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COMPARATIVE STUDY OF EFFECTIVENESS BETWEEN *PSEUDOMONAS AERUGINOSA* SIDEROPHORES AND DESFERAL IN IRON ACQUISITION

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Transfusion therapy of thalassemia major patients leads to progressive iron overload of vital organs. This ultimately fatal condition can only be treated by continuous chelating therapy with drugs, which increase the excretion of excess iron via the kidneys. From several hundred potential chelating agents tested only desferioxamine B (®Desferal) has proved to be safe and clinically effective. It is produced by fermentation using *Streptomyces pilosus* and is isolated by an elaborate and expensive recovery process. The objective of this study was to compare the effectiveness between siderophores produced from *Pseudomonas aeruginosa* and Desferal in iron acquisition in vitro.

Pyoverdin and pyochelin were extracted and purified from a culture of *P. aeruginosa* standard strain PAO1.The effectiveness in iron mobilization of the siderophores and Desferal were studied in Tris buffer pH 6.0, 6.5, 7.0 and 7.4 after 48 hours of incubation at 4 °C.

The results showed the siderophores and Desferal were capable of mobilizing ^{59}Fe from [^{59}Fe]-ferritin but ineffective in iron mobilization from [^{59}Fe]-transferrin.

The combination of pyoverdin and pyochelin at the concentration of 10 μg/ml each was effective in iron mobilization equal to that of pyoverdin alone. When compared with Desferal at equal molar ratio, Desferal was more effective than pyoverdin in iron mobilization from [^{59}Fe]-ferritin. On the other hand, pyochelin was the least effective siderophore. At various concentrations of pyoverdin and pyochelin tested in iron mobilization, the concentration of pyoverdin, which appeared to be effective in the test system, was more than 3 μg/ml and more than 5 μg/ml for pyochelin. The most effective concentrations of pyoverdin and pyochelin are 10 μ g/ml. In growth assay, pyoverdin, pyochelin and Desferal were effective in promoting growth of *P. aeruginosa* standard strain PAO1 in glucose minimum medium (GMM) or in GMM containing 10% heat inactivated human serum.

The data of positive hemoculture of thalassemia patients was also studied. The positive hemoculture of thalassemia patients admitted to Siriraj Hospital during 1994-1998 was 14.84%.

The siderophore produced by *P. aeruginosa* i.e. pyoverdin might be used in place of Desferal in the future upon more advanced research. The development of this siderophore might allow an alternative therapeutic agent for thalassemia patients, who need red cell transfusions. Which can be given to thalassemia patients orally and mustn't be given only by intravenous or subcutaneous infusion as Desferal.

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สุมลรัตน์ มีจั่นเพ็ชร : การศึกษาเปรียบเทียบประสิทธิภาพระหว่างซิเดอโรฟอร์ที่ผลิตขึ้นเองจาก *Pseudomonas aeruginosa* กับ Desferal ในการดึงเหล็ก (COMPARATIVE STUDY OF EFFECTIVENESS BETWEEN *PSEUDOMONAS AERUGINOSA* SIDEROPHORES AND DESFERAL IN IRON ACQUISITION) คณะกรรมการควบคุมวิทยานิพนธ์: สมพร ศรีเฟื่องฟุ้ง, Ph.D., โกวิทย์ พัฒนปัญญาสัตย์, Ph.D.,มลุลี ตัณฑวิรุฬห์, M.S., 106 หน้า ISBN 974-663-845-9

ภาวะเหล็กเกินเป็นภาวะแทรกซ้อนที่พบเสมอในผู้ป่วยธาลัสซีเมียชนิดรุนแรงทำให้ผู้ป่วยถึงแก่กรรมก่อนวัย ภาวะเหล็กเกินนี้เกิดจากได้รับเลือดที่ถ่ายให้เพื่อรักษาความซีด เหล็กที่เกินนี้จะไปสะสมตามอวัยวะต่างๆทำให้เกิดพยาธิสภาพต่อเนื้อเยื่อนั้นๆ วิธีแก้ภาวะเหล็กเกินคือการให้สารที่สามารถดึงเหล็กออกจากร่างกาย โดยสารเหล่านี้สามารถสร้างได้โดยจุลชีพ เช่น แบคทีเรีย รา เรียกว่า ซิเดอโรฟอร์ ซึ่งมีเพียงชนิดเดียวที่สามารถนำมาใช้กับผู้ป่วยธาลัสซีเมีย คือ Desferioxamine B ซึ่งผลิตจาก *Streptomyces pilosus* (ชื่อทางการค้าคือ Desferal) งานวิจัยนี้ได้ทำการศึกษาเปรียบเทียบประสิทธิภาพระหว่างซิเดอโรฟอร์ที่ผลิตขึ้นเองจาก *Pseudomonas aeruginosa* กับ Desferal ในการดึงเหล็กจากเฟอริทินและทรานสเฟอรินในหลอดทดลอง โดยการสกัด pyoverdin และ pyochelin จาก *P. aeruginosa* ซึ่งพบว่าซิเดอโรฟอร์ที่ความเข้มข้น 10 µg/ml ที่ผลิตจาก *Pseudomonas aeruginosa* เองนี้มีประสิทธิภาพในการดึงเหล็ก (Fe^{59}) ออกจากเฟอริทิน ใน Tris buffer pH 6.0, 6.5, 7.0 และ 7.4 หลังจาก incubate ที่ 4°ซ 48 ชั่วโมง ได้ตามลำดับ แต่ไม่มีประสิทธิภาพในการดึงเหล็กออกจากทรานสเฟอริน

ผลการศึกษาประสิทธิภาพของ pyoverdin และ pyochelin ในการดึงเหล็กเมื่อเปรียบเทียบกับ Desferal ยังพบว่า ประสิทธิภาพในการดึงเหล็กของ pyoverdin และ pyochelin รวมกันที่ความเข้มข้น 10 µg/ml นั้น ให้ผลใกล้เคียงกับเมื่อใช้ pyoverdin เพียงอย่างเดียว สำหรับผลการศึกษาประสิทธิภาพของซิเดอโรฟอร์และ Desferal เมื่อใช้ Molar ratio ระหว่าง chelator และ iron ที่เท่ากันนั้นพบว่า pyoverdin มีประสิทธิภาพในการดึงเหล็กใกล้เคียงกับ Desferal ส่วน pyochelin มีประสิทธิภาพน้อยกว่า และผลการศึกษาเกี่ยวกับความเข้มข้นของ pyoverdin และ pyochelin ที่เหมาะสมสำหรับประสิทธิภาพในการดึงเหล็กนั้น คือความเข้มข้นที่มากกว่า 3 µg/ml สำหรับ pyoverdin และ มากกว่า 5 µg/ml สำหรับ pyochelin แต่ความเข้มข้นที่เหมาะสมของซิเดอโรฟอร์ทั้ง 2 ชนิดนี้ คือ 10 µg/ml นอกจากนี้ pyoverdin, pyochelin และ Desferal ยังสามารถกระตุ้นการเจริญเติบโตของ *P. aeruginosa* standard strain PAO1 ใน glucose minimal medium (GMM) ได้ด้วย ส่วน pyoverdin และ Desferal ยังสามารถกระตุ้นการเจริญของ *P.aeruginosa* ในภาวะที่มี human transferrin ได้ และศึกษาข้อมูลเกี่ยวกับการติดเชื้อแบคทีเรียในเลือดของผู้ป่วยธาลัสซีเมียที่เข้ารับการรักษาในโรงพยาบาลศิริราช พบว่าการติดเชื้อในเลือดของผู้ป่วยธาลัสซีเมียของโรงยาบาลศิริราชจากปีคศ. 1994-1998 พบว่ามีการติดเชื้อร้อยละ 14.84%

จากการค้นพบดังกล่าว pyoverdin และ อนุพันธ์น่าจะนำมาใช้ทดแทน Desferal ได้ต่อไปในอนาคต ถ้ามีการทดลองในขั้นต่อไปที่มากกว่านี้ การพัฒนาซิเดอโรฟอร์ชนิดนี้อาจได้ยาตัวใหม่ที่นำมาใช้ในผู้ป่วยธาลัสซีเมียที่ต้องมีการถ่ายเลือด โดยทางการกิน ไม่ต้องลำบากยุ่งยากในการให้ทางเส้นเลือดหรือใต้ผิวหนังเหมือน Desferal

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28. Effects of added siderophores or Desferal on growth stimulation in glucose minimal medium. 81

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
° C	Degree Celsius
CAA broth	Casamino acid broth
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
g	gram
GMM	Glucose minimal medium
min	Minute
mg	Milligram (10^{-3} gram)
ml	Milliliter (10^{-3} liter)
mM	Millimolar (10^{-3} molar)
μg	Microgram (10^{-6} gram)
μl	Microliter (10^{-6} liter)
M.W.	Molecular weight
N	Normal
nm	Nanometer (10^{-9} meter)
nM	Nanomolar (10^{-9} molar)
OMPs	Outer membrane proteins
UV	Ultraviolet
rpm	Round per minute

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CHAPTER I

INTRODUCTION

Lifelong transfusion therapy of thalassemia major patients leads to progressive iron overload of vital organs. This ultimately fatal condition can only be treated by continuous chelation therapy with drugs, which increase the excretion of excess iron via the kidneys. From several hundred potential chelating agents tested only desferioxamine B (®Desferal) has proved to be safe and clinically effective. It is produced by fermentation using the microorganism *Streptomyces pilosus* and is isolated by an elaborate and expensive recovery process. The complex mode of production and the need for frequent administration in gram amounts by slow infusion all contribute to the considerable overall cost of treatment (1).

Thalassemia is a group of disorders, each of which results from an inherited abnormality of globin production. These conditions form part of the spectrum of disorders known collectively as the hemoglobinopathies. Hemoglobin is made up of four polypeptide chains called globin chains which are held together by noncovalent interactions. There are four binding sites for oxygen on the hemoglobin molecule because each chain contains one heme group. Hemoglobin A, the principal hemoglobin in adults, consists of two globin chains of one kind, called α chains, and two of another kind, called β chains. Sickle cell anemia, results from an inherited structural alteration in one of the globin chains. Although such abnormal hemoglobins may be synthesized less efficiently or broken down more rapidly than normal adult

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hemoglobin, the associated clinical abnormalities result from the physical properties of the abnormal hemoglobin. The other major subdivision of the hemoglobinopathies, the thalassemias, is constituted by inherited defects in the rate of synthesis of one or more of the globin chains. This causes ineffective erythropoiesis, hemolysis, and a variable degree of anemia (2).

Thalassemia occurs widely throughout Africa, the Mediterranean countries, the Middle East and Southeast Asia including Thailand (2). Now, the thalassemia is one of important problems in the world such as health, social and economic problems. Incidence rate of thalassemia in Thai people is more than 1 percent of the population and increases when includes thalassemia carriers. Thalassemia is a genetic disease, which is one of the important health problems in Thailand.

The only forms of treatment available for thalassemic children are regular blood transfusion, iron-chelation therapy in an attempt to prevent iron overload, the use of splenectomy in cases complicated by hyperspleenism, and a general pediatric care. Bone marrow transplantation may have a limited role in highly selected case (2). Since every child maintained on a high-transfusion regimen will ultimately develop iron overload and die of siderosis of the myocardium. It is vital, where possible, to start these children on a program of iron chelation sometimes within the first 5 years of life (3). Despite extensive searches for an oral chelating agent, desferrioxamine is currently the only drug of real value for the treatment of thalassemia. It is best administered by a 12 hours-overnight infusion into the subcutaneous tissues of the anterior abdominal wall (4,5). Therefore, desferioxamine suffers from the disadvantage that it cannot be given orally and requires enough iron excretion to keep pace with the transfusion regimens when given either subcutaneously or intravenously

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over 8-12 hours several times a week. For this reason, many patients find it difficult to comply with the treatment and some even stop taking the drug altogether and so develop the complications of iron overload. Therefore it is no doubt that an orally active chelating agent is needed to treat patients on life-long transfusion programs such as those with thalassemia major, other severe congenital or acquired anemias as well as some patients with sideroblastic anemias, all conditions for which desferioxamine is now used. The development of an oral iron chelator might also allow the extension of the therapeutic use of red cell transfusions in sickle cell anemia (6).

Desferal (trade name for desferioxamine produced by Ciba-Geigy Co., Ltd.), the only generally used iron chelator on the market, meets most of the above requirements and is a highly effective drug with low toxicity when used with care. The disadvantage is lacking oral activity. It is a bacterial siderophore, delivering iron to some bacteria and enhancing their pathogenicity. Although it can penetrate hepatocytes, it is slow at removing iron from ferritin, and due to its hydrophilic properties may penetrate hepatocyte lysosomes only slowly. This property also limits access to chelatable iron. Desferal is an expensive drug and although it is available to most patients in the Western world, only a small proportion of patients in poorer countries is treated with it. However this drug remains the important compound by which new iron chelators must be compared both in terms of iron excretion and toxicity (6).

Development of easier forms of administration for Desferal and the search for cheaper, orally active drugs have been prime targets of many research groups. Several interesting candidates have been described and investigated further. None has yet been shown to be effective in man and tolerated as well as Desferal (1).

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The objective of this study was to compare the effectiveness between siderophores produced from *Pseudomonas aeruginosa* and Desferal in iron acquisition.

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CHAPTER II

LITERATURE REVIEW

Pseudomonas aeruginosa is free living bacteria, widely distributed in nature, plants, soil and water. *P. aeruginosa* sometimes colonizes human and is the major human pathogen of the group. It is invasive and toxigenic, producing infection inpatients with abnormal host defenses and is an important nosocomial pathogen (7). It has been classified in the order of Pseudomonadales and the family of Pseudomonadaceae (8). *P. aeruginosa* was first named by Schroeter in 1872 (9). The term "pseudo", presumably the variable cellular morphology of the organism either curved or straight rods gave early observers a sense of spuriousness about their shape and thus account for the prefix "pseudo". The term "monad" is derived from the Greek word meaning "unit" (10). In the past, the basic etiologic unit causing infection was refers to as a monad (11). The designation "aeruginosa" is clearly defined from the Latin word for copper rust (12), and hence green, and obviously refers to the distinguishing pigment of the species.

General Characteristics of *P. aeruginosa*

1. Morphology

P. aeruginosa is short, unicellular, straight or slightly curved, gram-negative rods that measure 0.5-1 μm by 1.5 to 4 μm. It does not form spore. When motile, it has single polar flagellum. The flagellum is unsheathed in this species. With electron microscopy, it is evident that this bacterium has a typical multi-layered cell wall of

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gram-negative bacteria, similar in appearance to the cell wall of *Enterobacteriaceae*. In addition, with proper fixation, outer slime layer can be detected surrounding individual cells (13,14).

2. Culture Appearance

P. aeruginosa is an obligate aerobe that grows readily on many types of culture media, sometimes producing a sweet or grape-like odor. Some strains hemolyze blood (7). Many strains of *P. aeruginosa* grow well on simple nutrient agar and on common selective media such as EMB and MacConkey agars. The colonial morphology of *P. aeruginosa* is quite diverse. There are six colony types on nutrient agar: 1) Flat, with irregular edges, a grey-green metal sheen; 2) Raised, smooth, with entire edges resembling *Enterobacteriaceae* colony; 3) Raised, rough; 4) Rugous; 5) Dwarf; and 6) Mucoid. Type 1 and 2 account for approximately 90 percent of isolates. Mucoid strains do not usually maintain this form when passed on culture media (14).

3. Growth Characteristics

P. aeruginosa is a nonfermentative aerobe that derives its energy form oxidation rather than fermentation of carbohydrates. Although able to use more than 75 different organic compounds, it can grow on media supplying only acetate for carbon and ammonium sulfate for nitrogen. This organism grows well at 25°C to 37°C, but can grow slowly or at least survive at higher and lower temperatures. The ability to grow at 42°C distinguishes it from many other *Pseudomonas* species. In addition to its nutritional versatility, *P. aeruginosa* resists high concentrations of salt, dyes, weak antiseptics, and many commonly used antibiotics. These properties help explain its ubiquitous nature and contribute to its prominence as a cause of nosocomial infections (7,15).

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4. Pigment Formation

Most strains of *P. aeruginosa* produce one or more pigments. Under suitable conditions, it produces water or chloroform soluble fluorescent and nonfluorescent pigments that diffuse into agar. Pyocyanin (a phenazine pigment) is a distinctive blue pigment made by most strains of *P. aeruginosa*. It is not fluorescent. This pigment is responsible for the characteristic blue pus seen in wounds infected with this organism. Pyocyanin is a bacteriocin that may allow P. *aeruginosa* to exist in nature. It may play a role in nutrition by functioning as a means of acquiring inorganic phosphate. Pyoverdin and pyochelin are yellow-green, fluorescent pigments made by most strains of *P. aeruginosa* and they may play a role in nutrition by accumulating iron. Additional work is needed to clarify the role of these pigments in *P. aeruginosa* infections. Strains of *P. aeruginosa* that make pyoverdin, pyochelin and pyocyanin are common. Approximately 5 percent of strains of *P. aeruginosa* make rust-red pigments (pyorubins) or black pigments (pyomelanin) (7,13,14,15). The important requirement for fluorescein production is that the content of free iron in the medium should be restricted.

5. Metabolic and Biochemical Characteristics.

P. aeruginosa is a strict aerobe. It derives its energy from oxidation and does not ferment sugars. However, it can oxidize glucose. The growth requirement for *P. aeruginosa* is extremely simple. It can grow in aqueous solution of mineral salts with ammonium ions as the only nitrogen source and can use a variety of simple organic compounds as energy sources. It can utilize carbohydrates, alcohol, saturated and unsaturated fatty acids, amino acids, amines and amides. The ability to metabolize such a wide variety of substrates implies many specialized catabolic pathways, which

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are regulated in response to environmental demand (14). According to these properties, it explains the ability of *P. aeruginosa* to survive in environment like an excellent "scavenger" of essential nutrients. It can grow in distilled water and hospital environment (16,17).

Most *P. aeruginosa* strains are identified on the basis of the characteristic grape-like odor of aminoacetophenone, colonial morphology and production of pyocyanin (a blue, water-soluble, nonfluorescent, phenazine pigment) that is soluble in chloroform. *P. aeruginosa* produces pyocyanin and the yellowish pyoverdins, often impart a greenish color to culture media (7). The characteristics necessary for identification of most *P. aeruginosa* strains are shown in Table 1 (7).

6. Antigenic Structure

The somatic or O antigen has been used to group various strains for epidemiological purposes. Serological typing of the O antigen is less cumbersome and less variable system of strain characterization than is pyocin or phage typing, although the latter systems may be necessary for complete characterization of strains isolated during outbreaks (13). Various vaccines using commonly isolated O types have been developed for prevention of *P. aeruginosa* infections.

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Table 1. Characteristics for identification of *P. aeruginosa* (84 strains) (7).

Characteristics	Reaction	% Positive
Polar monotrichous, fewer than three flagella per pole	+	97
Motility	+	97
OF* glucose OF, oxidation	+	98
OF* lactose, sucrose media, acid	-	0
Indophenol oxidase	+	100
Pyoverdin	+ or -	71
L-Arginine dihydrolase	+	99
L-Lysine decarboxylase	-	0
L-Ornithine decarboxylase	-	0
Hydrogen sulfide production	-	0
Gas from nitrate	+ or -	61
Growth at 42°C	+	100

* OF = Oxidation-Fermentation

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7. Therapy

Third generation cephalosporins are used primarily against gram-negative rods e.g., *P. aeruginosa*. The drugs function by interfering with cell wall synthesis in bacteria.

Polymyxin B is effective against *P. aeruginosa* and other gram-negative bacilli, particularly those causing superficial infections in wounds, abrasions and burns. Polymyxin B functions by injuring bacterial membrane (18).

8. Resistance

P. aeruginosa is one of the most adaptable vegetative bacteria known. When adequate moisture is provided, it can survive with only minimum nutrients. It can be isolated from a number of sites in the hospital environment e.g., respiratory care equipment, baths, water faucets, cold water, humidifiers, bed pans, and floors. *P. aeruginosa* is very resistant to chemical disinfection and has even grown in certain types of quaternary ammonium compounds, hexachlorophene, soaps and iodine solutions. Phenolics and β–gluteraldehyde usually are effective disinfectants for *Pseudomonas*. Boiling kills the organism, as does desiccation. Most commonly used antibiotics, particularly beta-lactam antibiotics e.g. penicillins are not effective against *Pseudomonas* (13). Thus *P. aeruginosa* is highly resistant to most antibiotic used nowadays.

9. Genetics

Gene transfer between *P. aeruginosa* strains can occur through conjugation, transduction and transformation. Serologic typing of the somatic (O) antigen, phage typing and pyocin (bacteriocin) typing can detect strain differences. Pyocins are rod-shaped protein particles that resemble phage tail; they attach to specific receptors on

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the cell wall of susceptible bacteria causing bacterial lysis. Lysogeny is common and most strains are lysogenic for at least one bacteriophage. Comparisons of genetic maps of pseudomonads to that of the enteric bacteria demonstrate fundamental differences in the arrangement of functionally related genes. The gene arrangement of the pseudomonads is non-contiguous, whereas the enterics have a contiguous arrangement of similar genes. Furthermore, bacteriocinogenic determinants found in plasmids of the *Enterobacteriaceae* are inserted into the *Pseudomonas* chromosome (13).

10. Extracellular products associated with pathogenicity

Pseudomonads are notorious for their ability to synthesize and secrete a large number of extracellular products. Among the enzymes secreted by *P. aeruginosa* are fibrinolysin, collagenase, elastase, lecithinase, lipase, proteases and hemolysins. A leukocidin, an enterotoxin and two exotoxins, designated A and S is also produced (19).

Table 2 lists extracellular products of *P. aeruginosa*. The most important ones in pathogenesis are mentioned, along with products of importance to the organism in its natural habitat as follows.

10.1 The mucoid extracellular polysaccharide slime layer, identified in 1964 as sodium alginate, seems to act as an adherence factor. It may form a protective barrier around organisms, enabling them to multiply more quickly in environment. Some factors in the mucous secretions of cystic fibrosis patients induce production of this exopolysaccharide.

10.2 Proteases, including elastase, contribute to the invasiveness of the organism and to its ability to destroy tissue. One type of clinical manifestation,

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necrotic skin lesions called "ecthyma gangrenosum" is probably mediated in part by proteases.

10.3 Lipases and lecithinases also destroy tissue and blood cells, enhancing invasiveness and potentiating the inflammatory response.

10.4 Exotoxin A inhibits protein synthesis by blocking the activity of elongation factor 2 (EF-2), in a manner similar to that of diphtheria toxin. This important virulence factor, exclusive to *P. aeruginosa*, contributes to the high mortality associated with septicemia involving this species.

10.5 Bacteriocins. They have specific name as pyocins and sometimes are used in typing schemes for epidemiological purpose. Even though they are not acting as major virulence factors in human disease, they are important colonization resistance factors in the environment or host tissue. These enzymes may act to prevent colonization of the environment or infected sites with other less virulent bacteria (13,15,19).

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Table 2. Extracellular products of *P.aeruginosa* that are important in pathogenicity (20).

Product	Activity
Alginate	Increase viscosity of mucous secretions in lungs of cystic fibrosis patients and thus interfere with phagocytosis and other immune responses and decreases the ability of the patient to control the infection.
Cytotoxin (Leukocidin)	Acts on cell membranes of eukaryotic cells; inhibits and eventually destroys polymorphonuclear leukocytes; may induce capillary endothelial cell damage.
Elastase	May dissolve elastic lamina of blood vessels, leading to ecthyma gangrenosum. Also inactivates C3b and C5a complement components, inhibiting opsonization and elaboration of chemotactic factors, which serves to dampen the inflammatory response.
Exoenzyme S	Inhibits eukaryotic protein activity, contributing to tissue damage.
Exoenzyme A	Important toxin mediating local and systemic effects; acts in same manner as diphtheria toxin as a ribosyltransferase, inhibits EF-2 and thus protein synthesis in infected tissue, produces necrosis locally, and contributes to lethality in *Pseudomonas* sepsis.
Glycolipid hemolysin	Acts synergistically with phospholipase to break down tissue lipids and lecithin; mediates local necrosis and tissue invasion.
Phospholipase C	Destroys pulmonary surfactant, a lecithin containing lipoprotein that contributes to alveolar integrity, and destroys tissue lipids, contributing to destructive lesions and spread of the disease process.

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DESFERIOXAMINE B

Desferrioxamine B is marketed as the methanesulphonate salt under the brand name ®Desferal. Ciba-Geigy Co., Ltd. produces it by large-scale fermentation of *Streptomyces pilosus* (1). Chemically it belongs to the class of trihydroxamate siderophore.

Desferrioxamine is composed of one molecule of acetic acid, two molecules of succinic acid and three molecules of 1-amino-5-hydroxylaminopentane. The organic units of which desferrioxamine is built up are interlinked to form a chain, in which there are three hydroxamic acid groups inside, and one free amino group at the end. The free amino group accounts for the basic character of the compound, which enables it to form salts with organic and inorganic acids (Figure 1).

When a ferric ion comes into contact with desferrioxamine, the straight-chained molecule twines itself around the ion, becoming attached to the latter by means of its three-hydroxamic acid groups (Figure 2). A shell of organic material, which results in an iron complex of very great stability, thus surrounds the iron (21).

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1Mol	CH_3-COOH	Acetic acid
2Mol	$HOOC-(CH_2)_2-COOH$	Succinic acid
3Mol	$NH_2-(CH_2)_5-NHOH$	1-Amino-5-hydroxylamino-pentane

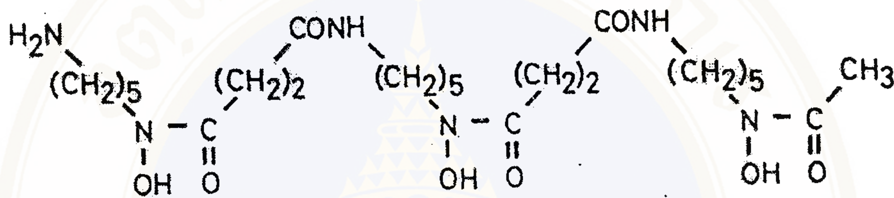

Figure 1. Desferrioxamine B (21)

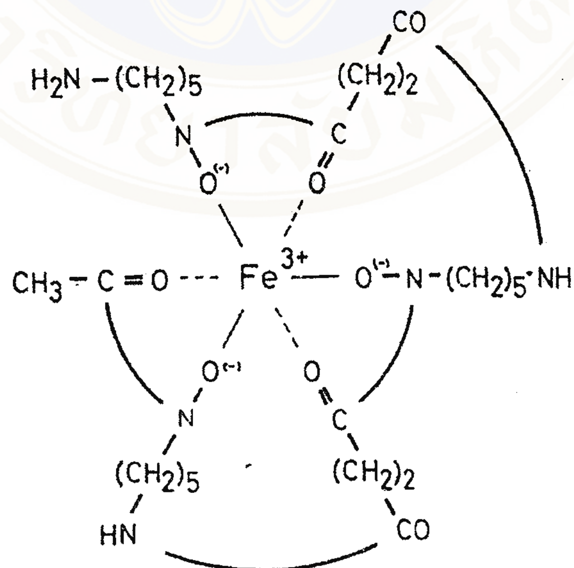

Figure 2. Ferrioxamine B (21)

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The high affinity for ferric ions is demonstrated in Table 3. The complex-formation constant of $10^{30.6}$ or 10^{31} desferrioxamine B to compete effectively with transport proteins for iron. The much higher formation constants of certain bacterial siderophores such as enterobactin or agrobactin might bring about the risk of a highly undesirable interference with functional iron-containing protein (1).

The stability constants for various desferrioxamine-metal complexes compared with the constants for other chelating agents are listed in Table 4. The higher the constant, the more stable the complex. The highest figure here, 10^{31}, is for the iron complex of desferrioxamine. All the other constants for this compound and other metallic ions are lower. This means that desferrioxamine is not only the most potent, but also the most specific ferric complexing agent of those listed in the table. Desferrioxamine shows no significant affinity for ferrous ion (21).

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Table 3 Complex-formation constants of various siderophores with ferric ions (1).

Siderophores	Complex-formation constants with ferric ions
Enterobactin	10^{52}
Desferrioxamine E	$10^{32.5}$
Rhodotorulic acid	$10^{31.2}$
Mycobactin	$>10^{30.6}$
Desferrioxamine B	$10^{30.6}$
Desferri-ferrithiocin	$10^{29.6}$
Desferri-ferrichrome A	$10^{29.6}$
Desferri-ferrichrome	$10^{29.1}$
Acetohydroxamic acid	$10^{28.3}$
Aerobactin	$10^{22.9}$
For comparison: Ferric hydroxide	10^{39}
Transferrin (iron transport protein containing two iron-binding sites)	10^{30}

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Table 4 Stability constants of some chelating agents (21).

Metallic ion	**Desferioxamine**	**Ethylene diamine tetra-acetic acid (EDTA)**	**Diethylene triamine penta-acetic acid (DTPA)**	**Transferrin**
Fe^{3+}	10^{31}	10^{25}	10^{29}	10^{30}
Ca^{2+}	10^{2}	10^{11}	10^{10}	
Mg^{2+}	10^{4}	10^{9}	10^{9}	
Sr^{2+}	10	10^{9}	10^{10}	
Zn^{2+}	10^{11}	10^{16}		
Ni^{2+}	10^{10}	10^{19}	10^{20}	
Co^{2+}	10^{11}	10^{16}	10^{19}	
Cu^{2+}	10^{14}			
Fe^{2+}	10^{10}			

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In order to discover what type of iron desferrioxamine would be capable of removing from the organism, the iron protein e.g., ferritin, hemosiderin, transferrin, and hemoglobin were incubated with desferrioxamine and then dialyzed. Desferrioxamine removes iron from ferritin and hemosiderin and continues to do so until its maximum theoretical binding capacity is attained. The situation in the case of transferrin, however, is different. Desferrioxamine was able to take up only some 10-15 percent of the iron from totally saturated transferrin. On the other hand, there was no demonstrable exchange of iron from ferrioxamine to transferrin. This finding shows that, under conditions of equilibrium, desferrioxamine is not capable of removing all the iron from transferrin. The incubation tests performed with hemoglobin yielded completely negative results. Desferrioxamine cannot withdraw iron from the porphyrin system (21). Therefore, it may be concluded that desferrioxamine in vivo is only able to attack the depot iron. In view of the excellent tolerability and low toxicity of desferrioxamine its ability to remove iron from the organism can also be demonstrated in animal experiments (14,21).

From the animal experiments, it was founded that desferrioxamine is poorly absorbed from the gastrointestinal tract. For this reason when the compound is given orally, the urinary excretion of iron remains very low, even in rabbits subjected to iron loading (21).

As described above desferrioxamine does indeed meet all the criteria of a therapeutically useful iron chelator, but two disadvantages prevent it from being an ideal iron chelator i.e., poor oral absorption, therefore it must be given intravenously and the relatively high cost of the drug. Therefore considerable effort is being

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investigated in many laboratories around the world to find better forms of administration or alternative orally active chelator.

SIDEROPHORES

P. aeruginosa has a strong iron requirement and the mechanisms by which it fulfills its iron needs in vitro are well known. As a free-living organism, it is able to excrete large amounts of chemically unrelated siderophores (22). Siderophores are bacterial products which bind iron and increase the rate of bacterial iron transport.The results of siderophore function are best observed in low-iron media as a more rapid onset of growth and possibly an increase in growth rate. Some iron-binding compounds are also known to function in medium containing animal or human serum. Since an extension of this phenomenon is the stimulation of bacterial growth during infections, siderophore synthesis has been linked to bacterial virulence (23).

Siderophores function as powerful iron chelators, solubilizing and transporting iron through the bacterial membranes via specific receptor proteins at the level of the outer membrane (24,25), and have a TonB-like system for the translocation of iron through the cytoplasmic membrane (26). When inside the cell, iron is released from the ferrisiderophores by a reductive process before it reaches its targets (27,28).

Siderophores are low-molecular-weight chelating agents synthesized and released by microorganism in response to conditions of iron [Fe (III)] deficiency (29). *P. aeruginosa* is an opportunistic human pathogen which requires iron for multiplication both in the environment and in the tissue of the infected host (29,30), like other members of the first genetic homology group of genus *Pseudomonas* (31).

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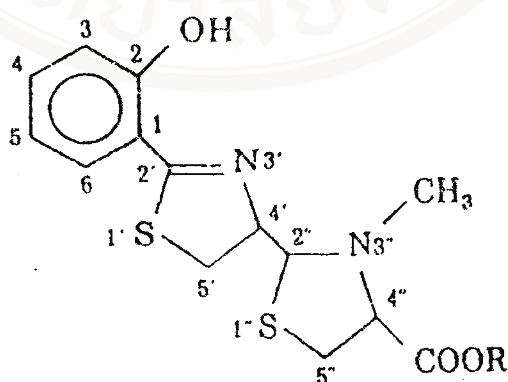
Siderophores are often classified according to the phenolic or hydroxamic acid composition of the compound (32). The standard strain of *P. aeruginosa* American Type Culture Collection (ATCC) 15692 (PAO1) has been shown to synthesis two distinct siderophores pyochelin and pyoverdin into its environment (33).

Pyochelin is an unusual siderophore in that it is a poorly water-soluble, low-molecular weight of 325 (33). It is a phenolic iron-binding compound, which can be extracted into ethyl acetate from culture media of *P. aeruginosa* (32). The structure of pyochelin as shown in Figure 3 is thiazoline derivative [2-(2-o-hydroxy-phenyl-2-thiazolin-4-yl)-3 methyl thiazolidine-4-carboxylic acid] which binds Fe (III) with a stoichiometry of two molecules per iron atom and a remarkably low stability constant of approximately 5×10^5 M^{-2} (34). Ferripyochelin transport has been shown to involve two distinct outer membrane proteins (OMPs) M.W. 14,000 and 75,000 (24,35), the latter characterized by a higher affinity i.e., operating at Fe (III) concentrations 5 to10 fold lower than the former (24,33,36).



1: R = H

2: R = CH_3

Figure 3 Structure of pyochelin.(37)

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Pyoverdin is a yellow-green water-soluble fluorescent pigment. It is a more complex molecule, M.W. of 1,500. This molecule is hydroxamate siderophore composed of a 6,7-dihydroxyquinoline containing fluorescent chromophore joined to the N-terminus of a partly cyclic octapeptide (D-Ser-L-Arg-D-Ser-N5-OH-Orn-L-Lys-L-N5-OH-Orn-L-Thr-L-Thr, in *P. aeruginosa* PAO1) (38). The hydroxamate groups formed by these two residues participate in the binding of iron together with the catecholate group of the chromophore. While the chromophore is conserved with minor structural variations in different pyoverdins from a number of fluorescent pseudomonads, the peptide composition varies in a strain-dependent manner (39), with difference reflecting uptake specificity of the ferripyoverdin complex (40). The structure of pyoverdin is shown in Figure 4. It is a powerful chelator of ferric iron, which is bound, with stoichiometry of 1:1 (23,41) and a stability constant of approximately 10^{24} M^{-1} at neutral pH (41,42). Both features favor Fe (III) binding to pyoverdin with respect to pyochelin (34,43). Two high-molecular-mass outer membrane proteins (OMPs) of 80,000 and 90,000 (25,44) have been shown to function in ferripyoverdin uptake (33). Moreover, an 85,000 Fe (III)-regulated protein, which functions as the pyocin receptor also, appears to be involved in ferripyoverdin uptake (45). As a general rule, pyoverdins (or pseudobactins from plant-relate isolates) contain one or two residues of L-N5-OH-Orn, which participate in Fe (III) coordination with the quinoline hydroxyl of the chromophore. The close structural relationships among pyoverdins and pseudobactins are reflected at the DNA level (46). The genetic loci involved in pyoverdin synthesis and uptake have been mapped in *P. aeruginosa* and found to be located within a 103 kb DNA region at about 47 min of the PAO chromosome map (47-50).

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Figure 4 The structure of pyoverdin (41).

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IRON METABOLISM IN MAN

Iron is an indispensable element essentially for all forms of life. The tendency of iron to oxidize, hydrolyzes, and polymerizes into insoluble ferric hydroxide has forced living cells to develop unique storage form for the metal. In humans excess iron was deposited in the protein, ferritin. This provides a soluble, nontoxic and biologically accessible form of the metal (51).

Iron metabolism in man is shown in Figure 5, which is highly conservative. There is a considerable daily exchange of iron between various body compartments. The epithelial cells of the gut take up only 1-1.5 mg per day from food (52). The overall balance is regulated by a complex mechanism controlling iron uptake. This mechanism has not yet been fully elucidated. It involves elaborate interaction of receptors and transport proteins in the mucous membrane of the gut. Under normal circumstances only approximately 10-15% of iron in food is absorbed. Iron deficiency in various forms of anemia leads to an increase in the rate of iron absorption. On the other hand absorption is decreased in the case of iron saturation or excess (53). Some iron is lost in cells shed from skin and also particularly in those, which are shed from and rapidly replaced by the gastrointestinal tract. Further losses occur in certain secretions and through occasional bleeding. No specific mechanism exists whereby excessive iron could be excreted. As a consequence, any disturbance on the uptake side invariably leads to a disturbance in overall iron balance (1).

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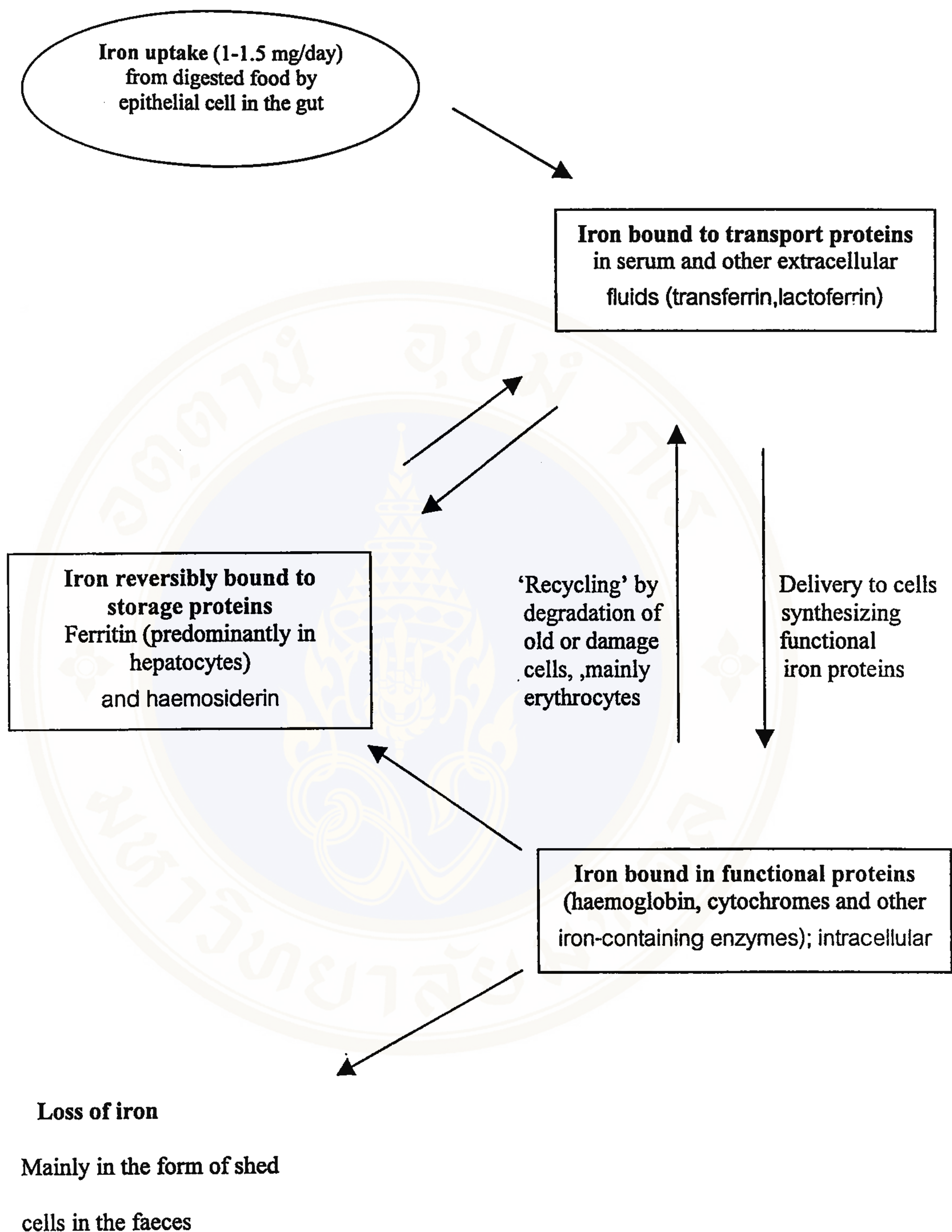

Figure 5 Iron balance in human body. Arrows indicate daily exchange of iron with quantities in mg (1)

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FERRITIN

The ferritin molecule has an important role in the overall physiology of iron metabolism as a result of (i) its ability to maintain iron in a soluble, non-toxic and biologically useful form and (ii) its capacity to sequester vast quantities of iron (54).

Ferritin is composed of an apoprotein shell (M.W. approximately 480,000) which surrounds a core of up to 4,500 atoms of iron in the form of the mineral ferrihydrite (54). The ferritin molecule is large, roughly spherical 8 nm diameter iron-storage cavity encompassed by a protein shell of 2.5 nm thickness and composed of 24 subunits of two structurally distinct subunit types (55). The heavy or H-subunit has a more acid isoelectric pH (pI) and has a molecular weight of approximately 21,000. The light or L-subunit is more basic than the H-subunit and has a molecular weight of approximately 19,000 as shown in Figure 6 (56,57). Different proportions of each subunit in a ferritin molecule give rise to isoferritins, which have characteristic pI and specific tissue distributions. Isoferritins with a higher number of L-subunits are predominantly found in the liver, spleen and placenta. The administration of iron results in a preferential synthesis of L-subunits, thereby favoring the assemblies of L-rich isoferritins in iron-overload condition (58). Isoferritins with an increased percentage of H-subunits are found in tissue such as heart (59,60), red blood cells (61), lymphocytes and monocytes (62) and HeLa cells (63,64), which are not primarily involved in iron storage. H-rich ferritins may be primarily involved in iron detoxification, while L-rich ferritins are mainly involved in long-term iron storage.

The ferroxidase site functions in true catalytic manner, accepting ferrous iron, promoting its oxidation by molecular dioxygen, and releasing the resulting ferric iron for deposition in the core as shown in Figure 7 (65).

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There are some differences in iron incorporation when iron is added as Fe (III) or Fe (II) in the presence of an oxidant. In the formation of ferritin from apoferritin and Fe (II) the first step is almost certainly the binding of Fe (II) by the protein (66,67). However, there are evidences for conclusion that the firmly bound Fe (III) lies inside the molecule attached to its iron core (68). There was a report (69) concerning the uptake of iron by horse spleen apoferritin, by using as an iron source the i.e., ferric dihydrolipoate complex, which represents the major product in the anaerobic removal of ferritin-bound iron by dihydrolipoate at neutral pH. The ferric dihydrolipoate complex was chemically synthesized and used as an iron donor to apoferritin.

The release of iron from the ferritin molecule in vivo is thought to occur in lysosomes or endosomes with acid pH. Xanthine oxidase may directly catalyse the reduction of ferric iron to ferrous iron (70). Other investigators suggested that the flavins might offer a more plausible explanation of the reduction of ferric iron. Degradation of ferritin may be necessary for the release of its iron (71).

HAEMOSIDERIN

Haemosiderin is the major iron storage protein present when excessive iron accumulates in the tissues of patients with genetic haemochromatosis or iron overload secondary to anemia as in thalassemia. Ferritin is water-soluble crystalline while haemosiderin is observed as an amorphous deposit. In normal liver, ferritin is the major iron-protein detected but in conditions of iron overload, haemosiderin has been shown to predominate.

Haemosiderin appears to consist of degraded ferritin protein and ferric hydroxide polymers or cores of varying sizes. Histological and also immunological studies have

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demonstrated a close relationship between ferritin and haemosiderin. Thus antisera raised against ferritin react with haemosiderin indicating that the major peptide in haemosiderin is derived from ferritin (54).

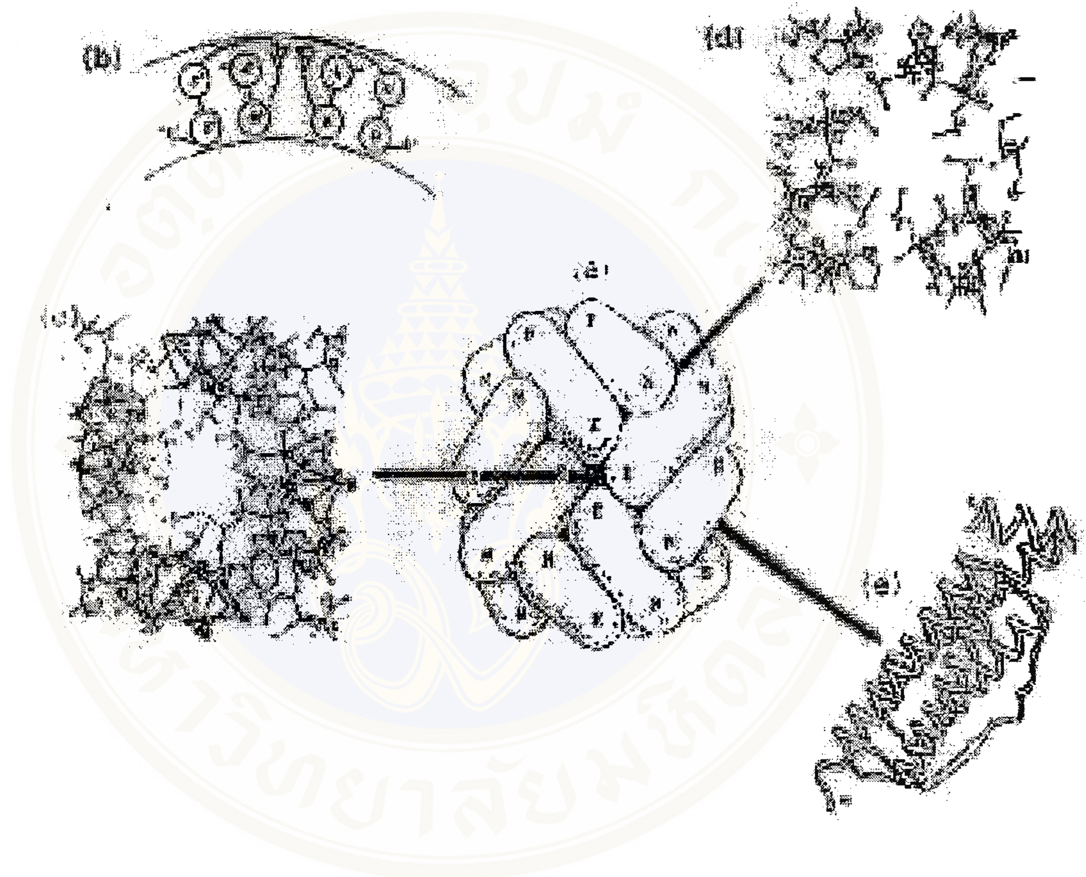

Figure 6 Structure of apoferritin. (a) Schematic representation of the 24-subunit molecule is showing 432 symmetry. (b) Section through shell depicting two subunits related by the 2-fold axis. (c) View down the hydrophobic channel around the 4-fold axis. (d) View down the hydrophilic channel ground the 3-fold axis. (e) Ribbon diagram of a single subunit (60).

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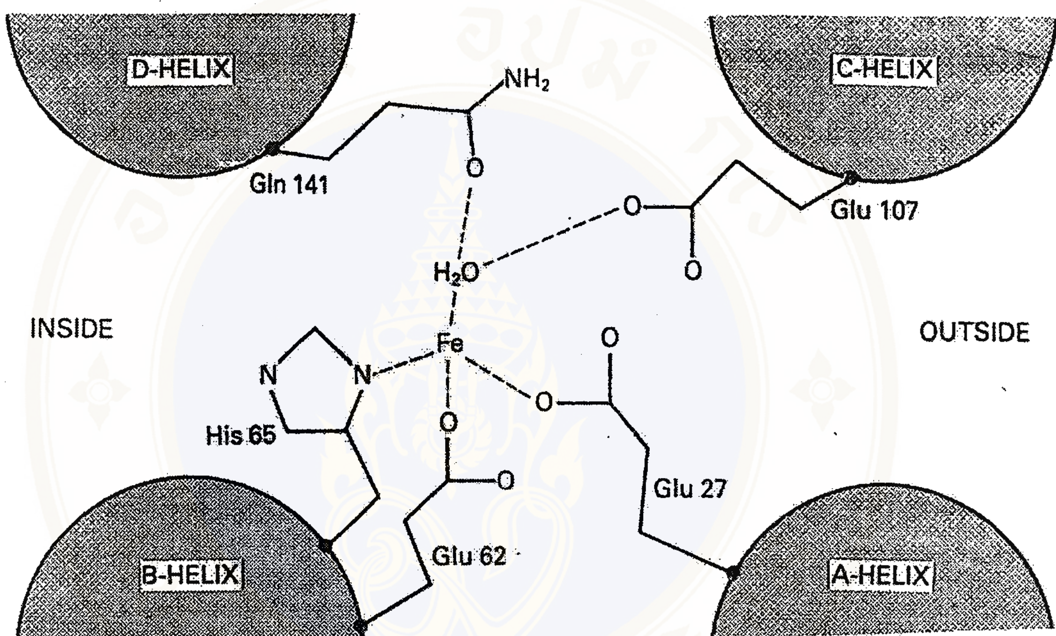

Figure 7 Ferroxidase site of human ferritin H-subunit (65).

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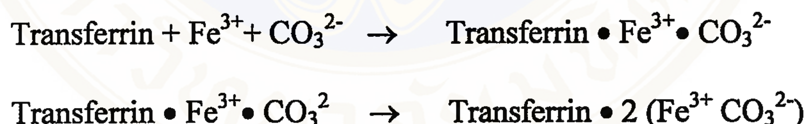
TRANSFERRIN

Transferrin is the major iron-transport protein in plasma. It has two binding sites for iron, one at the amino terminal and one at the carboxyl terminal ends of the molecule. This protein has an important role in iron metabolism returning iron derived from the catabolism of haemoglobin and other proteins to haemopoietic tissue. Unlike some other transport proteins, it is returned to circulation after unloading its iron at the cell membrane (72).

Transferrin, a glycoprotein, consists of a single polypeptide chain M.W. of 78,000 with two iron binding sites. Several metals bind to transferrin; the highest affinity is for Fe^{3+}. Ferrous ion is not bound. The binding of each Fe^{3+} ion is absolutely dependent on the coordinate binding of an anion, which in the physiological state is carbonate as indicated below (73).



$$\text{Transferrin} + Fe^{3+} + CO_3^{2-} \rightarrow \text{Transferrin} \bullet Fe^{3+} \bullet CO_3^{2-}$$

$$\text{Transferrin} \bullet Fe^{3+} \bullet CO_3^{2} \rightarrow \text{Transferrin} \bullet 2\,(Fe^{3+}\,CO_3^{2-})$$

Crystallographic studies have shown that transferrins are bilobal proteins (74) (Figure 8 (75,76)) consisting of nearly 700 amino acids, which there are approximately 40% amino acid sequence homology between the lobes (77,78). Homology between corresponding halves of transferrins from different species generally exceeds homology between N- and C- lobes of the same transferrin (79). Each lobe is further organized into two dissimilar sub-domains of approximately 160 amino acids, separated by a cleft that contains the metal-binding site.

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Human transferrin bears two carbohydrate chains, accounting for approximately 6% of the proteins mass, both attached to the C-terminal lobe at residues N414 and N611. No specific function of the carbohydrate is known although partial losses of terminal sialic residues are a distinctive finding in alcoholism (54).

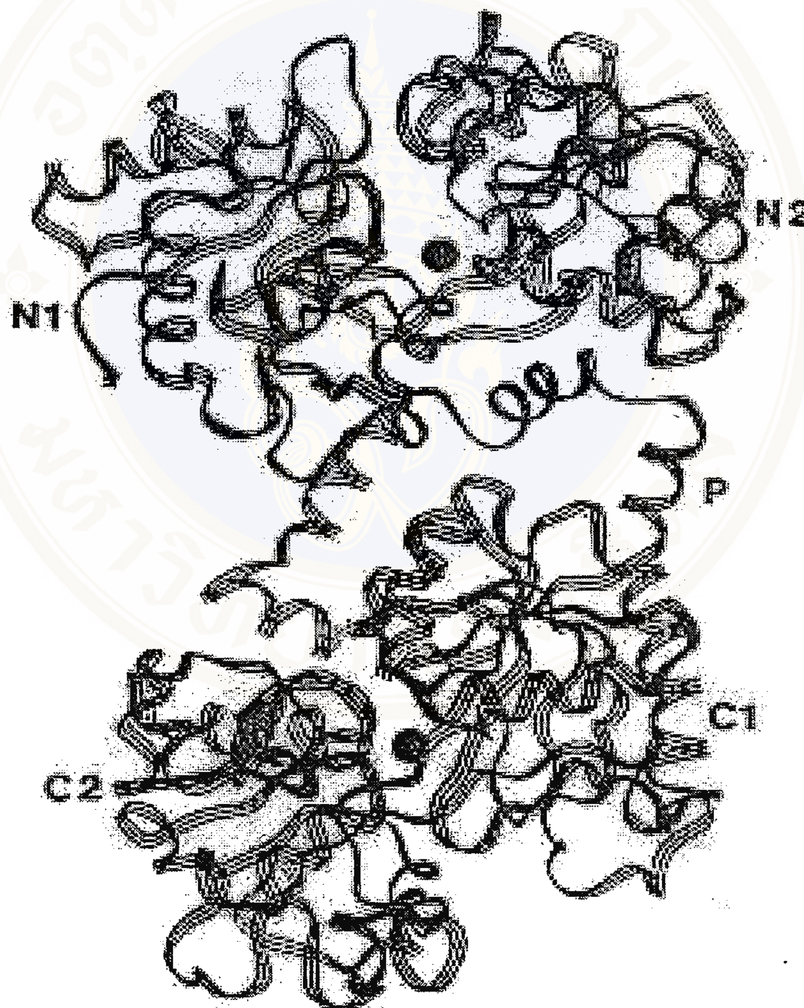

Figure 8 Ribbon diagram showing the characteristic folding of transferrins.(74).

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Estimates of the association constants for the binding of Fe^{3+} to transferrin from different species range from 10^{19} to 10^{31} M^{-1}, indicating for practical purposes that wherever there is excess transferrin, free ferric ions will not be found. In the normal physiological state, approximately one-ninths of transferrin molecules have iron at either site; and four-ninths of circulating transferrin are free of iron. Two iron-binding sites show differences in sequences and in affinities for other metal. Transferrin binds to specific cell surface receptors that mediate the internalization of the protein (73).

The structure of the human transferrin receptor is presented diagrammatically in Figure 9. It is a disulfide-linked dimer of two identical transmembrane subunits, each of M.W. approximately 90,000 and containing 760 amino acid residues. Three domains of each subunit can be recognized, and intracellular N-terminus consisting of 61 amino acids, a 28 residue membrane spanning part and extracellular C-terminal domain of 671 residues (80-84).

The interaction between transferrin and its receptor is reversible, pH-dependent and influenced by the iron content of transferrin. At near neutral pH the receptor has a much higher affinity for diferric transferrin than for apotransferrin and an intermediate affinity for monoferric transferrin. Furthermore diferric transferrin has a competitive advantage over monoferric transferrin with respect to binding to the receptor and an even greater advantage in its ability to deliver iron to cells. Hence cells bathed in a mixture of iron-transferrin and apotransferrin, such as in the extracellular fluids of the body, selectively bind iron-transferrin, especially diferric transferrin, and achieve maximal gain from receptor function with respect to iron uptake (85-89).

The major function of the transferrin receptor is to mediate cellular uptake of transferrin-bound iron (90).

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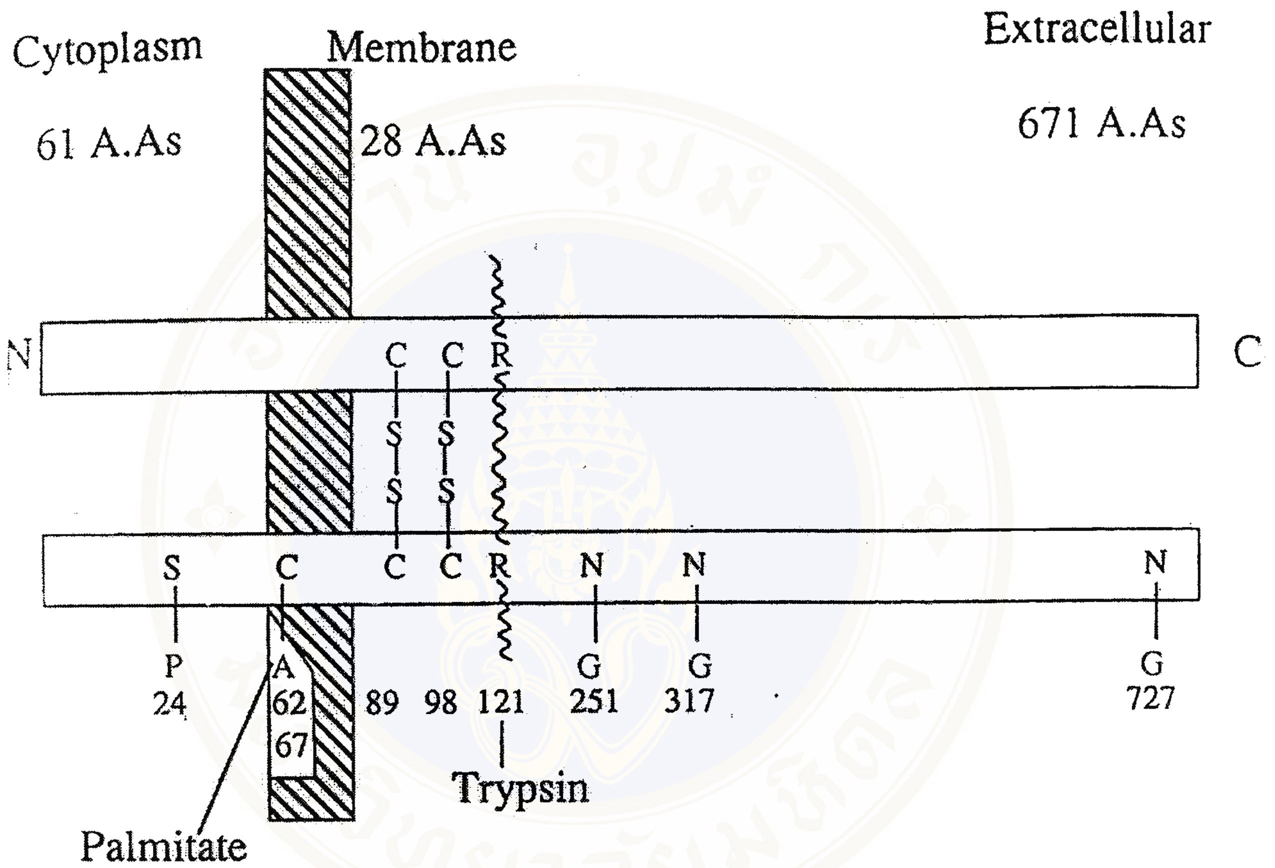

Figure 9 Diagram of the human transferrin receptor (90).

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Normally the plasma iron concentration is 10-30 μmole/l (mean approximately 20 μmole/l) and the plasma transferrin concentration 22-35 μmole/l (mean 30 μmole/l) so that the transferrin is approximately 30% saturated with iron (range 20-50%). Hence the plasma and interstitial fluid contain considerable iron-free transferrin which is able to bind iron absorbed from the intestine or released from cells elsewhere in the body. The plasma concentrations of iron and transferrin vary from the normal adult values at different stages during the life cycle and in many diseases.

Transferrin, like other plasma proteins, is distributed throughout most of the extracellular fluid of the body, with a continuous circulation from plasma to interstitial fluid and then back to the blood via the lymphatic vessels. Almost 100% of the plasma transferrin circulate through the lymphatic each day.

Major functions of transferrin are to maintain extracellular iron in a soluble form, which is suitable for cellular uptake, and to regulate the supply of iron to cells by influencing its distribution within the body and its availability to individual cells. The latter function is determined mostly by the cell component of transferrin receptors, which varies greatly from the cell type and at various times in the life cycle in any type of cell. Under normal conditions transferrin not only ensures an adequate supply of iron to meet the individual needs of different cell types but also restricts the uncontrolled entry of excessive amount of iron into cells resulting in cellular damage (90).

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GROWTH

Bacteria have generation times that range from 12 minutes to many hours. Generation time is the interval between divisions of a growing microorganism. In microbiology it is determined as the time required for the population of cells in a culture to double, since each organism produces two offspring per generation. A population of growing bacteria increases as a geometric progression. The generation time of a bacterial culture is not constant with time but changes as the composition of the culture medium changes. Generation time is also dependent on the initial medium, pH and the temperature at which the culture is grown (91).

Growth of culture can be measured with time by determining (1) the cell mass of the culture, (2) the total number of cells, or (3) the number of viable cells over the lifetime of the culture (91).

A typical growth curve for a population of bacterial cells, as shown in Figure 10, illustrates some of the dynamics that effect the population over the course of time. Four distinct phases of the curve are recognized as follows: (18)

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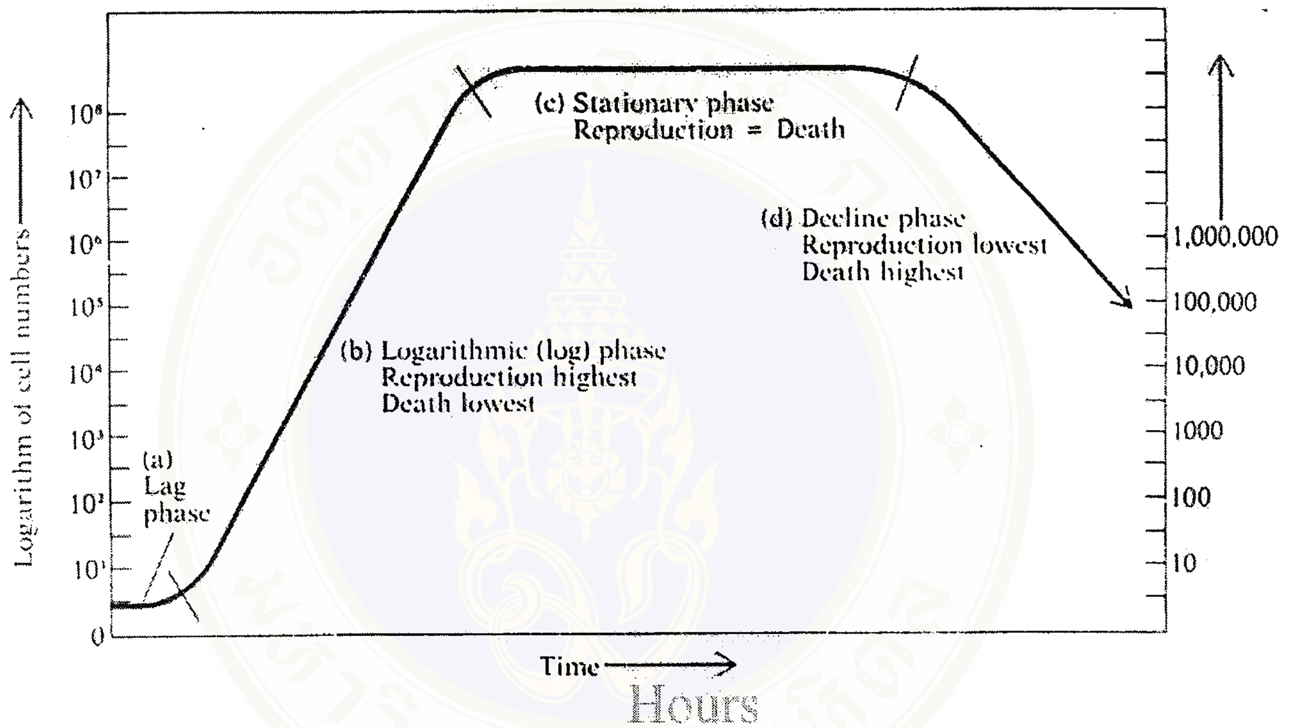

Figure 10 The growth curve for a bacterial population (18).

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1. Lag Phase

The cells do not immediately reproduce in a new medium. This period of little of no cell division is called the lag phase and it can last for an hour or several days. The microbial population is undergoing a period of intense metabolic activity involving, in particular DNA, enzyme synthesis and preparation for binary fission (92). The curve remains at a plateau, balanced by early reproduction in some cells and death in others (18). The length of the lag phase depends on the type of bacteria, the size of the inoculum, the nature of the medium from which they were taken, and the nutrients present in the new medium (93).

2. Log Phase

Eventually, the cells begin to divide and enter a period of growth or logarithmic increase called the log or exponential phase. Cellular reproduction is most active during this period. Because the generation time is constant, a logarithmic plot of growth during the log phase is a straight line. The log phase is the time when cells are most metabolically active (18). The rate of cell division depends on the type of organism, the components of the medium, the temperature and for aerobic organism, the aeration rates (93). In this phase the mass of each cell increases rapidly and reproduction follows (18). The increase in the number of cells is homogeneous, and no aggregates of cells are present.

When a plate count is performed, it is important that only a limited number of colonies develop on the plate. When too many colonies are present, some cells are overcrowded and do not develop; these conditions cause inaccuracy in the count. Generally, only plates witch form 30 to 300 colonies are counted. To ensure that some

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colony counts will be within this range, the original inoculum is diluted several times in a process called serial dilution (Figure 11) (92).

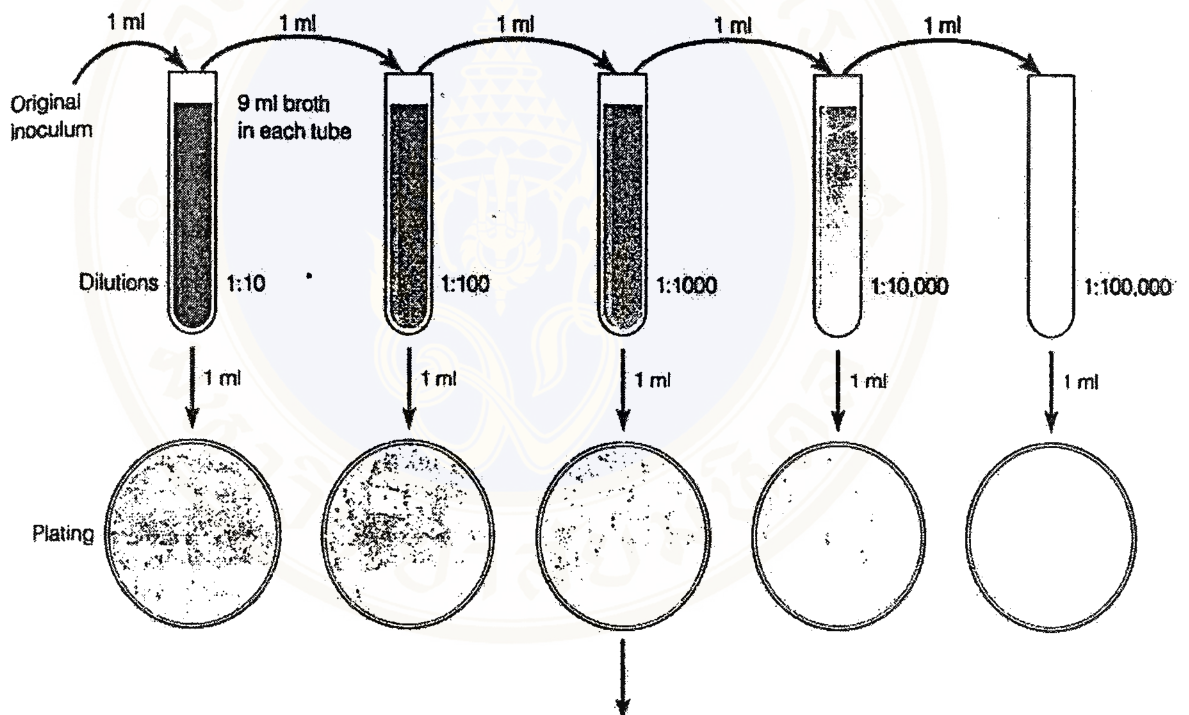

Figure 11 Plate counts and serial dilutions (92).

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3. Stationary Phase

Logarithmic growth eventually slows because of accumulation of waste products, exhaustion of nutrients, change in pH or a decrease in oxygen concentration. The population then enters the stationary phase in which the number of viable cells remains approximately constant. Usually, there is a division of some cells and the death of others. It is worth noting that entry into the stationary phase or even starvation of a required nutrient does not result in the killing of most bacteria (93).

4. Death Phase

Eventually the rate of death exceeds the rate of reproduction and the number of viable cell declines. The length of time before all cells died differs markedly for various organisms. Some species have a very short death phase, whereas for others it may take weeks before all cells in a culture died. During this phase cells often assume unusual shapes, making it difficult to recognize the bacteria in old cultures (93).

DIRECT MEASUREMENT of MICROBIAL GROWTH

The growth of microbial populations can be measured in various ways. Some methods measure cell numbers; other methods measure the population's total mass, which is often directly proportional to cell numbers. Population numbers are usually recorded as the number of cells in a milliliter of liquid or in a gram of solid material. Since bacterial populations are usually very large, most methods of counting them are based on direct or indirect counts of very small samples; calculations then determine the size of the total population.

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PLATE COUNTS

The most frequently used method for the measurement of bacterial population is the plate count. An important advantage of this method is that it measures the number of viable cells. One disadvantage may be that it takes time usually 24 hours or more for visible colonies to form. This can be a serious problem in some applications, such as quality control of milk, when it is not possible to hold a particular lot for this amount of time.

The plate count is based on two assumptions: that each bacterium grows and divides to produce a single colony, that the original inoculum through 10 divisions beginning with a single cell as is 1024 cells. Plotting this data on an arithmetic graph results in a curved line (Figure 12a). Plotting the same data on a semilogarithmic graph (Figure 12b) results in a straight line which is another way of demonstrating that bacterial growth during this phase can be represented as an exponential function. During exponential growth all cells in the culture are physiologically identical for all practical purposes. If all components (proteins, DNA, etc.) of these cells are changing at the same constant rate, the cells are said to be in balanced growth. Exponential growth cannot continue for an indefinite time because the cultures run out of essential nutrients and the cells produce toxic metabolic products that retard growth. This leads to a reduction in the growth rate of the culture (91).

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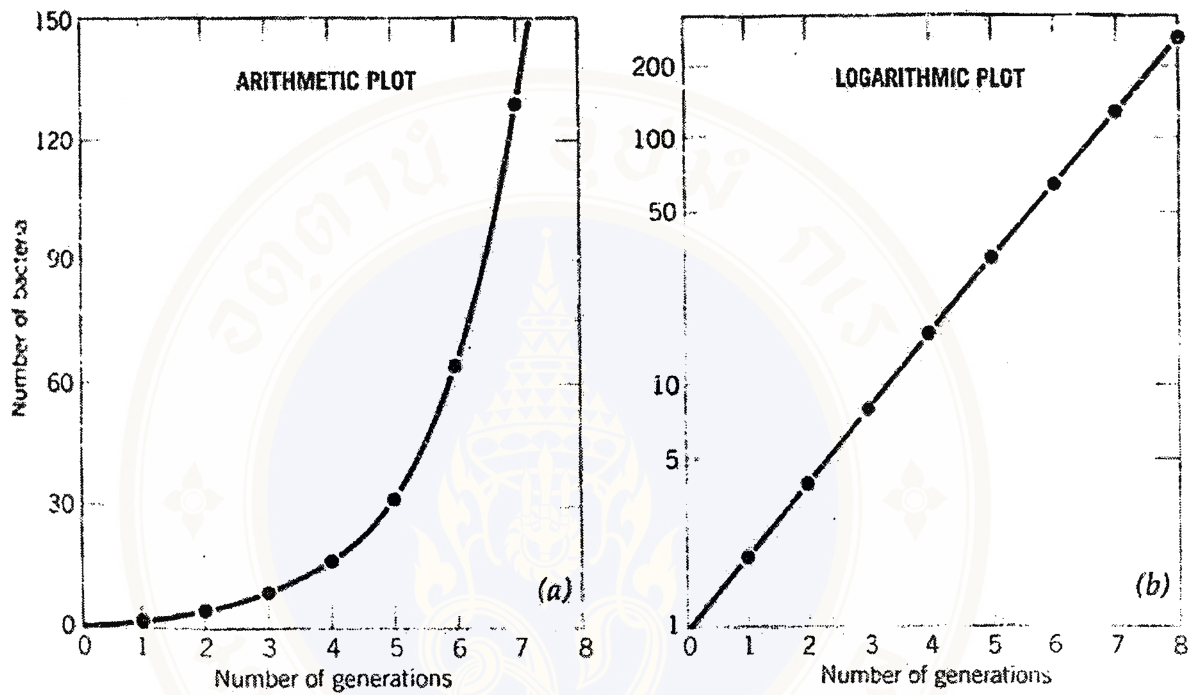

Figure 12 The (a) arithmetic and the (b) logarithmic plot of the data showing that populations of bacteria increase exponentially (91).

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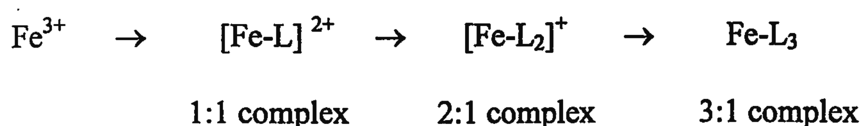
CHEMICAL PROPERTIES for MAXIMAL IRON (III) AFFINITY

To achieve effective chelation in vivo, a compound with a high iron binding constant and a high degree of specificity in relation to other metals is required. Iron exists in two oxidation states, iron (III) or ferric iron, and iron (II) or ferrous iron. Under aerobic conditions, iron (III) is more stable and chelators with high affinity for this form of iron are therefore of much greater use. Iron (III) possesses an extremely high charge density as a result of its tripositive charge. It therefore binds tightly to atoms bearing a similar high charge density and in particular to oxygen species such as carboxylates, catecholates and hydroxypyridones (Table 5). The number of covalently linked arms on the chelator also influences the stability of the metal complex; hexadentate ligands are more stable than bidentate ligands. Thus the affinity constants of iron for the bidentate acetohydroxamic acid and the hexadentate Desferal are 10^{28} and 10^{33} respectively (Figure 13). Hexadentate ligands also have greater binding power at low concentration (<20 μM) and therefore are less likely to dissociate. A further disadvantage with bidentate ligands is that partially dissociated complexes (2:1 and 1:1) form at low concentrations, and these can generate hydroxyl radicals. Thus, from chemical consideration alone, hexadentate chelators are more suitable than a bidentate or tridentate analogue as a scavenger of iron. The co-ordination of iron by a bidentate ligand occurs as follows: (6)



$$Fe^{3+} \rightarrow [Fe\text{-}L]^{2+} \rightarrow [Fe\text{-}L_2]^{+} \rightarrow Fe\text{-}L_3$$

1:1 complex 2:1 complex 3:1 complex

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Table 5. Structures of functional groups of various types of iron chelators and net charges at pH 7.4 (6).

Functional group	**Example of iron chelator**	**Net charge of iron (III) complex (pH7.4)**	**Charge of iron-free chelator (pH 7.4)**
Hydroxamate	Desferioxamine	1^{+}	1^{+}
	Rhodotorulic acid	0	0
Catecholate	Enterobactin	3	0
	2,3-dihydroxybenzoic acid	6	1
Amino-carboxylate	DTPA	2^{-}	5^{-}, 2^{+}
	EDTA	1	4^{-}, 2^{+}
Orthosubstituted phenolate	PIH	1	0
	Desferrithiocin	1^{-}	1^{-}
Mixed phenolate and amino carboxylate	HBED	1	2^{-}, 2^{+}
Hydroxypyridinon	CP 20	0	0
	CP 51	0	0

DTPA: Diethylene triamine pentaacetic acid

EDTA: Ethylene diamine tetraacetic acid

PIH: Isonicotinyl hydrazone

HBED: N,N'-bis (2-hydroxy-benzy)-ethylenediamine-N-N'-diacetic acid

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Bidentate ligand 3 : 1

Hexadentate ligand 1 : 1

N-methylacetohydroxamic acid

Desferrioxamine

Figure 13 Comparison of bidentate and hexadentate ligands (6).

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CHAPTER III

MATERIALS AND METHODS

1. BACTERIAL STRANDARD STRAIN

Pseudomonas aeruginosa standard strain PAO1 (ATCC15692) was obtained from the American Type Culture Collection, USA. It was used to prepare siderophores. It was stored as the stock culture in 20% glycerol+5% Tryptic soy broth solution and kept at –20 °C until used.

2. CHEMICALS and GLASSWARE

Apotransferrin and apoferritin were purchased from Sigma Chemical Co.Ltd., St. Louis Missouri, USA. Desferal (desferrioxamine mesylate, USP) was obtained from Ciba-Geigy Pharmaceutical Co. Ltd., Summit, New Jersy, USA.

Standard pyoverdin and pyochelin were obtained from Professor Charles D Cox, Department of Microbiology, University of Iowa, USA and were purified as previously described (32,33,95).

In every experiment, all glasswares were rendered iron free by 24 hours of treatment with 0.1 N HCl, then rinse three times with deionized water before used or sterilization if they were used in bacterial growth study. All media and solutions were prepared with deionized, double-distilled water (33).

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3. CULTURE MEDIA

Blood agar containing Tryptic soy agar plus 5% human blood was used for culturing *P. aeruginosa* strain ATCC15692 from the stock culture.

The medium for siderophore production was CAA which contained 0.5% Casamino acid (Difco, Detroit, Michigan, USA) supplemented with 0.4 mM $MgCl_2$ (23).

Glucose minimal medium (GMM) contained 20 mM glucose, 40 mM ammonium acetate, 0.4 mM $MgCl_2$ and 5 mM potassium phosphate buffer (pH 7.4) (36).

Precultures were prepared by inoculating 100 ml flasks containing 25 ml of CAA with strain ATCC15692 from nutrient agar slant and incubated overnight at 35 °C in ambient atmosphere. A 200 μl volume of the preculture broth was used to inoculate 1000 ml flasks containing 500 ml of CAA. Incubation was carried out with shaking at 200 rpm in the orbital incubator shaker (GYROMAX™ 707R) at 35 °C for 48 hours. The broth culture was used to prepare and purify pyochelin, pyoverdin and was also used in bacterial growth assays.

4. PURIFICATION and ASSAY of PYOCHELIN

P. aeruginosa, strain PAO1 was grown for siderophore production in CAA medium and pyochelin was extracted from spent culture medium.

Pyochelin was extracted from 48 hour-bacterial culture in CAA medium into methylene chloride containing 10% acetic acid (at 2:1 ratio with the medium) (36). The mixture was agitated for 4 hours at room temperature by using magnetic stirrer. The mixture was then put into separating funnel and left standing at room temperature overnight. The methylene chloride layer was collected and concentrated by rotary

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evaporation. After evaporation of the organic phase under vacuum, the dry residue was solubilized in a small volume of methylene chloride and purified by applying onto a Silica Gel G (Merck, Germany) thin-layer chromatography (TLC) plate. The TLC plate was run in a glass tank containing chloroform-acetic acid-95% ethanol (at 90:5:5 ratio) as the development solvent. Pyochelin from TLC plates was qualitatively characterized by (i) yellow green fluorescence band under ultra violet light (MODEL UVL-21 UVP UPLAND, California, USA.) (94), (ii) Iron-binding capacity resulting in red brown spot when sprayed with iron reagent i.e., 0.1 M $FeCl_3$ in 0.1 N HCl (33), (iii) chromatographic mobility (Rf = 0.35 to 0.40 in the above development solvent (96)).

Quantitative determinations of pyochelin were performed by scraping the fluorescent band from TLC plates loaded with 500 ml culture extracts. Pyochelin was eluted from the silica gel by extracting twice with methylene chloride. The methylene chloride extracts were dried under vacuum for 4 hours, and the pyochelin was measured by weight. When in use, dried pyochelin powder was solubilized and maintained in 95% ethanol as 1 mg/ml solution. In every experiment, the ethanol in the pyochelin solution was removed under vacuum and the pyochelin was dissolved in Tris buffer (36).

5. PURIFICATION and ASSAY of PYOVERDIN

Pyoverdin was extracted from 48 hour-bacterial culture in CAA medium. Bacteria were removed from the culture by centrifugation at 13,000 rpm for 2 minutes. This was followed by filtration through membranes (pore size, 0.45 μm). The pelleted cells were discarded and the supernatant was concentrated by rotary evaporation (23).

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The concentrated solution was passed through Sep-pak (Waters Associates, Inc., Milford, Massachusettes, USA.) C18 cartridges. The cartridges were subsequently washed with 40 ml of distilled water per cartridge and then with 5 ml of water-95%ethanol (1:1) mixture. The pyoverdin elute was chilled in an ice bath and was dried by using lyophillizer (42). The dried pyoverdin was measured by weight and kept at –20 °C until use. In each experiment pyoverdin was solubilized and maintained in sterile deionized water at a concentration of 1 mg/ml solution.

Pyoverdin was qualitatively characterized by (i) yellow green fluorescence band under ultraviolet light (MODEL UVL-21 UVP UPLAND, California, USA.), (ii) iron-binding capacity resulting in blue-violet spots when sprayed with iron reagent i.e., 0.1 M $FeCl_3$ in 0.1 N HCl (33,94).

6. DIALYSIS MEMBRANE ASSAYS

The ability of bacterial siderophores and Desferal to mobilize ^{59}Fe from [^{59}Fe]-ferritin was tested by placing either siderophores or Desferal only on the dialysate side. Since they are small molecular weight compounds i.e., 325 for pyochelin, 1333 for pyoverdin and 638 for Desferal, they passed through the dialysis membrane to the [^{59}Fe]-ferritin side. If they mobilized iron, they would pick up radioactivity from [^{59}Fe]-ferritin and carry the radioactivity back to the dialysate side to establish the equilibrium. Thus, the radioactivity would appear in samples taken from the dialysate side.

Since many dialysis chambers were needed for comparative assays, dialysis chambers were developed from plastic vials (4.5 by 1.5 cm) with the bottoms cut off.

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The smooth top of the vial was used to seal a dialysis membrane (M.W. size exclusion 8,000) between the vial cap.

6.1 Dialysis Membrane Assays (equal concentration)

The bottom of vial cap containing 450 μl of total volume of [^{59}Fe]-ferritin solution (which compose of ferritin (final concentration of 100 μg/μl) 45 μl, ^{59}Fe 5 μl, Tris buffer (final concentration of 50mM) 45 μl and distilled water 355 μl) and the vial itself, containing 450 μl of 50 mM Tris buffer to which were added either siderophores or Desferal at a final concentration of 10 μg/ml. The smooth edge of the vial and the cap trapped the membrane in a liquid-tight seal, and the open end of the vial was closed with a rubber stopper. The vial was shaken at an angle, which permitted both fluid phases to mix on the surface of the dialysis membrane. All experiments were conducted at 4 °C in shaking incubator at 30 rpm. After 48 hours of incubation 400 μl samples were removed from the upper (dialysate) fluid to measure the amounts of ^{59}Fe in the low-molecular-weight chelates which diffused through the membrane by scintillation gamma-counter.

Control was used to assay the amount of iron appearing in the dialysate in the presence of buffer without siderophores or Desferal. The buffer used in the dialysis experiments containing 50 mM Tris (Hydroxymethyl aminomethane) at various pHs (pH 6, 6.5, 7.0 and 7.4).

6.2 Dialysis Membrane Assays (equal molar ratio between chelator and iron)

Calculated molar ratio between chelator and iron, which in this study pyoverdin, pyochelin and Desferal were added in the dialysate at a final concentration of 15 nM. After that using the same method as described in 6.1.

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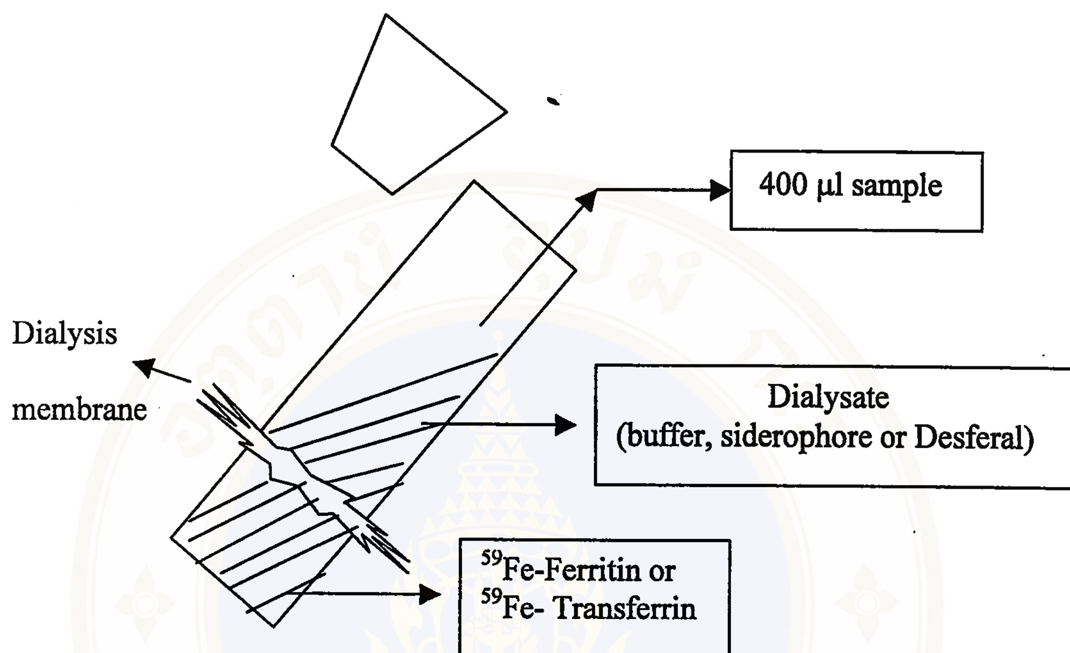

Figure 14 Dialysis assay for siderophore or Desferal activity in iron mobilization.

For 450 μl of total volume of [^{59}Fe]- Transferrin solution, it was compose of transferrin (final concentration of 100 μg/μl) 45 μl, $NaHCO_3$ (final concentration of 20 mM) 45μl, ^{59}Fe 5 μl, Tris buffer (final concentration of 50mM) 45 μl and distilled water 310 μl) and the vial itself, containing 450 μl of buffer to which were added either siderophores or Desferal at a final concentration of 10 μg/ml.

6.3 Dialysis Membrane Assays (at various pyoverdin or pyochelin concentrations)

In this study, pyoverdin or pyochelin was added in the dialysate at various concentrations i.e., 1, 3, 5, 10, 20, 40 μg/ml. After that using the same method as described in 6.1.

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7. GROWTH CONDITION

7.1 Growth Assays of *P. aeruginosa* by Siderophores in GMM Medium.

7.1.1 Growth assays were conducted in glucose minimal medium (GMM) (36). This medium allowed bacterial growth with concomitant siderophore production. The glucose solution was sterillized by cellulose acetate membrane filtration with 0.45 μm in pore diameter (Millipore filter Co., Ltd, USA.).

7.1.2 Purified siderophores were added to GMM medium each at a final concentration of 10 μg/ml (Siderophore; Pyoverdin (Pvd), Pyochelin (Pch), Pyoverdin and Pyochelin (Pvd+Pch)). Desferal was added to GMM medium at a final concentration of 10 μg/ml.

7.1.3 Bacteria prepared for inoculum by using CAA culture medium. Bacteria were harvested from the culture by centrifugation at 7,500 rpm for 20 minutes. They were then washed three times in sterile deionized water and inoculated at a final concentration of approximately 3×10^2 CFU/ml into GMM medium.

7.1.4 Bacterial growth was assayed by measuring absorbance at 600 nm against GMM or the serum controls without bacteria by using a Spectrophotometer. The data was then used for plotting graph on a semilogarithmic paper.

7.2 Growth Assays of *P. aeruginosa* by Siderophores in Medium Containing Transferrin or Heat-Inactivated Normal Human Serum.

7.2.1 Prepare GMM medium as described in [3] but was supplemented with 20 mM sodium bicarbonate for growth experiments involving transferrin or heat-inactivated normal human serum.

7.2.2 Heat-inactivated (56 °C for 30 min) normal human serum from normal donor was added to GMM medium at a final concentration of 10%.

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7.2.3 Purified siderophores were added to GMM medium each at a final concentration of 10 μg/ml (Siderophores; Pyoverdin (Pvd), Pyochelin (Pch), Pyoverdin and Pyochelin (Pvd+Pch)). Desferal was added to GMM medium at a final concentration of 10 μg/ml.

7.2.4 Prepare bacteria for inoculum in this GMM medium by using the same method as described in 7.1.3

7.2.5 Growth was assayed by diluting culture medium and determining viable bacteria by colony plate counts. Viable bacteria in suspensions were counted as colony-forming unit per ml by triplicate plating on agar surface (Mueller Hinton agar; MHA; Becton Dickinson, Cockeysville, Maryland, USA.) at 0 and 18 hours of incubation at 35 °C and then analyzed as shown in Table 16.

In some experiments it was important to reduce the iron content of the medium by mixing the medium constituents with magnesium carbonate solution ($MgCO_3$) at the concentration of 20 g per liter of the medium (95). Then the medium was centrifuged and filtered through membrane with 0.45 μm in pore diameter to remove the $MgCO_3$. This treatment has been reported to lower the iron concentration in the medium from 2.3 μM to 0.4 μM (36).

8. STUDY the DATA of POSITIVE HEMOCULTURE of THALASSEMIA PATIENTS that ADMITTED to SIRIRAJ HOSPITAL during 1994-1998.

Hemoculture from thalassemia patients who had fever and admitted to Siriraj Hospital during January 1994 – December 1998 were analyzed.

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CHAPTER IV

RESULTS

I. Extraction, purification and characterization of pyochelin

Purification of pyochelin from 500 ml spent culture medium (CAA broth) yielded dry weight of 5 mg yellow powder.

The purified pyochelin was qualitatively characterized by

1. Yellow-green fluorescence emission under UV light.
2. The iron reactivity of the pyochelin was verified (33,94) by spraying the pyochelin with an iron reagent (0.1 M $FeCl_3$ in 0.1 N HCl). The result was red-brown as shown in Figure 15 and Figure 17.
3. Comparative study on the TLC chromatography between the purified pyochelin and the standard pyochelin obtained from Professor CD. Cox at University of Iowa, USA concerning mobility by using chloroform-acetic acid-ethanol (90:5:5) as the development solvent gave the same result as shown in Figure 16.

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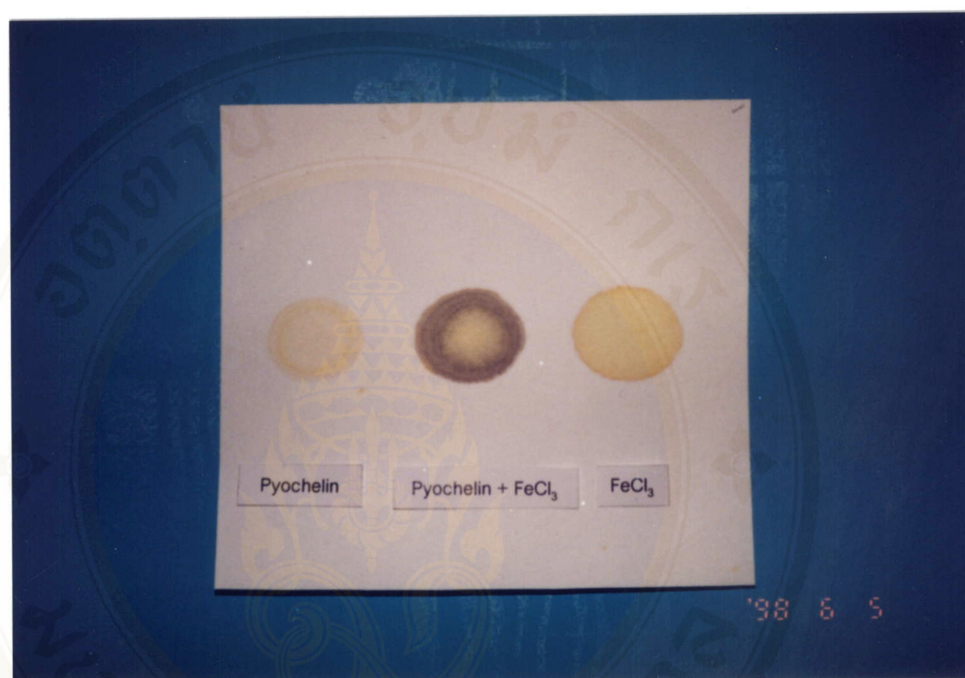

Figure 15 The iron reactivity of the pyochelin was verified by spraying the pyochelin with an iron reagent (0.1 M $FeCl_3$ in 0.1 N HCl) The result was red brown.

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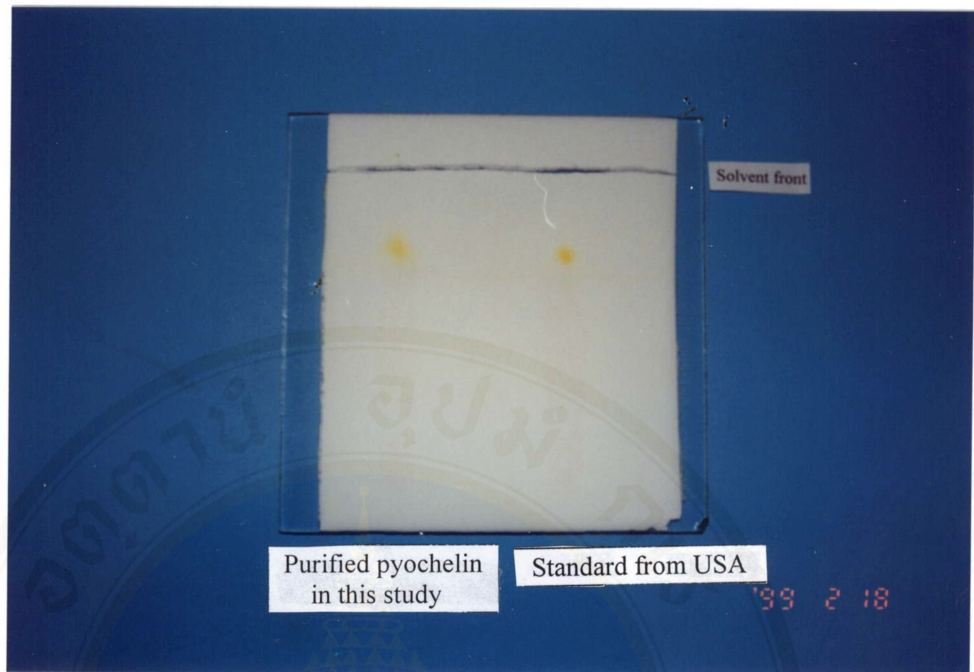

Figure 16 Comparative study on the thin layer chromatography, between purified pyochelin in this study and the standard pyochelin obtained from Prof. CD. Cox, USA.

Figure 17 Iron reactivity of purified pyochelin in this study after spraying with an iron reagent.

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II. Extraction, purification and characterization of pyoverdin

Purification of pyoverdin from 500 ml spent culture medium (CAA broth) yielded dry weight of 8 mg yellow powder.

The purified pyoverdin was qualitatively characterized by

1. Yellow-green fluorescence emission under UV light.
2. The iron reactivity of the pyoverdin was verified (33,94) by spraying the pyoverdin with an iron reagent (0.1 M $FeCl_3$ in 0.1 N HCl). The result was blue-violet as shown in Figure 18 and Figure 20.
3. Comparative study on the paper chromatography between the purified pyoverdin and the standard pyoverdin obtained from Professor CD. Cox at University of Iowa, USA concerning mobility by using H_2O-acetic acid-ethanol (8:1:1) as the development solvent gave the same result as shown in Figure 19.

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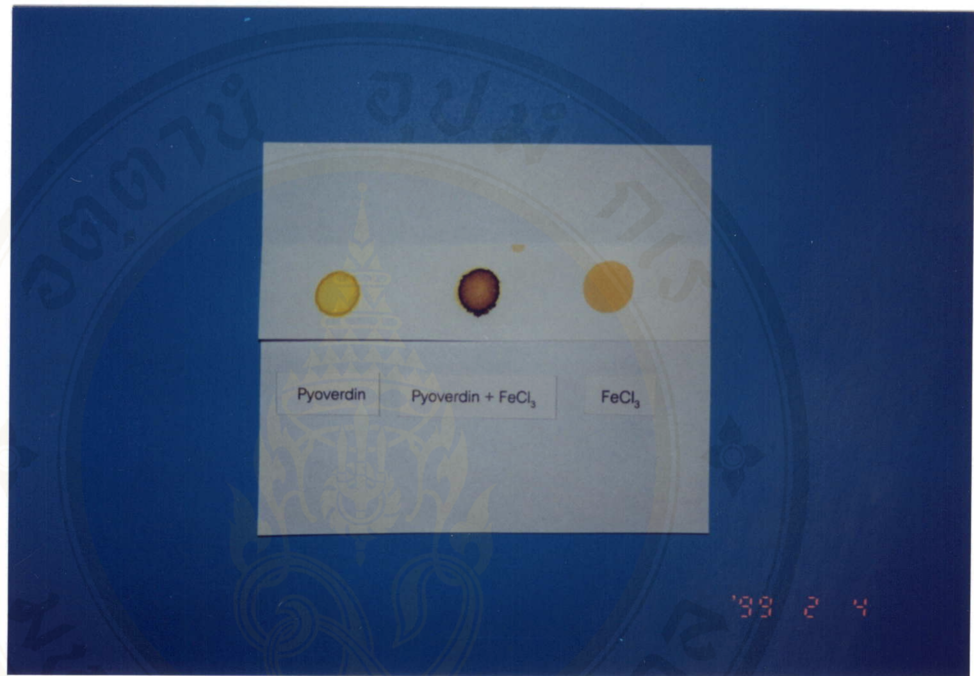

Figure 18 The iron reactivity of the pyoverdin was verified by spraying the pyoverdin with an iron reagent (0.1 M $FeCl_3$ in 0.1 N HCl) The result was blue-violet.

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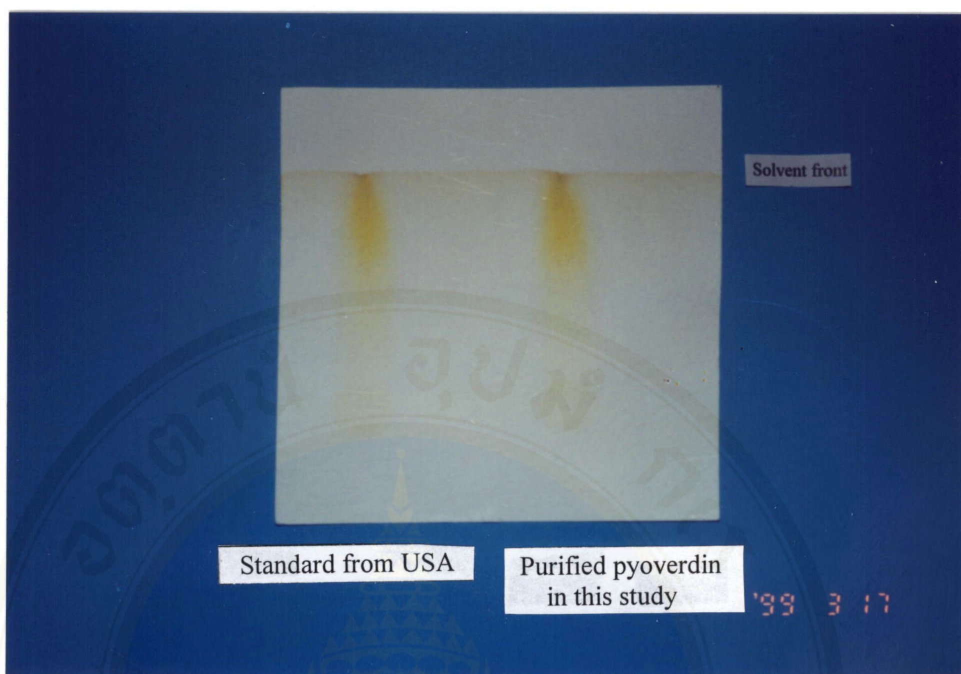

Figure 19 Comparative study on the paper chromatography, between the standard pyoverdin obtained from Prof. CD. Cox, USA and purified pyoverdin in this study.

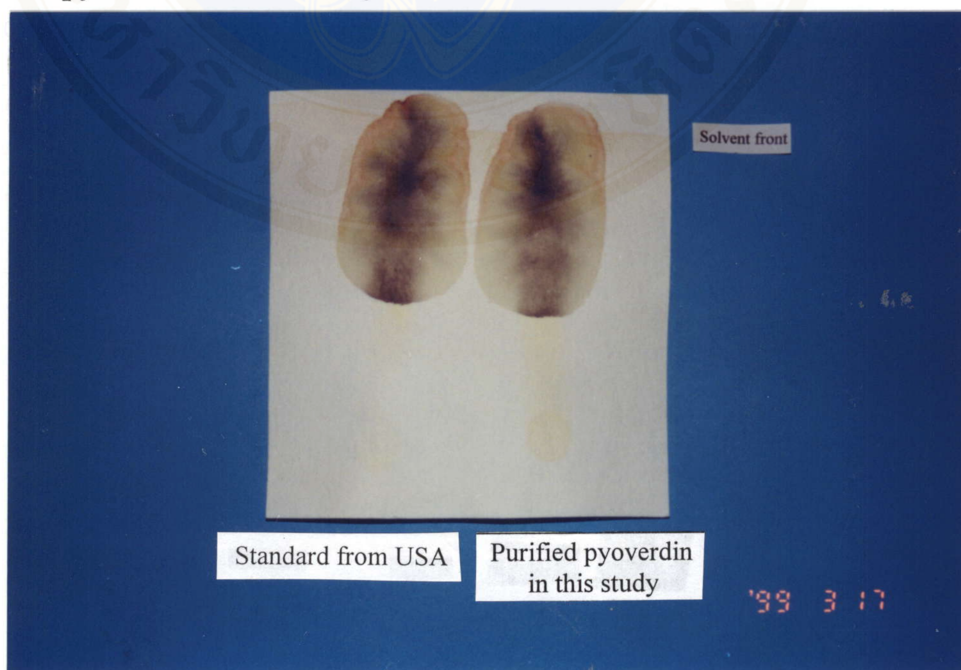

Figure 20 Iron reactivity of standard pyoverdin from USA and purified pyoverdin in this study after spraying with an iron reagent.

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III. Effect of pyoverdin, pyochelin and Desferal on iron mobilization from ^{59}Fe-ferritin in Tris buffer at pH 6.0 after 48 hours of incubation

It was found that the siderophores and Desferal were capable of iron mobilization from [^{59}Fe]-ferritin. Desferal was most effective in iron mobilization, followed by pyoverdin and pyochelin respectively. It was noticeable that pyochelin was very little effective in iron mobilization. Interestingly, the combination of pyoverdin and pyochelin at the concentration of 10 µg/ml each gave an effect of iron mobilization equal to that of pyoverdin alone. The iron mobilization was shown as the amount of radioactivity in counts per minute appeared in the dialysate side. The results were shown in Figure 21 and Table 6.

The effectiveness in iron mobilization of the siderophores and Desferal were also studied in Tris buffer at pHs 6.5, 7.0 and 7.4 after 48 hours of incubation at 4 °C. The results showed similar trends as that of experiment performed in Tris buffer at pH 6.0. The results were shown in Figures 22, 23, 24 and Table 7, 8, 9 respectively. Interestingly, the siderophores and Desferal had more significant effects on iron mobilization at lower pHs compared to pH 7.4. This might be due to a decrease in the values of the association constants of iron with ferritin when the pH of buffer was lowered from 7.4. Therefore, the siderophores and Desferal may mobilize iron easier at these acid pHs (6.0-7.0) than at pH 7.4.

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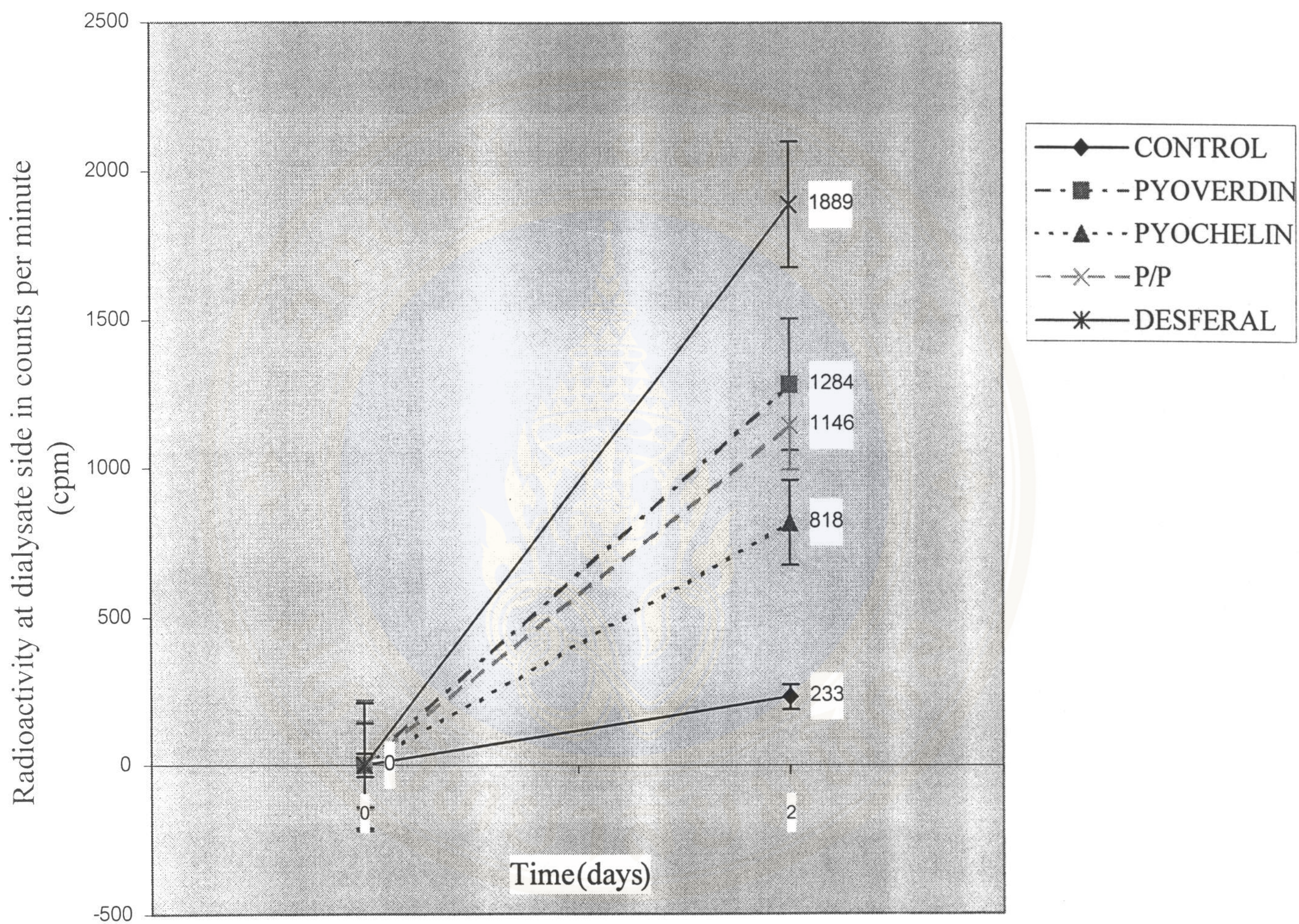

Figure 21 Effect of pyoverdin, pyochelin and Desferal on iron mobilization from [^{59}Fe]-ferritin in Tris buffer pH 6.0 after 48 hours of incubation.

* P/P = Pyoverdin+Pyochelin

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Table 6. Radioactivity in counts per minute (cpm) at dialysate side in Tris buffer at pH 6.0 after 48 hours of incubation.

	Control	Pyoverdin (Pvd)	Pyochelin (Pch)	Pvd+Pch	Desferal
Tube 1	199	1380	667	1208	2119
Tube 2	222	1441	949	973	1696
Tube 3	280	1031	839	1259	1853
Mean ± S.D.	234 ± 42	1284 ± 221	818 ± 142	1147 ± 153	1889 ± 214
t-test		-7.69	-6.837	-10	-7.743
p-value		< 0.05	< 0.05	< 0.05	< 0.05

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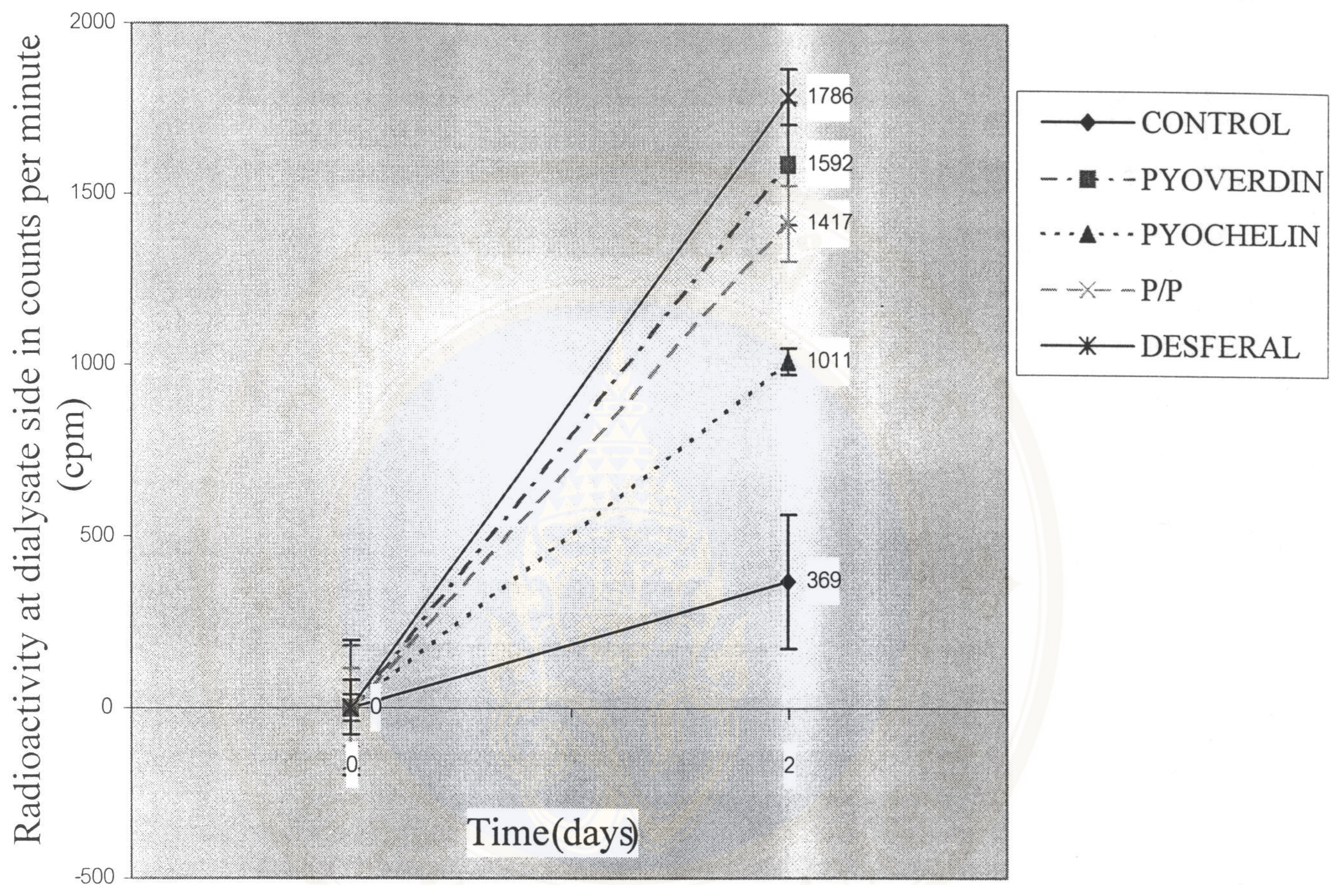

Figure 22 Effect of pyoverdin, pyochelin and Desferal on iron mobilization from [^{59}Fe]-ferritin in Tris buffer pH 6.5 after 48 hours of incubation.

* P/P = Pyoverdin+Pyochelin

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Table 7. Radioactivity in counts per minute (cpm) at dialysate side in Tris buffer at pH 6.5 after 48 hours of incubation.

	Control	Pyoverdin (Pvd)	Pyochelin (Pch)	Pvd+Pch	Desferal
Tube 1	202	1401	1000	1384	1816
Tube 2	319	1616	1054	1543	1696
Tube 3	586	1760	979	1325	1848
Mean ± S.D.	369 ± 197	1592 ± 181	1011 ± 39	1147 ± 113	1786 ± 80
t-test		-7.931	-5.544	-8.005	-11.55
p-value		< 0.05	< 0.05	< 0.05	< 0.05

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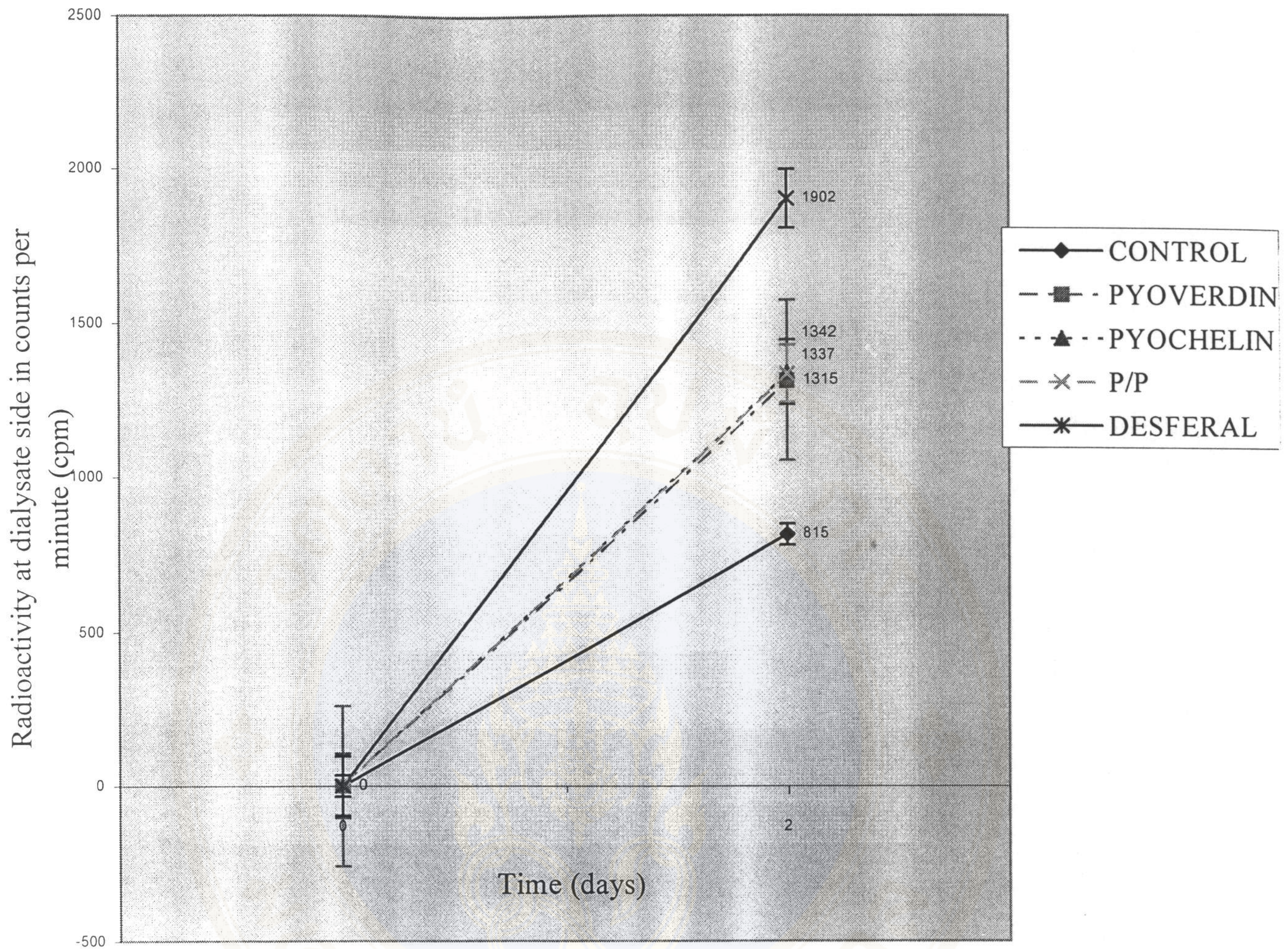

Figure 23 Effect of pyoverdin, pyochelin and Desferal on iron mobilization from [^{59}Fe]-ferritin in Tris buffer pH 7.0 after 48 hours of incubation.

* P/P = Pyoverdin+Pyochelin

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Table 8. Radioactivity in counts per minute (cpm) at dialysate side in Tris buffer at pH 7.0 after 48 hours of incubation.

	Control	Pyoverdin (Pvd)	Pyochelin (Pch)	Pvd+Pch	Desferal
Tube 1	855	1525	1422	1367	1797
Tube 2	803	1025	1381	1234	1987
Tube 3	789	1396	1223	1412	1922
Mean ± S.D.	816 ± 35	1315 ± 260	1342 ± 105	1338 ± 93	1902 ± 97
t-test		-3.304	-8.238	-9.145	-18.337
p-value		< 0.05	< 0.05	< 0.05	< 0.05

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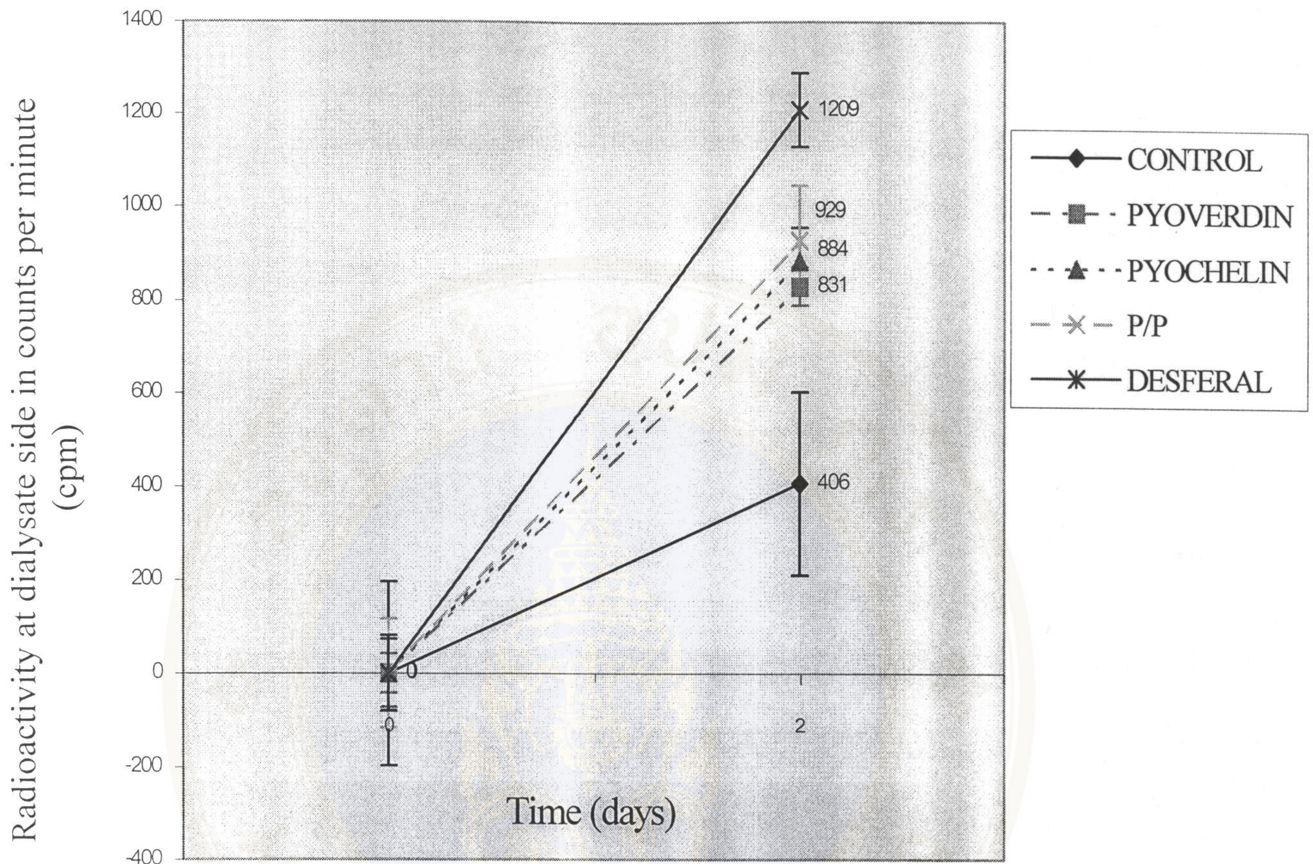

Figure 24 Effect of pyoverdin, pyochelin and Desferal on iron mobilization from [^{59}Fe]-ferritin in Tris buffer pH 7.4 after 48 hours of incubation.

* P/P = Pyoverdin+Pyochelin

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Table 9. Radioactivity in counts per minute (cpm) at dialysate side in Tris buffer at pH 7.4 after 48 hours of incubation.

	Control	Pyoverdin (Pvd)	Pyochelin (Pch)	Pvd+Pch	Desferal
Tube 1	632	812	823	1052	1244
Tube 2	272	803	866	914	1117
Tube 3	315	879	964	821	1266
Mean ± S.D.	406 ± 197	831 ± 42	884 ± 72	929 ± 116	1209 ± 80
t-test		-3.66	3.95	-3.941	-6.545
p-value		< 0.05	< 0.05	< 0.05	< 0.05

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IV. Effect of pyoverdin, pyochelin and Desferal at equal molar ratio in iron mobilization from [^{59}Fe]-ferritin in Tris buffer at pH 6.0 after 48 hours of incubation

The equal molar ratio of pyoverdin, pyochelin and Desferal were used in the experiment i.e., 15 nM in Tris buffer at pH 6.0. After 48 hours of incubation, it was found that they were effective in iron mobilization form [^{59}Fe]-ferritin compared to the control, which contained only buffer. On the other hand, pyochelin gave little effective in iron mobilization. The results were shown in Figure 25 and Table 10.

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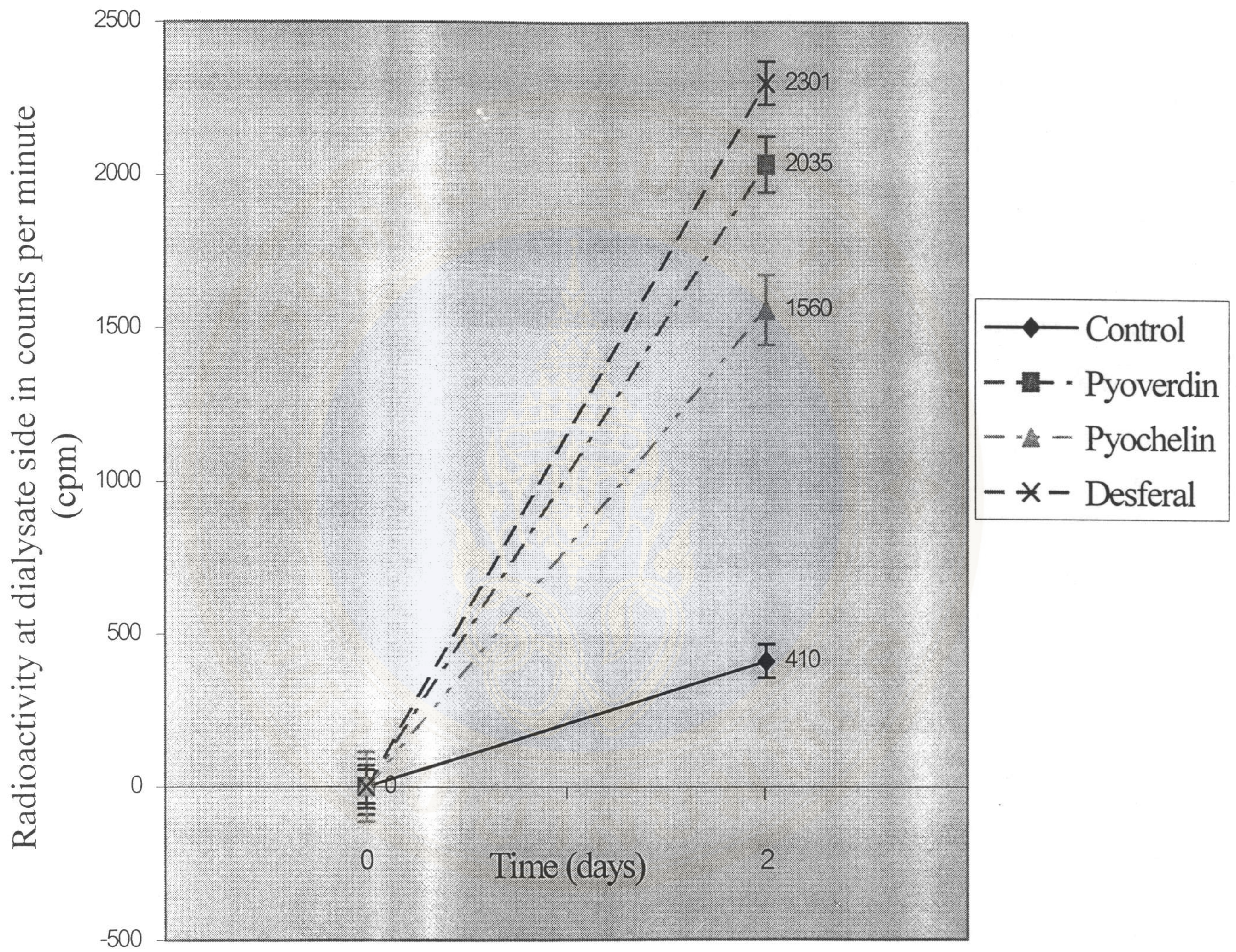

Figure 25 Comparison between the activities of pyoverdin, pyochelin and Desferal at equal molar ratio in iron mobilization from [^{59}Fe]-ferritin.

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Table 10. Radioactivity in counts per minute (cpm) at dialysate side of pyoverdin, pyochelin and Desferal at equal molar ratio in Tris buffer at pH 6.0 after 48 hours of incubation.

	Control	Pyoverdin	Pyochelin	Desferal
Tube 1	403	2022	1668	2230
Tube 2	358	1952	1661	2302
Tube 3	468	2132	1468	2371
Mean ± S.D.	410 ± 55	2035 ± 91	1560 ± 114	2301 ± 71
t-test		-26.498	-15.736	-35.561
p-value		< 0.05	< 0.05	< 0.05

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V. Effect of siderophore and Desferal in iron mobilization from [^{59}Fe]-transferrin in Tris buffer at pH 6.0 and 7.4 after 48 hours of incubation

From the experiment; Desferal, pyoverdin, pyochelin each at 10 μg/ml or the combination of pyoverdin and pyochelin were not effective in iron mobilization from [^{59}Fe]-transferrin both in Tris buffer pH 6.0 and 7.4 after 48 hours of incubation. Their radioactivities were not significantly different from the control, which contained only buffer. The results were shown in Tables 11 and 12.

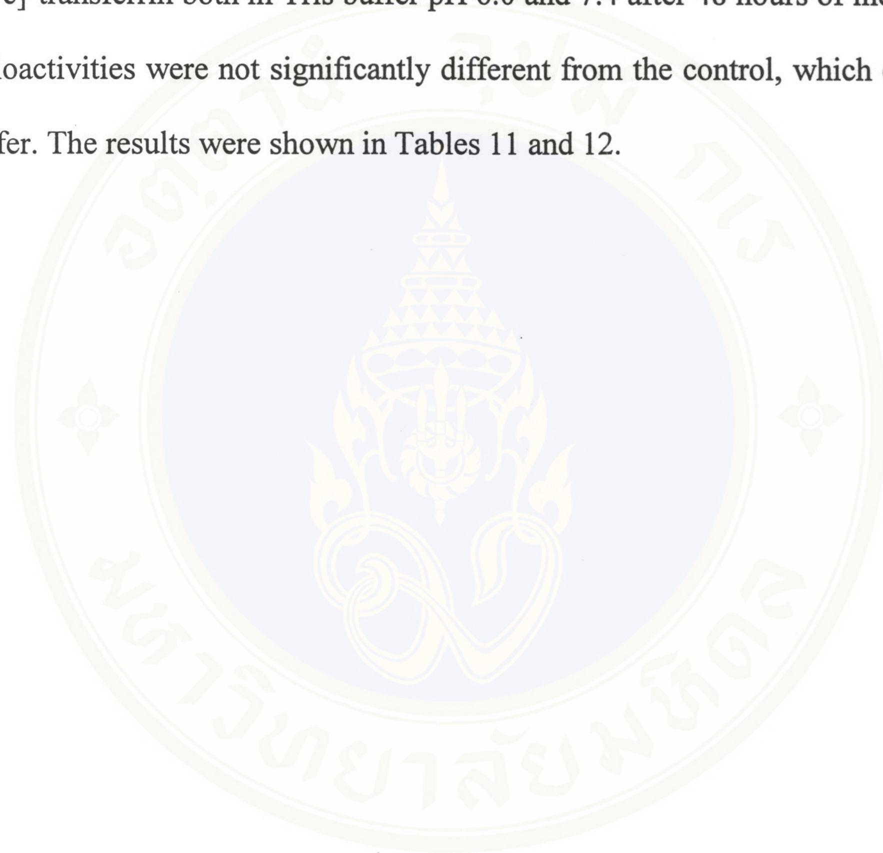

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Table 11. Radioactivity of Fe^{59} in counts per minute (cpm) from [^{59}Fe]-transferrin at dialysate side in Tris buffer at pH 6.0 after 48 hours of incubation.

	Control	Pyoverdin (Pvd)	Pyochelin (Pch)	Pvd+Pch	Desferal
Tube 1	1845	1936	1795	1980	1818
Tube 2	1588	2102	1775	2339	1903
Tube 3	1951	1856	1784	1815	1882
Mean ± S.D.	1795±187	1965±125	1785±10	2045±268	1868±44
t-test		-1.309	0.092	-1.325	-0.658
p-value		> 0.05	> 0.05	> 0.05	> 0.05

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Table 12. Radioactivity of Fe^{59} in counts per minute (cpm) from [^{59}Fe]-transferrin at dialysate side in Tris buffer at pH 7.4 after 48 hours of incubation.

	Control	Pyoverdin (Pvd)	Pyochelin (Pch)	Pvd+Pch	Desferal
Tube 1	2057	2005	2314	2684	2804
Tube 2	2308	2608	1831	2873	2860
Tube 3	2362	2718	2477	2250	2474
Mean ± S.D.	2242±163	2444±284	2207±336	2602±319	2713±209
t-test		-1.068	0.162	-1.741	-3.078
p-value		> 0.05	> 0.05	> 0.05	> 0.05

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VI. Effect of various pyoverdin concentrations for iron mobilization from [^{59}Fe]-ferritin in Tris buffer at pH 6.0 after 48 hours of incubation.

The effects of pyoverdin at various concentrations i.e., 1, 3, 5, 10, 20 and 40 μg/ml on iron mobilization from [^{59}Fe]-ferritin were shown in Figure 26 and Table13 respectively. Pyoverdin at concentration of 1 μg/ml had no effect on iron mobilization. However, pyoverdin at higher concentrations i.e., 3 or 5 μg/ml showed approximately 3 folds in increasing iron mobilization from [^{59}Fe]-ferritin in comparison to the control which did not contain pyoverdin. It was found that pyoverdin at 10 μg/ml increased iron mobilization 8 folds in comparison to the control. Interestingly, pyoverdin at concentrations of ≥ 20μg/ml showed the same effectiveness on iron mobilization as that of pyoverdin at 10 μg/ml. Therefore, the following experiments in this research, pyoverdin was used at 10 μg/ml. In addition, this concentration was used because it was possible to find this concentration of pyoverdin in the supernatant of *P. aeruginosa* in CAA broth culture after incubation at 35 ° C for 2 days (Prof. C.D. Cox, Univ. of Iowa, USA, personal communication).

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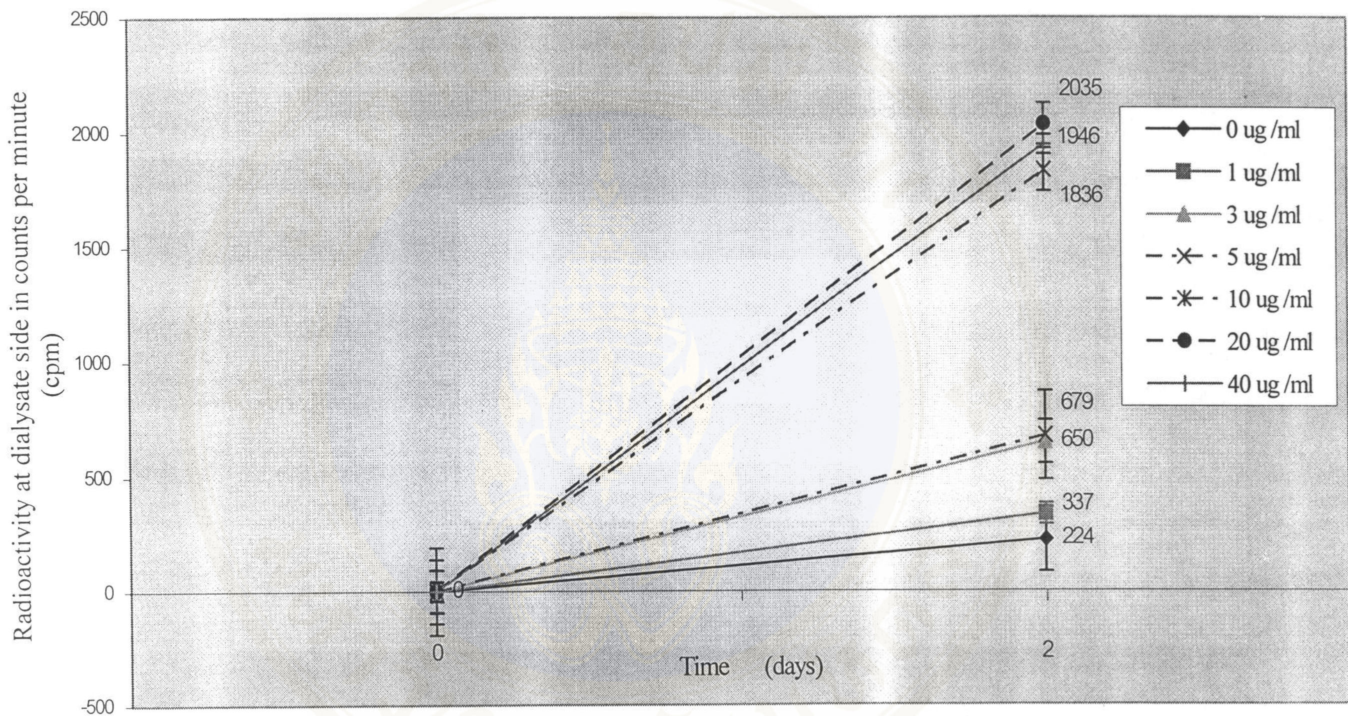

Figure 26 Effect of various pyoverdin concentrations for iron mobilization from [^{59}Fe]-ferritin in Tris buffer at pH 6.0 after 48 hours of incubation.

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Table 13. Radioactivity in counts per minute (cpm) at dialysate side of various pyoverdin concentrations in Tris buffer pH 6.0 after 48 hours of incubation.

Pyoverdin (μg/ml)	0	1	3	5	10	20	40
Tube 1	150	337	715	861	1899	2132	1966
Tube 2	137	429	694	698	1730	1952	1975
Tube 3	387	367	543	478	1881	2022	1989
Mean ± S.D.	225±141	338±47	651±94	679±192	1837±93	2035±91	1946±42
t-test		-1.319	-4.363	-3.303	-16.562	-18.728	-20.3
p-value		> 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

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VII. Effect of various pyochelin concentrations for iron mobilization from [^{59}Fe]-ferritin in Tris buffer at pH 6.0 after 48 hours of incubation.

The effects of pyochelin at various concentrations i.e., 1, 3, 5, 10, 20 and 40 μg/ml on iron mobilization from [^{59}Fe]-ferritin were shown in Figure 27 and Table14 respectively. Pyochelin at concentration of 1 μg/ml or 3 μg/ml had no effect on iron mobilization. However, pyochelin at higher concentrations e.g., 5 μg/ml showed approximately 1.5 folds in increasing iron mobilization from [^{59}Fe]-ferritin in comparison to the control, which did not contain pyochelin. It was found that pyochelin at 10 μg/ml increased iron mobilization 2.5 folds in comparison to the control. Interestingly, pyochelin at concentrations of ≥ 20μg/ml showed the same effectiveness on iron mobilization as that of pyochelin at 10 μg/ml. Therefore, the following experiments in this research, pyochelin was used at 10 μg/ml. In addition, this concentration was used because it was possible to find this concentration of pyochelin in the supernatant of *P. aeruginosa* in CAA broth culture after incubation at 35 ° C for 2 days (Prof. C.D. Cox, Univ. of Iowa, USA, personal communication).

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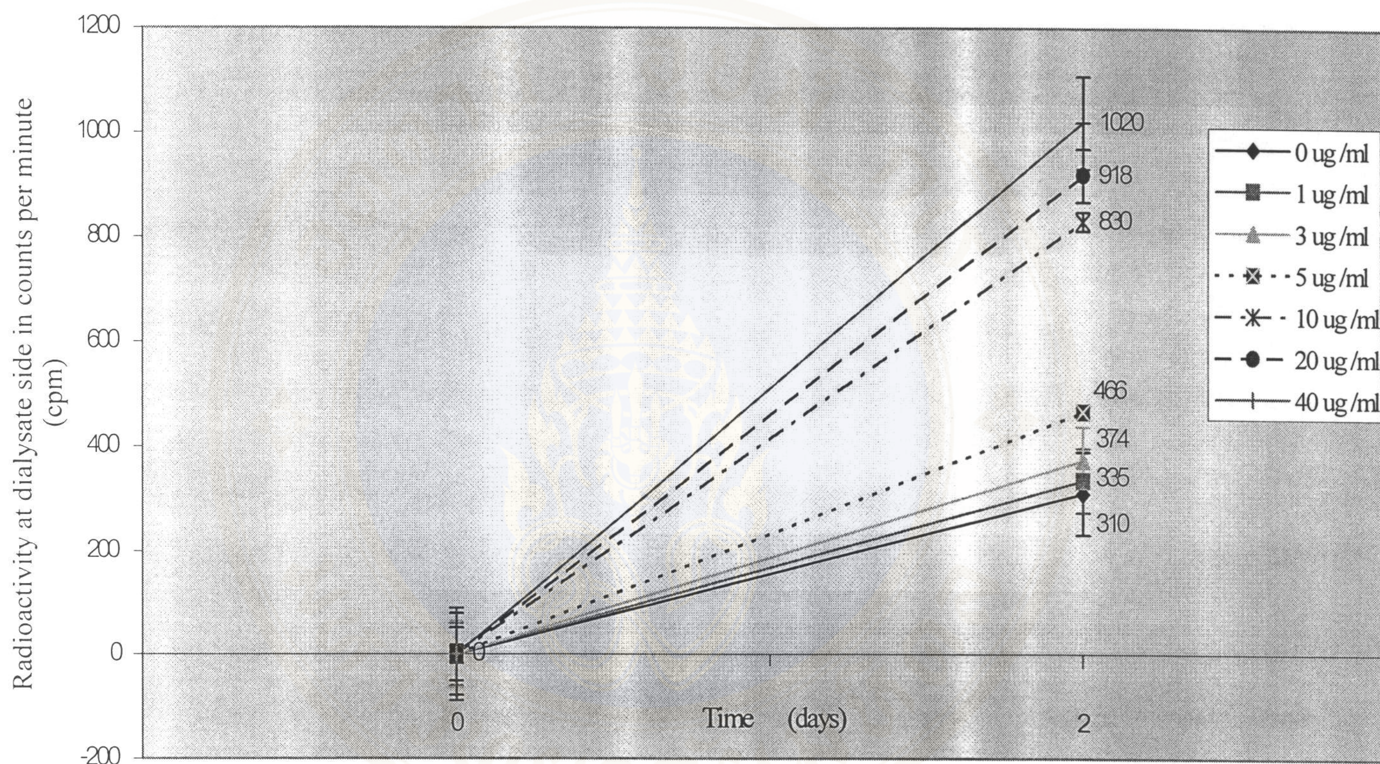

Figure 27 Effect of various pyochelin concentrations for iron mobilization from [^{59}Fe]-ferritin in Tris buffer at pH 6.0 after 48 hours of incubation.

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Table 14. Radioactivity in counts per minute (cpm) at dialysate side of various pyochelin concentrations in Tris buffer pH 6.0 after 48 hours of incubation.

Pyochelin (µg/ml)	0	1	3	5	10	20	40
Tube 1	326	354	309	473	816	924	1034
Tube 2	381	268	376	456	824	967	925
Tube 3	225	385	437	466	850	865	1102
Mean ± S.D.	311±79	336±61	374±64	466±9	830±18	919±51	1020±89
t-test		-0.433	-1.078	-3.379	-11.092	-11.172	-10.283
p-value		> 0.05	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05

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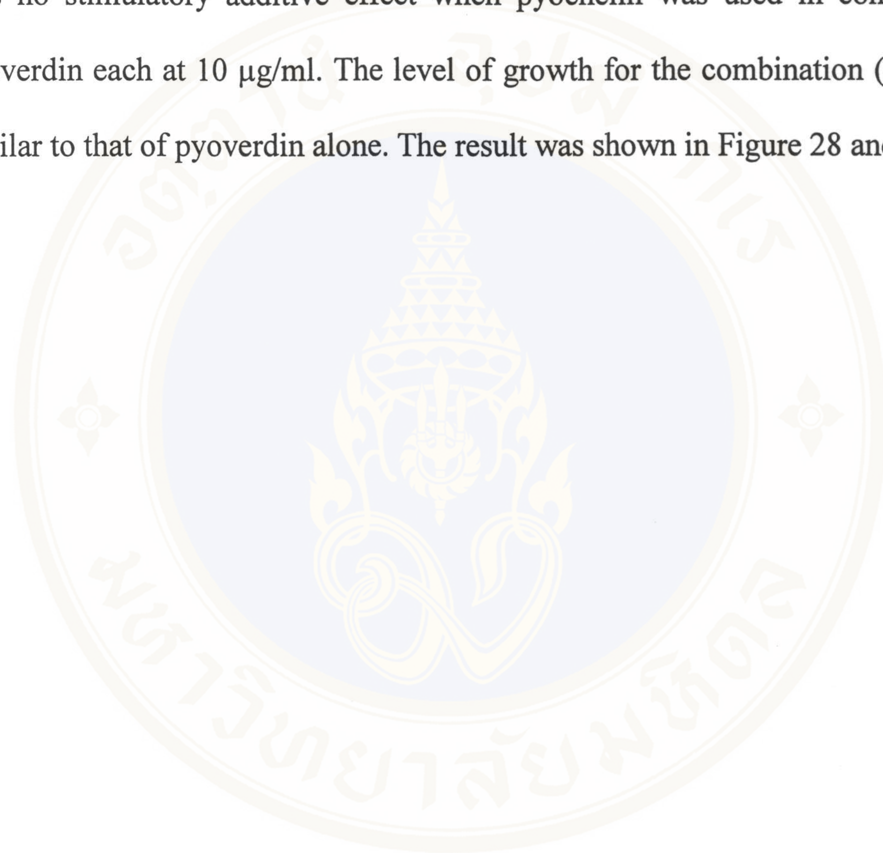
VIII. Growth assay of *P. aeruginosa* standard strain PAO1 by siderophores in glucose minimal medium.

Pyoverdin, pyochelin and Desferal were effective in promoting growth of *P. aeruginosa* standard strain PAO1 in glucose minimal medium (GMM). However there was no stimulatory additive effect when pyochelin was used in combination with pyoverdin each at 10 μg/ml. The level of growth for the combination (Pvd+Pch) was similar to that of pyoverdin alone. The result was shown in Figure 28 and Table 15.



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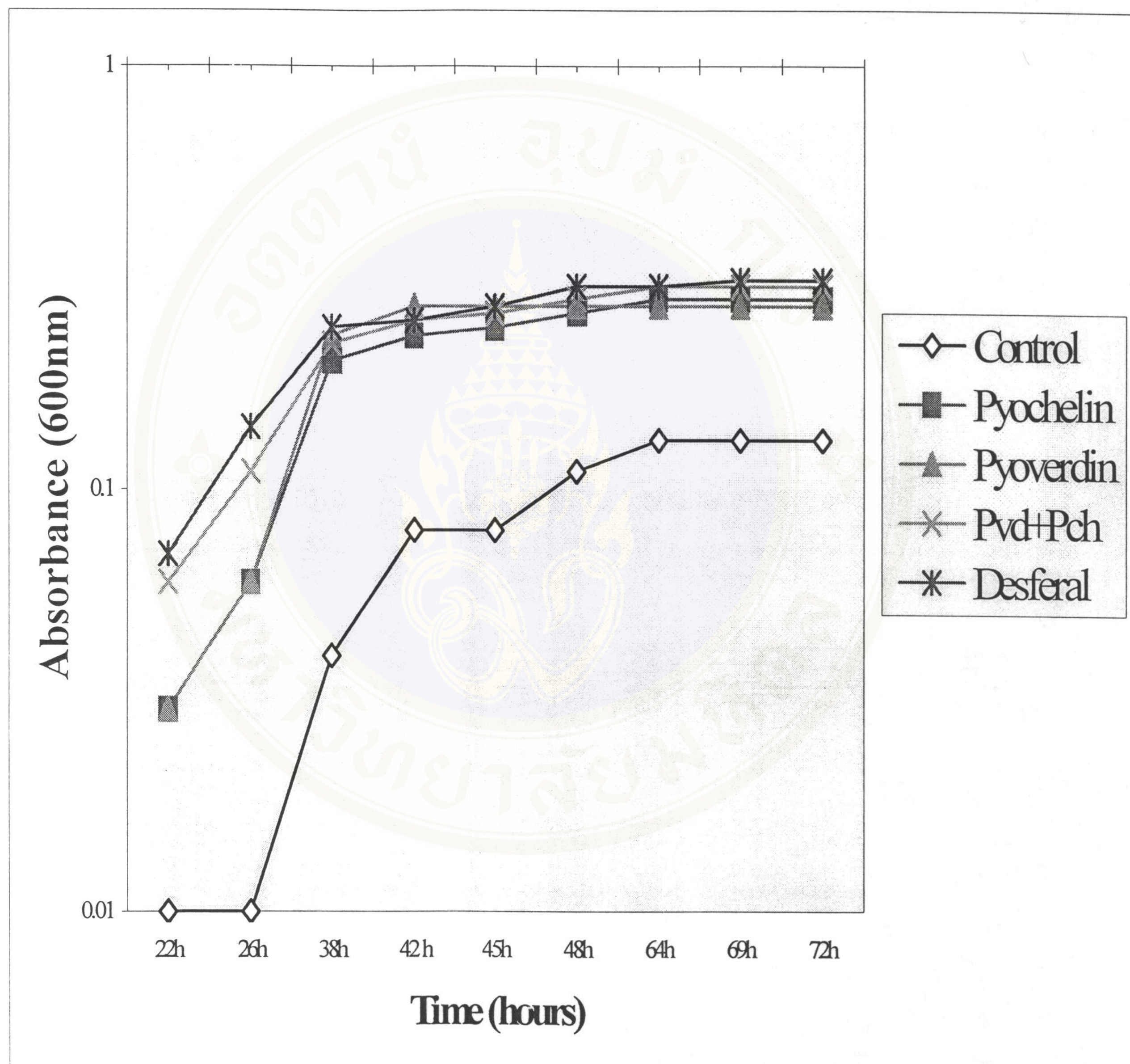

Figure 28 Effects of added siderophores or Desferal on growth stimulation in glucose minimal medium

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Table 15. Effect of added siderophores or Desferal on growth stimulation in glucose minimal medium (GMM)

Time of incubation (hours)	Absorbance (600 nm)				
	Control	Pyochelin (Pch)	Pyoverdin (Pvd)	Pvd+Pch	Desferal
0	0	0	0	0	0
22	0.01	0.03	0.03	0.06	0.07
26	0.01	0.06	0.06	0.11	0.14
38	0.04	0.20	0.23	0.22	0.24
42	0.08	0.23	0.27	0.25	0.25
45	0.08	0.24	0.27	0.26	0.27
48	0.11	0.26	0.27	0.28	0.30
64	0.13	0.28	0.27	0.30	0.30
69	0.13	0.28	0.27	0.30	0.31
72	0.13	0.28	0.27	0.30	0.31

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IX. Growth assay of *P. aeruginosa* standard strain PAO1 by siderophores in medium containing transferrin or heat-inactivated normal human serum.

Presumably, pyoverdin was active in promoting bacterial growth in normal human serum by providing iron to the bacteria. To test this presumption, *P. aeruginosa* standard strain PAO1 was inoculated at a final concentration of 10^2 CFU/ml into glucose minimal medium (GMM) containing 10% (V/V) heat-inactivated normal human serum. Purified pyoverdin and Desferal stimulated the bacterial growth which was detected by spreading 0.1 ml of each suspension for determining plate counts on triplicate plates of Mueller Hinton agar and incubated for 18 hours at 35°C. Pyochelin was also effective but to a lesser extent. The results were shown in Table 16. Pyoverdin, pyochelin or the combination was added each at a final concentration of 10 μg/ml, a level that was below the visible detection of yellow color but was detectable by fluorescence after excitation by hand-held UV lamp.

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Table 16. Colony plate counts of *P. aeruginosa* standard strain PAO1 at 0 and 18 hours of incubation in medium containing heat-inactivated human serum.

Time of incubation	Colony plate count (CFU/ml)			
	Control	Pyoverdin	Pyochelin	Desferal
At 0 hour	3.1×10^{2}	2.9×10^{2}	2.7×10^{2}	2.8×10^{2}
At 18 hours	1.73×10^{8}	3.53×10^{12}	8.86×10^{9}	4.3×10^{12}

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X. Bacterial pathogens isolated from hemoculture of thalassemia patients admitted to Siriraj Hospital in 1994-1998.

When the data of hemoculture obtained from thalassemia patients admitted to Siriraj Hospital was analyzed during the five-year periods (1994-1998). From 364 thalassemia patients, the positive culture was 14.84% (57/364). It was found that the five most common bacteria isolated were *Salmonella* (group D, A and C) (22.50%) followed by coagulase negative staphylococci (12.06%), *Escherichia coli* (8.62%), Nonfermentative gram-negative rod (8.62%).

Noticeably, *Pseudomonas aeruginosa*, which is one of the important nosocomial pathogens, appeared to be the fourth rank pathogen isolated from hemoculture of the thalassemia patients in this study. The result was shown in Table 17.

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Table 17. Five most common bacteria isolated from hemoculture of thalassemia patients admitted to Siriraj Hospital in 1994-1998

Bacteria	**Percent (%)**
Salmonella (group D, A and C)	22.50
Coagulase-negative staphylococci	12.06
Escherichia coli	8.62
Non-Fermentative gram-negative rod	8.62
Klebsiella pneumoniae	5.17
Pseudomonas aeruginosa	5.17
Vibrio parahaemolyticus	3.44
Group A streptococci	3.44

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CHAPTER V

DISCUSSION

In this investigation the relative activities of siderophores produced by *P. aeruginosa* and Desferal produced by *Streptomyces pilosus* were tested concerning the mobilization of iron from [^{59}Fe]-ferritin and [^{59}Fe]-transferrin. The growth assay of *P. aeruginosa* standard strain PAO1 by siderophores and Desferal was also studied.

Pyoverdin in this study was purified by modification of the method described by Professor CD Cox at University of Iowa, USA. For pyochelin purification the original method was used (34).

The result of purified pyoverdin and pyochelin in this study have the same qualification as the standard pyoverdin and pyochelin obtained from Professor CD Cox at University of Iowa, USA.

The dialysis assay, which was modified from the method described by Simmonson et al. (98) was used to determine the activities of *P. aeruginosa* siderophores. The dialysis membrane with M.W. cut off 8,000 prevented the diffusion of [^{59}Fe]-ferritin or [^{59}Fe]-transferrin into the dialysate side. Therefore, the increasing in radioactivity in the dialysate side when the siderophores (pyoverdin or pyochelin) was added into the system indicated the iron mobilization effect. This could be seen in Figures 21, 22, 23, 24 or Tables 6, 7, 8, 9 which showed that ^{59}Fe was detectable in the dialysate side. By using two siderophores, pyoverdin and pyochelin, which were

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produced by *P. aeruginosa*, the activity to mobilize iron from [^{59}Fe]-ferritin across the dialysis membrane was statistically significant ($p<0.05$) at pH 6.0, 6.5, 7.0 and 7.4

The siderophores and Desferal had more significant effects on iron mobilization from [^{59}Fe]-ferritin at lower pHs compared to pH 7.4. This might be due to a decrease in the values of the association constants of iron with ferritin when the pH of buffer was lowered from 7.4. Therefore, the siderophores and Desferal may mobilize iron easier at this acid pH (6.0-7.0) than at pH 7.4.

For [^{59}Fe]-transferrin; the siderophores lacked activities to mobilize iron across the dialysis membrane at pH 6.0 and 7.4 (Tables 11, 12). When other types of buffer e.g. sodium phosphate buffer at various pHs were tested the results were still the same (data not shown). The siderophores showed no activity to mobilize iron from [^{59}Fe]-transferrin across the dialysis membrane. It is probably explained that normal human serum, which was used in this assay, the Fe saturated in transferrin was only 30% when compared with thalassemia patients Fe saturated in transferrin is 100%. Therefore the trend of mobilizing Fe^{59} from [^{59}Fe]-transferrin may be increased, if sera from thalassemia patients were used.

Although both siderophores were active in mobilizing iron from [^{59}Fe]-ferritin, pyochelin did not appear to add to the activity of pyoverdin when the siderophores were combined during dialysis membrane assays. In other words the combination of pyoverdin and pyochelin gave an effect equal to pyoverdin alone.

We used the siderphores at concentrations, which are present in most culture media and at equal molarity to compare the activities of iron mobilization from [^{59}Fe]-ferritin. The optimal concentrations of pyoverdin and pyochelin were 10 μg/ml.

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The interesting and important result from bacterial growth assay was that pyoverdin was active in promoting growth in human serum to the same extent as Desferal (96). Pyochelin was less capable of stimulating bacterial growth. The results were correlated with the binding coefficients of the siderophores for iron. The iron binding coefficient of pyoverdin and Desferal are 10^{32} (41) and 10^{31} (1) respectively which are much higher than that of pyochelin which is approximately 10^{5} (34). Therefore, pyoverdin and Desferal are capable of providing iron to bacteria more than pyochelin. Studying the growth stimulating factors were interesting since a majority of people suffering from *P. aeruginosa* infections have impaired defense mechanisms.

In growth assay; pyoverdin, pyochelin and Desferal were effective in promoting growth of *P. aeruginosa* standard strain PAO1 in glucose minimal medium (GMM). However there was no stimulatory additive effect when pyochelin was used in combination with pyoverdin each at 10 μg/ml. The level of growth for the combination (pyoverdin+pyochelin) was similar to that of pyoverdin alone. Therefore, pyoverdin appeared to by more effective than pyochelin in these assays.

When activities of pyoverdin and Desferal were compared. Pyoverdin demonstrated a little less activity than Desferal. In summary pyoverdin demonstrated greater activity than pyochelin in the dialysis membrane assay and growth assay in glucose minimal medium, when the two siderophores were tested separately (Table 17). This may correspond to previous report (36).

From this study the data of hemoculture obtained from thalassemic patients admitted to Siriraj Hospital during the 5 year period (1994-1998) was analyzed. It was found that the most bacteria isolated was *Salmonella* (group D, A and C) (22.50%) followed by coagulase-negative staphylococci (12.06%), which only positive cultures

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2/3 or 3/3 of hemoculture bottles were chosen for this bacterium. If there was positive culture 1/3, the result would be disregarded. The exception was when there was only 1 hemoculture bottle from 1 pediatric patient. The positve result in this case would be counted.

From the data of hemoculture on Table 17 and growth assay, showed that pyoverdin, pyochelin and Desferal are also capable of donating iron to a range of bacteria to activated growth and division of bacteria in bacterial infection patients receiving them. Consequently it is essential to design a chelator capable of forming an iron (III) complex e.g., pyoverdin analogue that does not bind to iron receptors on outer membrane of microorganism.

Form the qualification and structure of pyoverdin that was purified in this study, it may be orally active because the structure is hydrophillic.

This study is a part of preliminary development for iron chelating drugs. Therefore many properties required for an ideal iron chelator need to be studied. The siderophore produced by *P. aeruginosa* i.e. pyoverdin might be used in place of Desferal in the future upon more advanced research. The development of this siderophore might allow an alternative therapeutic agent for thalassemia patients, who need red cell transfusions. Pyoverdin is cheap and easy to produce. If pyoverdin or its analogue can be given to thalassemia patients orally and are not toxic, they will be very useful. Desferal is difficult to prepare, must be given only by intravenous or subcutaneous infusion slowly for 8-12 hours per day and very expensive.

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CHAPTER VI

CONCLUSION

The present study can be concluded as follows:

1. Two types of siderophores i.e., pyoverdin and pyochelin were extracted and purified from casamino acid broth culture of *P. aeruginosa* standard strain PAO1 (ATCC15692).
2. Desferal appeared to be most effective in iron mobilization from [^{59}Fe]-ferritin, followed by pyoverdin and pyochelin respectively. The combination of pyoverdin and pyochelin at the concentration of 10 μg/ml each was effective in iron mobilization equal to that of pyoverdin alone. Both pyoverdin and pyochelin were virtually ineffective in iron mobilization from [^{59}Fe]-transferrin in this study.
3. The effectiveness in iron mobilization of the siderophores and Desferal at the concentration of 10 μg/ml were studied in Tris buffer pH 6.0, 6.5, 7.0 and 7.4 after 48 hours of incubation at 4°C in shaking incubator at 30 rpm. The results showed the siderophores and Desferal were able to mobilize ^{59}Fe from [^{59}Fe]-ferritin (dialysis membrane assay) dramatically at lower pHs compared to pH 7.4.
4. At equal molar ratio; Desferal was more effective than pyoverdin in iron mobilization from [^{59}Fe]-ferritin. On the other hand, pyochelin was the least effective siderophore.
5. At various concentrations of pyoverdin and pyochelin tested in iron mobilization (dialysis membrane assay). The concentration of pyoverdin, which appeared to be

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effective in the test system, was more than 3 μg/ml and more than 5 μg/ml for pyochelin. The most effective concentrations of pyoverdin and pyochelin are 10 μg/ml.

6. Pyoverdin, pyochelin and Desferal were effective in promoting growth of *P. aeruginosa* standard strain PAO1 in glucose minimum medium (GMM). This property is in accordance with the general characteristic of microbial siderophore.
7. Pyoverdin, pyochelin and Desferal were effective in promoting bacterial growth in GMM containing 10% heat inactivated normal human serum.
8. The percentage of positive hemoculture from 364 thalassemia patients admitted to Siriraj Hospital during 1994-1998 was 14.84%. It was found that the most common bacteria isolated from total thalassemia patients was *Salmonella* (group D, A and C) (22.50%) followed by coagulase-negative staphylococci (12.06%) and *Escherichia coli* (8.62%), Nonfermentative gram-negative rod (8.62%).

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APPENDIX A

Culture Media

1. Trypticase Soy Broth

Dissolve 30 g of the powder in one liter of distilled water. Mix throughly and then dispense exactly 5 ml in 15 × 150 ml test tube and autoclave at 121 ° C and 15 pound/inch^2 pressure for 15 minutes.

2. Mueller-Hinton Agar

Suspend 38 g of medium in one liter of distilled water. Mix throughly and sterile in the autoclave at 121 ° C and 15 pound/inch^2 pressure for 15 minutes. Pour plate to a uniform dept of 4 mm.

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APPENDIX B

Statistical Method

Unpaired t-test

When (n1 = n2)

$$t = \frac{(\bar{X}_1 - \bar{X}_2) - (\bar{\mu}_1 - \bar{\mu}_2)}{S(\bar{X}_1 - \bar{X}_2)}$$

$$S(\bar{X}_1 - \bar{X}_2) = \sqrt{(S\bar{x}_1)^2 + (S\bar{x}_2)^2}$$

$$df = (n_1 - 1) + (n_2 - 1)$$

$$Sx = SD / \sqrt{n}$$

Statistical significant difference was indicated at p-value < 0.05

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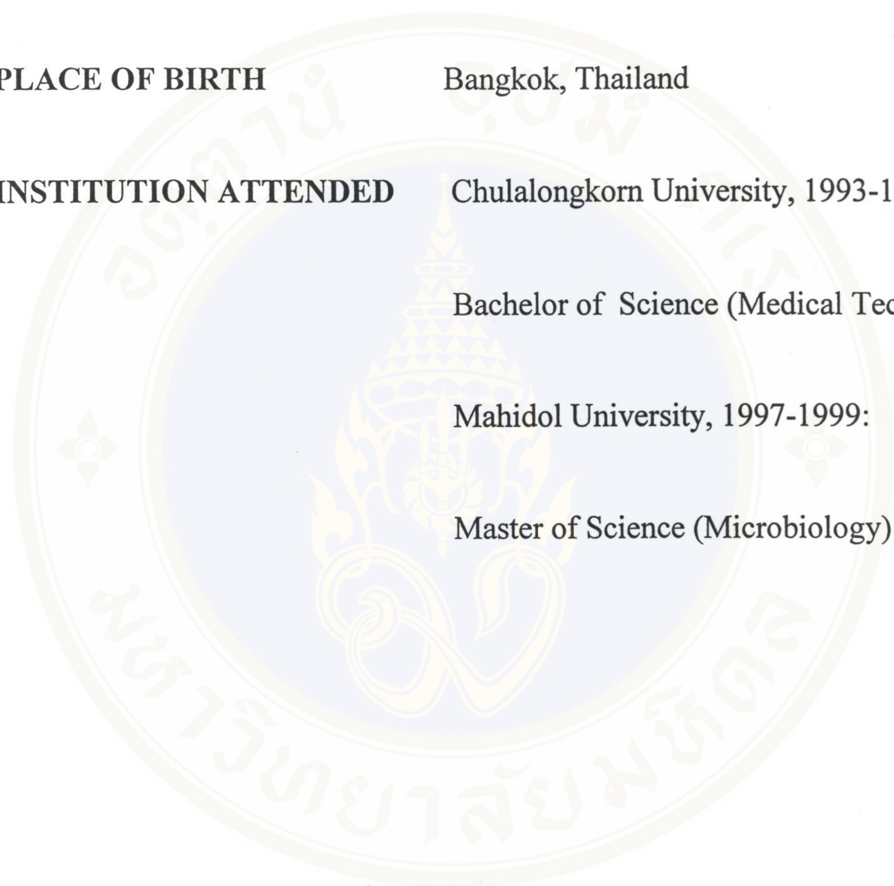

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