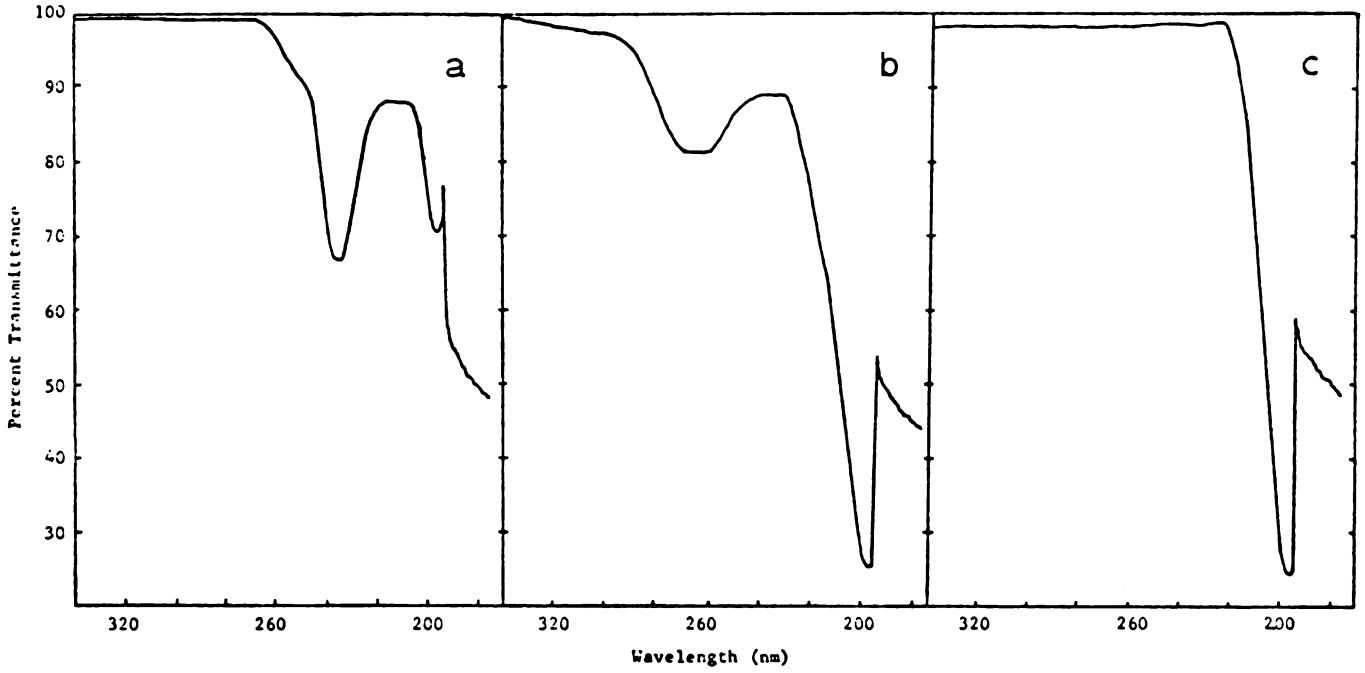


Figure 13.--Ultraviolet absorption spectra of ferri-dextrose broth containing 8-HQ (a), deferri-dextrose broth diluted to volume with doubly distilled deionized water (b), and deferri-dextrose broth diluted to volume with doubly distilled deionized water further demineralized with 8-HQ (c).

diluted to volume with doubly distilled deionized water a small absorption peak appears near 270 nm (Fig. 13b). This peak is eliminated by the dilution of the concentrated solution with doubly distilled deionized water which had been further demineralized by the method of Waring and Werkman (1942) (Fig. 13c). Because a small peak occurs at 200 nm even in the absence of chloroform (Fig. 13a), the large peaks at 200 nm found in deferri-dextrose broth (Fig. 13b and Fig. 13c) probably do not represent a high concentration of chloroform.

When penassay broth is deferrated by the method of Waring and Werkman (1942) a whitish precipitate occurs. This precipitate probably contains chloroform-soluble components such as lipids, fats, and hydrophobic short-chain polypeptides.

Growth Study Results

Preliminary Studies

Preliminary studies at 25 C in deferri-penassay broth of P. mirabilis, P. morganii, P. rettgeri, P. vulgaris, Y. pseudotuberculosis, Staph. aureus BB, Staph. aureus ONT 6, Staph. aureus UNH 10, Staph. aureus Smith's diffuse, and the two strains of Y. pestis showed viable counts in these cultures of approximately 10^9 CFU/ml after 24 hours of incubation. With all these organisms, assays performed at 24 and 48 hours of incubation for hydroxamate and phenolate compounds were negative. For phenolate compounds, the assay was positive only for identical cultures of K. pneumoniae, Sal. enteriditis, Ser. marcescens, Sh. sonnei, and Sh. boydii. For identical cultures of Sh. sonnei, Sh. boydii, Ps. aeruginosa, and Ps. fluorescens the Csáky assay yielded positive results. In all cases when either or

both the Arnow and Csáky assays yielded positive reactions, the $FeCl_3$-ITC assay detected the presence of a compound or compounds which chelated iron.

Growth Studies in Deferri-dextrose Broth

K. pneumoniae, Ps. aeruginosa, Sal. enteriditis, Ser. marcescens, and Sh. sonnei were all tested for growth patterns and synthesis of phenolates and/or hydroxamates in deferri-dextrose broth. In all cases detectable accumulation of these compounds was repressed in cultures containing an iron concentration of 10^{-4} M. The two iron-deficient cultures (4.25×10^{-7} M iron and 5.25×10^{-7} M iron) showed no differences in the synthesis of suspected ITC's except in the case of Sh. sonnei. In all cases the phenolate compounds were not detected until cultures had reached populations of 2×10^8 CFU/ml. All recorded results in this section are averages of duplicate cultures. The concentrations of phenolate or hydroxamate compounds are reported as DHB or desferal equivalent micrograms per 10^8 CFU, respectively. Iron-deficient cultures containing 4.25×10^{-7} M iron were tested only at 25 C and not at 37 C.

The curves of growth and phenolate synthesis of K. pneumoniae incubated at 25 C and 37 C are represented in Fig. 14 and Fig. 15. There were no significant differences in the growth patterns between iron-sufficient and iron-deficient cultures at 25 C; however, after sixteen hours of incubation at 37 C in the iron-deficient culture, large numbers of organisms began to die. Production of phenolate compounds at 37 C reached much higher levels than at 25 C.

Neither differences in the growth patterns nor in the levels of phenolate compounds produced by Sal.

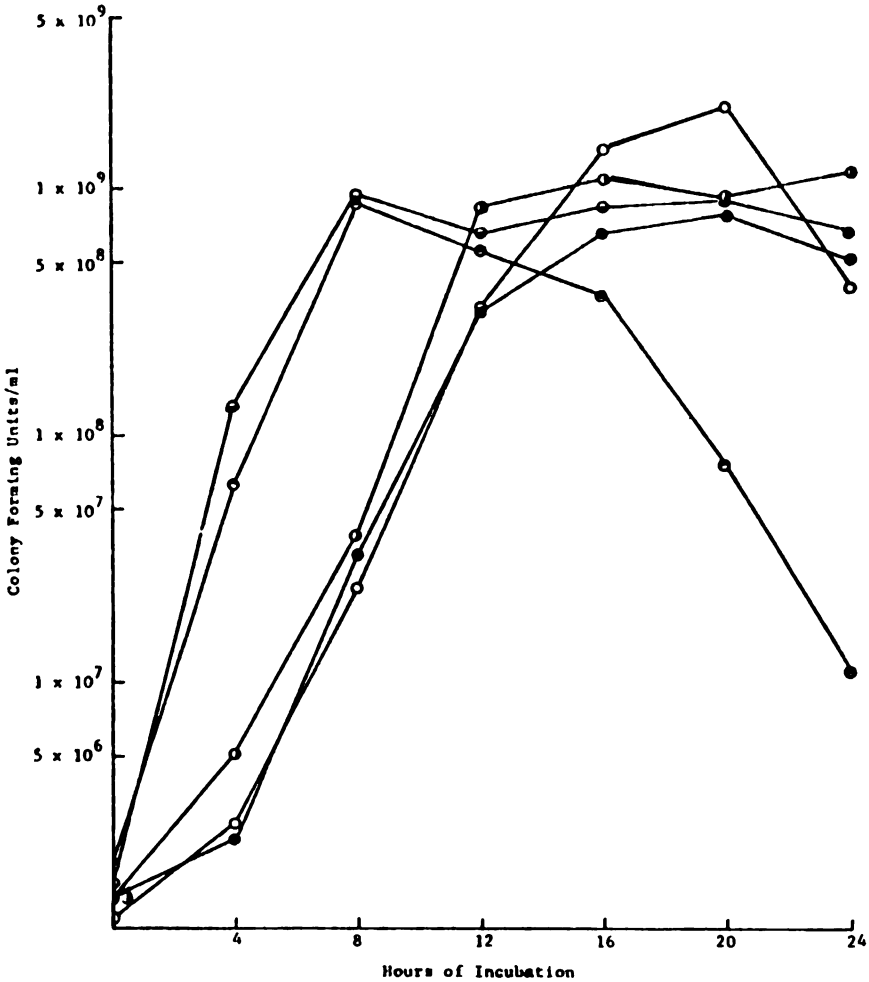


Figure 14.--Growth curves of iron-sufficient and iron-deficient dextrose broth cultures of K. pneumoniae at 25 C and 37 C. Symbols: ●, 1×10^{-4} M Fe culture at 37 C; ●, 5.25×10^{-7} M Fe culture at 37 C; ●, 1×10^{-4} M Fe culture at 25 C; ○, 5.25×10^{-7} M Fe culture at 25 C; and ●, 4.25×10^{-7} M Fe culture at 25 C.

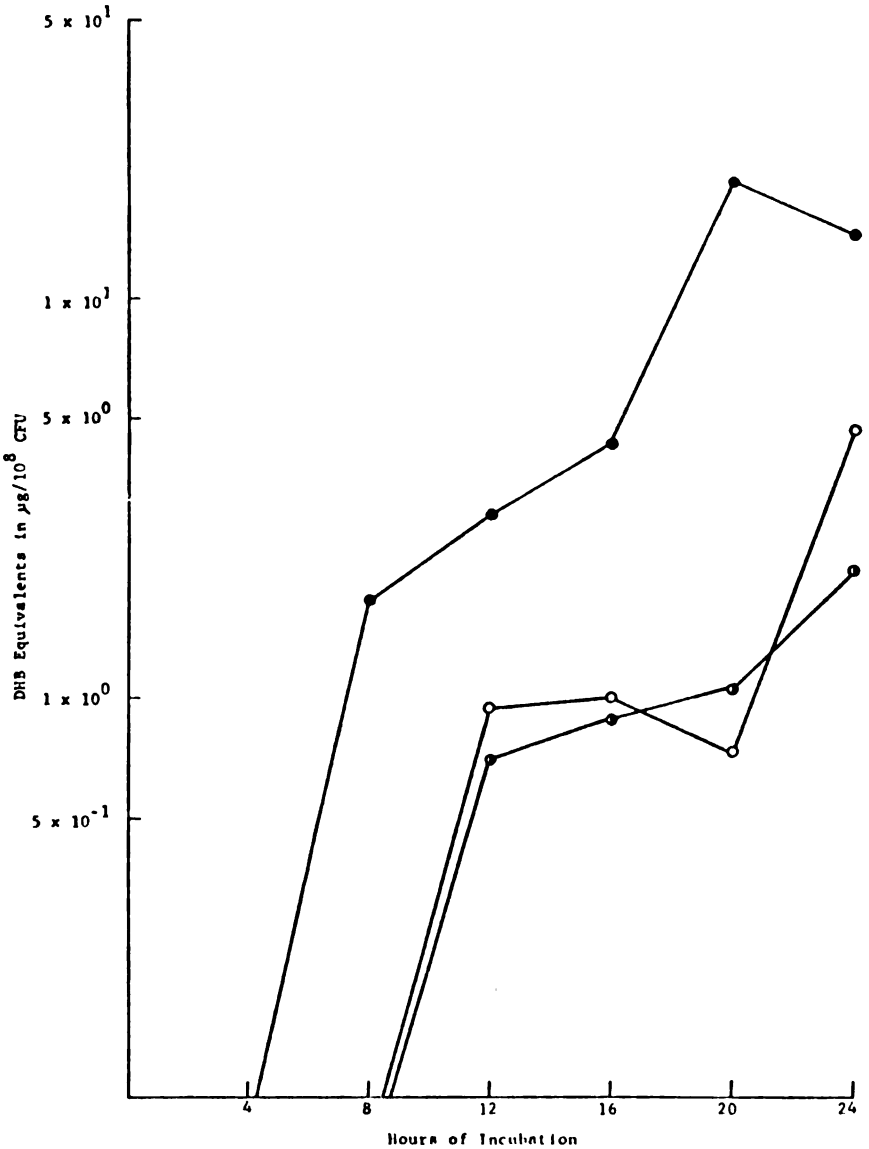
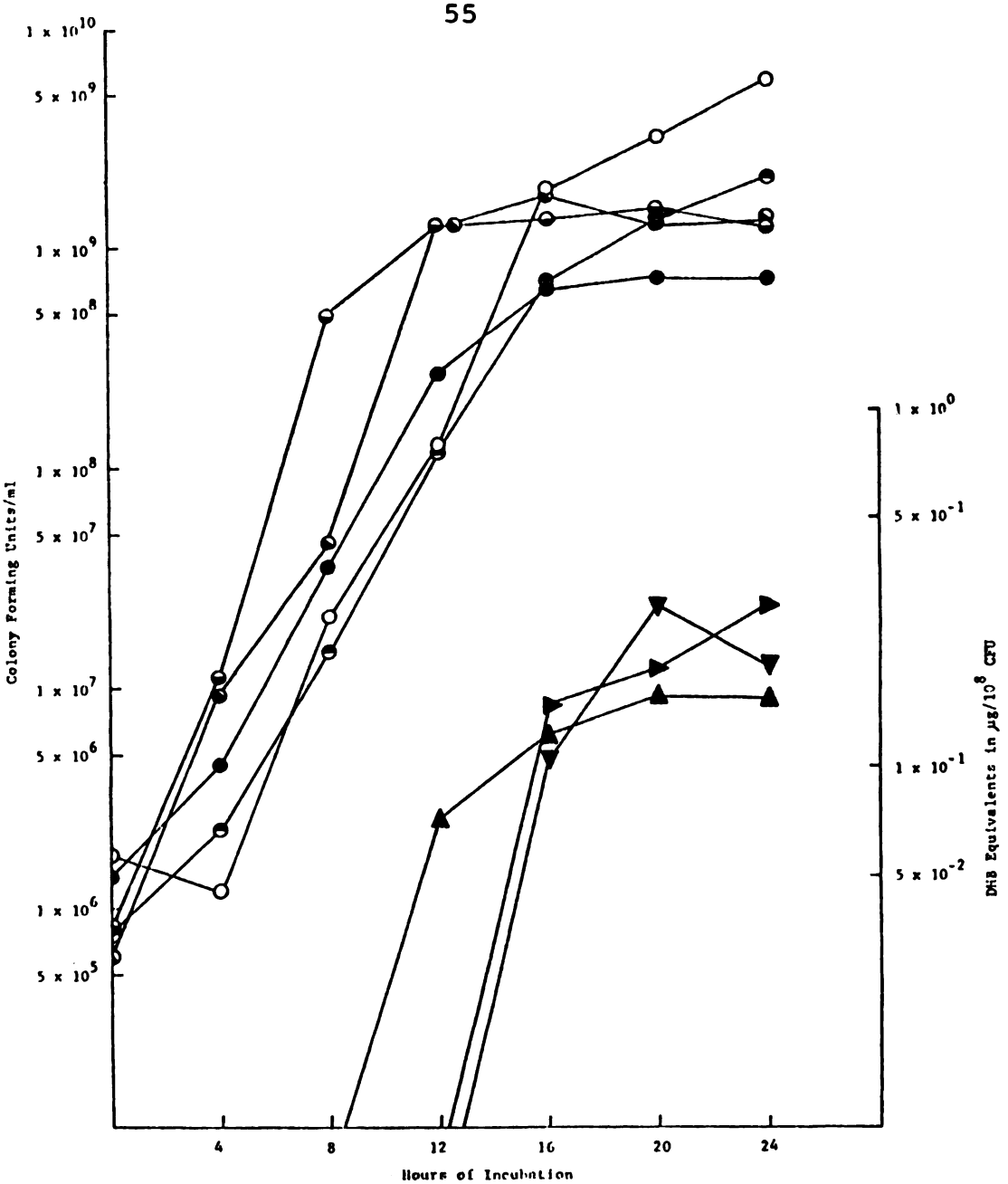


Figure 15.--Phenolate accumulation curves of iron-deficient dextrose broth cultures of K. pneumoniae at 25 C and 37 C. Symbols: —●—, 5.25 x 10^{-7} M Fe culture at 37 C; —○—, 5.25 x 10^{-7} M Fe culture at 25 C; and —◐—, 4.25 x 10^{-7} M Fe culture at 25 C.

enteriditis were noted between the two temperatures. However, at 25 C iron-deficient cultures containing 4.25 x 10^{-7} M iron attained lower cell populations (a full log unit) than did the iron-deficient cultures containing 5.25 x 10^{-7} M iron (Fig. 16). Cultures of Ser. marcescens were incubated only at 25 C and grew equally well in iron-deficient and iron-sufficient conditions (Fig. 17). For Ps. aeruginosa, iron-sufficient and iron-deficient cultures showed equivalent growth patterns at 37 C (Fig. 18). Accumulation of significant levels of hydroxamate compounds by the iron-deficient culture was delayed until sixteen hours of incubation when the culture had been at a population exceeding 5 x 10^{8} CFU/ml for four hours.

Within twenty hours of incubation at 25 C and in the presence of 4.25 x 10^{-7} M iron, cultures of Sh. sonnei exhibited a significantly lower population than the iron-sufficient and 5.25 x 10^{-7} M iron cultures. In this instance phenolate compounds were detected four hours earlier (at twelve hours of incubation) in cultures containing 4.25 x 10^{-7} M iron compared to those with 5.25 x 10^{-7} M iron. However, at both iron-deficient concentrations, the accumulation of phenolate compounds eventually reached nearly equal levels (Fig. 19). At 25 C hydroxamate compounds were detected at both iron-deficient concentrations, but only after 24 hours of incubation when the cultures with 4.25 x 10^{-7} M iron had reached populations of 5 x 10^{8} CFU/ml four hours earlier, and in the case of cultures containing 5.25 x 10^{-7} M iron, a minimum of eight hours earlier. The growth patterns of iron-sufficient and iron-deficient cultures at 37 C were similar. At 37 C the levels of phenolate compounds accumulated were higher than at 25 C, while hydroxamate compounds were detected after twelve hours of incubation at 37 C; the latter eventually reached a level not significantly higher than those

Figure 16.--Growth curves and phenolate accumulation curves of iron-sufficient and iron-deficient dextrose broth cultures of Sal. enteriditis at 25 C and 37 C. Symbols for growth curves: ●, 1 x 10^{-4} M Fe culture at 37 C; ●, 5.25 x 10^{-7} M Fe culture at 37 C; ○, 1 x 10^{-4} M Fe culture at 25 C; ●, 5.25 x 10^{-7} M Fe culture at 25 C; and ●, 4.25 x 10^{-7} M Fe culture at 25 C. Symbols for phenolate accumulation curves: ▲, 5.25 x 10^{-7} M Fe culture at 37 C; ▼, 5.25 x 10^{-7} M Fe culture at 25 C; and ▶, 4.25 x 10^{-7} M Fe culture at 25 C.


1 x 10^10
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Colony Forming Units/ml
1 x 10^0
5 x 10^-1
1 x 10^-1
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DHB Equivalents in µg/10^8 CFU
4
8
12
16
20
24
Hours of Incubation

Figure 16

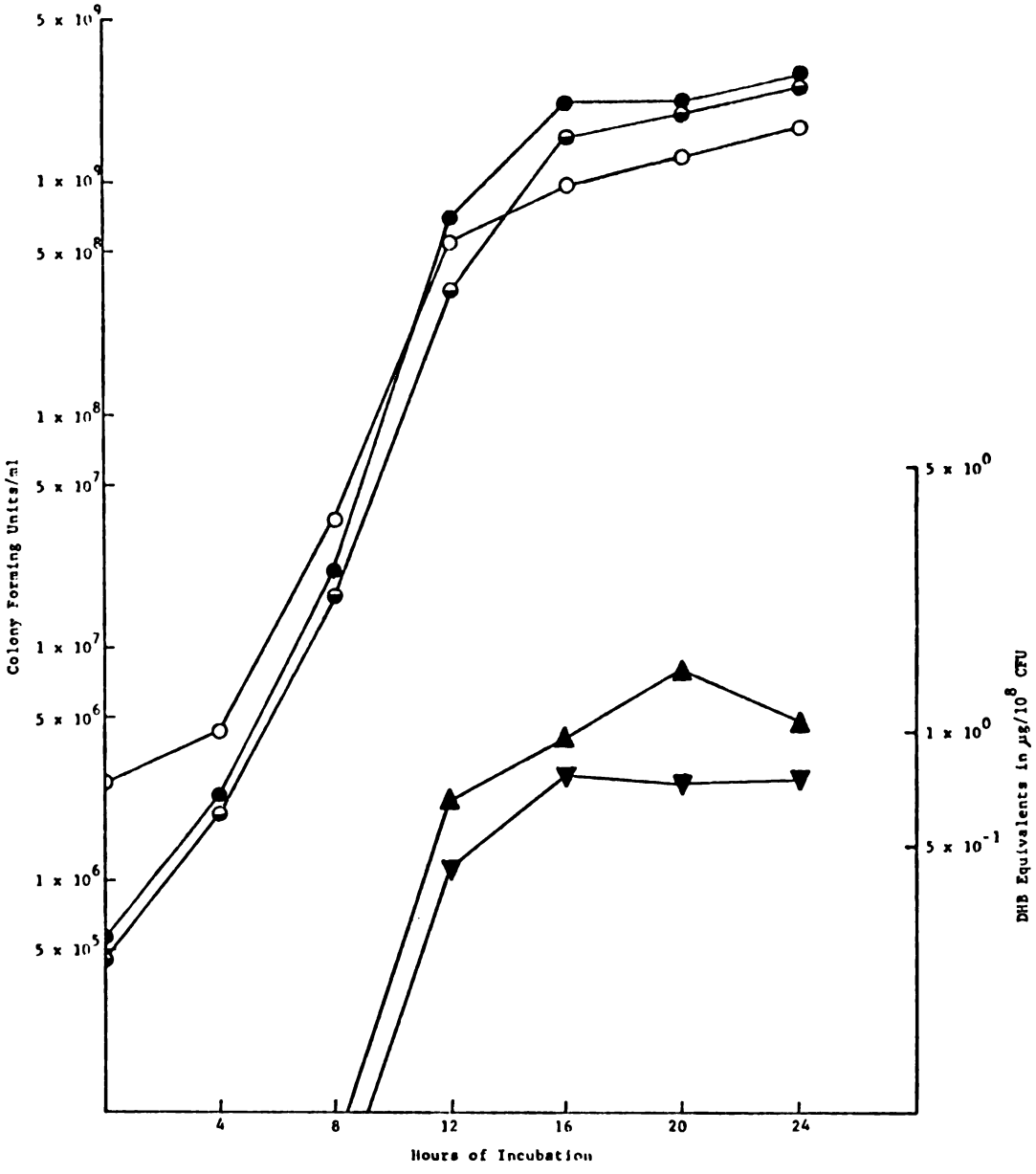


Figure 17.--Growth curves and phenolate accumulation curves of iron-sufficient and iron-deficient dextrose broth cultures of Ser. marcescens at 25 C. Symbols for growth curves: -●-, 1 x 10^{-4} M Fe culture; -⊖-, 5.25 x 10^{-7} M Fe culture; and -○-, 4.25 x 10^{-7} M Fe culture. Symbols for phenolate accumulation curves: -▲-, 5.25 x 10^{-7} M Fe culture; and -▼-, 4.25 x 10^{-7} M Fe culture.

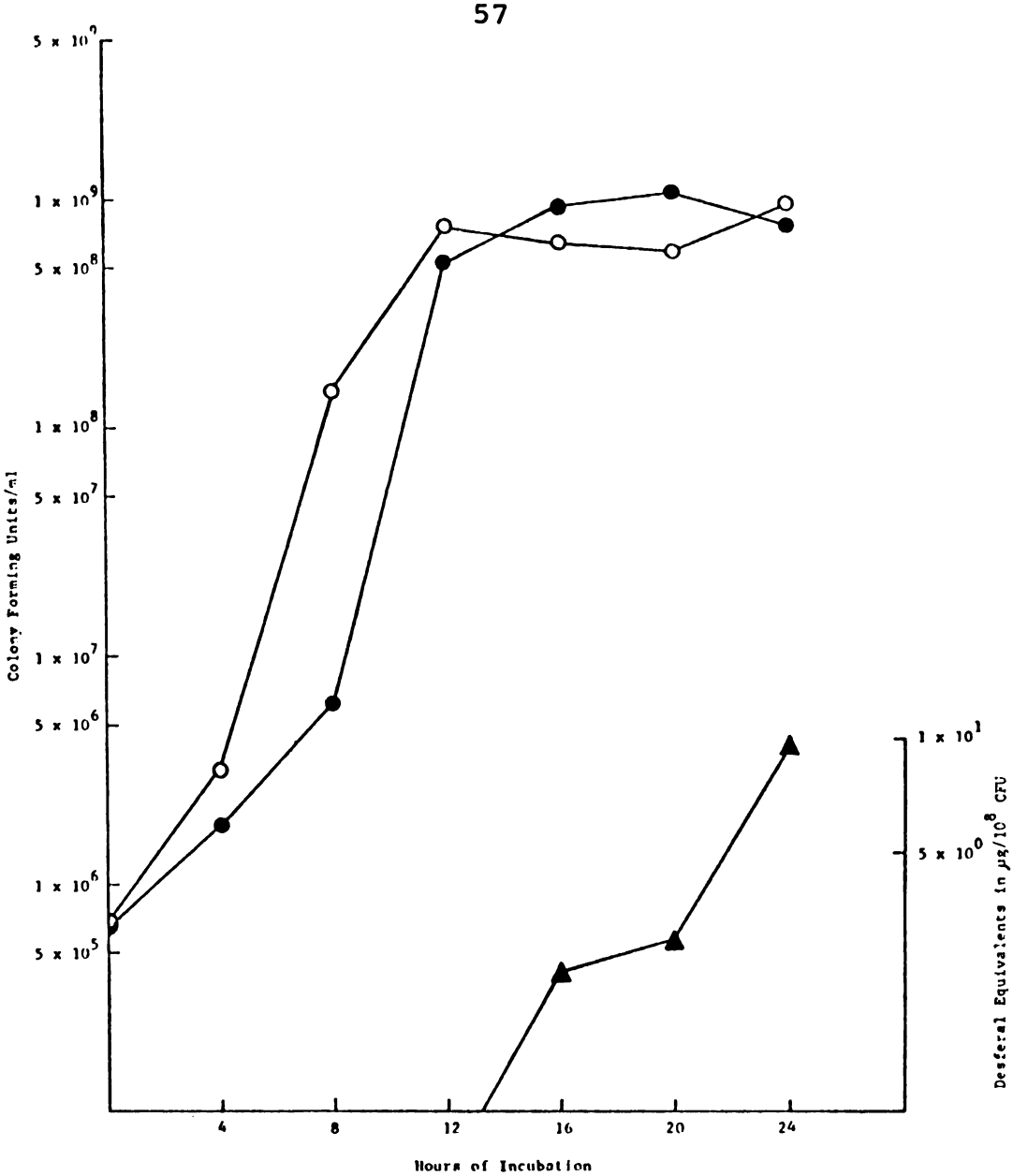
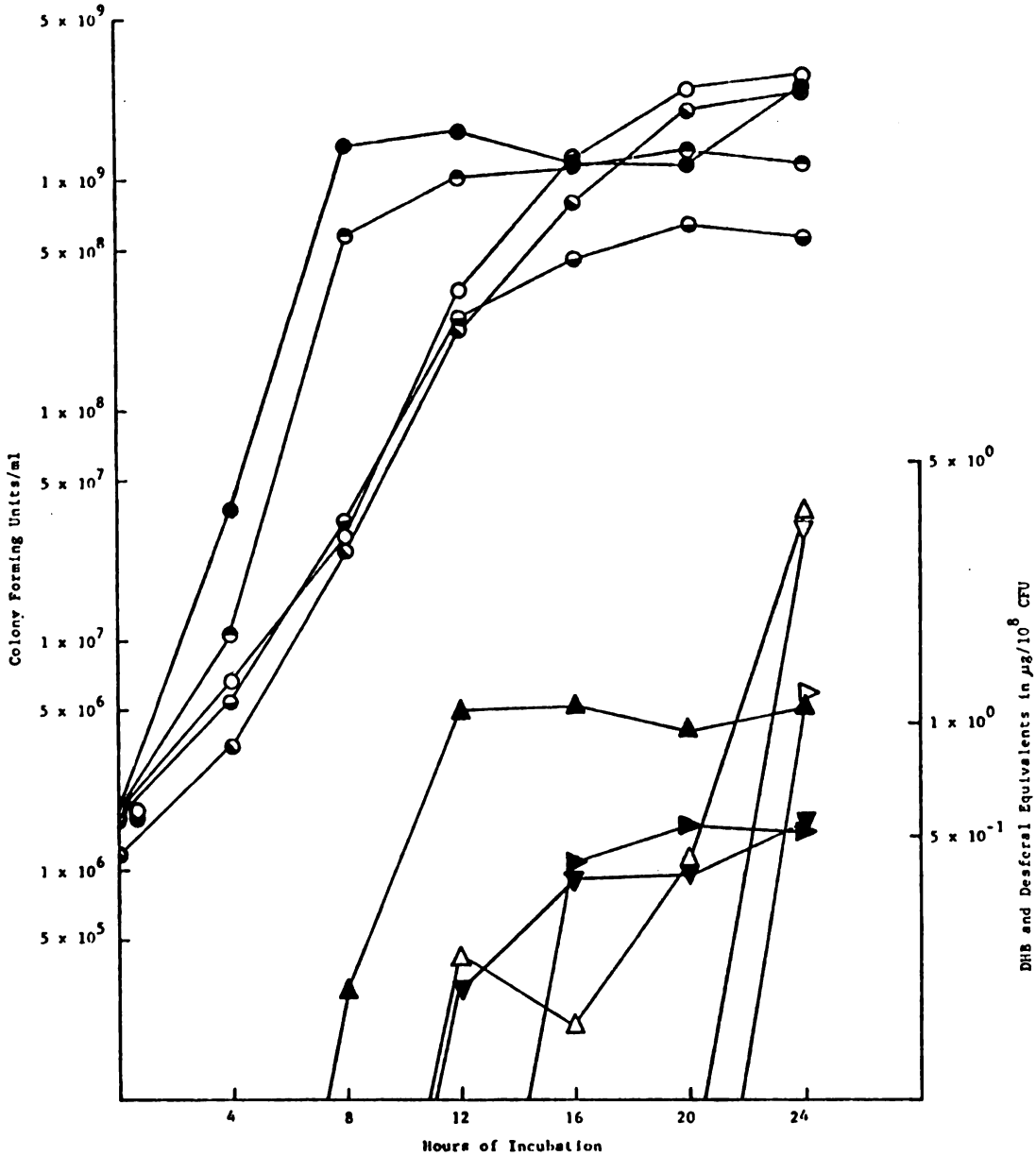


Figure 18.--Growth curves and hydroxamate accumulation curves of iron-sufficient and iron-deficient dextrose broth cultures of Ps. aeruginosa at 37 C. Symbols: -o-, growth curve of 1 x 10^{-4} M Fe culture; -●-, growth curve of 5.25 x 10^{-7} M Fe culture; and -▲-, hydroxamate accumulation curve of 5.25 x 10^{-7} M Fe culture.

Figure 19.--Growth, phenolate, and hydroxamate curves of iron-sufficient and iron-deficient dextrose broth cultures of Sh. sonnei at 25 C and 37 C. Symbols for growth curves: ●, 1 x 10^{-4} M Fe culture at 37 C; ⊖, 5.25 x 10^{-7} M Fe culture at 37 C; ⊖, 1 x 10^{-4} M Fe culture at 25 C; ○, 5.25 x 10^{-7} M Fe culture at 25 C; and ⊖, 4.25 x 10^{-7} M Fe culture at 25 C. Symbols for phenolate accumulation curves: ▲, 5.25 x 10^{-7} M Fe culture at 37 C; ▼, 5.25 x 10^{-7} M Fe culture at 25 C; and ▶, 4.25 x 10^{-7} M Fe culture at 25 C. Symbols for hydroxamate accumulation curves: △, 5.25 x 10^{-7} M Fe culture at 37 C; ▷, 5.25 x 10^{-7} M Fe culture at 25 C; and ▽, 4.25 x 10^{-7} M Fe culture at 25 C.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Colony Forming Units/ml
5 x 10^0
1 x 10^0
5 x 10^-1
DHB and Desferal Equivalents in μg/10^8 CFU
4
8
12
16
20
24
Hours of Incubation

Figure 19

detected at 25 C (Fig. 19).

A comparison of phenolate accumulation curves (Fig. 15, 16, 17, and 19) shows that at 25 C K. pneumoniae reaches the highest level of accumulation followed by Ser. marcescens, Sh. sonnei, and Sal. enteriditis. At 37 C the differences between K. pneumoniae, Sh. sonnei, and Sal. enteriditis are more pronounced while maintaining the same order of phenolate concentrations observed at 25 C. A comparison of the hydroxamate accumulation curves (Fig. 18 and Fig. 19) indicates that at 37 C hydroxamate compounds are detected four hours after populations of both Sh. sonnei and Ps. aeruginosa had reached 5 x 10^8 CFU/ml, or greater. The level of hydroxamate compounds accumulated by Ps. aeruginosa is clearly larger than that accumulated by Sh. sonnei.

Growth Study in Deferri-penassay Broth

Only Sh. sonnei (incubated at 30 C for three days) was examined for possible ITC synthesis in the deferri-penassay broth (Fig. 20). The growth curves of iron-sufficient and iron-deficient cultures were nearly identical as were the hydroxamate and phenolate accumulation curves of the two iron-deficient cultures. Phenolate compounds were detected six hours after the iron-deficient cultures had attained populations of 2 x 10^8 CFU/ml or greater, while hydroxamate compounds were detected two hours after the appearance of phenolate compounds. Hydroxamate compounds quickly reached much greater concentrations than those attained by phenolate compounds (Fig. 20). By the third day of incubation, there was an unexplained disappearance of the phenolate compounds (Fig. 20).

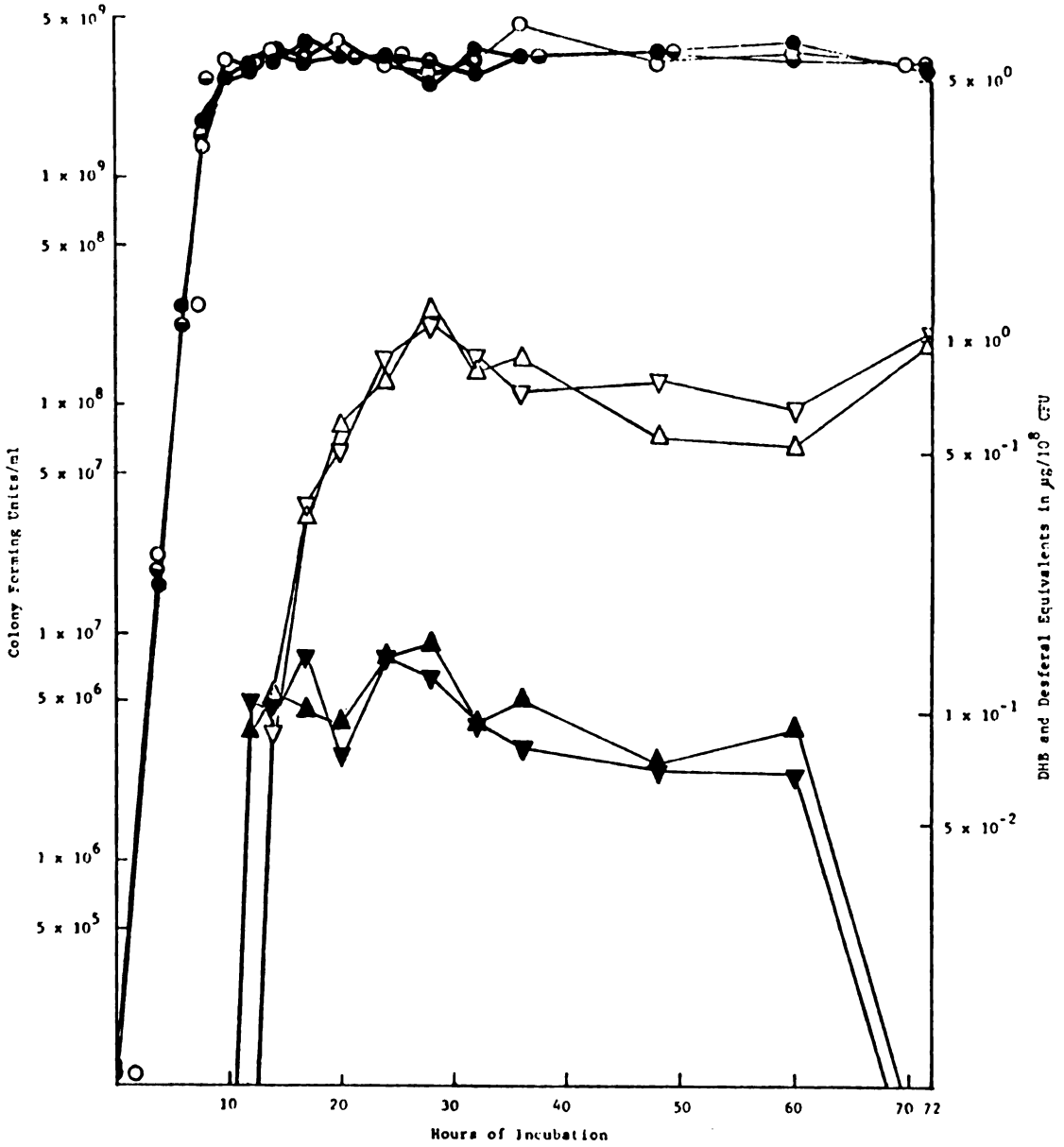

Figure 20.--Growth, phenolate, and hydroxamate curves of iron-sufficient and iron-deficient penassay broth cultures of Sh. sonnei at 30 C. Symbols for growth curves: ⊸, 1 x 10^{-4} M Fe culture; ⊸, 4.68 x 10^{-7} M Fe culture; and ⊸, 5.6 x 10^{-7} M OP culture. Symbols for phenolate accumulation curves: ⊸, 4.68 x 10^{-7} M Fe culture; and ⊸, 5.6 x 10^{-7} M OP culture. Symbols for hydroxamate accumulation curves: ⊸, 4.68 x 10^{-7} M Fe culture; ⊸, 5.6 x 10^{-7} M OP culture.

DISCUSSION

The simultaneous production of phenolate and/or hydroxamate compounds and the occurrence of iron-chelating compounds during iron deficiency suggests that the phenolate and hydroxamate compounds synthesized by the bacteria studied herein are ITC's. Definite proof of their involvement in the transport of iron requires that these compounds first be isolated and characterized. Subsequent studies employing ^{3}H-labelled compounds complexed with ^{59}Fe will clarify their role in the uptake of iron by iron-deficient cells.

Previous studies have shown that increasing amounts of iron decrease ITC production and will completely halt ITC synthesis at concentrations above 10^{-4} M to 10^{-5} M (Young and Gibson, 1969; Lankford, 1973; Brot et al., 1966; Emery, 1965; Emery, 1971b). Only in the case of Sh. sonnei were any differences detected between the two iron-deficient cultures (4.25×10^{-7} M iron and 5.25×10^{-7} M iron). This result is not unexpected because of the minute difference between the two iron-deficient concentrations. Later investigations will use iron concentrations of approximately 10^{-6} M, 10^{-7} M, and 10^{-8} M.

Decreased synthesis of ITC's at elevated temperatures has been noted only in a fluorescent pseudomonad and a number of species of Salmonella (Garibaldi, 1971; 1972; personal communication). In the case of Sal. typhimurium Tm-1 synthesis was decreased at 36.9 C and no growth was observed at 40.3 C (Garibaldi, 1972). Garibaldi (personal communication) also observed decreases in synthesis of phenolates by a number of species of Salmonella when the incubation temperature was raised from 30.6 C to 34 C and 38.6 C. This effect was not observed in any of the bacteria employed in this study. On the contrary K.

pneumoniae and Sh. sonnei accumulated higher levels of phenolate compounds at 37 C than at 25 C. This increased accumulation may be partially due to the decreased generation time at 37 C and consequently the higher total number of organisms which have grown, synthesized phenolate compounds, and died. This may be an important factor in the observed slight increase in phenolate accumulation at 37 C by Sh. sonnei. However, its importance in the case of K. pneumoniae is probably negligible in comparison to the large increases observed at 37 C. These throw into doubt the hypothesis that decreased synthesis of bacterial ITC's at elevated temperatures is a generally occurring phenomenon.

With K. pneumoniae, the increased accumulation of the phenolate compound(s) did not allow longevity at 37 C comparable to the culture with 10^{-4} M iron. After sixteen hours of incubation at 37 C, the iron-deficient culture had begun to show a significant death phase whereas the iron-sufficient culture remained in stationary phase. The increased phenolate accumulation observed at 37 C compared to 25 C could have been partially due to increased synthesis when the available iron had become limiting enough to initiate a death phase.

In the deferri-penassay broth, the observed lack of synthesis of either phenolate or hydroxamate compounds, coupled with good growth by species of Proteus, Yersinia, and Staphylococcus may be explained by two possibilities. Firstly, these species may synthesize an ITC which does not contain either the phenolate or hydroxamate moiety. Another explanation might be that the deferri-penassay broth contained sufficient contaminating iron complexed with proteins to repress the synthesis of any ITC's. The observation by Knüsel et al. (1969) that the growth of Staph. aureus is inhibited by some sideromycins suggests that

this organism possesses the ability to transport hydroxamate compounds if not to synthesize them.

The deferri-dextrose broth is a minimal medium which will not support the growth of many bacterial species. For example, Proteus, Staphylococcus and Yersinia species would not grow in the ferri-dextrose broth even with the addition of various cofactors and vitamins. Since ITC synthesis is linked to amino acid biosynthesis, hydroxamate and phenolate synthesis might be increased by growth in a more complex deferri-broth containing exogenous amino acid components. In any case a complex deferri-broth will be required to continue studies with fastidious organisms. A number of alternate procedures are available for the extraction of contaminating iron from more complex media (Donald et al., 1952; Wawszkiewicz et al., 1971). Passage of penassay broth through a Chelex 100 column resulted in a residual iron concentration in this medium of approximately 10^{-8} M (Wawszkiewicz, personal communication). Chelex 100 treatment of media or chelation of contaminating iron by addition of transferrin would be preferred alternatives to the method of Waring and Werkman (1942) used in this study.

A successful pathogen will mimic as many of the biological characteristics of its host as possible, thereby reducing the number of specific bacterial metabolic reactions which would be susceptible to attack by host defense factors. Indeed, the major goal in therapeutic medicine is to achieve maximal damage to the pathogen and none to the host. To achieve this end, one needs to identify a unique metabolic reaction, absolutely essential for the survival of the pathogen, which is absent in or of minimal importance to the host.

The extreme importance of iron in the metabolism of microorganisms and mammals is evidenced by the extra-

ordinary iron chelating and transport systems which both groups possess. Whereas microbial iron transport systems utilize low molecular weight organic compounds for the sequestering of iron, such sequestering compounds in mammalian systems are large proteins such as transferrin, lactoferrin, and ferritin. The *in vivo* survival of several pathogens has been correlated with their capacity for ITC synthesis (Kochan, 1973; Lankford, 1973). Extension of this observation to include a wide variety of pathogens will serve to define a novel area of bacterial metabolism critical in establishing an infection. Exploiting the vast differences in the chemical natures of microbial and mammalian iron sequestering compounds as well as their distinct biosynthetic pathways and control mechanisms may be an approach to an antimicrobial therapy with practical applications.

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