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PRODUCTION OF UNIQUE METABOLITES BY THE MARINE DINOFLAGELLATE *PROROCENTRUM MINIMUM*

by

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Abstract

Marine phytoplankton produce extracellular metabolites which may be important in controlling the interactions among species or the competition for a limiting nutrient. While the absolute amount of these metabolites may be small compared to the primary organics released by the phytoplankton cell, the control of the production of these unique metabolites may be an important factor in the ecology of the producing species. These compounds have not been extensively studied due to the difficulty in isolating these minute quantities from seawater.

In this thesis, two externally produced metabolites have been investigated. The concentration of 1-(2,6,6-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione, a nor-caroteniod commonly referred to as the β-diketone, was quantitatively determined during the exponential and senescent stages of growth of Prorocentrum minimum in P-, N-, and iron-deficient batch cultures. The β-diketone was released extracellularly in a single 'pulse' during the stationary stage of growth. Several factors such as temperature, irradiance, type of nutrient-deficiency (N, P, or Fe), and the ambient nitrate concentration were important in establishing the amount of the β-diketone produced. The environmental factors did not influence the temporal pattern of production, only the absolute amount of the β-diketone produced. The limits of the range of production of the β-diketone were narrower than the range of maximum growth for any enviromental influence. The inhibition of growth and the heterotrophic uptake of glucose by marine bacteria,

demonstrated the antibacterial properties of the β -diketone.

The second extracellular organic examined was prorocentrin. Prorocentrin is the extracellular siderophore produced by Prorocentrum minimum, P. mariae-lebouriae, and P.gracile. Functionally similar compounds are produced by Thalassiosira pseudonana and Dunaliella tertiolecta. This study is first to characterize this type of high-affinity iron(III)-transport system in marine eukaryotic phytoplankton.

The pattern of siderophore production by all species is the same, although the absolute amount of the material produced is species specific. There is no intracellular or extracellular siderophore production under iron-sufficient culture conditions. When iron was deficient there was a short period of rapid extracellular siderophore production during the stationary stage of growth. The intracellular prorocentrin concentration was very low which suggests that de novo synthesis of the prorocentrin occurs just prior to extracellular release. The persistence of the extracellular siderophore in the culture medium was brief. There was an increase in the in vivo fluorescence following the loss of the siderophore from the medium. The increase in in vivo fluorescence was not accompanied by an increase in cell concentration. An hypothesis concerning the mechanism of the iron-uptake system is proposed.

Procedures for the isolation and characterization of prorocentrin are presented. Prorocentrin appears to be a tri-hydroxamate siderophore with a molecular weight between 560 and 590 daltons. The iron-prorocentrin complex is stable over a

wide pH range.

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I. INTRODUCTION

A. GENERAL INTRODUCTION

The study of extracellular organics produced by phytoplankton is of considerable ecological interest. These compounds are important in the cycling of organic matter by bacteria, the establishment and maintenance of symbiotic relationships and in the chelation or binding of nutrients.

Extracellular organics from phytoplankton can be separated into artificial groupings.

1). The low molecular weight intermediates of metabolism. These are compounds which are found intracellularly and are released extracellularly as a cellular response to stress or through diffusive leakage from the cell. Compounds of this type include glycolic acid, glycerol, mannitol, and amino acids and comprise the majority of the cellular exudate (Hellebust, 1965, 1974).

2). High molecular weight compounds which are produced and released as a cellular response to the environment (Fogg, 1975).

This second group of extracellular metabolites is quantitatively less important than the low molecular weight intermediates and little research has been done to characterize the components of this fraction. Compounds in this group may be important in influencing the vital activity of the producing organism (e.g. production of extracellular alkaline phosphatase or vitamin B_{12} binding factor) or in influencing the quality of the water (e.g. production of antibacterial or antialgal

organics). To display an important role in the ecology of the producing species, a quantitatively large amount of material is not necessary.

This thesis examines the control of production of organic compounds from this second group. Specifically, two extracellular biologically active metabolites from Prorocentrum minimum and related species will be considered. The first metabolite, 1-(6,2,6-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione, given the trivial name, the β-diketone, is an antibacterial metabolite. The second, prorocentrin, is an extracellular iron-specific chelator (siderophore). Both are representative of compounds in the extracellular, biologically active organic fraction.

B. HISTORICAL BACKGROUND

The production of biologically active metabolites by phytoplankton and the role of these compounds in altering or delimiting the ecology of marine phytoplankton is not a new concept. Quoting Johnson et al. (1924): "...we are pretty sure that the plankton communities influence each other... that there are what we can call group symbiosis on the grand scale so that the kind of plankton which we may expect to be present in a certain sea area must depend to some extent, on the kind of plankton which was previously present." (p.275, Lucas, 1947). Papers by Lucas (1944, 1947, 1955) have provided the philosophical basis for the control of marine relationships by the production of organic compounds.

There are many reviews concerning various aspects of the production of biologically active organics by phytoplankton (Wilson, 1951, 1954, 1981; Saunders, 1957; Lefevre, 1964, Sieburth, 1968; Hellebust, 1974). The production of biologically active metabolites by phytoplankton, to a certain extent, is supported by field studies. The inhibitory nature of bloom water has been noted for a long time (Lefevre, 1964) and allelopathic compounds have been implicated in establishing the sequence of species occurring within the bloom (Bentley, 1960; Pratt, 1966; Brockmann et al., 1977; Keating, 1978; Uchida, 1977).

Much of the supportive work for phytoplankton-phytoplankton relationships has been obtained through laboratory studies. These studies involved either measuring the growth response of species in multispecies cultures (Kayser, 1979; Sharp et al., 1979) or by examining the influence of cell-free filtrate on the growth responses of the assay species ('conditioned' medium)(Uchida, 1977; Iwasaki, 1979). In two instances metabolite action has been characterized as; (1) extracellular vitamin B_{12} binders (Kristensen, 1956; Pintner and Altmeyer, 1979), and (2) chelating compounds, especially extracellular hydroxamic acids (Spencer et al., 1973; Murphy et al., 1976).

With regard to the production of antibacterial compounds by phytoplankton, there is well established antibacterial activity of seawater (Sieburth, 1968), often associated with declining phytoplankton blooms (Moebus, 1972). Bell and Mitchell (1972), Bell et al. (1974) and Chan et al. (1980) have noted the

production of antibacterial compounds by phytoplankton grown under laboratory conditions.

The relationship between phytoplankton and organisms in higher trophic levels is an important aspect in biological oceanography. It is thus surprising that little work has been performed in the area of phytoplankton-zooplankton chemical interactions. However, recent work by Tomas (1980) has suggested that Olisthodiscus luteus out-competes Skeletonema costatum by producing a chemical grazing deterrent. Secondary metabolites from higher plants growing in a salt marsh have been shown to deter detritus feeders (Valiela et al., 1979).

While many papers describe the role of extracellular metabolites in controlling the interactions between species, the approaches of many researchers have failed to differentiate between the activity of extracellular metabolites and other factors:

1) In many studies, there was a failure to separate the competition for nutrients from the inhibition by extracellular metabolites (Lam and Silvester, 1979);

2) Other studies failed to differentiate between the effects of pH and other non-metabolites from the effects of inhibitory substances, even though considerable evidence indicates that factors such as pH and light may be more important than extracellular products in explaining certain interactions (Kroes, 1973; Elbrächter, 1976; D'Elia et al., 1979; Nelson et al., 1979);

3) Many studies have involved the inhibitory nature of compounds

released on cell lysis (Proctor, 1957; Duff et al., 1966; Bruce and Duff, 1967);

4) Algal cultures are not always axenic and this condition is necessary to establish the source of the compound (Spencer et al., 1973);

5) Many experiments have used bioassay organisms from environments removed from that of the producing species. Ecological implications can not be drawn due to environmentally controlled physiological differences between strains (Fisher et al., 1973; Fisher, 1977; Murphy and Belastock, 1980);

6) Finally, one can not be satisfied with establishing the basic chemical characteristics (molecular weight, heat sensitivity, etc.) of the compound(s). This provides little information on essential quantitative questions related to the extent of production, critical concentrations for effectiveness, predicted concentrations in nature or the activity of structurally similar compounds.

To fully appreciate and understand the possible role of extracellular, biologically active metabolites, the responsible extracellular organic must be characterized and the pattern of production of this metabolite under the influence of various environmental factors be established. By understanding which organics are present, the extent of production and the mode of action, one can start envisioning possible situations where extracellular, biologically active organics may occur. Research aimed at answering these questions will provide information necessary to assess the validity of extracellular metabolites as

important factors in biological interactions in marine waters.

C. TAXONOMIC CONSIDERATIONS

The differentiating feature between Prorocentrum and other members of the Dinophyceae are the two anteriorly inserted flagella and a theca consisting of two large valves with a small field of platelets surrounding two, unequal flagella pores (Taylor, 1980). Loeblich et al. (1979) have examined the position of the two flagella and have suggested that both flagella emerge from the larger of the two pores, rather than one flagella per pore. The two valves are covered with minute spines (Dodge, 1975; Toriumi, 1980) and a large apical tooth may form near the anterior pore.

At least 21 species of Prorocentrum have been described. Criteria for the separation of species include size, shape, covering of thecal plate, and the extent of the apical tooth (Dodge, 1975). P. minimum can be distinguished from the other species as the only species with spiny ornamentation on the valves, anterior pores and an apical tooth (Toriumi, 1980). Closely related species include P. balticum (Lohmann) Loeblich and P. mariae-lebouriae (Parke et Ballentine) Hulburt. P. minimum can be separated from P. balticum based on size and shape (P. minimum is ovate and larger)(Toriumi, 1980). The distinction between P. minimum and P. mariae-lebouriae is based entirely on the size of the apical tooth near the flagellar pore. Hulburt (1965) has suggested that all three are the same

species since each shows a large variation in cell size and the form of the anterior tooth within a single sample. P. balticum and P. mariae-lebouriae retain varietal status.

Synonymous species include Exuviaella minima Pavillard, E. mariae-lebouriae Parke & Ballantine, P.triangulatum Martin, and P.cordiformis Bursa (Dodge, 1975).

D. DISTRIBUTION OF PROROCENTRUM SPECIES

Members of the genus Prorocentrum are ubiquitous. The most studied species is P.micans , which is a cosmopolitan neritic species (Dodge, 1975). Holligan et al. (1980) recognized P. micans in over one-half of the samples collected around the British Isles.

P. minimum is commonly found in waters along the west coast of North America. This species is a common component of the phytoplankton of the Gulf of Mexico, English Channel, Caspian Sea, and the Mediterranean Sea (Dodge, 1975). This species comprises about 20% of the fall bloom phytoplankton in Indian Arm and Port Moody Arm in British Columbian waters (Stockner and Cliff, 1979). Large non-toxic blooms of P. minimum are a year-round component of the phytoplankton population of Chesapeake Bay (Tyler and Seliger, 1978) and in Japan (Okaichi, 1975).

Toxic P. minimum blooms have been reported but the nature of this "toxicity" has not been clarified. Tangen (1980) recorded a toxic P. minimum bloom in the outer fjord of Oslofjord in the fall of 1979. The unique bloom was initiated

by the upwelling of deep water. The toxic nature was not described.

Kat (1979) recorded an outbreak of gastrointestinal illness associated with a bloom of P. minimum in the Netherlands. While P. minimum were not the direct cause of the outbreak, bacteria associated with P. minimum was thought to be the causative agent.

E. EXTRACELLULAR PRODUCTS FROM PROROCENTRUM SPECIES

The extracellular products of most marine phytoplankton have not been studied thoroughly. This is especially true in the case of Prorocentrum species. Hellebust (1965) examined the photoexcretion of organic carbon by P. minimum (Exuviaella sp. = P. cordiformis , Bursa, 1957) and found low amounts of organic carbon excreted over a wide range of irridiances. Less than 10% of the total carbon assimilated was released. Of the released material, nearly one-third was glycolic acid. For comparison, Olisthodiscus sp. and Skeletonema costatum released 38 and 52% of the total assimilated carbon, respectively.

While the absolute amount of extracellular organics produced by Prorocentrum species may be small, the quality of the exudate may be important. P.micans has been shown to produce several biologically active organic compounds. Uchida (1977) characterized an extracellular diatom-inhibiting substance which was non-dialyzable and was denatured on autoclaving. Aubert and Pesando (1971) and Gauthier et al.

(1978) examined an extracellular protein which inhibited the production of extracellular antibacterial substances (a fatty acid and nucleosides) by Asterionella japonica and Chaetoceros lauderi. Inhibition of the production of the antibacterial substances had no influence on the growth of the diatom cultures. Several other researchers have attempted to show species interactions involving P. micans, but interpretation in each case was complicated by competition for nutrients (Elbrachter, 1976; Kayser, 1979).

F. PURPOSE

This thesis contains results of the examination of unique extracellular metabolites from P. minimum and related species. Emphasis has been placed on two compounds: 1-(2,6,6-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione (the β-diketone), a unique extracellular antibacterial compound (Chapters II and III) and prorocentrin, an extracellular iron-specific chelator (Chapters IV to VIII).

II. A QUANTITATIVE EXAMINATION OF THE RELEASE OF AN EXTRACELLULAR METABOLITE BY THE MARINE DINOFLAGELLATE PROROCENTRUM MINIMUM

A. ABSTRACT

Marine dinoflagellates produce extracellular secondary metabolites which may play a role in the ecology of the producing species. The concentration of one such external metabolite, 1-(2,6,6-trimethyl-4-hydroxycyclohexenyl)-1,3 butanedione, produced by the marine dinoflagellate, Prorocentrum minimum, was determined quantitatively during exponential growth and during senescence in phosphate starved batch cultures. The pattern of production is similar to the production of many bacterial toxins. There was little production of the β-diketone during the exponential growth period and highest production occurred within one week after cessation of cell division. Approximately 50% of the total β-diketone produced, was excreted on a single day, five days after phosphate became limiting to growth. Cell lysis or photodestruction of carotenoids do not appear to be the cause of release of this compound.

B. INTRODUCTION

Marine phytoplankton excrete a wide variety of organic compounds; however, the magnitude and the conditions causing excretion are still matters of controversy (Smith and Wiebe, 1976; Sharp, 1977; Fogg, 1977; Mague et al., 1980). To-date most of the excreted material has been reported to be simple compounds such as organic acids, amino acids, and carbohydrates (Hellebust, 1974). Excretion of large molecular weight compounds has been reported and the inhibitory or stimulatory effects observed during species interaction experiments have been attributed to these compounds (Uchida, 1977; Pintner and Altmeyer, 1979).

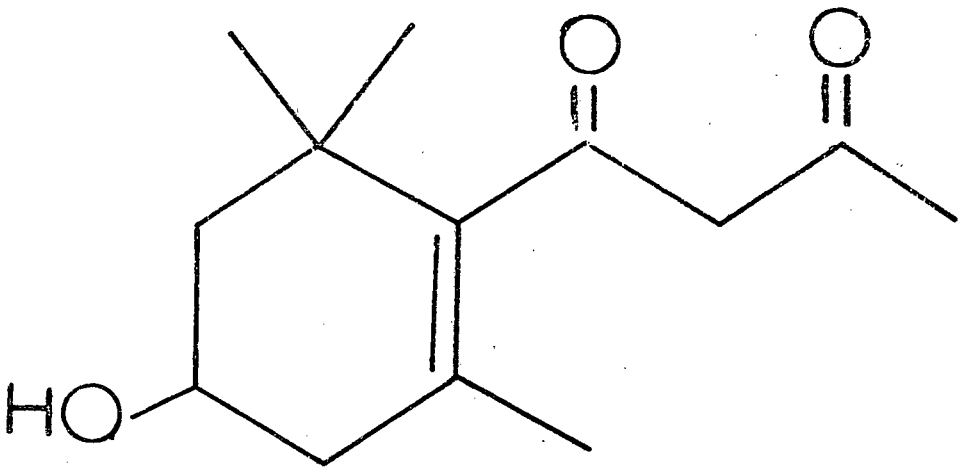
Very few extracellular secondary metabolites from marine phytoplankton have been chemically characterized. Most of the compounds investigated prior to this work have been intracellular toxins and sterols from dinoflagellates (Shimizu et al., 1976; Finer et al., 1978; Alam et al., 1979; Schantz, 1979; Withers et al., 1979) and toxins, alkaloids and novel lipids from marine cyanobacteria (Moore, 1977; Mynderse and Moore, 1978; Cardellina et al., 1978, 1979; Cardellina and Moore, 1980). There is increasing interest in interactions among species in the field and in culture. Extracellular metabolites are thought to play an important role in many of the observed competitive interactions among species (Pratt, 1966). The control of production of individual excreted compounds by environmental and physical factors, has not been examined previously.

Recent work has provided conflicting results on the ability of Prorocentrum species to produce compounds inhibitory to other algal species (Elbrächter, 1976; Uchida, 1977; Iwasaki, 1979; Kayser 1979). An extracellular antibiotic from filtrates of Prorocentrum culture has been isolated and its chemical structure shown to be the nor-carotenoid, 1-(2,6,6,-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione (Fig. 1; Andersen et al. 1980). Compounds of this type have also been shown to be produced by Cyanidium (Rhodophyceae) in mass culture (Jüttner 1979a). As part of a larger investigation to evaluate the bioactive nature or extracellular metabolites from marine dinoflagellates, the kinetics of production of this external secondary metabolite by Prorocentrum minimum grown in the laboratory during exponential growth and during defined conditions of phosphate starvation were examined.

C. MATERIALS AND METHODS

Prorocentrum minimum Schiller was originally isolated from English Bay, B.C. Canada and has been maintained in the Northeast Pacific Culture Collection (NEPCC #96) since 1971. An axenic isolate was grown in sterile enriched artificial seawater, modified by the replacement of Na glycerophosphate with Na_2HPO_4 and $Fe(NH_4)_2SO_4$ with $FeCl_3$ (Harrison et al., 1980). The amount of phosphate in the enrichment solution was reduced to 6 μM (N:P = 27:1) to ensure that cultures became P-starved during senescence. Continuous light was provided by daylight fluorescent bulbs at an irradiance of 160 $\mu E \cdot m^{-2} \cdot s^{-1}$ and a

Figure 1 - The structure of the extracellular metabolite, β-diketone, (1-(2,6,6-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione) from the marine dinoflagellate, Prorocentrum minimum.



temperature of 18°C. Cells were grown axenically in 6-L batch cultures with constant stirring (60 or 120 rpm).

Cultures were tested frequently to determine if they remained axenic throughout the experiment by plating on an organic-containing sterility medium. Disappearance of phosphate from the medium was monitored to ensure phosphate deficiency. Cultures were examined daily and cells were counted using a Palmer-Maloney chamber.

Several identical cultures were set up and each culture was completely harvested at different stages of growth. Production during the growth cycle was a compilation of three distinct experimental repetitions. Cells were removed from the medium by passing the 6-L cultures through a continuous centrifuge (Sharples, type TL). The cell-free medium was membrane filtered (0.45 μ m) and adjusted to pH 2.0. A dissolved organic fraction was collected by passing the acidified filtrate through a 25 x 2 cm column of precleaned (Soxhlet extraction with methanol, 72 h) XAD-2 resin (Mallinckrodt). Remaining salts were removed by washing the column with 200-300 ml deionized, distilled water. The collected organic fraction was eluted with 150 ml methanol.

The methanol extract was concentrated by rotary evaporation at 35°C *in vacuo* and the residue was partitioned between $CHCL_3$ and H_2O. Evaporation of the $CHCl_3$ *in vacuo* at room temperature provided the crude filtrate extract.

Pure β-diketone (Fig. 1) was used as an analytical standard (Andersen *et al.*, 1980). All crude extracts were quantitatively determined via HPLC equipped with a silica gel

column (LiChrosorb Si60) using isocratic separation (2 ml·min^{1} ; hexane: isopropanol, 83:17) and UV detection (287 nm).

To determine the efficiency of the extraction procedure, a known amount of standard compound was introduced into two replicate flasks with 6-L of seawater medium and, after extraction, assayed by HPLC and by absorbance in methanol (287 nm).

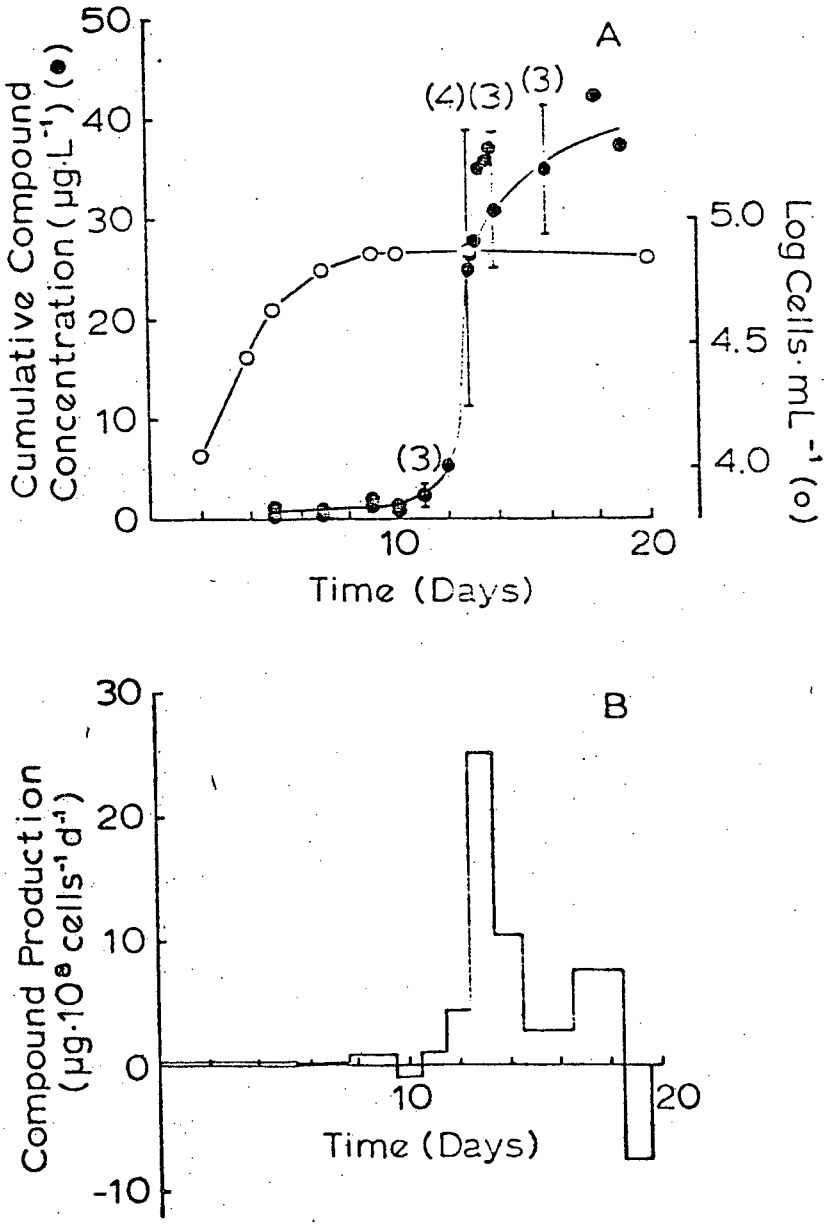
In order to estimate the importance of the β-diketone in relation to the total organic excretion by the culture, excretion was measured using two different methods. Dissolved organic carbon (DOC) in the culture filtrate was determined by wet oxidation with potassium persulfate, with correction for reagent and medium blanks. Rates of excretion were estimated using the $H_2^{14}CO_3$ technique of Mague et al. (1980). Radioactivity was measured in a Nuclear-Chicago Unilux III liquid scintillation counter. Cells were incubated for 4 h under experimental conditions.

D. RESULTS AND DISCUSSION

Only two compounds were found in the extract of the culture filtrate from Prorocentrum minimum. The main compound was identified to be the β-diketone (Fig. 1; Andersen et al., 1980), while the second compound was only present in minor concentrations and was not identified or examined further.

The production of the β-diketone during culture growth is shown in Fig. 2. Little β-diketone was produced during exponential growth. Cells became phosphate limited after seven

Figure 2 - Production of the β-diketone in batch culture. Prorocentrum minimum. A: Changes in β-diketone concentration and cell numbers during exponential growth and during senescence in phosphate-starved batch cultures. Values are single experiments unless noted. Replicate compound concentration values are mean ± one SE, where n is noted in brackets. B: Rates of compound production.



days of growth and 50% of the compound was produced in a single 'pulse' six days later. Decreasing amounts were produced during the later stages of senescence under phosphate starvation.

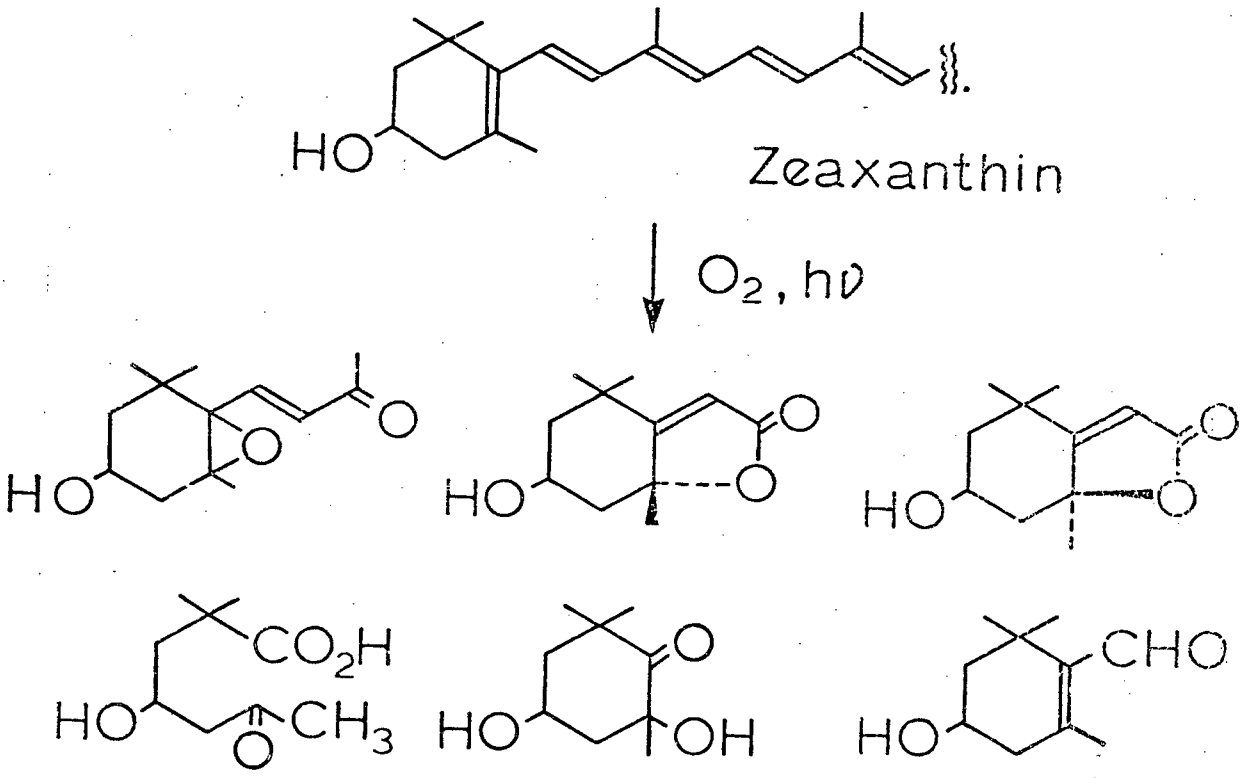
Based on rates of excretion determined using the $H^{14}CO_3^-$ technique the β-diketone comprises approximately 2.8% of the total amount of carbon produced on Day 13 (the period of maximum β-diketone production). The average production over the entire period of β-diketone production (Day 11 through Day 19) was much lower, with the β-diketone accounting for an average of 0.4% of the carbon released per day. This was similar to calculations based on direct DOC determinations where the β-diketone comprised 0.6% of the total DOC at the end of the experimental period.

The observed pattern of production and the relatively high light intensities utilized, prompted the concern that the occurrence of this compound in the medium was a result of either cell lysis or the photochemical oxidation of carotenoids. It is unlikely that the compound was released as a result of cell lysis since loss of cells based on cell count was only noted 18 days after most of the β-diketone had been released. In addition, intracellular β-diketone in the Prorocentrum isolate has never been found. Furthermore, the HPLC technique indicated the production of only one major and one minor compound in this extracellular fraction throughout the entire growth period except for Day 30, when a variety of compounds were collected, indicating possible cell lysis at this later date.

A second consideration was whether this compound was the

result of *in vitro* photooxidation of carotenoids. The structure of the β-diketone suggested a possible carotenoid origin and preliminary data indicated the compound was produced only at saturating irradiances It has been shown that nor-carotenoids of similar carbon skeleton were produced by photooxygenation of carotenoids *in vitro* (Isoe *et al*. 1969; Isoe *et al*. 1972). This suggests that the β-diketone is a product of an enzyme controlled metabolism, perhaps involving a photobiochemical step, rather than a non-specific photooxidation for the following reasons: a) *Prorocentrum minimum* produced only one compound of this type. If non-enzymatic chemical breakdown of carotenoids was occurring, one would expect all carotenoids to undergo degradation and several compounds would be produced in direct proportion to the carotenoid composition. The precise carotenoid composition of *P. minimum* is not known; however, members of this genus have been reported to contain two major xanthophylls (peridinin and diadinoxanthin), with β-carotene constituting less than 10% of the total carotenoid content (Johansen *et al*., 1974). Zeaxanthin and violaxanthin, two likely precursors of the β-diketone have not been reported in members of the Dinophyceae (Goodwin, 1979), while on a structural basis peridinin does not appear to be a likely precursor. However, the β-diketone could have originated from diadinoxanthin by hydration of the acetylene bond followed by oxidative rupture of the carotenoid chain at $C_8 - C_9$; b) Isoe *et al*. (1972) have shown that the *in vitro* photooxidation of zeaxanthin produces a complex mixture of products (Fig. 3),

Figure 3 - Zeaxanthin and products of photooxygenation (from Isoe et al., 1972).



while the isolation of a single non-carotenoid argued against such non-specific photooxidations; and c) the low rate of production during the initial period of phosphate starvation, followed by a 'pulse' of excretion during later stages, suggested some form of biological control.

While this is the first report on the pattern of production of an extracellular secondary metabolite from phytoplankton, this pattern was not unique among microorganisms. The production pattern was very similar to that observed for many bacterial toxins, where there is no production during exponential growth, low production as cells undergo adjustment from exponential growth to the stationary phase, high production during the early stationary phase, and a rapid cessation of production after a short period of time (Weinberg 1970). Usually the length of production was between one half to two times the length of the exponential growth period in bacterial cultures (Weinberg 1970). In this study, 50% of the material was produced in a single pulse suggesting a readily available precursor pool or a rapid exhaustion of available precursors.

In conclusion, the first pattern of production of an identifiable extracellular secondary metabolite produced by a marine dinoflagellate has been established. Since the data show production only under nutrient starved conditions and a low yield of compound when compared to total organic excretion determined by other techniques, the production of this compound would have negligible influence on organic carbon cycling under open ocean conditions. However, the important influence this

compound may exert on species succession, especially under bloom conditions in which Prorocentrum minimum has occasionally been found to be abundant (Nakazima 1978; Kat 1979), cannot be ignored.

III. THE INFLUENCE OF ENVIRONMENTAL FACTORS ON THE PRODUCTION OF AN ANTIBACTERIAL METABOLITE FROM PROROCENTRUM MINIMUM

A. ABSTRACT

Prorocentrum minimum, an ubiquitous non-toxic red-tide forming dinoflagellate, produces biologically active extracellular metabolites. One such extracellular organic compound is 1-(2,6,6-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione, a nor-carotenoid commonly referred to as the β-diketone. The β-diketone was released extracellularly in a single 'pulse' during the stationary phase of growth. Factors such as irradiance and temperature are important environmental factors controlling the production of this metabolite. Different extracellular concentrations were obtained depending on the type of limiting nutrient. If cells were P-deficient the production was twice that of the N-deficient cultures. Iron-deficient growth further reduced the amount of the β-diketone produced. The ambient nitrate concentration in the medium had a strong influence on the amount of the β-diketone produced. The pattern of production and the control by environmental factors suggest that the production of this metabolite is directly controlled by the physiological state of the cell, and not merely a result of the photodestruction of carotenoids. The speculative role of the β-diketone in the natural environment is discussed.

B. INTRODUCTION

Phytoplankton convert large amounts of the fixed carbon into extracellular metabolites. The majority of these extracellular metabolites are primary organic compounds which play an important role in the heterotrophic cycle of aquatic ecosystems (Aaronson, 1971; Hellebust, 1974). A smaller amount of less well defined secondary, or unique, metabolites are also produced. Compounds in this latter group may be important in controlling interactions among phytoplankton and other organisms, or in regulating intracellular metabolism.

An example of these less well studied, unique metabolites include acrylic acid, first noted by Sieburth (1959). The precursor of acrylic acid (DMPT: dimethyl-B-proprothetin), was found internally in colonial forms of Phaeocystis pouchetii (Haptophyceae). Evidence by Guillard and Hellebust (1971) suggested that acrylic acid should exist extracellularly (2% of total excreted carbon) and they calculated external acrylic acid concentrations in natural blooms to reach 7 $\mu g \cdot L^{-1}$.

Another unique extracellular metabolite involved in marine phytoplankton ecology is 'aponin', a group of base-labile organics produced by the cyanobacterium Gomphasphaeria aponina. At low concentrations this compound causes lysis of the dinoflagellate Gymnodinium breve (Martin and Martin, 1976; Eng-Wilmt, et al., 1979).

Of increasing interest is the production by phytoplankton of nor-carotenoids, a group of organics related to carotenoids and some of their breakdown products. Nor-carotenoids have been

recognized as anti-fungal and antibacterial agents (Zajic-Seeret and Kuehn, 1961) and have been found in waters associated with blooms of fresh water cyanobacteria (Tabachek and Yurkowski, 1976). Natural populations of Microcystis wesenberg, produce the nor-carotenoid, β-cyclocitral, which gives the bloom a tobacco-like odor (Jüttner, 1979a). The fragrant, volatile nature of nor-carotenoids led Juttner to isolate four nor-carotenoids produced extracellularly by Cyanidium caldarium (Rhodophyceae) under large scale culturing conditions. These included β-ionone, geranylacetone, methylheptenone, and dihydrotrimethylnaphthalene (Jüttner, 1979a).

The antibiotic nature of the nor-carotenoids produced by C. caldarium has been established. Of the four nor-carotenoids, β-ionone and dihydrotrimethylnapthalene were inhibitory to cyanobacteria at 10 ppm. Geranylacetone reduced growth of cyanobacteria at 50 ppm. Methylheptenone was the least inhibitory to the cyanobacteria species examined, since concentration greater then 100 ppm were required to reduce the growth of the assay species (Jüttner, 1979b). The antibacterial nature of nor-carotenoids in aquatic systems has also been examined by Reichardt (1981). Methylheptenone, inhibited colonial growth of all bacterial species examined and was inhibitory to glucose uptake at 2 ppm (18 μM). Conversely β-ionone and geranylacetone inhibited only pigmented bacteria. The mechanism of inhibition involves the blockage of pigment production by the bacteria. This is a similiar mechanism to the inhibition of carotenoid synthesis in the cyanobacterium

Synechococcus by the addition of geranylacetone (Jüttner, 1979b).

Recently, Andersen et al. (1980) characterized the extracellular nor-carotenoid[1] 1-(2,6,6,-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione, from laboratory cultures of the marine dinoflagellate Prorocentrum minimum. This compound, given the trivial name, the β-diketone, is an example of an antibacterial, nor-carotenoid which imparts a tobacco-like odor to P. minimum cultures. The production of the β-diketone by P. minimum was examined in Chapter 2. Production was dependent on the physiological state of the cells and not simply a by-product of the photooxygenation of carotenoids.

Since the β-diketone is antibacterial under both laboratory (Andersen et al., 1980) and field conditions (Appendix A), understanding environmental factors which control the production and release of this metabolite may provide insight into the possible role of nor-carotenoids in controlling the interactions among microorganisms in aquatic ecosystems.

In this chapter, the influence of enviromental factors on the amount of β-diketone produced is examined. Factors such as irradiance, temperature, and salinity are considered for phosphate-deficient batch cultures. Since the β-diketone is a potential trace metal chelator (Andersen et al., 1980), production under iron-limited growth conditions was also investigated.

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[1] The traditional definition, norcatotenoids as possible carotenoid breakdown products, is employed.

C. MATERIALS AND METHODS

All Prorocentrum species were grown in enriched artificial seawater medium (Harrison et al., 1980) , at 18 °C with the exception of P. mariae-lebouriae which required an enriched natural seawater-based medium of lower salinity (15 ‰) and elevated temperatures (22-24 °C) for growth. Both media were modified by the replacement of Na glycerophosphate with $Na_2 HPO_4$ and $Fe(NH_4)_2 SO_4$ with $FeCl_3$. The amount of phosphate in the enrichment solution was reduced to 6 μM (N:P 27:1 by atoms) to ensure that the batch cultures became phosphate-starved during senescence.

To achieve iron-starved growth, P. minimum was grown in the following media: 1) charcoal-treated natural seawater followed by the addition of all required nutrients (ESNW) (Harrison et al., 1980); 2) Charcoal-treated natural seawater followed by the removal of most of the available iron by the procedure of Lewin and Chen (1971), and then supplemented with all nutrients except $FeCl_3$ and EDTA (ESNW-Fe); and 3) charcoal- and Chelex-100 resin-treated natural seawater supplemented with AQUIL nutrients minus $FeCl_3$ and EDTA (Morel et al., 1979). The first medium provided an iron-sufficient control while the other media provided iron-limited growth conditions.

Vanadium was added as NH_4VO_3 to alleviate the effect of iron-limition on growth in one set of experiments.

Continuous lighting was provided by daylight fluorescent bulbs. Irradiance was reduced to 120, 90, 60 and 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ by wrapping cultures in neutral density screening in certain

experiments.

Cells were grown axenically in 6 L or 10 L flat bottomed boiling flasks with constant stirring (60 rpm). The axenic nature of the cultures was verified visually using the acridine orange direct epifluorescent count technique (Hobbie et al., 1977), or by plating on an organic sterility medium and examining for growth after 48 hours. If bacterial contamination was discovered, the cultures were discarded.

Cultures were harvested and the β-diketone collected using the procedure stated previously (Chapter II) (Fig. 4). Briefly: cells were removed from the entire 6 or 10 L culture by continuous centrifugation. The remaining cells were removed by filtering the medium through a glass fiber filter (GF/A), followed by a 0.45 μm membrane filter. The cell-free filtrate was acidifided (pH 2.0) with HCl. Organics were removed from the acidified filtrate by passage over a 25 X 2 cm column of XAD-2 resin (Mallinckrodt). The XAD-2 resin collected a portion of the extracellular organics which included the β-diketone. The column was washed with 150 ml H_2O and the organics eluted with 300 ml methanol. The methanol fraction was reduced to dryness by rotatary evaporation and the residue partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was reduced to dryness and the amount of β-diketone in the crude fraction determined using HPLC (Chapter II). Using this procedure recovery efficiency of the β-diketone was 80% (Table I). All reported values have been corrected accordingly.

Figure 4 - The isolation procedure for the β-diketone from cultures of P. minimum.

Details are given in the text.

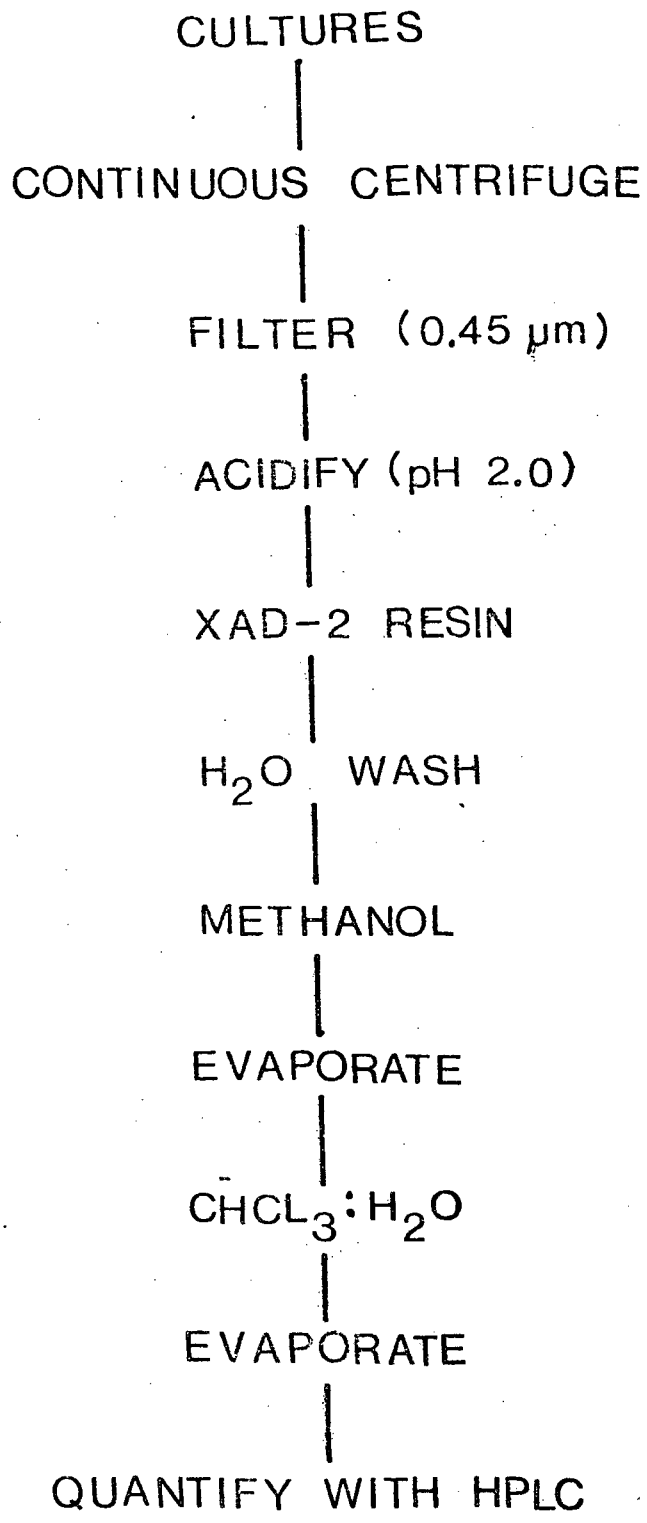


Table I - Recovery of the β-diketone using XAD-2 resin. Six different amounts of the β-diketone were added separately to 5 L of ESNW medium. The acidified medium was passed over a column of XAD-2 resin and the collected β-diketone analysed by HPLC. The average recovery (± one SE) was 78.7 % (2.8)% (n = 6).

Initial amount of β-diketone added to medium (μg)	Recovered amount of β-diketone (μg)	Recovery (%)
10	7.4	74.3
20	16.2	81.0
30	24.0	80.0
40	30.9	77.2
60	46.7	77.9
100	82.0	82.0

Cell concentrations were determined using an electronic particle counter (Coulter Counter Model TA-II), and were used to determine growth rates.

D. RESULTS

1) Production under P-limited growth conditions

Of the six species examined (Table II), *P. minimum* var. *balticum*, *P. gracile* and *P. maximum* showed no detectable extracellular β-diketone production and were not examined further. *P. micans* and *P. mariae-lebouriae* had lower amounts of β-diketone production (10 $\mu g \cdot L^{-1}$) compared to *P. minimum* (40 $\mu g \cdot L^{-1}$) (Table II), the original producer of the β-diketone material (Chapter II).

a) Influence of irradiance

The influence of irradiance on the production of β-diketone by *P. minimum* and *P. mariae-lebouriae* is shown in Fig. 5. Small variations in the irradiance had a substantial influence on the amount of β-diketone produced by *P. minimum* (Fig. 5A). A reduction from 160 to 120 $\mu E \cdot m^{-2} \cdot s^{-1}$ reduced the amount of β-diketone produced by 50%, but had no influence on the maximum growth rate or final cell yield (not shown). Reducing the irradiance to 90 $\mu E \cdot m^{-2} \cdot s^{-1}$ had no effect on the growth parameters but reduced the β-diketone production to only 3 $\mu g \cdot L^{-1}$. There was no β-diketone produced at 60 $\mu E \cdot m^{-2} s^{-1}$, a level at which light became limiting to the growth of *P. minimum*.

While the absolute amount of β-diketone produced by *P. mariae-lebouriae* was lower than that produced by *P. minimum*,

the influence of irradiance on the temporal pattern of the β-diketone production was similar (Fig. 5B). In P. mariae-lebouriae, reduction of the irradiance from 160 to 120 $\mu E \cdot m^{-2} \cdot s^{-1}$ had no significant influence on the amount of -diketone produced. An increase or decrease in irradiance to 270 or 90 $\mu E\ m^{-2} s^{-1}$ inhibited β-diketone production.

At each irradiance where β-diketone was detectable, the temporal pattern of production was the same regardless of the absolute amount of material produced. At the onset of phosphate starvation (Day 11 for P. minimum and Day 9 for P. mariae-lebouriae), the β-diketone was produced rapidly and maintained at a constant level in the medium. Similar patterns of production were seen under all other experimental conditions.

The production of the β-diketone under a light/dark cycle (18:$\bar{6}$) suggests the production of the β-diketone is light controlled (Fig. 6). While only one experiment was performed, there was no evidence of β-diketone production during the dark period. At the onset of the light, there was rapid production during the first few hours, shifting to a gradually slower rate of release. The rapid release of the β-diketone may be due to the conversion of an intracellular precursor pool which forms during the dark period. While the kinetics of production cannot be fully appreciated with a single experiment, the link of production with the onset of the light period suggests a photocontrol mechanism.

Table II - Species examined for the production of the β-diketone.

Final cell yield and the maximum recorded β-diketone concentration (± one SE, n = 3) are presented for cells grown at 160 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 18 °C; except P. mariae-lebouriae which was grown at 22-23 °C. All cultures were grown in P-deficient batch cultures. The extracellular concentration of the β-diketone was monitored daily. North East Pacific Culture Collection number is given in brackets for the appropriate species.

Species	Final Cell Yield (10^{-7} cell·L^{-1})	Maximum -diketone ($\mu g\ NH_2OH \cdot L^{-1}$)
P. minimum (Pavillard) Schiller (#96)	3.3	39.6 ± 7.2
P. minimum var. balticum (Pavillard) Schiller (#115)	0.7	N.D.
P. micans Ehrenb. (#33)	2.1	10.3 ± 2.7
P. gracile Schuett (#104)	3.0	N.D.
P. maximum (Gourret) Schiller (#250)	1.7	N.D.
P. mariae lebouriae (Parke et Ballentine) Hulburt*	3.5	10.1 ± 3.4

N.D. not detectable

* Kindly supplied by Prof. H.H. Seliger, The Johns Hopkins University, Baltimore, Maryland

Figure 5 - Production of the β-diketone in batch culture - influence of irradiance.

The influence of irradiance on the maximum growth rate (upper graph) and the temporal production of the extracellular β-diketone (lower graph) for: A) P. minimum, and B) P. mariae-lebouriae. Batch cultures eventually became P-deficient. Replicate values are mean ± one SE where n is noted in brackets.

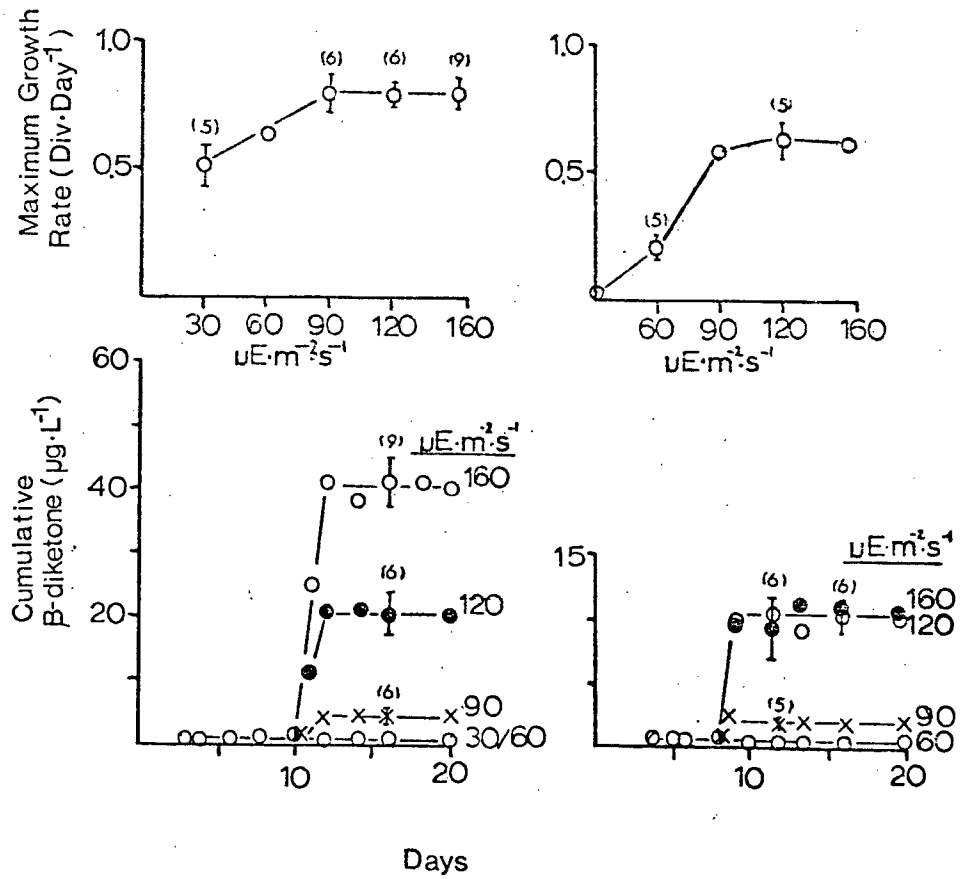
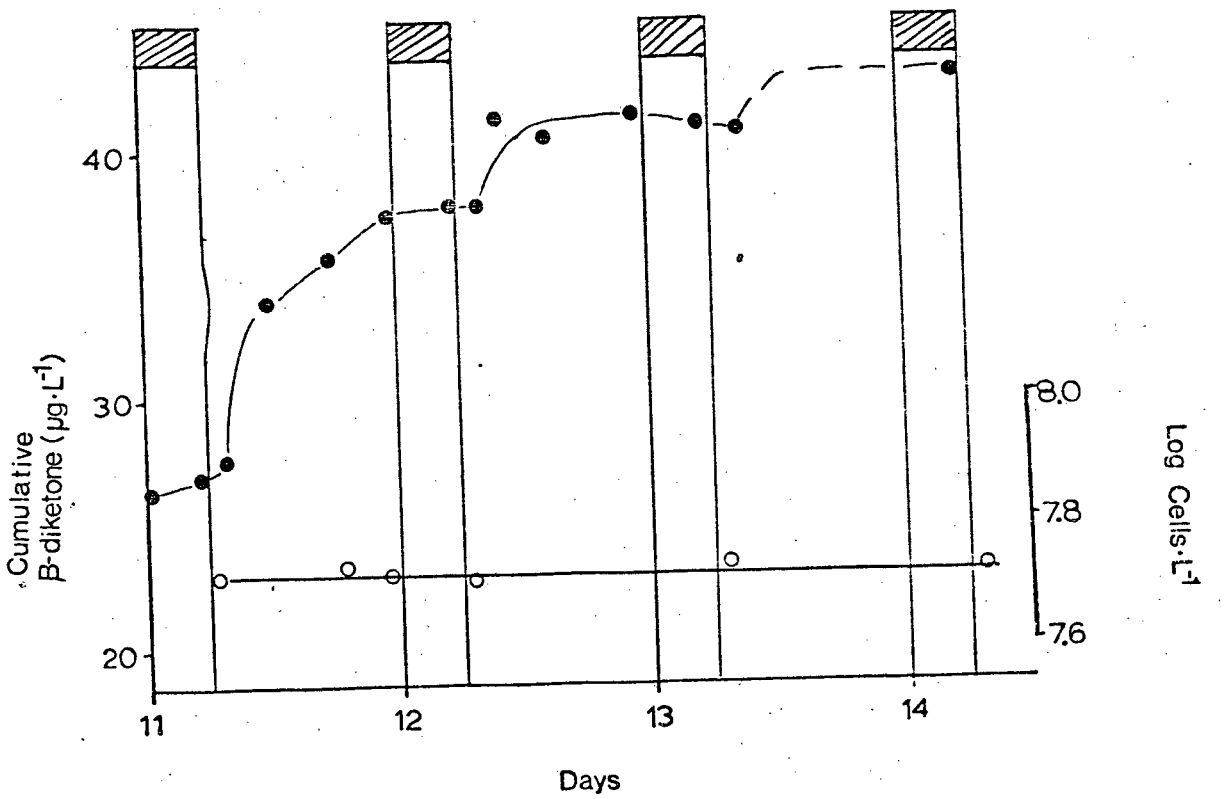


Figure 6 - Production of the β-diketone in batch culture - influence of a light:dark regime.

The cumulative extracellular β-diketone concentration for P. minimum grown under a 18:$\overline{6}$ light:dark regime. Only the senescent phase of batch culture growth is shown.



b) Influence of salinity

Salinity had a significant influence on the final extracellular β-diketone concentration. P. minimum had maximum growth rates over a wide range of salinities (from 15‰ to 30‰) (Fig. 7A). Maximum β-diketone production had a restricted salinity range of 20-30‰. At salinities less than 20‰, the amount of extracellular β-diketone produced decreased rapidly.

P. mariae-lebouriae had a very different salinity tolerance range. Salinites of 10 to 15‰ were required to give maximum growth rates (Fig. 7B). Growth in full strength medium (27‰) reduced the growth rate by more than 50% and nearly eliminated the β-diketone production. Growth rates and β-diketone production were also reduced at salinities less than 10‰. Maximum extracellular β-diketone concentrations were achieved at salinities below those providing maximum growth rates.

c) Influence of temperature

Temperature also had a profound effect on extracellular β-diketone production (Fig. 8). Temperatures less then 15°C reduced the amount of β-diketone produced from 50 $\mu g \cdot L^{-1}$ to less then 3 $\mu g \cdot L^{-1}$. There was no detectable β-diketone when cells were grown at 5°C.

2) Production under different N:P ratios

The influence of the initial nitrogen:phosphorus atomic ratios on the production of the β-diketone is shown in Fig. 9. P. minimum was grown at N:P ratios ranging from 2 to 100. The

Figure 7 - Production of the β-diketone in batch culture - influence of salinity.

The influence of salinity on the growth rate (upper graphs) and the maximum β-diketone concentration (lower graphs) for: A) P. minimum and B) P. mariae-lebouriae. Replicate values are means (± one SE), where n is noted in brackets.

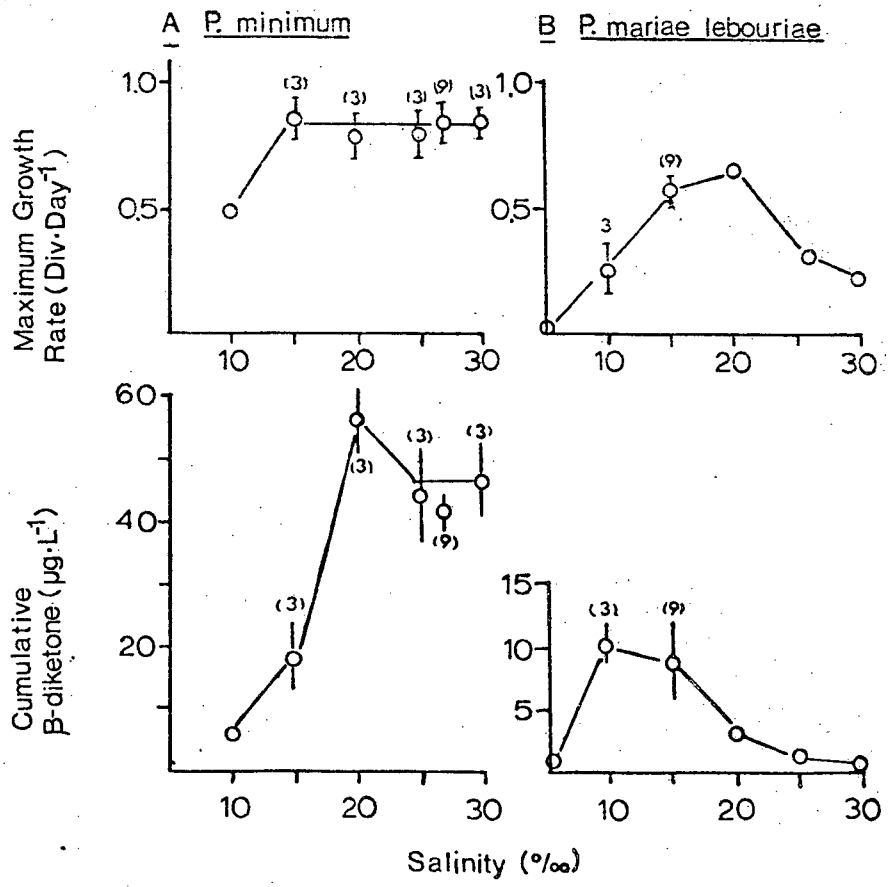


Figure 8 - Production of the β-diketone in batch culture - influence of temperature.

The influence of temperature on the growth rate (upper graph) and the maximum β-diketone concentration (lower graph) for P. minimum. Replicate values are mean ± one SE where n is noted in brackets.

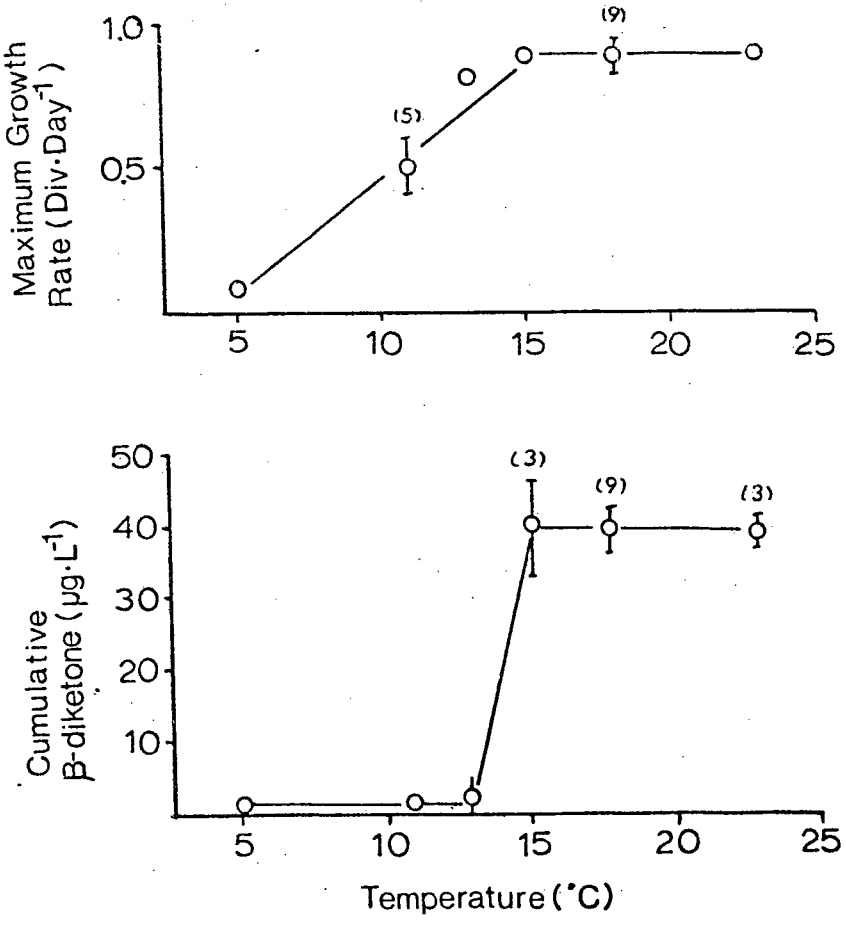
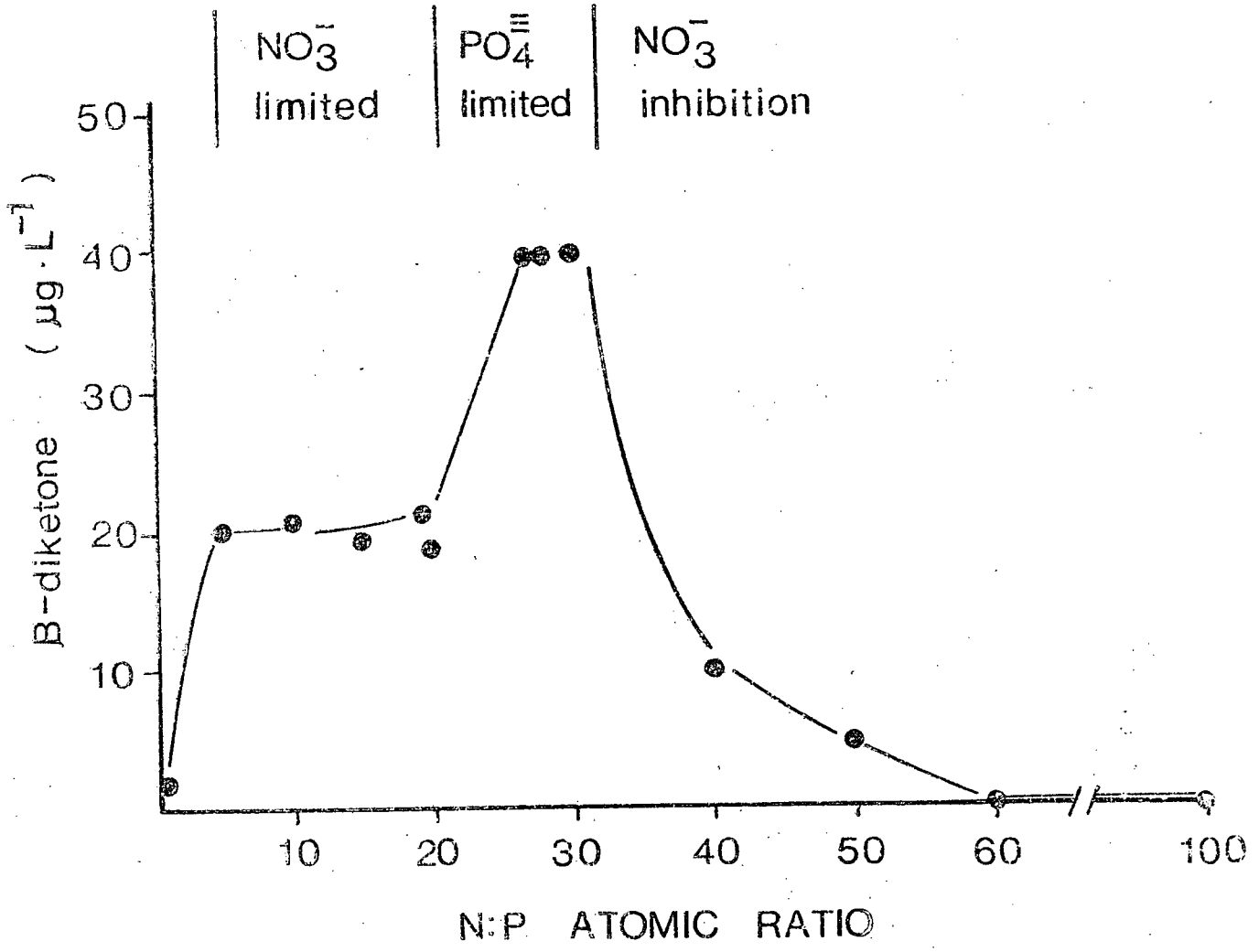


Figure 9 - Production of the β-diketone in batch culture - influence of the initial N:P atomic ratio in the medium. Final cell yields for all experiments were equivalent.



atomic ratio of N:P = 25 was chosen as the theoretical ratio where N and P were both limiting to growth, based on the disappearance of nitrate and phosphate from the batch culture medium. To achieve these experimental nutrient ratios without decreasing the final cell yield of the cultures, the amount of non-limiting nutrient was increased in the medium. For example, N:P ratios greater than N:P = 25 were achieved by maintaining the limiting nutrient (P) at 6 μM and increasing the amount of added N until the desired ratio was achieved.

At very low N:P ratios (N:P = 2) a large amount of inorganic precipitate formed (C = 2.8%, H = 1.4%, N = 0.3%, P = 6.9%). Since autoclaved N:P = 2 medium, without cells, failed to show any precipitate, formation of this precipitate is either stimulated by the presence of the cells or the activity of the cells. The β-diketone was not produced at this ratio.

At N:P ratios from 5 to 22 (N-deficient growth), the level of β-diketone produced was constant at 20 $\mu g \cdot L^{-1}$. As the cells shifted to P-deficient growth conditions (N:P = 27 to 35), production doubled to about 40 $\mu g \cdot L^{-1}$. Phosphate-deficient cells exposed to increasing concentrations of ambient nitrate, produced decreasing amounts of β-diketone. At N:P ratios greater then 60, no β-diketone was produced.

To test whether the reduction in β-diketone production at N:P = 40 was a function of the actual ratio or the amount of excess nitrate in the culture medium, a series of phosphate-deficient culture experiments was performed where the N:P atomic ratio was kept constant at 40, but the amount of phosphate added

was increased or decreased. As the amount of excess nitrate in the culture medium decreased, the amount of β-diketone normalized on a per cell basis, increased, reaching values similar to the maximum amount of the β-diketone produced under P-limitation (Table III). Conversely, an increase in the amount of excess nitrate, at a constant N:P ratio of 40, completely inhibited the β-diketone production.

A similar experiment to examine the influence of excess phosphate at N:P = 2 failed to show an increase in the β-diketone production. In all experiments, a large amount of precipitate formed which may have interferred with cellular processes and reduced the amount of excess phosphate, since the precipitate contained large amounts of phosphate. The amount of precipitate could not be quantified, since much of the material adhered tenaciously to the glass flask.

The methanol extracts of P. minimum cells grown at N:P = 5 were examined by TLC to determine if the difference in extracellular β-diketone concentration was due to intracellular storage when cells were nitrate limited. Cells from N:P = 5 and N:P = 27 were extracted with methanol and run with a β-diketone standard in a $CHCl_3$:ethyl acetate (1:1) TLC system. Neither of the cell extracts contained the β-diketone, although there were obvious differences in the cellular profiles. Cells grown at N:P = 5 had large amount of $FeCl_3$ positive material with Rf values greater than the -diketone (0.6 and 0.89 vs. 0.37 for the β-diketone)(Fig. 10). Neither of the $FeCl_3$ positive spots gave the characteristic red-brown color of the β-diketone.

Table III - The influence of increasing ambient nitrate concentration in the medium on the production of the β-diketone by P. minimum.

Cells were grown in N:P = 40 medium.

P added (μM)	N added (μM)	Calculated * excess N in the medium (μM)	Extracellular β-diketone, (μg · 10^{-7} cells)
21.9 (control)	591.3	43.8	6.3
2.7	109.8	36.1	5.8
5.4	219.6	82.4	3.7
10.9	439.2	164.7	0.9
21.9	878.4	330.9	0.8
43.9	1756.8	657.8	0.0

* Calculated based on a theoretical nutrient equilibrium ratio of N:P = 25.

Figure 10 - Examination of cell contents for the presence of the β-diketone.

Diagramatic representation of thin layer chromatography strips (SIL G/UV 254) of the intracellular, chloroform-soluble fraction of P. minimum grown at two different N:P atomic ratios. 1) cells grown at N:P = 27; 2) β-diketone standard; and 3) cells grown at N:P = 5. The solvent system used was chloroform:ethyl acetate (1:1). Shaded areas give a positive reaction to FeCl (2% in ethanol) spray.

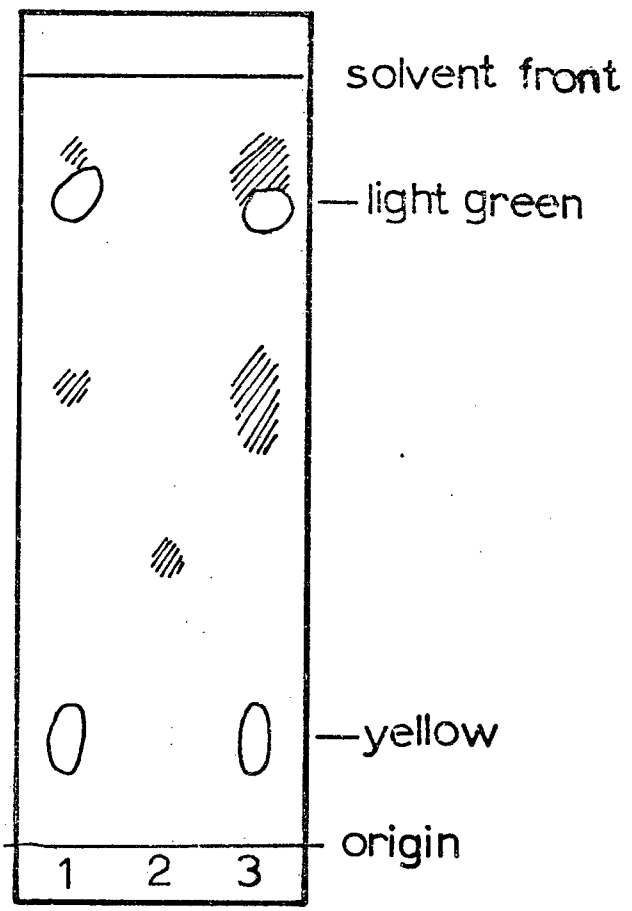


Figure 11 - Production of the β-diketone in batch culture - influence of iron-deficiency.

Changes in the β-diketone concentration and cell numbers during exponential growth and during senescence in iron-starved batch cultures. Replicate values are mean ± one SE where n is noted in brackets.

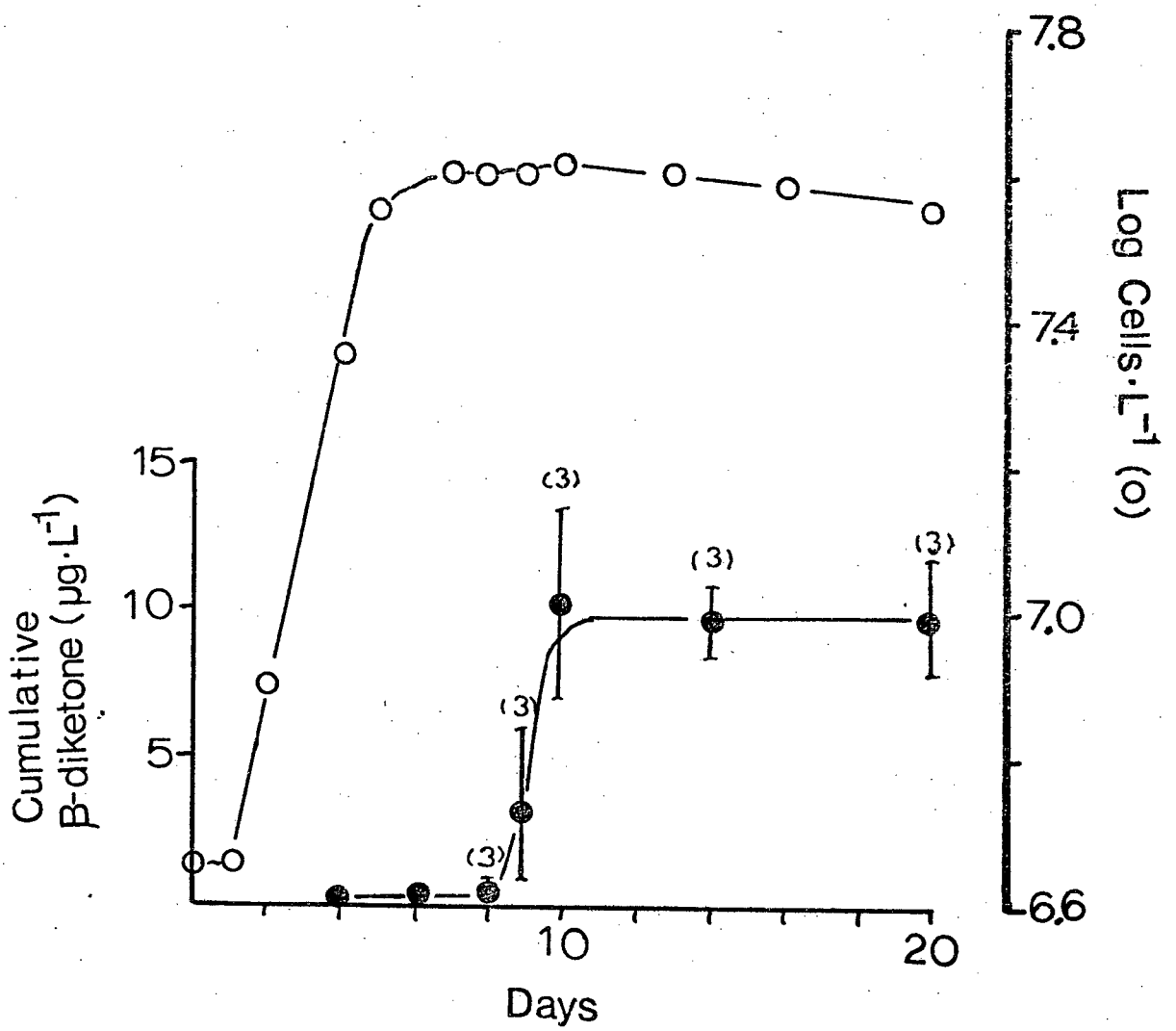


Table IV - Influence of the addition of vanadium on the β-diketone production in iron-starved batch cultures of *P. minimum*.

Cultures were harvested at Day 15 of batch culture growth.

Growth Conditions	Maximum β-diketone produced ($\mu g \cdot L^{-1}$)
iron limited (ESNW-Fe)	10.9
iron limited with 4×10^{-8}M NH_4VO_3	0.0
iron limited with 8×10^{-8}M NH_4VO_3	0.0
iron sufficient (control)	38.3

3) Production under iron-deficient growth conditions

P. minimum cells grown under iron-limited culture conditions had a pattern of extracellular β-diketone production similar to the pattern from phosphate-deficient cultures, but the absolute amount of the β-diketone produced was substantially lower (Fig. 11). Only cells grown in the ESNW-Fe medium produced the β-diketone. Cells grown in the AQUIL-Fe medium failed to produce the extracellular β-diketone.

The addition of NH_4VO_3 (a trace metal used to alleviate the iron limitation; Meisch and Bielig, 1975) had no effect on the growth parameters measured (growth rate and final cell yield were equivalent to the iron-sufficient controls), but it completely inhibited the production of the β-diketone (Table IV). The addition of iron to the treated medium reestablished maximum extracellular β-diketone concentrations.

E. DISCUSSION

The production of the nor-carotenoid 1-(2,6,6-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione by P. minimum is under direct physiological control and cannot simply be considered as a photooxygenation product of carotenoid degradation.

Several lines of evidence indicate that the control of the β-diketone production is under the same control processes as the total extracellular carbon release (composed mostly of primary organics). Production of the β-diketone is related to the physiological state of the cell. All production is limited to

the stationary phase of growth. This is the case for many extracellular organics (Guillard and Wangersky 1958; Marker 1965; Bolze and Soeder 1978; Melkonian 1979). However, in the case of the β-diketone produced by P. minimum, the production in the stationary phase is tightly controlled. Release of the β-diketone is not gradual, suggesting a "leaking" of cellular contents, but rather, there is a short period of rapid production followed by an equally rapid cessation of β-diketone production after a short (1-2 days) period of time. The restricted period of production suggests the exhaustion of cellular precursors available for β-diketone production.

The de novo synthesis of the -diketone is supported by the production under light/dark cycles. Release of the β-diketone does not occur in the dark. Since there was no evidence of accumulated intracellular β-diketone in cells collected from the dark immediately prior to the start of the light cycle, we must assume that precursors have accumulated intracellularly and that the β-diketone is formed by photochemically mediated biosynthesis.

Irradiance was a strong influence in controlling the β-diketone production. Increased irradiances favor the release of DOC by marine phytoplankton (Fogg et al., 1965; Hellebust, 1965; Nalewajko,1966) and the same can be seen for the production of the β-diketone. In the latter case, the extent of production increased non-linearly over the range of irradiances. Similar light control of DOC from Skeletonema has also been shown (Ignatiades and Fogg, 1973). The production of β-diketone did

not occur if light was limiting to growth. This is not necessarily the case if one examines DOC release. Kuenzler (1970) found DOC release to be constant over a wide range of irradiances, including light levels limiting to growth rates. The amount of the β-diketone production was not controlled extensively by temperature, although production of the β-diketone ceased when temperatures reduced the growth rate of P. minimum.

The influence of nutrient deficiency on the production of the extracellular β-diketone suggests a strong control by the physiological state of the cell. Shifting from N-deficient to P-deficient growth conditions, shifts the carbon metabolism of the cell. Enzymes (such as polyphosphokinase, polyphophatase, and alkaline phosphatase), storage products, and energy intermediates (such as adenylate charge and adenosine triphosphate) are all controlled by the deficient nutrient (Healey, 1975). The influence of the limiting nutrient on the composition of the extracellular exudate is less well understood. Myklestad (1977) clarified the control of N:P ratios on cellular processes. Exponentially growing cells show little variation in the cellular compostion since neither nutrient is limiting, but as the stationary phase is reached, the composition of the medium has profound effects. The cellular composition changes to reflect the composition of the medium. Examining the influence of N:P ratios on growth and carbohydrate metabolism, Myklestad (1977) noted little difference in the extracellular polysaccharides at

concentrations over the N:P range of 0.4 to 100 for Skeletonema costatum. However, Myklestad did find increased levels of extracellular polysaccharide during the senescent stage of growth if cells were P-deficient instead of N-deficient. Phosphate-deficient cells produced two to three times the amount of extracellular polysaccharide compared to nitrate-deficient cells. A similar trend was shown for extracellular polysaccharide production in stationary Chaetoceros affinis cultures (Myklestad, 1977).

The influence of the N:P ratio on the extracellular production of the β-diketone is similar in several significant ways. When P.minimum was N-deficient, a constant amount of β-diketone was produced at 20 $\mu g \cdot L^{-1}$. When cells shift to P-deficiency there was an increased amount of the β-diketone produced (40 $\mu g \cdot L^{-1}$). But, at N:P values greater than N:P = 30, it was the amount of excess nitrate which inhibited the amount of the β-diketone produced. The control of extracellular metabolites by excess nutrients has been shown for many bacterial and fungal metabolites (Weinberg, 1970; Drew and Demain, 1977; Martin and Demain, 1980).

The reduced amount of the β-diketone produced under iron-deficient growth conditions compared to P-deficient cultures suggests that the β-diketone was not directly linked to the high affinity iron transport system, even though the β-diketone forms an iron complex and has the potential to chelate metals (Andersen et al., 1980).

The addition of vanadium to iron-limited cultures of

P. minimum is a critical experiment. Vanadium is a trace metal found commonly as a constituent of marine algae (O'Kelley, 1974), but it is not necessarily an essential nutrient. The addition of vanadium to the culture medium inhibits the formation of δ-aminolevulinic acid (δ-ALA), which is a key step in the regulation of chlorophyll production (Meisch and Bielig, 1975). This is the same point at which iron limitation affects the cell's physiology (Jones, 1976). A second influence of vanadium on the cell's physiology was the stimulation of the photoinduction of carotenoidgenesis (Shropshire, 1980). The addition of vanadium to P. minimum inhibited the β-diketone production rather than stimulated the nor-carotenoid release, providing further evidence against the β-diketone as a non-selective photooxygenation product of carotenoids.

It is apparent from the limiting nutrient experiments that the two processes controlling the β-diketone production are regulated by two nutrient-related criteria: 1) the type of limiting nutrient (P, N, or Fe, and 2) the amount of excess nitrate available in the culture medium.

Several points can be stressed. The production of the extracellular β-diketone is a normal physiological process related to the carbon flow within the cell. The β-diketone was formed immediately prior to release and was never found intracellularly. The production was a function of the nutrient environment of the cells and was mediated by photochemical control of compound formation.

Relating the production of the β-diketone to the ecology of

P. minimum is difficult. Unlike prorocentrin (the siderophore which will be discussed in Chapters V and VII of this thesis), the role of the β-diketone cannot be directly related to the physiological response of the cell.

The link between the production of extracellular β-diketone and the formation of P. minimum blooms is especially open to speculation. Researchers examining natural phytoplankton blooms have noticed that even though cells are severely stressed or senescent, there is no resulting increase in the amount of extracellular carbon release (Herbland and Dandonneau, 1975; Mague et al., 1980). While the quantity of extracellular organics may not change significantly, the quality might change. The β-diketone comprises only a small proportion of the total released organic and is produced at levels which will not result in an increase in DOC. But the β-diketone shows antibacterial activity when tested at the concentrations recorded in culture. If the amount of the β-diketone produced in batch cultures is similar to that in the field, it is predicted that sufficient β-diketone will be produced to inhibit natural populations of bacteria (Appendix A).

Part of the problem in evaluating the role that the β-diketone may play in controlling bacterial populations is our poor understanding of the link between phytoplankton and bacteria. Researchers in the past have suggested that antibacterial metabolites from algae are not important (Carlucci and Pramer 1960), but recent work by Aubert and co-workers (Aubert et al. 1968; 1970; Aubert and Pesando, 1971), has

provided substantial evidence of strong algal-bacterial interactions. Metabolites which influence algal-bacterial interactions can be non-specific inhibitors such as tannins or organic acids (Lucas, 1955; Nigrelli, 1958), or specific, uncharacterized metabolites produced by laboratory cultures (Bell et al., 1977; Kogure et al., 1979) or by natural populations (Sieburth, 1971; Moebus, 1972). Characterized antibacterial agents produced by phytoplankton include chlorellum from Chlorella (Pratt et al., 1944), acrylic acid from Phaeocystis pouchetii (Guillard and Hellebust, 1971) and Phaeodactylum tricornutum (Brown et al., 1977), and goniodomin from the marine dinoflagellate Goniodoma (Sharma et al., 1968).

There are several ways of viewing the organic carbon link between phytoplankton and bacteria. The link can be a one-way carbon exchange where the phytoplankton exudates are the source of organic material for the bacteria (Smith, 1974; Smith and Higgins,1978; Larsson and Hagstrom, 1979; Meffert and Overbeck, 1979). The extent of carbon transfer to the bacteria can be substantial. Estimates indicate that from 25% to 50% of the total fixed carbon is incorporated into the bacteria (Andrews and Williams, 1971; Hagstrom et al., 1979; Larsson and Hagstrom, 1979). These estimates suggest that a large part of the bacterial carbon requirement is met by phytoplankton exudates. To strengthen the link between phytoplankton and bacteria, Larsson and Hagstrom (1982) have reintroduced the concept of the "phycosphere". The phycosphere, as it was originally described by Bell and Mitchell (1972), is a zone surrounding each

phytoplankton cell. This zone is composed of a high concentration of algal exudates. The zone contains all extracellular organics including bacterial attractants (Kogure et al., 1982) and antibacterial agents. Thus, the epiphytic bacterial population of a phytoplankton cell would be a function of the control of extracellular organic production.

This relationship has been poorly characterized. Bacteria gain by having a high concentration of readily available organics, and a constant source of material. The phytoplankton may gain required organics such as vitamins or growth factors from the bacteria but almost no evidence exists for the transfer of organics from bacteria to phytoplankton (Haines and Guillard, 1974).

Utilizing the concept of the phycosphere, exponentially growing cells would develop a metabolically active bacterial population, especially if the bacteria show a chemotactic response to algal exudates (Bell and Mitchell, 1972; Sjoblad and Mitchell, 1979). As phytoplankton become nutrient deficient, the amount of organics released also decreases. The bacteria become organic-stressed and may stimulate the degradation and remineralization of the associated phytoplankton by the production of extracellular enzymes. Using the concept of a phycosphere around the phytoplankton cell, the bacteria could produce sufficient concentrations of extracellular enzymes to degrade the host.

A phytoplankton cell which forms a resting stage may alter its structure to resist bacterial attack. However species such

as P. minimum , for which no alternate stage is known, could produce antibacterial metabolites such as the β-diketone to reduce the degradative activity of the associated bacterial population.

The pattern of production of the β-diketone lends itself to this hypothesis. Prior to the -diketone production, bacteria associated with P. minimum would have a favorable environment for heterotrophic activity. With the heterotrophic mechanism intact , the β-diketone is produced in a single rapid pulse, creating a toxic phycosphere. Associated bacteria are inhibited prior to excretion of extracellular degradative enzymes. The activity of the associated bacteria is influenced by the chemical compostion of the substances released and the pattern of release. Strict control of production is necessary to ensure that the β-diketone is released in sufficient quanitities to eliminate the associated bacterial activity under physiological conditions which are temporally severe.

It has been argued that extracellular organics serve no purpose to the producing species and that antibacterial compounds, such as the β-diketone, are produced at concentrations too low to be active in the marine environment. Under certain conditions, the production is hardly insignificant and the absolute concentration of the β-diketone may be much higher than initially thought if one examines the pattern of production and the existence of a physosphere.

The study of the role of heterotrophic bacteria and microflagellates in degrading and remineralizing the organics in

marine waters is of primary importance in the understanding of microbial interrelationships (Strickland, 1971). To understand the phytoplankton-bacteria relationships not only must the quantity of organics be considered, but also the quality. The production of the nor-carotenoid from P. minimum may be an example of the severing of organic links between trophic levels by extracellular phytoplankton exudates.

IV. GENERAL INTRODUCTION TO SIDEROPHORES

A. ABSTRACT

A general review of siderophores (ferric ion-specific chelators) is presented with emphasis on siderophore production by higher plants and phytoplankton. Siderophores, by strict definition, have never been verified in higher plants. Higher plants may rely on the production of organics which increase the solublization of particulate iron, but these compounds have no role in the transport of iron into the roots. Cyanobacteria are well known producers of siderophores, but the production of siderophores by eukaryotic phytoplankton is controversial. Evidence, both for and against the siderophore production by eukaryotic marine algae, is reviewed.

B. INTRODUCTION

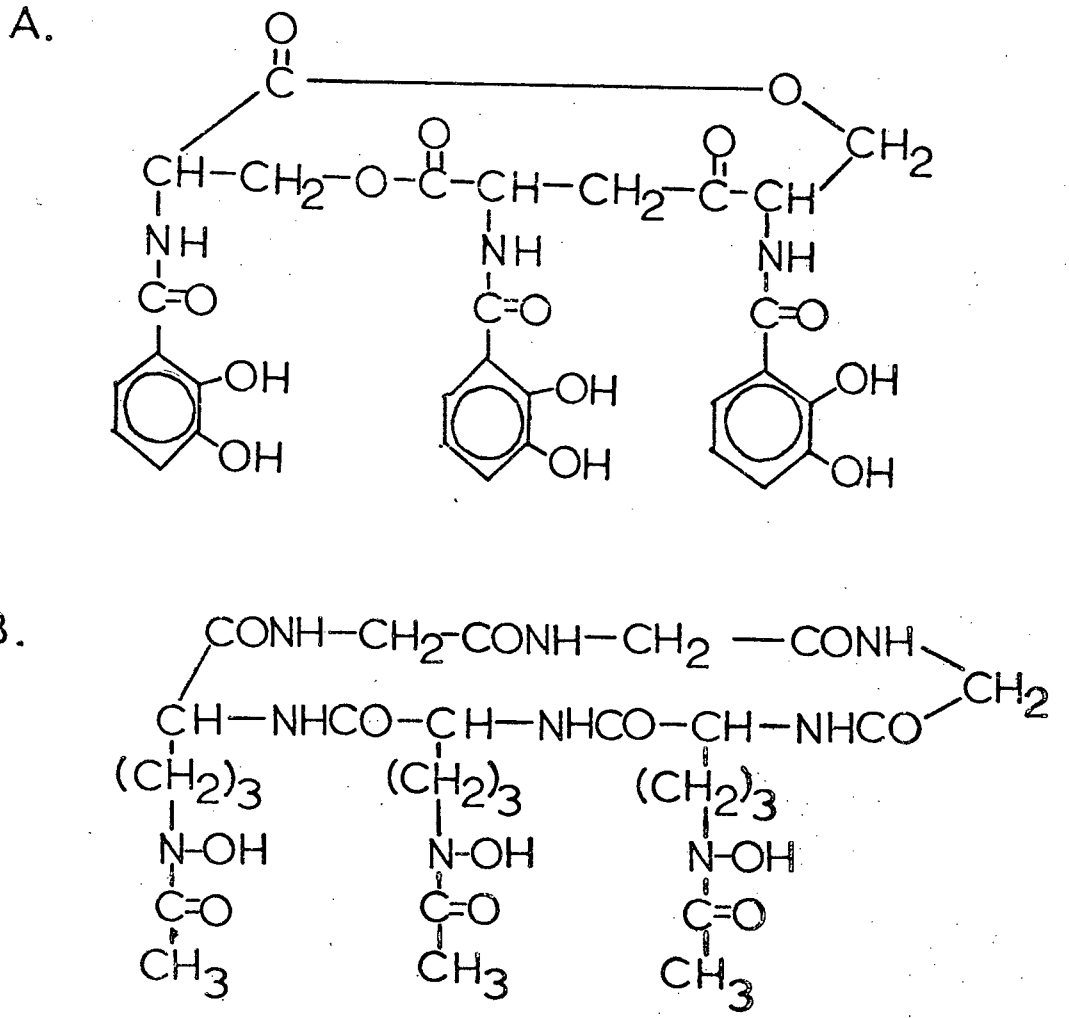
Evidence for siderophores as high-affinity iron-aquisition systems is well documented. Recent reviews have covered most general aspects of siderophores such as classification and properties of siderophores (Neilands, 1981a), microbial iron uptake (Lankford, 1973; Neilands, 1980; Neilands, 1981b), siderophore-mediated iron-transport mechanisms (Raymond and Carrano, 1979; Bezkorovainy, 1980), and the evolutionary development of siderophore systems (Neilands, 1966).

Siderophores are low molecular weight organics, which coordinate and solublize iron(III) by forming a highly stable complex. Siderophore production is stimulated by growth in low iron conditions (Garibaldi and Neilands, 1956). The mechanism of metabolic control has only been examined in a few systems but it appears that the biosynthetic pathway for the production of siderophores is repressed by available iron. It does not appear to be a system controlled simply by enzyme inhibition (Neilands, 1973).

Siderophores are of two chemical types. The catechol-type, of which enterobactin is an example, binds iron *via* three bidentate catechol-functionalities. The iron complex of the catechol siderophore is red to purple in color. The second group of siderophores is the hydroxamate-type siderophore. Three hydroxamate groups are required to fill the hexacoordinate ferric complex (Neilands, 1973). Ferrichrome is an example of a trihydroxamate siderophore (Fig. 12).

As discussed by Neilands (1973), this dichotomy of

Figure 12 - The structures of representative siderophores. A: Enterobactin (tri-catechol siderophore). B: Ferrichrome (tri-hydroxamate siderophore)(Adapted from Cooper et al., 1978).



siderophore types is misleading. Siderophores are a highly diverse group of iron-chelators. Many siderophores contain a combination of hydroxamate and catechol functionalities or contain hydroxamate/catechol functionalities with another unrelated group (eg. citric acid) forming the final coordination site.

Given the present evidence, the separation of siderophores into the two groupings is sufficient for the examination of siderophores from eukaryotic phytoplankton. The only microorganisms known to produce catechol-type siderophores are the true bacteria, such as Aerobacter aerogenes, Escherichia coli, and Salmonella typhimurium (Neilands, 1973). Species with mitochondria produce siderophores with a hydroxamate-type ligand, although hydroxamate-type siderophores are produced by some bacteria. The specificity of the hydroxamate-type siderophores in eukaryotic microorganisms may be a result of a specific transport mechanism required to provide iron to the mitochondria (Neilands, 1972).

C. TRANSPORT OF IRON BY SIDEROPHORES

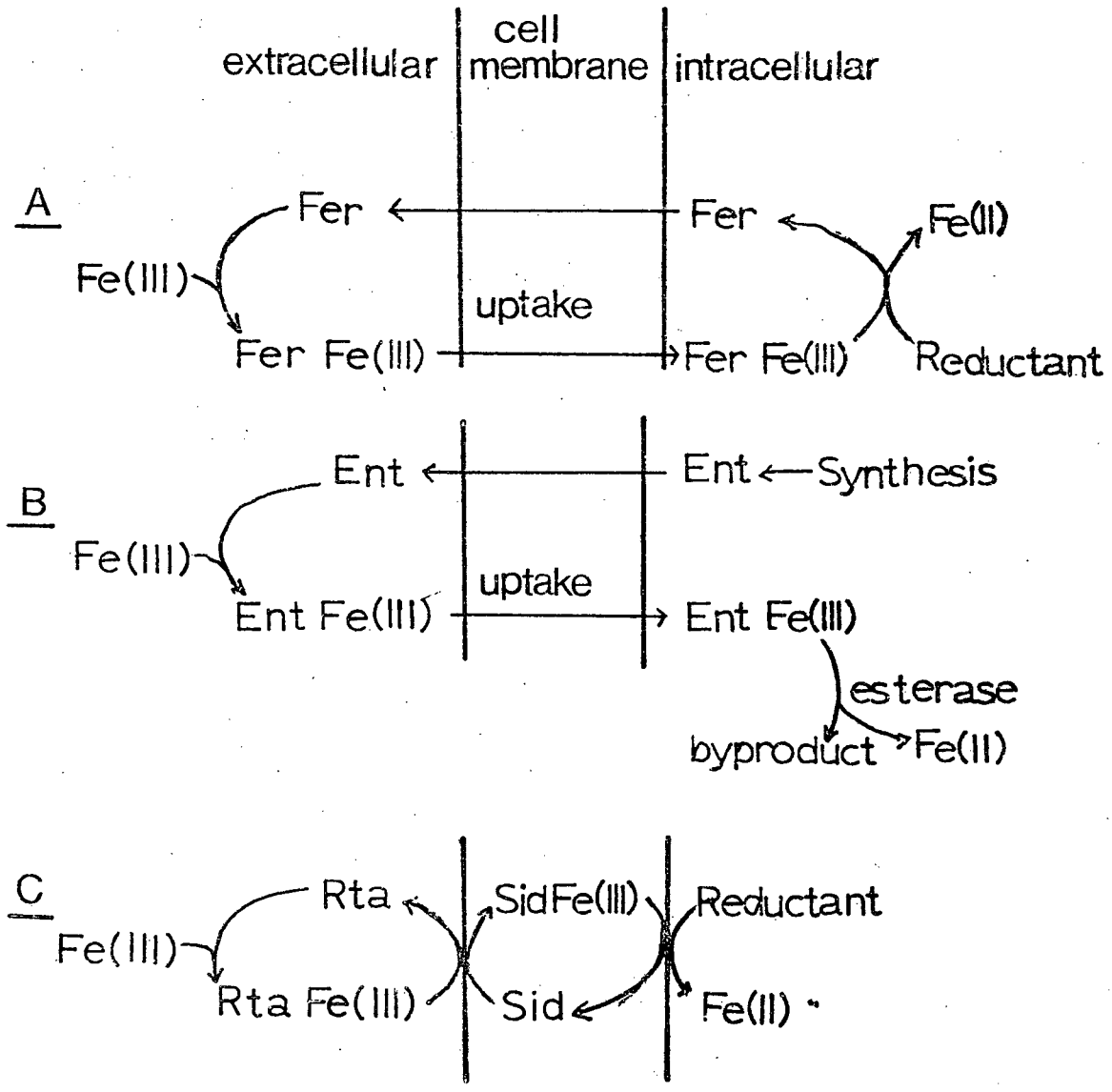
Production of siderophores by microorganisms has been extremely well documented in the literature. Less well studied is the mechanism of siderophore mediated iron transport. While only a small number of organisms has been examined, certain trends have begun to emerge. First, the uptake of the siderophore-iron complex requires energy (Lankford, 1973).

Second, the iron-siderophore transport system is highly specific for a given chelator. For each siderophore which can be transported into the cell there is a specific, separate transport system (Frost and Rosenberg, 1973; Hayden et al., 1973; Aswell et al., 1977). Of equal importance is the fact that these specific transport systems are inducible by the presence of the specific siderophore-iron complex. These are important criteria when one considers the ecological role of siderophores. The selectivity of these transport systems could provide the basis for competition between species during iron-limited growth conditions.

The cellular management of the siderophore-iron complex has been studied in several diverse siderophore systems. Generally, hydroxamates are taken across the cell membrane as the ferri-hydroxamate complex. The ferri-hydroxamate complex is strong; therefore to release the Fe(III), the cell must reduce the iron to Fe(II) which is only weakly bound to the siderophore. The iron(II) is released and the hydroxamate recycled back into the medium (Fig. 13A). This recycling has been referred to as the "European approach" (Bezkorovainy, 1980).

Conversely, enterobactin (a catechol-type siderophore) transfers one iron atom into the cell and is destroyed. There is no recycling of the siderophore. This has been referred to as the "American approach" (Fig. 13B). This system is obviously more expensive than the recycling hydroxamate-type siderophore. Cooper et al. (1978) has suggested that the enterobactin-iron complex has a reduction potential which is too

Figure 13 - Representative transport mechanisms. A: Ferrichrome (hydroxamate){Fer} - "European Approach". B: Enterobactin (phenolate){Ent} - "American Approach". C. Rhodotorulic Acid (di-hydroxamate){Rta}{'Sid' is a membrane bound iron-transport compound} (Adapted from Bezkorovainy, 1980; Cooper et al., 1978).



low for reductive iron release. These authors estimated a redox potential of the enterobactin-iron complex of -750 mV. This can be compared to the redox potential of NADPH (-300 mV). Thus, to release the iron, enterobactin is broken down intracellularly with an esterase. Recently, Lodge et al. (1980) have challenged this rationale and have shown that iron can be reduced off enterobactin and utilized without the hydrolysis of the siderophore.

A third mechanism has been shown in mycobacteria and species which produce rhodotorulic acid (Fig. 13C). This system can be easily distinguished from the previous systems since it involves two siderophores, one extracellular and one membrane bound. In this system, iron bound to the extracellular siderophore, is transferred to the cell wall where the membrane-bound siderophore accepts the iron and transports the iron to a reductant within the cell (Bezkorovainy, 1980). While the system has not been fully characterized, Rhodotorula pilmanae, which produces rhodotorulic acid (Atkin et al., 1970), also appears to tranfer iron in this manner (Carrano and Raymond, 1978).

D. SIDEROPHORES IN HIGHER PLANTS

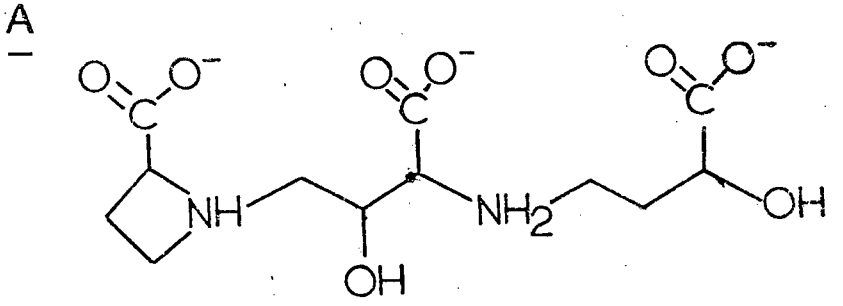
There is no evidence of siderophores being produced by higher plants. This is not to suggest that iron does not become limiting to these plants (Price, 1968), but that higher plants may depend on the production of siderophores and iron-transporting compounds by epiphytic bacteria. While higher

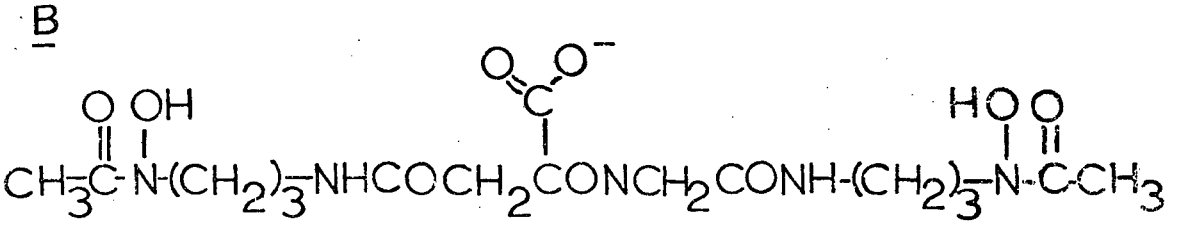
plants have been less fully studied than bacteria or fungi, there appears to be two processes to solublize iron. Plants may depend on the humic content of soil, derived from decaying plant material, to increase the availability of iron. The second mechanism involves the release of low molecular weight organics, such as citric acid, malic acid, or mugineic acid, from the roots. Each of these compounds plays an important role in iron acquisition but none have the characteristics of siderophores. Citric acid, for example, is a common constituent in plants which poorly binds iron when compared to microbial siderophores. The ferric iron is thought to form a complex with the alcohol and carboxylic acid functionalities of the citrate (Neilands, 1973). Under iron-limited conditions citrate accumulates in the roots of soybeans (Brown, 1966), but there was no reported stimulation of extracellular citrate.

Similarly, malic acid is a common component of the TCA cycle and forms relatively weak complexes with iron(III). Ojima and Ohira (1980) report the release of malic acid and citric acid by cell suspension cultures of rice. These compounds appeared to stimulate the conversion of iron(III) to iron(II). Release of these organics was not stimulated by iron stress. The authors suggest that the release of these two organics was a cellular response to adjust the intracellular ion balance. As the pH dropped from 6.0 to 5.5 there was a shift in metabolic pathways resulting in the release of these two organic acids. Again, the production of the organics and a need for iron could not be related.

Figure 14 - Representative iron chelating agents from photosynthetic organisms.

A: Mugineic acid, a iron chelating agent from higher plants (from Sugiura et al., 1981). B: Schizokinen, a dihydroxamate siderophore from cyanobacteria (from Simpson and Neilands, 1976).





Takemota et al. (1978) and Sugiura et al. (1981) have suggested that mugineic acid is a possible phytosiderophore. While originally isolated from barley, similar compounds have been found in rice and oats (Takagi, 1976; Fushiya et al., 1980). Examination of the structure of this compound (Fig. 14A) shows that mugineic acid is not a hydroxamate nor a phenolate. Sugiura et al. (1981) provided evidence of mugineic acid forming a 1:1 complex with iron(III) with the ligands consisting of the carboxyl, amine, and hydroxyl groups, The formation constant is very low (log K =18.1) for iron(III), but the formation constant for iron(II) was very high (log K =8.1) compared to microbial siderophores. There was no evidence of mugineic acid being positively linked with iron-stress; although unpublished work by Takagi (cited by Sugiura et al., 1981) has shown mugineic acid to stimulate iron-uptake in barley.

In conclusion, while there is no evidence for the production of siderophores (strictly defined) by higher plants, there is evidence to suggest that iron-chelating agents are produced by higher plants. These organics occur either intracellularly (accumulated in the roots) or extracellularly, but there is no evidence of the stimulation of production under iron-stressed conditions. Higher plants may rely on these lower affinity iron-complexing reagents as iron-solubilizing factors which aid in iron removal from humic materials and from microbial siderophores (Lindsay, 1972; Lankford, 1973).

E. SIDEROPHORE PRODUCTION BY PHYTOPLANKTON

Siderophore production by phytoplankton has received increasing attention in recent years. Lange (1974) found natural chelator production from 6 out of 10 planktonic freshwater cyanobacteria. Cultures produced sufficient chelating material to grow without the addition of artificial chelators. These natural chelators were thought to be large molecular weight peptides or polysaccharides which chelate trace metals (Fogg and Westlake, 1955; Fogg, 1966).

Murphy *et al.* (1976) found hydroxamate-type siderophores in cultures of *Anabaena flos-aquae* and in field samples from blooms of *Anabaena* sp. Hydroxamate-type siderophores have also been reported from *Anabaena* sp. by Bailey and Taub (1980). Simpson and Neilands (1976) have isolated the dihydroxamate siderophore (schizokinen, Fig. 14B) from the culture supernatant of iron-starved *Anabaena* sp.

Several authors have provided evidence to support the production of hydroxamate-type siderophores by marine cyanobacteria. Estep *et al.* (1975) found cyanobacteria collected from two different habitats (cyanobacterial mats and seagrass beds) to contain extracellular compounds which promoted the growth of the siderophore auxotroph, *Arthrobacter* JG-9.

Using the same siderophore auxotroph bioassay, Armstrong and Van Baalen (1979) isolated a siderophore from the marine cyanobacterium, *Agmenellum quadruplicayum*. The siderophore was positive to the Csaky test (a colorimetric test for secondary hydroxamate functionalities) and was found intracellularly when

cells were cultured under low iron conditions.

While there is some supporting evidence for the production of siderophores by prokaryotic phtoplankton, production of similar iron-specific chelators by eukaryotic phytoplankton is controversial. Murphy et al. (1976) hypothesize that cyanobacteria can outcompete eukaryotic phytoplankton during iron-limited growth conditions since the latter group cannot produce siderophores. This inability was further supported by work of McKnight and Morel (1979), who could not induce siderophore production in any of the 13 eukaryotic phytoplankton investigated. However, all seven prokaryotic phytoplankters that were examined produced hydroxamates.

Evidence in support of siderophore production by eukaryotic phytoplankton includes Spencer et al. (1973), who isolated a trihydroxamate-type siderophore from a bacterized Chaetoceros socialis culture. The isolated compound reacted with $FeCl_3$, had a broad absorption band between 425 and 525 nm for the ferri-siderophore complex, and contained secondary hydroxamate groups based on the infra-red spectrum. These are all criteria for a hydroxamate-type siderophore (Neilands, 1981a).

Researchers have been critical of this work since the cultures contained bacteria, and bacteria are excellent sources of hydroxamate-type siderophores (Goyne and Carpenter, 1974). Key papers dealing with siderophore production in phytoplankton have chosen not to cite this work, due, most likely, to the ambiguous origin of the siderophore (Murphy et al., 1976; McKnight and Morel, 1979; Anderson and Morel, 1980; Huntsman and

Sunda, 1980).

Further evidence for the production of siderophores from eukaryotic phytoplankton is presented by Armstrong and Van Baalen (1979). The authors found that intracellular and extracellular extracts of the marine diatom Cylindrotheca sp. stimulated the growth of the siderophore auxotroph, Arthrobacter JG-9. The extracellular supernatent was not reactive to the Csaky test. Thus, it does not fit the criterion for a hydroxamate-type siderophore. The existence of this siderophore will have to be confirmed, since the fractions which produced stimulation in the bioassay test were concentrated 1000 fold and the bioassay will respond to non-hydroxamate siderophores present in high concentrations (Morrison et al., 1965).

The following chapters describe prorocentrin, a hydroxamate-type siderophore isolated from the marine dinoflagellate, Prorocentrum minimum. Production of hydroxamate-type siderophores in other species is also established.

V. PROROCENTRIN:AN EXTRACELLULAR SIDEROPHORE PRODUCED BY THE MARINE DINOFLAGELLATE PROROCENTRUM MINIMUM

A. ABSTRACT

Prorocentrin is a Csaky positive metabolite that can be extracted from the filtrates of Prorocentrum minimum cultures by XAD-2 resin. Production of prorocentrin can be stimulated by culturing P. minimum under conditions of iron deficiency. The iron (III) complex of prorocentrin has a UV-visible absorption spectrum typical of hydroxamate siderophores.

B. INTRODUCTION

Marine phytoplankton, like most other living organisms, have a nutritional requirement for iron (Price, 1968; O'Kelly, 1974). Iron in seawater is either associated with organic chelators (Sugimura et al., 1978) or present as aggregates of the almost totally insoluble iron (III) hydroxide ($K_{sp}=10^{-38}$) (Neilands, 1972). A portion of the chelated iron (III) may be utilized by phytoplankton, however, the large pool of insoluble iron (III) hydroxide is presumed to be unavailable as a nutritional source.

Aquatic microorganisms, for example bacteria (Gonye and Carpenter, 1974) and cyanobacteria (Murphy et al., 1976; Simpson and Neilands, 1976; Armstrong and Van Baalen, 1979), have been shown to produce extracellular iron (III) chelating agents (siderophores) that enable them to solublize and therefore acquire the iron present in the iron (III) hydroxide aggregates.

It is not clear whether eukaryotic phytoplankton acquire iron (III) in a similar manner. Spencer et al. (1973) characterized a possible siderophore from a non-axenic marine diatom, Chaetoceros socialis. The isolated substance had chemical properties similar to a hydroxamate-type siderophore but definitive evidence for the origin of the compound (from the diatom and not the bacteria) was not presented. More recently, McKnight and Morel (1979) have concluded that a number of axenic eukaryotic phytoplankton are unable to produce iron (III) specific chelators. They have suggested that the diatom Thalassiosira weissflogii utilizes iron (II) rather than iron

(III) (Anderson and Morel, 1980), because ferrous iron is more soluble in seawater and therefore it should be more nutritionally accessible. Since iron (II) is extremely labile in the marine environment, transformation of iron (III) to iron (II) at the cell membrane may be required for efficient uptake. Armstrong and Van Baalen (1979) showed that a concentrated chloroform extract of the marine diatom Cylindrotheca was Csaky positive (Csaky, 1948; Gillam et al., 1981) and that it stimulated the growth of the siderophore auxotroph Arthrobacter flavescens JG-9 (Burnhard and Neilands, 1961).

Circumstancial evidence for the utilization of siderophores by phytoplankton comes from numerous observations that either synthetic or natural chelators stimulate phytoplankton growth in culture and in the field, possibly by enhancing the availability of iron (Johnston, 1964; Barber and Ryther, 1969; Barber et al., 1971; Barber, 1973).

C. MATERIALS AND METHODS

In an attempt to clarify whether marine eukaryotes do produce siderophores, a survey was conducted of marine phytoplankton species in the North East Pacific Culture Collection (NEPCC) for the production of either intracellular or extracellular metabolites which were Csaky positive (a quantitative test for hydroxamate siderophores) (Gillam et al., 1981) and which would satisfy the requirements of the siderophore auxotroph A. flavescens JG-9 (Burnhard and Neilands, 1961). The survey (Gillam et al., in prep.) revealed

that the cell and filtrate extracts of the non-toxic, red tide dinoflagellate Prorocentrum minimum Schiller (NEPCC #96) showed considerable promise as a source of hydroxamate siderophores.

It is well documented that the microbial production of siderophores is stimulated by growth in iron-limited medium (Garibaldi and Neilands, 1956). Six L axenic cultures (Chapter 2) of P. minimum were grown in the following media:

1) charcoal-treated natural seawater followed by addition of all required nutrients (ESNW)(Harrison et al.,1980);

2) charcoal-treated natural seawater followed by the removal of most of the available iron by the procedure of Lewin and Chen (1971), and then supplemented with all nutrients, except $FeCl_3$ and EDTA (ESNW-Fe); and

3) charcoal- and Chelex-100 resin-treated natural seawater supplemented with AQUIL nutrients (Morel et al., 1979), minus $FeCl_3$ and EDTA (AQUIL-Fe).

The first medium provided an iron sufficient control while the other media provided iron-stressed conditions.

All cultures were harvested when extracellular siderophore concentration was maximal. This occurred approximately three days after the culture reached senescence. Cells were harvested by continuous centrifugation and membrane filtration (0.45 μm). The cell-free filtrates were acidified to pH 2 and passed over XAD-2 resin (Sugimura et al., 1978) to extract the Csaky positive metabolites. Methanol elution removed the organics from the XAD-2 resin. In vacuo evaporation of methanol followed by partitioning the resulting residue between chloroform and

water generated an aqueous fraction which contained the Csaky activity.

D. RESULTS AND DISCUSSION

The influence of the media on growth parameters and Csaky positive metabolite production is shown in Table V. The reduction in growth rate and final cell yield is attributed to the iron-deficient culture medium. Cells grown under iron-stressed conditions produced more Csaky positive compound. The addition of freshly prepared $FeCl_3$ to alleviate the iron deficiency in ENSW-Fe and AQUIL-Fe reestablished maximum growth rates (1.01± 0.07, 0.94± 0.01 divisions/day, respectively) and reduced the production of Csaky positive compound to less than 5 $\mu g\ NH_2OH \cdot 10^{-8}$ cells .

A 40 L culture of P. minimum grown in ESNW-Fe medium and harvested shortly after the cessation of growth, provided a sufficient amount of the extracellular Csaky positive compound to enable purification and preliminary chemical characterization; this compound has been named prorocentrin. Culture filtrates were extracted as described above. Ferri-prorocentrin was formed by adding freshly prepared iron (III) hydroxide (Neilands, 1966) to the resulting aqueous fraction and heating the suspension at 80°C for 2 h. Excess iron (III) hydroxide was removed by filtration and the deep red filtrate was reduced in volume by rotary evaporation in vacuo. A white precipitate that formed in the concentrated filtrate was

Table V - Influence of culture medium on growth rate of *Prorocentrum minimum* and production of Csaky positive compounds.

Medium and nutrient status	Growth rate (divisions · day^{-1})	Final cell yield (10^7 cells · L^{-1})	Csaky test (µg NH_2 OH·10^8 $cells^{-1}$)
ESNW, iron sufficient	1.04 ± 0.08	6.27	5.9
AQUIL-Fe, iron deficient	0.42 ± 0.01	4.01	127.2
ESNW-Fe, iron deficient	0.75 ± 0.02	3.95	133.0

Figure 15 - The UV-visible absorption spectra of desferri-prorocentrin (solid line) and ferri-prorocentrin (broken line).

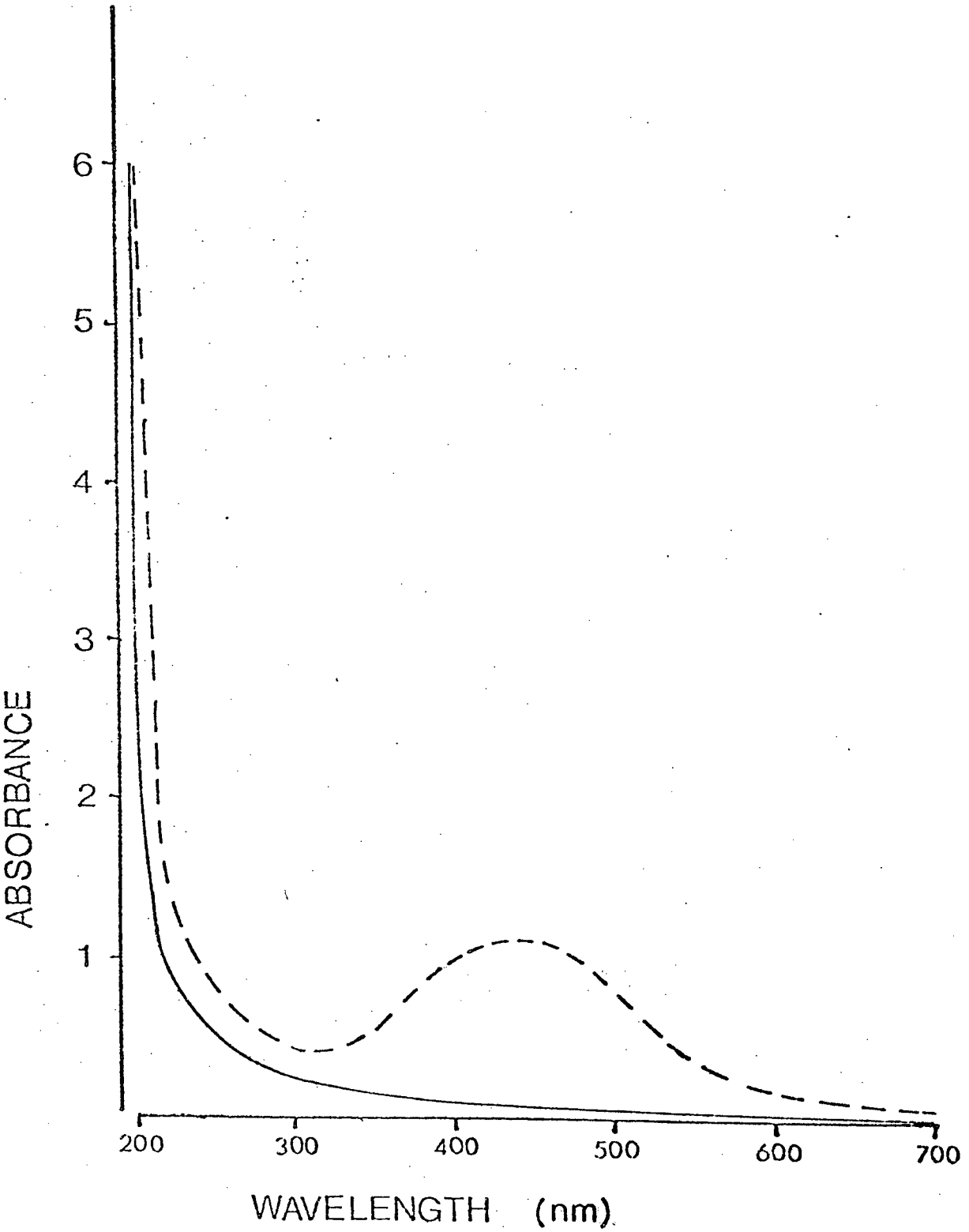


Table VI - Comparison of ferri-prorocentrin with desferri-prorocentrin.

Test or parameter	Ferri-prorocentrin	Desferri-prorocentrin
R_f (But:AcOH:H_2O)	0.60	0.18 - 0.20
Reaction to I_2 vapor	positive	positive
Reaction to $FeCl_3$ spray	none	light pink color
Reaction to perchlorate test	positive	positive
Reaction to Csaky test	positive	positive
Estimated molecular weight (daltons)	not determined	560-590

removed and the mother liquor was applied to a rotary thin layer chromatography plate (Chromatotron-Harrison Research, HF Silica Gel with a starch binder). Fractionation was achieved by elution with a series of solvents (MeOH, 1:24 H_2O:MeOH, 3:7 H_2O:MeOH, 60:25:15 Butanol:H_2O:Acetic acid). Several red-orange bands were collected in each solvent pass. Following concentration by rotary evaporation each fraction was assayed by the Csaky test. A single orange red fraction from the Butanol:H O:Acetic acid pass was positive.

Analytical thin layer chromatography of the Csaky positive fraction on silica gel (SIL G/UV254 - Butanol:H_2O:Acetic acid-60:25:15) showed a single iodine positive spot at Rf=0.6 corresponding to ferri-prorocentrin. The iron could be removed from the complex by treatment with 1N NaOH. Thin layer chromatography of the desferri-prorocentrin showed a single spot at Rf=0.2 which took up iodine and gave a pink spot with ferric chloride spray (2% in EtOH). Prorocentrin was desalted on a Bio Gel P-2 column to give 7 mg of a white solid. The Bio Gel column was calibrated with several low molecular weight standards. The estimated molecular weight for prorocentrin is 560--590 daltons. The molecular weight for ferri-prorocentrin could not estimated because it streaked on the column.

Prorocentrin shows only strong end absorption in its UV-visible spectrum (Fig. 15). Formation of the iron (III) complex at neutral pH, generates a new absorption band with a = 440 nm (H_2O), which shifts to a = 450 nm upon acidification to pH 2. The UV- visible absorption spectrum of ferri-prorocentrin

is typical of tri-hydroxamate siderophore such as ferrichrome (Neilands, 1966).

Several observations support the claim that prorocentrin is a hydroxamate containing siderophore produced by the dinoflagellate P. minimum. First, the origin of the metabolite cannot be questioned. The axenic condition of our cultures and the isolation of the same compound (identical TLC) from cell extracts eliminates the possibility of a bacterial origin. Secondly, the positive Csaky test shown by pure prorocentrin (Table VI) and the UV-visible absorption spectrum of ferri-prorocentrin are characteristic of hydroxamate siderophores. Thirdly, increased production of prorocentrin in iron-limited culture medium is the required physiological manifestation of a siderophore-based iron acquisition mechanism. Finally, it is a routine matter to interconvert the desferri and ferri forms of prorocentrin.

Success in isolation of the siderophore can be attributed to the use of iron-deficient medium and the concentration of prorocentrin on XAD-2 resin. While the recovery using XAD-2 is less than ideal (50%), it is a necessary step in isolating and quantifying extracellular metabolites from this marine dinoflagellate (Andersen et al., 1980; Chapter II). Without concentration, iron (III) specific chelators from marine eukaryotes may remain undetected, whereas these compounds from marine cyanobacteria can be measured (Armstrong and Van Baalen, 1979). Whether or not marine eukaryotic phytoplankton produce quantitatively less siderophore than marine cyanobacteria is

unclear. It is well documented that cyanobacteria have a higher specific requirement for iron (especially when fixing nitrogen) (Stewart, 1980) than marine eukaryotic phytoplankton, however, quantitative comparisons between the groups cannot be made until further information is available on the production, excretion and cellular uptake of siderophores.

Prorocentrin is the first siderophore to be isolated from a eukaryotic marine phytoplankton. The discovery suggests that the iron uptake mechanism of dinoflagellates may closely parallel that of prokaryotic siderophore producing organisms. The production of a strong trace metal chelator by a red tide organism has many important ecological implications. Blue-green algae can effectively eliminate competing algal species in fresh water lakes by sequestering all the available iron as a siderophore complex (Murphy et al., 1976) and there is some indication that exogenous siderophores can both positively and negatively affect the growth of marine phytoplankton (Bailey and Taub, 1980). Strong trace metal chelators have also been shown to reduce the toxicity of cupric ions to sensitive marine phytoplankton species (Sunda and Guillard, 1976). It will be interesting to test whether this dinoflagellate siderophore provides iron to prokaryotic organisms.

VI. METHODOLOGY USED IN ISOLATING THE HYDROXAMATE SIDEROPHORE, PROROCENTRIN, FROM PROROCENTRUM MINIMUM

A. ABSTRACT

The experimental procedure for the isolation of prorocentrin, a hydroxamate-type siderophore, from cell-free Prorocentrum minimum culture is critically examined. The siderophore adsorbs poorly to the Amberlite XAD-2 resin but with a rigidly defined experimental procedure a constant recovery of approximately 60% can be achieved. This recovery allows for an accurate quantitative estimate of the total amount of prorocentrin produced. The isolated prorocentrin is a trihydroxamate with a molecular weight of 560-590 daltons. The prorocentrin-iron complex is stable over a wide pH range.

B. INTRODUCTION

The isolation of the extracellular hydroxamate-type siderophore, prorocentrin, from the marine eukaryotic marine phytoplankton, Prorocentrum minimum, has recently been undertaken (Chapter V). Similar compounds are produced by marine bacteria (Gonye and Carpenter, 1974), marine cyanobacteria (Estep, et al., 1978; Armstrong and Van Baalen, 1979), and other eukaryotic phytoplankton such as Thalassiosira pseudonana (Chapter VIII) and Chaetoceros socialis (Spencer et al., 1973).

In an initial paper describing prorocentrin (Chapter V) it was stressed that the success of isolation was primarily due to the concentration technique utilized. This technique is based on the concentration of the hydroxamate-type siderophore on XAD-2 resin, prior to analysis for hydroxamate-containing compounds (Csaky test).

This chapter critically examines the isolation steps of prorocentrin. Since prorocentrin does not bind strongly to the XAD-2 resin, carefully defined procedures must be followed to achieve constant recovery efficiencies for quantitative studies. This chapter also reports the attempted purification of prorocentrin. Various separation techniques were utilized and prorocentrin was characterized with respect to molecular size and chromatographic behavior.

C. MATERIALS

Prorocentrum minimum was obtained from the North East Pacific Culture Collection (NEPCC #96). Axenic cultures were grown in 20 L batch cultures, using enriched natural seawater medium which had been treated to remove residual iron (ESNW-Fe; Chapter V). Culture conditions were as follows: constant daylight fluorescent lighting at 120 $\mu E \cdot m^{-2} \cdot s^{-1}$, 18°C, continuous stirring at 60 rpm.

Since maximum intracellular prorocentrin concentrations occur prior to maximum extracellular prorocentrin concentrations (Chapter VII), cultures were harvested 8 to 10 days after inoculation to obtain intracellular prorocentrin and 10 to 12 days after inoculation if extracellular prorocentrin was required. The harvesting procedure has been described previously (Andersen, *et al.*, 1979; Chapter II). Cells were harvested using a Sharples continuous flow centrifuge. The culture medium was then filtered through a glass fiber filter (GF/A) followed by a 0.45 µm membrane filter. The cell-free medium was adjusted to pH 2.0 with HCl. The dissolved organic fraction was collected by passing the acidified filtrate through a column of precleaned (Soxhlet extraction with methanol, 72 h) XAD-2 resin (Mallinckrodt). Remaining salts were removed by washing the column with 150 ml deionized, distilled water The adsorbed organic fraction was removed using 300 ml of methanol. The organic fraction was dissolved in water and repeatedly washed with chloroform until the water fraction was colorless.

To quantify the amount of prorocentrin, the modified Csaky

test of Gillam et al. (1981) was employed. The Csaky test is a colorimetric test specific for secondary hydroxamates. The test requires a long acid hydrolysis step (4 h, 121°C, 15 psi). The hydroxy-amino compound released is oxidized with I_2 to nitrite which is measured colorimetrically after reaction with sulfanilic acid and α-naphthylamine. For each 5 ml sample, a 2 ml aliquot was used for the test and a second, 2 ml sample was used for the color and reagent blank. All concentrations are presented as the amount of product produced by the Csaky test, corrected for subsampling and the initial volume of seawater concentrated (e.g. $NH_2OH \cdot L^{-1}$).

Certain procedures such as gel permeation chromatography, required the examination of a large number of samples. Since this was technically difficult to do by the Csaky test, initial screening of samples by the addition of ferric perchlorate served to detect the presence of organics which formed colored ferric complexes (Atkin et al., 1970). The procedure was modified slightly to facilitate analysis of small sample volumes (2.5 ml reagent, 0.5 ml sample). Fractions positive to the ferric perchlorate test were further examined using the Csaky test to confirm the presence of prorocentrin.

The thin layer chromatography solvent systems employed to isolate prorocentrin were butanol:acetic acid:water (60:15:25) (BAW), methanol:water (70:30), isopropanol:water (70:30) and methanol:water (98:2). Samples were run on silica strips with a fluorescecnce indicator (SIL G/UV 254) or using a rotary chromatography system with a silica-starch plate (Chromatotron,

Harrison Research). Organics were visualized by fluorescence or by reaction to any of the following: $FeCl_3$ spray (2% in ethanol), $FeCl_3$ spray with monochloroacetic acid (Bernhard et al., 1964), ninhydrin spray, or I_2 vapor. The acidified ammonium vanadate test, a chemical test for hydroxamates, was also employed for aqueous samples (Abbasi, 1976).

Purification and estimation of molecular size were performed by gel chromatography using a 55 X 1.3 cm column slurry-packed with BioGel P-2 (200-400 mesh). The gel was swollen and washed withdeionized distilled water. Bio Gel P-2 has a molecular size exclusion limit of 1800 daltons. Samples of 1 to 2 ml were applied to the top of the column and chromatographed with deionized, distilled water. The flow rate was maintained at 15 $ml \cdot h^{-1}$ using a peristaltic pump. Fractions were collected every 5 ml. Standards for molecular size calibration (blue dextran, vitamin B_{12}, riboflavin, and lumichrome) were detected by absorbance at 260 nm. Desferrioxamine B (Ciba-Geigy) and prorocentrin were detected using the ferric perchlorate test for iron-binding organics.

Absorption spectra in the visible and UV ranges were obtained with a Model 2200 Bausch and Lomb double-beam spectrophotometer.

The formation of a ferri-prorocentrin complex was performed by addition of freshly precipitated ferric hydroxide (Carrano and Raymond, 1978), or by addition of freshly prepared $FeCl_3$. The addition of iron using ferric acetylacetonate as a source was also attempted.

To convert ferri-prorocentrin to desferri-prorocentrin the iron was removed using a strong base following the methods of Emery and Neilands (1960). An equal volume of 1.0 N KOH was added to the solution containing ferri-prorocentrin. The reaction mixture was chilled for 30 minutes, then centrifuged for 20 minutes. The supernatant containing iron-free prorocentrin was decanted from the ferric hydroxide precipitate and neutralized with 1.0 N HCl.

D. RESULTS

1. Recovery of Prorocentrin Using XAD-2 Resin

The procedure for the isolation and concentration of dissolved organics from the cell-free culture medium is summarized in Fig. 16. To standardize all experiments, the bed-volume of the XAD-2 resin was constant (25 X 2 cm). A flow rate of 1 $L \cdot h^{-1}$ was employed. To determine the maximum efficiency of prorocentrin collection using XAD-2 resin, four variables were tested as follows: 1) the volume of the cell-free, acidified filtrate passed through the column; 2) the volume of the deionized, distilled water used to desalt (wash) the column; 3) the amount of methanol used to removed the collected organics from the column; and 4) the initial concentration of the siderophore in the culture medium.

a) Influence of Filtrate Volume

Table VII shows the influence of increasing volumes of

acidified filtrate on the efficiency of recovery. Csaky-positive material was collected from 50 L of cell-free filtrate using the procedure from Fig. 16. A subfraction of the XAD-2 collected organics was taken to determine the concentration of collected Csaky positive material. The remaining XAD-2 collected organics were added to ESNW-Fe medium to give a final reactive Csaky concentration of 6.0 ug $NH_2OH \cdot L^{-1}$. Volumes ranging from 1 to 20 L were passed through the standard XAD-2 column. The column was washed with 150 ml water and the adsorbed organic eluted with 300 ml methanol. Recovery is presented as a percentage of the recovered reactive material (based on the Csaky test) compared to the concentration in the initial sample before it was passed through the column. The maximum efficiency of recovery was in the range of 60%. Using the standard column, organics from 6 L could be scavenged without further loss in recovery. Passage of more than 6 L of medium per column, provided little further binding of reactive material and the efficiency of recovery declined rapidly.

b) Washing or Desalting the Column

The next step in the analysis involves adjusting the volume of the water for the column wash. The collected water-soluble organic fraction was added back to 40 L of acidified ESNW-Fe medium. Initial concentration of hydroxamate-type siderophore was equivalent to 3.6 $\mu g\ NH_2OH \cdot L^{-1}$. The 40 L was split into eight, 5 L fractions. Each fraction was passed over a standard XAD-2 column which was then washed with either 50, 150, 1000, or

Figure 16 - Summary of the procedure for the isolation of the aqueous fraction containing the hydroxamate-type siderophore, prorocentrin.

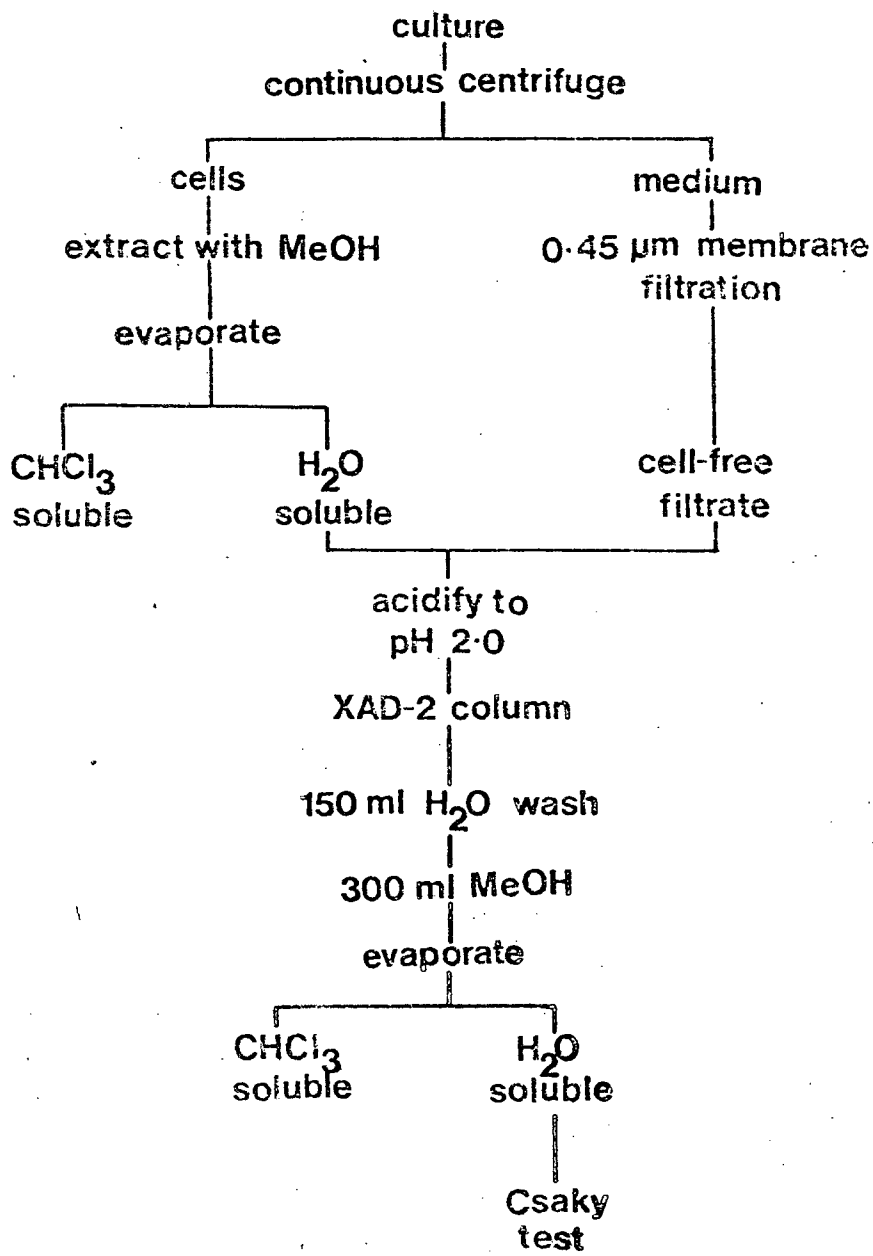


Table VII - The influence of filtrate volume on the efficiency of extraction and recovery of prorocentrum on XAD-2 resin.

The concentration of the hydroxamate-type siderophore in the initial seawater was 6.0 μg $NH_2OH \cdot L^{-1}$ (Csaky test equivalents).

Volume (L)	Csaky Absorbance	Prorocentrin (μg NH_2 OH)	Recovery (%)
1	0.068	3.6	59.4
3	0.197	11.2	61.9
5	0.331	18.8	62.6
6	0.383	21.8	60.3
10	0.420	23.9	39.6
20	0.443	25.2	20.8

Table VIII - Influence of the volume of deionized, distilled water washes on the recovery of prorocentrin.

Five L of seawater containing 3.6 μg NH_2OH equivalents of Csaky positive material was passed through each column. Values are efficiency of recovery of the original material (expressed as a %). Experiments were performed in duplicate. (*-These samples contained too much salt to enable a Csaky test to be performed.)

Wash Volume (L)	Csaky Absorbance	Prorocentrin Collected (μg $NH_2OH \cdot L^{-1}$)	Recovery (%)
0.050	*	------	-----
0.050	*	------	-----
0.150	0.105	2.3	63
0.150	0.098	2.1	59
1.0	0.070	1.5	41
1.0	0.069	1.4	40
2.0	0.048	1.0	27
2.0	0.038	0.8	21

Table IX - Removal of prorocentrin from XAD-2 resin by successive 100 ml methanol washes.

N.D.-none detected.

Methanol Wash (mL)	Csaky Absorbance	Prorocentrin Collected (µg NH_2OH)
000-100	0.188	10.4
100-200	0.197	11.2
200-300	0.037	2.1
300-400	<0.010	ND
400-500	<0.010	ND
500-600	<0.010	ND
600-700	<0.010	ND

Table X - Recovery of prorocentrin from XAD-2 resin with increasing concentrations of Csaky positive material.

Recovery (%) is based on the original concentration of prorocentrin added to 5 L of acidified ESNW-Fe medium. Average recovery (and one standard deviation) was 62.2 (±4.2) %.

Initial Csaky Material ($\mu g\ NH_2OH \cdot L^{-1}$)	Csaky Absorbance	Prorocentrin Collected ($\mu g\ NH_2OH$)	Recovery (%)
5	0.057	2.9	58
10	0.117	6.3	63
20	0.215	11.9	60
30	0.328	18.3	61
50	1.524	34.5	69

2000 ml deionized, distilled water. The adsorbed organics were removed from the column with 300 ml methanol. Each experiment was run in duplicate. The recovery of Csaky positive material is presented in Table VIII. Washing the column with 50 ml was unsatisfactory as the large amount of salt remaining interferred with the concentration step prior to performance of the Csaky test. Recovery after washing the column with 1000 ml or 2000 ml of water was very low, indicating prorocentrin did not bind strongly to the XAD-2 column.

c) Volume of Methanol to Elute the Column

Organics from 10 L of cell-free filtrate were collected on a standard XAD-2 column. The column was washed with 150 ml of deionized, distilled water. Organics were eluted from the column in seven successive 100 ml methanol passes. The amount of Csaky reactive material was measured in each fraction. All of the Csaky reactive material was eluted in the first 300 ml (Table IX).

d) Initial Concentration of Siderophore

Based on the previous experiments, the following standard procedure was adopted for this test. Six liters or less of sample was passed through a 25 X 2 cm column of Soxhlet-cleaned XAD-2 resin. The column was washed with 150 ml deionized, distilled water and the adsorbed organics eluted with 300 ml methanol. Siderophore was collected from 100 L of P. minimum

culture. The eluted aqueous fraction from the XAD-2 concentration procedure was added back to five, 5 L aliquots of acidified ESNW-Fe medium. Each flask was inoculated at one of the following Csaky equivalent concentrations: 5, 10, 20, 30, or 50 μg $NH_2OH \cdot L^{-1}$. Recovery was constant over the range of 5 to 50 μg $NH_2OH \cdot L^{-1}$ (Table X).

2. Isolation and Characterization of Prorocentrin From XAD-2 Collected Fractions

a) Detection of Prorocentrin in Collected Aqueous Fractions

To follow the isolation and purification of prorocentrin, two methods were routinely employed. For accurate analysis the previously described Csaky test was used. The addition of ferric perchlorate to the aqueous fractions (collected from the gel permeation chromatography column) was used to detect the presence of organics which form colored ferric complexes (Atkin, et al., 1970). A third procedure to characterize prorocentrin in aqueous samples was attempted but was unsuccessful. The reaction with ammonium metavanadate is a sensitive test for microgram amounts of some hydroxamates (Abbasi, 1976). This technique involves forming the vanadium-hydroxamic acid complex and the extraction of the violet-colored complex into a water-immiscible organic. The detection of prorocentrin by this procedure was unsuitable since a colored vanadate-prorocentrin complex could not be detected. Either prorocentrin did not form appreciable amounts of the vanadium-complex or the

vanadium-prorocentrin complex was not soluble in the chloroform, butanol, or ethyl acetate organic layers tested.

b) Detection of Prorocentrin by Thin Layer Chromatography

Of the thin layer chromatography solvent systems examined only butanol:acetic acid:water (60:15:25) {BAW} suitably chromatographed prorocentrin from the orgin (Rf= 0.18-0.20 for the desferri-prorocentrin). Prorocentrin remained at the origin in all the other thin layer chromatography solvent systems. The other solvent systems separated other organics away from the origin (and, thus, away from prorocentrin).

To detect the presence of prorocentrin on thin layer chromatography plates, a $FeCl_3$ spray (2% in ethanol) was utilized. Prorocentrin was visible immediately after spraying as a light pink spot which quickly faded. To reduce the speed at which the iron-prorocentrin lost its color, the modified FeCl spray with chloroacetic acid was used. This spray is considered to form a more stable complex (Bernhard et al., 1964), but it was unsuitable because a colored prorocentrin-product failed to form.

Prorocentrin on thin layer chromatography plates could be also visualized by reaction with ninhydrin spray (brown, yellow color) or by I vapor. These reactions could only be used in conjunction with the $FeCl_3$ spray, as neither is specific for iron-binding organics.

c) Fractionation by Gel Permeation Chromatography

Initial separation of prorocentrin from other XAD-2 collected water-soluble organics, was attempted on a gel permeation chromatography column. The column was packed with BioGel P-2, a polyacrylamide gel with a functional molecular size separation range between 100 and 1800 daltons. Profiles of the collected fractions are presented in Fig. 17. In each case several compounds or groups of compounds were reactive to the ferric perchlorate. Four major ferric perchlorate peaks could be distinguished, but only one of the peaks contained Csaky-positive compounds (peak III in Fig. 17C).

Figure 17 also serves as a comparison of the organic profiles from various experimental treatments. Based on these profiles, cells grown in large-scale polyethylene barrels contained the same type of intracellular organics as did axenic cells grown in glass carbouys. In contrast, the profiles of the extracellular XAD-2 collected organics differed significantly. The extracellular compounds of cells grown in the barrels always had a strong peak of high molecular weight and perchlorate-positive compounds at the void volume fractions (peak I in Fig. 17C and 17D). This was evident regardless of whether natural or artificial seawater was employed (data not shown). This peak was virtually eliminated from the

Figure 17 - Comparison of profiles of the separation of collected metabolites based on molecular size. Collected organics were isolated from: a) cells grown in polyethylene barrels; b) cells grown in glass carbouys; c) cell-free culture medium from polyethylene barrels; and d) cell-free culture medium from glass carbouys.

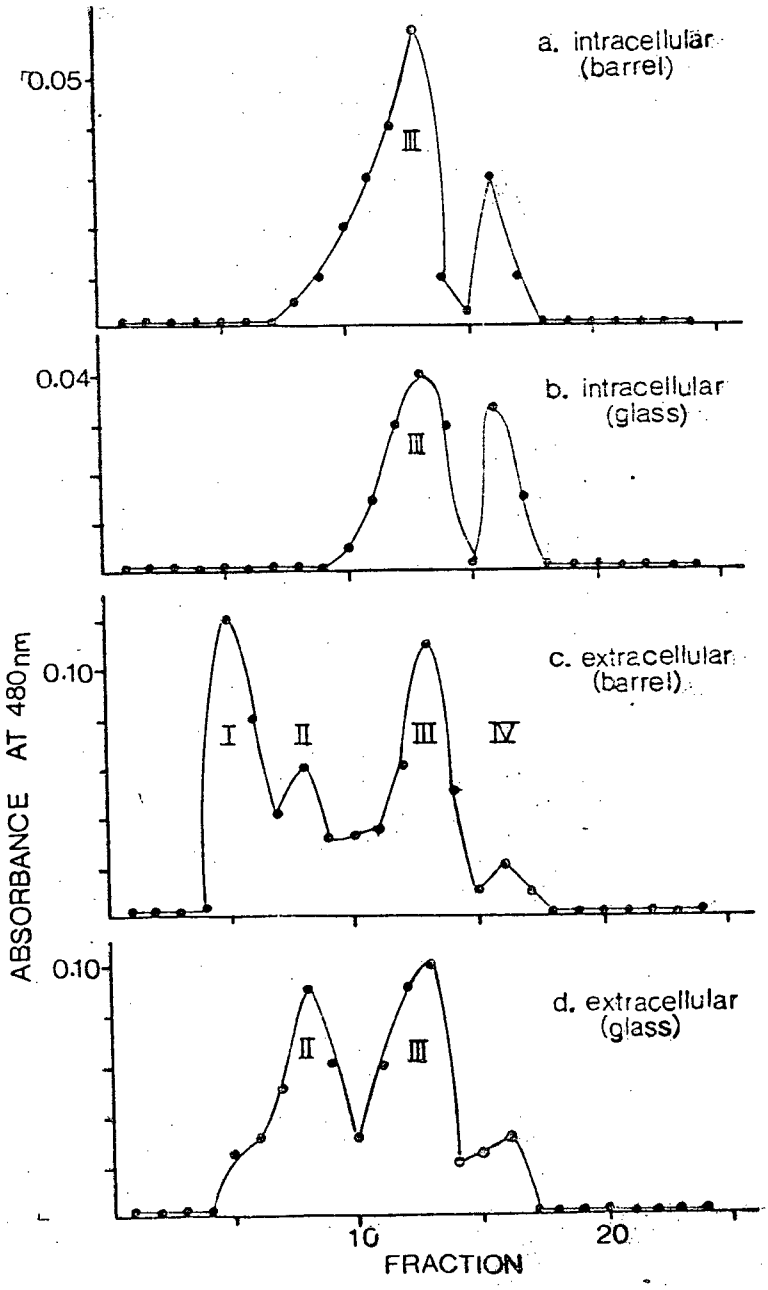
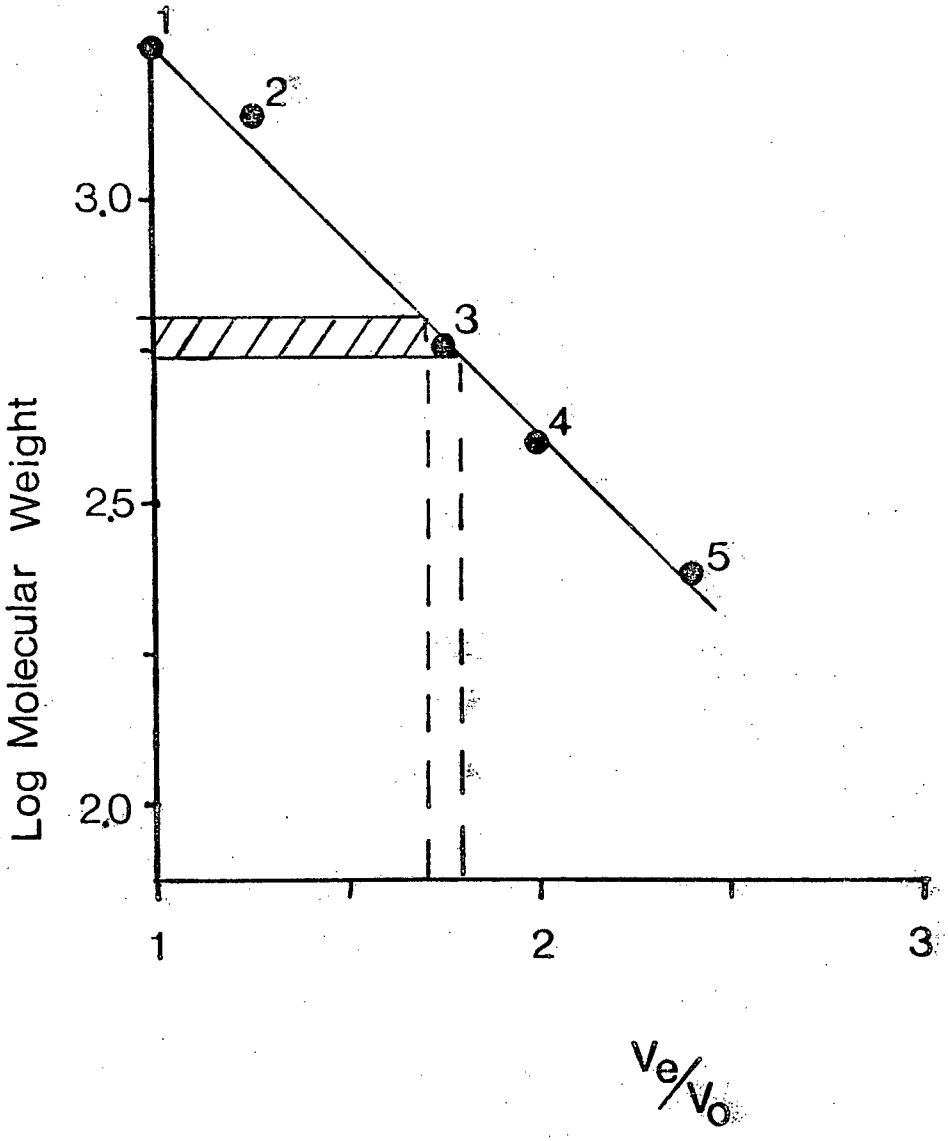


Figure 18 - Standard curve for the estimation of the molecular weight of procentrin.

Standards include: dextran blue (1), vitamin B_{12} (2), desferrioxamine B (3), riboflavin (4), and lumichrome (5). The shaded area represents the best estimate of prorocentrin based on the collection of a fraction positive to the perchlorate test and the Csaky test. Ve/Vo is the ratio of the collected volume containing the standard compound compared with the void volume of the column.



extracellular compounds of the glass-grown cells (Fig. 17C), suggesting that these compounds originated either from the barrel or as a cellular response to some component of the barrel. There was no evidence of peak I in either of the intracellular profiles (Fig. 17A and 17D). The only organic fraction that was consistently observed was the Csaky-positive, peak III. Examination of all fractions by the BAW thin layer chromatography solvent system and $FeCl_3$ spray, verified peak III as the only fraction with appreciable amounts of prorocentrin.

d) Molecular Size Determination

To determine the molecular size of prorocentrin the previously discussed BioGel P-2 column was employed. The column was calibrated using a series of compounds (molecular weights, daltons): blue dextran (>1800), vitamin B_{12} (1350), riboflavin (376), desferrioxamine B (560), and lumichrome (242). Prorocentrin was run separately on the same column immediately prior to running the standards. While the standards gave discrete estimates of molecular weight, prorocentrin spread somewhat while descending the column. The best estimate of molecular weight is between 560 and 590 daltons (Fig. 18).

e) Fractionation by Rotary Thin Layer Chromatography

The prorocentrin-containing fraction from the gel permeation chromatography column (peak III) was further fractionated using the rotary thin layer chromatography system.

Rotary thin layer chromatography is functionally different from traditional thin layer chromatography in that there is no limit to the solvent front. Thus, separation of prorocentrin is suited to this system since prorocentrin has a low Rf in BAW and remains at the origin with the other solvent systems examined.

Material from peak III was dissolved in a small volume of methanol:water (50:50). This sample was applied to the origin of the rotary thin layer chromatography plate and allowed to dry overnight. The plate was washed with methanol (100%), followed by water:methanol (2:98) to remove unwanted organics away from the origin. Prorocentrin was then recovered from the plate with the BAW solvent system. Any organics remaining on the plate were removed with 100 ml wash with hexane. Examination of each fraction by traditional thin layer chromatography and $FeCl_3$ spray confirmed prorocentrin only in the BAW fraction.

Fractionation by rotary chromatography was the best way to isolate prorocentrin, but it was not without certain problems. The high amount of water in the BAW solvent system weakened the support. A small amount of material (probably silica) was collected along with prorocentrin. While this contaminating material could be removed by passing the collected fraction back over the gel permeation chromatography column, the additional pass resulted in a very reduced yield of prorocentrin.

f) Addition of Iron

Since the iron-prorocentrin complex is colored (as shown by the reaction with ferric perchlorate), it was thought that the

formation of the complex prior to the application on the rotary chromatography plate might result in a more efficient separation technique. Several procedures to add iron were attempted. While prorocentrin did readily form the iron-prorocentrin complex, the separation of this complex from the non-complexed iron was a more difficult task.

The simplest technique of adding iron as $FeCl_3$ was unsuccessful. The ferri-prorocentrin could not be separated from the residual colloidal ferric hydroxide which formed at the experimental pH (7 - 9). Application of the iron-complex and iron hydroxide system to either of the thin layer chromatography systems resulted in the iron adsorbing to the plate. None of the solvent systems was successful in releasing the ferri-prorocentrin complex from the plate-bound iron hydroxide. Addition of the complex to a gel permeation chromatography column was unsuccessful since the iron and the ferri-prorocentrin bound irreversibly to the column.

Addition of iron using freshly precipitated iron hydroxide was more successful, but it could not be routinely performed. Following the procedure of Carrano and Raymond (1978), freshly precipitated and washed ferric hydroxide was added to the aqueous solution containing prorocentrin. The mixture was stirred for two hours at 80°C. The non-bound colloidal iron was removed by filtration {glass fiber (GF/A), followed by filtration through a 1 μm membrane filter}. The ferri-prorocentrin complex remained in solution.

The aqueous sample was applied to the rotary chromatography

plate and allowed to dry overnight. The same series of solvents were employed to remove organics associated with the prorocentrin. During the BAW wash a red-orange band chromatographed away from the origin. This band was collected and its UV/VIS absorption spectrum obtained. The material had the characteristic absorption spectrum of a ferri-hydroxamate. The spectrum has been described elsewhere in the thesis (Chapter V).

To verify that this compound was the iron-complex of prorocentrin, a sample was run on the traditional thin layer chromatography strip. Using the BAW solvent system, the sample showed a single spot with an Rf of 0.6. Based on short-wavelength fluorescence and reaction to I_2 vapor and $FeCl_3$ spray, there was no evidence of contaminating compounds. To remove the iron, a small subsample was basified with 1 ml 1N KOH. The reagent mixture was chilled and the ferric hydroxide removed by centrifugation. Chromatography of the colorless supernatant showed a single, $FeCl_3$ positive spot, corresponding to the original desferri-prorocentrin. Thus, the interconversion of the ferri-form and desferri-form of prorocentrin was verified.

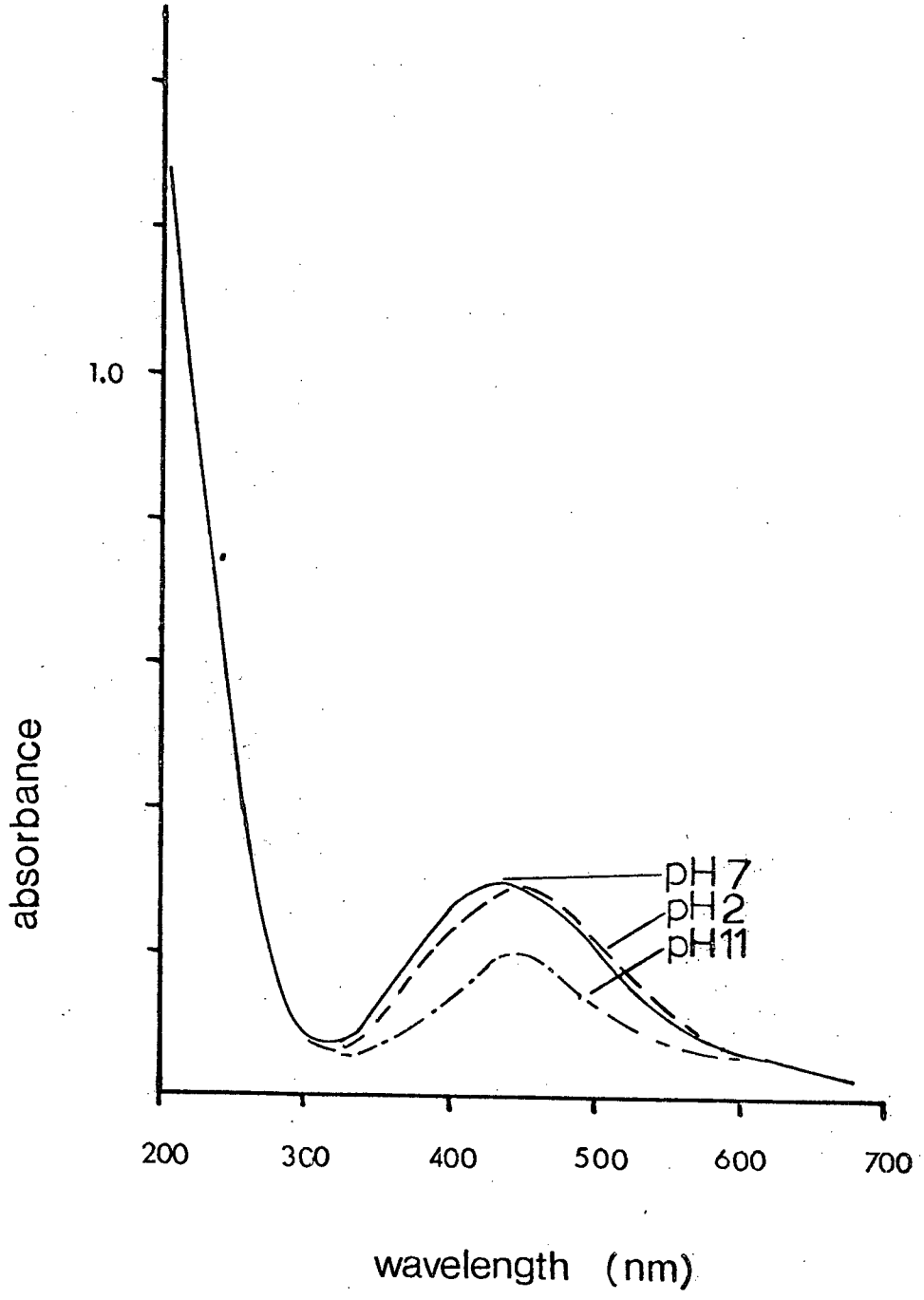
The addition of iron by ferric hydroxide was not always successful. Of the five attempts, only two provided sufficient transfer of iron to prorocentrin. It seems the preparation of the freshly precipitated and washed ferric hydroxide is a critical step which is not entirely reproducible.

g) Analysis of Isolated Prorocentrin

Several analyses were performed on the small amount of ferri-prorocentrin. The isolated ferri-prorocentrin gave a positive Csaky test and did not react to the addition of ferric perchlorate. The isolated ferri-prorocentrin had a Rf = 0.6 in the BAW solvent system. However, when ferri-prorocentrin was co-spotted with either ferric chloride or ferric hydroxide, the iron-prorocentrin complex remained at the origin. This confirms the previous suggestion that difficulties in isolating the iron complex may be due to our inability to separate the complex away from the origin, rather than our inability to form the iron complex. The unbound iron must be removed from the sample before ferri-prorocentrin will chromatograph.

A subfraction of the collected ferri-prorocentrin was examined spectroscopically to determine the influence of pH on the iron-complex. The influence of a wide range of pH values is documented in Fig. 19. For each trial, the same amount of ferri-prorocentrin was dissolved in pH-adjusted water. The shift in the absorption was recorded immediately and after 24 hours. While the absorbance shifted and stabilized immediately, some siderophores require longer incubations before a stable absorbance is obtained (Ong et al., 1979). In the case of ferri-prorocentrin, acidification of the sample had little effect on either the intensity of absorbance or in the absorbancy maximum. Basifying the sample to pH 11 did diminish the intensity of the absorbance but an iron-prorocentrin complex still existed with an absorbance maximum at 440 nm.

Figure 19 - The influence of pH on the UV-visible spectrum of prorocentrin.



The remaining material was submitted for C:N:Fe analysis. Based on thin layer chromatography, the ferri-prorocentrin sample appeared to be pure, however, the actual percentage of organic material was low (10%). The sample contained a large amount of contaminating inorganic material (probably silica from the rotary thin layer chromatography plate). The low amount of ferri-prorocentrin did not allow for an accurate measurement of the iron content, although iron was verified as a component of the sample. The measured atomic ratio of C:N was 2.4:1. This is lower than the 3:1 ratio for the trihydroxamate siderophore, ferrichrome, and much lower than the 3.6:1 ratio for rhodotorulic acid (a dihydroxamate siderophore) (Neilands, 1981).

E. DISCUSSION

Examination of the total isolation procedure clarifies the difficulties in isolating the highly water-soluble hydroxamate siderophore. Special care must be taken during the XAD-2 extraction of prorocentrin from the culture supernatant. Under the most rigorous procedure, up to 40% of the prorocentrin was unrecoverable. However, for the quantitative analysis of the production of prorocentrin, the standard isolation procedure consistently recovered 60-65% of the extracellular siderophore. This recovery was maintained over the entire range of siderophore concentrations recorded. Thus, as a quantitative analysis, the XAD-2 technique as outlined in Fig. 16, provides

a good approximation of the actual amount of prorocentrin produced.

The procedure for isolating pure prorocentrin was not satisfactory. Each step did separate prorocentrin from other organics, but the final product contained a large amount of colorless inorganic material. While this was only verified for the ferri-prorocentrin sample (by elemental analysis), one must suspect an inorganic contaminant in the desferri-prorocentrin samples also. The inorganic contaminant is of little consequence since its presence did not interfere with the analysis of the isolated material.

In Chapters V and VII, the role of prorocentrin as a functional hydroxamate-type siderophore was established. In review, the rationale was that prorocentrin gave a positive Csaky test, formed an iron-complex with an absorption maximum near 440 nm, and production was stimulated by low-iron medium.

Neilands (1981a) has characterized hydroxamate-type siderophores as having the following properties: formation of a colored complex by addition of ferric chloride or ferric perchlorate, positive Csaky test, formation of a colored complex with acidified ammonium vanadate, and a colored iron-complex with maximum absorbancy within 425-450 nm. In contrast, a catechol-type of siderophore forms an iron-complex in an absorbancy range closer to 500 nm and does not react to the Csaky test.

Of the four criteria , three were exhibited by prorocentrin. The fourth, the formation of a colored complex

with vanadium, was not met. However, not all hydroxamate-sideophores give a positive ammonium vanadate test (Abbasi, 1976).

The absorption spectra presented in this paper provide evidence for prorocentrin being specifically a tri-hydroxamic siderophore. Tri-hydroxamic siderophores are characterized by a strong, broad absorption band between 425 and 440 nm. The band is not strongly shifted by acidification from pH 7 to pH 2 and this agrees with earlier observations by Warren and Neilands (1964). There is only a slight loss in absorbancy which has been described in other tri-hydroxamates (Anderegg, et al., 1963). Similarly the resistance to decomposition of the iron-complex under alkaline conditions is a characteristic of some tri-hydroxamates (Neilands, 1966). While some tri-hydroxamates lose iron easily at elevated pH values (eg. ferrichrome; Emery and Neilands, 1970), the stability of the iron-prorocentrin complex at basic pH values would be essential for this compound to function as a siderophore in the marine environment.

In conclusion, the water-soluble nature of prorocentrin makes isolation a challenging problem. With care, XAD-2 could be used as a suitable method of concentration of prorocentrin. However, the limits are easily exceeded. The procedure used to isolate prorocentrin would not be suitable if large amounts of material were necessary. The large number of steps resulted in significant loss of material. The presence of the inorganic contaminant would hamper any further characterization. The absorbance spectra of the ferri-prorocentrin, provides evidence

of prorocentrin being a tri-hydroxamic siderophore. The stability of the iron-complex at elevated pH values may be an important criterion for extracellular siderophores produced in marine waters.

VII. THE CONTROL OF THE PRODUCTION OF A SIDEROPHORE BY THE MARINE DINOFLAGELLATE, PROROCENTRUM MINIMUM.

A. ABSTRACT

Factors which influence the production of prorocentrin by species of Prorocentrum were investigated. Prorocentrin, the extracellular siderophore isolated from P. minimum, was also produced by P.mariae-lebouriae and P.gracile . P.gracile was susceptible to iron-stress and was not considered further. Under iron-saturated conditions there was no measurable siderophore found either intracellularly or extracellularly. Under iron-deficient culture conditions, prorocentrin was produced 1 to 2 days after the cessation of growth in the stationary phase. Production was over a short period of time (1 to 2 days) and the prorocentrin did not remain in the medium. The rate of prorocentrin disappearance from the medium was similar to the rate of production. Immediately following the removal of prorocentrin from the medium, there was a rapid increase in in vivo fluorescence. There was no increase in cell numbers and the increase was not seen in iron-sufficient cultures. An hypothesis on the iron uptake mechanism is proposed.

B. INTRODUCTION

The relationship between trace metals and chelators in controlling primary productivity in the ocean has received an increasing amount of attention in recent years. Of particular importance is the role of iron. In a series of early experiments, Johnston (1964) found that the growth of natural phytoplankton was often controlled by the availability of chelating agents. Barber and Ryther (1969) suggested that production of natural chelators stimulated phytoplankton growth rates in upwelled seawater. Since the stimulation of growth could be mimicked by the addition of $FeCl_3$, the initial interpretation was that iron became available for growth by forming iron-organic complexes (Barber et al., 1971; Barber, 1973). Most of the iron in seawater appears to be unavailable for the direct utilization by phytoplankton because it is associated with large organic particles (Sugimura, et al., 1978) or it forms insoluble, $Fe(OH)_3$ aggregates (Lewin and Chen, 1971). The addition of a chelator is thought to enhance the availablity of iron (III) by solublizing the ferric hydroxide. Thus, available iron in marine waters may be low enough to limit growth (Menzel and Ryther, 1961; Menzel et al., 1963; Tranter and Newell, 1963; Glover, 1978).

In general, microorganisms can scavenge iron from a low iron environment by producing extracellular low molecular weight iron (III) specific chelators (siderophores). Siderophores are of two general types. Those containing secondary hydroxamate groups and those with catechol functionalities (Neilands, 1980).

High affinity iron acquisition systems have been demonstrated for marine bacteria (Gonye and Carpenter, 1974) and cyanobacteria (Simpson and Neilands, 1976; Armstrong and Van Baalen, 1979). Until recently, siderophore production by eukaryotic phytoplankton was unknown. Barber and co-workers (Spencer et al., 1973) characterized a possible siderophore from a non-axenic culture of the marine diatom Chaetoceros socialis The isolated substance had chemical properties similar to a hydroxamate-type siderophore but the possibility of a bacterial origin for this compound was not rigorously excluded. Recent work aimed at stimulating the production of siderophores in axenic eukaryotic phytoplankton has demonstrated they are unable to produce iron (III)-specific chelators (Swallow et al., 1978; McKnight and Morel, 1979). This has led to the suggestion that eukaryotic phytoplankton may not utilize iron (III), but rather iron (II), which is more readily transported across the cell membrane, but is extremely labile in marine waters (Anderson and Morel, 1980).

Recently the isolation of prorocentrin, a low molecular weight extracellular hydroxamate-type siderophore produced by the marine dinoflagellate Prorocentrum minimum was described (Chapter V). Prorocentrin represents the first example of an extracellular siderophore produced by a marine eukaryotic phytoplankter. This paper presents the results of an experimental investigation of the factors controlling the production of prorocentrin in culture.

C. MATERIALS AND METHODS

The five Prorocentrum species examined and the source of each culture are presented in Table 11. Cells were grown in sterile enriched natural seawater, modified by the replacement of Na glycerophosphate with Na_2HPO_4 and $Fe(NH_4)_2SO_4$ with $FeCl_3$ (ESNW) (Harrison et al.,1980) Experiments were conducted in 6 L or 10 L flat bottomed boiling flasks in iron sufficient or iron deficient media. To reduce iron contamination from culture glassware, all culture flasks were repeatedly rinsed with 3N NaOH, followed by 3N HCl and finally, deionized, distilled water.

Seawater was collected at a depth of 60 m and initially filtered through a glass fiber pre-filter followed by another glass fiber filter (GF/A). The seawater was treated with activated charcoal (24 h, constant stirring) to remove dissolved organics. Charcoal was removed by passage through a glass fiber filter followed by a 0.45 μm membrane filter.

Two methods for removal of residual iron in charcoal treated seawater were compared: 1) seawater was heated and filtered by the procedure of Lewin and Chen (1971), and then the medium was supplemented with ESNW nutrients, minus $FeCl_3$ and EDTA (ESNW-Fe); and 2) seawater was passed through a column of Chelex-100 resin to remove trace metals (procedure of Morel et al., 1979), and then supplemented with AQUIL nutrients, minus EDTA. A small known amount of iron was added back (1 μM $FeCl_3$) in order to provide some growth under this iron-limited condition (AQUIL-Fe). Both the ESNW-Fe and AQUIL-Fe media

provided iron-stressed growth conditions. Iron sufficient controls were the above media supplemented with freshly prepared FeCl (ESNW + Fe and AQUIL + Fe, respectively).

Experimental flasks were incubated at 18 C using continuous light provided by daylight fluorescent bulbs at an irradiance of 160 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cultures were stirred continuously (60 rpm). Growth was monitored by daily cell counts using an electronic particle counter (Coulter Counter TA-II). The axenic nature of all cultures was tested frequently by inoculating onto an organic-containing sterility-medium plate and examining the plate for growth of colonies after 48 h of incubation. The axenic nature was also verified by acridine orange direct staining (Hobbie et al.,1977).

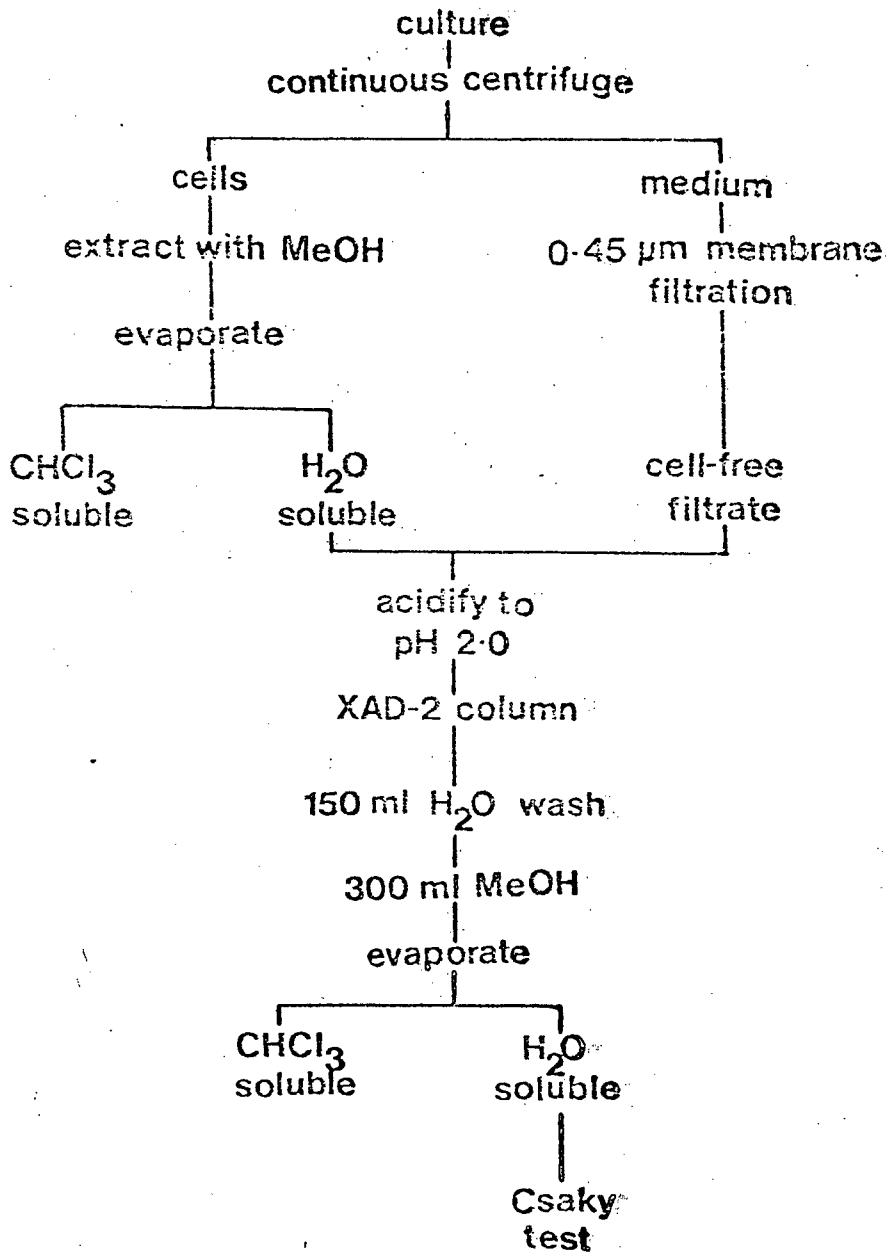
Cells were harvested using a Sharples continuous centrifuge followed by passage of the supernatant through glass fiber filters (GF/C) (Fig. 20). Remaining cells were removed from the filtrate by passage through a 0.45 μm membrane filter under a gentle vacuum (<200 mm Hg). Both the cells and the filtrate were tested for prorocentrin. A crude fraction containing prorocentrin was obtained in the following manner. The cells were extracted by stirring with MeOH (2 X 100 ml, 16 h, 8 h), followed by filtration through a glass fiber filter (GF/A). The MeOH filtrates were evaporated to dryness in vacuo and the residue was partitioned between 50 ml H_2O and 100 ml $CHCl_3$. The aqueous layer was repeatedly extracted with $CHCl_3$ until a nearly colorless layer was achieved. The H_2O layer was evaporated in vacuo to a volume of 5 ml. Extracellular

Table XI - Prorocentrin production by *Prorocentrum* species. Maximum extracellular prorocentrin concentrations and final cell yield of six *Prorocentrum* species grown in natural, enriched, iron-deficient medium (ESNW-Fe).

Species and NEPCC Identification No.	Final Cell Yield ($10^7 \cdot L^{-1}$)	Maximum Extracellular Prorocentrin ($\mu g\ NH_2OH \cdot L^{-1}$)
P. minimum #96 (Pavillard) Schiller	3.2	37.5
P. mariae lebouriae * (Parke et Ballantine) Loeblich	3.0	41.0
P. gracile #104 (a) Schuett	3.0	44.9
P. maximum #250 (Gourret) Schiller	1.7	5.7
P. micans #33 Ehrenb.	1.9	3.4

* Kindly supplied by Prof. H.H. Seliger, The Johns Hopkins University, Baltimore, Maryland.

Figure 20 - Procedure for the isolation of the aqueous fraction containing prorocentrin.



prorocentrin was collected by adjusting the cell-free filtrate to pH 2.0 and passing it over a 25 X 2 cm column of precleaned (Soxhlet extraction with methanol,72 h) XAD-2 resin (Mallinckrodt). The column was subsequently washed with 150 ml of deionized and distilled H_2O (pH 2.0) and the adsorbed organics were eluted with 300 ml MeOH. The MeOH was reduced to dryness by rotary evaporation and the residue was partitioned between water and $CHCl_3$. The aqueous fraction was evaporated *in vacuo* to a final volume of 5 ml.

To quantify the amount of prorocentrin, a modified Csaky test which determines the concentration of hydroxamate functionalities colorimetrically (Gillam *et al.*, 1981) was used. In the initial studies where all fractions were examined, only fractions containing prorocentrin gave positive Csaky results. The presence of prorocentrin was verified by thin layer chromatography of each fraction (Chapter V). For each sample, a 2 ml aliquot was used as a test and a second, 2 ml sample was used as a color and reagent blank.

D. RESULTS

Three of the five *Prorocentrum* species showed high amounts of extracellular Csaky positive compound when grown in iron-deficient medium. *Prorocentrum gracile* produced the largest amount of Csaky positive material but was intolerant of repetitive transfers and was not studied further (Table 10). All subsequent experiments were performed with a local isolate of *P. minimum*. This isolate is the original source of

prorocentrin (Chapter V), although thin layer chromatography indicated that prorocentrin was present in the other two species that gave strong positive Csaky tests (P. mariae-lebouriae and P. gracile).

In order to provide accurate absolute values of the amount of prorocentrin produced, the efficiency of isolation of this material from culture filtrates was quantified. Figure 20 shows a schematic of the isolation procedure. The critical step is the desalting of the column with a distilled water wash. If the column was washed with a minimum amount of distilled, deionized water (150 ml) recovery based on successive transfers was 60%. Using this wash volume a small amount of salt remained, but it had no influence on the Csaky test. Desalting the column with larger volumes of water (1 L and 2 L) eliminated residual salt but also stripped the resin of prorocentrin (45% and 23% recovery, respectively). Columns washed with less than 50 mL of distilled water retained large quantities of salt which interferred with the evaporation step. As a result of the above observations, all columns were washed with 150 ml H_2O and values have been corrected for a 40% loss.

The production of prorocentrin under iron-sufficient and iron-deficient conditions is presented in Fig. 21. There was little difference in prorocentrin production between the ESNW-Fe and AQUIL-Fe media, although growth rates were significantly different (75% and 40% of the control, respectively).

The pattern of production of prorocentrin is of interest. Under iron-sufficient conditions a small amount of prorocentrin

was produced both extracellularly and intracellularly at the onset of nutrient deficiency. A similar small pulse in intracellular prorocentrin production occurred under iron-deficiency (ESNW-Fe). Iron-deficient cells produced most of the extracellular prorocentrin within three days of the cessation of growth. There was no corresponding extracellular production of prorocentrin in medium fully supplemented with iron. Prorocentrin existed extracellularly for only a short period of time before it rapidly disappeared from the medium. The loss of extracellular prorocentrin was not followed by an increase in intracellular prorocentrin. Within two to three days after maximum extracellular prorocentrin concentrations were observed, neither the cells nor the medium contained prorocentrin.

The effect of iron-deficiency can be clearly seen by examining the growth rates of the cultures. Iron-sufficient cells grew at maximum growth rates whereas cells grown in the medium without added iron had significantly reduced growth rates and slightly reduced cell yields. Since the final cell yields of ESNW-Fe and AQUIL-Fe approached maximum yields, it appeared that there was sufficient residual iron in the two cultures unsupplemented with iron to provide growth, but the form of the iron could only be slowly utilized by the cells.

To test this hypothesis, varying amounts of EDTA were added to the iron-deficient medium (ESNW-Fe) and maximum prorocentrin concentration and cell yields were measured. If iron was in the medium, but not in an available state, then the addition of an

Figure 21 - Comparison of the growth rates and prorocentrin production in batch cultures of P. minimum.

Prorocentrin values have been corrected for loss in the recovery procedure. Medium used was: a) ESNW+Fe; b) ESNW-Fe; c) AQUIL+Fe; and d) AQUIL-Fe.

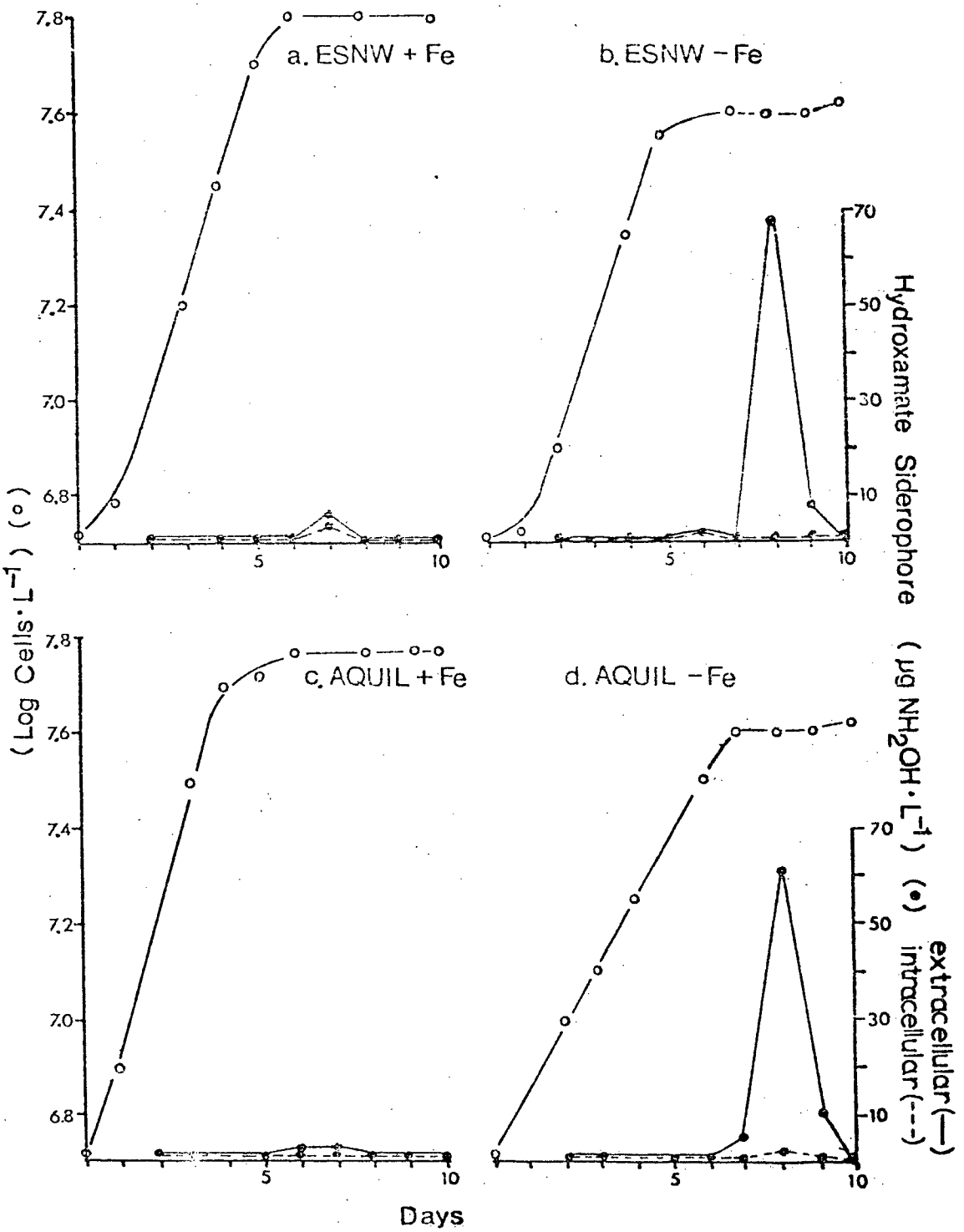


Figure 22 - Influence of the addition of various concentrations of EDTA on prorocentrin production. A) Final cell yield. B) Maximum intracellular or extracellular prorocentrin concentration produced by P. minimum. Replicate values are mean (± one SE), for n = 3.

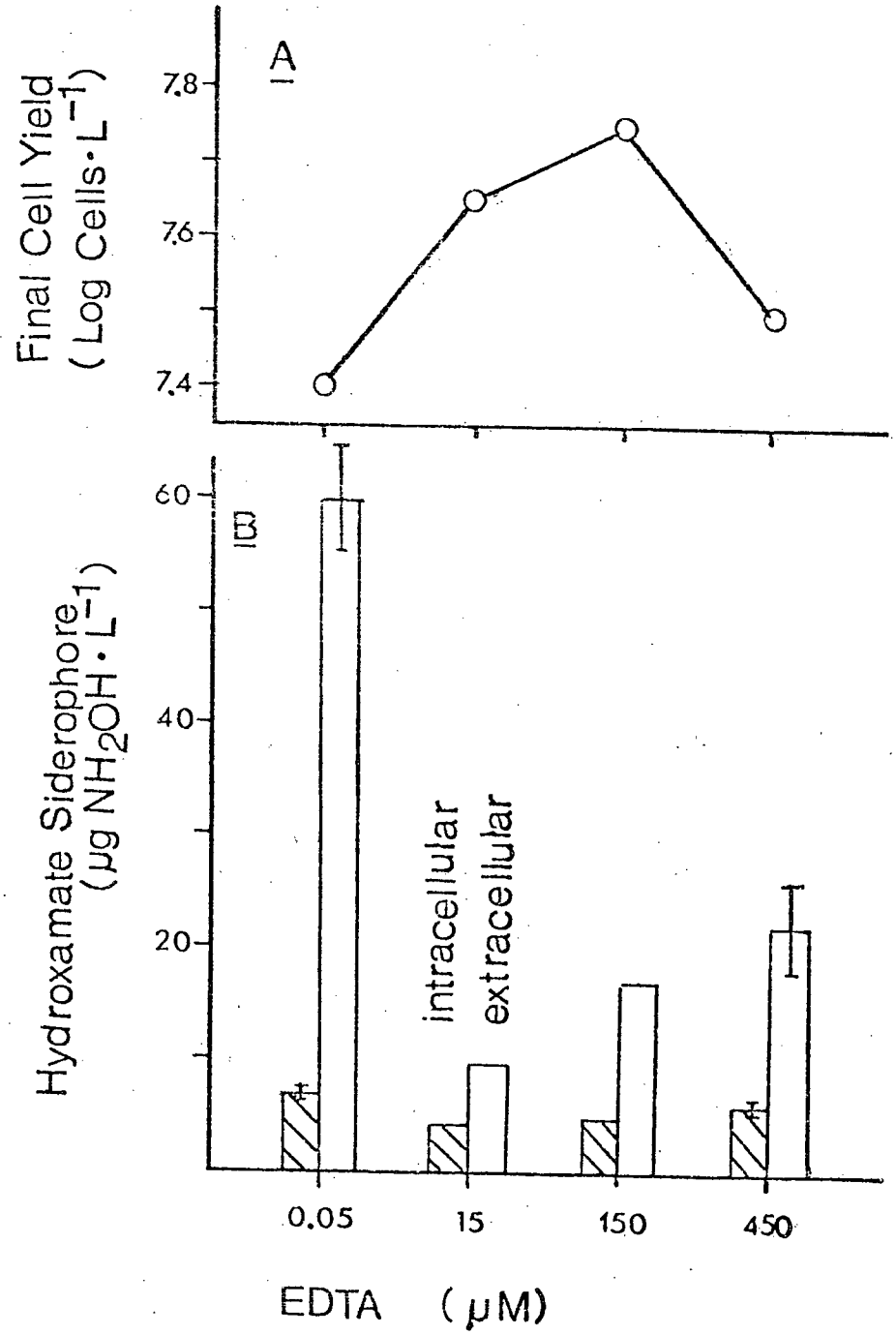
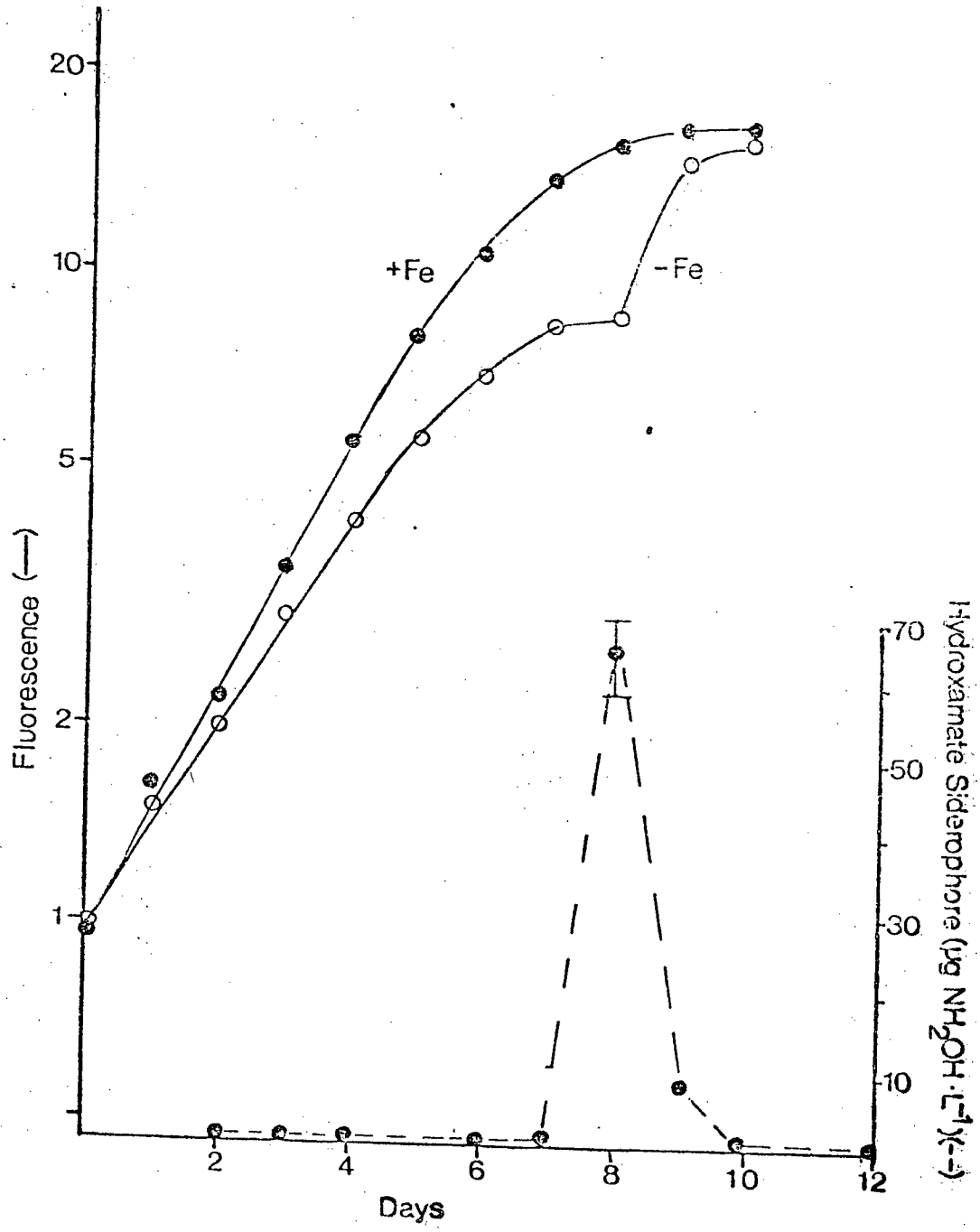


Figure 23 - Influence of iron on the growth of P. minimum. Comparison of growth, measured by in vivo fluorescence (log scale) for iron-sufficient and iron-deficient batch cultures of P. minimum. Temporal changes in extracellular prorocentrin concentration for the iron-deficient culture are also included.



artificial chelator such as EDTA should alleviate the iron stress. Increasing the available iron should inhibit siderophore production and re-establish a maximum growth rate and final cell yield. The addition of a low amount of EDTA (0.05 μM) had no effect on these parameters (Fig. 22). As the EDTA concentration was increased all parameters attained values similar to those of the iron-sufficient cultures. Using prorocentrin production as an indicator of iron stress the addition of 15 μM EDTA was sufficient to remove iron-limitation, even though iron was not added to the medium. Further increases in EDTA concentration (450 μM) reduced cell yields and growth rates to values similar to the iron-deficient values. This was accompanied by an increased production of prorocentrin suggesting that iron availability was reduced due to excessive chelation.

The influence of iron deficiency on growth was measured by fluorescence and is presented in Figure 23. Fluoresence values provide a significantly different picture than growth monitored by cell numbers (Fig. 21). Cell counts provided the traditional growth curve (exponential growth, followed by a plateau due to nutrient limitation) for both iron-sufficient and iron-deficient cultures. The same pattern was seen for growth in iron-sufficient medium monitored by <u>in vivo</u> fluorescence. However, growth in medium without added iron provided a bimodal fluorescence pattern. There was an initial increase in fluorescence as cell numbers increased. As cells became iron-limited the fluorescence values plateaued.

Fluorescence increased significantly again, coincidental with the disappearance of extracellular prorocentrin. Cell numbers showed no corresponding change.

E. DISCUSSION

Successful examination of siderophore production by marine eukaryotic phytoplankton is dependent upon two processes: the successful creation of iron-limited growth medium and the concentration of the siderophore prior to analysis. Both of the procedures used to reduce or eliminate available iron from seawater were successful, albeit to different degrees. The procedure of Lewin and Chen (1971) reduced the amount of available iron to concentrations limiting to growth rate and final cell yield. The final cell yield was , however, high enough for adequate accumulation of extracellular products. Repetition of the treatment failed to remove enough additional iron to significantly reduce either cell yield or growth rate or to further stimulate prorocentrin production.

The preparation of iron-sufficient controls was equally important. To achieve maximum growth rates and yields and a minimum production of prorocentrin, it was essential to use FeCl which had been freshly prepared. Iron stocks which were stored in polyethylene bottles for as short as 2 to 3 days formed enough unavailable iron upon autoclaving to stimulate prorocentrin production; however, final prorocentrin concentrations were far less than the values obtained using ESNW-Fe or AQUIL-Fe. No noticeable change in growth rate or

final cell yield was recorded. The reduction in available iron in an aged iron stock solution has been shown previously (Lewin and Chen 1973).

A concentration procedure was also necessary to detect and quantify the production of prorocentrin. As discussed previously (Chapter V), past researchers have concluded that marine eukaryotic phytoplankton are unable to produce siderophores. An alternative hypothesis is that production is at a level which requires concentration for detection using present assays.

XAD-2 resin has been used previously in marine systems to isolate humic substances (Stuermer and Harvey, 1974; Mantoura and Riley, 1975; Lyons et al., 1979) and novel extracellular metabolites (Andersen et al., 1980; Trick et al., 1981). Sugimura et al. (1978) used XAD-2 to isolate iron-organic complexes from seawater. This resin has also been used in the isolation of siderophores from other organisms (Horowitz et al., 1976). While XAD-2 resin has been used in many related applications, the recovery of prorocentrin was less than ideal. The highly water soluble prorocentrin binds ineffectively to the resin and could easily be lost through excessive desalting of the XAD-2 column. Using the defined conditions of sample preparation (150 mL H_2O wash) recovery was consistent and therefore the total prorocentrin concentration could be estimated with confidence.

The stimulation of prorocentrin production under low iron growth conditions satisfies the traditional test for a high

affinity iron transport system (Garibaldi and Neilands, 1956). Growth of P. minimum in either of the two low iron media stimulated extracellular prorocentrin production and the effect could be reversed by the addition of freshly prepared FeCl . While this result points to prorocentrin being a siderophore, the pattern of production is equally important. All of the prorocentrin was produced in the early stationary phase of batch culture. Stimulation of extracellular organic production during stationary phase is common for both primary organics (Hellebust, 1974) and unique extracellular metabolites. (Trick et al., 1981). A similar period of production has been reported for the copper complexing agents from marine and freshwater phytoplankton (McKnight and Morel, 1979). Production of prorocentrin in the stationary phase differs from the pattern of siderophore production by the marine cyanobacterium, Agmenellum quadruplicatum , grown in batch culture (Armstrong and Van Baalen, 1979). Extracellular siderophore from the blue-green alga accumulated during exponential growth and was removed from the medium as cells approached stationary phase. Whether this difference in production is a characteristic divergence between the eukaryotic and prokaryotic phytoplankton remains to be verified.

Our analysis of prorocentrin production in batch culture provides further insight into the unsuccessful attempts of other researchers to verify the production of extracellular siderophore by eukaryotic phytoplankton. Under iron limitation, the period of high extracellular prorocentrin concentration is

very short and could easily be missed in a rigid, single point sampling schedule. This is also true for intracellular prorocentrin, although the differences are less dramatic due to a lower absolute concentration for cellular prorocentrin.

The low internal prorocentrin concentration compared to the large amount of extracellular siderophore produced, is evidence for the de novo synthesis of prorocentrin immediately prior to release. This rapid formation of prorocentrin after the onset of iron stress in batch culture is similar to the de novo synthesis of the β-diketone, 1-(2,6,6-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione, a unique extracellular metabolite excreted by P. minimum (Trick et al., 1981). In the case of the β-diketone, production occurred in a narrow time period in the stationary growth phase. Maximum β-diketone concentrations were rapidly achieved but they were maintained at maximal levels. There was no indication of reabsorption or breakdown. In the case of prorocentrin , maximum extracellular concentration was exhibited for only a short period of time (less than one day). The reduction in extracellular concentration was as rapid as the initial production, with almost no Csaky positive material remaining in the medium or in the cells two days after maximum extracellular concentration.

The loss of extracellular prorocentrin without a simultaneous increase in cellular concentration suggests a possible mechanism of iron acquisition by P. minimum. It is hypothesized that the high, affinity iron transport system in P. minimum functions as follows. The extracellular prorocentrin

forms the iron-prorocentrin complex which is then taken up by the cells. The iron is removed and a modified prorocentrin by-product, not reactive to the Csaky test, is produced. The by-product is either maintained intracellularly or released back into the medium. The free iron is utilized in the biosynthesis of chlorophyll resulting in the corresponding increase in fluorescence. A similar mechanism of iron acquisition (termed the "American approach") has been shown in organisms producing the catechol-type siderophores (Bezkorovainy, 1980). The mechanism is unique to eukaryotic microorganisms. Further studies of the mechanism of iron aquisition are necessary. It is clear, however, that the mechanisms for hydroxamate-type iron transport for fungi and molds (termed the "European approach") which involves the release of the intact, Csaky positive siderophore back into the medium (Raymond and Carrano, 1979; Bezkoravainy,1980) could not account for the rapid disappearance of prorocentrin.

The stimulation of cell fluorescence after the disappearance of extracellular prorocentrin is an interesting feature. There have been many reportssuggesting that cholorophyll a synthesis is governed by the supply of iron (Myers 1947; Hayward, 1968; Glover, 1978). Davies (1970) suggested that it is the presence of chelated iron which stimulated chlorophyll production. The strong correlation between production of Csaky positive compounds and stimulated fluorescence may be of use as a screening procedure for siderophore producing phytoplankton. This procedure could

perhaps detect siderophore production by cells which produced siderophore during the stationary phase of growth, but it would not be suitable for detecting siderophore production during exponential growth. Further work is required to confirm the relationship between siderophore production and cellular fluorescence.

Our data characterize the production of prorocentrin by the marine dinoflagellate, Prorocentrum minimum. Two other species P. mariae-lebouriae and P. gracile, although less thoroughly studied , showed similar patterns of production. Our inability to stimulate prorocentrin production in the two other Prorocentrum species may be due to our inability to create suitable conditions for iron-limited growth.

The verification of an active, high affinity, iron transport system in marine dinoflagellates is an important step in our understanding of trace metal control of phytoplankton growth. If these compounds are species specific, then the ecological advantage of prorocentrin production under iron-stress is immediately evident. However, the role of a siderophore as a factor controlling interspecies competition is more complicated. Certain algal excretion products, of which prorocentrin is one example, form complexes with other metal ions at low concentrations. This will serve either to detoxify or increase the availability the metals (Degens, 1970; Barber, 1973; Smith, 1976). This most certainly is the case for hydroxamate-type siderophores. Compounds like prorocentrin, while highly selective for iron, also form stable complexes with

Cu(II) (Anderegg et al., 1963). The ability to detoxify copper would most certainly be important in areas where exchangeable iron is very low.

VIII. EXTRACELLULAR HYDROXAMATE-SIDEROPHORE PRODUCTION BY NERITIC EUKARYOTIC MARINE PHYTOPLANKTON.

A. ABSTRACT

The production of hydroxamate-type siderophores by eukaryotic marine neritic phytoplankton is presented. Four species were investigated for siderophore production under iron-sufficient and iron-limiting culture conditions. Thalassiosira pseudonana and Dunaliella tertiolecta produced extracellular siderophores under iron-limiting culture conditions. There was no siderophore production in two species, Olisthodiscus luteus and Skeletonema costatum. An increase in intracellular siderophore concentration was not observed. In species which produced siderophores, there was an increase in the in vivo fluorescence associated with the disappearance of the extracellular siderophore from the culture medium. While the extent of siderophore production varied between these two species, the pattern of production and the associated rapid increase in in vivo fluorescence indicate that the iron-acquisition system is similar to the system in Prorocentrum minimum.

B. INTRODUCTION

Iron is an essential micronutrient for all phytoplankton. In marine waters, iron may limit the growth of phytoplankton, since most of the iron is in a biologically unavailable form, either associated in large organic complexes (Sugimura et al., 1978) or as almost totally insoluble ferric hydroxide aggregrates. Under iron limiting growth conditions most microorganisms utilize a high affinity iron acquisition system. The mechanism of iron acquisition involves the production of iron(III)-specific extracellular organics (siderophores) to scavenge iron at low concentrations. The iron-siderophore complex is actively taken up by the cell. The iron is released and utilized in cellular metabolism (Neilands, 1974). It has been suggested that all aerobic microorganisms can produce siderophores (Neilands, 1974), yet controversy surrounds the existence of siderophore production by marine eukaryotic phytoplankton.

Marine bacteria (Goyne and Carpenter,1974) and marine cyanobacteria (Estep et al., 1975; Armstrong and Van Baalen, 1979) can produce siderophores. Murphy et al.,(1976) suggested that hydroxamate siderophores produced by fresh water cyanobacteria enabled them to out compete associated eukaryotic phytoplankton, which were unable to produce a siderophore. However, there exists some evidence for the production of siderophores by eukaryotic phytoplankton. Spencer et al. (1973) isolated a hydroxamate-like compound from a non-axenic culture of the marine diatom, Chaetoceros socialis. Critics

have suggested that the siderophore may have originated from the bacteria, rather than the diatom. Similarly, Armstrong and Van Baalen (1979) found hydroxamate-type compounds produced by the marine diatom, Cylindrotheca sp. This material was not characterized further.

Other researchers have claimed that axenic eukaryotic phytoplankton, unlike prokaryotic phytoplankton, are unable to produce siderophores (Swallow et al., 1978; McKnight and Morel, 1979). Since, the production of low molecular weight iron (III) chelators was considered unlikely by eukaryotic phytoplankton, Anderson and Morel (1980) suggested the acquisition of iron (II) as a alternative mechanism.

A siderophore from the marine dinoflagellate, Prorocentrum minimum has recently been isolated (Chapter V). This compound, given the trivial name prorocentrin, had chemical and spectroscopic characteristics typical of low molecular weight hydroxamate-containing siderophores and it was found both intracellularly and extracellularly. Iron-limited growth conditions stimulated the production of extracellular prorocentrin.

It was argued that the inability of past researchers to detect hydroxamate siderophore in cultures of eukaryotic marine phytoplankton was due to: 1) The inability to achieve iron-limited growth conditions and 2) the failure to concentrate siderophores prior to analysis for hydroxamate.

In this chapter, siderophore production in a few species of eukaryotic phytoplankton from three major algal classes is

examined. It has been suggested that neritic phytoplankton have a larger requirement for iron than oceanic species (Menzel and Ryther, 1961; Ryther and Kramer, 1961; Johnston, 1964; Lewin and Chen, 1971). In order to facilitate achieving iron-limited growth conditions only neritic species were chosen.

C. MATERIALS AND METHODS

All isolates were obtained from the North East Pacific Culture (NEPCC), University of British Columbia. Species examined included Skeletonema costatum (NEPCC #18(a)), Thalassiosira pseudonana (clone 3H) (NEPCC #58), Olisthodiscus luteus (NEPCC #278) and Dunaliella tertiolecta (NEPCC #1). cultures, with the exception of O. luteus, were axenic (Chapter III). Cells were grown in iron-deficient enriched, natural seawater medium (ESNW-Fe) described in Chapter V. The seawater was treated with charcoal (24h, constant stirring) to remove dissolved organics. To reduce the biologically available iron, the charcoal treated seawater was repeatedly heated and filtered by the procedure of Lewin and Chen (1971) prior to enrichment. Cells were cultured in 6 L or 10 L flasks with constant stirring (60 rpm) and were incubated at 18 °C under constant daylight fluorescent lamps providing 120 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cell growth was monitored by in vivo fluorescence using a Turner Designs Model 10 fluorometer.

Cells were harvested by filtering 1 L of culture through glass fiber filters (GF/C). The remaining cells were removed by passing the filtrate through 0.45 μm membrane filters. Filtration pressure was kept below 250 mm Hg vacuum.

Dissolved organics were collected from the cell-free filtrate by the procedure outlined in Chapter 7 and briefly outlined as follows. The filtrate was acidified to pH 2.0 with concentrated HCl and passed over a 25 X 2 cm column of XAD-2 resin. The column was desalted with 150 ml of deionized, distilled water and the absorbed organics were removed from the column with 300 ml of MeOH. The MeOH fraction was reduced to dryness by rotary evaporation. Organics were partitioned between $CHCl_3$ and H_2O.

To test for siderophores, a modified Csaky test (Gillam, et al., 1981) was employed on the water soluble organic fraction. Thorough testing has shown that the Csaky test is diagnostic for hydroxamate-type siderophores, as opposed to catechol-type siderophores.

D. RESULTS

Growth results are presented in Figs. 24-27. For two of the four species, there was little difference between the fluorescence of the cells growing in iron-sufficient medium and iron-deficient medium. Skeletonema costatum (Fig. 24) and O. luteus (Fig. 25) showed exponential increases in fluorescence and a plateau of fluorescence after iron limitation at day 6 and day 5, respectively.

The two other species, T. pseudonana (Fig. 26) and D. tertiolecta (Fig. 27), showed fluorescence patterns of iron-deficient cultures which differed significantly from the increase in fluorescence in iron-sufficient medium. When iron

Figure 24 - Influence of iron on the growth of S. costatum. Comparison of growth and the production of extracellular hydroxamate siderophores of S. costatum grown in batch culture. Cells were grown in: a) iron-sufficient, or b) iron-deficient media.

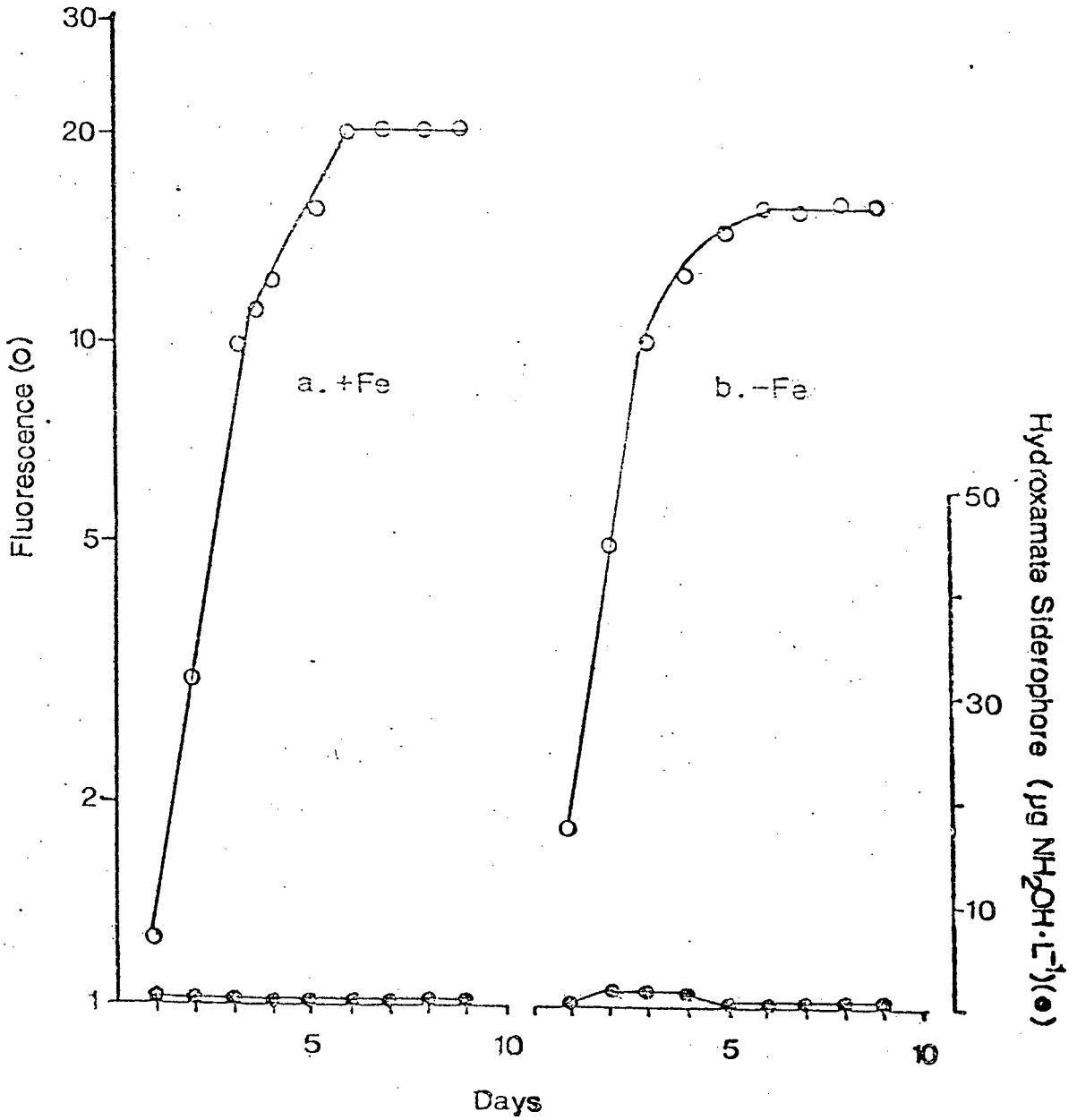


Figure 25 - Influence of iron on the growth of O. luteus. 'Comparison of growth and the production of extracellular hydroxamate siderophores of O. luteus grown in batch culture. Cells were grown in: a) iron-sufficient, or b) iron-deficient media.

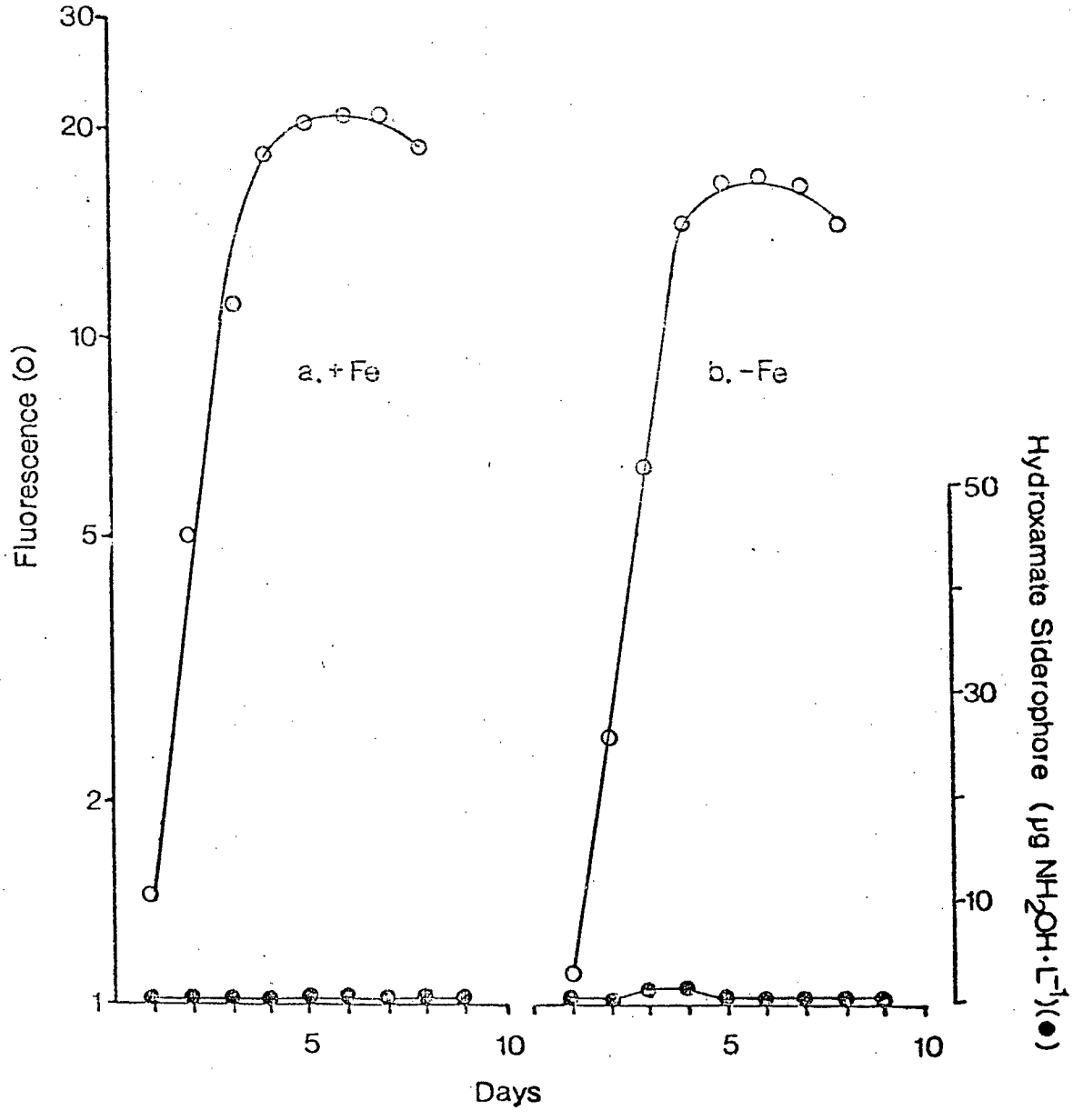


Figure 26 - Influence of iron on the growth of T. pseudonana.

Comparison of growth and the production of extracellular hydroxamate siderophores of T. pseudonana grown in batch culture. Cells were grown in: a) iron-sufficient, or b) iron-deficient media.

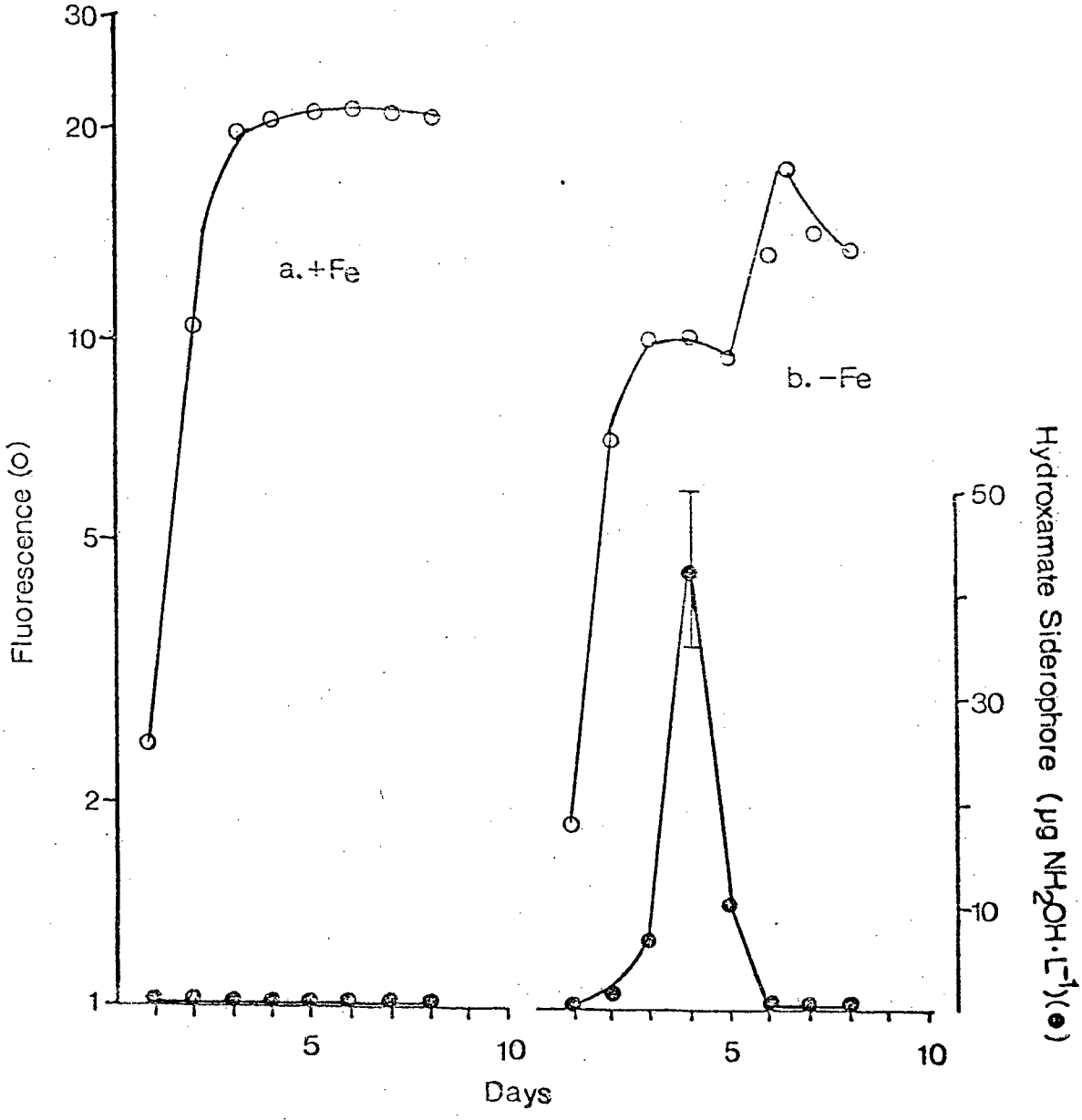
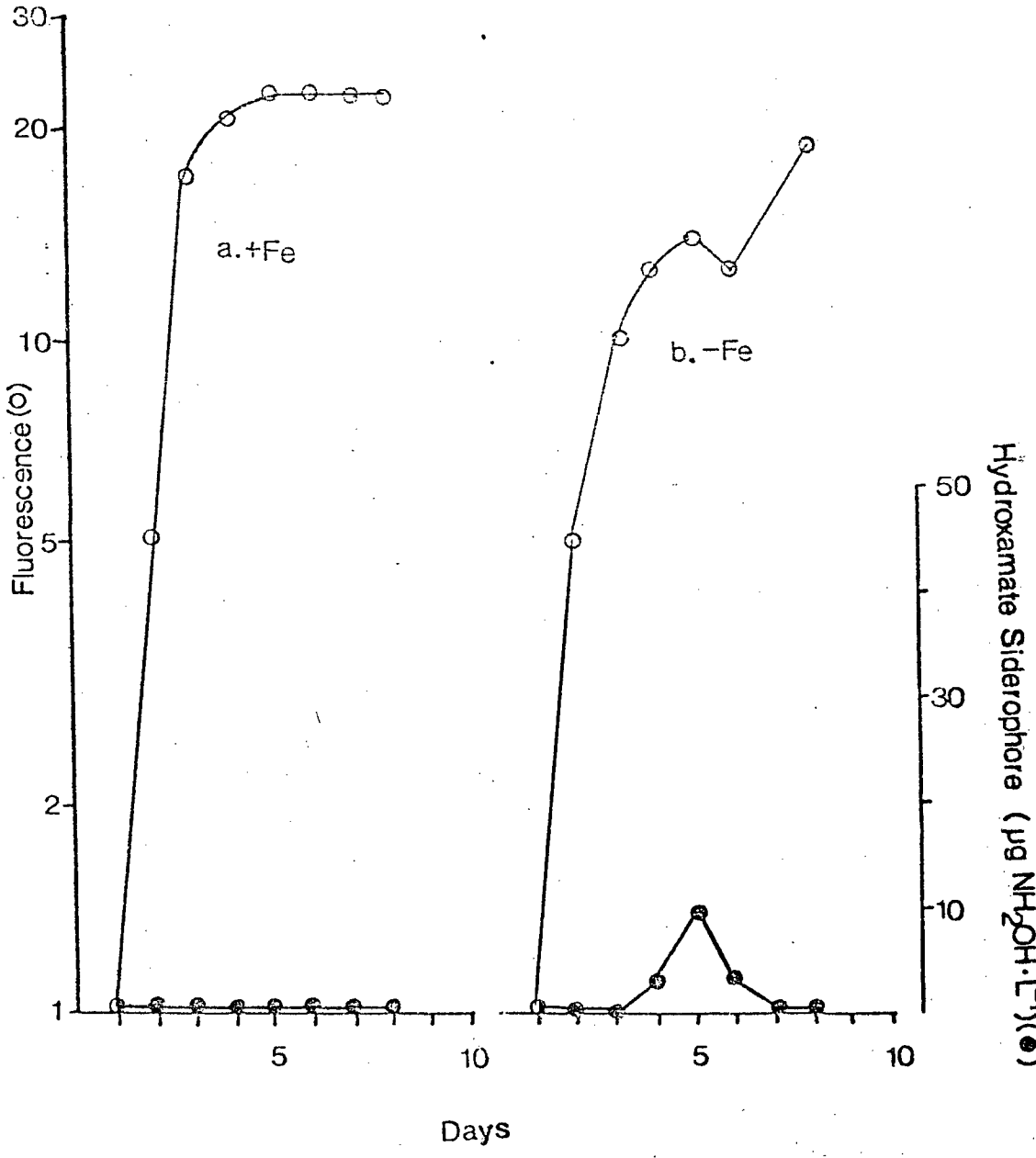


Figure 27 - Influence of iron on the growth of D. tertiolecta.

Comparison of growth and the production of extracellular hydroxamate siderophores of D. tertiolecta grown in batch culture. Cells were grown in: a) iron-sufficient, or b) iron-deficient media.



was sufficient both species exhibited the traditional pattern of an exponential increase in fluorescence with time, followed bya plateau when iron limitation occurred. Fluorescence for iron-limited growth was similar but exhibited a second increase in fluorescence about three days later, probably due to the onset of iron limitation. In the case of T. pseudonana, the second increase in fluorescence was short-lived and fluorescence decreased within the next two days.

A pattern of fluorescence similar to T. pseudonana and D. tertiolecta was seen in iron-limited cultures of P. minimum (Chapter 7). It was suggested that the rapid increase in fluorescence during the stationary growth period may be used as a diagnostic feature to estimate the temporal pattern of siderophore production in batch culture, providing iron-limited growth was achieved.

To investigate the link between extracellular hydroxamate-type siderophore production and the resulting increase in fluorescence, we analyzed for the production of extracellular siderophores by growing the species under iron-deficient conditions. The patterns of extracellular hydroxamate-type siderophore for the two species which did not show the stimulation of fluorescence are presented in Figs. 24 and 25. Neither S. costatum nor O. luteus produced significant amounts of Csaky positive siderophore. Thalassiosira (Fig. 26), however, produced a large amount of extracellular, Csaky positive material immediately before the increase in fluorescence. D. tertiolecta (Fig. 27) produced less

extracellular Csaky-positive material, than T. pseudonana, but the relationship to the shift in in vivo fluorescence was the same. There was no intracellular Csaky positive material produced by any of the species. None of the cultures produced significant amounts of Csaky positive siderophore when iron was sufficient.

E. DISCUSSION

In Chapter VII it was suggested that the pattern of growth measured by in vivo fluorescence, could be used as a diagnostic feature to predict hydroxamate-type siderophore production. Critical examination of T. pseudonana, S. costatum, O. luteus, and D. tertiolecta, combined with previous analysis of P. minimum (Chapter VII), strengthens the reliability of the predictive value of this method. Since fluorescence values require no concentration or isolation steps, this feature should aid in large surveys of siderophore production by eukaryotic phytoplankton.

The inability of S. costatum and O. luteus to produce Csaky positive siderophore may be due to our inability to decrease the available iron to low enough concentrations significantly low to stimulate siderophore production. Since all of the species were grown in identical medium, this may suggest that species which did not produce siderophores have a low requirement for iron, rather than the species' inability to produce siderophore. Further examination of these two species is required to differentiate between these two processes.

The production of a hydroxamate-type siderophore by D. tertiolecta and T. pseudonana is of great interest. McKnight and Morel (1978) were unable to induce the same Thalassiosira clone to produce significant quantities of high affinity chelators. They noted that micromolar amounts of a weak chelating ligand was produced. Our results confirm the necessity to concentrate the organics prior to analysis and to induce production by careful control of available iron.

The amount of Csaky positive compound produced by T. pseudonana is in the same range as the maximum values recorded for Prorocentrum species (Chapter VII). As discussed in the previous chapter, the absolute amount of material will be a function of the number of hydroxamate units per siderophore, the efficiency of oxidation during the Csaky test and the efficiency of recovery from the medium. Thus, little significance can be attached to the absolute amount recorded for T. pseudonana. The nature of the uncertanties in our estimate is such that siderophore concentrations would be underestimates.

The pattern of production of Csaky positive compounds by T. pseudonana and D. tertiolecta are identical in nature to the pattern of prorocentrin (the hydroxamate-type siderophore) production by P. minimum. The mechanism for iron acquisition is similar to that discussed for P. minimum (Chapter VII). This mechanism involves de novo synthesis of extracellular siderophore after the onset of iron limitation. The residence time of this extracellular material is short because the Csaky reactive material is rapidly removed from the medium.

Several conclusions are clear. The production of the extracellular hydroxamate-type compounds by T. pseudonana and D. tertiolecta verifies that siderophore production is not unique to dinoflagellates (or to Prorocentrum species). Phytoplankton from three major algal classes, Bacillariophyceae, Chlorophyceae, and Dinophyceae, can be induced to produce similar, but not necessarily chemically identical, iron (III) chelators.

Newly acquired iron may stimulate in vivo fluorescence by removing the iron-inhibition of chlorophyll biosynthesis. Iron deficiency inhibits the activity of α-amino-levulinic acid synthetase, the first enzyme of the heme biosynthetic pathway (Light and Clegg, 1974). Iron from the transported iron-siderophore complex could remove the inhibition, initiating de novo chlorophyll biosynthesis. Since iron acts at the enzyme level of biosynthesis, only a small increase in available iron would be necessary to significantly increase in vivo fluorescence.

The catalytic stimulation of fluorescence appears to be a reliable indicator of temporal patterns of siderophore production under the iron- deficient batch culture conditions. Initial screening by fluorescence reduces the time-consuming isolation of the siderophore containing water fraction and enables the time of production to be predicted under defined growth conditions. Fluorescence patterns corresponded to siderophore production in P. minimum , T. pseudonana and D. tertiolecta. The use of other growth parameters, such as

cell concentrations, does not provide the sensitivity of the fluorescence technique.

Two species failed to produce siderophore in our experiments. The lack of significant amounts of extracellular Csaky positive compounds was verified for both O. luteus and S. costatum. Further research is necessary to verify whether these two species lack the ability to produce siderophore or whether the medium contained unsuitably high amounts of available iron. Since all species were grown in the same batch of treated seawater, the lack of siderophore production may only indicate differences in cellular iron quotas for the two species. Further information on the level of iron required to control the shift from the low affinity to the high affinity iron acquisition system in eukaryotic phytoplankton is required before the full significance of this work can be recognized.

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APPENDIX A - ANTIBACTERIAL PROPERTIES OF THE β-DIKETONE USING MARINE BIOASSAY SPECIES

A. Introduction

To establish the β-diketone as a biologically important metabolite, the antibacterial nature must be examined. Preliminary observations indicated that the β-diketone was inhibitory to Staphylococcus aureus (Andersen et al., 1980). Since one of the criticisms against previous research (see Chapter 1) was the use of bioassay species from habitats that were widely different than the metabolite-producing species, the antibacterial nature of the β-diketone against marine bacteria was investigated.

b. Materials and Methods

Four marine species were chosen as bioassay organisms. Two marine species, Pseudomonas acidivaris and Vibrio sp. were obtained from the American Type Culture Collection (ATCC). The other two species, Flavobacterium sp. and a Chromobacterium sp., were isolated from Scrippsiella sweeneya cultures. Initial taxonomic identification is tentative, based entirely on colony shape and color.

Bacterial cultures were maintained at 18°C in liquid broth cultures (ISOL medium; Bell and Mitchell, 1972). Growth was monitored by recording changes in absorbance of the culture at 750 nm.

Initial experiments were similar to those of Chan et al. (1980). ISOL medium with 1.5% agar was made into plates and inoculated with a lawn of the bioassay species. A single 1 cm glass fiber filter disc, which had been inoculated with 100 μg of the β-diketone and autoclaved, was placed in the center of the plate. Plates were incubated at 18 C and examined after 24 and 48 hours. After 48 hours the size of the zone of inhibition, which is seen as a clear zone around the disc, was recorded.

To examine the influence of known concentrations of the β-diketone on growth of Chromobacterium, liquid ISOL medium cultures were employed.

The heterotrophic activity of natural, bacterial populations was determined as follows. A subsurface sample was collected from two stations from the Fraser River plume in the Strait of Georgia, off Vancouver, B.C. (March 18, 1982). The sample was separated into 50 ml aliquots and different amounts of β-diketone were added. Two ml of ^{14}C-glucose (sp. act.=

4.43) was added and all samples were placed in running surface water (7.5 C). After one hour of incubation in the dark, samples were filtered through 0.22 μm Nucleopore filters. The filters were washed with 10 ml filtered (0.22 μm) seawater, and placed in scintillation fluor. Counts were taken after 40 hours using a Unilux III scintillation counter. Since bacterial counts were not taken, results are given as the percent inhibition compared to the control (no added β-diketone).

C. Results

All bacteria formed smooth, uniform lawns within 48 hours of inoculation. The influence of discs impregnated with 100 μg β-diketone on bacterial growth is presented in Table 12. One marine isolate (Pseudomonas acidivaris) was insensitive to β-diketone. Chromobacterium sp. was the most sensitive based on the size of the zone of inhibition.

Since the disc bioassay is not a reliable predictor of the effective concentration of inhibition, Chromatobactium sp. was grown in liquid culture with β-diketone concentrations ranging from 0 to 100 μg L^{1} . At β-diketone concentrations greater than 25 μg L^{1}, cell yield was inhibited but the growth rate was unaffected (Fig. 28). Complete supression of growth was not recorded in this range of β-diketone concentrations. Growth was monitored for 14 days to ensure that the bacteria could not adapt to the β-diketone concentration.

The influence of increasing β-diketone concentrations on the heterotrophic uptake of glucose is presented in Fig. 29. For a 50% inhibition of the short-term heterotrophic uptake, 100 μg L^{1} β-diketone was required. However, concentrations as low as 20 μg L^{1} inhibited the heterotrophic uptake (10% reduction) and at β-diketone concentrations (50 μg L^{1}) typically produced by laboratory cultures of P. minimum, heterotrophic uptake was inhibited by about 15%. The β-diketone concentrations from laboratory cultures would overestimate the actual concentration of extracellular β-diketone produced because of the large discrepancies in cell concentrations.

These preliminary experiments have established the β-diketone as an antibiotic metabolite active against marine bacteria. The inhibition of growth of three of four marine isolates and the inhibition of the heterotrophic uptake of glucose is sufficient evidence to verify the antibacterial properties of the β-diketone.

Table XII - Description and origin of bacterial species examined.

Qualitative results of the disc bioassay in terms of the size of the zone of inhibition (0-no inhibition; +-<0.5 cm; ++->0.5 cm). ATCC-American Type Culture Collection.

Species	Origin	Size of zone of Inhibition
Pseudomonas acidivaris	ATCC # 9355	0
Vibrio sp.	ATCC # 14400	+
Flavobacter sp.	*Scrippsiella sweeneya* culture	++
Chromobacterium sp.	*Scrippsiella sweeneya* culture	++

Figure 28 - Growth characteristics of Chromobacterium sp. Growth was measured by changes in absorbance at 750 nm, in ISOL medium with varying concentrations of the β-diketone. (Control=● ; 10 $\mu g \cdot L^{-1}$=o; 25 $\mu g \cdot L^{-1}$ =x; 40 $\mu g \cdot L^{-1}$=▲; 75 $\mu g \cdot L^{-1}$ =△).

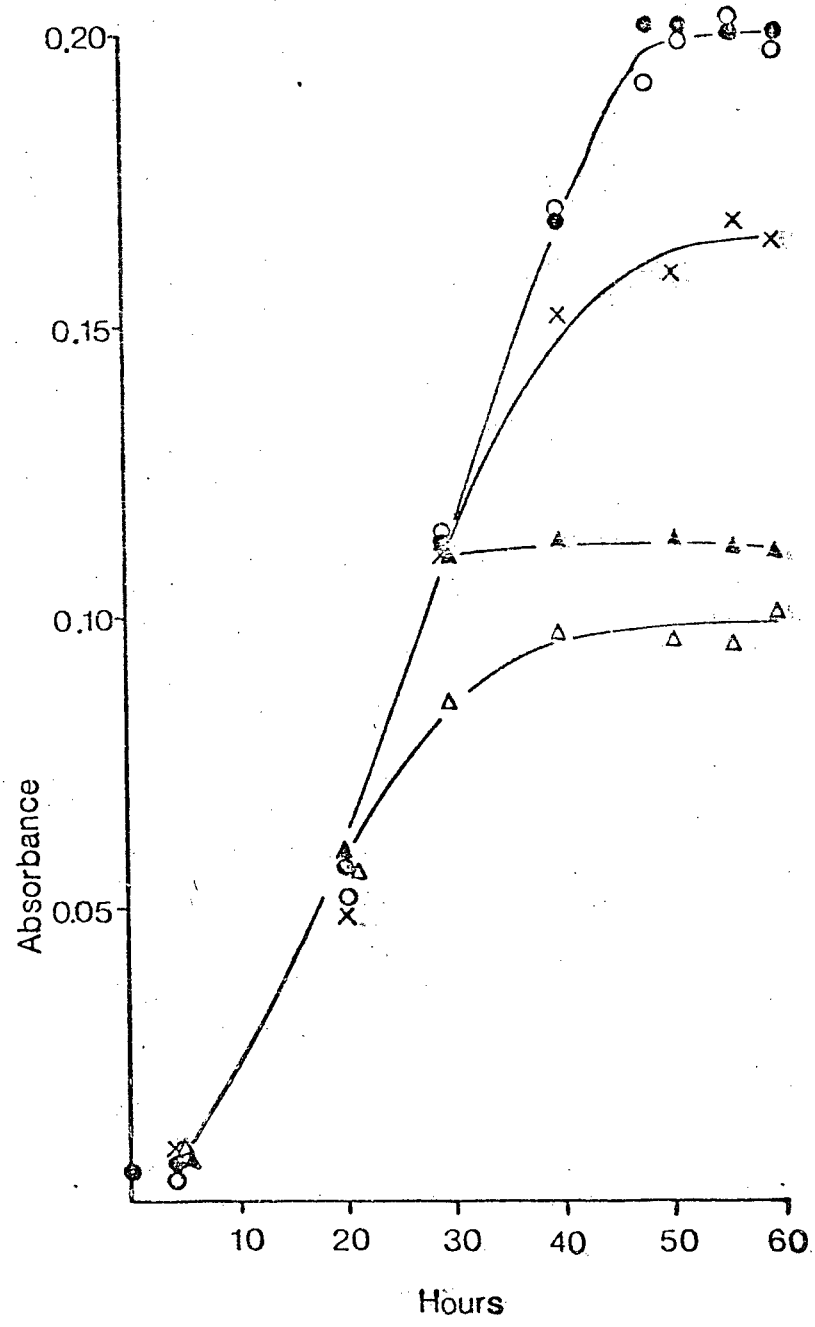

Figure 29 - The effect of the β-diketone on the heterotrophic uptake of ^{14}C-glucose by a natural population. Bacteria were collected from the Fraser River plume, Strait of Georgia, Vancouver, Canada. Arrow indicates the relative amount of the β-diketone produced by laboratory cultures.