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THE EFFECT OF PYOVERDINE$_{P.F.}$ ON THE GROWTH OF *SALMONELLA TYPHIMURIUM* IN THE HEN'S EGG

by

Nalini Shiwnarain

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

WINNIPEG, MANITOBA

OCTOBER 1981

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ACKNOWLEDGEMENTS

The author wishes to acknowledge the advice and direction offered by Dr. G. Blank, thesis supervisor, and to Drs. R.R. Pereira and R. Lyric for their criticisms in the preparation of this thesis.

Appreciation is extended to Dr. E.D. Murray, Head, Department of Food Science for the provision of financial support.

Special appreciation is also extended to fellow students for their friendship and encouragement.

This thesis is dedicated to my parents and my brother, Raj, in sincere appreciation for their loving and constant support.

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ABSTRACT

Shiwnarain, Nalini. M.Sc., The University of Manitoba. October, 1981. The Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in the Hen's Egg.

Major Professor: Dr. Gregory Blank.

Pyoverdine$_{p.f.}$, a water-soluble, yellow-green, fluorescent pigment produced by Pseudomonas fluorescens was isolated and partially purified. Pyoverdine$_{p.f.}$, a known siderochrome, involved in the possible transport and, or metabolism of iron within the bacterial cell was shown to inhibit the growth of Salmonella typhimurium by disc assay. During the course of this investigation, involving both in vivo and in vitro studies with egg albumen, pyoverdine$_{p.f.}$ was shown to inhibit and/or promote the growth of S. typhimurium.

It was found that levels of pyoverdine$_{p.f.}$ greater than 0.4mg/ml albumen (in vitro) exhibited a growth promoting effect on S. typhimurium. Results from in vivo studies using whole eggs indicated that this growth promoting effect of pyoverdine$_{p.f.}$ decreased as the age of the egg increased. This effect, however, was not observed in studies using whole eggs, in vitro. Storage of pyoverdine$_{p.f.}$ in egg albumen (in vivo), also resulted in a decrease in the growth promoting effect on S. typhimurium. Thus it appeared that pyoverdine$_{p.f.}$ was capable of acting as both a sideromycin since, initially, pyoverdine$_{p.f.}$ inhibited the growth of

S. typhimurium via disc assay and, as a sideramine, by exerting a growth promoting effect on S. typhimurium when grown in the egg albumen (in vitro), at levels greater than 0.4 mg/ml pyoverdine$_{p.f.}$/ml albumen.

INTRODUCTION

Fluorescent Pseudomonads are comprised of Pseudomonas aeruginosa, the type species of the genus, P. fluorescens (four biotypes), P. putida (two biotypes), P. chlororaphis, P. aureofaciens, and the two plant pathogenic species P. cichorii and P. syringae (Doudoroff and Palleroni, 1974).

P. fluorescens is the primary spoilage organism of fresh shell eggs and causes "fluorescent sour" of eggs (Elliot, 1958). Under certain conditions, all representatives of the fluorescent Pseudomonads produce water-soluble, yellow-green, fluorescent pigments which have been referred to as bacterial fluoroscein, fluoroscein or pyoverdine. $\text{Pyoverdine}_{\text{p.f.}}$, the fluorescent pigment of P. fluorescens, is produced when the bacterium is cultured in an iron-deficient medium. This pigment is capable of chelating iron by forming a stable Fe (III) complex (Meyer and Abdallah, 1978).

A possible role of pyoverdine in the metabolism of Pseudomonads has been linked to iron transport (Meyer and Abdallah, 1978). To date, whether this pigment plays any role in the infectivity and/or the invasiveness of the host organism or other organisms has not been investigated.

Since P. fluorescens is an ubiquitous pathogen both for humans and plants, it was of interest to investigate and determine the effect of $\text{pyoverdine}_{\text{p.f.}}$ on, and its possible interactions with, other opportunistic micro-

organisms such as Salmonella typhimurium. The hen's egg was chosen since it represents an active ecosystem wherein both P. fluorescens and S. typhimurium can survive and grow (Yadav and Vadehra, 1977).

REVIEW OF LITERATURE

SIDEROCHROMES

Iron exists predominantly as insoluble complexes of Fe(III) in aerobic environments and microorganisms which inhabit these environments must have the means of making iron soluble for transport into the cells for metabolic purposes (Cox and Graham, 1978). Microbial iron transport compounds have been referred to by various authors as siderochromes (Bickel et al., 1960), ferric ionophores (Pressman, 1968) ironophores and ferriphores (Hutner, 1972).

Bickel et al (1960), Prelog (1963), and Keller-Schierlien (1964) were among the first investigators who reported the isolation of iron-containing substances from microbial cells. These substances were similar to each other since they exhibited a maximum absorption band between 420 and 440 nm, and were designated as siderochromes (Bickel et al., 1960) since subsequent chemical studies indicated that they were Fe(III) hydroxamate complexes. Zahner et al. (1962) subdivided the siderochromes into three classes based on their biological properties: sideromycins, are siderochromes which display antibiotic activity; sideramines, are siderochromes which competitively antagonize the antibiotic effect of the sideromycins exerting a growth promoting effect; and siderochromes are those whose biological properties are as yet unknown. The sideromycins were the first type of siderochromes to be reported (Nuesch and Knusel, 1965).

1. MICROBIAL IRON TRANSPORT

Several mechanisms have been reported for the uptake of iron by microorganisms, including the production of siderochromes by aerobic and facultative aerobic microorganisms. These transport systems are known to be part of a "high affinity" mechanism which are only expressed when the organism is, in one way or another, deprived of iron (Neilands, 1974). Analyses of several microbial transport systems suggest that the uptake of most nutrients depends on a reversible combination between the transported substrate and a plasma membrane component (carrier) having a high recognition affinity for the substrate (Harold, 1972). This membrane-associated substance generally considered to be a protein(s) may be deformed by combination with the substrate thereby producing a sufficient thermodynamic shift in which the substrate is exposed to the inner surface of the lipid boundary where it is then released. This is accomplished either because the carrier now has less affinity for the molecules of the substrate, or by transfer of the substrate to a second acceptor molecule. This process is described as facilitated diffusion and does not require nor produce a concentration of nutrients against a gradient (Neilands, 1974). On the other hand, the coupling of a carrier molecule with metabolic energy can initiate the accumulation of the transported solute against an electrochemical or osmotic gradient. This process is referred to as active transport. Bacterial transport

systems, in this manner, are able to achieve very high concentration gradients with some nutrients being reported to reach a 0.2M concentration within cells (Harold, 1972). The formation of an iron-attracting surface site by embedding a protein with a high affinity for iron in the membrane, would be advantageous since this phenomenon could be linked to a high energy source facilitating the rapid concentration of iron. Studies performed on energy-consuming microbial transport systems involving the following cations K^+ , (Zarlengo and Schultz, 1966), Mn^{2+} , (Silver and Kralovic, 1969), Mg^{2+} , (Lusk and Kennedy, 1969), (Silver et al., 1970) have suggested that separate transport systems may be involved for essential cations. Studies performed on the solubility characteristics of iron in aqueous solutions have suggested that membrane transport proteins with recognition capacity for ionic iron may still be inadequate for the satisfaction of an internal iron requirement (Chaberek and Martell, 1959). Dissociation of iron readily occurs in solutions which are neutral or slightly acidic; various metal hydroxide species are formed which may polymerize and precipitate from these solutions. It is known that some free metal iron in solution always exist, however, the concentration of the ferric ion in equilibrium with insoluble ferric hydroxide ($Fe(OH)_3$) is quite small when compared to the more soluble metal hydroxides such as manganese hydroxide ($Mn(OH)_2$). Moreover, substances such as phosphates which are found normally in a biological

environment can combine with iron to form insoluble macromolecular lattices (Neilands, 1974).

2. SIDEROCHROMES IN MICROBIAL TRANSPORT

Microbial cells are known to secrete chelating agents which show a high specificity for iron for the purpose of transport. Two chemical categories of siderochromes which are now though to be involved in this iron transport system include the secondary hydroxamic acid compounds (i.e. ferroxamine B) and the phenolic acids (catechols i.e. enterochelin). Originally the term "siderochromes" was applied to the secondary hydroxamates (Zahner et al. 1962). Neilands (1978) suggested that the definition of siderochromes should be extended to include the catechol-type chelators. The siderochromes are comprised of all memebrs of the ferrichrome series of compounds which are cyclohexapeptide, containing a tripeptide sequence of N-acyl-N-hydroxyornithine, including the ferroxamines which contain alternating units of succinic acid and 1-amino-ω-aminoalkane. The siderochromes which are produced by bacteria and which contain catechol groups coordinated to iron, must also be placed in the all-oxygen ligand group. This all-oxygen ligand is commonly found in the siderochromes constituting a functional requirement in these compounds for a structure which will strongly bind ferric ion. A sparcity of information regarding the coordination chemistry of members of the enterobactin (enterochelin) class has been reported although it is

known that these iron coordination compounds are difficult to reduce (Neilands, 1974).

In spite of the large number of siderochromes isolated, ferrichromeA is the only member for which a crystal structure is known (Nuesch and Knusel, 1965). Other siderochromes which have been reported and isolated are reviewed by Nuesch and Knusel (1965) and Prelog (1963). Despite their structural differences, all the reported siderochromes have one feature in common - the iron-free siderochromes can selectively bind ferric ions and display a very high complex-stability constant; the complexing of other metallic ions by siderochromes, however, yields low stability constants (Nuesch and Knusel (1965). Meyer and Abdallah (1978), for example, found that $pyoverdine_{p.f.}$, a siderochrome belonging to the phenolate group displayed a stability constant of the order of 10^{32} which is characteristic of a highly stable Fe(III) complex. This stability constant was measured using the sodium salt of ethylene-diamine-tetra-acetic-acid (EDTA) as a competitive chelator of the ferric ion. The standard methods for measuring stability constants have been summarized by Rosotti and Rosotti (1961).

3. PURIFICATION OF SIDEROCHROMES

It is usually very difficult to purify siderochromes contained in crude extracts, due mainly to the low yields produced in the culture media. The crude extracts have been shown to contain large quantities of inactive components from the nutrient solution, as well as fermentation products displaying similar physico-chemical properties (Nuesch and Knusel, 1965). The siderochromes are readily soluble in strongly polar solvents such as water, glycol, and dimethylformamide; some are also soluble in methanol; insoluble in less polar solvents, and some are highly soluble in phenol as well as in mixtures of phenol and lipoid solvents, i.e. chloroform. Adsorption on charcoal and the use of ion-exchange materials are reported to be suitable for the isolation of siderochromes (Elliot, 1958; Nuesch and Knusel, 1965). Often combinations of various methods are employed in the isolation and purification of siderochromes (Reynolds and Waksman, 1948; Bickel et al, 1960; Tsukiura et al., 1964). Some of the siderochromes have been reported to be quite unstable over a wide range of pH levels (Nuesch and Knusel, 1965; Meyer and Abdallah, 1978); the iron-complexes, however, are stable over a broad pH range (3-10). As a rule, the least destructive method of removing iron from siderochromes is by the use of 8-hydroxyquinoline or cupferron, (Meyer and Abdallah, 1978).

4. SIDEROCHROMES AS MICROBIAL GROWTH FACTORS

In 1957, Neilands reported that ferrichrome compounds acted as "coenzymes" for the transport of iron in microbial metabolism. Lochead et al (1952) described a microbial growth factor for Arthrobacter terregens which was termed the "terregens factor" while Parge (1952) found that the compound hemin was a growth factor for the Pilobolus fungus. Hesseltine et al (1952) described the isolation of coprogen, an iron-containing compound from an Actinomycetes fermentation which was an effective growth factor for Pilobolus. Although ferrichrome, the terregens factor and coprogen were all shown to exhibit biological activity, they were reported to have different chemical and physical properties (Neilands, 1974). Enterobactin, an iron transport compound excreted by Salmonella typhimurium into culture medium containing low iron concentrations, was also found to be a physiologically active, iron-sequestering agent (Pollack and Neilands, 1970). Examples of other siderochromes include mycobactins which are produced by Mycobacterium species and schizokinens which are produced by Bacillus species (Beyer et al., 1967). Cox and Graham (1979) isolated an iron-binding compound from Pseudomonas aeruginosa which exhibited growth-promoting activity. They reported that this compound, named pyochelin, when added to iron-poor cultures of P. aeruginosa, not only promoted the growth of the bacteria but also reversed any growth inhibition by the iron chelator ethylene-di-(o-hydroxy phenylac-

atic acid) (EDDA). It was, therefore, suggested that pyochelin was an iron chelator which made iron available to the bacteria in a usable form (Cox and Graham, 1979). Meyer and Hornsberger (1978) observed that the yellow-green, water-soluble pigment, pyoverdine$_{p.f.}$ was the physiological iron carrier of P. fluorescens. P. stutzeri, a non-fluorescent pseudomonad belonging to the same homology group as the fluorescent Pseudomonads, produced a colourless compound which could complex strongly with the ferric ion and was identified as nocardamine (Meyer and Abdallah, 1978).

Since the siderochromes are so widely encountered and generally serve as non-specific interchangeable growth factors for the heterotrophic microorganisms, it was suggested that the sideramines and their antagonists, the sideromycins, play some role in the metabolism of iron of these microorganisms (Nuesch and Knusel, 1965). This supposition was further supported by the general observation that larger quantities of siderochromes are not produced by these organisms except when there is an iron deficiency in their growth medium (Neilands, 1974). It is known that the total quantity of iron within the living microbial cell is far greater than that which can be accounted for by the iron containing compounds (Neilands, 1957). The bulk of the iron is engaged in electron transport processes of some sort or another (Green and Beinert, 1955, Neilands, 1974). The location and/or metabolic path-

ways of the remaining residual iron is still unknown although the discovery of large numbers of microbial iron containing growth factors may serve as the starting point for extensive studies on iron metabolism in microorganisms.

THE PYOVERDINES

A somewhat variable but distinctive property of fluorescent Pseudomonads is the production of a yellow-green, water-soluble pigment which diffuses into the growth medium and is fluorescent under ultra-violet light. These bacteria do not require growth factors and do not synthesize polyβ-hydroxy-butyrate as a cellular reserve. An example of such a bacterium is P. fluorescens which produces a fluorescent pigment when grown in an iron-deficient medium.

In 1942, Turfriejer proposed the term "pyoverdine" for the yellow-green, fluorescent, water-soluble pigments produced by P. fluorescens. This name was chosen by analogy to that of the phenazine pigment pyocyanine, produced by P. aeruginosa. The term "pyoverdine" is preferable to the designations of "bacterial fluorescein" or "fluorescein" which are likely to be confused with the chemically synthesized fluorescein (resorcinolphthalien). The name of pyoverdine has been extended to include all pigments produced by fluorescent Pseudomomads. The pyoverdines formed by different species of fluorescent Pseudomonads may be distinguished by a suffix indicating the producing

species. For example, pyoverdine$_{p.f.}$ represents the pyoverdine produced by P. fluorescens.

It has been shown that the yellow-green, water-soluble pigment, pyoverdine$_{p.f.}$ is the physiological iron carrier of P. fluorescens (Meyer and Hornsberger, 1978). Pyoverdine$_{p.f.}$ possesses the two essential characteristics shown by siderochromes: it is synthesized in large amount and excreted into the culture medium only when bacteria have insufficient iron, and forms a very stable complex with the ferric ion (Meyer and Abdallah, 1978).

1. SYNTHESIS OF PYOVERDINE$_{P.F.}$

Many different environmental factors have been reported to affect the synthesis of pyoverdine$_{p.f.}$ by the host organism, notably the chemical nature of the organic carbon and energy source (Lepierre, 1895; Sullivan, 1905; Blanchetiere, 1920; Giral, 1936; Gouda and Chodat, 1963; Gouda and Greppin, 1965), the degree of aeration in the culture medium (Elliot, 1958; Lenhoff, 1963), the pH and presence of light (Greppin and Gouda, 1965) and the presence of the cations, especially Mg^{2+} (Georgia and Poe, 1931), Zn^{2+} (Baghdiantz, 1952; Chakrabarty and Roy, 1964) and Fe^{3+} (King et al, 1948; Totter and Moseley, 1953; Lenhoff, 1968; Palumbo, 1972, Lluch et al, 1973). Meyer and Abdallah (1978) proposed that the concentration of Fe^{3+} is the sole factor that regulates the pyoverdine synthesis by P. fluorescens.

These authors also suggested that any organic substrate could support pyoverdine synthesis and that the absolute level of Fe^{3+} at which growth becomes limited may vary with the nature of the substrate.

The biosynthesis of pyoverdine by P. fluorescens was reported to occur when the organisms were grown in a standard succinate medium (Meyer and Abdallah, 1978). The pigment produced formed a very stable Fe(III)-complex and could be purified in this form (Zahner et al, 1963; Meyer and Abdallah, 1978).

2. CHEMICAL STRUCTURE OF PYOVERDINES

Due to the difficulties encountered during the isolation and purification of the fragile molecules, their chemical structures remain largely unknown. Several hypotheses as to the nature of the fluorescent chromophore have been proposed. Lenhoff (1963) and Greppin and Gouda (1965) have postulated a pyrolle derivative by analogy with the cytochromes. A riboflavin component of bacterial fluorescein was postulated by Birkofer and Birkofer (1943), and a pteridine chromophore was suggested by Giral (1935), Knaves (1955), and Chakrabarty and Roy (1964).

In these earlier studies, the molecular weights of the fluorescent pigments were presumably underestimated and the fluorescent compounds isolated may have been the degradation products of pyoverdines or other fluorescent metabolites produced by Pseudomonads (Leisinger and

Margraff, 1979). More recent studies have revealed that pyoverdines have molecular weights in the range of 1000. In partially purified preparations, obtained by gel filtration, the fluorescent pigments were found to consist of a peptide associated with a fluorescent chromophore. The peptide from P. mildenbergii, for example, contained serine, threonine, glutamic acid and lysine (Hulcher 1968; Newkirk and Hulcher, 1969). Michea and Greppin (1974) found that the peptide from P. fluorescens (Migula) consisted of serine, glycine, glutamic acid, ornithine and lysine residues. Although the chemical structure of pyoverdine has not been fully established, it is thought to consist of a quinoline chromophore associated with a cyclic peptide and a short aliphatic chain. Hydrolysis of the peptide moiety yielded serine, glycine, alanine and N^5-hydroxyornithine (meyer and Abdallah, 1978). N^5-hydroxyornithine is a constituent of many siderochromes, including the polypeptide siderochrome, ferribatin, isolated from cultures of P. fluorescens (Migula) by Maurer et al. (1968). Meyer and Abdallah (1978) observed that $pyoverdine_{p.f.}$ formed an extremely stable complex with ferric ions and could be purified in this form. P. fluorescens produced only one molecular species of pyoverdine with a molecular weight of 1500, which at slightly alkaline pH was transformed into a number of degradation products.

3. ROLE OF PYOVERDINES

Reports to date, indicate no clear physiological role for the pyoverdines. Newkirk and Hulcher (1969) proposed that pyoverdine$_{p.f.}$ was able to replace ferrous ion in activating 6-phosphogluconate dehydrase from P. mildenbergii, although they were unable to show the presence of iron in pyoverdine$_{p.m.}$. Newkirk et al, (1960) also proposed that pyoverdine$_{p.m.}$ may be the product of a biosynthetic pathway interrupted by an iron deficiency. A third possibility suggested was that the pyoverdines may act as iron scavengers (Neilands, 1978). Meyer and Hornsberger (1978) found that pyoverdine$_{p.f.}$ is the physiological iron carrier of P. fluorescens.

COMPOSITION OF THE HEN'S EGG

On the average, nearly one-half of the albumen of a newly laid egg consists of a gel, the thick white, interposed between two liquid fractions, the outer thin white and the inner thin white. The two shell membranes separate at the broad end of the egg to enclose the air cell. The yolk of the egg is suspended in the albumen by the chalazae at approximately the centre of the egg and is contained by the vitelline (yolk) membrane. As an egg ages, the yolk tends to take up water, and becomes larger. The yolk membrane weakens, thus giving the yolk a flattened shape.

The egg has various ways of protecting itself

from microbial invasion. The shell and the thin surface layer of proteinaceous material known as the cuticle or bloom are the first line of defense and serve to retard entry. However, the shell is porous for gas exchange during embryonic development. The membranes inside the shell also tend to serve as a mechanical barrier. This barrier is probably only temporary and also offers no protection against the infiltration of mold hyphae through the membrane pores. This fact emphasizes the need to store eggs in a way that will avoid accumulation of moisture on the surface of the shell. A rapid change in storage temperature may permit bacteria to overcome the physical barrier of the shell membrane. If a warm egg is placed in a cold environment, microorganisms on the shell surface may be drawn in through the pores upon contraction of the egg contents. Changes in the membrances occur with aging and favour rapid bacterial multiplication. The rates of physical and chemical changes in the eggs depend upon the time and temperature of holding, the relative humidity, and the composition of the atmosphere about the eggs. In addition to the physical barrier of the shell and its membranes, numerous studies have shown that the albumen is an inadequate growth medium for many microorganisms (Board, 1960). The characteristics of egg albumen (Table 1) that hinder microbial growth include a pH of 9 to 10 that may be reached during storage; low levels of simple nitrogenous compounds; apoproteins, which ties up riboflavin; avidin,

TABLE 1 The Biological Properties of Components of the Albumen of the Hen's Egg.

Component	Action
Lysozyme	Lysis of cell walls of certain bacteria. Flocculation of bacterial cells. Hydrolysis of β1-4-glycosidic bonds
Conalbumin	Chelation of iron, zinc, and copper
Ovomucoid	Inhibition of trypsin
Avidin	Combination with biotin
Riboflavin	Chelation of cations
Uncharacterized proteins	A Inhibition of trypsin and chemotrypsin
	B Inhibition of fungal protease
	C Combination with riboflavin
	D Combination with vitamin B_6
	E Chelation of calcium

which ties up biotin; antiproteolytic factors, which might prevent bacterial proteinases from releasing nitrogenous compounds necessary for growth; conalbumin (ovotransferrin) which chelates iron; and lysozyme, an enzyme which hydrolyzes the cell wall of most Gram-negative bacteria (Board, 1968).

CONALBUMIN

Conalbumin is a glycoprotein which accounts for approximately 10% of the total egg-albumen solids (Board, 1964). It is widely distributed throughout the albumen and forms complexes with iron, zinc and copper. Feeney and Nagy (1952) found that the chelating agent must be stoichiometrically in excess of iron before microbial growth is inhibited. The extent of microbial inhibition by conalbumin has been demonstrated to increase with decreasing concentration of hydrogen ions (Schade and Caroline, 1944). Microbial inhibition of hen's egg due to the large amounts of conalbumin is evidenced by both an increase in the lag phase of growth and generation time, resulting in a marked reduction in the size of the final microbial population (Theodore and Schade, 1965). Studies performed by Garibaldi (1960) on whole egg-white, in vitro, have shown that conalbumin will normally support the growth of the common contaminants found in rotten eggs when the pH is poised at neutrality or the chelating potential of conalbumin is satisfied. Likewise, the addition of iron to the albumen of whole egg

resulted in the rapid growth of bacteria present in the shell membranes (Board, 1964). Conalbumin represents the principal antimicrobial component in hen's egg demonstrating an efficiency which increases as the pH of the albumen drifts to the alkaline side of neutrality (Board, 1964).

IRON AND BACTERIAL SPOILAGE OF EGGS

The practice of washing soiled eggs has been carried on for many years. Thompson (1952) reported that washing increases the risk of spoilage in eggs destined either for the retail market or storage. Jenkins and Pennington (1919) showed that there was a higher incidence of rots in washed eggs than in eggs which were not washed; the genera Pseudomonas and Proteus were largely responsible for these rots (Haines, 1938). Garibaldi and Bayne (1960) have suggested that sufficient iron may be introduced into shell eggs during washing to affect both the rate and extent of subsequent bacterial spoilage. It has been reported by Smith et al (1952) that well waters contain iron in concentrations of 1-5μg/ml, with the higher concentrations being more prevalent; the washing of eggs in such waters may therefore introduce iron in quantities sufficient to affect both the rate and extent of subsequent spoilage. Board (1968) reported that the effect of contaminating iron, introduced during the washing of the egg, on the course of bacterial infection into egg was to promote multiplication of bacteria, particularly at the site of the shell membrane.

Schade and Caroline (1944) believed that conalbumin inhibited bacterial growth because of the formation of an iron-conalbumin complex. Waring and Werkman (1942), and Fraenkel-Conrat and Feeney (1960) stated that *Pseudomonas* cultures required iron at a concentration of 0.1μg/ml for optimum growth. The concentration of total iron in egg-white is in the range of 0.10-0.25 μg/ml with the average being 0.15 μg/ml (Fraenkel-Conrat and Feeney, 1960). Contamination with iron may influence spoilage of the egg in another manner, the microorganisms having penetrated the shell membranes can now grow more readily because of the presence of this essential trace nutrient. Concurrently, with growth at the membranes, bacteria may excrete metabolic products which are able to diffuse into the albumen creating a more favourable medium for their growth (Board, 1973). Among these products excreted, iron-binding compounds may be found which would allow the bacteria to satisfy their iron requirements even in the presence of a large excess of conalbumin. Garibaldi and Bayne (1960, 1962), labelled these compounds as iron-transport compounds. Garibaldi (1970) proposed that such compounds excreted by *Salmonella typhimurium* and *Pseudomonas ovalis* play a significant role in reversing the bacteriostatic action of conalbumin on their growth in egg-white. $Pyoverdine_{p.f.}$, an iron sequestering compound which is produced by *P. fluorescens* is said to function in the iron transport of this microorganism (Meyer and Hornsberger, 1978).

SALMONELLA IN EGGS

Salmonellosis is one of the most frequently encountered bacterial diseases infecting poultry (Williams et al., 1968; Simmons and Byrnes, 1972). World-wide, Salmonella typhimurium is the predominant serotype found in both man and domestic animals, including poultry (Williams et al., 1968). The major source of salmonella in the infection of egg contents is through shell contamination resulting from fecal contact (Forsythe et al., 1967). Salmonella organisms are also found in the feed and water supplies, contaminating litter and nest materials, and are frequently deposited in a moist medium on the shells of the eggs at the time they are laid (Williams et al., 1968; Stuart and McNally, 1943). The total number of micro-organisms per shell is estimated to vary from 9.5×10^3 to 3.1×10^6 (Board and Wilson, 1956). Gram-negative bacteria were found to predominate on badly soiled eggs. Pseudomonas spp. accounted for 22.5% of the microflora of cleaned, shelled eggs (Board et al. 1964). Fluorescent Pseudomonads seem to be the most frequent cause of rots in shell eggs (Moats, 1980). Both Salmonellae and Pseudomonads can survive and grow in the egg albumen and as such, these micro-organisms may be found in egg products (Yadav and Vadehra, 1977).

In Canada, Salmonellosis accounted for 39% of all reported cases of food - borne disease during the period from 1973-1975 (Bryan, 1981). The number of reported cases

and outbreaks of food-borne disease represents only a fraction of the actual number that occurs. Hauschild and Bryan, (1980) concluded that for every laboratory-confirmed case of Salmonellosis, an average of 29.5 other cases are identified when thorough investigations are conducted. Therefore during the ten year period, 1968-1978, it is estimated that an average of 150,000 food or water-borne cases of human Salmonellosis occured annually in Canada (Bryan, 1981). A major source of the "Salmonella problem" in man is still derived from foods of animal origin, especially poultry, beef and pork. Silliker (1980) found that eggs accounted for 6% of 500 reported cases of human Salmonellosis outbreaks during the period 1966-1975. Some factors which usually contribute to the outbreaks of Salmonellosis include improper cooling of food material, inadequate cooking or processing, ingestion of contaminated raw ingredients and cross-contamination (Bryan, 1980).

MATERIALS AND METHODS

I. SOURCE AND MAINTENANCE OF MICROORGANISMS

The bacterial cultures used in this study were:

1. Pseudomonas fluorescens ATCC 13525 (American Type Culture Collection, Rockville, Maryland, U.S.A.)

Isolates of P. fluorescens grown on Trypticase Soy Agar (Difco, TSA) plates were transferred into 5 ml volumes of Trypticase Soy Broth (Difco, TSB), and incubated at 20°C for 24 hours. The organisms were then streaked on TSA slants which were subsequently incubated at 20°C for an additional 24 hours. Following incubation, the slants were stored at 4°C. The cultures were transferred to fresh TSA slants every week to maintain activity and verify purity.

2. Salmonella typhimurium LHO 67891 (Laboratory of Hygiene, Ottawa, Canada)

Isolates of S. typhimurium grown on TSA plates were transferred into 5 ml volumes of TSB, and incubated at 37°C for 24 hours. The organisms were then streaked on TSA slants, which were subsequently incubated at 37°C for an additional 24 hours. Following incubation, the slants were stored at 4°C. The cultures were transferred to fresh TSA slants every week to maintain activity and verify purity.

The eggs used in this study were obtained from 28-week old Shavers hens raised at the Poultry Barn, Department of Animal Science, University of Manitoba. The eggs had an average total volume (yolk and albumen) of 50 ml and an average weight of 55 gm.

II. PRODUCTION OF PYOVERDINE$_{P.F.}$

1. Preparation of Inoculum

Discrete colonies of P. fluorescens, grown on TSA at 20°C for 24 hours were inoculated into 200 ml TSB contained in 500 ml Erlenmeyer flasks and incubated at 25°C in a New Brunswick gyratory shaker incubator operating at 200-250 rpm, for 24 hours. The resulting culture was then centrifuged at 150 x g for 20 minutes at 5°C using a Sorval Superspeed RC-2B centrifuge. (This method of centrifugation was used throughout this study, unless otherwise indicated.) The supernatant was discarded and the pellet washed with ca. 100 ml of sterile physiological saline solution. This procedure was repeated at least three times until the supernatant appeared clear. The pellet was then resuspended to a final volume of 80 ml saline (2/5 volume). To obtain a 5% (v/v) inoculum level, 25 ml of this bacterial stock saline suspension was added to 475 ml of a Standard Succinate Medium (SSM) described by Meyer and Abdullah (1978). The composition of this medium is as follows: (g/litre distilled water) K_2HPO_4, 6.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, $7H_2O$, 0.2; Succinic acid, 4.0. The pH was adjusted to 7.0 by addition of 1 N NaOH prior to sterilization. All growth media were routinely sterilized at 121°C for 15 min.

2. Production of Pyoverdine$_{p.f.}$

Pyoverdine$_{p.f.}$ was produced by inoculating a 5% (v/v) inoculum of P. fluorescens obtained from the pre-

inocula (stock saline culture) into 1-litre Erlenmeyer flasks each containing 475 ml of SSM. The flasks were then incubated at 25^{o}C on a gyratory shaker operating at 200-250 rpm, for 40 hours. A growth period of 40 hours was chosen since preliminary studies indicated that there was maximum pigment production at this time period. Maximum pigment production produced by these growing cultures was assayed via a time-course study in which 25 ml samples were aseptically removed and centrifuged. The resulting supernatants were then monitored for absorbance at 400 nm (Meyer and Abdallah, 1978) using a Perkin-Elmer Coleman 111 Spectrophotometer. Cellular growth was monitored by absorbance at 500 nm. After a growth period of 40 hours, Fe Cl_3 was added to the culture media yielding a final concentration of 200 mg Fe^{3+}/litre of fermentation broth (Meyer and Abdallah, 1978). Addition of Fe^{3+} to the culture medium resulted in a colour change from yellow-green to a reddish brown due to the formation of an iron-pigment complex. The bacteria were then removed by centrifugation. The supernatants were pooled and stored at 4^{o}C. The final volume of the collected supernatants was 23.5 litres.

3. Purification of $Pyoverdine_{p.f.}$

The isolation and purification of $pyoverdine_{p.f.}$ was carried out as outlined by Meyer and Abdallah (1978) with some minor modifications:

(a) Concentration of supernatant containing the

iron-pigment complex. Several methods were employed in an effort to concentrate the volume of supernatant, including a method of ultrafiltration using a Hollow Fiber Concentration/Desalting System, Model DC2. This method was rejected since the iron-pigment complexes were not retained by the silicone rubber fibres (Membrane size 2000, inside diameter of fibre: 0.313", outside diameter: 0.439"). The method used for the concentration of the supernatant involved adsorption of the iron-pigment complex on activated carbon followed by elution with alcohol (Elliot, 1958). A three-gram sample size of Darco Activated Carbon (Atlas Chemical Ind.) was added to each litre of collected supernatant and subsequently agitated on a rotary shaker for 12 hours. The iron-pigment complex adsorbed to the activated carbon was then removed from the aqueous solution. This was achieved by filtration using a Buchner funnel equipped with a Whatman #1 Filter Paper Pad (W & R Balston Ltd.), attached to a Duo-Seal Vacuum Pump (Welch Scientific Co.). The iron-pigment complex was then eluted from the activated carbon with 95% ethanol. The ethanol solution containing the iron-pigment complex was concentrated to a final volume of 1 litre by flash evaporation on a Bulcher Flash Evaporator at 30^{o}C equipped with a Dewar condenser, (Model No. PF-10DN), stored at 4^{o}C.

(b) Ion-Exchange Chromatography. Five millilitre (5 ml) aliquots of concentrated iron-pigment complex were applied to a CM-sephadex C-25 (2.5 x 90 cm) column and

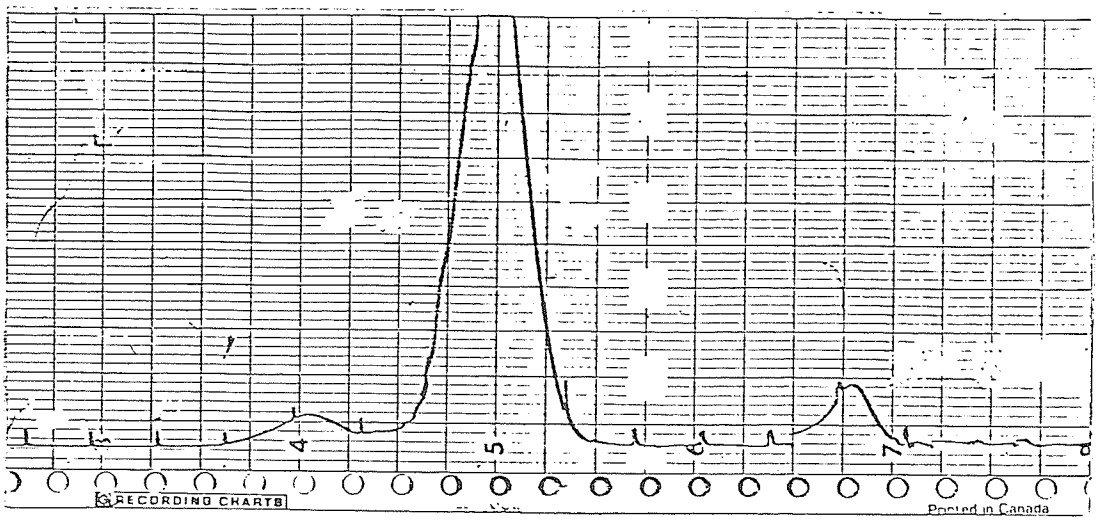


ILLUSTRATION 1. Elution Diagram of Chromatography of Material containing Pyoverdine-Iron Complex Measured at 410 nm

Column Type: CM- Sephadex C-25 (2.5 x 90 cm);
Buffer : Citrate-Phosphate pH6.56
Flow Rate : 60 ml/hour

Absorbance Monitor (Instrumentation Specialities Co. Inc.) set at a wavelength 410 nm

eluted with citrate-phosphate buffer pH 6.56, temp. 21°C, at a flow rate of 60 ml/hour. The eluant was monitored using an Absorbance Monitor (Instrumentation Specialties Co. Inc.) set at 410 nm (Meyer and Abdallah, 1978) and samples which exhibited a maximum absorbance at this wavelength were pooled and stored at 4°C (Illustration 1). The samples were collected using a 7000 Ultrarac R Fraction Collector, L.K.B. The samples collected were lyophilized using a Virtis Freeze Drier. The lyophilized materials were then stored at 4°C until further use.

4. Preparation of iron-free pigment

All glass-ware used in this preparation was rinsed with concentrated HCl, followed by deionized water (Rand, Greenberg, and Taras, 1975) to remove any deposits of iron-oxide. During the course of this investigation, iron-free pigment was prepared on the same day as required. A 100 mg sample size of lyophilized iron-pigment complex was re-suspended in 5 ml deionized water. The pH of this suspension was then adjusted to pH 4.0 with 10% (v/v) acetic acid using a Fisher Acumet (Model 520) Digital pH/ion Meter. This solution was then extracted with 15 ml of 5% (w/v) 8-hydroxiquinoline (Baker reagent) in chloroform, using a 30 ml separatory funnel. The aqueous phase was retained, and re-extracted twice with 15 ml aliquots of the solvent. The aqueous phase was finally washed twice with 15 ml aliquots of chloroform. The resultant iron-free pigment,

pyoverdine$_{p.f.}$, was a clear, yellow-green liquid which fluoresced under UV light.

5. Analysis of Pyoverdine$_{p.f.}$

(a) Absorption Spectrum. An aliquot of 1 ml of pyoverdine$_{p.f.}$ (20 mg/ml) was added to 4 ml of deionized water and was used to reestablish the Absorption Spectrum of pyoverdine$_{p.f.}$ at 400 nm.

III. THE EFFECT OF PYOVERDINE$_{P.F.}$ ON THE GROWTH OF SALMONELLA TYPHIMURIUM IN THE HEN'S EGG.

1. Preparation of Standard Inoculum of S. typhimurium.

Colonies of S. typhimurium grown on TSA were transferred into 5 ml TSB, and incubated at 37°C for 24 hours. After incubation, the culture was transferred aseptically into 250 ml Erlenmeyer flasks each containing 100 ml TSB and incubated at 37°C for 24 hours. The cultures were then centrifuged; the supernatant was discarded and the pellets were resuspended with 50 ml aliquots of saline and re-centrifuged until the supernatant became clear. The cells were then resuspended to a final volume of 10 ml saline and served as the stock bacterial culture. One millilitre aliquots of the bacterial stock culture were adjusted to an O.D. of .125 by the addition of sterile saline solution. Total viable counts vs. O.D. = 0.125 at 340 nm were performed via serial dilution pour-plate technique using saline as the diluent and TSA as the plating medium.

2. Sensitivity of S. typhimurium to Pyoverdine$_{p.f.}$ - Preliminary Study.

A disc assay method was initially used to determine the sensitivity of S. typhimurium to pyoverdine$_{p.f.}$. Sterile blank discs, 1/4" in diameter, (Difco), wetted by capillary action from a freshly-prepared pyoverdine$_{p.f.}$ solution (20 mg/ml) were gently layered on solidified TSA contained in 100 x 15 mm Petri plates seeded with 24 hour culture of S. typhimurium (2×10^7 cells/ml assay medium). The Petri plates were incubated at 37°C for 5 hours; zones of inhibition were then recorded.

3. Effect of Pyoverdine$_{p.f.}$ on the Growth of S. typhimurium in Egg Albumen (in vitro).

One-day old and eight-week old eggs were used in this experiment. Each egg was swabbed with 95% ethanol, cracked open, and the yolk material aseptically separated from the albumen by means of a sterile egg-separator. The total quantity of albumen was placed in a sterile blender jar and blended for ca. 30 seconds in order to break-up the chalazae. Ten millilitre aliquots of albumen were then placed in sterile Whirl-Pak bags (6oz capacity) and were then inoculated using 1 cc. Tuberculin Syringes (Plastipak Non-Toxic-Non Pyrogenic, Becton-Dickinson & Co.) fitted to sterile Yale Needles (22G 1½, Becton-Dickinson & Co.) with 0,0.2, 0.4 0.8 and 1.0 ml aliquots of pyoverdine$_{p.f.}$ (20 mg/ml) respectively. The bags were then set aside for two hours at room temperature. Two inoculum levels of S.

typhimurium were used for each pyoverdine level assayed (0.7×10^1, 3.2×10^2 viable cells); the bags were then incubated at 37°C for 24 hours. Aliquots of 1.0 ml were removed from each bag, serially diluted with saline, and enumerated for S. typhimurium with Brilliant Green Sulphadiazine (BGS, Difco) agar. The Petri plates were inverted and incubated at 37°C for 24 hours. Salmonellae colonies were then enumerated. On BGS agar medium, typical S. typhimurium colonies appear reddish or pink.

4. Effect of Conalbumin on the Growth of S. typhimurium in Egg Albumen (in vitro).

Whirl-Pak bags each containing 10 ml aliquots of albumen obtained from one-day old and eight-week old eggs were prepared as described in Section III.3. These bags were then supplemented with 0.1 ml aqueous aliquots containing 0, 5 and 10% conalbumin (Type 1, obtained from Chicken Egg White, Sigma Chem. Corp.) and then set aside for 2 hours at room temperature. Two inoculum levels of S. typhimurium (1.0×10^1, 2.9×10^2) were inoculated into specified bags, which were then incubated at 37°C for 24 hours. The number of S. typhimurium were determined as described in Section III.3.

5. Effects of $\text{Pyoverdine}_{\text{p.f.}}$ on the Growth of S. typhimurium (in vivo) in Eggs of Varying Ages.

Two dozen (3 hour-old) eggs were collected and maintained at 4°C for a 3-week storage period. After specified periods of storage, six eggs were randomly removed,

three of which were inoculated with both a pyoverdine$_{p.f.}$ solution (20 mg/ml) and a S. typhimurium culture, while the remaining three eggs were inoculated only with a S. typhimurium culture. These eggs served as a control. All eggs were swabbed on the blunt side with 95% ethanol. Aliquots of 0.1 ml of pyoverdine$_{p.f.}$ (20 mg/ml) were injected into theeggs by means of separate Sterile Single use 1 cc. Tuberculin Syringes. After inoculation with pyoverdine$_{p.f.}$, the point of entry was sealed by placing a drop of melted candle wax, which solidified after a few seconds. The eggs were then set aside for a 2-hour period at room temperature ($20^{o}C$) after which the entire set of eggs was then inoculated with 0.1 ml S. typhimurium culture (ca. 2×10^{7}/ml) using the same procedure as described for the inoculation with pyoverdine$_{p.f.}$. The point of entry for the Salmonellae organisms in eggs previously inoculated with pyoverdine$_{p.f.}$ was ca. 0.5 cm away from the entrance used for pyoverdine$_{p.f.}$. The eggs were then incubated at $37^{o}C$ for 24 hours. After this period of incubation, each egg was again swabbed with 95% ethanol, cracked open, and the entire contents placed in previously sterilized Mason jars (300 ml capacity) fitted with sterile heads (cover and blades) and blended for 30 seconds. An aliquot of 1 ml was removed aseptically from the blended material and enumerated for S. typhimurium as described in Section III.3.

6. Effect of Pyoverdine$_{p.f.}$ on the Growth of S. typhimurium (in vitro) in Eggs of Varying Ages.

Two dozen (3-hour old) eggs were collected and maintained at 4°C for a two week period. After specified periods of storage, six eggs were randomly removed, swabbed with 95% ethanol, cracked open and the entire contents of each egg was placed in sterile Whirl-Pak bags. Aliquots of 0.2 ml of pyoverdine$_{p.f.}$ (20 mg/ml) were added by means of Tuberculin Syringes to three of the six bags, and set aside for two hours at room temperature; an inoculum level of ca. 4×10^5 S. typhimurium cells was then added to each bag, and the bags were then incubated at 37°C for 24 hours. The contents of each bag were transferred aseptically into sterile Mason jars and blended. The number of Salmonellae organisms was then determined by the same procedure as described in Section III.3.

7. The Growth of S. typhimurium in Pyoverdine-Inoculated Eggs.

Thirty-six (3-hour old) eggs were collected, eighteen of which were inoculated with 0.1 ml aliquots of pyoverdine$_{p.f.}$ (20 mg/ml) as described in Section III.3. The entire set of 36 eggs were then placed at 4°C, and on specified days during a three week period, six eggs (3 from pyoverdine inoculated and 3 from the non-inoculated set) were removed, and then inoculated with 0.1 ml S. typhimurium culture (ca. 2×10^6/ml) as described in Section III.3 The eggs were then incubated at 37°C for 24 hours. The

number of Salmonellae organisms was determined as described in Section III.3.

8. Effect of Iron on the Growth of S. typhimurium in Standard Succinate Medium.

Sterile test-tubes containing 10 ml aliquots of iron-deficient SSM were supplemented with Fe^{3+} in the form of Ferric Ammonium Sulphate (FAS) (10 µg/ml) as follows:

Tube #	Fe^{3+} (µg) added as 10 µg/ml FAS	SSM (ml)	Deionized H_2O (ml)	Fe^{3+} (µg/ml) final
1	0	10	2.5	0
2	0.5	10	2.0	0.4
3	1.0	10	1.5	0.8
4	1.5	10	1.0	1.2
5	2.0	10	0.5	1.6
6	2.5	10	0	2.0

All glass-ware used in this series of experiments was rinsed with concentrated HCl, followed by deionized water, in order to remove any deposits of iron oxide deposits. (Rand, Greenberg and Taras, 1975) Aliquots of 0.1 ml of S. typhimurium culture containing ca. 1.0×10^1 cells/ml were inoculated into each tube. The tubes were then incubated at 37°C for 24 hours. Resultant growth was determined by serial dilution with saline and pour-plated with BGS agar. The Petri plates were then inverted, incubated at 37°C for

an additional 24 hours and the number of S. typhimurium were enumerated.

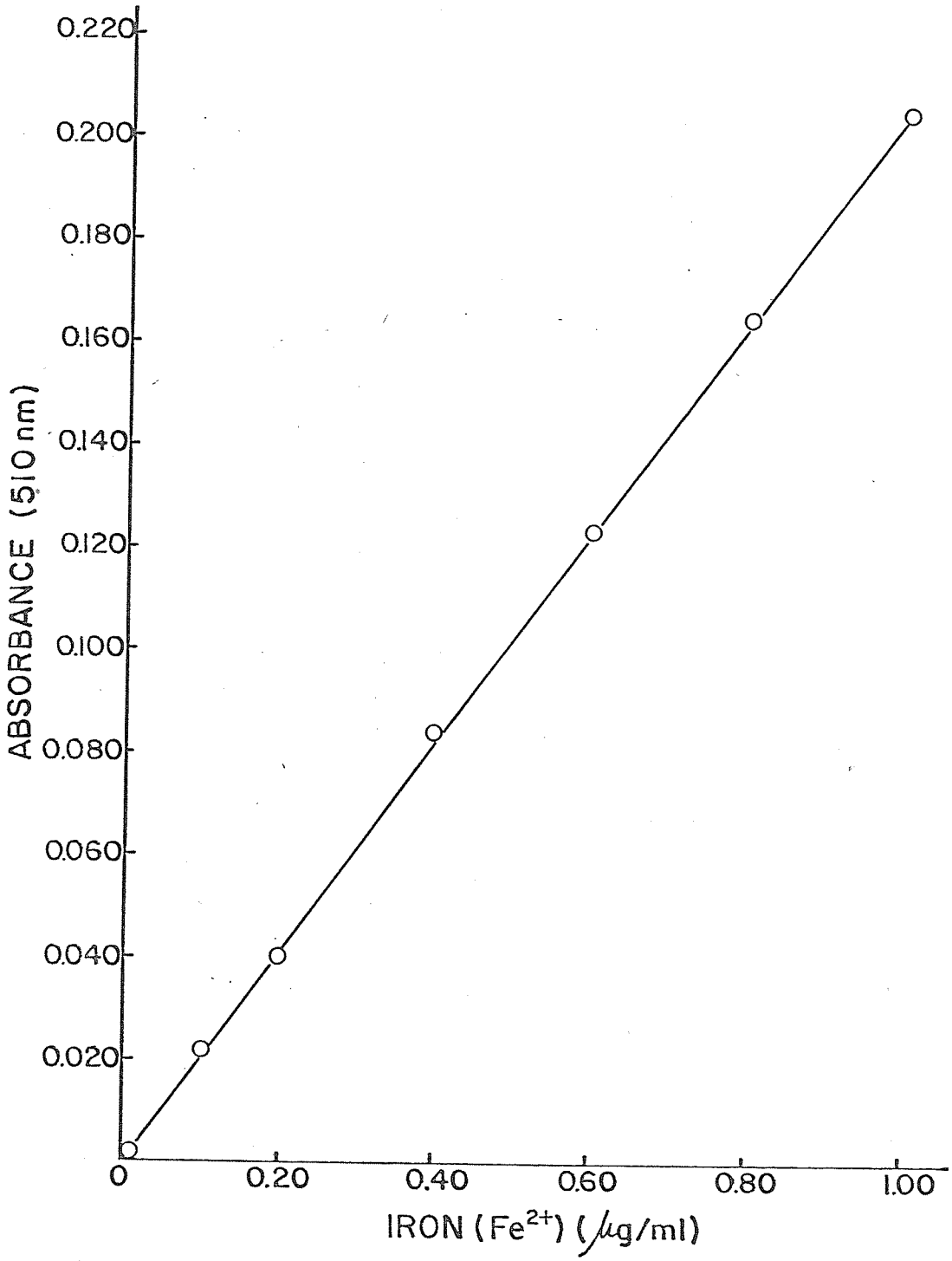
9. (a) Calibration of a Standard Curve for Iron.

The 1,10-phenanthroline method as described in "The Standard Methods for the Examination of Water and Wastewater" was employed, without modifications, to calibrate a standard curve for iron expressed as the Fe^{2+} ion. Absorbance readings at a wavelength of 510 nm were determined on a Perkin-Elmer Coleman 111 Spectrophotometer.

(b) Determination of Residual Iron and the Growth of Salmonella typhimurium in Standard Succinate Media containing Varying Concentrations of Iron Supplemented with Different Iron Chelators.

Six series of test-tubes containing SSM were prepared as described in Section III.9. Two series of these test-tubes (A), were aseptically inoculated with 0.1 ml aliquots of $pyoverdine_{p.f.}$ (20 mg/ml), two series (B), were inoculated with 0.1 ml aliquots of 10% conalbumin, and the remaining two series (C), were inoculated with 0.1 ml aliquots of $pyoverdine_{p.f.}$ (20 mg/ml) in addition to 0.1 ml aliquots of 10% conalbumin. On series from each of (A), (B) and (C) were inoculated with ca. 1.2×10^1 S. typhimurium cells, then incubated at $37^{o}C$ for 24 hours. One millilitre aliquots were then removed aseptically from each tube, serially diluted with saline and pour-plated with BGS agar. The Petri plates were inverted, incubated at $37^{o}C$ for 24 hours and the resulting Salmonellae organisms colonies enumerated.

FIGURE 1 Standard Curve for the Determination of Iron (Fe^{2+}).


0.220
0.200
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
ABSORBANCE (510 nm)
0
0.20
0.40
0.60
0.80
1.00
IRON (Fe^{2+}) (μg/ml)

The remaining series of tubes were then analyzed for residual iron by the 1,10-phenanthroline method (Rand, Greenberg and Teras, 1975).

RESULTS AND DISCUSSION

In common with the other species of the genus Pseudomonas, the fluorescent Pseudomonads are Gram-negative, strictly aerobic, polarly flagellated rods. This group of organisms is quite heterogeneous and, apart from the ability to produce water-soluble pigments of unknown structures, there are no phenotypic properties common to all members of the group (Leisinger and Margraff, 1979).

1. Production of Pyoverdine$_{p.f.}$

The water-soluble, yellow-green fluorescent pigment of P. fluorescens was produced when the organisms were grown in an iron-deficient media, (SSM). A characteristic of secondary metabolism is the temporal relationship between product formation and cell growth in batch cultures (Castric et al, 1979). As shown in Figures 2 and 3, the production of the water-soluble, fluorescent pigment of P. fluorescens occurred during the exponential phase of growth with maximum pigment production at approximately 40 hours. This time period corresponded to the early stationary phase of growth, thereafter the pigment production gradually decreased. Weinberg (1971) noted that secondary metabolites are produced for only a discrete period of the log or the early stationary growth phase. Castric (1979) concluded that the inception of the production of secondary metabolites is sudden and is followed by a linear rate of

FIGURE 2 Growth Curve of Pseudomonas fluorescens in Standard Succinate Media.

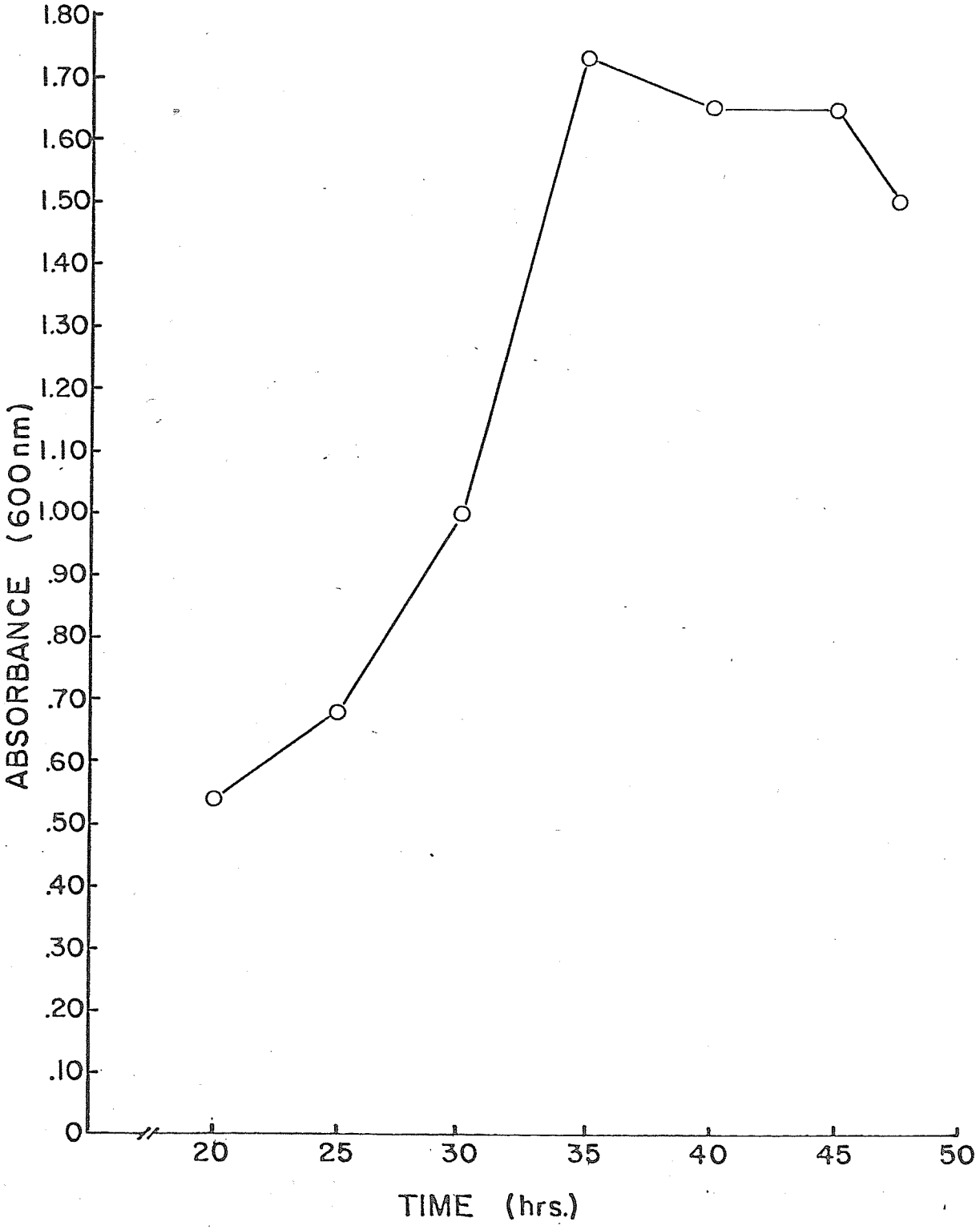


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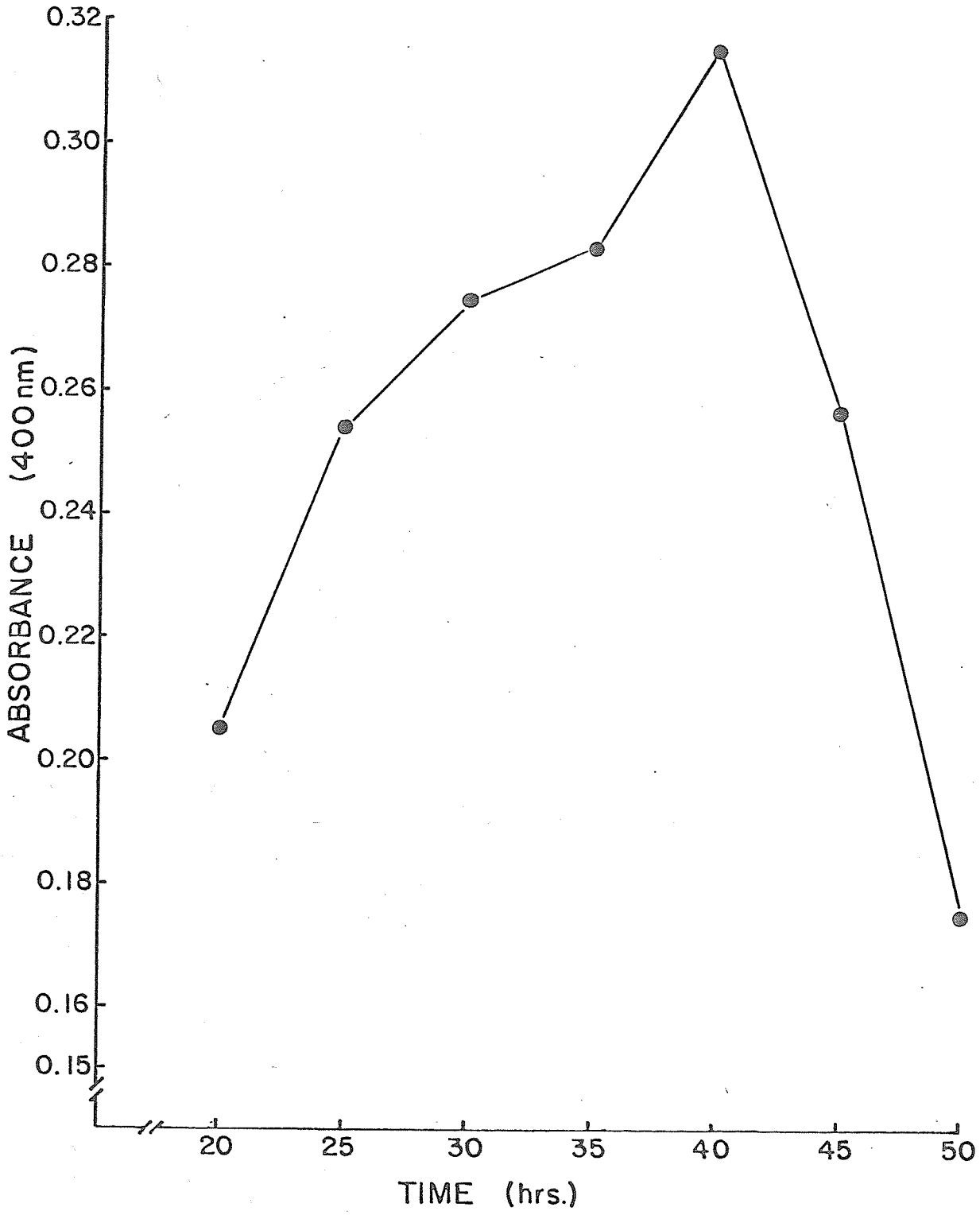
FIGURE 3 Pigment Production by Pseudomonas fluorescens in Standard Succinate Media.


0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.15
ABSORBANCE (400 nm)
20
25
30
35
40
45
50
TIME (hrs.)

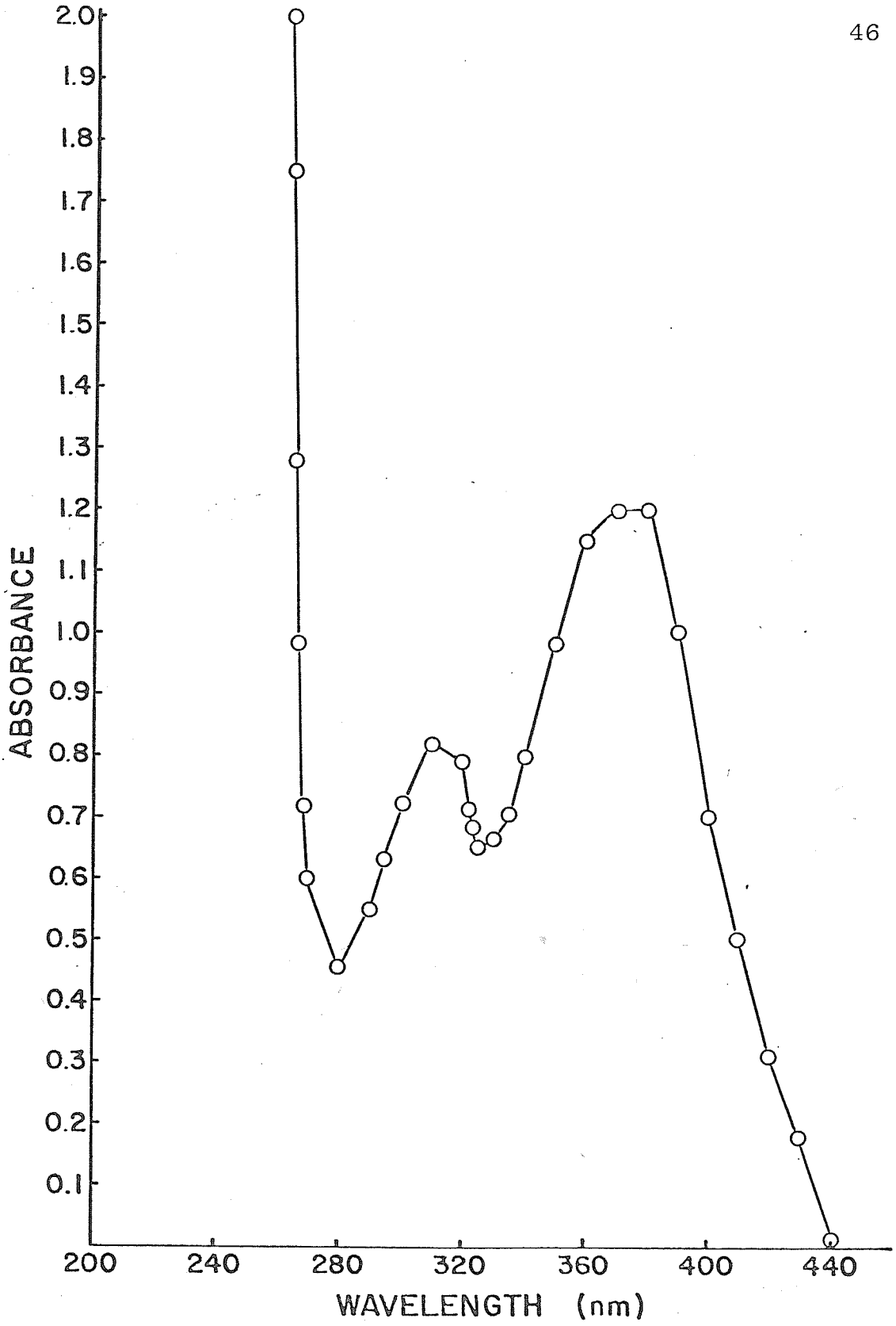
production that ends abruptly.

The absorption specturm of pyoverdine$_{p.f.}$ in water (Figure 4) exhibited a maximum absorbance peak between the wavelengths 380-395 nm. A characteristic of siderochromes is a maximum absorbance at a wavelength of 400 nm (Nuesch and Knusel, 1965). Work performed by other researchers with crude preparations of pyoverdines, have also observed an absorption maximum at approximately 400 nm (Elliot, 1957; Bonde et al, 1957; and Newkirk and Hulcher, 1969).

Throughout this investigation, iron-free pyoverdine$_{p.f.}$ was prepared as required from iron-bound pyoverdine since iron-bound pyoverdine$_{p.f.}$ is more stable than its iron-free counterpart and thus could be stored in a lyophillized form for extended periods of time. Cox and Graham (1979) found that pyochelin, the iron-binding compound produced by P. aeruginosa, could be stored in a dried form for months without any deterioration. The pyoverdine$_{p.f.}$ used in this study was partially purified, and was considered as a single entity.

To date no physiological role has been assigned to the pyoverdines (Leisinger and Margraff, 1979). Since the concentration of the ferric ion in the medium is the sole factor that regulates the synthesis of pyoverdine$_{p.f.}$ and since pyoverdine$_{p.f.}$ is a strong chelator of the ferric ion (Neilands, 1974; Meyer and Abdallah, 1978), it has been suggested that this pigment, pyoverdine$_{p.f.}$, might play a

FIGURE 4 Absorbance Spectrum of $Pyoverdine_{p.f.}$ in Water. (4 mg/ml)

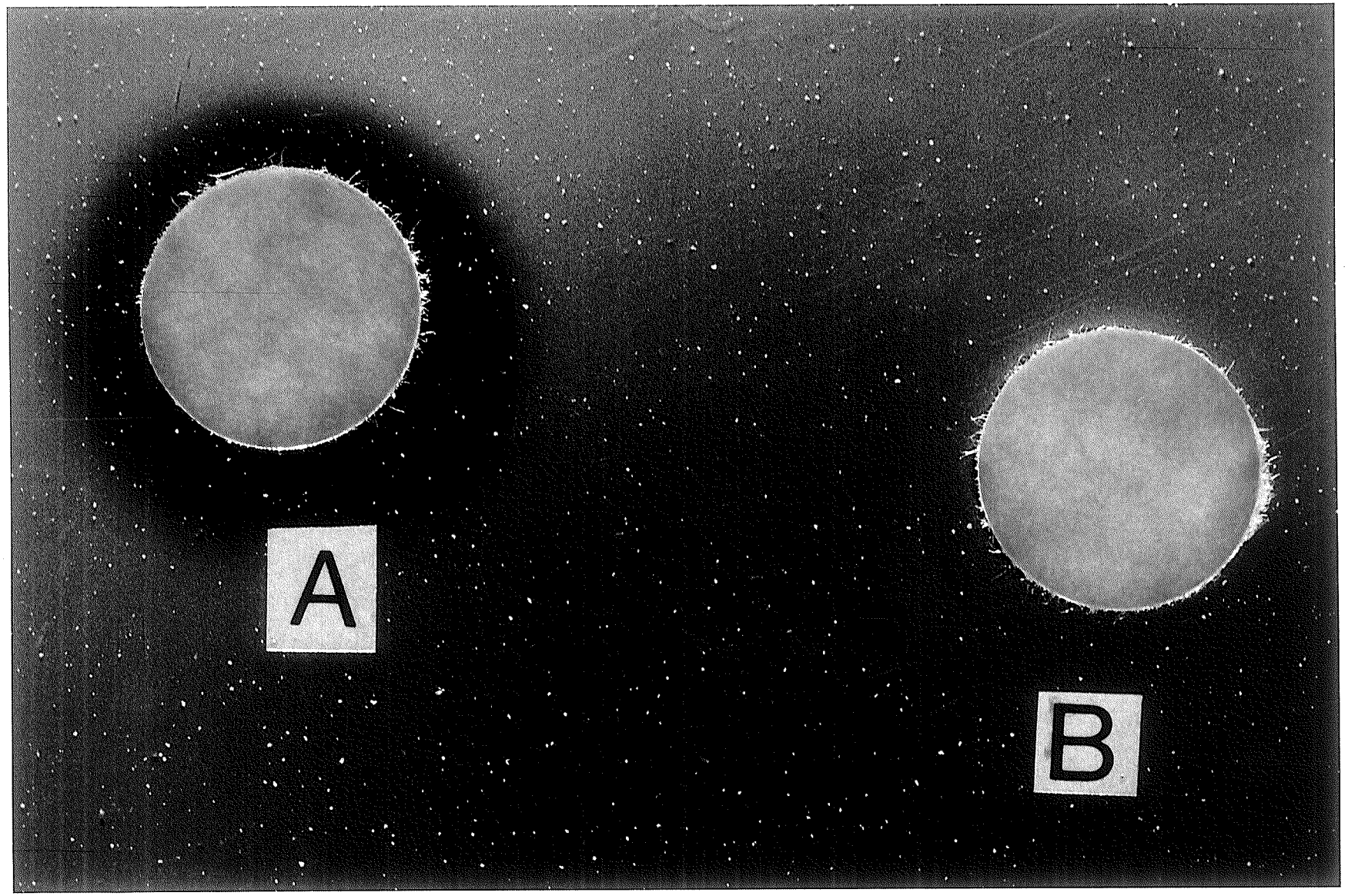

ABSORBANCE
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
200
240
280
320
360
400
440
WAVELENGTH (nm)

role in either the transport and/or the metabolism of iron for the producing organism (Meyer and Hornsberger, 1978).

2. Sensitivity of Salmonella typhimurium to Pyoverdine$_{p.f.}$ - Preliminary Study

A preliminary study was performed using a disc assay method to determine the effects of pyoverdine$_{p.f.}$ on the growth of S. typhimurium as shown in Illustration 2. Sterile discs impregnated with pyoverdine$_{p.f.}$ (A)(20 mg/ml) resulted in a zone of inhibition on plates seeded with S. typhimurium on TSA. A similar disc without pyoverdine$_{p.f.}$ impregnation (B), showed no such inhibition. Since pyoverdine$_{p.f.}$ is a known iron chelator, it is apparent that the zone of inhibition observed on these plates may be due to the unavailability of iron caused by the complexing of iron. The amount of iron in TSA was not determined, however, it was likely that the concentration of pyoverdine$_{p.f.}$ was greater than the concentration of iron in the medium since the iron in the medium surrounding the disc was chelated. The effect of pyoverdine$_{p.f.}$ on the growth of S. typhimurium was then investigated both in vivo and in vitro using the hen's egg as the typical ecosystem. Since both P. fluorescens and S. typhimurium are frequently implicated in rot spoilage and pathogenicity in eggs, respectively, the effects of pyoverdine$_{p.f.}$ on the growth of S. typhimurium in eggs was considered to be a major focal point of this study.

ILLUSTRATION 2 Sensitivity of Salmonella typhimurium to $Pyoverdine_{p.f.}$ (A, $Pyoverdine_{p.f.}$: 20 mg/ml; B, Control-distilled water).


A
B

3. Effect of Pyoverdine$_{p.f.}$ on the Growth of *Salmonella typhimurium* in Egg Albumen (in vitro)

The growth of *S. typhimurium* in egg albumen in vitro is shown in Figure 5. In this study, pyoverdine$_{p.f.}$ exhibited a growth promoting effect on *S. typhimurium* when grown in egg albumen. The age of the albumen did not appear to affect the action of pyoverdine$_{p.f.}$. As the level of pyoverdine$_{p.f.}$ increased, there was a subsequent increase in the growth of *S. typhimurium* in both the one-day old and eight-week old albumen. Since pyoverdine$_{p.f.}$ was previously shown to inhibit the growth of this organism when grown on a synthetic medium, it would appear that the growth promoting effect of pyoverdine$_{p.f.}$ was due to its ability to reverse bacteriostatic effects of the albumen, Since conalbumin is a known bacteriostatic agent present in egg albumen, it is possible that increasing concentrations of pyoverdine$_{p.f.}$ exerted a nullifying effect on conalbumin. Varying concentrations of conalbumin (5-10% w/v) similarly employed using inoculum levels of 10^1 and 10^2 (Table 2) did not show any growth, thus confirming the bacteriostatic effect of conalbumin and the plausible reversion effect of this compound in the presence of pyoverdine$_{p.f.}$. Frankel-Conrat and Feeney (1950) observed that the addition of conalbumin in excess of the amount necessary to bind the iron in a specified growth medium did not prevent growth of *Micrococcus pyogenes* var. *albus*, but greatly increased the

FIGURE 5 Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in Egg Albumen (in vitro). (1-day old, ●–●; 8-week old, ○–○.)

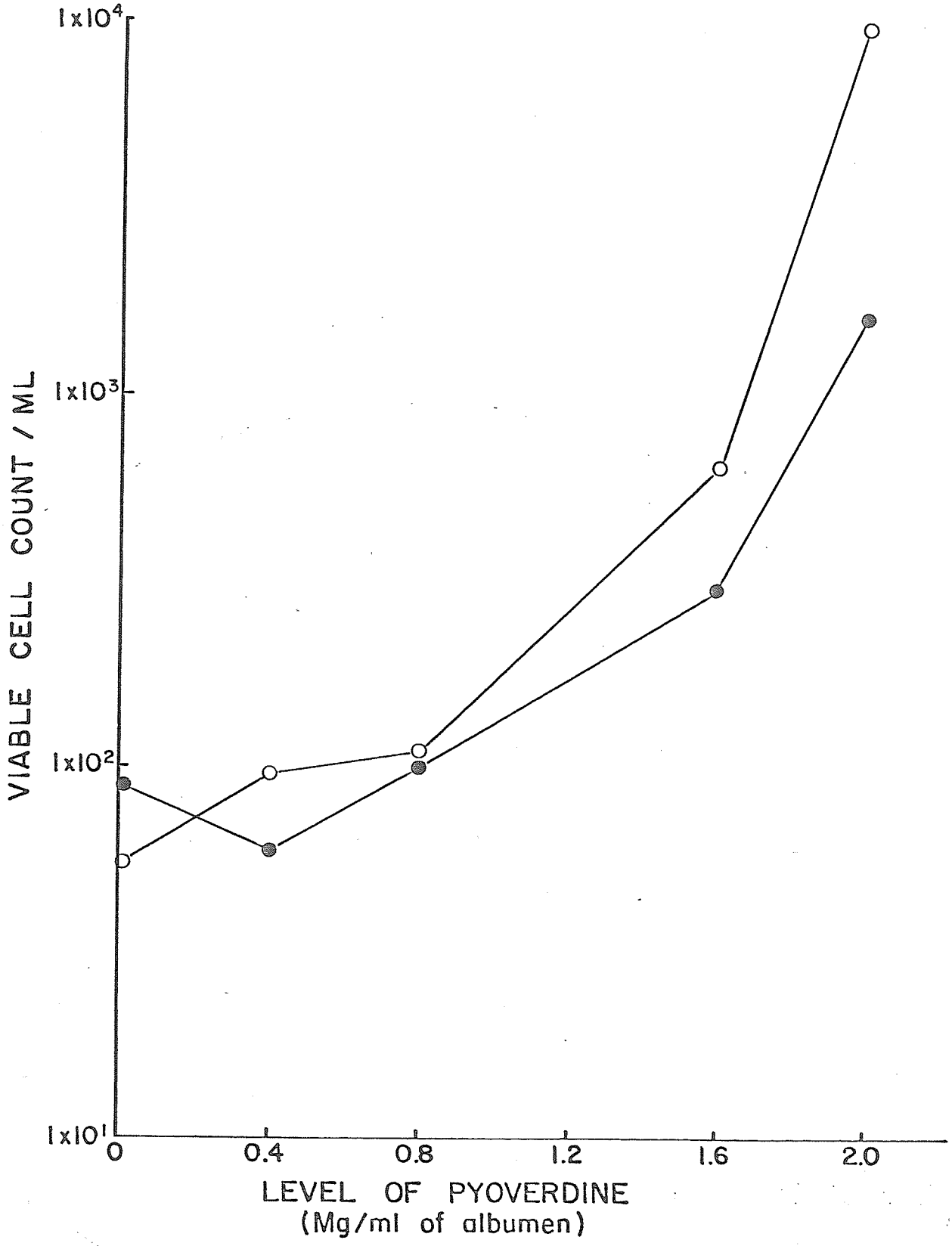

1x10⁴
1x10³
1x10²
1x10¹
VIABLE CELL COUNT / ML
0
0.4
0.8
1.2
1.6
2.0
LEVEL OF PYOVERDINE
(Mg/ml of albumen)

TABLE 2. Effect of Conalbumin on the Growth of *Salmonella typhimurium* in Egg Albumen (in vitro).

Level of Supplemented Conalbumin (%)	Viable Cell Count/ml			
	1-day old albumen		8-week old albumen	
	Inoculum: 10^1	10^2	Inoculum: 10^1	10^2
0	0	2×10^1	0	4×10^1
5	0	0	0	0
10	0	0	0	0
15	0	0	0	0

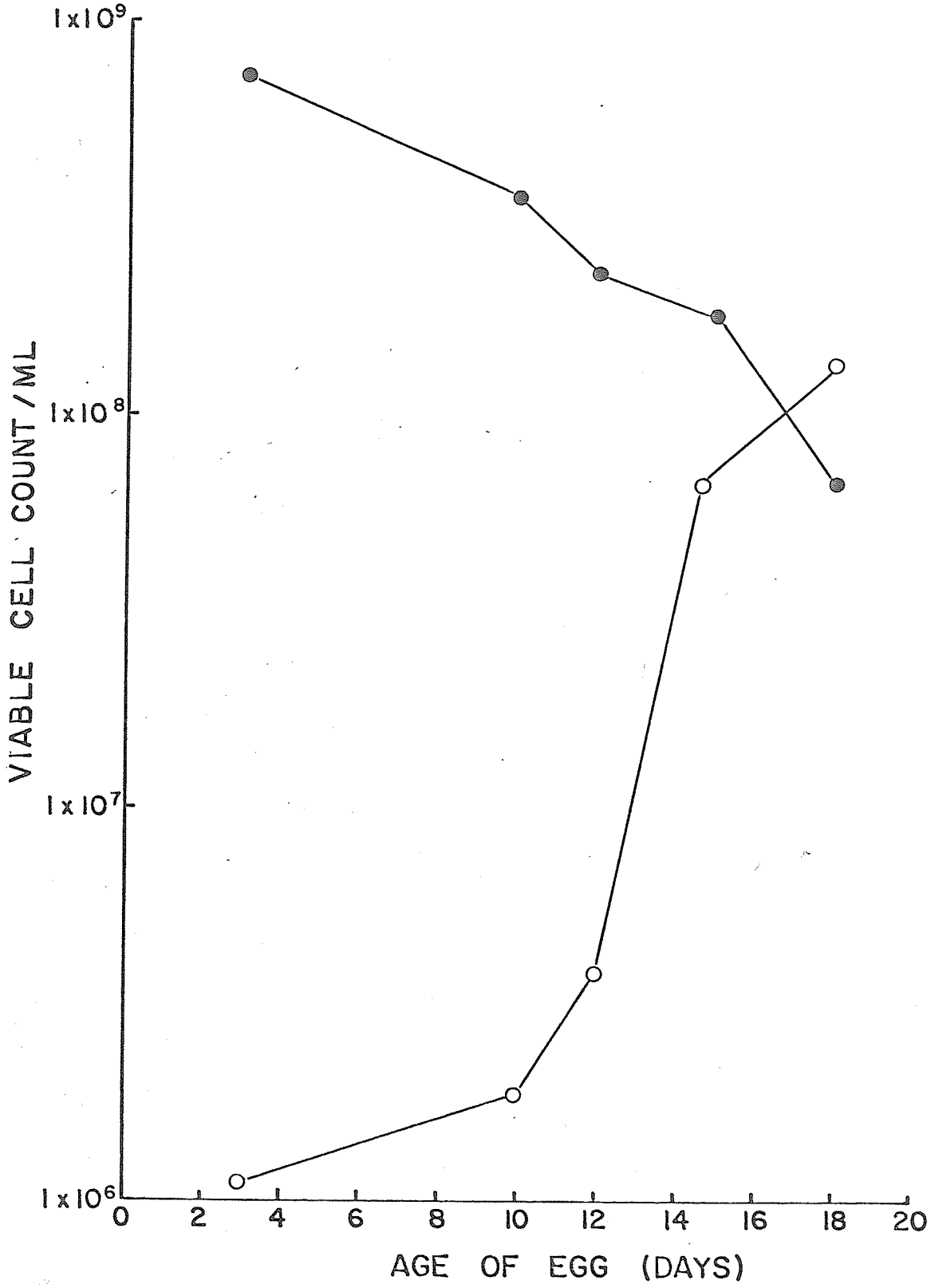
lag period and reduced the rate of subsequent growth. They proposed that the bacteria were slowly dissociating the iron-conalbumin complex by assimilation of the free iron in equilibrium with the complex or by a shift of the equilibrium through reactions such as lowering the pH at the surface of the bacterial cell. Work performed by Garibaldi (1970) using iron-transport compounds produced by S. typhimurium and P. ovalis in egg albumen, showed that these compounds were capable of reverting the bacteriostatic effect of conalbumin thereby promoting the growth of these organisms in egg albumen. This author also proposed that these bacteria excreted iron-transport compounds into the albumen which then diffuse and facilitate the multiplication of the bacteria therein. The results obtained using an initial inoculum of 10^1 viable cells showed no growth upon addition of $pyoverdine_{p.f.}$; this low inoculum was initially chosen to better represent indigenous and incipient contamination by Salmonella, however, it was shown to be too low for the detection of any effect that may be caused by $pyoverdine_{p.f.}$. From the results shown in Figure 5, it appears that $pyoverdine_{p.f.}$ acted as a sideramine, neutralizing the antibiotic effect of native conalbumin thereby exerting a growth promoting effect. Studies performed by Feeney (1951) indicated that the inhibition of the growth of several Gram-positive bacteria caused by either conalbumin or 8-hydroxyquinoline, both of which are iron chelators, did not occur when these two substances were added together to the

test culture media and that the lack of this inhibition was intimately related to theiron and cobalt contents of the media.

4. Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in Eggs of Varying Ages (in vivo).

The explanation that pyoverdine$_{p.f.}$ acts as a sideramine can also be applied to the growth promoting effect which was observed in Figure 6. Using a 0.1 ml aliquot of pyoverdine$_{p.f.}$ (20 mg/ml) inoculated into the albumen of whole eggs (in vivo), pyoverdine$_{p.f.}$ showed a growth promoting effect as compared to eggs inoculated with S. typhimurium alone. This growth promoting effect decreased as the age of the egg increased. Since it is speculated that pyoverdine$_{p.f.}$ and conalbumin chelate iron in a yet undefined stoichiometric relationship, it is plausible that the diffusion of the nutrients such as iron, and inorganic phosphate from the yolk to the albumen may cause conalbumin and/or pyoverdine$_{p.f.}$ to exist as limiting factors thereby altering the stoichiometric relationship. It was found by Luckey et al. (1972) that S. typhimurium was capable of scavenging iron complexed with siderochromes secreted by other organisms. Neilands (1974) proposed that these bacteria used what appeared to be several genetically determined transport systems of varying specificity. Hence, it is possible that pyoverdine$_{p.f.}$ was binding the free iron, presenting it to the bacteria in a usable form. The

FIGURE 6 Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in Eggs of Varying Ages (in vivo). (Pyoverdine$_{p.f.}$ (2 mg)●–●; Control, ○–○).

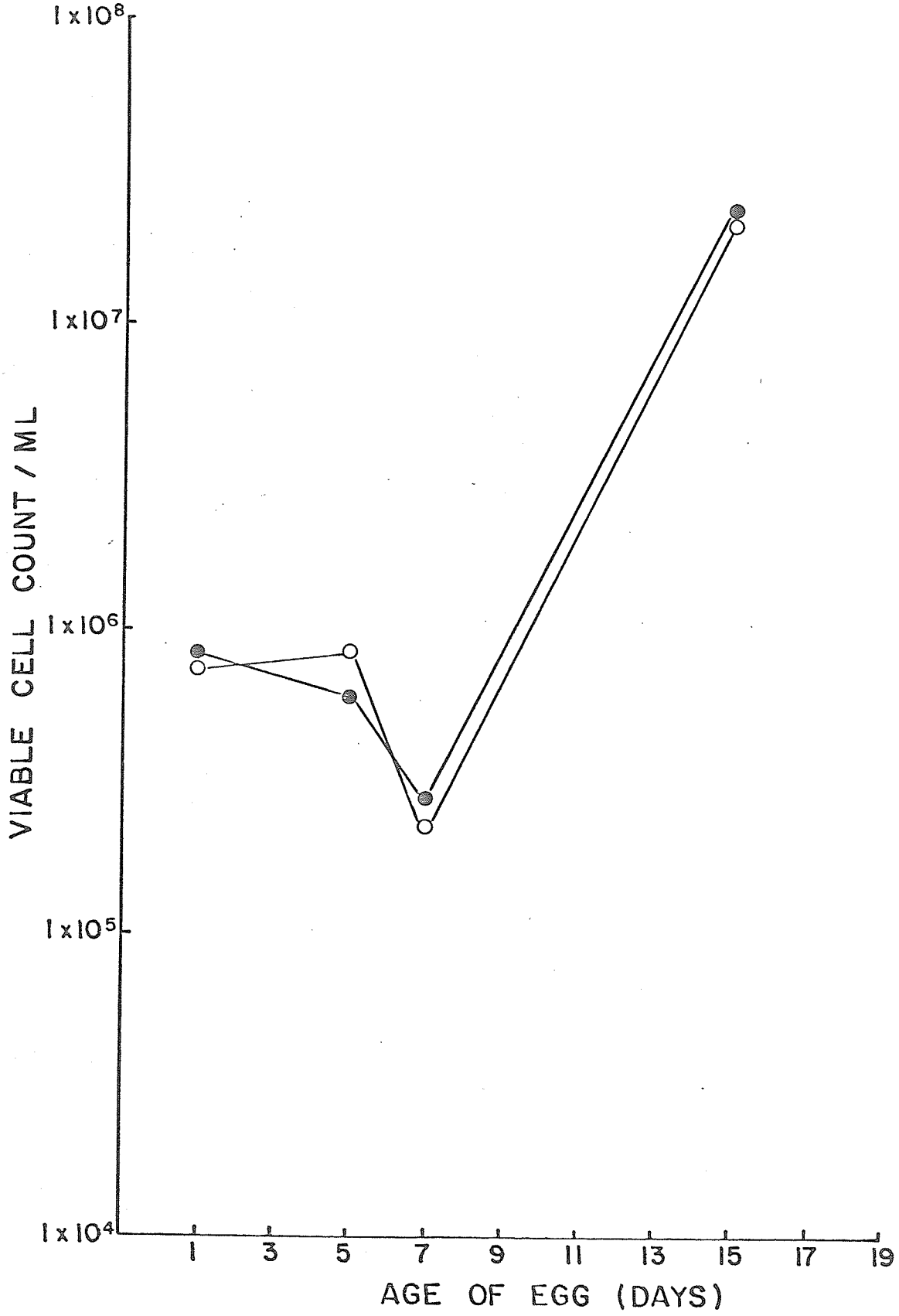

1x10⁹
1x10⁸
1x10⁷
1x10⁶
VIABLE CELL COUNT / ML
0
2
4
6
8
10
12
14
16
18
20
AGE OF EGG (DAYS)

increase in bacterial numbers in the control eggs as the age of the egg increased, was perhaps due to a decrease in the antimicrobial activity of the albumen as well as the diffusion of iron, inorganic phosphate, free amino acids and biotin from the yolk even though the rate of diffusion is low (Brooks, 1960). Brooks (1960) proposed that the age of the egg had little or no effect on the ability of the albumen to sustain bacterial growth regardless of whether the albumen had been kept in vitro or in the intact egg. In this investigation, it was found that in, in vitro studies, age had little effect on bacterial growth (Figure 5), however, when studies were performed in vivo, there was an increase in bacterial growth versus the age of the egg (Figure 6).

5. Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in Eggs of Varying Ages (in vitro).

The effects of pyoverdine$_{p.f.}$ on the growth of S. typhimurium in eggs of varying ages (in vitro) is shown in Figure 7. In this study, it appears that pyoverdine$_{p.f.}$ did not affect the growth of S. typhimurium. It is possible that the level of pyoverdine$_{p.f.}$ did not affect the growth of S. typhimurium. It is possible that the level of pyoverdine$_{p.f.}$ was insufficient to show any effect and/or that these results are inexplicable at this point in time. Further studies will be necessary to clarify this result.

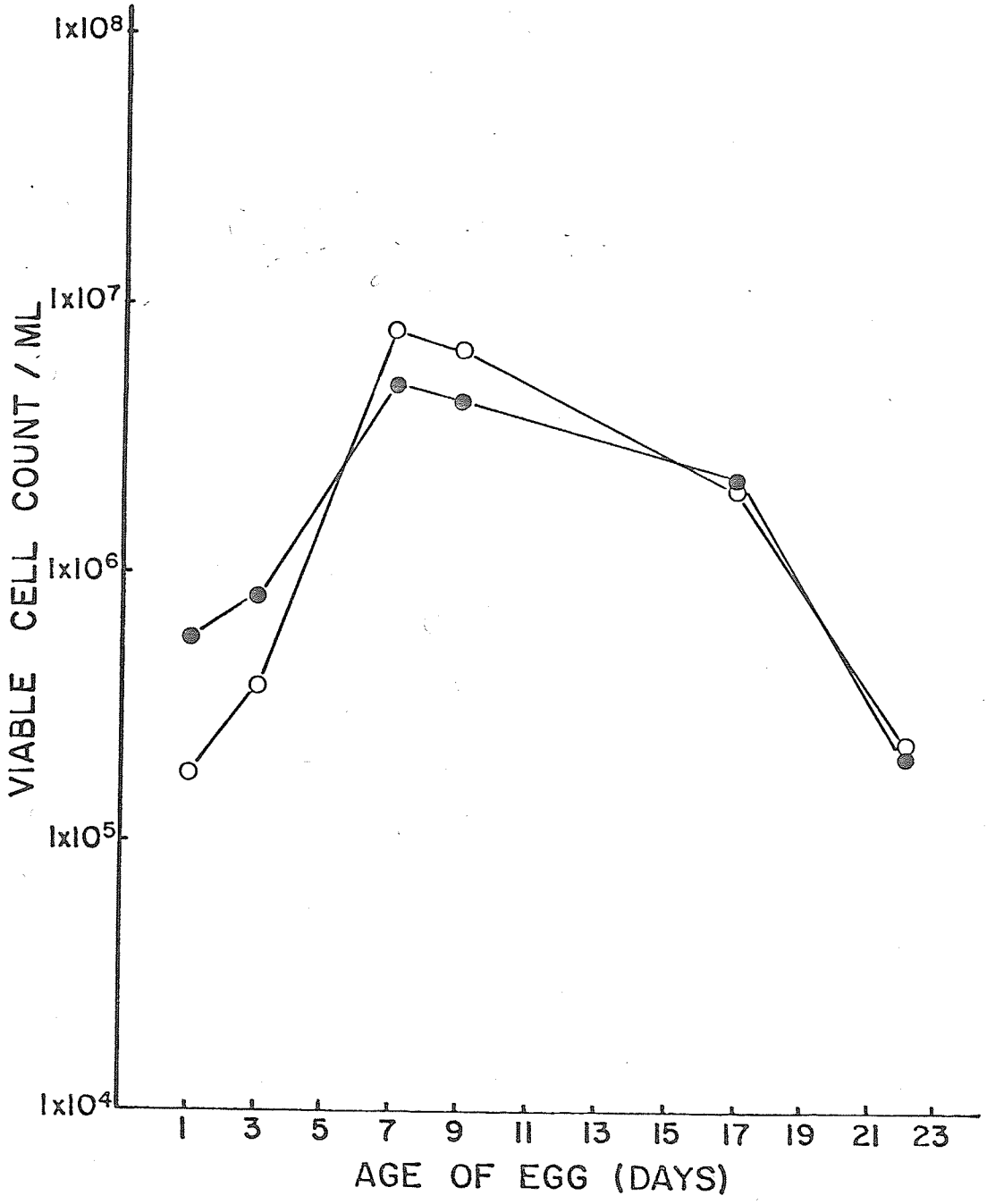
FIGURE 7 Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in Eggs of Varying Ages (in vitro). (Pyoverdine$_{p.f.}$,(4 mg), ●–● ; Control, ○–○).


1 x 10^8
1 x 10^7
1 x 10^6
1 x 10^5
1 x 10^4
VIABLE CELL COUNT / ML
1
3
5
7
9
11
13
15
17
19
AGE OF EGG (DAYS)

6. The Growth of Salmonella typhimurium in Pyoverdine$_{p.f.}$ - Inoculated Eggs.

The growth of S. typhimurium in pyoverdine-inoculated eggs is shown in Figure 8. This investigation was performed to simulate the growth and the production of pyoverdine$_{p.f.}$ by P. fluorescens followed by an invasion of S. typhimurium. The eggs were pre-inoculated with pyoverdine$_{p.f.}$ (20 mg/ml) and were then stored at 4^{o}C. The eggs which were pyoverdine-inoculated exhibited a somewhat similar growth pattern as the control (Figure 8). Up to and including the fourth day of storage, pyoverdine$_{p.f.}$ appeared to have a growth promoting effect as shown by the increase in recoverable cell counts. This growth promoting effect was to progressively decrease during ensuing storage. The control eggs initially showing lower recoverable counts exhibited an increase in S. typhimurium at approximately 5 days of storage, thereafter, increasing and decreasing during storage. The results presented in Figure 8 are highly interpretive, however, two main explanations may be offered. Firstly, the data does not indicate any difference between pyoverdine-inoculated and control eggs. Secondly, that pyoverdine$_{p.f.}$ which was inoculated into the eggs did offer a growth promoting effect as was shown in previous studies, however, this effect decreased due to some breakdown of pyoverdine$_{p.f.}$ during storage. It has been reported that pyoverdine$_{p.f.}$ is labile at alkaline pH values (Meyer and Abdallah, 1978), therefore storage of

FIGURE 8 The Growth of Salmonella typhimurium in Pyoverdine$_{p.f.}$ - Inoculated Eggs (in vivo). (Pyoverdine$_{p.f.}$, (2 mg)●–●; Control, O–O).

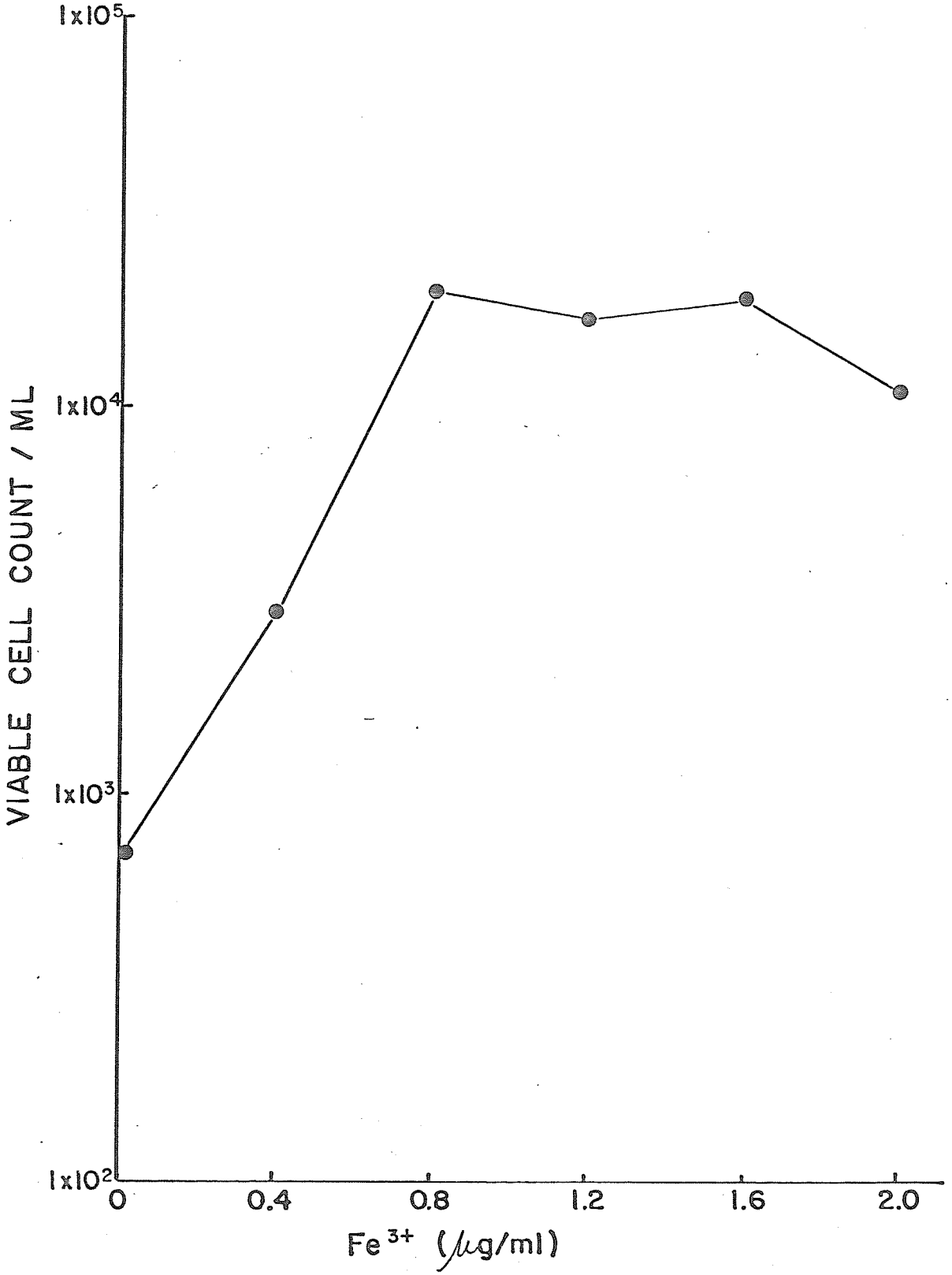

1x10⁸
1x10⁷
1x10⁶
1x10⁵
1x10⁴
VIABLE CELL COUNT / ML
1
3
5
7
9
11
13
15
17
19
21
23
AGE OF EGG (DAYS)

pyoverdine$_{p.f.}$ in the egg albumen at pH 8.8 (Appendix Table 11) for extended time periods might have led to some decomposition of pyoverdine$_{p.f.}$.

7. Effect of Iron on the Growth of Salmonella typhimurium in Standard Succinate Medium.

The effects of iron on the growth of S. typhimurium is shown in Figure 9. This study was performed to verify the need for iron by S. typhimurium. Prior to using SSM as the iron deficient medium, TSB was made iron deficient following the method outlined by Waring and Werkman (1942) which involved extraction with 8-hydroxyquinoline (oxine). However, the bacteria failed to grow in this oxine-treated media despite the addition of copper, zinc, and manganese back to the media (Waring and Werkman, 1942). This lack of growth may be due to the presence of toxic traces of oxine on oxine-treated media when iron and copper were added back into the growth medium (Rubbo, 1950). The optimum concentration of iron for the growth of S. typhimurium in SSM was ca. 0.8 µg/ml. The concentration of iron between 0.8-1.6 µg/ml showed no enhancement of growth. Concentrations greater than 1.6 µg/ml showed a decrease in growth. It is possible that at these higher iron concentrations, the formation of ferric hydrate precipitates were responsible for the inhibition of bacterial growth. Waring and Werkman (1942) postulated that the minute particles of the solid hydrate probably adsorb on the outer surface of the

FIGURE 9 Effect of Iron (Fe^{3+}) on the Growth of Salmonella typhimurium in Standard Succinate Media.

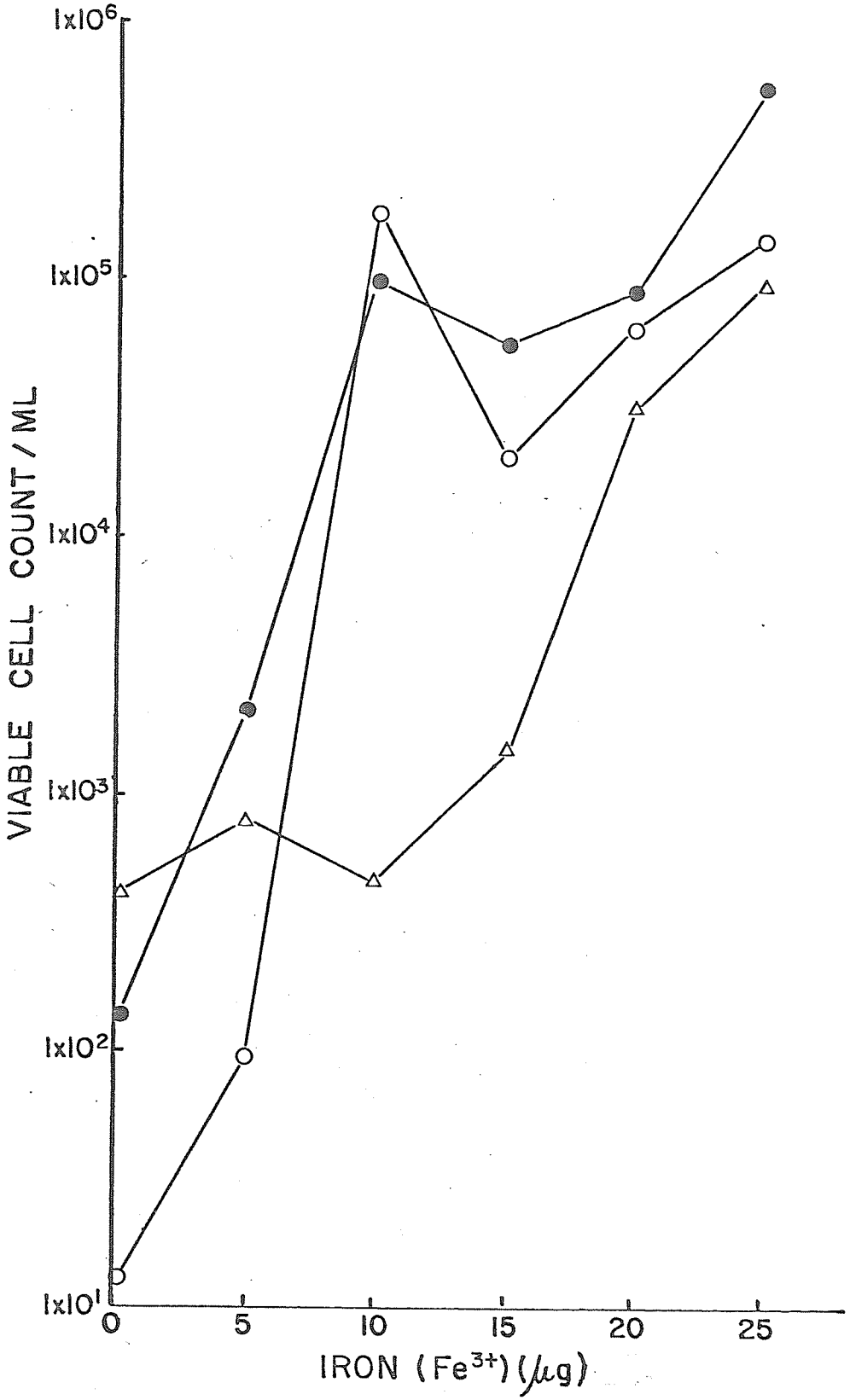

1x10⁵
1x10⁴
1x10³
1x10²
VIABLE CELL COUNT / ML
0
0.4
0.8
1.2
1.6
2.0
Fe 3+ (µg/ml)

cell membrane, thereby interfering with cell wall permeability, the nutrition of the cell, excretion and possibly respiration. Excess solid iron in close proximity to the cell may also cause an increase in solution of the iron brought about by the acidic products of fermentation. The excess of dissolved iron might then enter the cell and bring about an ionic imbalance within the protoplasm itself (Waring and Werkman, 1942). Analysis of SSM showed that this medium contained 0.04 µg/ml iron (Fe^{2+}). Since there was growth of S. typhimurium in this medium without any supplementation of iron, it is likely that the iron requirements of S. typhimurium are less than 0.04 µg/ml.

8. Determination of Growth of Salmonella typhimurium in Standard Succinate Media containing Varying Concentrations of Iron Supplemented with Different Iron Chelators.

The growth of S. typhimurium in SSM containing varying concentrations of iron supplemented with $pyoverdine_{p.f.}$ (20 mg) conalbumin (10%) and a combination of $pyoverdine_{p.f.}$ (20 mg) and conalbumin (10%) is shown in Figure 10. The growth promoting effect of $pyoverdine_{p.f.}$ is observed at the zero level of iron. At this level, samples containing the combination of $pyoverdine_{p.f.}$ and conalbumin yielded the highest recoverable cell counts, followed by $pyoverdine_{p.f.}$ and lastly by conalbumin. It would appear that in the presence of $pyoverdine_{p.f.}$ the

FIGURE 10 Growth of Salmonella typhimurium in Standard Succinate Media containing Varying Concentrations of Iron Supplemented with Different Iron Chelators. (Pyoverdine$_{p.f.}$: 20 mg ●—●; Conalbumin, 10% ○—○; Pyoverdine$_{p.f.}$: 20 mg and Conalbumin, 10% △—△).


1x10^6
1x10^5
1x10^4
1x10^3
1x10^2
1x10^1
VIABLE CELL COUNT / ML
0
5
10
15
20
25
IRON (Fe3+) (μg)

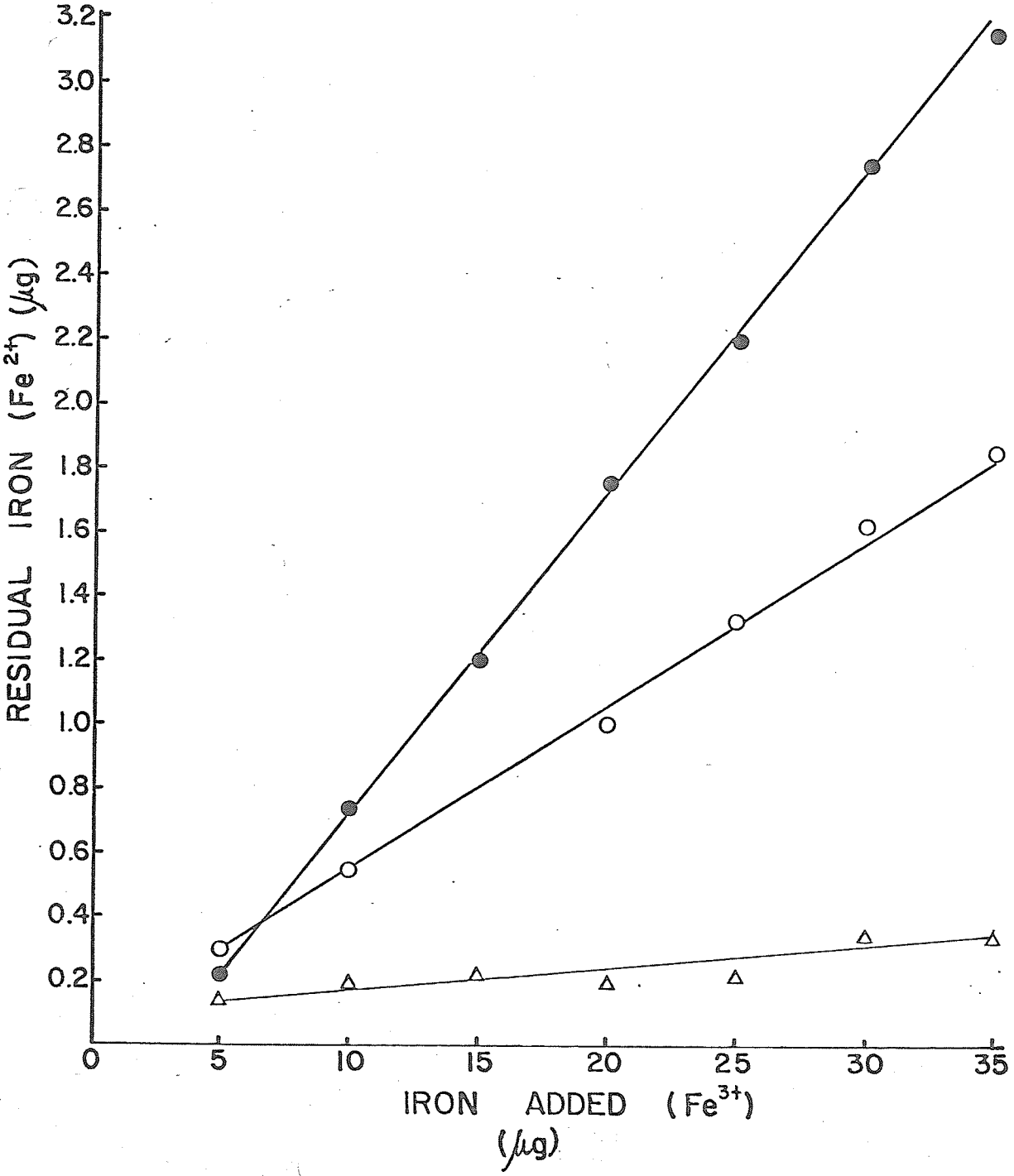
bacteriostatic effect of conalbumin is nullified. However, as the concentration of iron increased, this trend was not observed. There was an increase in the recoverable cell counts for pyoverdine$_{p.f.}$ as well as for conalbumin. In these two samples, the level of iron was high enough to counteract any growth promoting effect and/or inhibitory effect. Feeney (1951) noted that conalbumin had to be present in excess of the amount of iron in order for inhibition by conalbumin to occur. As the concentration of iron increased, samples containing both pyoverdine$_{p.f.}$ and conalbumin exhibited recoverable cell counts which remained somewhat stationary, then increased at concentrations higher than 10 µg/ml iron. It would be expected that these counts would be higher than those of either pyoverdine$_{p.f.}$ or conalbumin. It is possible that the stoichiometric relationship between pyoverdine$_{p.f.}$ and conalbumin may have been slightly altered at increasing concentrations of iron.

The concentration of iron in egg albumen and egg yolk are reported to be 0.0009% and 0.005-0.011% respectively (Powrie, 1973). From the studies which were performed during this investigation, it appeared that the growth promoting effect of pyoverdine$_{p.f.}$ in the presence of conalbumin occured when the level of iron was in a limiting supply. The fact that siderochromes are produced by microorganisms when grown in iron-deficient conditions would suggest that these compounds are beneficial to the organisms rather than detrimental. However, as the concentration of

iron is increased, these iron chelating compounds are no longer produced by the organisms. From the results obtained in this investigation, the growth promoting effect of pyoverdine$_{p.f.}$, if any, is probably masked by the presence of an excess amount of iron (Figure 10).

A preliminary investigation was performed to verify the binding of iron by pyoverdine$_{p.f.}$, conalbumin and a combination of pyoverdine$_{p.f.}$ and conalbumin. From these results (Figure 11), it appeared that conalbumin is a more efficient iron chelator than pyoverdine$_{p.f.}$, however, there was 5 times as much conalbumin as pyoverdine$_{p.f.}$ (Appendix 12). A more detailed investigation is required to determine the binding capacity of pyoverdine$_{p.f.}$ and conalbumin.

FIGURE 11 Residual Iron in Standard Succinate Media Containing Varying Concentrations of Iron Supplemented with Different Iron Chelators. $Pyoverdine_{p.f.}$: 20 mg ●–●; Conalbumin, 10%, ○–○; $Pyoverdine_{p.f.}$: 20 mg and Conalbumin, 10%, △–△.


RESIDUAL IRON (Fe^{2+}) (μg)
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
35
IRON ADDED (Fe^{3+})
(μg)

SUMMARY AND CONCLUSIONS

Pyoverdine$_{p.f.}$, a water-soluble, yellow-green, fluorescent pigment was produced by Pseudomonas fluorescens when grown in an iron-deficient medium, SSM, (Meyer and Abdallah, 1978). This pigment was partially purified following the method outlined by Meyer and Abdallah (1978) with some minor modifications.

The role of pyoverdine$_{p.f.}$ on the growth of Salmonella typhimurium in the hen's egg was then investigated. The following results were obtained in this investigation.

1. Maximum pigment production by P. fluorescens in SSM occured during the early stationary phase of growth (40 hours).
2. Pyoverdine$_{p.f.}$ (20 mg/ml) was shown to inhibit the growth of S. typhimurium using a disc assay method in a synthetic medium.
3. Pyoverdine$_{p.f.}$ exhibited a growth promoting effect on S. typhimurium in egg albumen at levels greater than 0.4 mg/ml pyoverdine$_{p.f.}$/ml albumen (in vitro).
4. The age of the egg albumen (in vitro) did not appear to affect the action of pyoverdine$_{p.f.}$. In, in vivo studies, it appeared that the growth promoting effect of pyoverdine$_{p.f.}$ decreased as the age of the egg increased.

5. The results obtained from the study on the growth of S. typhimurium in pyoverdine$_{p.f.}$ - inoculated eggs indicated that either pyoverdine$_{p.f.}$ probably decomposed during storage in the egg albumen at 4°C, or that the concentration of pyoverdine was too low to detect any significant changes.
6. The optimum iron concentration for S. typhimurium in SSM was 0.8 µg/ml (Fe^{3+}) . Minimum iron requirement for S. typhimurium was less than 0.04 µg/ml (Fe^{3+}) since there was growth of S. typhimurium in SSM which was not supplemented with iron.
7. The growth promoting effect of pyoverdine$_{p.f.}$ was not exhibited when there was an excess of iron present in the growth medium.

Pyoverdine$_{p.f.}$ is an iron chelator and may inhibit or promote the growth of S. typhimurium depending upon the growth medium. In the hen's egg, in particular, the albumen which contains conalbumin, pyoverdine$_{p.f.}$ exhibited a growth promoting effect on S. typhimurium (in vitro). This effect was masked, however, in the presence of egg yolk. Thus, it appeared that pyoverdine$_{p.f.}$ was capable of reversing the bacteriostatic effect of egg albumen and thus promoted the growth of S. typhimurium therein.

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APPENDIX TABLE 1 Standard Curve for the Determination of Iron (Fe^{2+})*.

Level of Iron (µg/ml)	Absorbance (510 nm)
0	-
0.1	0.022
0.2	0.041
0.4	0.084
0.6	0.123
0.8	0.165
1.0	0.205

* Rand, Greenberg and Taras, 1975.
Refer to FIGURE 1.

APPENDIX TABLE 2 Growth Curve of Pseudomonas fluorescens in Standard Succinate Media.

Time (hours)	Absorbance (500 nm)
20	0.54
25	0.67
30	1.00
35	1.73
40	1.65
45	1.65
50	1.50

Refer to FIGURE 2.

APPENDIX TABLE 3 Pigment Production by Pseudomonas Fluorescens in Standard Succinate Media.

Time (hours)	Absorbance (hours)
20	0.20
25	0.25
30	0.27
35	0.28
40	0.31
45	0.25
50	0.17

Refer to FIGURE 3.

APPENDIX TABLE 4 Absorbance Spectrum of Pyoverdine$^{*}_{p.f.}$ in Water.

Wavelength (nm)	Absorbance	Wavelength (nm)	Absorbance
200	2.00	323	0.68
260	2.00	325	0.65
263	2.00	330	0.66
264	1.75	335	0.70
265	1.28	340	0.80
266	0.98	350	0.98
268	0.72	360	1.15
270	0.60	370	1.20
280	0.46	380	1.20
290	0.55	390	1.00
295	0.63	400	0.70
300	0.72	410	0.50
310	0.82	420	0.31
320	0.79	430	0.17
321	0.72	440	0
322	0.71	500	0

* Concentration of Pyoverdine 4 mg/ml H_2O

Refer to FIGURE 4.

APPENDIX TABLE 5 Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in Egg Albumen (in vitro).

Level of Pyoverdine$_{p.f.}$ (mg/ml of albumen)	Visible Cell Count/ml	
	1-day old albumen	8-week old albumen
0	9.0×10^1	5.5×10^1
0.4	6.0×10^1	9.5×10^1
0.8	1.0×10^2	1.1×10^2
1.6	3.0×10^2	6.3×10^2
2.0	1.6×10^3	9.7×10^3

Refer to FIGURE 5.

APPENDIX TABLE 6 Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in Eggs of Varying Ages (in vivo).

Age of Egg (days)	Viable Cell Count/ml	
	Pyoverdine*	Control
3	7.2×10^8	1.1×10^6
10	3.6×10^8	1.8×10^6
12	2.4×10^8	3.8×10^6
15	1.8×10^8	6.7×10^7
18	6.9×10^7	1.4×10^8

* Level of Pyoverdine$_{p.f.}$ 2.0 mg.

Refer to FIGURE 6.

APPENDIX TABLE 7 Effect of Pyoverdine$_{p.f.}$ on the Growth of Salmonella typhimurium in Eggs of Varying Ages (in vitro).

Age of Egg (days)	Viable Cell Count/ml	
	Pyoverdine*	Control
1	8.5×10^5	7.5×10^5
5	6.1×10^5	8.1×10^5
7	2.8×10^5	2.3×10^5
15	2.3×10^7	2.2×10^7

* Level of Pyoverdine$_{p.f.}$ - 4.0 mg.

Refer to FIGURE 7.

APPENDIX TABLE 8 The Growth of Salmonella typhimurium in Pyoverdine$_{p.f.}$ - Inoculated Eggs (in vivo).

Age of Eggs (days)	Viable Cell Count/ml	
	Pyoverdine$_{p.f.}$*	Control
1	5.9×10^5	1.8×10^3
3	8.2×10^5	3.9×10^5
7	5.5×10^5	8.1×10^6
9	2.5×10^4	6.8×10^6
17	2.2×10^6	2.2×10^6
22	2.1×10^5	2.4×10^5

* Level of Pyoverdine$_{p.f.}$ - 2.0 mg.

Refer to FIGURE 8.

APPENDIX TABLE 9 Effect of Iron (Fe^{3+}) on the Growth of *Salmonella typhimurium* in Standard Succinate Media

Iron (final) (μg/ml)	Iron Added (μg/ml)	Viable Cell Count/ml
0	0	1.7×10^2
0.4	5	3.0×10^3
0.8	10	2.0×10^4
1.2	15	1.7×10^4
1.6	20	1.9×10^4
2.0	25	1.1×10^4

Refer to FIGURE 9.

APPENDIX TABLE 10 Growth of Salmonella typhimurium in Standard Succinate Media Containing Varying Concentrations of Iron Supplemented with different Iron-Chelators.

Iron Added (µg)	Viable Cell Count/ml		
	Pyoverdine$_{p.f.}$ (20 mg/ml)	Conalbumin (10%)	Pyoverdine$_{p.f.}$ (20 mg/ml)+ Conalbumin (10%)
0	1.4×10^2	1.2×10^1	1.4×10^2
5	2.1×10^3	9.7×10^1	7.7×10^2
10	9.9×10^4	1.8×10^5	4.6×10^3
15	5.6×10^4	2.0×10^4	1.5×10^3
20	9.0×10^4	6.1×10^4	3.1×10^4
25	5.5×10^5	1.4×10^5	9.7×10^5

Refer to FIGURE 10.

APPENDIX TABLE 11 Change in pH versus Age of Egg.

Age of Egg (days)	pH
1	7.91
3	8.84
7	8.78
9	8.80
17	8.82
20	8.71

APPENDIX 12

Refer to FIGURE 11.

Linear Regression Equations:

$Pyoverdine_{p.f.}$: $y = 0.099 x - 0.266$

Conalbumin : $y = 0.053 x - 0.023$

$Pyoverdine_{p.f.}$

and

Conalbumin : $y = 0.0068 x + 0.109$

where y = residual iron (μg/ml)

x = iron added (μg/ml)

Quantity of Iron Chelator Used:

$Pyoverdine_{p.f.}$: 2mg/12.5ml SSM which is equivalent to 0.16 mg $pyoverdine_{p.f.}$/ml SSM

Conalbumin : 10mg/12.5 ml SSM which is equivalent to 0.83mg conalbumin/ml SSM.

Ratio of $Pyoverdine_{p.f.}$: conalbumin is 1:5 .

APPENDIX TABLE 13 Residual Iron in Standard Succinate Media Containing Varying Concentrations of Iron Supplemented with Different Iron Chelators.

Level of Iron (µg/ml)	Residual Iron (µg/ml)		
	Pyoverdine p.f.	Conalbumin	Pyoverdine p.f. and Conalbumin
0	Blank	Blank	Blank
5	0.22	0.30	0.15
10	0.73	0.54	0.20
15	1.20	0.68	0.22
20	1.76	1.00	0.20
25	2.20	1.32	0.22
30	2.75	1.62	0.36
35	3.17	1.86	0.36

Refer to FIGURE 11.