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STUDIES ON THE RESPIRATORY METABOLISM OF THE MARINE BACTERIUM *Alteromonas haloplanktis*

by

Marie-Claire Bonin Aly Hassan

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the
degree of Doctor of Philosophy

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Short title: Respiration in *Alteromonas haloplanktis*

Marie-Claire B. Aly Hassan

TO MY HUSBAND OMAR AND MY SON SHARIF

The growth of our knowledge is the result of a process closely resembling what Darwin called "natural selection"; that is, the natural selection of hypothesis: our knowledge consists, at every moment, of those hypotheses which have shown their [comparative] fitness by surviving so far their struggle for existence. . . .

Karl Popper

ABSTRACT

Ph.D. MARIE-CLAIRE BONIN ALY HASSAN Microbiology

STUDIES ON THE RESPIRATORY METABOLISM OF THE MARINE BACTERIUM *Alteromonas haloplanktis*

The respiratory metabolism of *Alteromonas haloplanktis* strain 214, was investigated spectrophotometrically and polarographically and was found to contain substrate-specific dehydrogenases, flavoproteins and cytochromes. Spectral studies showed that the whole cells, the cell-free extracts and the membrane fractions contained *b*- and *c*-type cytochromes reducible by NADH, succinate and ascorbate/TMPD. With the exception of succinate, the other substrates reduced the periplasmic/soluble fraction of the cell-free extracts. Oxidase activities were measured in all the cell fractions with the corresponding substrates. Only ascorbate/TMPD oxidase activity could be detected in the periplasmic fraction. The cytochrome content of the marine bacterium was measured in cells grown in media of various complexity. *In situ* dissolved oxygen content was monitored in cells grown in complex and chemically defined media.

The nutrient composition of the growth media, the availability of dissolved oxygen, the growth rate and the population density governed the synthesis of the *b*- and *c*-type cytochromes bound to the membrane. The synthesis of the periplasmic cytochrome *c* responded specifically to nutrients and to the concentration of dissolved oxygen in the growth media. Kinetic and CO-binding studies revealed that at least two terminal oxidases accounted for the reduction of the final electron acceptor, molecular oxygen. Spectral and polarographic measurements in the presence of the respiratory inhibitors amytal, rotenone, TTFA, cyanide and azide confirmed the

existence of inhibitor-sensitive and inhibitor-resistant pathways. An assessment of all the experimental data accumulated led to the proposal of an electron transport chain composed of a major membrane-bound and a minor periplasmic route for electron transport.

RESUME

Ph.D. MARIE-CLAIRE BONIN ALY HASSAN Microbiologie

ETUDE DU METABOLISME RESPIRATOIRE DE LA BACTERIE MARINE *Alteromonas haloplanktis*

Le métabolisme respiratoire de la souche 214 d' *Alteromonas haloplanktis* a été étudié par spectrophotométrie et polarographie. Il se compose de déshydrogénases spécifiques aux substrats, de flavoprotéines et de cytochromes. Des études spectrophotométriques ont montré que les cellules entières, les extraits cellulaires et la fraction membranaire contenaient des cytochromes de type b et c pouvant être réduits par le NADH, le succinate et l'ascorbate/TMPD. Les activitées enzymatiques ont été mesurées dans toutes les fractions cellulaires avec les substrats correspondants. Seule l'activité enzymatique de l'ascorbate/TMPD a pu être démontrée dans la fraction périplasmique. Le contenu cytochromique de la bactérie marine a été mesuré dans les cellules cultivées en milieux complexe et chimiquement défini.

La synthèse des cytochromes de type b et c liés à la membrane était gouvernée par la composition nutritive des milieux de culture, la concentration d'oxygène dissout, le taux de croissance et la densité de population. La synthèse du cytochrome c logé dans l'espace périplasmique de l'enveloppe cellulaire a répondu spécifiquement aux éléments nutritifs et à la concentration d'oxygène dissout. Des études cinétiques et des études impliquant le monoxyde de carbon ont révélé la présence d'au moins deux oxydases terminales impliquées dans la réduction de l'accepteur final d'électrons, l'oxygène moléculaire. Des mesures spectrophotométriques et polarographiques en présence des inhibiteurs respiratoires amytal, rotenone, TTFA, .

cyanure et azide ont confirmé l'existence de chaînes sensible(s) et résistante(s) aux inhibiteurs. Sur la base des données expérimentales accumulées, une chaîne respiratoire composée d'un segment majeur membranaire et d'un segment mineur périplasmique a été proposée.

(traduit par l'auteur)

ACKNOWLEDGEMENTS

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Difference absorption spectrophotometry using dithionite as reductant and oxygen gas as oxidant were recorded with whole cells, cell-free extracts, membrane and periplasmic fractions of *Alteromonas haloplanktis*. They revealed the presence of substrate-specific dehydrogenases, flavoproteins, and five cytochromes, mainly two b-type cytochromes and two c-type cytochromes firmly bound to the membrane as well as one c-type cytochrome located in the periplasmic space of the cell envelope.

2. Nutritional studies in batch culture showed that nutrients influenced the cytochrome composition of the marine organism: as the complexity of the growth media increased, the concentration of cytochromes increased.

3. A synthetic mixture of 4 amino acids (cystine, glycine, tyrosine, tryptophane) plus nicotinic acid was shown to specifically enhance the cytochrome synthesis in bacteria grown in succinate chemically defined medium.

4. Chemostat cultures of A. *haloplanktis* in complex and chemically defined media revealed that the growth rate influenced the synthesis of the membrane-bound cytochromes but not the synthesis of the periplasmic cytochrome.

5. Dissolved oxygen concentration was directly monitored in cells grown in different media with an O_2 electrode covered with a Teflon membrane.

6. Cytochrome patterns were followed *in situ* throughout the growth cycle of the marine bacterium as it consumed the ambient dissolved oxygen.

7. Oxygen was recognized as a dominant factor controlling the synthesis of cytochromes in A. *haloplanktis*.

8. Aeration of the growth media repressed the synthesis of the periplasmic and the membrane-bound cytochromes in complex medium grown cells but stimulated the synthesis of the membrane-bound cytochromes in chemically defined medium.

9. Molecular oxygen was the sole electron acceptor in A. *haloplanktis*.

10. Kinetic analysis of the dependency of the respiratory rates upon the oxygen concentration for various substrates in whole cells, cell-free extracts, membrane and periplasmic fractions revealed that the respiratory system of the marine bacterium obeyed Michaelis-Menten saturation kinetics.

11. Oxygen gas, hydrogen peroxide and ferricyanide oxidized the periplasmic and the membrane-bound cytochromes of A. *haloplanktis*.

12. Sodium dithionite, NADH, succinate and ascorbate/TMPD reduced the membrane-bound cytochromes of the marine bacterium. With the exception of succinate, the former substrates reduced as well the periplasmic cytochrome.

13. Detailed study of the electron transport chain using a series of specific respiratory inhibitors was undertaken to resolve the sequence of the respiratory components.

14. Inhibitor-sensitive and inhibitor-resistant pathways carried reducing equivalents from the reduced end to the oxidized end of the aerobic respiratory chain as revealed by the double reciprocal plots of the fractional inhibition *versus* the reciprocal of the inhibitor concentrations.

15. CO-binding studies showed the presence of three CO-binding cytochromes: a *b* (*o*) cytochrome and a c cytochrome bound to the membrane and a cytochrome c in the periplasmic space.

16. On the basis of NO-binding studies the periplasmic cytochrome c_{549} was classified as a c' cytochrome.

17. Based on the spectrophotometric and polarographic data accumulated, two pathways, one major, membrane-bound, and one minor, periplasmic, were proposed to account for the transport of electrons in the respiratory chain of A. *haloplanktis*.

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CHAPTER I

1. INTRODUCTION

Oxygen utilization by obligate aerobes occurs exclusively through the respiratory chain. The most important function of the respiratory chain is to act as a proton pump producing a proton motive force across the cytoplasmic membrane of the cells which, among other things, serves to give rise to the energy-rich molecule, ATP. Other important functions of the respiratory chain are to maintain the necessary level of oxidized and reduced forms of NAD, regulate the energy metabolism by controlling the phosphorylation level of ADP and provide an efficient oxygen-scavenging oxidase network to meet the metabolic demands of the aerobic cell.

In view of the fact that rather fragmentary information is available on the respiratory metabolism of *Alteromonas haloplanktis* and that the majority of the active transport processes characterized to date in the marine bacterium implies the existence of a functional respiratory chain, our primary concern in this thesis was to ascertain the composition of the cytochrome segment of the respiratory chain and assign functions to the respiratory carriers. A second concern in this research was to determine to what extent the concentration and distribution of the membrane-bound and the periplasmic cytochromes were affected by the nutrients, the growth phase and the dissolved oxygen concentration of the growth media.

2. LITERATURE REVIEW

Respiration and Energy Conservation

The vital energy necessary for bacterial life as well as for other forms of life is conserved in the form of adenosine 5'triphosphate (ATP), (Lipman, 1941), an energy-rich molecule that can be synthesized via substrate level phosphorylation or by oxidative and photophosphorylation. No membrane structure is necessary for the substrate mode of ATP synthesis, but it is fundamentally required for the oxidative mode. Aerobic heterotrophs utilize oxidative phosphorylation as a primary mode of ATP synthesis. In this case, ATP formation is coupled to electron transfer reactions which are driven by the oxidation of organic compounds (in organoheterotrophs) or inorganic ions (in chemolithotrophs) from negative redox donors to more positive redox acceptors.

The coupling mechanisms of redox reactions to ATP synthesis have been proposed and developed in the 1960's. Three theories among which the chemical (Slater, 1953) and the conformational (Boyer, 1965) were put forward to explain energy-linked reactions but did not embody the unifying concept of the chemiosmotic theory of Peter Mitchell (1961a,b, 1966).

According to the chemiosmotic theory, the enzymes responsible for oxidative phosphorylation are asymetrically organized in the membrane so as to catalyze vectorial chemical reactions that bring about the translocation of molecules, ions and chemical groups across a closed insulating coupling membrane. In brief, the essential features of electron transport - dependent ATP synthesis are triple: (*i*) a protolytic reaction involving the oxidation of an electron donor and the reduction of an electron acceptor, (*ii*) a translocation of protons accompanying the oxido-reduction creating a proton gradient across the membrane, (*iii*) a concomitant generation of a proton motive force ($\Delta\mu_{H^+}$) made up of an electrical ($\Delta\Psi$) and a chemical (ΔpH) gradient.

Figure 1 represents graphically a proton translocating segment of the respiratory chain leading to the synthesis of ATP. Exact stoichiometry of protons translocated per energy conserving site is still debated (Chance, 1977; Lehninger *et al.*, 1975; Meijer *et al.*, 1977; Williams, 1961, 1978; Papa, 1976, 1982; Mitchell, 1980; Sone and Hinkle, 1982; Papa *et al.*, 1982, 1983; Lehninger, 1984). All stoichiometries higher than 2 are not in agreement with the loop mechanism described by Mitchell (1961, 1966, 1976). Nevertheless, the basic principle of the chemiosmotic theory remains valid. Experimental proofs to the effect that the bacterial ATPase also couples the inward movement of protons to the synthesis of ATP are numerous (Cole and Aleem, 1973; Gould, 1979; Maloney *et al.*, 1974; Maloney and Wilson, 1975; Maloney, 1977, 1978; Tsuchiya, 1977; Singh and Bragg, 1979; Azzone *et al.*, 1984; Ferguson and Parsonage, 1984; Kell and Hitchens, 1984).

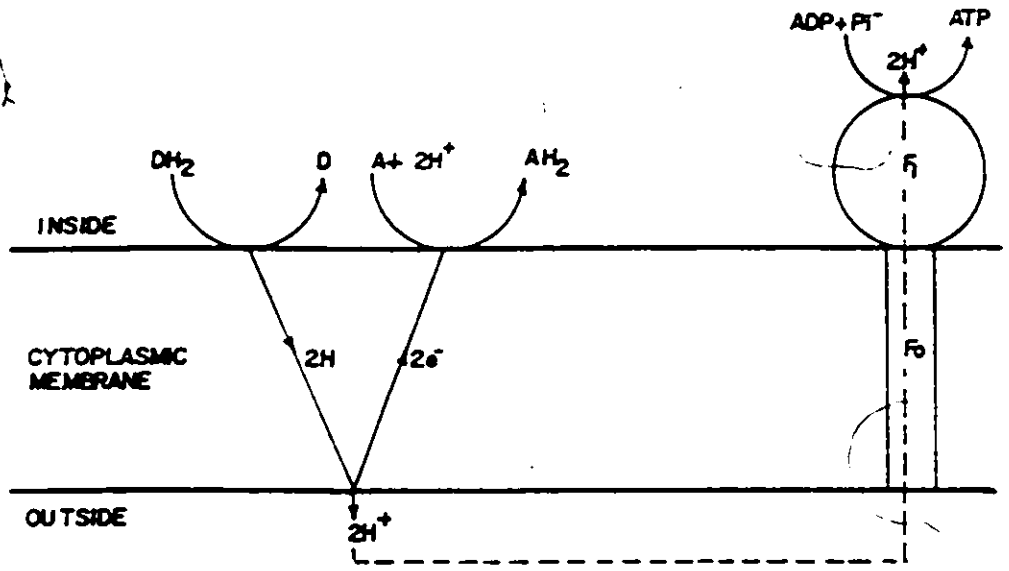
Bacterial Respiration

Several features differentiate the mitochondria from the bacteria and so the composition and effectiveness of their respiratory systems. While the mitochondrial electron transport chain has only one link with oxygen through the cytochrome oxidase aa_3, the bacterial system can synthesize multiple cytochrome oxidases (aa_3, o, a_1, $d(=a_2)$, cd_1) to accommodate the final electron acceptor (Meyer and Jones, 1973c; Jurtshuk *et al.*, 1975; Degn *et al.*, 1978; Ludwig, 1980; Knowles, 1980a; Poole, 1983b).

Moreover, while oxygen is the sole electron acceptor in eukaryotes, a number of other compounds (organic and inorganic) can function as terminal electron acceptors in prokaryotes (Knowles 1980a). The multiplicity of terminal oxido-reductases leads irrevocably to terminal branching of the respiratory chain (White and Sinclair, 1971; Jones, 1977; Knowles, 1980a; Kim and Hageman, 1981; Poole, 1983b). Branching has also been noticed at the level of primary dehydrogenases (Appleby, 1969a,b; Jones, 1977), and cytochrome

Figure 1. Simplified scheme of a redox loop and the proton translocating ATP_{ase} according to the chemiosmotic theory.

DH_2: electron donor; A: electron acceptor; F_0F_1: coupling factors. (Refer to the text for discussion.)


ADP + Pi⁻
ATP
2H⁺
DH₂
D
A + 2H⁺
AH₂
F₁
INSIDE
CYTOPLASMIC MEMBRANE
2H
2e⁻
Fo
OUTSIDE
2H⁺

b (Arima and Oka, 1965; Heinen, 1971; van Verseveld and Stouthamer, 1978; Ensley and Finnerty, 1980; Cypionka and Meyer, 1982).

Differentiation at the level of synthesis of components is mainly reflected at the level of cytochromes: some bacteria are deficient in cytochrome c (Dietrich and Biggins, 1971; Heinen, 1971; Meyer and Jones, 1973b; Haddock and Jones, 1977; Webster and Orii, 1978; Willison and John, 1979; DeMaio *et al.*, 1983) while others synthesize soluble cytochromes (Iwasaki and Shidara, 1969; Moriarty and Nicholas, 1969; Jones, 1972; Sapshead and Wimpenny, 1972; Weston and Knowles, 1973; Tonge *et al.*, 1974, 1977; Niven *et al.*, 1975; Anthony, 1975; Shidara, 1980; Cross and Anthony, 1980; Beardmore-Gray *et al.*, 1983; Ward *et al.*, 1983; Niven, 1984).

In addition, mutations (Haddock and Schairer, 1973; Cox and Gibson, 1974; Orth *et al.*, 1980; Cole *et al.*, 1980; Hacket and Bragg, 1983; Green and Gennis, 1983; Green *et al.*, 1984; McInerney *et al.*, 1984; Kajie *et al.*, 1984; Booth *et al.*, 1984) and growth conditions can greatly affect the composition of the bacterial respiratory chain (Smith, 1954; Richmond and Maaløe, 1962; White 1963a; Peterson, 1970; Oelze and Drews, 1972; Haddock *et al.*, 1976; Cox *et al.*, 1978; Ward *et al.*, 1983). Also worth considering, the response of the bacterial proteins toward classical respiratory inhibitors is not always as sensitive and predictable as in the case with mitochondria (Jones, 1977). But as a whole, the differences between the mitochondrial and the bacterial respiratory systems lie more at the structural organization level than at the mechanistic level (Yamanaka *et al.*, 1984).

Finally, the bacterial respiration fails to exhibit respiratory control as depicted in the mitochondrial system. Low P/O ratios (the amount of phosphate esterified per atom of oxygen consumed) have been time and again reported in prokaryotes (Stouthamer and Bettenhaussen, 1976; Eilermann, 1970; Knoblock *et al.*, 1971). Since it has been recognized that bacterial cytochromes are of importance in relation to growth

efficiency because of the presence of high potential cytochrome c as a prerequisite for a high P/O ratio in bacteria (Jones *et al.*, 1975). In addition, alteration in the redox patterns expressed in bacteria is not without effect on the vectorial organization of the respiratory chain within the coupling membrane, hence lowering the efficiency of oxidative phosphorylation. It seems that the respiration rate, under conditions of active bacterial growth is not ADP-limited as in mitochondria (Harrison, 1976a). The ATP content of the cell is controlled in such a manner that any changes in its value cause reactions that tend to bring it back to the steady state and probably elicit a response in the metabolic rate to restore the balance between ATP generation and its utilization (Niven *et al.*, 1977; Erecinska *et al.*, 1979; Rosenberg and Friedberg, 1984). The essential point remains that bacterial respiratory chains are more flexible both in terms of components and organization than the mitochondrial respiratory chain.

Bacterial Cytochromes

The name cytochrome was given by Keilin to a group of haemoproteins which in the reduced form showed a marked absorption spectrum in the visible region. Keilin (1925) confirmed and extended earlier observations made in 1866 by MacMunn and showed the fundamental role of the cytochrome system in the cell respiration.

The cytochromes fall into four groups differing in the nature of the haem prosthetic group: cytochrome *a* (non covalently bound formyl haem), cytochrome *b* (non covalently bound protohaem), cytochrome *c* (covalently bound mesohaem), cytochrome *d* (non covalently bound chlorin haem) (Lemberg and Barret, 1973). Common structural features and evolutionary links exist between the cytochromes of many different bacterial species and the mitochondrial cytochromes, particularly so for the cytochrome *c* (Almassy and Dickerson, 1978; Dickerson, 1980; Barber, 1984) suggesting a common evolutionary origin of the electron transport chains.

The functions of a number of cytochromes are unknown, but they all appear to act by undergoing oxidation and reduction. Some of those whose functions are known are enzymes, while the remainders are simply redox carriers. A terminal oxidase function has been assigned to cytochromes aa_3, a_2, a_1, o, o' (Castor and Chance, 1959; Sasaki *et al.*, 1970; Jurtshuk *et al.*, 1975; Degn *et al.*, 1978; Smith, 1978; Knowles, 1980a; Yamanaka *et al.*, 1984; van Verseveld *et al.*, 1983; Cypionka and Meyer, 1983; DeMaio *et al.*, 1983; Poole, 1983b; Miller and Gennis, 1983, 1984; Poole *et al.*, 1983; Yang, 1984). Spectral studies of the redox centers of the cytochrome oxidase aa_3 suggested that the bacterial cytochrome may be composed of other heme group than aa_3 (Ludwig, 1980), differentiating the molecule from its mitochondrial counterpart (Azzi and Casey, 1979). Combinations of hemes have also been identified as integrating parts of major terminal oxidases: this is the case for cytochrome cd_1 (Sapshead and Wimpenny, 1972; Koronen *et al.*, 1975; Barber *et al.*, 1978; Mitra and Bersohn, 1980), cytochrome *bd* (Watanabe *et al.*, 1979; Reid and Ingledew, 1980), cytochrome *o* and *c* (Mueller and Jurtshuk, 1972; King and Drews, 1976; Jurtshuk *et al.*, 1978; Matsushita *et al.*, 1982; Carver and Jones, 1983), cytochrome c_4 and c_5 (Jurtshuk *et al.*, 1979), and cytochrome caa_3 (Sone *et al.*, 1984).

The cytochromes of micro-organisms are usually found in the particulate fraction of the cell-free extracts and are part of the cell membrane (Alexander, 1956; Horio and Kamen, 1970; Lemberg and Barret, 1973; Poole *et al.*, 1980). Cytochrome b/NO_3^- reductase is a documented example of a membrane-bound cytochrome having an enzymatic function (MacGregor and Bishop, 1977). However, a number of soluble cytochromes have been characterized in many different bacterial species: *Haemophilus parainfluenzae* (Smith and White, 1962), *Escherichia coli* (Fujita, 1966; Haddock and Schairer, 1973), *Rhizobium japonicum* (Appleby, 1969a,b), sulfate-reducing bacteria (Yagi and Maruyama, 1971; Jones, 1972), *Thiobacillus neapolitanus*

(Sadler and Johnson, 1972), *Beneckea natriegens* (Weston and Knowles, 1974), *Pseudomonas extorquens* (Tonge *et al.*, 1974), *Pseudomonas* AM1 (Anthony, 1975), *Pseudomonas aeruginosa* (Parr *et al.*, 1976), *Methylosinus trichosporium* (Tonge *et al.*, 1975, 1977), *Paracoccus denitrificans* (van Verseveld and Stouthamer, 1978), *Neisseria meningitidis* (Yu *et al.*, 1979; Yu and De Voe, 1980), *Alcaligenes sp* (Shidara, 1980), *Campylobacter sputorum* (ElKurdi *et al.*, 1982), *Chromatium vinosum* (Gray *et al.*, 1983; Gaul *et al.*, 1983), *Haemophilus parasuis* (Niven, 1984), some of which are associated with enzymatic activities such as: nitrate reductase (Gauthier *et al.*, 1970), formatehydrogen lyase (Haddock and Schairer, 1973), nitrite reductase (Parr *et al.*, 1976), nitrite and nitric oxide reductase (Liu *et al.*, 1983), methane oxygenase (Tonge *et al.*, 1975, 1977), methanol: cytochrome c oxidoreductase (Beardmore-Gray *et al.*, 1983), methanol dehydrogenase (van Verseveld and Stouthamer, 1978; Alefounder and Ferguson, 1981; Quilter and Jones, 1984) and methanol oxidase (Froud and Anthony, 1984).

Still other cytochromes have been specifically localized in the periplasmic space of the cell envelope. This is the case for the periplasmic c_2 in facultative phototrophs (Hauska and Baccarini-Melandri, 1980; Meyer and Kamen, 1982), the periplasmic c_{CO} in a marine bacterium (Knowles *et al.*, 1974), the periplasmic c_{552} in extreme thermophile (Lorence *et al.*, 1981), the periplasmic c_{552} of the cyanobacterium *Anacystis nidulans* (Peschek *et al.*, 1982), the periplasmic c_{552} in anaerobically grown enterobacteriaceae (Fujita, 1966), the periplasmic c in hydrogen bacteria (Probst and Schlegel, 1976), the periplasmic c in cyanide-evolving bacteria (Niven *et al.*, 1975), the periplasmic c_{552} in hydrogen-utilizing bacteria (Macy *et al.*, 1976; Yamamoto and Ishimoto, 1978), the periplasmic c_3 in sulfate-reducing bacteria (Bell *et al.*, 1974; Wood, 1978a,b), the periplasmic cytochrome associated with the nitrate reductase of *Spirillum itersonii*

(Gauthier *et al.*, 1970; Garrard, 1971), the hydrogenase of *Desulfovibrio gigas* (Bell *et al.*, 1974), the sulphide-linked NO_2 reductase of *Thiobacillus denitrificans* (Sawhney and Nicholas, 1978), the nitrite and nitrous oxide reductase of *Paracoccus denitrificans* (Meijer *et al.*, 1979; Alefounder and Ferguson, 1980; Boogerd *et al.*, 1981; van Verseveld *et al.*, 1983), the cytochrome c_{551} sulfide: acceptor oxidoreductase of *Ectothiorhodospira abdelmalekii* (Then and Trüper, 1983), and the c-type cytochromes of some methylotrophs (Jones *et al.*, 1982; Kasprzak and Steenkamp, 1983; Burton *et al.*, 1983; Quilter and Jones, 1984).

The variety of bacterial pigments and their relative proportions vary from species to species (Lascelles, 1961; Meyer and Jones, 1973a; Jones and Meyer, 1976) and within species of the same genera (Bernard *et al.*, 1974; Faller *et al.*, 1980). Marked changes in the amount and types of cytochromes have been correlated with the physiological age and growth rate of the cultures as well as with the composition of the growth media (Scholes and Smith, 1968; Smith, 1968; Jones, 1977; Ensley and Finnerty, 1980). Such changes have been carefully studied in *Haemophilus parainfluenzae* (White, 1962, 1963a,b; Smith and White, 1962), *Escherichia coli* (Rice and Hempfling, 1978; Reid and Ingledew, 1979; Van Wielink *et al.*, 1983; Ingledew and Poole, 1984), *Pseudomonas putida* (Sweet and Peterson, 1978), *Spirillum itersonii* (Clark-Walker *et al.*, 1967).

The terminal oxidoreduction reaction leading to the formation of water has not yet been fully explained in bacteria nor in mitochondria. In eukaryotic cells, the formation of three categories of functional intermediates has been established in the primary events of the reaction of reduced cytochrome oxidase aa_3 with O_2 (Chance and Leigh, 1977). Likewise, active intermediates in the reaction of bacterial cytochrome *o* (oxidase) and cytochrome *d* with molecular O_2 have been documented in *Escherichia coli* (Poole *et al.*, 1979, 1983), *Azotobacter vinelandii* (Jurtshuk *et al.*, 1979),

Pseudomonas aeruginosa (Shimada and Orii, 1976; Parr *et al.*, 1976; Greenwood *et al.*, 1978) and in the filamentous myxobacterium *Vitreoscilla* (Webster and Orii, 1977, 1978).

This thesis has been divided into five chapters in order to give full coverage to some main aspects of the respiratory metabolism of *Alteromonas haloplanktis*, strain 214. Likewise, it shall be noticed that pertinent and specific literature review has been covered at the beginning of every chapter.

3. OBJECTIVES AND SCOPE

Among living organisms, microbial life exhibits the greatest diversity in the assembly of the respiratory chain components. Fluctuation in the synthesis of the cytochromes, the back-bone components of the respiratory chain is particularly evidenced under various environmental and growth conditions. As a result, detailed analysis of the sequence of hydrogen and electron transfer intermediates must be looked at in a wide variety of bacterial systems in order to identify the components that are the most affected and explain the reason(s) for the occurrence of such variation.

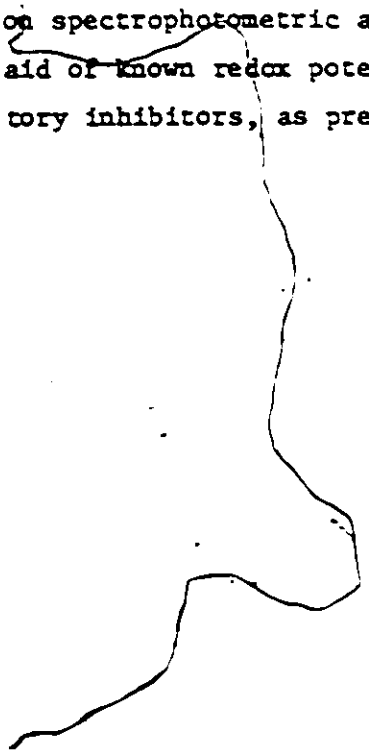
The micro-organism under study is the Gram negative bacterium *Alteromonas haloplanktis*, strain 214. This marine bacterium was shown to possess five cytochromes: two b-type and two c-type firmly attached to the cytoplasmic membrane and one c-type located in the periplasmic space of the cell envelope.

The experiments presented in this thesis aimed at analyzing the factors responsible for the variation in the cytochrome synthesis observed at the level of the periplasm and the cytoplasmic membrane. Such factors include mainly the availability of nutrients and the dissolved oxygen concentration are dealt with in Chapters II and IV.

Kinetic studies to determine the affinity constants of cellular and sub-cellular fractions towards O_2, the final electron acceptor, were undertaken in order to assign terminal oxidase functions to particular cytochromes in the respiratory chain of *A. haloplanktis*. Those studies are reported in Chapter III.

The synthesis of the cytochromes was also examined in the physiological context of the growth phases of the microorganism in a complex and a simple growth environment with particular attention given to pinpoint the cytochrome species which are the most flexible under various growth environments, as discussed in Chapter IV.

Finally, sequencing of the electron transfer intermediates and proposal of an electron transport chain were attempted based on spectrophotometric and polarographic results obtained with the aid of known redox potential substrates and conventional respiratory inhibitors, as presented in Chapter V.



CHAPTER II

NUTRITIONAL STUDIES

1. INTRODUCTION

Bacteria which upon initial isolation require a medium containing sea water as a diluent are classified as marine bacteria. In contrast to halophiles, marine bacteria do not tolerate high salt concentrations in the growth medium (MacLeod *et al.*, 1954; MacLeod, 1965). An extensive survey of nutritional requirements for marine bacteria revealed that no amino acids or vitamins were required in addition to a carbon and a nitrogen source to support the growth of A. *haloplanktis*, formerly referred to as marine pseudomonad B-16 (MacLeod *et al.*, 1954; MacLeod and Onofrey, 1957). The marine milieu would also supply the bacterium with the essential inorganic ions such as Na^{+}, K^{+}, Mg^{++}, Ca^{++}, PO_4^{---} and $SO_4^{=}$ which it has been shown are required for growth (MacLeod and Onofrey, 1957; Tomlinson and MacLeod, 1957). It was subsequently demonstrated that Na^{+} and K^{+} were vitally important ions for metabolic functions such as respiration (Sedgwick and MacLeod, 1980; Khanna *et al.*, 1984) and active transport of metabolites across the cytoplasmic membrane of this Gram negative bacterium (Thompson and MacLeod, 1974, a,b; Fein and MacLeod, 1975; Niven and MacLeod, 1978, 1980; Sprott *et al.*, 1975).

On the other hand, preliminary studies on the cytochrome distribution in this marine organism revealed that the cellular extracts contained periplasmic as well as membrane-bound cytochromes (Knowles *et al.*, 1974). The unusual location of a cytochrome in the periplasmic space of the cell envelope led us to investigate the growth conditions which were affecting its synthesis in order to subsequently explain its role in the respiratory metabolism of the aerobic micro-organism.

To test whether the synthesis of the periplasmic cytochrome was governed by a specific nutrient, a variety of simple and complex nutrient supplements were added to a succinate chemically defined medium (CDM).

During the course of these experiments, it was observed that the periplasmic cytochrome was easily leached from cells grown in complex nutrient environments although normally living organisms retain vital compounds when incubated at optimal growth temperature or washed under physiological conditions. Exceptions to this rule are found in Gram negative bacteria harboring periplasmic enzymes (Heppel, 1967; Forsberg *et al.*, 1970a,b; Ingram *et al.*, 1973; Thompson and MacLeod, 1974a,b; Bhatti *et al.*, 1976; Bhatti and Ingram, 1982).

The effects of growth rate and population density were also looked at in an attempt to determine their impact on cytochrome synthesis.

2. MATERIALS AND METHODS

Organism: Maintenance, Harvest, Fractionation

Alteromonas haloplanktis, strain 214, variant 3, derives its name from the classification of Reichelt and Baumann (1973). It was previously referred to as marine pseudomonad B-16, variant 3 (ATCC 19855 (NCMB 19) (Gow *et al.*, 1973). The culture was maintained by monthly transfer on agar slants of a medium containing 0.8% (w/v) nutrient broth (Difco Laboratories), 0.5% (w/v) yeast extract (Difco Laboratories) in a salt solution composed of 0.3M NaCl, 0.026M $MgSO_4$ and 0.01M KCl.

The organism was cultured at 25°C with a vigorous agitation. Cell turbidity was followed at 660 nm in a micro-sample spectrophotometer (Gilford Instrument 300-N). Stationary phase cells were harvested by centrifugation (16,000 xg, 10 min., Sorvall RC2-B, rotor GS-, 4°C) following 16 hours of growth in complex medium (CM) and 24 hours in the chemically defined medium (CDM). The harvested cells were washed twice in a physiological salt solution and resuspended in an appropriate volume of buffered salt solution containing .3M NaCl, .05M $MgSO_4$, .01M KCl *plus* Tris-PO_4 (50+1mM, pH7.4). The cells were subsequently disrupted by means of a French press, (14,000 to 16,000 psi., Power Laboratory Press, American Instrument Co. Ltd., Inc.). The crude extract was centrifuged at 39,000 xg for 20 min. (Sorvall RC2-B, rotor SS34, 4°C) and gave rise to a clear cell-free extract (CFE) preparation. The CFE was further ultracentrifuged (150,000 to 180,000 xg, 3 hours, Beckman L2-65B, rotor 60Ti, 4°) to obtain the (sedimented) particulate fraction and the (supernatant) periplasmic soluble fraction (Knowles *et al.*, 1974), hereafter referred to as the periplasmic fraction. The particulate fraction collected was enriched with respect to membrane-bound cytochromes absorbing at 552 and 559 nm in a reduced *minus* oxidized difference spectrum recorded at room temperature, while the supernatant fraction was enriched with respect to a periplasmic cytochrome with an α-peak absorbing at 549 nm.

Growth Conditions in Batch Culture

A. Complex medium

The medium referred to as complex medium (CM) contained 0.8% (w/v) nutrient broth (Difco Laboratories), 0.5% (w/v) yeast extract (Difco Laboratories) in a salt solution consisting of 0.3M NaCl, 0.026M $MgSO_4$, 0.01M KCl and 0.1 mM $FeSO_4(NH_4)_2\ SO_4$. The growth medium was sterilized by autoclaving (15 min, 15 psi., Barnstead Sterilizer).

B. Chemically defined medium

The chemically defined medium (CDM) consisted of 0.11M sodium succinate and 0.02M $(NH_4)_2\ SO_4$, as carbon and nitrogen sources respectively, in a salt solution containing 0.3M NaCl, 0.01M KCl, 5mM $MgCl_2$, 3mM K_2HPO_4, 26uM $FeSO_4(NH_4)_2\ SO_4$ and 10uM $CaCl_2$. The pH of the growth medium was adjusted to 7.4 by addition of small volumes of 40% NaOH and the carbon source autoclaved separately.

C. Amended chemically defined medium

The chemically defined medium outlined above was amended by the addition of the following supplements: casein hydrolysate (Sigma Chemical Co.), casein hydrolysate, vitamin-and salt-free (Nutritional Biochemicals Co.), bacto peptone (Difco Laboratories), bacto soytone (Difco Laboratories), all present at 1% (w/v) final concentration. Defined supplements included: a synthetic mixture of L-form amino acids based on the amino acid composition of casein and composed of: glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, arginine, aspartic acid, glutamic acid, proline, histidine, tyrosine, cystine, methionine; a mixture of six amino acids predominant in the amino acid sequence of several cytochromes c: threonine, tryptophan, tyrosine, lysine, cystine and glycine each added at 0.035% (w/v) final concentration; a variety of vitamins including nicotinic acid, thiamine, riboflavin, pyridoxine, pantothenic acid, biotin, folic acid and vitamin B-12. In addition, horse heart cytochrome c (Sigma Chemical Co.) and hemin (Sigma Chemical Co.) were tested at 10 ug/ml final concentration. The pH of these supplemented defined media were

adjusted to pH 7.4 by the addition of small volumes of 40% NaOH or 30% HCl whenever necessary. All the complex supplements were autoclaved separately and added aseptically to the basic succinate chemically defined medium. The heat sensitive compounds were membrane filtered in sterile millipore flasks using Metricel filters of 0.45 µm pore size (Gelman Instrument Co.) and added aseptically to the succinate chemically defined medium.

Growth Conditions in Continuous Culture

The media used for cultivation were the complex and the chemically defined medium outlined in the batch culture section. The cells were grown under controlled aeration at 25°C in a chemostat (New Brunswick Scientific Co., Model Bioflo C30) in 300 ml effective volumes at a variety of fixed dilution rates with pH controlled at 7.4 ± 0.1 by the addition of 2% H_2SO_4 (Titrator 11/PHA 942, Radiometer, Copenhagen, Denmark). The cultures were aerated with 0.4 l of sterile atmospheric air per liter of medium per min and rapidly agitated with propellers (500 rpm). The airflow was measured by a flowmeter accurate to ± 0.5%. Whole cells were harvested when a steady state had been attained for at least five doublings of the population. The doubling time of logarithmic phase cells in complex medium was approximately 35 min and approximately 150 min in chemically defined medium. The population density was frequently monitored on diluted portions of the respective cultures using a Gilford spectrophotometer (Model 300-N). Purity of the samples was checked by plating a loopful of the culture on complex agar media and Brain Heart Infusion agar which were incubated at 25°C for a period of 48 hr. Ordinarily in chemostat cultures, the level of a limiting nutrient in the inflow medium governs the population density in the reaction vessel. In the experiments described, the limiting nutrient is unknown. To test whether the optical density of the culture affected the level of the cytochromes synthesized, data from several runs were pooled where slight variations in the optical density at steady state were experienced.

Spectrophotometric Measurements

The cytochrome content of the various fractions was determined

spectrophotometrically using a two wavelength/double beam spectrophotometer (Perkin-Elmer, Model 356) operated in the split mode. The difference spectra were recorded by using 10 mm light path cuvettes, a spectral band width of 1.0 nm, a scanning speed of 1 nm/sec and the medium time-response setting. At high sensitivities and in low temperature difference spectra, the slow time-response setting was used and 2 mm light path cuvettes employed. The concentration of each cytochrome was calculated from dithionite-reduced *minus* ferricyanide-oxidized difference spectra using published extinction coefficients (Jones and Redfearn, 1966) and expressed as n moles cytochrome/mg protein. Since the spectra are recorded for a reference of equal turbidity, the quantitative measure of the cytochrome is expressed by the difference in absorption (ΔA) between a wavelength pair corresponding to an absorption peak (A_{max}) to a trough (or plateau) (A_{min}). The split beam spectrophotometer plots as a function of λ (wavelength) the difference of optical density between the 2 identical solutions that differ only by their chemical treatment (Chance and Williams, 1955). The protein content of the various fractions was estimated by the Biuret method (Gornall *et al.*, 1949) using lysozyme as the standard protein. Absorbancy was measured at 540 nm using a Gilford spectrophotometer (Model 300-N). The protein content was expressed in mg/ml.

3. RESULTS AND DISCUSSION

Much information has been obtained with regard to the chemical and physical identification of the various layers of the cell envelope in *Altermonas haloplanktis* (Forsberg *et al.*, 1970 a, b). Interestingly, the fractionation of the cell envelope by controlling the ion composition of the suspending medium and by differential centrifugation also revealed the presence of a cytochrome-like pigment in the underlying layer of the cell envelope (Forsberg *et al.*, 1970a). This early finding was later confirmed (Sprott and MacLeod, 1974) and substantiated by the work of Knowles *et al.*, (1974).

Table I outlines the absorbancy wavelengths (maxima and minima) of the cytochrome complement of *Alteromonas haloplanktis* as revealed by low and room temperature difference absorption spectrophotometry. The quantification of the various cytochromes is based on reduced *minus* oxidized difference spectra recorded at room temperature at the level of the α-band, using the wavelength pairs and the extinction coefficients given in the table. The dithionite-reduced *minus* ferricyanide-oxidized difference spectra were recorded in a number of whole cells, cell-free extracts, membrane and periplasmic fractions. No *a*-type cytochrome could be detected spectrophotometrically in any of the preparations scanned at room or at low temperature. In the cell-free extracts and the membrane fraction α-peaks in the Red/Ox difference spectra absorbed at 559 nm (*b*-type cytochrome) and at 552 nm (*c*-type cytochrome), when the spectra were run at room temperature. In the periplasmic fraction, only one peak absorbing at 549 nm (*c*-type cytochrome) could be detected. Analysis of the various fractions at liquid air temperature revealed the presence of two *b*-type cytochromes with α-peaks at 558 and 556 nm in whole cells and cell-free extracts and at 558.5 and 556.5 nm in the membrane fraction. The two *c*-type cytochromes bound to the membrane absorbed at 549 and 546 nm in whole cells and cell-free extracts and at 549.5 and 544 nm in the membrane fraction. Only one

TABLE I. Spectrophotometric characteristics of the cytochrome complement of *Alteromonas haloplanktis* grown in complex medium.

Cell fraction	Absorption spectra[†]	Cytochrome type	Absorption maxima (nm) (room temperature, 21°C) α	β	γ	Absorption maxima (nm) (low temperature, 81°K) α	β	γ	Wavelength pairs (nm)[**] (maxima and minima) room temperature	low temperature	Δε mM[***]
Whole cells	Red/Ox	b	no resolution at room temperature			558	526	421-425	no resolution at room temperature	558-560	± 1 nm 17.5
						556				556-566	
						549				549-532	± 1 nm 17.3
		c				546	520			546-532	
Cell-free extracts	Red/Ox	b	559	527		558	526		559-571 ± 1 nm	558-565	± 1 nm 17.5
						556				556-565	
		c	552	522	broad 422	549		broad 422	552-534 ± 1 nm	549-532	± 1 nm 17.3
						546	520			546-532	
						544*					
Membrane fraction	Red/Ox	b	559	527		558.5	526		559-571 ± 1 nm	565.5-565	± 1 nm 17.5
					broad 424	556.5		broad 422	552-534 ± 1 nm	556.5-565	
		c	552	521		549.5	520			549.5-532	± 1 nm 17.3
						544				544-532	
Periplasmic fraction	Red/Ox	c	549	519	414	546	518	412.5	549-532 ± 1 nm	546-530	± 1 nm 17.3

[†] Position of peaks in reduced minus oxidized difference spectra; the redox state of the sample and reference cuvette is indicated by the expression Red/Ox, where the numerator (Red=reduced) represents the sample cuvette the contents of which have been reduced by dithionite and the denominator (Ox=oxidized) represents the reference cuvette the contents of which have been oxidized by ferricyanide.

* Numbers marked with asterisk refer to shoulders on main peak.

** In the presence of inhibitors and/or substrates, the absorption peaks may shift slightly toward shorter wavelengths.

*** Extinction coefficients used in calculating the concentration of cytochromes (Jones and Redfearn, 1966).

c-type cytochrome could be detected in the periplasmic fraction. The identification of the heme portion of the cytochromes was accomplished previously by acid-acetone treatment and examination of the pyridine haemochrome spectra (Knowles *et al.*, 1974). The flavin moities of the substrate-linked dehydrogenases present in all the fractions give troughs (absorption minima) in the 510 - 465 nm region of the reduced *minus* oxidized difference spectra, using O_2 gas or H_2O_2 as oxidant instead of ferricyanide because of the strong absorption of this chemical below 510 nm. The millimolar extinction coefficient used for calculating the flavoprotein concentration is 11.0 (Jones and Redfearn, 1966).

Growth Experiments in Batch Culture

Figures 2 and 3 illustrate the cytochrome complement of *A. haloplanktis* grown in complex and chemically defined media respectively, as determined by difference absorption spectra scanned at low temperature. The α-absorption peaks at 558 and 556 nm in whole cells correspond to the b-type cytochromes bound to the membrane of both cell types and are the equivalents of the 559 nm absorption band of the same preparations scanned at room temperature. In the cells grown in complex media (Figure 2, upper trace), the α-absorption peaks at 549 and 546 nm account for the membrane-bound c-type cytochromes (both absorbing at 552 nm at room temperature) as well as the periplasmic cytochrome (absorbing at 549 nm at room temperature). In the cells grown in succinate chemically defined media (Figure 3, upper trace), the main absorption peak at 551 nm and a minor shoulder to the right represent the c-type cytochromes bound to the membrane (both absorbing at 552 nm at room temperature). The other a and b absorption peaks for the cytochromes present in the cell-free extracts, the membrane and the periplasmic fractions of the respective media (lower traces) are as outlined in Table I.

Upon variation of the growth media, it was observed that the cytochrome composition of the marine micro-organism could be altered

Figure 2. Low temperature difference absorption spectra of the cytochrome complement of *Alteromonas haloplanktis* grown in complex medium.

Dithionite-reduced *minus* ferricyanide-oxidized difference spectra. The fractions from top to bottom: whole cells (WC), particulate fraction (P), cell-free extracts (CFE) and soluble (S) fraction of the cell-free extracts were mixed (1:1) with sucrose (0.75M, final concentration). One half was reduced with solid crystals of dithionite, the other half oxidized with solid crystals of ferricyanide. The difference spectra were recorded at 83°K in cuvettes of 2 mm light path. The vertical bars represent one tenth of the absorbancy unit at which the spectra were recorded (A=0.03 for the top three traces, A=0.01 for the bottom trace) and the wavelengths are expressed in nanometers. The protein concentrations of the various fractions were: WC (22.0 mg protein/ml), P (16.5 mg protein/ml), CFE (14.8 mg protein/ml) and S (5.75 mg protein/ml).

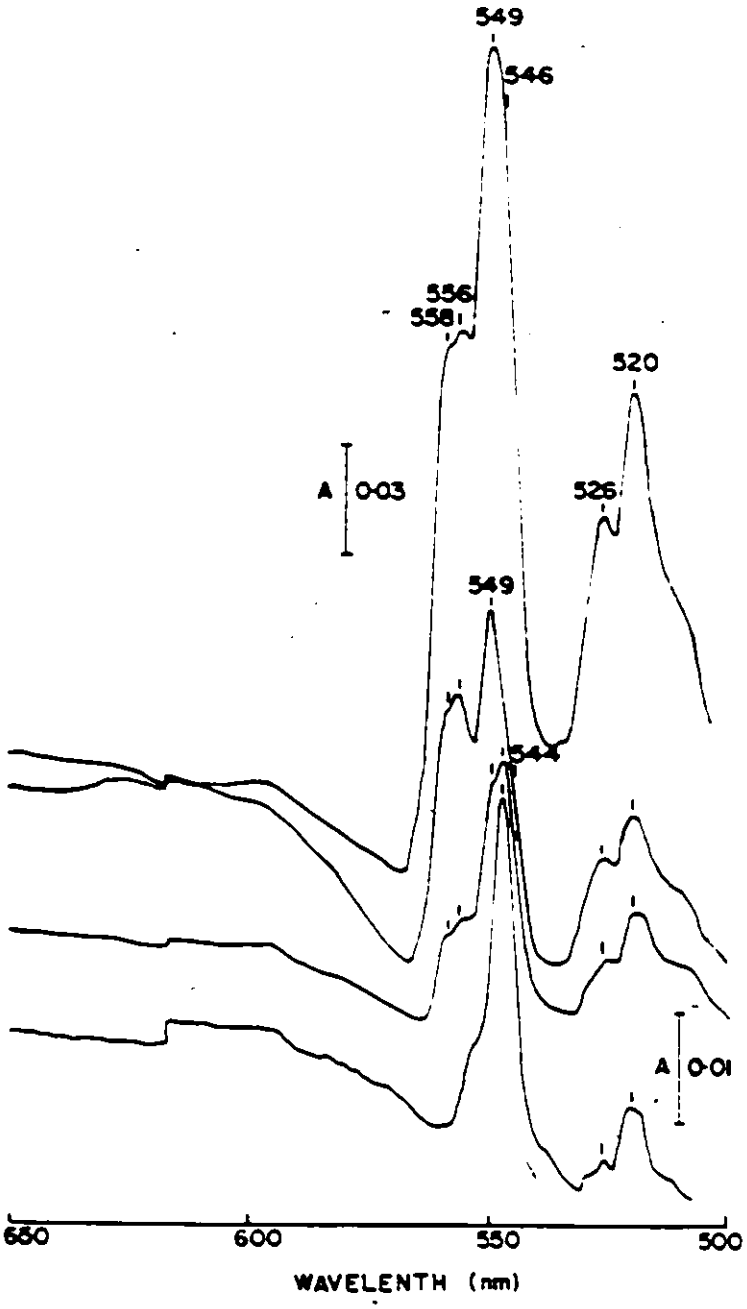
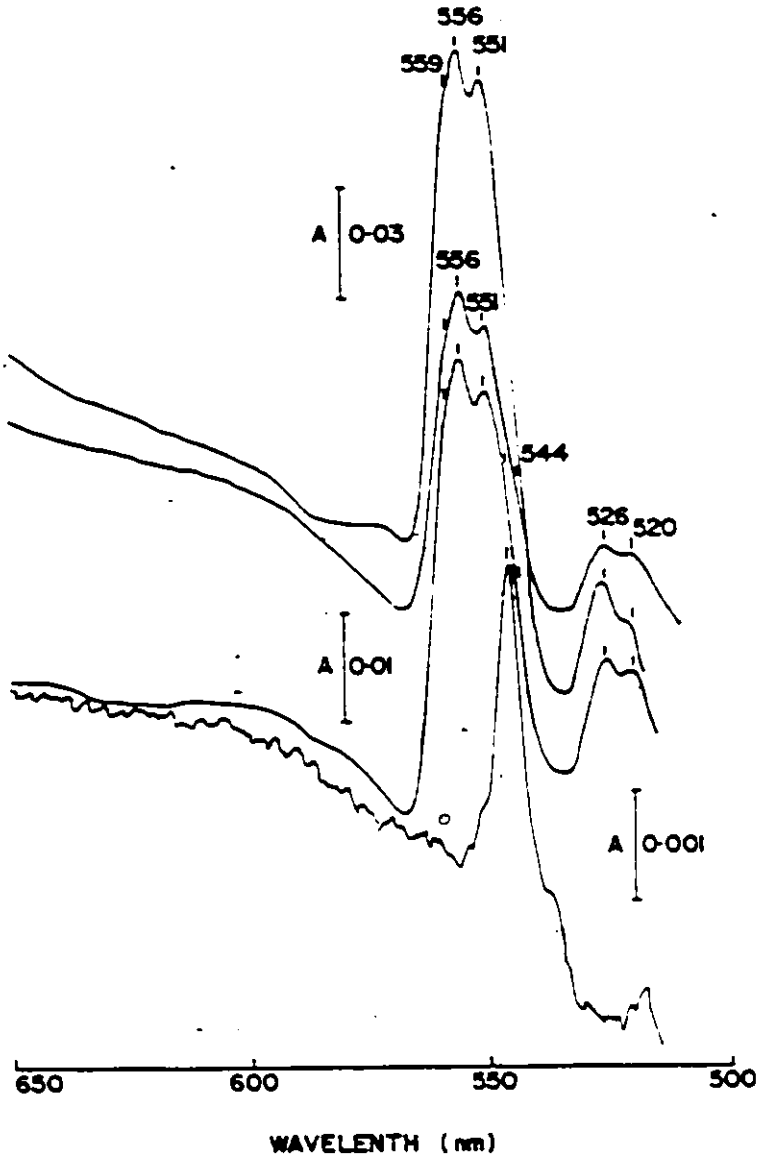

549
546
556
558
520
A 0·03
526
549
544
A 0·01
650
600
550
500
WAVELENTH (nm)

Figure 3. Low temperature difference absorption spectra of the cytochrome complement of *Alteromonas haloplanktis* grown in succinate chemically defined medium.

Dithionite-reduced *minus* ferricyanide-oxidized difference spectra. The fractions from top to bottom: whole cells (WC), particulate fraction (P), cell-free extracts (CFE) and soluble (S) fraction of the cell-free extracts were mixed (1:1) with sucrose (0.75M, final concentration). One half was reduced with solid crystals of dithionite, the other half oxidized with solid crystals of ferricyanide. The difference spectra were recorded at 83°K in cuvettes of 2 mm light path. The vertical bars represent one tenth of the absorbancy unit at which the spectra were recorded (A=0.03 for the top two traces, A=0.01 for the middle trace, A=0.001 for the bottom trace) and the wavelengths are expressed in nanometers. The protein concentrations of the various fractions were: WC (32.0 mg protein/ml), P (24.5 mg protein/ml, CFE (19.0 mg protein/ml) and S (7.5 mg protein/ml).


556
559
551
A 0·03
556
551
544
526
520
A 0·01
A 0·001
650
600
550
500
WAVELENTH (nm)

to the extent that large quantities of particularly cytochromes c (periplasmic and membrane-bound) could be synthesized in a complex nutrient medium, whereas only small quantities of the periplasmic protein could only be recovered from cells grown in a chemically defined medium without added supplements. In view of the different cytochrome patterns (*i.e.* different ratios of cytochromes *b* to cytochromes c in the cells from the two media), and particularly since the level of the periplasmic cytochrome c was higher in the cells grown in complex medium, it was thought appropriate to examine the factors which governed the regulation of the synthesis of these proteins.

Initially, a selection of non-defined, commercially available media were assessed for their ability to stimulate cytochrome synthesis. Each complex, non-defined supplement was added at 1% (w/v) final concentration to the succinate chemically defined medium (CDM). The experimental data are presented in Table II. As can be seen, the various supplements stimulated the synthesis of the cytochromes to different levels. Certain components, *e.g.* casein hydrolysate stimulated the synthesis of the cytochromes even above that normally obtained in the routine complex medium used (nutrient broth + yeast extract). Incidentally, casein hydrolysate is a complete acid hydrolysate of casein and specially suited as a source of amino acids for culture media. Other components such as bacto peptone gave poor stimulation of most cytochromes, particularly evident in the case of the periplasmic cytochrome c_{549}.

Considering the various layers of the cell envelope of *A. haloplanktis*, (Forsberg *et al.*, 1970a, b) the underlying layer (ULL) or periplasmic space and the embedded proteins would be among the first targets toward which environmental factors would exert their beneficial or detrimental actions. Therefore, it is conceivable that stimulation of cytochrome synthesis affects primarily the periplasmic cytochrome c_{549}.

TABLE II. The effect of complex supplements on the level of cytochromes in *A. haloplanktis*.

Growth medium succinate-CDM supplemented with:*	Number of experiments	Cytochrome content Periplasmic fraction c_{549}	Membrane fraction c_{552}	Membrane fraction b_{559}
		n moles/mg protein		
None	17	.035 ± .016	.071 ± .033	.070 ± .034
Bacto Peptone	4	.038 ± .019	.182 ± .031	.166 ± .030
Bacto Soytone	7	.228 ± .200	.475 ± .169	.305 ± .117
Yeast Extract	2	.320 ± .030	.270 ± .050	.180 ± .060
Nutrient Broth + Yeast Extract	4	.410 ± .120	.460 ± .300	.370 ± .280
Casein Hydrolysate	8	.474 ± .230	.566 ± .139	.414 ± .118
Casein Hydrolysate (vitamin- and salt-free)	4	.870 ± .356	1.23 ± .060	.833 ± .005

*Each supplement was added at the level of 1% (w/v) to the basic succinate chemically defined medium. Each entry is an average value of the cytochrome species measured in all the experiments ± the standard deviation of the mean.

Several factors might be responsible for the observed stimulation. The phenomenon could be due to a nutritional requirement for synthesis or induction of respiratory proteins supplied by the components of a complex medium. A further possibility is that differences in the growth rate of the cells could result in differences in the synthesis of cytochromes (Rosenberger and Kogut, 1958). Molar growth yields of bacteria are substantially higher when the cells are grown in complex than when they are grown in minimal medium (Stouthamer and Bettenhaussen, 1976).

To test these possibilities, two lines of approach were used. The first was to attempt to identify the component(s) responsible for the stimulation of cytochrome synthesis; the second approach was to use a continuous type of culture in which the growth rate and the final population density could be controlled.

Since casein hydrolysate was able to stimulate the synthesis of the periplasmic cytochrome c, a mixture of 16 crystalline amino acids, approximating the amino acid composition of casein hydrolysate, was tested at the amino acid concentrations which one would expect to be provided by 1% casein hydrolysate. As can be seen in Table III, this amino acid mixture failed to stimulate cytochrome synthesis, in contrast to results with casein hydrolysate (Table II).

Looking subsequently at the amino acid composition of several cytochromes, six amino acids were chosen because of their predominance in the amino acid sequence of the protein (Dickerson, 1980) and added to the succinate chemically defined medium. Careful study of the typical analysis of the commercially available growth media revealed that nicotinic acid was the most commonly found co-factor present in relatively high concentration in the complex supplements that stimulated the synthesis of cytochromes (Table II), with the exception of bacto peptone. It was thus

TABLE III. The effect of chemically defined supplements on the level of cytochromes in *A. haloplanktis*.

Growth medium Succinate-CDM supplemented with:*	Number of experiments	Cytochrome content Periplasmic fraction c_{549}	Membrane fraction c_{552}	Membrane fraction b_{559}
		n moles/mg protein		
None	17	.035 ± .016	.071 ± .033	.070 ± .034
16 amino acids	2	.069 ± .007	.092 ± .001	.097 ± .001
6 amino acids	4	.088 ± .067	.214 ± .084	.206 ± .057
5 µg/ml nicotinic acid	4	.032 ± .014	.090 ± .028	.092 ± .039
6 amino acids + nicotinic acid	2	.370 ± .082	.440 ± .090	.380 ± .095
10 µg/ml hemin	2	.099 ± .000	——— **	——— **
10 µg/ml cyt. c	3	.055 ± .028	.034 ± .020	.032 ± .028

* The 16 amino acids were as follows (g/l, final concentration):
glycine (.005), alanine (.021), valine (.020), leucine (.017), isoleucine (.013), serine (.048), threonine (.012), lysine (.018), arginine (.007), aspartic acid (.180), glutamic acid (.071), proline (.002), histidine (.003), tyrosine (.015), cystine (.001), methionine (.008).
The mixture of 6 amino acids (thr, try, lys, tyr, gly, cys) and 4 amino acids, (try, tyr, gly, cys) were added at the level of a 0.035% (w/v) to the basic succinate chemically defined medium.
The nicotinic acid was present at the level of 5 mg/l.

** The membrane-bound cytochromes could not be measured accurately due to significant binding of the hemin to the membrane.

Each entry is an average value of the cytochrome species measured in all the experiments ± the standard deviation of the mean.

added to the synthetic mixture of the 6 amino acids selected. Pyrrole-containing compounds such as hemin and horse heart cytochrome c were also tested for their ability to stimulate cytochrome synthesis. Both compounds contain four pyrrole rings surrounding a central iron atom. Results of these experiments are presented in Table III.

The mixture of 16 amino acids did not stimulate to a great extent the synthesis of the periplasmic or the membrane-bound cytochromes. The six selected amino acids failed to stimulate the synthesis of the periplasmic cytochrome although they did stimulate the synthesis of the membrane-bound ones to a certain degree. The nicotinic acid alone did not have any stimulatory effect. On the other hand, when the 6 selected amino acids were tested in the presence of nicotinic acid, the levels of periplasmic and membrane-bound cytochromes were enhanced to values comparable to the levels found in cells grown routinely in complex medium. The pyrrole-containing compounds were not very effective in promoting the synthesis of either cytochrome species. The somewhat greater stimulating effect of hemin, when compared to exogenous cytochrome c could partially be explained in terms of a molecular weight effect: the smaller hemin molecule (MW 652) may penetrate or be taken up more easily by the cells grown in chemically defined medium than the larger cytochrome c molecule (MW: 12, 384).

The next step was to see whether these six amino acids were all essential for the synthesis of the cytochromes. The cells were grown in the succinate-CDM with nicotinic acid plus five of the six amino acids (removing one at the time). The data obtained are presented in Table IV. As can be seen, tyrosine, glycine and cystine seemed to be required along with nicotinic acid, since omitting them singly significantly reduced the level of the periplasmic cytochrome; threonine seemed not to be required, only slightly reducing the level of the periplasmic c_{549} when left out. The results with

TABLE IV. The effect of removal of amino acids from the supplemented succinate-CDM on the level of cytochromes in *A. haloplanktis*.

Growth medium Succinate-CDM supplemented with:*	Number of experiments	Cytochrome content Periplasmic fraction c_{549}	Membrane fraction c_{552}	Membrane fraction b_{559}
		n moles/mg protein		
None	17	.035 ± .016	.071 ± .033	.070 ± .034
6 amino acids + nicotinic acid (thr, try, lys, tyr, gly, cys)	2	.370 ± .082	.440 ± .090	.380 ± .095
minus thr	2	.315 ± .064	.530 ± .141	.420 ± .085
minus try	3	.280 ± .070	.489 ± .118	.396 ± .072
minus lys	2	.229 ± .084	.454 ± .011	.359 ± .025
minus tyr	2	.147 ± .013	.225 ± .058	.205 ± .055
minus gly	2	.141 ± .008	.267 ± .035	.233 ± .027
minus cys	2	.071 ± .003	.284 ± .059	.264 ± .043
4 amino acids + nicotinic acid (cys, gly, tyr, try)	6	.347 ± .075	.328 ± .055	.372 ± .121
4 amino acids + 7 vitamins	2	.100 ± .009	.222 ± .004	.230 ± .014

*The mixtures of 6 and 4 amino acids were added at the level of 0.035% (w/v) to the basic succinate chemically defined medium. The mixture of vitamins contained at the indicated final concentrations in the medium (mg/l): thiamine (3.25), riboflavin (2.50), pyridoxine (1.37), pantothenic acid (0.37), folic acid (0.22), vitamin B-12 (0.008), biotin (0.45).

Each entry is an average value of the cytochrome species measured in all the experiments ± the standard deviation of the mean.

tryptophan and lysine were more difficult to interpret because of their different effect on periplasmic versus membrane-bound cytochromes. However, if lysine and threonine were omitted, then the level of the periplasmic cytochrome was over 90% of that when all the six were included. As a result of these experiments, a mixture of four amino acids consisting of cystine, glycine, tyrosine and tryptophan together with nicotinic acid was retained as a synthetic mixture which could specifically promote cytochrome synthesis in A. *haloplanktis*.

Also in Table IV are data indicating that a mixture of seven vitamins, together with four selected amino acids, were unable to stimulate the synthesis of the periplasmic cytochrome to the same extent as nicotinic acid with the 6 previously tested amino acids, thus conferring an important role to nicotinic acid in the synthetic mixture.

Growth Experiments in Continuous Culture

Several parameters are known to affect the growth of a microorganism in batch culture (Harrison, 1972; Pácá, 1976). Microbial cultures can only be efficiently maintained in a steady state growth environment such as in continuous culture if used metabolic products are constantly replaced by fresh nutrients (Pirt, 1975).
In the following experiments we made use of a chemostat to grow the cells. These chemostat cultures varied from the standard chemostat cultures in that the substrate concentration was not the growth limiting factor. Instead, the cells were simply allowed to grow continuously under conditions of controlled pH, aeration and dilution rate. Essentially, these were batch cultures run in a chemostat with the fundamental difference that metabolic products were flowing out of the reaction vessel at the same rate as fresh nutrients were flowing in. The relationship between the cytochrome content of A. *haloplanktis* and the dilution rate of the cells when grown in chemically

defined medium (CDM) and complex medium (CM) is illustrated in Figure 4.

As can be seen, the dilution rate had a profound effect on the level of the membrane-bound cytochromes (c_{552}, b_{559}) both in complex and chemically defined media. As a general trend, the lower the dilution rate, the higher the level of the membrane-bound cytochromes. On the other hand, it was interesting to note that the level of the periplasmic cytochrome c_{549} did not parallel the enhanced dilution rate recorded. When the population density was considered in those chemostat cultures, the following finding was made: as the dilution rate increased, the population density decreased, so did the synthesis of the membrane-bound cytochromes but again, the level of the periplasmic cytochrome c_{549} stayed relatively constant and was not affected by the population density in cultures where the metabolic products were constantly removed.

In batch cultures, the concentration of the periplasmic cytochrome c_{549} detected in cells grown in complex medium was consistently higher than in cells harvested from chemostat cultures indicating that one or more parameter(s) which governed the synthesis of the periplasmic cytochrome in chemostat cultures was/were not controlled in batch cultures. Still, in batch cultures, as the complexity of the growth media increased, the population density increased (as determined by optical density) (Table V). From this set of data, it could be seen that the cytochrome yield (measured as cytochrome concentration versus optical density of the culture) did not increase steadily as the optical density of the cultures was increasing. In other words, if a constant ratio (cytochrome synthesized/optical density) would have been observed in the various growth media, it would have meant that the population density was specifically regulating the amount of respiratory pigments synthesized, which was not found to be the case. Thus, even in batch cultures, other factor(s) but the population density specifically regulate the

Figure 4. Relationship between the cytochrome content in *A. haloplanktis* and the dilution rate of the cells when grown in complex medium (CM) and in chemically defined medium (CDM).

A. haloplanktis was grown at various fixed dilution rates in a chemostat under conditions of controlled pH and aeration. Whole cells were harvested when a steady state had been attained for at least five doublings of the respective populations. The population density was monitored frequently on diluted portions of the cultures. Harvested cells were fractionated and cytochromes assayed both in the periplasmic (c_{549}) and membrane fractions (c_{552}, b_{559}).

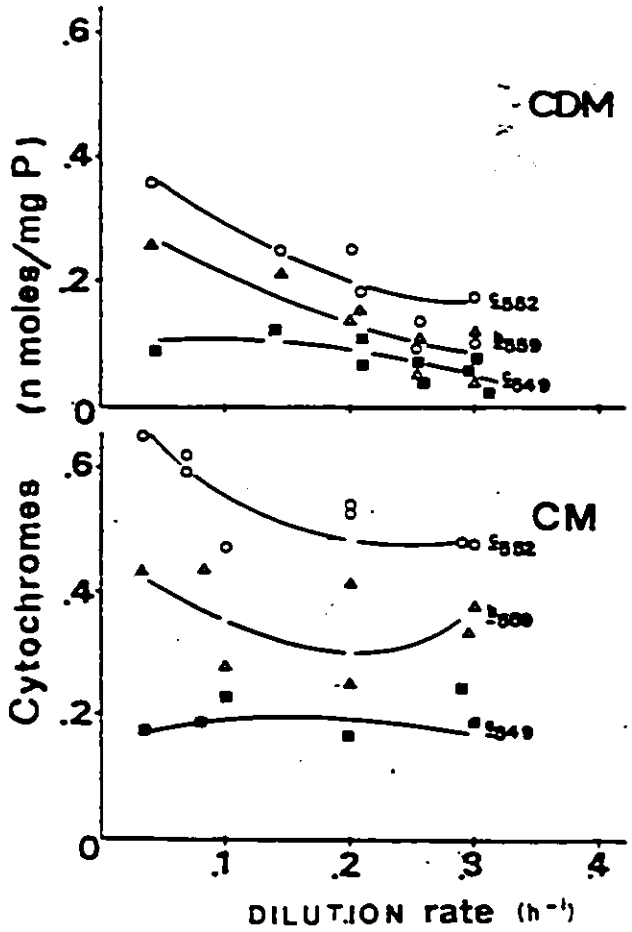

CDM
CM
Cytochromes (n moles/mg P)
.6
.4
.2
0
.1
.2
.3
.4
DILUTION rate (h⁻¹)

TABLE V. Cytochrome content and cytochrome yield of *A. hiloplanktis* harvested from various growth media of increasing population density in batch cultures.

Growth media succinate-CDM supplemented with:*	t_d	OD_{660}†	Cytochrome content			Cytochrome yield		
			c_{549}	c_{552}	b_{559}	$\frac{[c_{549}]}{OD}$	$\frac{[c_{552}]}{OD}$	$\frac{[b_{559}]}{OD}$
			n moles/mg protein					
none	150 min	.066	.016	.107	.116	.242	1.621	1.757
4 amino acids*	n.d.	.149	.021	.049	.049	.141	.329	.329
4 amino acids + nicotinic acid	115 min	.212	.344	.327	.274	1.615	1.535	1.286
6 amino Acids*	n.d.	.257	.063	.195	.204	.245	.759	.794
6 amino acids + nicotinic acid	117 min	.271	.428	.504	.447	1.579	1.860	1.649
1% Bacto Peptone	62 min	.432	.029	.161	.136	.067	.373	.315
1% Bacto Soytone	63 min	.475	.243	.511	.396	.512	1.076	.834
1% Yeast Extract	n.d.	.560	.338	.231	.139	.604	.413	.248
1% Casein Hydrolysate (vit.-and salt-free)	64 min	.624	.563	1.187	.835	.902	1.902	1.338
COMPLEX MEDIUM	35 min	.752	.474	.361	.306	.630	.480	.407

†Optical density values of the stationary phase cells were measured on diluted samples (1:10) at 660 nm in 1 cm cuvette light path with a Gilford spectrophotometer (Model 300-N).

*The mixtures of 4 and 6 amino acids were as specified in TABLE IV.

n.d. not determined

t_d doubling time

synthesis of cytochromes in A. *haloplanktis*. In addition, there is no obvious correlation between the doubling time and the amount of cytochrome synthesized. For instance, approximately 117 min are required for the bacterial population to double in succinate-CDM amended with 6 amino acids + nicotinic acid producing in average .428 n moles c_{549}/mg protein, whereas the cells growing in Bacto Peptone take half this doubling time to multiply but produce only .029 n moles c_{549}/mg protein. The same trend is expressed by the membrane-bound cytochromes c_{552} and b_{559}.

4. CONCLUSION

The cytochrome system of A. *haloplanktis* is basically composed of five cytochromes: 2 *b*-types and 2 *c*-types firmly attached to the membrane together with a *c*-type cytochrome located in the periplasm. In this section, experimental evidence was presented to indicate that the level of the cytochromes synthesized by the marine pseudomonad are governed by the presence of various nutrients in the growth medium. The greatest variation in terms of cytochrome concentration was expressed by the periplasmic cytochrome c_{549}.

As a general trend, the low content of the periplasmic cytochrome in the chemically defined medium contrasts with the relatively high content of membrane-bound cytochromes. As the complexity of the growth medium increases, the ratio is inverted and the synthesis of the periplasmic cytochrome is favoured. From our findings, a synthetic mixture composed of four amino acids mainly tyrosine, cystine, glycine and tryptophan plus nicotinic acid was effective in stimulating the synthesis of the periplasmic cytochrome.

The exact function(s) of the promoting factors have not been elucidated. However, there appears to be two alternatives: either they acted as a direct source of specific precursors for synthesis of the cytochromes or were degraded to form specific precursors. Conceivably, when the cells are grown in chemically defined medium where the concentration of organic-N is zero, the cells may be limited by the ability to synthesize these specific nutrients. Although the routes of degradation of the various components are not known in this bacterium, they have been elucidated in other organisms (Greenberg 1969a; Rodwell, 1969; Massey *et al.*, 1976). Most amino acids, for instance, are degraded to intermediates of the TCA cycle: α -ketoglutarate, succinate, fumarate or oxaloacetate.

While there are no data on the biosynthesis of amino acids in A. *haloplanktis*, the pathways operative in other bacteria are well

documented (Greenberg, 1969b; Rodwell, 1969; Hermann and Somerville, 1983). The major amino acids are synthesized by three routes, from α-ketoglutarate, oxaloacetate and pyruvate. All four amino acids which stimulate the synthesis of the periplasmic cytochrome belong to the pyruvate group. It appears also that a common precursor for all is phosphoenol-pyruvate (PEP). It is also worth noting that an amino acid such as glycine is vitally important in the synthesis of a cytochrome molecule *i.e.* at the first step of the biosynthetic pathway involving the condensation of 8 molecules of glycine and 8 molecules of succinic acid (Lascelles, 1961; Franck, 1979; Franck *et al.*, 1980). Since the chemically defined medium used in this study employs succinate as sole carbon and energy source, it seems likely that the synthesis of the tetrapyrrole-iron linked compounds is dependent on the availability of the carbon source in addition to the selected amino acids. Nicotinic acid on the other hand is primarily involved in the formation of pyridine nucleotide coenzymes and thus, of importance in the synthesis of respiratory proteins (Preiss and Handler, 1958).

Our experimental results do not allow us to conclude on the specific nature of the enzymic system involved in the induction or stimulation of the periplasmic cytochrome synthesis. Certainly, the synthesis of this cytochrome is intimately linked to growth parameters, such as concentration of nutrients, pH, phase of growth and partial pressure of oxygen.

The data support the possibility that one of the functions of the periplasmic respiratory protein may be to re-establish the balance between the bulk of the respiratory proteins synthesized in various growth environments, particularly so in growth environments where end metabolic products accumulate. As a consequence, the functional organization of the (aerobic) respiratory chain of *A. haloplanktis* might be expected to vary upon modification of the growth environment.

CHAPTER III

OXYGEN AFFINITY SYSTEMS

1. INTRODUCTION

The prokaryotes are capable of remarkable adaptation to facilitate growth when exposed to a variety of environmental changes (van Verseveld, 1979; Ingledew and Poole, 1984). The availability of dissolved oxygen is one environmental factor that can force bacteria to alter the composition of their respiratory system. For that reason, branched respiratory systems have been formed in several bacterial species (Smith, 1968; Jones, 1977; Knowles, 1980a). In most instances, each branch was terminated by a specific cytochrome oxidase (Degn *et al.*, 1978). Some oxidases have been shown to operate at very low oxygen concentrations: cytochrome a_1, cytochrome a_2 in *Escherichia coli* (Rice and Hempfling, 1978), cytochrome a_1 in *Haemophilus parainfluenzae* (White, 1963a); others preferentially operate when excess dissolved oxygen prevails in the growth medium such as cytochrome *c* in *Escherichia coli* (Rice and Hempfling, 1978). No upper or lower limit of critical oxygen concentration has been defined in a growing culture (Harrison, 1976b). The only practical technique for measuring low oxygen tensions in suspensions of cells or subcellular particles is polarography (Harrison, 1976b). This technique has the advantages of being simple and rapid. Experiments conducted in a closed system must however be of short duration and involve slow rates of respiration. Weston *et al.*, (1974) have obviated this problem by measuring the affinity for O_2 using various starting O_2 concentrations and determining K_m values by plotting initial uptake rates against initial oxygen concentration. If initial rates are not used the dissolved oxygen concentration changes during the experiment (Degn and Wohlrab, 1971). Linton *et al.*, (1977) were able to

measure K_m values for oxygen with an order of magnitude lower than the values reported by Weston *et al.*, (1974) using an "open" system in which oxygen is supplied continuously at a controlled rate while the dissolved oxygen tension is changed gradually in the reaction chamber. More recent studies concerned with the measurement of the O_2 affinity of bacterial oxidase systems have used O_2 carriers such as oxyleghaemoglobin and oxymyoglobin to supply very low dissolved concentrations of oxygen (Bergersen and Turner, 1979,1980). Examples of apparent affinity constants for oxidases toward the final electron acceptor, molecular O_2, are given in Table VI. The results presented in Table VI basically mean that the bacterial apparent affinity constants for oxygen (K_m) fall into four categories when defined by their order of magnitude: very low K_m values (under 0.01 µM), low K_m values (up to 1 µM), average K_m values (between 1 and 10 µM) and high K_m values (above 10 µM). In all the examples reported, except for *Azospirillum brasilense*, a minimum of two K_m values of different magnitude were recorded and accounted for at least two different functional terminal oxidases. Mitochondrial systems also exhibit a biphasic affinity system depending on the energy state: a K_m of 0.5 µM is expressed by coupled mitochondria, 0.05 µM for uncoupled mitochondria (Degn and Wohlrab, 1971).

We must understand however the conditions in which the microorganisms are grown. For instance, the values of K_m for O_2 tend to be lower in continuous cultures than in batch cultures. It was shown in continuous cultures that carefully controlled low levels of O_2 elicited the synthesis of multiple bacterial oxidases (Rice and Hempfling, 1978; Bergersen and Turner, 1980).

The respiration of biological systems at low oxygen concentration usually shows saturation kinetics and agrees with the Michaelis-Menten model of an enzyme reaction (White, 1963a,b; Harrison, 1976a; Weston *et al.*, 1974). Decreased electron flow to the oxidases by using submaximal substrate concentration (White, 1962) or by using

TABLE VI. Examples of apparent affinity constants for microbial oxidases towards the final electron acceptor, molecular O_2.

Micro-organism	K_m values (μM)					REFERENCES
	very low	low		average	high	
Beneckea natriegens				3.0	16.0	Weston *et al.*, 1974.
" "		0.15-0.25				Linton *et al.*, 1977.
Azotobacter vinelandii		0.48			35.0	Bergensen and Turner, 1980.
Klebsiella pneumoniae		0.11			25.0	Bergensen and Turner, 1980.
Acetobacter suboxydans				2.9	33.0	Daniel, 1970.
Escherichia coli	0.024	0.20				Rice and Hempfling, 1978.
Azospirillum brasilense	0.006					Bergensen and Turner, 1980.
Paracoccus denitrificans		0.35; 0.75;	0.85-1.0	4.33; 4.5		Henry and Vignais, 1979.
Rhizobium japonicum (bacteroids)	0.006; 0.015-0.028		0.17-0.2; 1.4			Bergensen and Turner, 1980.
Saccharomyces cerevisiae			0.73	2.84; 3.4		Johnson, 1967.
(MITOCHONDRIA)	0.05		0.5			Degn and Wohlrab, 1971.

respiratory inhibitors (Henry and Vignais, 1979) raises the critical oxygen concentration (the point at which respiratory activity is directly dependent on the oxygen present in the reaction medium). and thus raises the K_m.

To obtain a better knowledge of the dependency of the respiratory process on the concentration of its primary reactant, molecular O_2, the respiratory rates at various initial O_2 concentrations were measured in *A. haloplanktis*. These studies were designed to give some indication of the degree of branching of the aerobic electron pathway.

2. MATERIALS AND METHODS

Cultural Conditions and Preparation of Cell Fractions

The organism used in this study was the marine pseudomonad, *Alteromonas haloplanktis* strain 214, (ATCC 19855, NCMB 19) variant 3 (Gow *et al.*, 1973). The organism was grown for 16 hours at 25°C with vigorous aeration on a rotary shaker (200 rpm) in a complex broth containing 0.8% nutrient broth (Difco), 0.5% yeast extract (Difco), 0.3M NaCl, 0.026M $MgSO_4 \cdot 7\ H_2O$ and 0.01M KCl. The stationary phase cells were harvested by low speed centrifugation (16,000 xg for 10 min, Sorvall RC2-B, rotor GSA, 4°C) and washed twice in a physiological salt solution containing 0.3M NaCl, 0.05M $MgCl_2$, 0.01M KCl. The supernatant was removed by aspiration and the whole cell pellet resuspended in 20 ml of a buffered salt solution containing: 50 mM Tris-HCl (pH 7.2), 0.3M NaCl, 0.05M $MgCl_2$ and 0.01M KCl. Cell-free extracts were subsequently obtained by disrupting the intact cells in a French Pressure cell operating at 12-16,000 psi. (Power Laboratory Press, American Instrument Co.). The resulting suspension of crude extracts and cell debris was centrifuged (39,000 xg for 20 min, Sorvall RC2-B, rotor SS34, 4°C). The cell-free extract was further ultracentrifuged (150,000 to 180,000 xg for 3 hours, Beckman L2-65B, rotor 60Ti, 4°C) to obtain the (sedimented) membrane fraction and the (supernatant) periplasmic soluble fraction (Knowles *et al.*, 197-), hereafter referred to as the periplasmic fraction.

Protein Determination

The protein content of all the preparations, whole cells, cell-free extracts, membrane and periplasmic fractions was estimated by a Biuret method (Gornall *et al.*, 1949) using lysozyme (1 to 10 mg protein/ml, Sigma Chem. Co.) as the standard protein. Absorbancy was recorded at 540 nm using a Gilford spectrophotometer 300-N.

Measurement of Oxygen Consumption and Concentration

The kinetics of O_2 consumption of suspensions of whole cells and subcellular fractions as a function of dissolved oxygen concentration were determined by the use of a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, England). The reaction chamber containing a 50 mM Tris-HCl buffered salt solution (pH 7.2) was fitted with a serum cap through which was inserted two syringes: one syringe introduced the gas mixture and the other the respiratory proteins and the oxidizable substrates. The procedure has been described in detail by Weston *et al.*, (1974). The initial respiration rates of the proteins in suspension were measured following the addition of the substrates. The final concentration of the substrates used with all the preparations tested were as follows: NADH (2.5 mM), succinate (10.0 mM) and ascorbate (1.5 mM) plus (1.0 mM) N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). Ascorbate + TMPD oxidase activities were corrected for small non-enzymatic rates due to autooxidation. All chemicals utilized were reagent grade from Sigma Chemical Company.

The desired starting oxygen concentrations were obtained by flushing the electrode chamber with appropriate mixtures of O_2-free nitrogen and compressed air. The oxygen concentrations studied ranged from air-saturation (≈ 240 μM O_2, at 25°C) down to 1μM O_2, the lowest limit of detection of the O_2 electrode employed (Weston *et al.*, 1974; Linton *et al.*, 1977). The *apparent* K_m for oxygen was obtained from Lineweaver - Burk plots tracing the reciprocal of the respiratory rate 1/V (n atoms O/min/mg protein) *versus* the reciprocal of the (medium) dissolved oxygen concentration $1/O_2$ (mM). The levels of oxygen concentrations were allowed to stabilize before the addition of respiratory proteins and oxidizable substrates, both maintained under O_2-depleted conditions by a constant supply of O_2-free nitrogen gas. For further analysis of the kinetics of respiration, the reader is referred to the publication of Bergersen and Turner, 1980.

Statistical Analysis

In the present study, the data were subjected to statistical analysis. Double reciprocal plots (1/V *versus* 1/S) were drawn and the slopes and intercepts of the lines were calculated using groups of results for which linear correlation coefficients (r) were highly significant ($P < 0.05$; $P < 0.01$). The apparent affinity constant, K_m, was then estimated for every system studied. The statistical analysis was done on paired values of the variable x (the reciprocal of V, the dissolved oxygen concentration) and y (the reciprocal of S, the respiration rate) above and below 10uM O_2 to see how closely the equation ($y = a + bx$) fits the experimental data. All the calculations were performed with a programmable Hewlett-Packard HP-25 calculator.

Growth Media and Growth Conditions

A. Final Electron Acceptor

Alteromonas haloplanktis was grown in the complex medium described previously with and without $SO_4^=$ (26mM) or with and without NO_3^- (7.17 mM). The magnesium sulfate in the medium was replaced by magnesium chloride.

In the chemically defined medium, the ammonium sulfate, the magnesium sulfate and the ferrous ammonium sulfate were replaced by ammonium chloride, magnesium chloride and ferric chloride. Cysteine (10mM) was added to the medium depleted of sulfate. Bacto agar (1.5% (w/v), final concentration) was added to the liquid media for the experiments on solid media.

The inoculated solid media were incubated in air, under an atmosphere enriched in CO_2 and in an $H_2 + CO_2$ atmosphere provided by a generator system (BBL GasPak, Becton Dickinson and Co.) at 25°C. The corresponding broth cultures contained in 50 ml-side arm-flasks were shaken at 200 rpm in air or evacuated and flushed 3 times with one atmosphere of N_2 or He

(Liquid Carbonic Canada Ltd.). Growth was monitored by following the increase in optical density at 660 nm (Junior Coleman spectrophotometer) at daily intervals for a period of 10 days. The starter culture (stationary phase cells grown in complex medium) had been aseptically washed twice in sterile salt solution before being inoculated into the various media; the inoculum size was maintained at 2%. Two separate experiments were run in duplicate.

B. Oxidation-Fermentation (OF) Test

Commercially available Bacto OF Basal medium (Difco Laboratories, Detroit, Michigan) was utilized according to the procedure outlined in the Manual of Clinical Microbiology (Lennette *et al.*, 1980). To 100 ml of sterile medium was aseptically added 10 ml of sterile 10% sugar solution of dextrose, lactose, or saccharose. After thorough mixing, 5 ml aliquots were dispensed into sterile culture tubes. Soft agar (0.3% final concentration) had previously been added to the liquid medium, according to direction of the manufacturer. The OF basal medium was prepared either in complete salt solution or in distilled water, as were the stock solutions of sugars. The inoculated tubes were examined for the presence or absence of growth after 24, 48 and 72 hours; the results shown were recorded after 72 hours of incubation at 25°C or 37°C. A facultative anaerobe *Escherichia coli* (Macdonald Culture Collection, No: 85) was chosen as control organism. The inoculated soft agar media were incubated in ambient air to meet aerobic conditions or covered with a film of sterile paraffin oil to maintain anaerobic conditions. Two separate experiments were run in duplicate.

3. RESULTS AND DISCUSSION

Oxidizable Substrates at Various Oxygen Concentrations

In order to test whether more than one respiratory pathway existed and was expressed by more than one affinity system in the aerobic marine bacterium, we conducted experiments with intact cells and subcellular fractions in which we measured the rate of oxygen consumption at various initial dissolved oxygen concentrations.

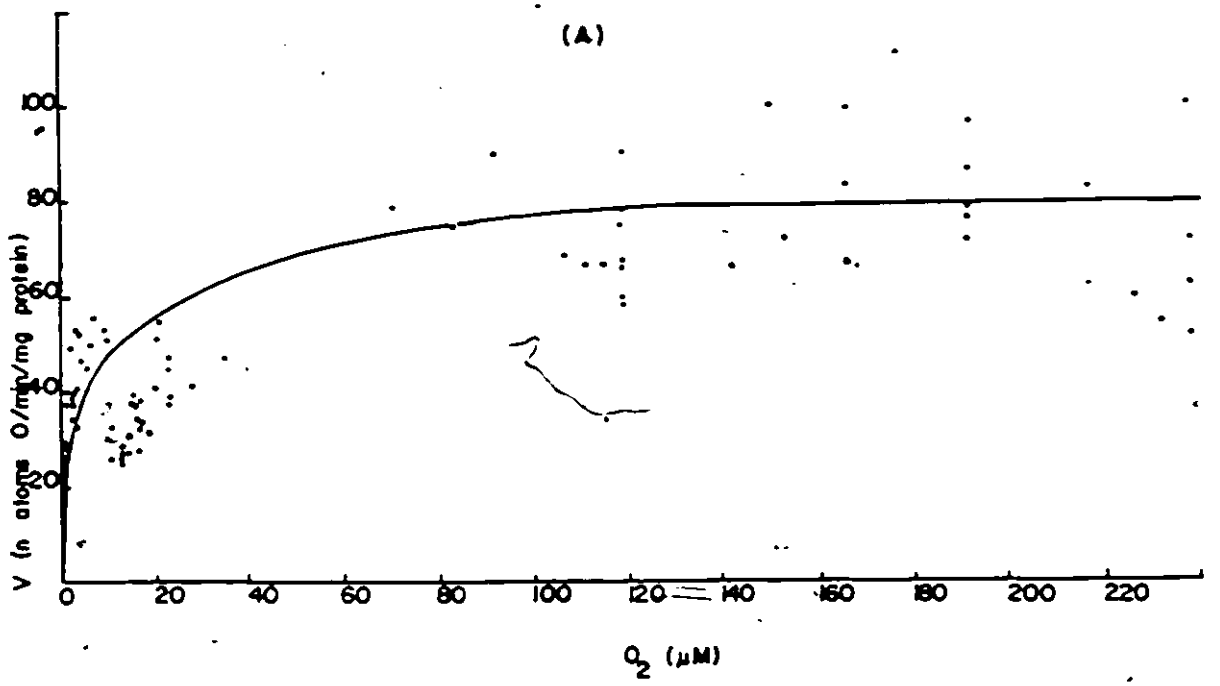
Analysis of the dependency of the respiratory rate upon the oxygen concentration revealed that *i*) the respiration of intact cells with endogenous substrates as well as exogenous substrates (*e.g.* NADH) showed Michaelis-Menten saturation kinetics when reducing the final electron acceptor, molecular oxygen (Figure 5A,B); *ii*) similarly, the oxidation of NADH by the cell-free extracts and the membrane particles showed the same saturation kinetics (Figure 6A,B); *iii*) the artificial electron donor ascorbate coupled to the dye TMPD was oxidized by the membrane-bound as well as by the periplasmic fraction in a similar fashion via saturation kinetics (Figure 7A,B).

The experimental data were analyzed above and below the dissolved oxygen concentration of 10 µM due to constraints imposed by the technique in use. To measure the oxygen consumption below 10 µM, an additional resistance was added to the recorder to increase the sensitivity of the measuring device. To obviate the obvious discrepancies resulting from plotting together all the values, below and above 10 µM O_2, the results are best understood if they are discussed separately than compared among themselves.

Additional information could be gained by tracing the double reciprocal plots to obtain the affinity constants (K_m) of the corresponding systems. The reciprocal plots presented in Figures 8 to 10 were obtained from the experimental data used to trace the saturation curves (Figures 5 to 7). The K_m values derived from the reciprocal plots

Figure 5. Effect of O_2 concentration on the rate of O_2 uptake by intact cells of *Alteromonas haloplanktis* oxidizing (A) endogenous reserves; (B) NADH.

Alteromonas haloplanktis was grown in complex medium to the stationary phase of growth. The cells were harvested, washed and resuspended in buffered salt solution as specified in Materials and Methods. The oxidase activities were measured in an O_2 electrode cell at various initial oxygen concentrations. The results are expressed by the Michaelis-Menten curves: V(n atoms O/min/mg protein) *versus* S (O_2 concentration, µM).


(A)
V (n atoms O/min/mg protein)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
220
O_2 (μM)

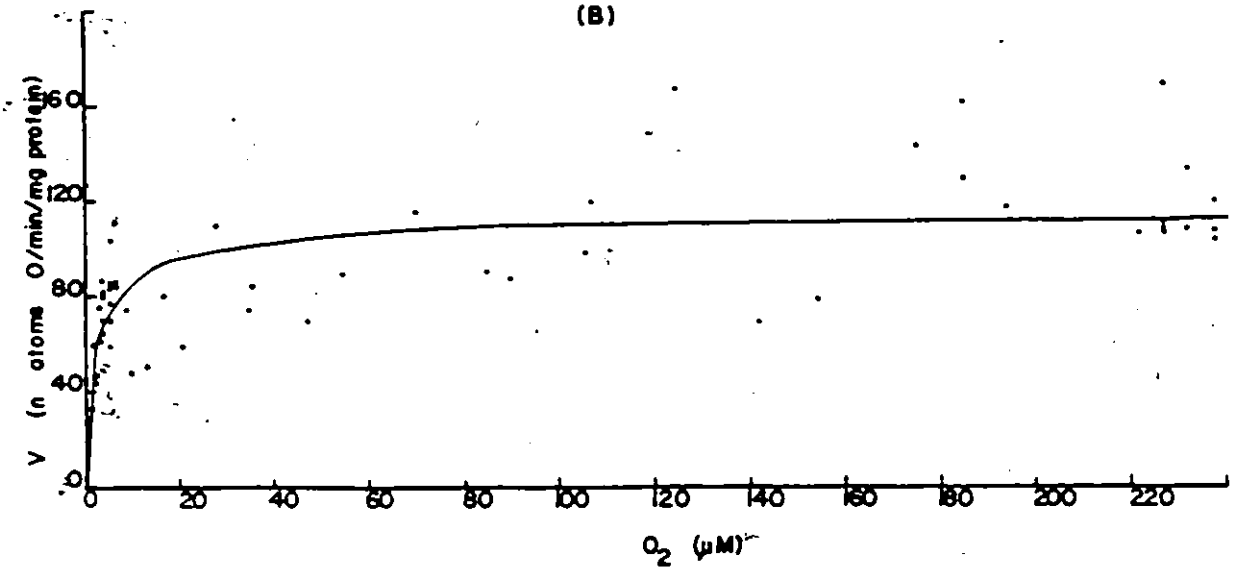
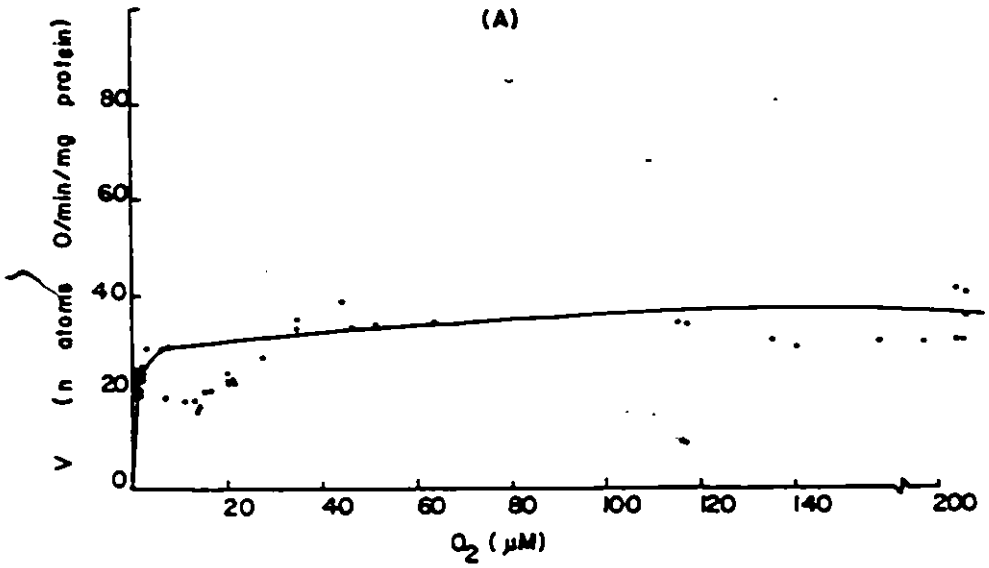

(B)
V (n atoms O/min/mg protein)
160
120
80
40
0
0
20
40
60
80
100
120
140
160
180
200
220
O_2 (μM)

Figure 6. Effect of O_2 concentration on the rate of O_2 uptake by (A) cell-free extracts and (B) membrane particles of *Alteromonas haloplanktis* oxidizing NADH.

Alteromonas haloplanktis was grown in complex medium to the stationary phase of growth. The cells were harvested, washed, resuspended in buffered salt solution and fractionated into cell-free extracts and membrane particles as specified in Materials and Methods. NADH oxidase activities were measured in an O_2 electrode cell at various initial oxygen concentrations. The results are expressed by the Michaelis-Menten curves: V (n atoms O/min/mg protein) *versus* S (O_2 concentration, μM).


(A)
V (n atoms O/min/mg protein)
0
20
40
60
80
20
40
60
80
100
120
140
200
O_2 (μM)

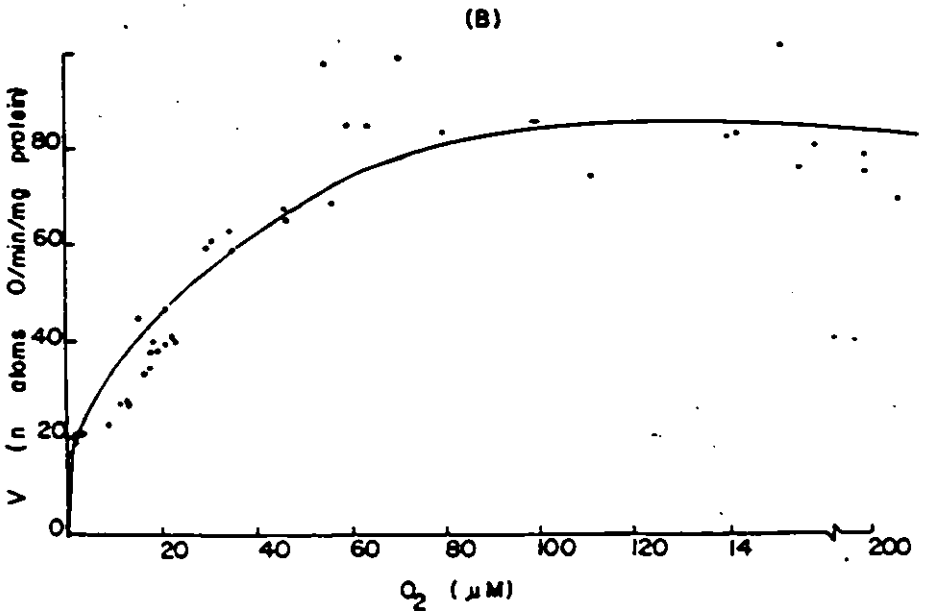
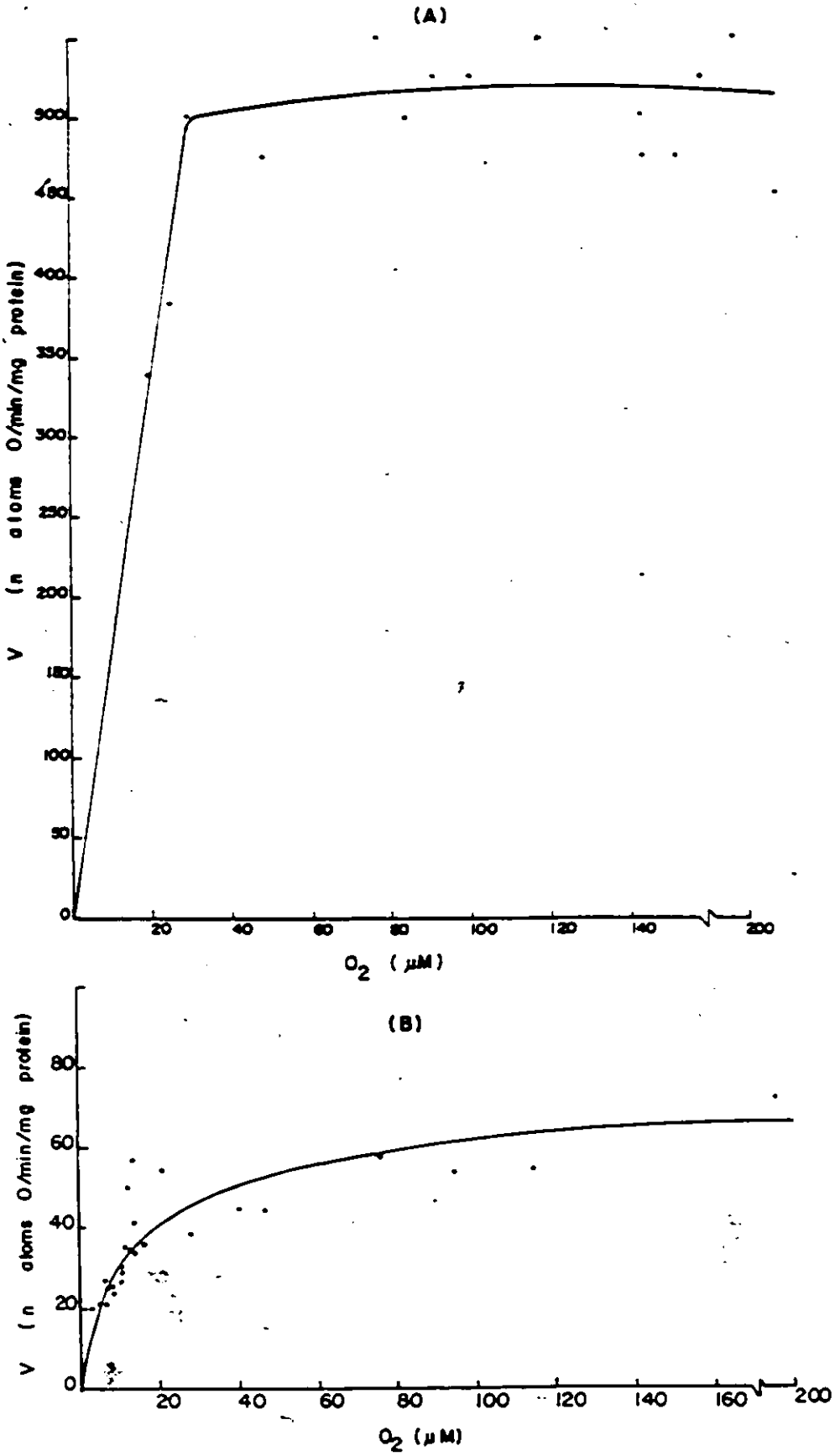

(B)
V (n atoms O/min/mg protein)
0
20
40
60
80
20
40
60
80
100
120
14
200
O_2 (μM)

Figure 7. Effect of O_2 concentration on the rate of O_2 uptake by (A) the membrane and (B) the periplasmic fraction of *Alteromonas haloplanktis* oxidizing ascorbate/TMPD.

Alteromonas haloplanktis was grown in complex medium. Cell-free extracts were prepared from stationary phase cells and fractionated into membrane and periplasmic fractions. Ascorbate/TMPD oxidase activities were measured in an O_2 electrode cell at various initial oxygen concentrations. The results are expressed by the Michaelis-Menten saturation curves: V (n atoms O/min/mg protein) *versus* S (O_2 concentration, µM).


(A)
V (n atoms O/min/mg protein)
0
50
100
150
200
250
300
350
400
450
500
20
40
60
80
100
120
140
200
O_2 (μM)
(B)
V (n atoms O/min/mg protein)
0
20
40
60
80
20
40
60
80
100
120
140
160
200
O_2 (μM)

were calculated by the method of least squares. In this analysis, the calculated regression constants a and b correspond respectively to the intercept with the y axis and the slope of the line in the equation $y = a + bx$, where x and y represented the reciprocals of V and S. The linear correlation coefficients (r) of the reciprocal plots were calculated and were found to be highly significant, indicating a close relationship between the experimental data and the linear regression constants as shown in Table VII.

For each of the substrates tested, the experimental results (respiratory rate as a function of oxygen concentration) were analyzed by means of double reciprocal plots. In the case of whole cells (Figure 8A), endogenous substrates were oxidized with biphasic kinetics (K_m values of 1.2 µM and 25.6 µM respectively). K_m values of 4.5 and 12.6 µM were recorded when the intact cells were oxidizing the exogenous substrate NADH (Figure 8B). The cell-free extracts also expressed biphasic kinetics when oxidizing NADH (Figure 9A): K_m values of 0.51 µM and 15 µM were obtained at oxygen concentrations below and above 10 µM O_2 respectively. The corresponding values of K_m for the membrane fraction oxidizing the same substrate were 0.40 and 31.2 µM (Figure 9B). With respect to the non-physiological substrate, ascorbate/TMPD, it was actively oxidized by both the membrane and the periplasmic fractions. One low affinity system with a K_m value of 8.1 µM was obtained with the membrane fraction (Figure 10A) and one high affinity system with a K_m value of 0.85 µM was recorded with the periplasmic fraction (Figure 10B). Since no accurate determination of oxidase activity with ascorbate/TMPD could be obtained below 10 µM of dissolved oxygen, it would be premature to conclude that no high affinity system is present in the membrane fraction. The K_m and V_{max} values determined for all the enzyme systems investigated are presented in Table VIII.

In separate experiments, whole cells and cell-free extracts were also shown to oxidize ascorbate/TMPD at various oxygen

TABLE VII. Statistical analysis of the kinetics of respiration of *Alteromonas haloplanktis*.

Cell fraction	Electron donor	Threshold	Regression constants* a	b	Correlation coefficients (r)** from experiments		from table†
Whole cells	Endogenous	> 10uM O_2	0.0117	0.0003	0.9281	(P.05)	0.273
						(P.01)	0.354
		< 10uM O_2	0.0169	0.0000207	0.9064	(P.05)	0.444
						(P.01)	0.561
Whole cells	NADH	> 10uM O_2	0.0079	0.0001	0.8572	(P.05)	0.349
						(P.01)	0.449
		< 10uM O_2	0.0068	0.0000310	0.9241	(P.05)	0.404
						(P.01)	0.515
CFE	NADH	> 10uM O_2	0.0262	0.0004	0.9121	(P.05)	0.367
						(P.01)	0.470
		< 10uM O_2	0.0327	0.0000169	0.8778	(P.05)	0.514
						(P.01)	0.641
Membrane fraction	NADH	> 10uM O_2	0.0096	0.0003	0.9396	(P.05)	0.349
						(P.01)	0.449
		< 10uM O_2	0.0414	0.0000166	0.9820	(P.05)	0.878
						(P.01)	0.959
Periplasmic fraction	Ascorbate/TMPD	< 10uM O_2	0.0445	0.000038	0.5282	(P.05)	0.468
						(P.01)	0.590
Membrane fraction	Ascorbate/TMPD	> 10uM O_2	0.0018	0.0000147	0.7812	(P.05)	0.497
						(P.01)	0.623

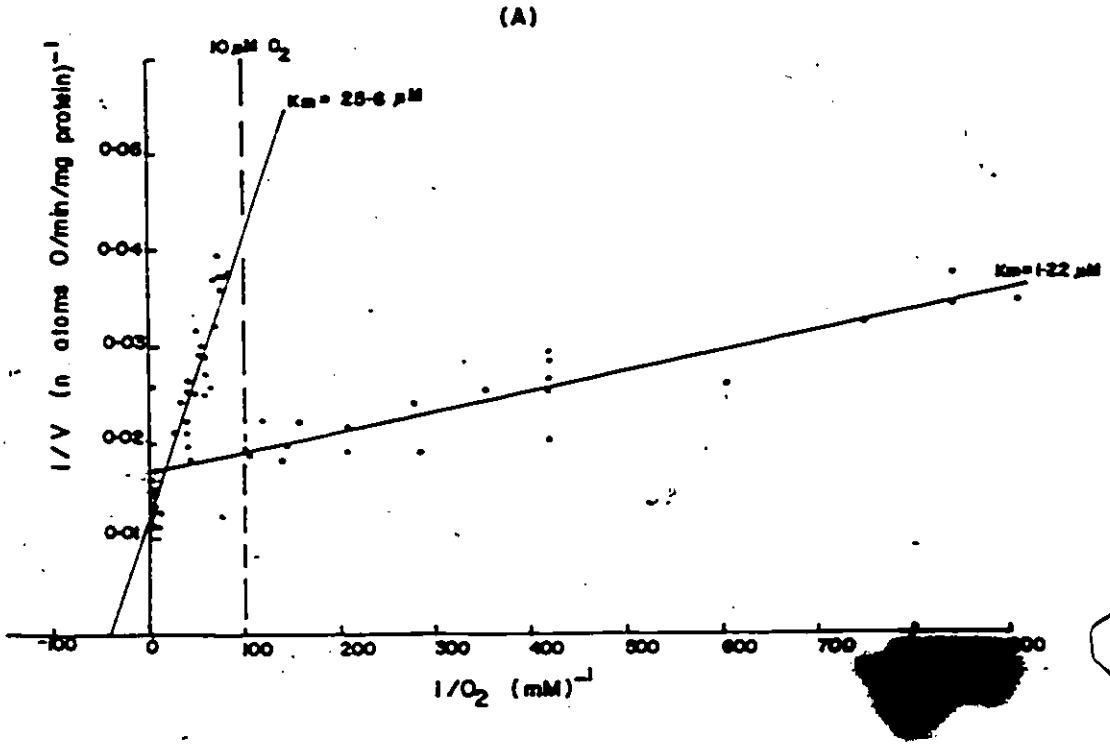
*According to the best fit curve $y = a + bx$, a and b are the regression constants and represent respectively the intercept with the y axis and the slope of the line.

**The correlation coefficient r for one variable; the respiratory rate was calculated from experimental data, and was found highly significant at P(.05) and P(,01).

†Table A.13 Steel, R.G.D., Torrie, J.H. Principles and Procedures of STATISTICS, with special reference to the Biological Sciences. McGraw-Hill Book Company, Inc., New York, 1960.

Figure 8. Lineweaver-Burk plot illustrating the effect of increasing O_2 concentration on the respiratory rate of intact *A. haloplanktis* cells oxidizing endogenous substrates (A) or NADH (B).

Same experimental protocol as in Figure 5. The results are expressed by a double reciprocal plot: $1/V$ (n. atoms O/min/mg protein)$^{-1}$ *versus* $1/O_2$ (mM)$^{-1}$. The apparent K_m values for oxygen were determined graphically by the method of Lineweaver and Burk (1934).


(A)
10 μM O2
Km = 25·6 μM
Km = 1·22 μM
1/V (n atoms O/min/mg protein)⁻¹
0·06
0·04
0·03
0·02
0·01
-100
0
100
200
300
400
500
600
700
800
1/O2 (mM)⁻¹

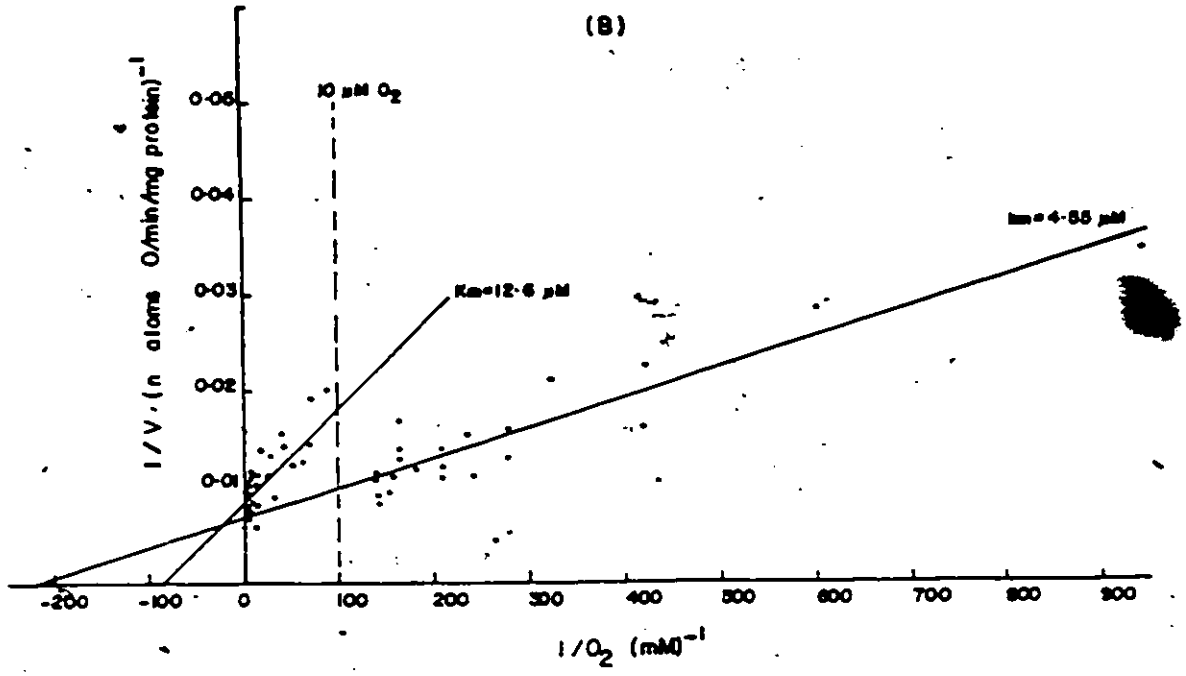
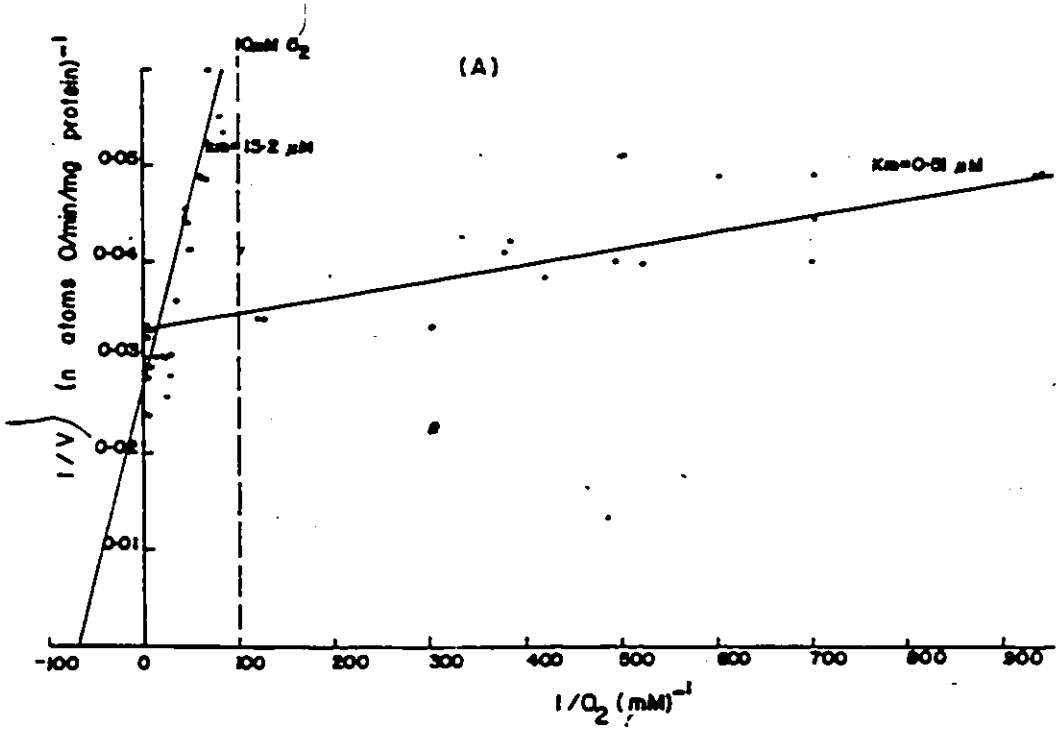

(B)
10 μM O2
Km = 4·55 μM
Km = 12·6 μM
1/V (n atoms O/min/mg protein)⁻¹
0·06
0·04
0·03
0·02
0·01
-200
-100
0
100
200
300
400
500
600
700
800
900
1/O2 (mM)⁻¹

Figure 9. Lineweaver-Burk plot illustrating the effect of increasing O_2 concentration on the respiratory rate of the cell-free extracts (A) and the membrane particles (B) of A. *haloplanktis* cells with NADH as substrate.

Same experimental protocol as in Figure 6. The results are expressed by a double reciprocal plot: $1/V$ (n atoms O/min/mg protein)$^{-1}$ *versus* $1/O_2$ (mM)$^{-1}$. The apparent K_m values for oxygen were determined graphically by the method of Lineweaver and Burk (1934).


(A)
10mM O_2
Km=15·2 μM
Km=0·61 μM
1/V (n atoms O/min/mg protein)$^{-1}$
0·05
0·04
0·03
0·02
0·01
-100
0
100
200
300
400
500
600
700
800
900
1/O_2 (mM)$^{-1}$

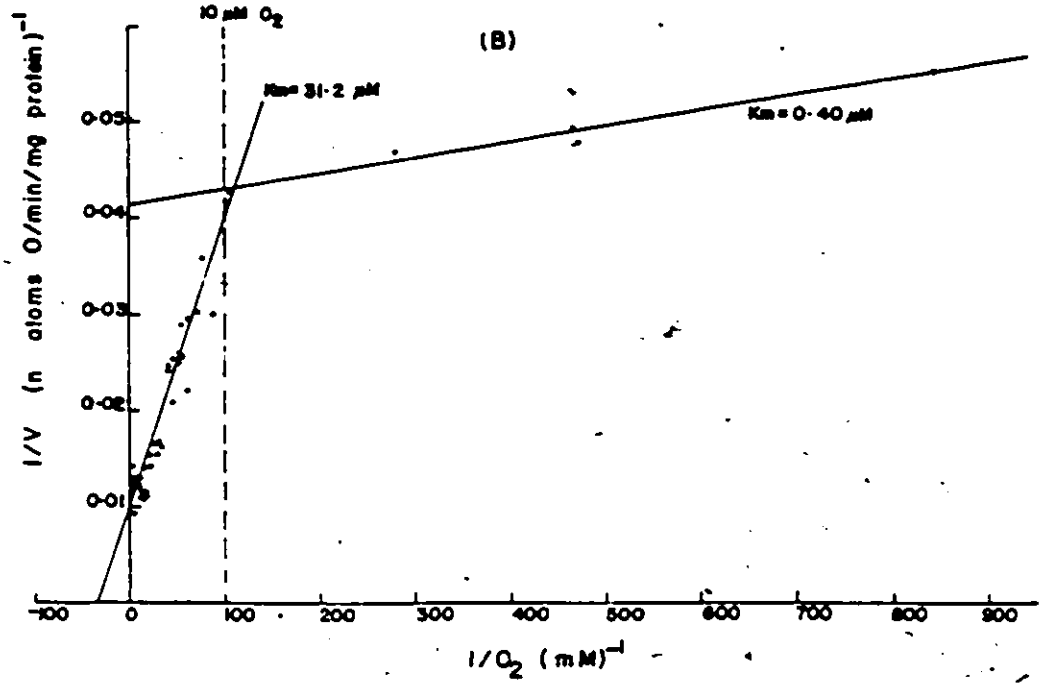
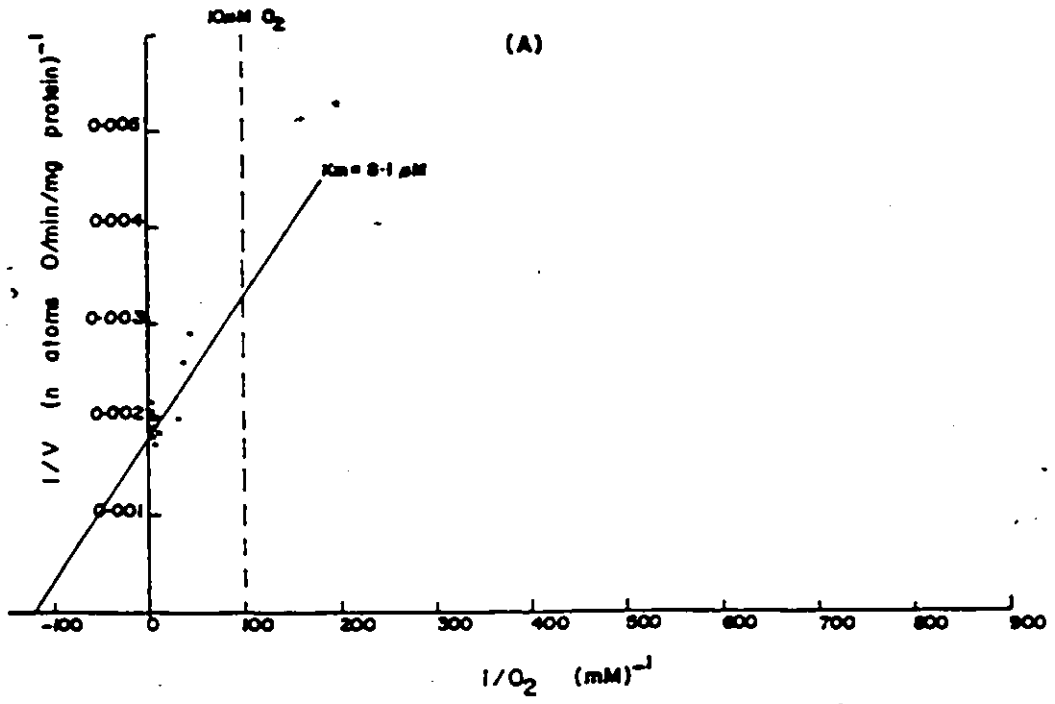

(B)
10 μM O_2
Km= 31·2 μM
Km = 0·40 μM
1/V (n atoms O/min/mg protein)$^{-1}$
0·05
0·04
0·03
0·02
0·01
-100
0
100
200
300
400
500
600
700
800
900
1/O_2 (mM)$^{-1}$

Figure 10. Lineweaver-Burk plot illustrating the effect of increasing O_2 concentration on the respiratory rate of the membrane fraction (A) and the periplasmic fraction (B) of A. *haloplanktis* cells with ascorbate/TMPD as substrate.

Same experimental protocol as in Figure 7. The results are expressed by a double reciprocal plot: $1/V$ (n atoms O/min/mg protein)$^{-1}$ *versus* $1/O_2$ (mM)$^{-1}$. The apparent K_m values for oxygen were determined graphically by the method of Lineweaver and Burk (1934).


(A)
10mM O_2
Km = 8·1 μM
0·006
0·004
0·003
0·002
0·001
1/V (n atoms O/min/mg protein)$^{-1}$
-100
0
100
200
300
400
500
600
700
800
900
1/O_2 (mM)$^{-1}$

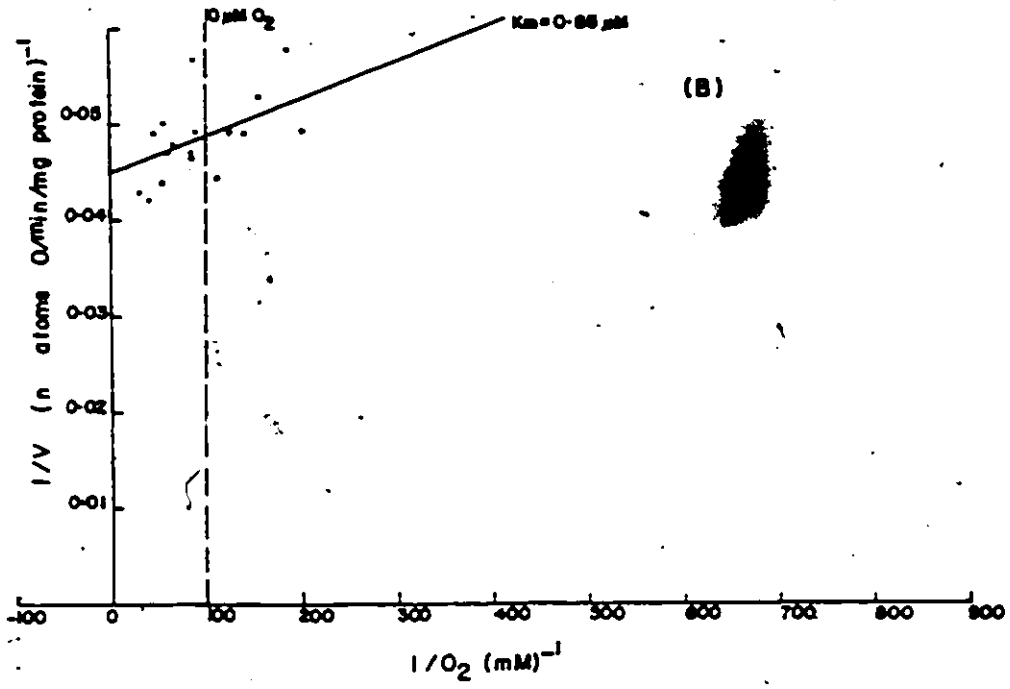

(B)
10 μM O_2
Km = 0·66 μM
0·05
0·04
0·03
0·02
0·01
1/V (n atoms O/min/mg protein)$^{-1}$
-100
0
100
200
300
400
500
600
700
800
900
1/O_2 (mM)$^{-1}$

TABLE VIII. Kinetic data of O_2-affinity systems in stationary phase cells of *Alteromonas haloplanktis* grown in complex medium.

Cell fraction	Electron donor	n*	apparent K_m (μM)		V_{max} (n atoms O/min/mg protein)	
			< 10μM O_2	> 10μM O_2	< 10μM O_2	> 10μM O_2
Whole cells	Endogenous	77	1.22	25.6	59.1	85.4
Whole cells	NADH	58	4.55	12.6	147.0	126.5
CFE	NADH	44	0.51	15.2	30.5	38.1
Membrane fraction	NADH	39	0.40	31.2	24.1	104.1
Periplasmic fraction	Ascorbate/TMPD	17	n.d.	0.85	n.d.	22.5
Membrane fraction	Ascorbate/TMPD	16	n.d.	8.1	n.d.	555.5

*represents the number of data points

n.d. not determined

concentrations. The number of determinations were not numerous enough to permit statistical analysis of the data and were for that reason omitted from the table. With intact cells, ascorbate/TMPD was oxidized very rapidly and at almost a constant rate irrespective of the oxygen concentration tested, indicating the possible presence of an extremely high affinity oxidase(s) with a K_m below the limit of detection of the polarographic technique utilized (K_m 0.1 μM, Degn and Wohlrab, 1971).

Although ascorbate/TMPD is a non-physiological substrate, its oxidation mediated by the cell-free extracts, the membrane and the periplasmic fraction is enzymatic in nature as demonstrated by its inhibition by the respiratory inhibitor, KCN, and by denaturation of the proteins at boiling temperature (Figures 11 and 12). (Note the oxygen consumption expressed in units/min and not in units/min/mg protein to permit the inclusion of the data recorded with ascorbate/TMPD alone.) The substrate was added to the respective fraction in the buffered salt solution and initial rates of oxygen consumption were determined polarographically (Figures 11 and 12, curve 1). When 10 mM KCN was allowed to incubate for a few minutes with the fractions before the substrate was admitted into the reaction chamber, oxidation was prevented to the extent of the autooxidation value observable when ascorbate/TMPD was mixed without further addition of respiratory proteins (Figures 11 and 12, curves 2,3). As expected, the fractions boiled for 5 minutes (curves 4,5) could not enzymatically oxidize the non-physiological substrate in the presence or absence of 10 mM KCN.

Final Electron Acceptor

Growth in living cells requires a continuous supply of energy obtained from a series of well controlled oxidation reactions. The final electron acceptor in this series of redox reactions determines the kind of respiratory chain existing in a given organism. Bacteria able to grow under anaerobic conditions make use of organic and/or inorganic final electron acceptors. Strict aerobes are confined to

Figure 11. Effect of KCN and boiling on the oxidation of ascorbate/TMPD by the particulate fraction of *Alteromonas haloplanktis*.

Cell-free extracts were prepared from stationary phase cells of A. *haloplanktis* grown in complex medium and fractionated into particulate and soluble fractions. The particulate fraction (0.63 mg protein) was tested for the presence of ascorbate/TMPD oxidase activity using an O_2 electrode cell at various initial oxygen concentrations. The enzyme activity was estimated in the absence and presence of 10 mM KCN (curves 1,2); autooxidation of the dye TMPD was also measured in the presence of the substrate ascorbate (curve 3). The activity of the particulate fraction after boiling, in the presence or absence of 10 mM KCN is shown in curves 4 and 5. The respiratory rates are expressed in n atoms O/min to permit inclusion of the data on ascorbate/TMPD alone. The initial oxygen concentrations in the reaction chamber are expressed both in terms of μM and % air saturation. The substrate concentration tested was (10 + 0.5 mM).

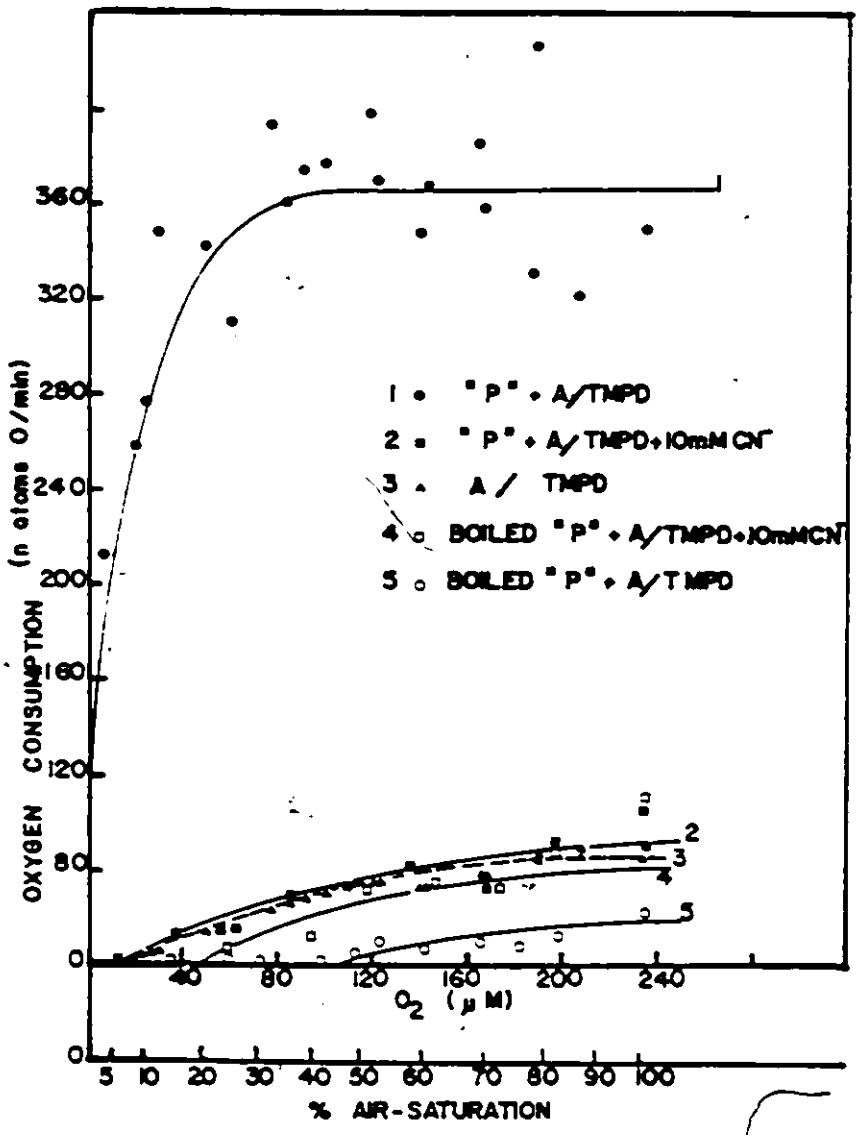
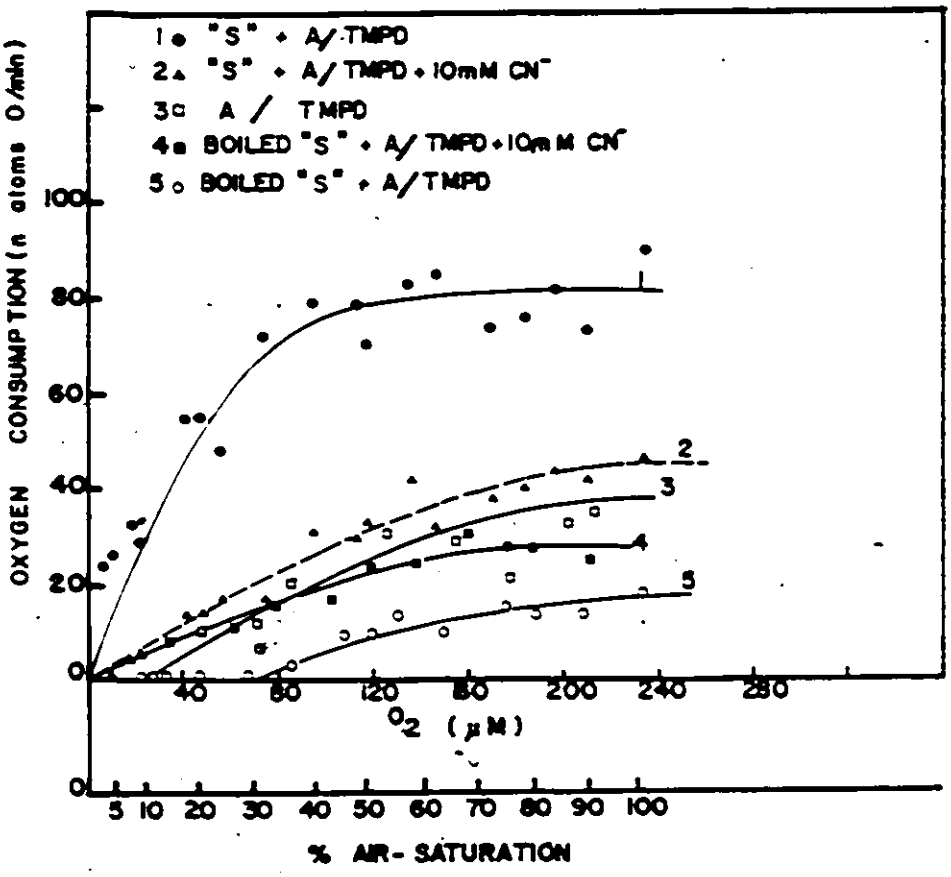

OXYGEN CONSUMPTION (n atoms O/min)
360
320
280
240
200
160
120
80
0
1 • "P" + A/TMPD
2 ▪ "P" + A/TMPD+10mM CN⁻
3 ▲ A / TMPD
4 ○ BOILED "P" + A/TMPD+10mMCN⁻
5 ○ BOILED "P" + A/TMPD
40 80 120 160 200 240
O_2 (μM)
0
5 10 20 30 40 50 60 70 80 90 100
% AIR-SATURATION

Figure 12. Effect of KCN and boiling on the oxidation of ascorbate/TMPD by the soluble fraction of *Alteromonas haloplanktis*.

Cell-free extract was prepared from stationary phase cells of *A. haloplanktis* grown in complex medium and fractionated into particulate and soluble fractions. The soluble fraction (1.2 mg protein) was tested for the presence of ascorbate/TMPD oxidase activity using an O_2 electrode cell at various initial oxygen concentrations. The enzyme activity was estimated in the absence and presence of 10 mM KCN (curves 1,2); autooxidation of the dye TMPD was also measured in the presence of the substrate ascorbate (curve 3). The activity of the soluble fraction after boiling, in the presence or absence of 10 mM KCN, is shown in curves 4 and 5. The respiratory rates are expressed in n atoms O/min to permit inclusion of the data on ascorbate/TMPD alone. The initial oxygen concentrations in the reaction chamber are expressed both in terms of μM and % air saturation. The substrate concentration used was (10 + 0.5 mM).


1 ● "S" + A/ TMPD
2 ▲ "S" + A/ TMPD + 10mM CN⁻
3 □ A / TMPD
4 ■ BOILED "S" + A/ TMPD + 10mM CN⁻
5 ○ BOILED "S" + A/ TMPD
OXYGEN CONSUMPTION (n atoms O/min)
0
20
40
60
80
100
1
2
3
4
5
40
80
120
160
200
240
280
O2 (μM)
0
5 10 20 30 40 50 60 70 80 90 100
% AIR- SATURATION

molecular oxygen as terminal electron acceptor (Knowles, 1980a).

To ascertain that *Alteromonas haloplanktis* is a strict user of molecular oxygen, the bacterium was grown with various electron acceptors on solid and liquid media and incubated under various gaseous atmospheres. The results of the experiments carried with solid media are reported in Table IX. The marine bacterium grew well in air on the complex and on the chemically defined media amended or not with sulfate or nitrate. Similarly, the micro-organism did grow in an atmosphere enriched with CO_2 (-4%). However, anaerobic growth under an atmosphere of $H_2 + CO_2$ was not recorded in any of the media tested, even after 10 days of incubation at optimal temperature. Upon exposure of these same plates to air, growth could be recorded within 48 hours of incubation at 25°C. The corresponding liquid media were shaken at 200 rpm in air and under an atmosphere of N_2 or He. No growth was detected in any of the media incubated under N_2 or He in the presence or absence of $SO^{=}_4$ or NO^{-}_3 after prolonged incubation (results not shown).

The marine bacterium was also screened for fermentative metabolism in the presence of lactose, dextrose (d-glucose), and saccharose (sucrose). *Escherichia coli* was chosen as the control-organism in these experiments. Both micro-organisms were tested for sugar utilization aerobically and anaerobically at their optimal temperatures. The sugars were dissolved in water or in complete salt solution. Results of the oxidation-fermentation (OF) tests are presented in Table X. Clearly, *Alteromonas haloplanktis* metabolized some of the sugars made up in physiological salt solution and under aerobic conditions. On the other hand, *Escherichia coli* utilized the sugars tested both aerobically and anaerobically, whether the sugars were dissolved in water or in complete salt solution. Production of gas was observed under anaerobic growth conditions with *E. coli*. Absence of growth was recorded in all the non-inoculated control tubes at both temperatures.

TABLE IX. Growth of *Alteromonas haloplanktis* under various gaseous atmospheres in the presence or absence of alternate electron acceptors.

Growth media	Alternate electron acceptor	Gas Atmosphere: Air (100%)	Carbon dioxide (-4%)*	Hydrogen (95%) plus carbon dioxide (5%)*
		Growth†		
Complex medium	none	++++	+++	-
	$SO_4^=$	++++	+++	-
	NO_3^-	++++	+++	-
Chemically defined medium	none	+++	+++	-
	$SO_4^=$	+++	+++	-
	NO_3^-	+++	+++	-

†luxuriant growth ++++; very good growth +++; good growth ++; visible growth +; absence of growth - .

*BBL Gas Generators were used for production of H_2+CO_2 and CO_2-enriched atmosphere.

TABLE X. Oxidation-Fermentation (OF) test in *Alteromonas haloplanktis* and *Escherichia coli*.

Organism	Incubation temperature	Incubation condition	Growth† in physiological salt solution of Lactose	d-Glucose	Sucrose	Growth† in aqueous solution of Lactose	d-Glucose	Sucrose
Alteromonas haloplanktis	25°C	Aerobic*	+++	+	-	-	-	-
		Anaerobic**	-	-	-	-	-	-
Escherichia coli	37°C	Aerobic*	+++	+++	+++	+++	++++	++++
		Anaerobic**	+++	+++	+++	++++	++++	++++

†Luxuriant growth ++++; very good growth +++; good growth ++; visible growth +; absence of growth -.

*Ambient air

**Under a film of sterile paraffin oil.

4. CONCLUSION

Well characterized bacterial electron transport chains of aerobes as well as facultative anaerobes exhibit more than one affinity system toward the final electron acceptor oxygen and harbor more than one cytochrome oxidase: one terminal oxidase operating under high concentration of the final electron acceptor, the other(s) operating under low or limiting concentration of the final electron acceptor. Combinations of up to four terminal oxidases have been observed in bacteria (Meyer and Jones, 1973c; Jurtshuk, 1975; Jones, 1977; Degn *et al.*, 1978; Poole, 1983a,b).

Kinetic analysis of the oxidase activities with NADH and ascorbate/TMPD as substrates in whole cells, cell-free extracts, membrane and periplasmic fractions of *A. haloplanktis* revealed that the respiratory system of this marine bacterium obeyed Michaelis-Menten saturation kinetics. As in most enzymatic reactions, the rate of oxygen utilization was independent of the O_2 concentration when the O_2 concentration was high, but became dependent as the dissolved oxygen concentration reached some critical value, this value being dependent on the rate of electron transfer to the oxidases as well as the rate of reoxidation of these oxidases by O_2 (Degn and Wohlrab, 1971). At low oxygen concentration, the initial reaction velocity was nearly proportional to the oxygen concentration and the reaction was thus approximately first order.

The experimental data presented are consistent with the hypothesis that indeed more than one terminal oxidase is operating in the aerobic oxidation of physiological and non-physiological electron donors, based on the finding that more than one affinity system is expressed toward the final electron acceptor, molecular O_2. One conclusion that can be drawn from such an observation is that the respiratory chain of *Alteromonas haloplanktis* would be branched at least at the oxygen end of the chain.

Several reasons could be invoked to explain the presence of high and low affinity systems in bacteria. Among them are listed survival, detoxification, protection against accumulated by- and end-products of the metabolism under excess or limited concentration of the final electron acceptor. The intrinsic nature of the branched bacterial respiratory pathways remains to be clarified. Rice and Hempfling (1978) attributed the existence of different terminal oxidase activities to different cytochromes based on kinetic data in which the K_m values vary in the order of 1 to 10. Henry and Vignais (1979) assessed their experimental data very similarly, with even less of a difference of magnitude between the K_m values recorded. The statistical analysis of the experimental data, below and above 10 µM O_2 (Figures 8 and 9) revealed the presence of biphasic kinetic systems for whole cells, cell-free extracts and membrane fractions oxidizing NADH. On the other hand, the data accumulated for the membrane and the periplasmic fractions showed monophasic kinetics with respect to the final electron acceptor when oxidizing ascorbate/TMPD above 10 µM O_2. Partition of the cell-free extracts which contain both high and low affinity systems into membrane and periplasmic fractions resulted in partition of the two oxidase systems. The membrane fraction appeared to possess the low affinity system toward oxygen with an apparent K_m of 8.1 µM, while the periplasmic fraction would contain the high affinity system expressed by a K_m value of 0.85 µM and be able to oxidize ascorbate/TMPD but not NADH as shown polarographically.

Different affinity constants were measured in the marine bacterium under different concentrations of O_2. One could imagine that branching of the electron transport system would provide the cell with a greater flexibility in changing growth environments where different respiratory needs ought to be met. It is worth mentioning that the concentration of dissolved oxygen as well as the concentration of reduced oxidase(s) present in the fraction influence the respiratory rate. In other words, if the ratio of reduced oxidase(s) to other respiratory proteins changes due to a change in growth, the

respiratory rate is expected to change. In a given environmental situation, under low oxygen tension, the aerobic marine organism would have to compensate for the lack of O_2 by either increasing the concentration of the high affinity oxidase(s), or by inducing synthesis of new respiratory proteins functioning at low oxygen concentration, or by branching its existing electron transport system in order to maximize the rate of O_2 consumption at oxygen concentration just slightly above the concentration producing half maximal respiratory rate.

From a survey of possible alternate electron acceptors in A. *haloplanktis*, it was concluded that only molecular oxygen was effective in supporting active growth of the marine micro-organism and was used as sole final electron acceptor. A recent publication on the taxonomy of *Alteromonas* confirms these findings. Among the 45 strains tested, *Alteromonas haloplanktis* strain 214 included, none was able to denitrify or to utilize H_2 as energy source and CO_2 as carbon source. The strains were all capable of respiratory but not fermentative metabolism and all used oxygen as the universal electron acceptor (Baumann *et al.*, 1984).

CHAPTER IV

DISSOLVED OXYGEN CONCENTRATION AND CYTOCHROME PATTERNS

1. INTRODUCTION

No micro-organism is indifferent to the presence of oxygen, whether it is an aerobe which actively exploits oxygen to generate the energy required for growth or an anaerobe to which oxygen is highly toxic. Pasteur (1861) recognized the importance of oxygen in the metabolism of microbes but real progress toward the elucidation of the response of micro-organisms to dissolved oxygen was not achieved for a century or more (Harrison, 1969,1976b). Research in this field was retarded by the lack of suitable techniques for the measurement of oxygen in solution (Johnson, 1959; Pácl, 1976) and for controlling the environment of micro-organisms sufficiently enough to reveal those effects due to oxygen (Harrison and Pirt, 1967; Rice and Hempfling, 1978).

How do bacteria respond to a change in dissolved oxygen concentration? Mainly by varying their cytochrome content and their potential respiratory rates as the dissolved oxygen concentration changes in the growth environment. Such a response has been observed in aerobes such as *Azotobacter vinelandii* (Ackrell and Jones, 1971a,b; Vignais *et al.*, 1981) as well as in facultative anaerobes such as *Beneckea natriegens* (Linton *et al.*, 1975). Induced synthesis of cytochromes a_1 and a_2, most particularly under limited aeration and unfavourable growth conditions, had been reported in several bacterial species: *Paracoccus denitrificans* (Henry and Vignais, 1979), *Achromobacter* (Arima and Oka, 1965), *Escherichia coli* (Moss, 1952,1956; Rice and Hempfling, 1978), *Klebsiella aerogenes* (Harrison and Pirt, 1967), *Haemophilus parainfluenzae* (White, 1963a), *Pseudomonas putida* (Sweet and Peterson, 1978), *Acinetobacter* sp.

(Ensley and Finnerty, 1980), thermophilic bacterium PS3 (Sone *et al.*, 1983), *Azotobacter vinelandii* (Yang, 1984), and aerobic marine pseudomonads (Stanier *et al.*, 1966; Sands *et al.*, 1967).

How does oxygen affect cytochrome synthesis? Pulse-labeling experiments have revealed that the assembly of functional cytochrome oxidase subunits in yeast is dependent on oxygen (Woodrow and Schatz, 1979). The molecular mechanism of this oxygen effect is at present unknown. One possibility could be that oxygen induces formation of heme *a*, another possibility could be that O_2 maintains heme *a* or copper, or both in an oxidation-reduction state which permits assembly of cytochrome *c* oxidase. In bacteria, the formation of heme prosthetic group is closely integrated with the synthesis of the protein moiety and oxygen might play a role in acting directly on heme synthesis by regulating the amount of succinyl-CoA available for the first enzyme of the tetrapyrrole biosynthetic pathway d-aminolevulinate (ALA) synthetase (Lascelles,1961; Clark-Walker *et al.*, 1967; Franck,1979; Knowles, 1980b).

In an attempt to understand better the effect of oxygen on the metabolism of a strict aerobe, and in view of the fact that qualitative and quantitative variations have been observed in the cytochrome complement of *Alteromonas haloplanktis*, experiments were undertaken in which dissolved oxygen concentration was monitored in the growth medium while cytochrome and protein concentrations were measured in the growing cells.

By looking at the cytochrome patterns throughout the growth cycle of the organism, we attempted to answer questions such as:

1) What is the effect of dissolved oxygen concentration on the growth of the aerobic marine bacterium?
2) What is the effect of dissolved oxygen concentration on the synthesis of membrane-bound and periplasmic cytochromes?
3) What are the actual and potential respiratory rates of the

bacterium growing in a given nutrient medium?

4) How does the marine micro-organism adapt to changing oxygen environments?

2. MATERIALS AND METHODS

Organism and Cultural Conditions

The organism used in the study was the marine pseudomonad *Alteromonas haloplanktis* strain 214 (ATCC 19855) variant 3 (Gow *et al.*, 1973). The aerobic gram negative bacterium was grown according to the schemes outlined in Figure 13 at 25°C on a gyrotory shaker (approx. 200 strokes/min). The pH was recorded in each sample using a Radiometer pH meter (Copenhagen, Denmark). The cell growth was monitored in a Gilford 300-N spectrophotometer by measuring absorbancy at 660 nm due to light scattering. The inoculum size was maintained at 2% (v/v) throughout the inoculation procedure. The incubation periods were of 24 hours for all the starter cultures. The ratio of volume of growth medium to growth flask capacity was maintained at 1:5 for all the starter cultures. The cultures were harvested following 16 hours of growth in the complex medium and 24 hours of growth in the chemically defined medium respectively. In a separate set of experiments, samples were withdrawn at various time intervals during the growth cycle.

In order to test the effect of dissolved oxygen concentration on the growth of *A. haloplanktis*, the cells were cultured in a final broth volume of 300 ml contained in erlenmeyers of 500 ml and 2000 ml - capacity (Figure 13, series A and B). Detailed composition of the growth media as well as the fractionation procedure are outlined in Figure 14.

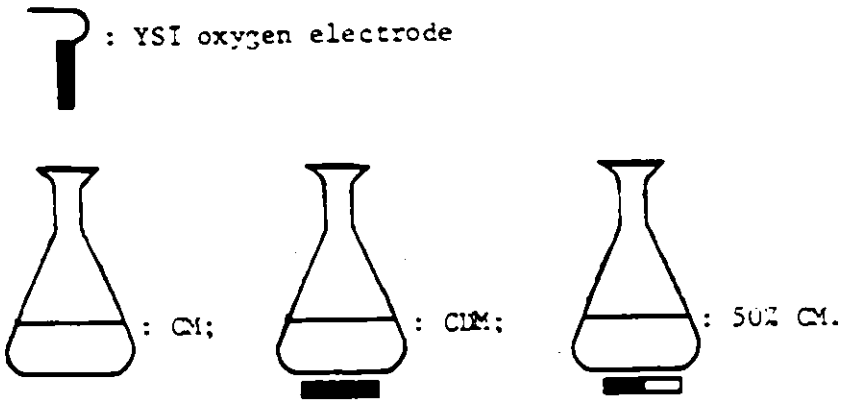
Sampling Procedure

At various time intervals during growth, suitable samples were aseptically withdrawn from the growing cultures. The culture samples were harvested by centrifugation (16,000 xg for 10 min, Sorvall RC2-B, rotor GSA) and washed twice in physiological salt solution. Purity of the samples was ascertained by streaking a loopful of the cultures into complex agar media.

Figure 13. Inoculation procedure for intact cells of *Alteromonas haloplanktis* under different conditions of oxygen supply in batch culture.

Normal and altered aeration and growth pattern of *Alteromonas haloplanktis*. The starter cultures were grown for 24 hr at 25°C on a gyrotory shaker (200 rpm) and samples of the cultures were aseptically harvested at time intervals during the growth, as shown on the diagrams. In a series of experiments (A, B), compressed air or [redacted] were sparged into the growing cultures through sterile Millipore device as specified in Materials and Methods. In another series of experiments (C), oxygen was monitored continuously in the growing cultures with a YSI oxygen electrode. The growth media used were: complex medium (CM), succinate chemically defined medium (CDM) and 50% CM complex medium (50% CM). The composition of the growth media is outlined in Figure 14. The growth flasks utilized were: * 50-ml capacity erlenmeyer flasks containing 10 ml sterile medium, ** 500-ml capacity erlenmeyer flasks containing 300 ml sterile medium, *** 2000-ml capacity erlenmeyer flasks containing 300 ml or 1000 ml sterile medium. The growth temperature was 25°C in all the cases. The starter cultures were maintained on complex medium throughout.

Symbols:



INOCULATION PROCEDURE OF INTACT CELLS OF

Alteromonas haloplanktis.

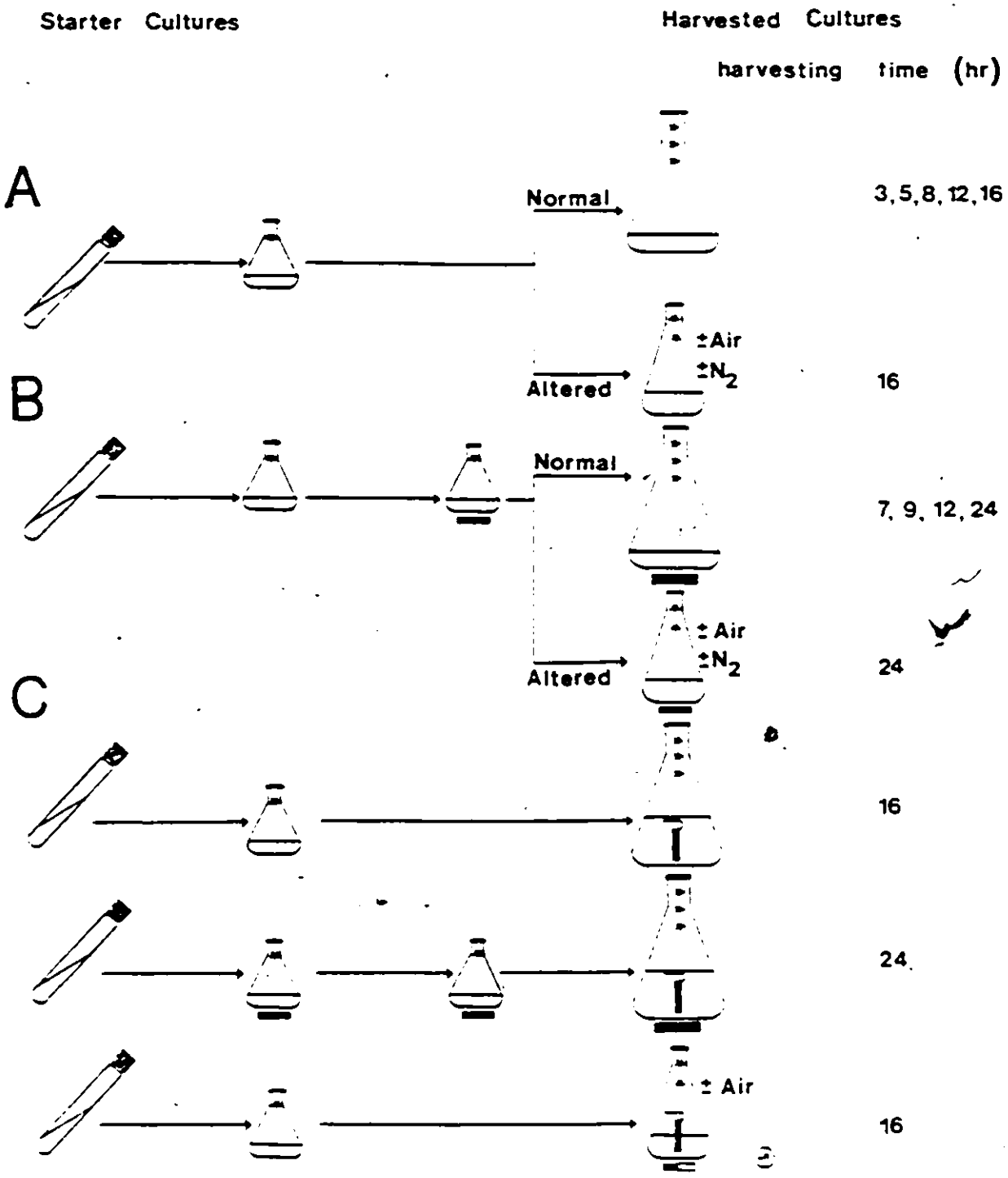
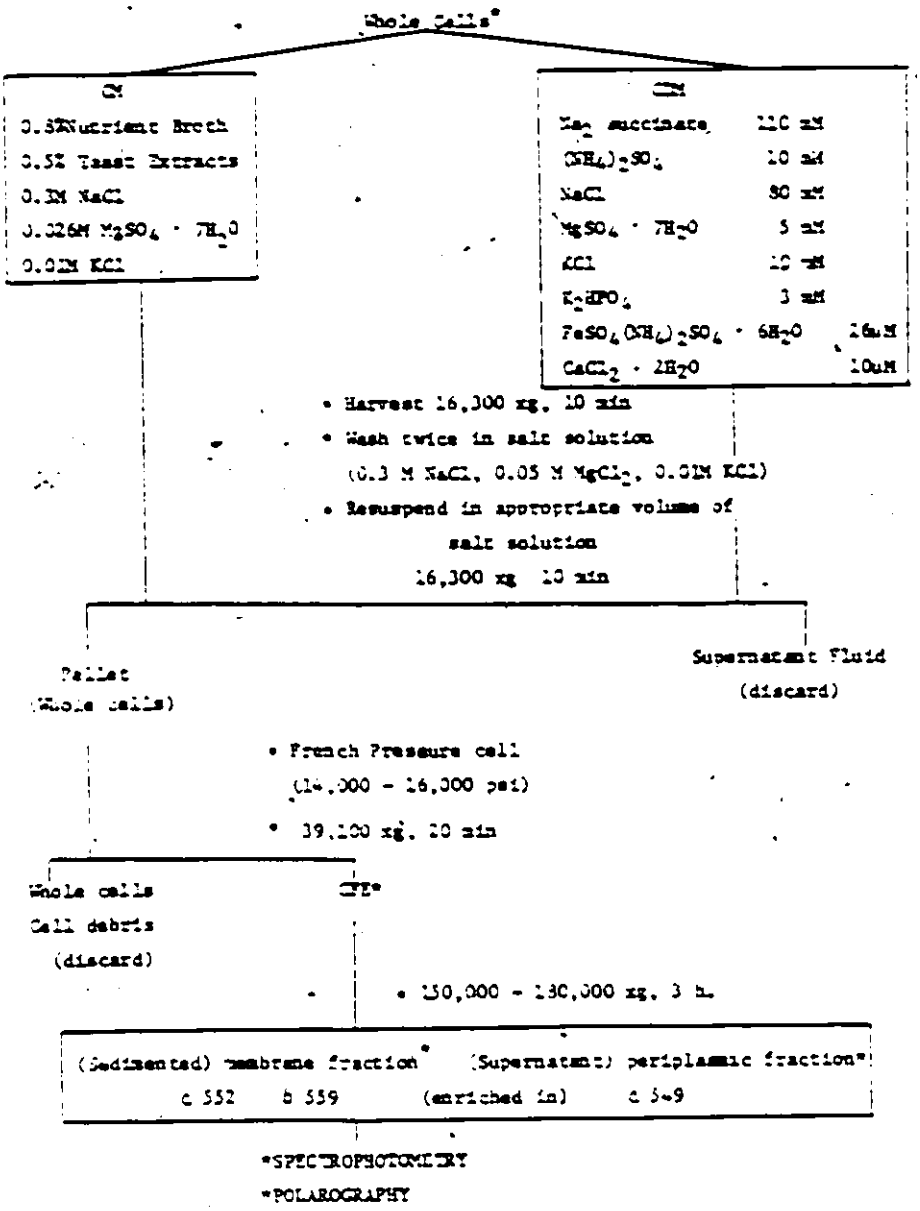


FIGURE 14

GROWTH CONDITIONS AND CELL FRACTIONATION



When occasionally contamination was detected after examination of the plates following 48 hours incubation at 25°C, the results were discarded.

Determination of Oxidase Activity and Cytochrome and Protein Contents

Whole cells were tested for endogenous and exogenous respiratory activity. Cellular extracts as well as membrane and periplasmic fractions were prepared according to a standardized procedure (Figure 14) and tested for cytochrome content and protein content and respiratory activity with ascorbate/TMPD as electron donor.

The respiratory activity was measured polarographically at 25°C in the reaction chamber of a precalibrated Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, England). The endogenous respiration was measured *in situ*, *i.e.* directly on the samples withdrawn from the culture medium. The exogenous respiration was also measured using 100µl of complex growth medium as oxidizable substrate(s).

The concentration of cytochromes was estimated by means of room temperature difference spectra using published values for extinction coefficients, (Jones and Redfearn, 1966) and expressed as n moles cytochrome/mg protein. Difference spectra were obtained by adding a few grains of sodium dithionite ($Na_2S_2O_4$) to reduce the contents of the sample cuvette (λ_1) and a few grains of potassium ferricyanide [K_3 Fe $(CN)_6$] to oxidize the contents of the reference cuvette (λ_2). The spectra were recorded using cuvettes of 10 mm light path at a chart speed of 60 mm/min and a scanning speed of 60 nm/min using an Hitachi Perkin-Elmer spectrophotometer (Model 356) coupled to a Perkin-Elmer chart recorder (Model 056). The protein content of every sample was determined in duplicate using the Biuret method (Gornall *et al.*, 1949). Lysozyme (Sigma Chem. Co.) was used as the protein standard.

Dissolved Oxygen Measurement

Oxygen concentration in growing cultures was measured using

an oxygen electrode (Yellow Springs Inst. Co., Model 5331) covered with a Teflon membrane fastened with an *O* ring. The assembled probe was chemically sterilized with the aid of a diluted solution of Zephiran chloride (Carr *et al.*, 1971) or soaked in acidic ethanol, pH 2.0 (Hempfling and Mainzer, 1975) for a period of 8 hours prior to use. In either case, the probe was thoroughly rinsed with sterile salt solution before being admitted into the growth broth. Sterility of the probe was ascertained by incubating the probe in the growth medium for 16 hours. The electrode was then allowed to equilibrate for one hour in the air-saturated growth medium at 25°C. The calibration was set to full scale at 100 following the equilibration period so that all changes in dissolved O_2 could be read directly in percent. The response of the electrode was checked periodically in distilled water flushed with compressed air and O_2-free nitrogen gas at 25°C. The electrode was connected to a biological oxygen monitor (Yellow Springs Inst. Co., Model 53, Ohio, U.S.A.) coupled to a 0-100 mV full scale chart recorder (Linear Inst. Corp., Irving, CA, U.S.A.) set at very low speed to monitor continuously the level of dissolved oxygen present in the growing culture.

In certain experiments, compressed air and nitrogen gas were sparged through a sterile air-stone added to the culture flasks which were constantly shaken to ensure equal distribution of gases. The gases were maintained free of contaminants by means of a Millipore set-up, using sterile filters of 0.45 µm pore size contained in a sterile Millipore filtering device (Swinnex-25, Millipore Corporation, Bedford, Mass., U.S.A.). All the apparatus was placed in a Puffer-Hubbard temperature control unit (Grand Haven, Michigan) operating at 25°C ± 0.2°C.

3. RESULTS AND DISCUSSION

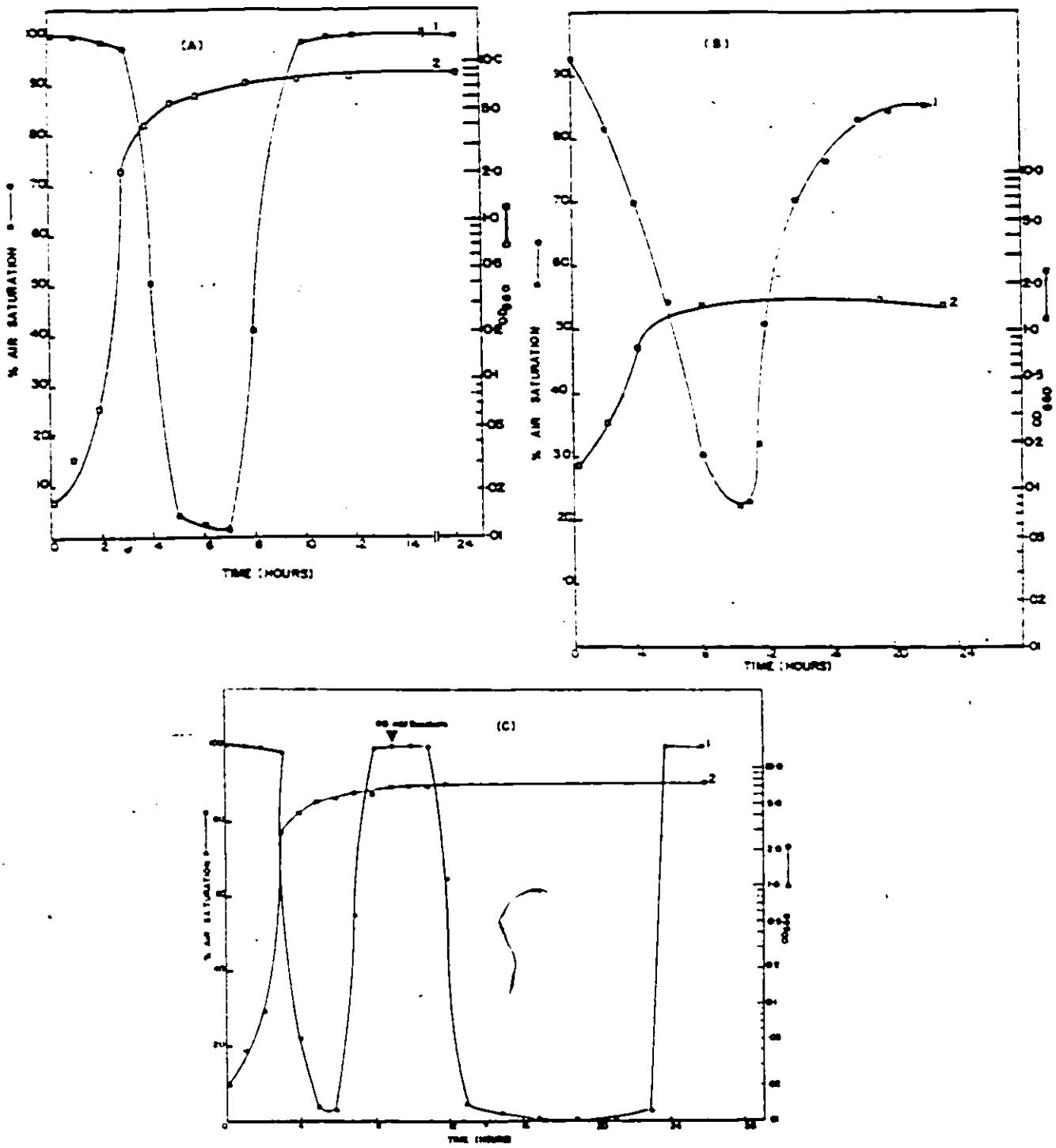
In the following series of experiments, an effort was made to determine if oxygen was a dominant factor in controlling the cytochrome synthesis of *Alteromonas haloplanktis*. Batch culture experiments were set up in which two growth environments were utilized: a complex and a chemically defined environment represented by the respective growth media. The dissolved oxygen concentration was monitored continuously as growth proceeded. By varying the size of the growth flask and by continuous sparging of the growing cultures, different conditions of oxygen supply were established in the batch cultures. Insights into the growth physiology and the cytochrome patterns of the aerobic marine bacterium were gained during the course of these experiments.

Growth Physiology and Dissolved Oxygen Concentration

The growth pattern of the marine bacterium *Alteromonas haloplanktis* follows the same course as the growth pattern of other bacterial species in batch culture. Figure 15A shows the growth responses of the marine bacterium in the complex medium. At the onset of growth and during the log phase, there is little consumption of oxygen. The rate of O_2 depletion accelerates in early stationary phase. In this O_2-restricted phase of growth, the culture consumes all the oxygen that can be dissolved in the growth medium and proceeds to the stationary phase. Presumably, the cells are growing slowly as the population density can be seen to increase after about 2 hours during which time the O_2 concentration remains low; the oxygen concentration suddenly rises to the starting rate. Since the same large number of cells are present, it seems likely that the oxidizable substrates in the medium have been used up, the respiratory activity is reduced to the endogenous rate and atmospheric oxygen diffuses back into the growth medium. By diluting the complex growth medium to one-third of initial strength (Figure 15B), it was observed that uptake of O_2 began at the onset of growth and was almost linear during the logarithmic phase. The lag phase was of the same duration

Figure 15. Dissolved oxygen and optical density curves of A. *haloplanktis* growing under normal aeration conditions in (A) full strength complex medium, (B) 30% complex medium and (C) full strength complex medium *plus* 110 mM succinate during the stationary phase.

Normal aeration and growth conditions refer to growth volumes of 300 ml contained in 2L erlenmeyers. The growth flasks were inoculated with CM-stationary phase cells and samples were withdrawn at time intervals. The cell density was monitored at 660 nm using cuvettes of 1 cm light path. Readings above OD = 0.5 were obtained by diluting the culture in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution to below this value and correcting for the dilution factor. The dissolved oxygen concentration was monitored in the growing cultures using a YSI oxygen electrode. The closed symbols indicate the dissolved oxygen content of the growth medium (curve 1) the open symbols the cell density (curve 2).


(A)
% AIR SATURATION
TIME (HOURS)
(B)
% AIR SATURATION
TIME (HOURS)
(C)
% AIR SATURATION

in both media, although the doubling time was twice the doubling time of the cells in the full strength medium. Also, since the concentration of readily available substrates was cut to one-third, the period of O_2-restriction, during which it was thought that remaining substrates were being fully oxidized, was also cut to approximately a third. In the diluted growth medium, nutrient-limitation was expressed before oxygen-limitation since the growth curve in stationary phase levelled off before the dissolved oxygen curve reached its lowest point (Figure 15B).

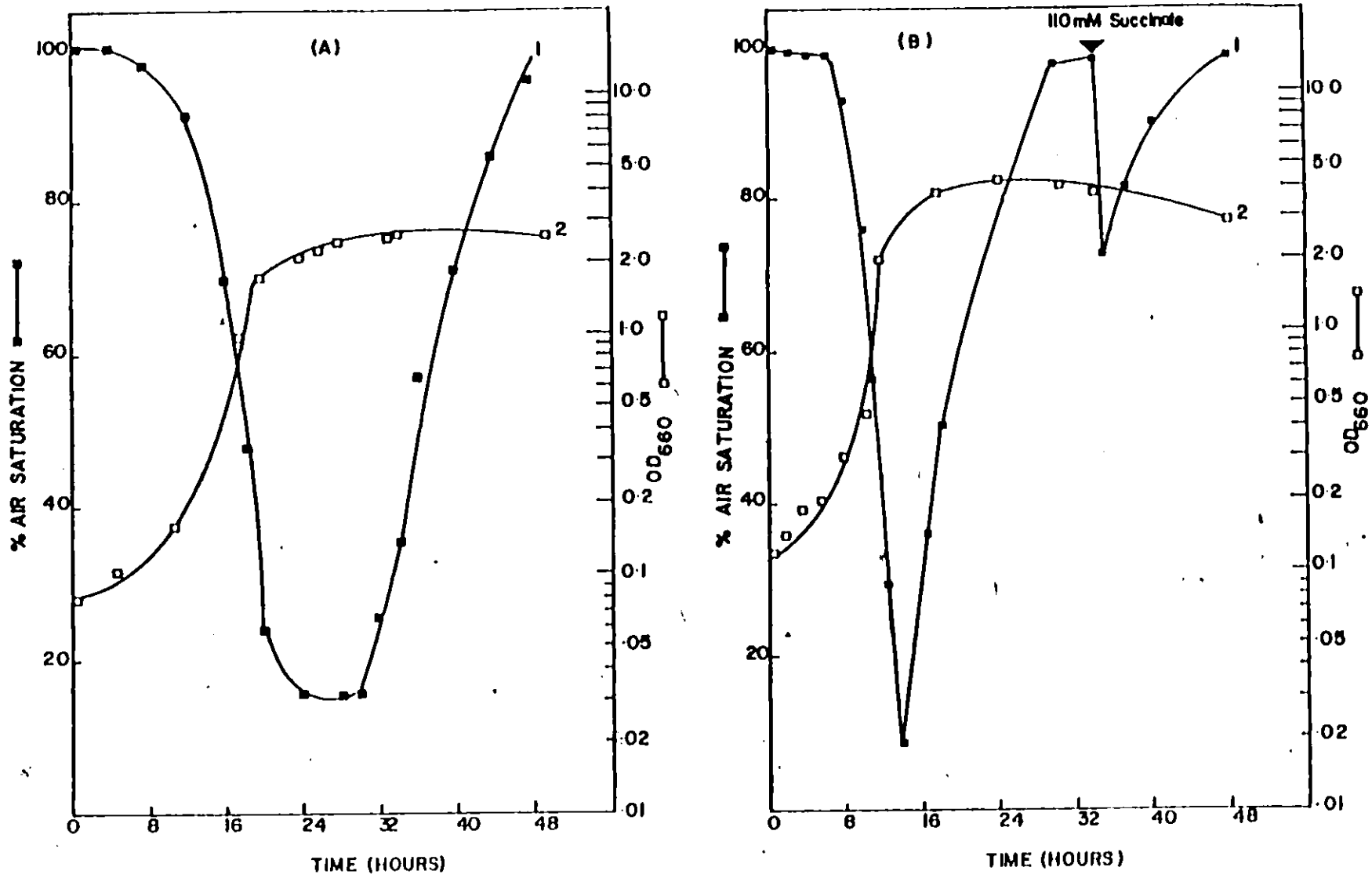
The assumption that at the onset of the stationary phase, the cells had oxidized through respiration, the bulk of the available substrates proved to be true when the following experiment was conducted (Figure 15C). Using the standard inoculation procedure, *Alteromonas haloplanktis* was allowed to grow for 8 hours; one hour later, sterile succinate (to 110 mM) was aseptically added to the culture, and the growth and dissolved oxygen were continually monitored. An intermediate of the tricarboxylic acid cycle such as succinic acid is considered to be a readily available substrate for the aerobic marine bacterium which was shown to possess all the functional enzymes of the TCA and the glyoxylate cycle, provided adequate salt concentrations were present in the assay medium (MacLeod and Hori, 1960). The addition of fresh oxidizable substrate triggered another cycle of cellular respiration which led to a second phase of O_2-depletion. Again shortly after cessation of substrate oxidation, the dissolved oxygen trace equilibrated back to levels of oxygen present in ambient air (Figure 15C).

The behavior of *Alteromonas haloplanktis* with respect to ambient dissolved oxygen is somewhat similar when the cells are grown in a chemically defined medium, using succinate as sole carbon source. Figure 16A represents the growth of the marine bacterium in a chemically defined medium. To ensure that the carbon source would not become the growth limiting factor during the 48 h experiment, the

Figure 16. Dissolved oxygen and optical density curves of A. *haloplanktis* growing under normal aeration conditions in (A) succinate chemically defined medium, (B) same as (A) *plus* 110 mM succinate during the stationary phase.

Normal aeration and growth conditions refer to growth volumes of 300 ml contained in 2L erlenmeyers. The growth flasks were inoculated with succinate-CDM stationary phase cells and samples were withdrawn at time intervals. The cell density was monitored at 660 nm using cuvettes of 1 cm light path. Readings above OD = 0.5 were obtained by diluting the culture in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution to below this value and correcting for the dilution factor. The dissolved oxygen concentration was monitored in the growing cultures using a YSI oxygen electrode. The closed symbols indicate the dissolved oxygen content of the growth medium (curve 1), the open symbols, the cell density (curve 2).

The growth media used were: In (A), the succinate chemically defined medium with 220 mM succinate as carbon source; in (B), the succinate chemically defined medium with 110 mM succinate as carbon source *plus* an additional 110 mM sterile succinate added after 34 hr of growth as pointed by the arrow. Refer to the text for discussion.


(A)
(B)
110mM Succinate
% AIR SATURATION
OD660
TIME (HOURS)
100
80
60
40
20
0
8
16
24
32
40
48
10·0
5·0
2·0
1·0
0·5
0·2
0·1
·05
·02
·01
1
2

concentration of substrate was doubled. During the approximate 10 h lag period (Figure 16A) little oxygen was consumed. Upon entry into the logarithmic phase of growth, the bulk of the dissolved oxygen was depleted from the growth medium. Since quite a substantial concentration of substrate was available, the O_2-depleted phase was extended. This was followed by an upward trend of the dissolved oxygen curve as its upper limit reached the dissolved O_2 level obtained in an ambient air-saturated medium.

In Figure 16B, the initial succinate concentration (110 mM) was doubled at the stationary phase of growth in chemically defined medium but did not lead to a second respiration cycle as observed in stationary phase cells grown in the complex medium (Figure 15C), presumably because the supply of the initial oxidizable substrate had not become depleted, or for other reasons.

Another difference between the behavior of cells grown in chemically defined as opposed to cells grown in complex medium is the lesser degree of dissolved O_2-depletion that occurs in chemically defined medium. For instance, levels of dissolved oxygen similar to those obtained in a chemically defined medium were maintained during the growth of cells supported by only 30% of the complex medium (Figure 15B), whereas low values close or equal to 1% air-saturation were achieved in the complex growth medium (Figure 15A). Definitely, the complex medium grown cells must alter their growth environment faster than their counterparts in chemically defined medium or possess a mechanism to support themselves in a more O_2-reduced environment.

Several factors influence the rate of oxygen transfer in submerged cultures (Pácá, 1976). The rate of oxygen transfer is a function of the concentration gradient between the concentration of oxygen in the air and the actual concentration of dissolved oxygen in the growth broth. By increasing the volume of growth broth while keeping the volume of growth flask constant and/or sparging a continuous stream of sterile compressed air through the growing

cultures, we could manipulate the aeration factor in batch cultures.

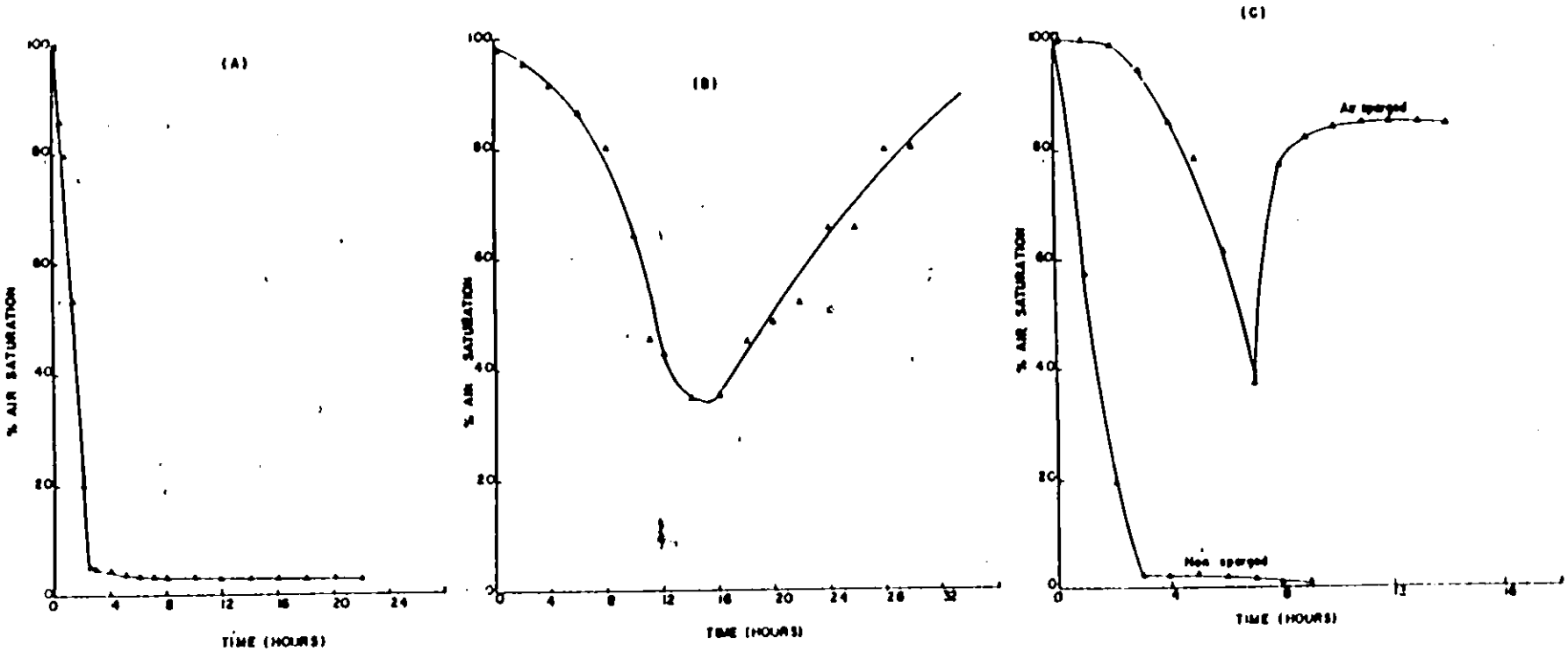
In Figure 17, the experiments were set as outlined in Figure 13, scheme C. The complex broth culture (Figure 17A) was allowed to grow for 24 hours and the dissolved oxygen was monitored throughout that period of time. Within three hours of growth, the dissolved oxygen concentration was brought down to levels equivalent to 3-4% air-saturation and did not equilibrate again with the O_2 concentration present in ambient air. In the chemically defined broth (Figure 17B), the growing culture was not able to bring the dissolved oxygen to air-saturation values lower than 25%, despite the fact that the ratio of broth volume to flask volume had been reduced from 1:5 to 1:2 to physically facilitate such a reaction; nevertheless, the dissolved oxygen curve took an upward trend following 15 hours of growth. Similar experiments were conducted in nutrient broths composed of only 50% of the nutrients normally found in the complex medium (Figure 17C). A set of cultures were constantly flushed with a mild stream of sterile compressed air (upper dissolved oxygen curve), the other set left non-sparged on the rotary shaker (Figure 17C, lower trace). In air-sparged cultures, the dissolved oxygen trace peaked down to approximately 40% air-saturation after 7 hours of growth and went up again to air-saturation levels close to 90%, whereas in non-sparged cultures, the dissolved oxygen trace maintained itself at approximately 1% air-saturation throughout.

Quadruplicate experiments of each type of cultures presented in Figure 17 allowed us to draw the following conclusions: (*i*) in any given growth environment, the concentration of dissolved oxygen can be manipulated; (*ii*) lower levels of dissolved oxygen were obtained in complex (100%) and semi-complex (50%) growth broths than in chemically defined broth, even when the experimental conditions were favouring low levels of dissolved oxygen; (*iii*) comparable levels of dissolved oxygen (approx. 35%) were achieved by air-sparged semi-complex cultures (Figure 17C, upper trace) and by non-sparged chemically defined cultures (Figure 17B); (*iv*) the final biomass

Figure 17. Dissolved oxygen curves of A. *haloplanktis* growing under altered aeration conditions in (A) full strength complex medium, (B) full strength succinate chemically defined medium and (C) in 50% complex medium.

Altered aeration and growth conditions refer to growth volumes of 1000 ml contained in 2L erlenmeyers (as in (A) or (B)) or 300 ml contained in 500 ml erlenmeyers (as in (C)).

The growth flasks were inoculated with CM-stationary phase cells (in (A) and (C)) or with succinate-CDM stationary phase cells (in (B)), according to the growth medium chosen. The dissolved oxygen concentration was monitored in the growing cultures using a YSI oxygen electrode. The growth media used were: in (A), the full strength complex medium; in (B), the full strength succinate chemically defined medium; in (C), 50% strength of the complex medium.


(A)
% AIR SATURATION
TIME (HOURS)
(B)
% AIR SATURATION
TIME (HOURS)
(C)
% AIR SATURATION
Air sparged
Non sparged
TIME (HOURS)

yield was affected by the rate of oxygen transfer in the given cultures; (v) other biochemical changes were directly related to the addition of air, for instance, the cytochrome content of the cultures.

Cytochrome Pattern and Dissolved Oxygen Concentration

In an attempt to answer the question as to what could maintain the cultures in a physiologically competent state of growth at low or high oxygen concentration, we investigated the cytochrome content of cells grown in complex and chemically defined media. Our interest in the hypothesis that reduced oxygen concentration in the growth medium could trigger or induce the synthesis of the periplasmic and membrane-bound cytochromes was maintained. It is well known that bacteria are capable of altering the composition of their respiratory systems in response to changes in the availability of dissolved oxygen (Jurtshuk *et al.*, 1975). For instance, induction of *a*-type cytochromes during the stationary phase of growth has been reported in a number of bacteria, both aerobes and facultative anaerobes.

The normal complement of cytochromes synthesized during the growth cycle of A. *haloplanktis* in complex and chemically defined media are presented in Figure 18 and Figure 19 respectively. The normal growth pattern refers to a standard inoculum of stationary phase cells into 300 ml of fresh broth contained in a 2L-erlenmeyer flask (Figure 13, schemes A and B). The concentration of membrane-bound (c_{552} and b_{559}) and periplasmic cytochrome (c_{549}) were measured in the respective fractions at room temperature by means of difference absorption spectrophotometry. When the cytochrome profiles were taken into consideration with the growth and dissolved oxygen curves characteristic of each growth environment (see Figures 15A and 16A), the following picture emerged: in the complex medium (Figure 18), the three species of cytochromes started to be synthesized during the lag phase of growth with a marked increase in concentration during the logarithmic phase. High levels of membrane-bound c_{552} and membrane-bound b_{559} were

Figure 18. Cytochrome pattern of A. *haloplanktis* grown in full strength complex medium, under normal aeration conditions.

Normal aeration and growth conditions refer to growth volumes of 300 ml contained in 2L erlenmeyers. Complex medium-grown cells were harvested at various points throughout the growth curve, washed, fractionated and assayed for the presence of cytochromes. The concentration of the periplasmic (c_{549}) and the membrane-bound (c_{552}, b_{559}) cytochromes was estimated by means of room temperature difference absorption spectrophotometry using solid crystals of dithionite as reductant and solid crystals of ferricyanide as oxidant, and expressed as n moles cytochrome/mg protein. The protein concentration was determined by the Biuret method.

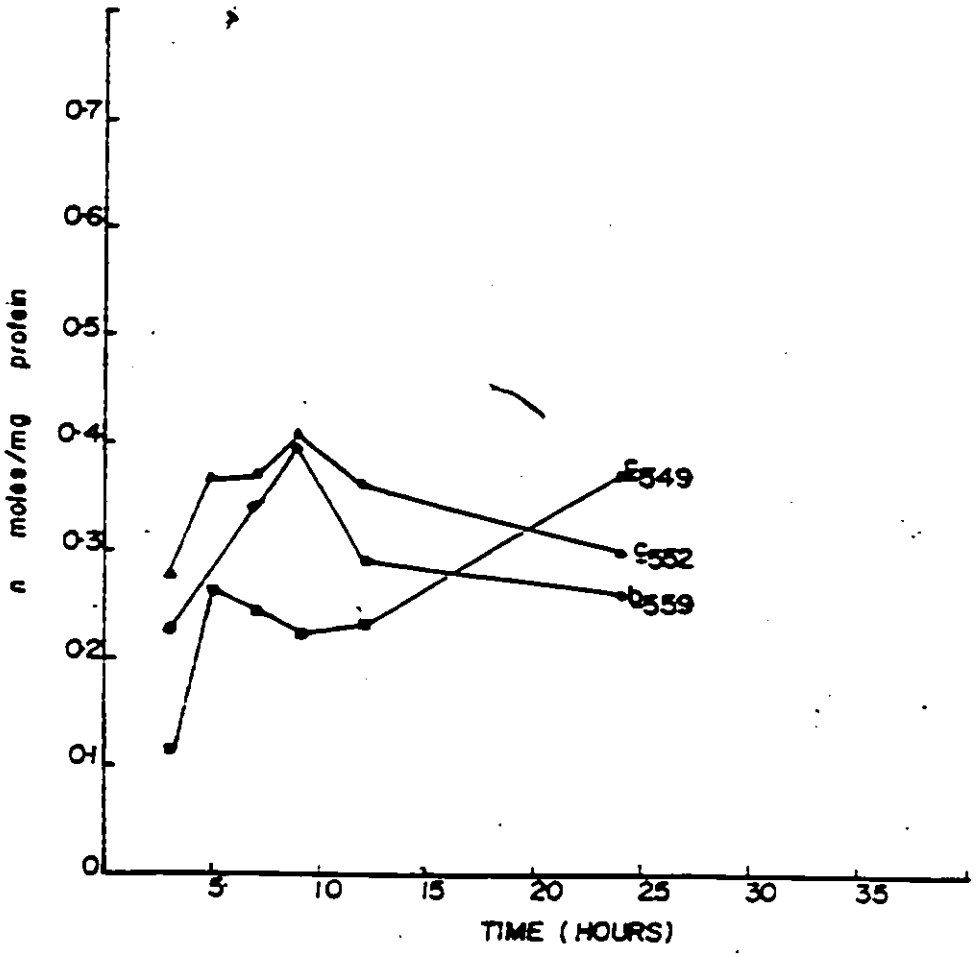
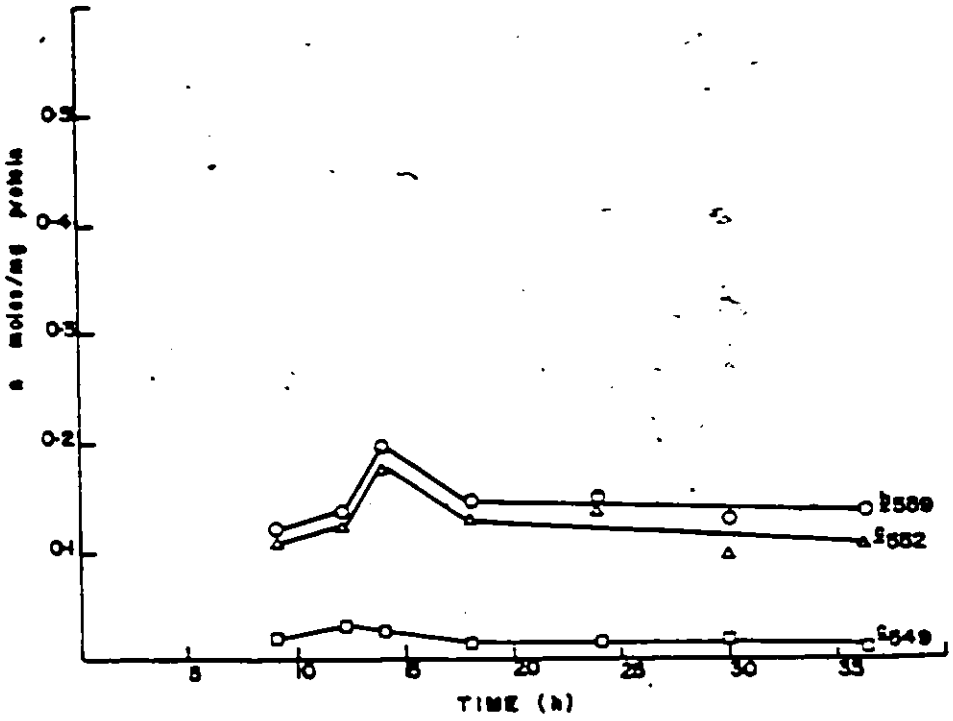

n moles/mg protein
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
5
10
15
20
25
30
35
TIME (HOURS)
c549
c552
b559

Figure 19. Cytochrome pattern of A. *haloplanktis* grown in full strength succinate chemically defined medium, under normal aeration conditions.

Normal aeration and growth conditions refer to growth volumes of 300 ml contained in 2L erlenmeyers. Succinate-CDM grown cells were harvested at various points throughout the growth curve, washed, fractionated and assayed for the presence of cytochromes. The concentration of the periplasmic (c_{549}) and the membrane-bound(c_{552}, b_{559}) cytochromes was estimated by room temperature difference absorption spectrophotometry using solid crystals of dithionite as reductant and solid crystals of ferricyanide as oxidant, and expressed an n moles cytochrome/mg protein. The protein concentration was determined by the Biuret method.


0.5
0.4
0.3
0.2
0.1
n moles/mg protein
5
10
15
20
25
30
35
TIME (h)
589
582
549

synthesized soon after the lag phase, the periplasmic c_{549} reached comparable levels only in the late stationary phase in the complex medium. The synthesis of membrane-bound cytochromes reached a peak at the onset of the stationary phase of growth, the membrane-bound c_{552} always exceeding the synthesis of the membrane-bound b_{559}. On the other hand, the concentration of the periplasmic c_{549} was maintained at relatively fixed values during the logarithmic phase of growth until the cytochrome content/mg protein of both membrane-bound species started to decline. Then in the late stationary phase, the situation was reversed with decreasing concentrations of membrane-bound cytochromes coupled with an increasing concentration of periplasmic cytochrome. In that respect, *Alteromonas haloplanktis* differed from the physiological grouping of aerobic Gram negative heterotrophs of the order of Pseudomonadales, in which cytochrome c is considered a minor component of their cytochrome complement, with major emphasis on the omnipresence of a-type cytochromes (a_1, a_2 or aa_3) synthesized after the logarithmic phase of growth. Cytochrome *o* is also a constant component of the cytochrome pattern of aerobic and facultatively anaerobic, Gram negative, heterotrophs (Meyer and Jones, 1973a).

The picture was different in cells grown on succinate in chemically defined medium, in which basically, throughout the entire growth cycle, the membrane-bound (c_{552}, b_{559}) cytochromes predominated (Figure 19). Here the increased synthesis of cytochromes was not visible before the end of the lag phase, a period of time supposedly required by the bacterium to start utilizing sufficient succinate, which is required for the biosynthesis of heme-containing respiratory pigments (Knowles, 1980b). One contrasting difference between the two growth environments was shown by the behavior of the periplasmic c_{549} cytochrome. In chemically defined succinate medium, its concentration did not increase in the stationary phase. Instead, the cytochrome content of c_{549}/mg protein decreased in cells grown on succinate, starting with the onset of the logarithmic phase of growth. Also worth mentioning, the cytochrome patterns in both growth environments

can change remarkably under conditions of little or no cell division as happened during the stationary phase of growth. In addition, the absence of absorption peaks in the region of 585 nm to 650 nm at any stage of growth indicated that no detectable quantities of a-type cytochromes were produced by the marine organism under the growth conditions tested.

The cytochrome patterns of A. *haloplanktis* have been investigated under normal aeration and growth conditions, *i.e.* 300 ml growth volumes contained in 2L-erlenmeyers and agitated at 200 rpm. When the cytochrome patterns of the marine bacterium were looked at under altered aeration and growth conditions, *i.e.* 300 ml growth volumes contained in 500 ml-erlenmeyers and sparged (or not) with a mild stream of sterile air (as schemed in Figure 13, series A,B,C, bottom), the following picture emerged (Table XI):

In the three growth media tested (succinate chemically defined, complex and semi(50%)complex medium), the concentration of the periplasmic cytochrome c_{549} was higher in non-sparged cultures than in air-sparged cultures. The results also show that in the succinate chemically defined medium, the membrane-bound cytochromes (c_{552}, b_{559}) were not markedly affected by the presence of a continuous stream of air as reflected in the total content of cytochromes, with a slight increase of cytochromes synthesized under sparging. On the contrary, the membrane-bound cytochromes synthesized by the cells grown in complex medium responded very differently to a continuous supply of air: over 3.5 times the amount of cytochromes bound to the membrane were produced under condition of no sparging of air.

The results presented in Table XI also show that the ratios of membrane-bound to periplasmic cytochromes were definitely higher in cells grown in chemically defined medium, irrespective of the oxygen supply, a feature characteristic of strict aerobes depending on their membrane-bound enzymes to function optimally.

TABLE XI. Cytochrome patterns in stationary phase cells of *A. haloplanktis* grown in different media under different conditions of O_2 supply.

Growth medium	Cultural conditions*	Cytochrome content (n moles/mg protein)						Cytochrome ratio				Total cytochrome content (n moles/mg protein)
		c_{549}		c_{552}		b_{559}		$\frac{c_{552}}{c_{549}}$	$\frac{b_{559}}{c_{549}}$	$\frac{c_{552}+b_{559}}{c_{549}}$	$\frac{c_{552}}{b_{559}}$	
		mean†	S.D.	mean†	S.D.	mean†	S.D.					
Succinate-chemically defined medium	No air-sparging	0.022	± .002	0.154	± .069	0.139	± .062	7.0	6.3	13.3	1.1	0.315
	air-sparging	0.013	± .003	0.225	± .027	0.178	± .061	17.3	13.6	31.0	1.3	0.416
Complex medium	No air-sparging	0.684	± .117	0.921	± .076	0.622	± .040	1.3	0.9	2.2	1.5	2.227
	air-sparging	0.307	± .282	.273	± .008	.163	± .072	0.8	0.5	1.4	1.7	0.743
50% Complex medium	No air-sparging	0.555	± .147	0.768	± .148	0.524	± .159	1.4	0.9	2.3	1.5	1.847
	air sparging	0.176	± .113	0.357	± .038	0.269	± .040	2.0	1.5	3.5	1.3	0.802
30% Complex medium	**	0.130	± .009	0.381	± .059	0.251	± .046	2.9	1.9	4.8	1.5	0.762

*300 ml broth cultures grown at 25°C, agitated at 200 rpm and contained in 500 ml erlenmeyers.

**300 ml broth cultures grown at 25°C, agitated at 200 rpm and contained in 2000 ml erlenmeyers.

†Each entry is an average of at least three replicates ± the standard deviation of the mean.

Looking at the ratio of membrane-bound c_{552} to membrane-bound b_{559}, it is clear that in all the growth media, the contribution of the membrane-bound c type exceeded the contribution of the membrane-bound b-type, particularly so in complex growth media

As far as the total amount of cytochromes synthesized is concerned, higher concentrations were recorded in complex media-grown cells, more particularly so in non-sparged cultures; the same aeration condition in chemically defined medium produced much less cytochromes. The cells from the two types of medium thus responded differently to a continuous supply of O_2.

In some species of pseudomonads, a-type cytochromes are induced under survival growth conditions (Stanier *et al.*, 1966; Sands *et al.*, 1967), when the concentration of dissolved oxygen becomes severely limited. Such growth conditions were tentatively reconstructed in the chemically defined and complex media through which a sterile stream of nitrogen gas containing 2000-5000 ppm O_2 (35 nM) was sparged continuously. The results obtained showed that (*i*) no a-type cytochrome could be spectrophotometrically detected in any growth medium tested; (*ii*) growth in succinate chemically defined medium was not possible under such conditions; (*iii*) survival in the complex medium was observed nevertheless, and both periplasmic and membrane-bound cytochromes could be detected in cells that survived such a harsh environment.

Dissolved Oxygen Concentration and Respiratory Rate

In addition to following the cytochrome pattern of the marine micro-organism throughout its growth cycle, specific rates of O_2 consumption were measured using endogenous and exogenous substrates in an attempt to visualize the physiological relationship between O_2 supply and O_2 consumption in growing cultures.

The marine bacterium was cultured in complex medium, a medium allowing synthesis of the full complement of cytochromes. Samples

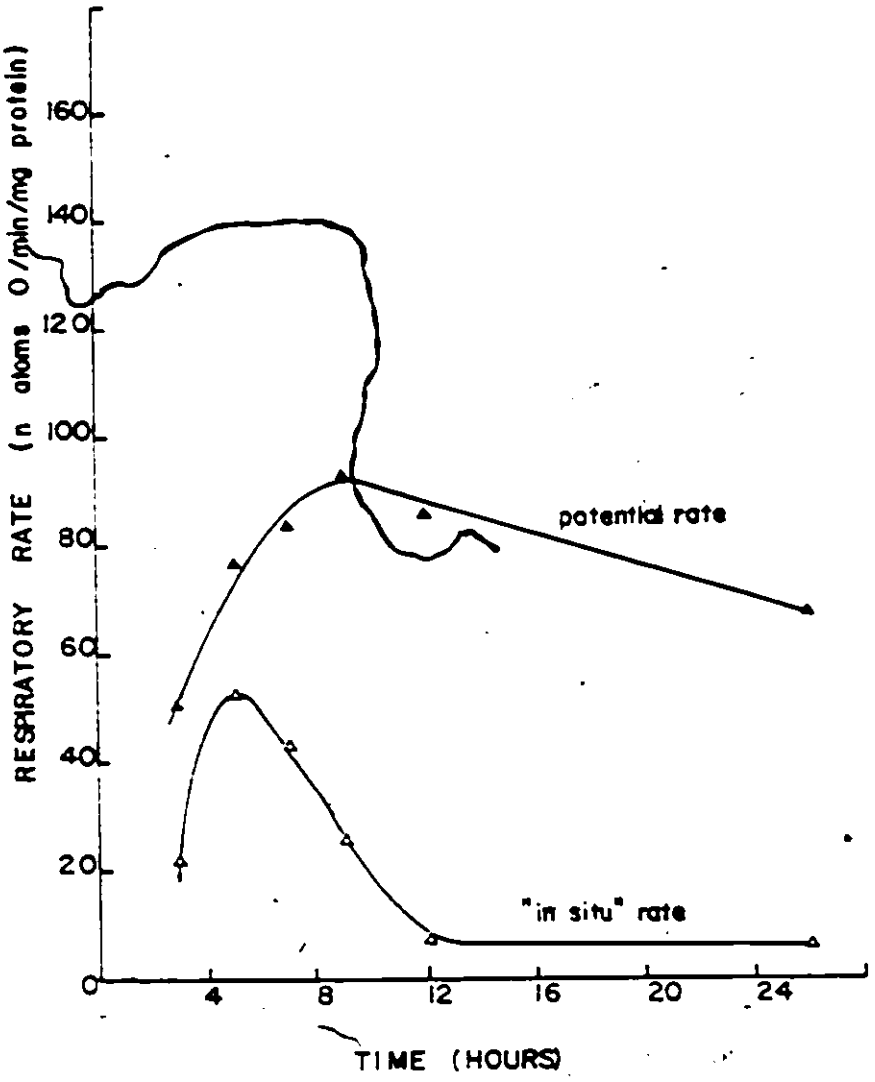
were withdrawn at various intervals during growth. *In situ* rates of respiration were measured directly in an O_2 electrode cell on non-washed, freshly harvested samples. In addition, portions of the samples removed were washed carefully and added to the O_2 electrode cell where the respiration rates were determined in the presence of excess substrate (100 µl fresh complex medium). These exogenous respiration rates are referred to as *potential* respiration rates to distinguish them from the respiration rates measured directly from the growing cells which are referred to as *in situ* or endogenous respiration rates. Results of such experiments are documented in Figure 20.

If one follows the growth and dissolved oxygen concentration curves typical of a culture growing in complex medium (Figure 15A), one sees that the *in situ* respiration rates measured in these cells (Figure 20, bottom trace) attained a maximal value at the beginning of the retarded or stationary phase of growth, a growth phase also characterized by its low content of dissolved oxygen. Since the respiratory rates were examined at ambient oxygen tension in the O_2 electrode cell, the change in *in situ* rates reflected the availability of substrates in the growth medium. As the availability of substrates decreased during the stationary phase, the *in situ* respiratory activity decreased.

As far as the *potential* rates of O_2 consumption were concerned (Figure 20, upper trace), they increased when the stationary phase was well established, with a minimum value found early on, at the onset of growth. The *potential* respiratory rates increased as more respiratory components were formed and the oxidation of the readily available substrate(s) became more efficient. As we may recall, there was increased synthesis of periplasmic cytochrome (c_{549}) at the stationary phase of growth. High production of cytochromes might have been necessary for dissimilatory purposes as well as for enabling the cell to maintain its respiration rate at fairly constant levels even under low O_2 concentration or altered growth conditions.

Figure 20. Change in *in situ* and potential respiratory activities with age of the culture.

At time intervals during the growth of *A. haloplanktis* in complex medium, samples were aseptically withdrawn from the growing culture and the respiratory activities measured polarographically at 25°C in an O_2 electrode cell. The *in situ* respiratory rates were measured on unwashed cell samples, directly harvested from the growth medium by centrifugation and resuspended in a small volume of complete salt solution (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1 mM, pH 7.4) and were not given any exogenous substrate to oxidize. The *potential* respiratory rates were measured on harvested, washed cell samples, resuspended in a small volume of complete salt solution (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1 mM, pH 7.4) and were given 100 µl of fresh sterile complex medium to oxidize. For the determination of protein by the Biuret method, both cell types were washed and resuspended in buffered salt solution.


RESPIRATORY RATE (n atoms O/min/mg protein)
160
140
120
100
80
60
40
20
0
4
8
12
16
20
24
TIME (HOURS)
potential rate
"in situ" rate

4. CONCLUSION

Bacteria have been found to harbor a wide variety of cytochrome components and the level of cytochromes produced by a given organism can fluctuate with growth conditions (Smith, 1968; Jones, 1977). The attention of a number of investigators has been focussed on these variations brought about by changes in oxygen concentration (see reviews Harrison, 1976a,b; Jurtshuk *et al.*, 1975; Poole, 1983a).

In general, the pattern that emerged from a good number of studies dealing with obligate aerobes as well as with facultative anaerobes showed that increased quantities of cytochromes were synthesized when the oxygen concentration dropped in the growth medium. Below a certain critical level of oxygen, facultative anaerobes switch to fermentative metabolism and the strict aerobes trigger the induction of a-type cytochromes. Possibly, a wider variety of cytochromes synthesized at oxygen tensions below the K_m for the cytochrome oxidase(s) enable the organism to maintain respiratory activity at lower than normal O_2 concentrations.

Our data revealed that the growth physiology in general and the formation of cytochromes in particular were indeed markedly influenced by the environmental growth conditions. As emphasized by the dissolved oxygen curves, the changes in the cytochrome patterns were to a large extent due to changes in dissolved oxygen concentration.

In a complex nutrient environment, at the onset of growth and during the logarithmic phase, the ratio of membrane-bound to periplasmic cytochromes was maintained at 1.5:1.0 until late stationary phase where the ratio was reversed, indicating that membrane-bound enzymes were playing a central role in physiologically active cells, leaving such a role to the periplasmic cytochrome at a later stage of growth when the dissolved oxygen concentration reached low values (= 1% air saturation).

In a simple nutrient environment, the ratio of membrane-bound to periplasmic cytochromes was relatively stable throughout the entire growth cycle and no important concentration of the periplasmic cytochrome c_{549} was detected at any stage of growth.

The cytochrome c_{549} is localized in the periplasmic space of the cell envelope of A. *haloplanktis* (Knowles *et al.*, 1974). This area of the Gram negative cell envelope defined by Mitchell (1961a) is known to contain a number of proteins including a variety of hydrolytic enzymes, binding proteins (Heppel 1971, 1972) and terminal reductases (Wood 1978a,b). The enzymes enclosed in the periplasm could be considered as integrated parts of an alternative feedback control mechanism, particularly when the cells are immersed in an osmotically or metabolically unfavourable milieu.

It is conceivable that in a complex nutrient environment, the respiratory control exerted by the bacterium would somehow be more complex and not assumed solely by the membrane-bound respiratory proteins. Controlled oxido-reduction reactions ought to take place at the level of the membrane and the periplasm, in order to ensure the growth and the maintenance of living cells which require a continuous supply of energy. In this respect, interesting information was gained from the analysis of the cytochrome ratios reported in Table XI. In complex media, the periplasmic cytochrome c_{549} significantly contributed to increase the total cytochrome content of the bacterium, especially under conditions of limited aeration. As the complexity of the growth medium increased, the contribution of the periplasmic cytochrome c_{549} increased. On the other hand, in chemically defined medium, where the complexity of the nutrient environment is much reduced, much higher ratios of membrane-bound to periplasmic cytochromes were recorded. Thus, not only the dissolved oxygen concentration, but also the nutrient composition of the growth milieu influenced the quantity and species of cytochromes synthesized.

Under microaerobic growth conditions, a cytochrome *d* (formerly a_2) which can function as an oxidase (Degn *et al.*, 1978) is induced in several bacterial species. Organisms in which this cytochrome species has been observed include: *Escherichia coli* (Moss, 1952; Shipp, 1972), *Klebsiella aerogenes* (Castor and Chance, 1959; Harrison, 1972), *Proteus vulgaris* (Moyed and O'Kane, 1956; Castor and Chance, 1959), *Achromobacter* Strain D (Arima and Oka, 1965), *Haemophilus parainfluenzae* (White, 1962; Smith *et al.*, 1970), *Alcaligenes faecalis* (Iwasaki and Shidara, 1971), *Micrococcus* (*Paracoccus*) *denitrificans* (Sapshead and Winpenny, 1972; Henry and Vignais, 1979), *Benechea natriegens* (Linton *et al.*, 1975), *Pseudomonas stutzeri* (Kodoma, 1970), *Pseudomonas putida* (Sweet and Peterson, 1978), *Acinetobacter* sp HO1-N (Ensley and Finnerty, 1980), *Azotobacter vinelandii* (Ackrell and Jones, 1971a,b; Nagai *et al.*, 1971). *Alteromonas haloplanktis* differed from the above by synthesizing only *b*-and *c*-type cytochromes throughout its growth cycle after the example of *Spirillum itersonii* which coped with microaerobic growth conditions by synthesizing predominantly *c*-type cytochromes and some *b*-types (Clark-Walker *et al.*, 1967 ; Lascelles *et al.*, 1969).

Under high aeration, synthesis of cytochromes *b* and *c* were repressed in *Spirillum itersonii* as was the case in *A. haloplanktis* grown in complex medium (Table XI). In other strict aerobes such as *Bacillus subtilis* (Chaix and Petit, 1956), *Pseudomonas fluorescens* (Lenhoff *et al.*, 1956) and *Azotobacter vinelandii* (Ackrell and Jones, 1971a,b), the presence of excess oxygen characteristically depressed cytochrome levels.

What places *Alteromonas haloplanktis* in a separate category is its ability to synthesize rather large quantities of soluble periplasmic cytochrome *c* (Knowles *et al.*, 1974) and share with other marine bacteria the unusual feature of not synthetizing *a*-type cytochromes (Krieg, 1976; Arcuri and Ehrlich, 1979, 1980). All

other Gram negative heterotrophic aerobes synthesize an *a*-type cytochrome either under the form of a_1, a_2, or aa_3 (Meyer and Jones, 1973a) and the cytochrome *c* is often absent in heterotrophic Pseudomonadales (Meyer and Jones, 1973a). On the other hand, the chemo-and photo-autotrophs of the order Pseudomonadales are characterized by the omnipresence of cytochrome(s) *c* (Meyer and Jones, 1973a). This observation along with the ability of the periplasmic cytochrome to bind carbon monoxide would confer on the marine bacterium a high degree of adaptability which could be beneficial in *in vivo* situations to sustain itself under highly unstable or even competitive environments.

Changes in cytochrome content as well as in respiratory rates throughout the growth cycle of A. *haloplanktis* were observed. The relationships between cytochrome synthesis and respiration rates were of two kinds: (*i*) the *in situ* respiratory rates were observed to reach a peak following five hours of growth in a complex nutritive medium (Figure 20, lower trace) while, at the same time, the concentration of the periplasmic cytochrome c_{549} was reaching its first peak in the same growth medium (Figure 18). (*ii*) The *potential* or exogenous respiratory rates achieved maximum values following approximately nine hours of growth in the complex medium (Figure 20, upper trace), a comparable lapse of time required by the membrane-bound cytochromes to achieve also maximal concentration, after which, both potential respiratory rates and membrane-bound cytochromes declined as growth proceeded through the stationary phase. In either case, it was obvious that any fluctuation in the dissolved oxygen concentration was translated into variations of the cytochrome complement of the marine micro-organism.

An aerobic micro-organism to which the concentration of dissolved oxygen is so vital must be able to exert some sort of respiratory control, in a broad sense, in order to maintain itself. A manifestation of such control can be expressed at the level of

primary dehydrogenases (White, 1964; Scholes and Smith, 1968; Anthony, 1975), at the level of terminal oxidases (Ackrell and Jones, 1971a,b; Poole, 1983a,b) or take into account the whole electron transport chain (Chance and Williams, 1955a,b,c,d). No typical mitochrondrial-type respiratory control (Chance and Williams, 1956) has so far been extensively documented nor fully demonstrated in bacterial systems. Recent work in that direction with *Escherichia coli* (Burnstein *et al.*, 1979) and *Paracoccus denitrificans* (Erecinska *et al.*, 1979) reached the following conclusions to the effect that respiratory control in bacteria was difficult to show because (*i*) the cell wall of the bacterium was impermeable to exogenous ADP and (*ii*) because growing cells were respiring at maximal rate, *i.e.* liberating continuously ADP plus phosphate through ATP-utilizing synthetic processes. However, with the use of starved cells and NO_3^- as electron acceptor, it was possible to show such control in the facultative anaerobic cells using a respiratory substrate which was not a carbon source mainly ascorbate plus phenazine methosulfate. In *P. denitrificans*, it was shown that in addition to the dependence of the respiratory control on intracellular (ATP/(ADP) P_i), the redox of cytochrome c was also involved in the respiratory control of the aerobically grown bacterium. Recently, the control of electron flow in the respiratory chain of *Micrococcus lysodeikticus* via ATP/ADP in intact bacterial cells was described for the first time (Rosenberg and Friedberg, 1984). The direct demonstration of respiratory control was possible due to the unique phosphate metabolism in *M. lysodeikticus*. The P:O ratio was calculated to be 1.0 in P_i-deprived cells, similar to the value reported earlier in membrane fragments of the same organism.

Cells of *A. haloplanktis* growing in a complex nutritive environment will allow themselves to bring down the dissolved oxygen concentration to values equal to or lower than 1% air-saturation, whereas cells from a simple growth environment will not go further down than 25% or so while presumably performing the same metabolic activities.

Cells grown in 30% complex medium compare well with cells grown in succinate chemically defined medium in terms of final cell yield and capacity to lower the dissolved oxygen content in the growth medium. Although the doubling time in 30% complex medium is half the doubling time in succinate chemically defined medium, the amount of membrane-bound cytochromes is twice as much and the amount of periplasmic cytochrome is six times as much as in cells grown in chemically defined medium (Table XI). It appears that the membrane-bound cytochromes tend to respond to a growth rate effect (half the doubling time, twice the amount of cytochromes synthesized), whereas the periplasmic species does not. Since all these cell types possess physiologically active membrane-bound cytochromes, the phase of growth beyond 25% air-saturation in complex medium must involve an alternative or a more branched and controlled respiratory chain. In that perspective, it might be conceivable that the periplasmic cytochrome of the marine bacterium, by virtue of its location and possible different redox state, when compared to its membrane-bound counterparts, is responsible for the compulsory function of eliminating or draining off large concentrations of reducing equivalents accumulating at certain stages of growth. As suggested by Meyer and Jones (1973b,c) and Jones *et al.*, (1975, 1978) multiple oxidases found in bacteria may function at different medium dissolved oxygen tensions and be associated with different efficiencies of phophorylation.

CHAPTER V

ELECTRON TRANSPORT CHAIN

1. INTRODUCTION

In aerobic micro-organisms, respiration is mediated through the electron transport chain which involves the cytochromes as electron carriers. The nature and order of the respiratory components, cytochromes included, can be elucidated through the selective use of respiratory substrates and inhibitors. During substrate reduction, a steady state of electron flow reducing the components of the respiratory chain according to their redox potentials is established according to their capacity to accept or give away electrons. During this steady state, the cytochromes become reduced and the extent of reduction is dependent on the relative activities of the dehydrogenases and terminal oxidases. If the electron flow is inhibited, then any component between the dehydrogenase and the point of inhibition would become more reduced, and any component on the oxygen side of the inhibitory block would become more oxidized.

Spectrophotometric and polarographic studies making use of respiratory inhibitors as well as substrates with known redox potentials have been undertaken with intent to establish the sequence of the electron transfer intermediates in the respiratory chain of *Alteromonas haloplanktis*.

2. MATERIALS AND METHODS

Preparation of Cells and Cellular Fractions

Alteromonas haloplanktis was grown at 25°C under vigorous agitation for a period of 16 hr in the complex medium and for 24 hr in the succinate chemically defined medium. The composition of the growth media and the inoculating procedure have been detailed in Chapter IV. The whole cells were harvested by centrifugation (Sorvall RC2-B, 16,000 xg for 10 min, rotor GSA, 4°C), washed twice in a complete salt solution (.3M NaCl, .05M $MgCl_2$ + .01M KCl), suspended in complete salt buffered with Tris-PO_4 (50 + 1mM respectively, pH 7.4) and disrupted in a French Press at 14-16,000 psi. (American Instrument Co.). The cell debris were removed by centrifugation (Sorvall RC2-B, 39,000 xg for 20 min, rotor SS34, 4°C) to yield clear cell-free extract. The cell-free extract was further ultracentrifuged at 150,000 to 180,000 xg for 3 hours, Beckman L2-65B, rotor 60 Ti, 4°C) to obtain the (sedimented) membrane fraction and the (supernatant) periplasmic fraction (Knowles *et al.*, 1974), hereafter referred to as the periplasmic fraction.

Starvation Procedures

According to similar procedures used for *Escherichia coli* (Berger, 1973; Berger and Heppel, 1974), stationary phase cells of *A. haloplanktis* were depleted of their endogenous reserves by vigorous agitation in a sterile salt solution containing .3M NaCl, .05M $Mg\ Cl_2$ and .01M KCl.

Difference Absorption Spectrophotometry

The technique of difference absorption spectrophotometry as devised by Chance and Williams (1955a,b,c,d, 1956) was utilized throughout the course of the different manipulations. The chemical reduction and oxidation of the respiratory pigments were measured

in whole cells, cell-free extracts, membrane and periplasmic fractions using dithionite $(S_2O_4)^{-2}$ as reducdant and ferricyanide $[Fe(CN)_6]^{-3}$ as oxidant. The Hitachi Perkin Elmer (Model 356) spectrophotometer was attached to a Perkin Elmer recorder (Model 056) and equipped with a Dewar cryogenic cell to permit the recording of absorption spectra at low (liquid air) temperature (83°K).

The biological reduction of the respiratory pigments was also carried out with substrates such as: NADH, succinate, ascorbate, and ascorbate/TMPD; likewise O_2 gas, compressed air and hydrogen peroxide (H_2O_2) were utilized to oxidize biologically the same pigments. The gases were flushed into the suspensions through a disposable Yale syringe.

The difference spectra were recorded at various absorbancy scales in order to register minor as well as major absorption peaks. The scanning speed was set at 60 nm/min and the chart recorder speed at 60mm/min, such that every mm on the chart paper corresponded to one nm. The slit opening was adjusted to 1.0 nm. Room temperature difference spectra were measured in 10 mm quartz cuvettes and low temperature spectra in 2 mm cryogenic cells, in the presence of 0.75M final sucrose concentration.

Difference Absorption Spectrophotometry with Inhibitors

The enzyme preparations were incubated for 3 min at room temperature in the presence of selected inhibitors prior to the addition of substrates. The final volume of the reference cuvette was made up to 500µl by addition of a buffered salt solution. All the reactions were allowed to reach steady state at room temperature before the spectra were recorded at room temperature or at low temperature. The concentrations and specificity of the inhibitors tested are outlined in the results section. Depending on the solubility of the inhibitors, buffer, methanol (Fisher Sci. Co.) or dimethylformamide (Fisher Sci. Co.) were employed as solvents

in a final volume of 50 µl. Whenever necessary, correction for solvent effects due to solvent interaction with the enzyme preparation was made by adding the solvent to the contents of both cuvettes.

The final reaction volume was 500 µl in all the cases and was made up of: 50 µl inhibitor, 350 µl protein suspension and 100 µl of substrate in buffered salt solution (.3M NaCl, .05M $MgSO_4$, .01M KCl) *plus* Tris-PO_4 (50 + 1mM, pH 7.4). Addition of inhibitor and/or substrate into the sample cuvette was compensated by an equal addition of complete salt Tris-PO_4 to the content of the reference cuvette. Stock solutions of sucrose, substrates and inhibitors (whenever possible) were all made up in complete salt Tris-PO_4, pH 7.4.

In some difference spectra, treatment of the contents of the sample or reference cuvette is indicated directly either by the subscript letter (S) or (R) in parenthesis beside the spectrum or by a numerator (e.g. $(S_2O_4)^{-2}$) representing the contents of the sample cuvette reduced by solid crystals of dithionite and a denominator (/ $[Fe(CN)_6]^{-3}$) representing the contents of the reference cuvette oxidized by solid crystals of ferricyanide.

Difference Absorption Spectrophotometry with Carbon Monoxide

Reduced + CO *minus* reduced difference spectra were recorded with chemical and biological reductants. The contents of the sample and the reference cuvettes were reduced with appropriate substrate, then CO (Matheson Co.) was slowly bubbled for 3 min in the sample which was sealed off with a parafilm paper and allowed to incubate in darkness for an additional 10 min. All these manipulations were performed at room temperature under a fume hood. The difference spectra were recorded at room or at low temperature depending on the fractions investigated.

Oxidase Assay Procedures

NADH, succinate and ascorbate/TMPD oxidase activities were measured polarographically at 25°C using an O_2 electrode cell (Rank Bros., Bottisham, Cambridge, England) calibrated according to the specifications of Chappell (1964) such that a final volume of 3.0 ml of air-saturated buffer contained 1422 n atoms O. In the absence of respiratory inhibitors, the reaction mixture contained 2.90 ml of complete salts buffered with 50mM Tris and 1mM PO_4, pH 7.4 and 0.10 ml of enzyme preparation originating from cells or subcellular fractions. In the presence of inhibitors, the volume of buffer was reduced to 2.85 ml and the inhibitor was added in a final volume of 50µl. The consumption of O_2 was recorded with and without inhibitors to establish the percentages of oxidation and inhibition of oxidation. Appropriate controls were run to cancel the solvent effect whenever detected. Depending on the solubility of the inhibitors tested, buffer, methanol or dimethylformamide were utilized as solvents. As in the spectrophotometric study, an incubation period of three minutes was allowed for the enzyme preparation to react with the inhibitors prior to the addition of substrate. Initial oxidation rates were then recorded for a period of 10 minutes with a recorder output operating at 10 mV and a chart speed fixed at 1cm/min (Cole Parmer Instrument Co.).

Catalase Assay Procedure

The disappearance of exogenous hydrogen peroxide was estimated polarographically with a Clark oxygen electrode covered with a thin Teflon membrane and maintained at the constant temperature of 25°C by use of a water jacket. During the breakdown of hydrogen peroxide by the enzyme catalase, oxygen was evolved. The amount of oxygen released was used to detect the amount of catalase present in the sample tested according to the relationship: $2H_2O_2 \longrightarrow 2H_2O + O_2$ (Rørth and Jensen, 1967; Goldstein, 1968; Mahler and Cordes, 1971). O_2 evolution was initiated by adding 10 µl of H_2O_2

(0.58mM, final concentration) to the 2.99 ml reaction mixture containing the enzyme preparation in the buffered salt solution.

Chemicals

In the spectrophotometric and the polarographic studies, the following substrates were used at the concentrations indicated in parenthesis or otherwise specified in the text: sodium hydrosulfite or dithionite, $Na_2S_2O_4$ (Fisher Sci. Co., few grains), sodium ferricyanide, $Na_3Fe(CN)_6$ (ICN Pharmaceuticals Inc., Plainview, N.Y., few grains), reduced nicotinamide adenine dinucleotide, NADH (Sigma Chem. Co., 2.5mM), sodium succinate (Sigma Chem. Co., 10mM), sodium ascorbate (Sigma Chem. Co., 10mM), N,N,N',N' tetramethyl-p-phenylenediamine 2HCl, TMPD (Eastman Kodak Co., 0.5mM), compressed air (Liquid Air Co.), oxygen gas (Matheson Co.), hydrogen peroxide, H_2O_2 (Fisher Sci. Co., 1.0mM), carbon monoxide, CO O_2-free (Matheson Co.), nitric oxide, NO, (Matheson Co.).

The following inhibitors were tested at different concentrations: sodium azide (Sigma Chem. Co.), potassium cyanide (Fisher Sci. Co.), atabrine (Sigma Chem. Co.), rotenone (Sigma Chem. Co.), 2-heptyl 4-hydroxyquinoline-N-oxide, HOQNO (Sigma Chem. Co.), antimycin A (Sigma Chem. Co.), amytal (Sigma Chem. Co.), thenoyl-trifluoracetone, TTFA (Fisher Sci. Co.).

The substrate stock solutions were freshly prepared daily in buffered salt solution; likewise the inhibitor stock solutions were made up in buffer, in methanol or in dimethylformamide, according to their solubility. All the chemicals used were reagent grade. All the stock solutions were prepared with glass distilled water.

Protein Determination

The protein content of the various fractions tested were determined according to a Biuret method (Gornall *et al.*, 1949), using lysozyme (1 to 10 mg protein/ml, Sigma Chem. Co.) as the protein standard.

3. RESULTS AND DISCUSSION

A. SPECTROPHOTOMETRIC STUDY OF THE ELECTRON TRANSPORT CHAIN

Oxidation of Cytochromes at Steady State

The steady state oxidation of the cytochromes was investigated in cellular extracts, a subcellular fraction comprising both the periplasmic and the membrane fractions.

Time course for the oxidation of cellular extracts using H_2O_2 as an oxidizing agent is presented in Figure 21. Five to ten minutes were required for the oxidizing agent to react with the enzyme preparation and cause the development of a difference spectrum. Figure 22 shows a series of difference spectra in which the content of the sample cuvette was exposed for 10 min to a mild stream of O_2 gas before the spectra were recorded; the reference cuvette contained an identical concentration of cell extracts untreated. Ten minutes exposure to the oxidizing agent was sufficient to maintain the enzyme preparation in an oxidized state. Longer exposure to the oxidizing agent did not ensure more complete oxidation. After 60 minutes of incubation at room temperature, some *b* and *c* cytochromes became reduced (30% and 45% respectively). As reported for other bacterial systems (Lanyi, 1968; Ishaque and Kato, 1974; Ishaque, 1984), the cytochromes are eventually reduced by endogenous reserve material in the absence of a continuous supply of O_2. However, the enzyme preparation was still fully redox competent since initial levels of oxidation were restored upon flushing O_2 gas through the sample cuvette (Figure 22, upper trace).

Although the physiological oxidant, O_2 gas, was efficient in oxidizing the enzyme preparations, a more pronounced difference in absorption was recorded when ferricyanide was utilised in the reference cuvette. Figures 23 and 24 illustrate room temperature difference spectra recorded with the membrane and periplasmic

Figure 21. Development of H_2O_2–oxidized *minus* endogenously reduced difference spectrum of cell-free extracts of *A. halo-planktis* with time.

H_2O_2–oxidized *minus* endogenously-reduced difference spectra of cell-free extracts. The extracts were initially reduced with endogenous substrate(s) and divided into the sample and the reference cuvette. The curves show the difference absorption spectra at 0,5,10,15, and 30 min after addition of H_2O_2 (1.0 mM) to the sample cuvette. An identical volume of buffered salt solution was added to the reference cuvette. The spectra were recorded at room temperature in cuvettes of 10 mm light path. The baseline was obtained by scanning the same concentration of untreated extracts (13.4 mg protein/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded. The spectra were scanned from 600 to 500 nm and the wavelengths (in nanometers) are as shown.

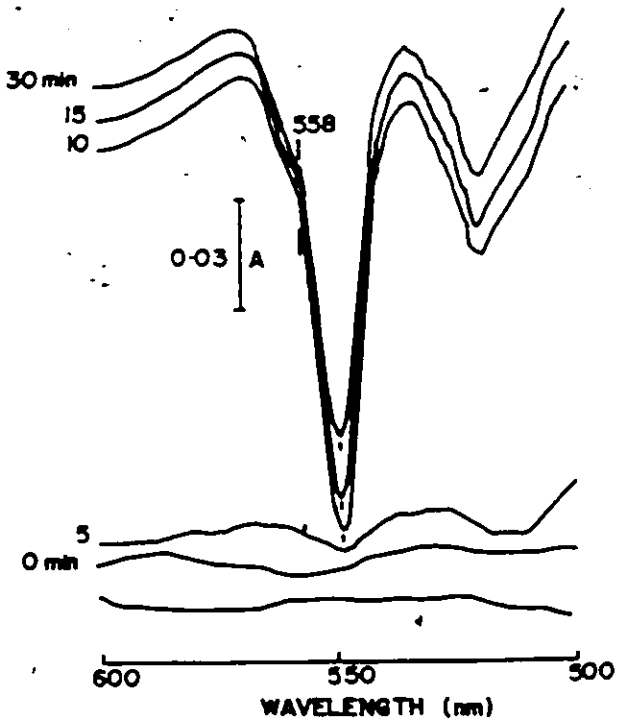

30 min
15
10
558
0·03 A
5
0 min
600
550
500
WAVELENGTH (nm)

Figure 22. Stability of the O_2-oxidized *minus* endogenously reduced difference spectrum of *A. haloplanktis* with time.

O_2-oxidized *minus* endogenously-reduced difference spectra of cell-free extracts. The extracts were initially reduced with endogenous substrate(s) and divided into the sample and the reference cuvette. The curves show the difference spectra at 3,10,20, and 30 min after bubbling O_2 gas into the contents of the sample cuvette for 10 min. The spectra were recorded at room temperature in 10 mm cuvettes light path. After 60 min of reaction, O_2 gas was bubbled again for an additional 10 min into the sample cuvette and another difference spectrum was recorded (upper trace). The baseline was obtained by scanning the same concentration of untreated extracts (15.0 mg protein/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.

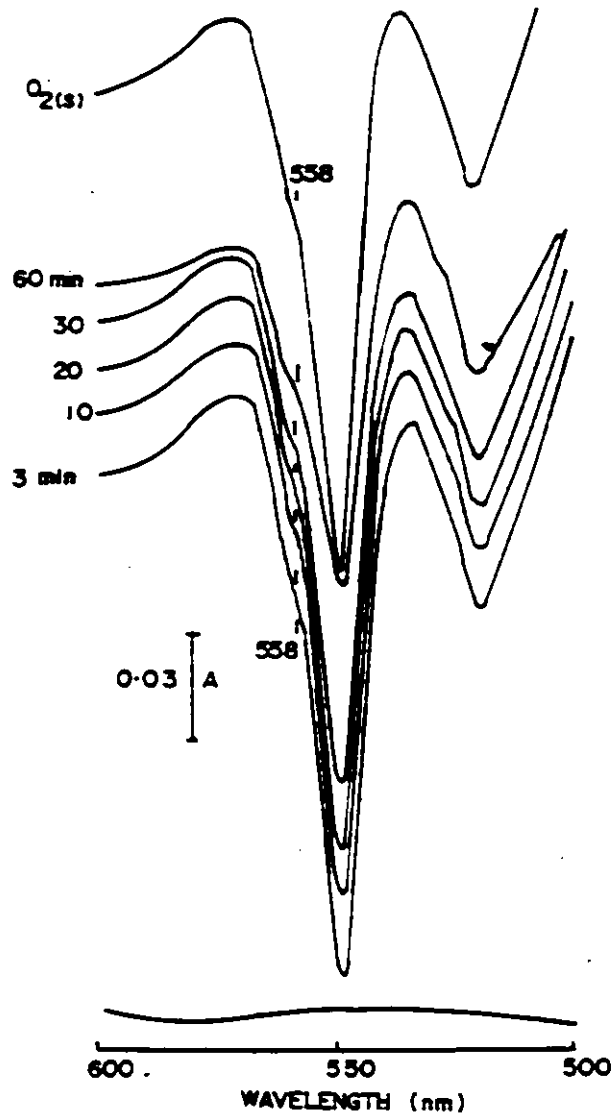

$O_{2(s)}$
558
60 min
30
20
10
3 min
558
0·03 A
600
550
500
WAVELENGTH (nm)

Figure 23. Dithionite-reduced *minus* oxidized difference spectra of the membrane fraction using various oxidants at room temperature.

The room temperature difference spectra are dithionite-reduced *minus* oxidized difference spectra. The oxidants used were: O_2 gas, H_2O_2 (1.0 mM), $[Fe(CN)_6]^{-3}$. The exposure time to the various oxidants was 10 min. The treatment of the contents of the cuvettes, the sample and the reference, is indicated by the numerator ($S_2O_4^{-2}$) and the denominator ($/O_2$) beside each curve. The baseline was obtained by scanning the same concentration of untreated protein (11.2 mg/ml) present in the sample and the reference cuvettes. The vertical bars represent one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.

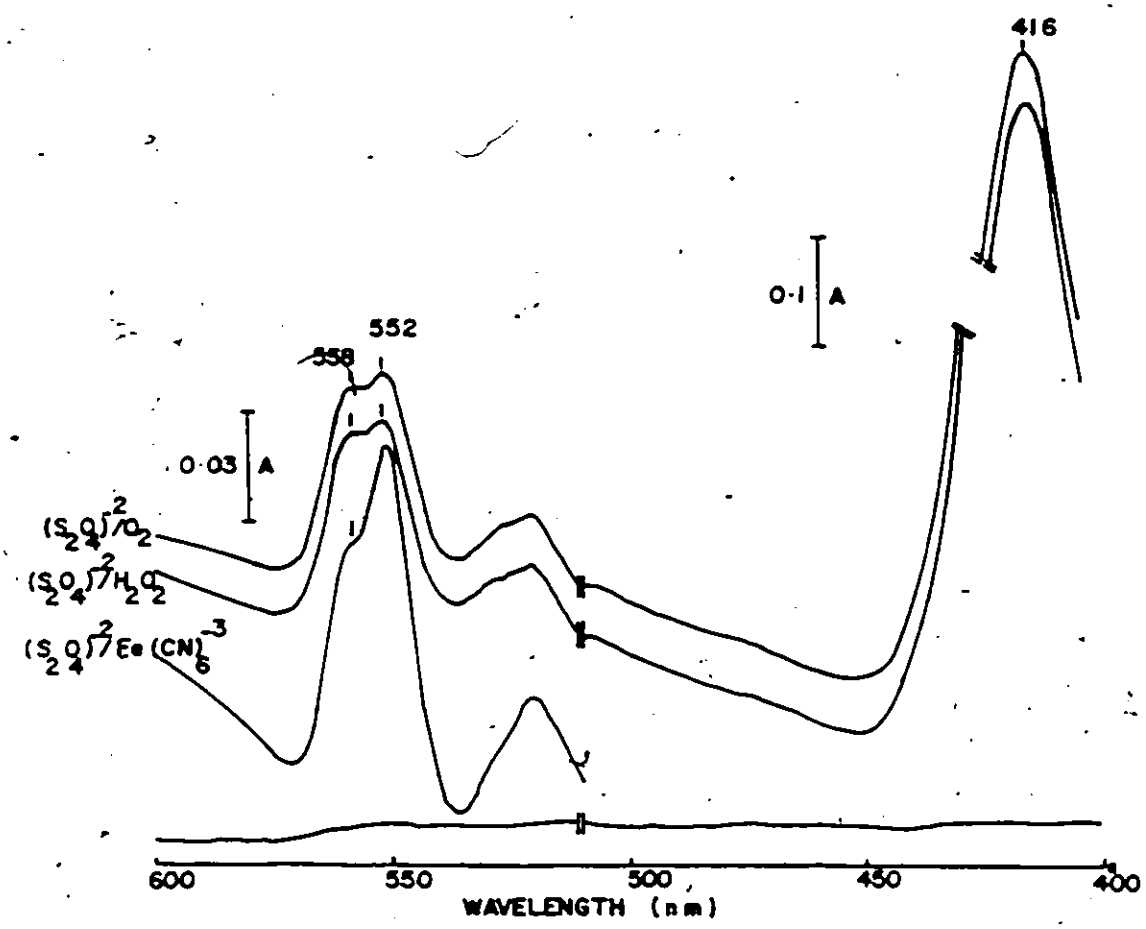
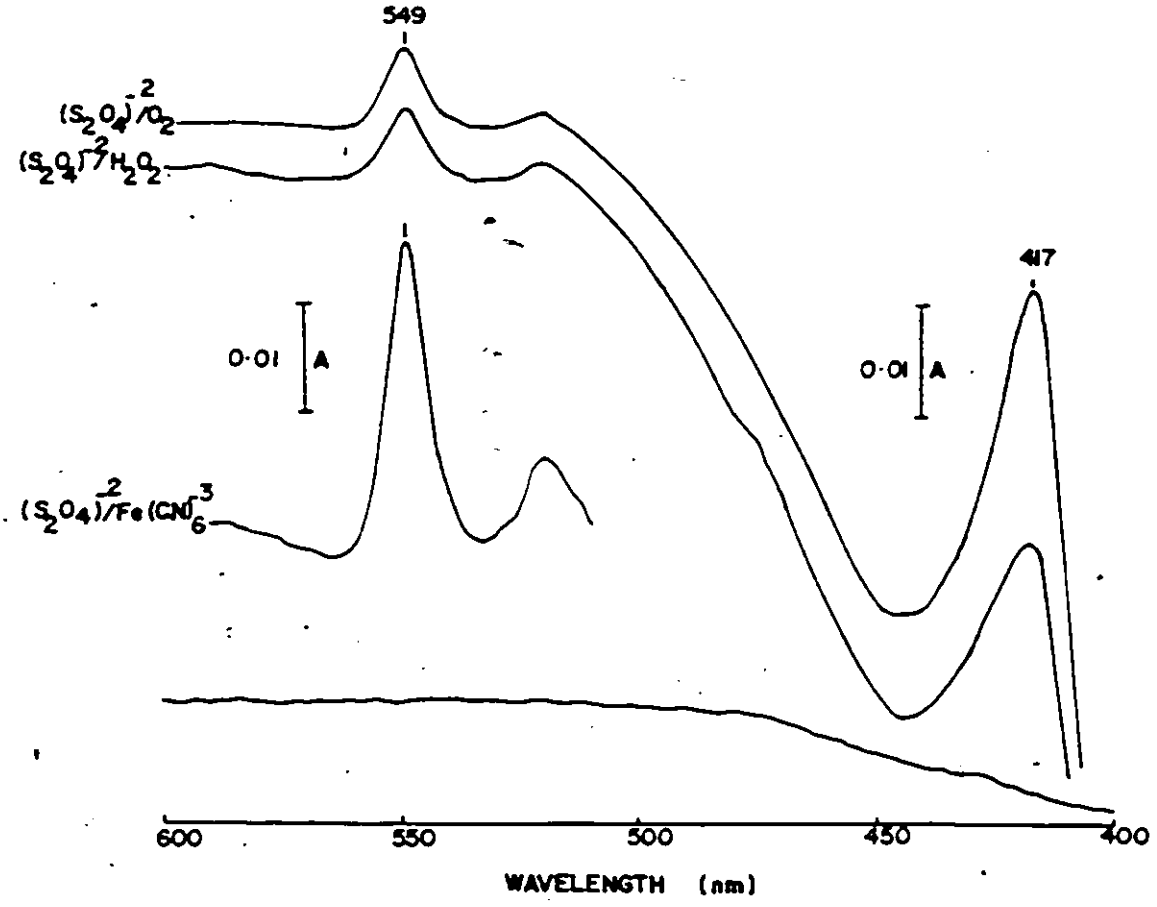

416
0·1 A
552
558
0·03 A
$(S_2O_4)^{-2}/O_2$
$(S_2O_4)^{-2}/H_2O_2$
$(S_2O_4)^{-2}/Fe(CN)_6^{-3}$
600
550
500
450
400
WAVELENGTH (nm)

Figure 24. Dithionite-reduced *minus* oxidized difference spectra of the periplasmic fraction using various oxidants at room temperature.

The room temperature difference spectra are dithionite-reduced *minus* oxidized difference spectra. The oxidants used were: O_2 gas, H_2O_2 (1.0 mM) and $[Fe(CN)_6]^{-3}$. The exposure time to the various oxidants was 10 min. The treatment of the contents of the cuvettes, the sample and the reference cuvette, is indicated by the numerator ($S_2O_4^{-2}$) and the denominator ($/O_2$) beside each curve. The baseline was obtained by scanning the same concentration of protein (3.36 mg/ml) present in the sample and the reference cuvettes. The vertical bars represent one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.


549
417
$(S_2O_4)^{-2}/O_2$
$(S_2O_4)^{-2}/H_2O_2$
0·01 A
0·01 A
$(S_2O_4)^{-2}/Fe(CN)_6^{-3}$
600
550
500
450
400
WAVELENGTH (nm)

fractions reduced by dithionite and oxidized by O_2 gas, hydrogen peroxide (H_2O_2) and ferricyanide $[Fe(CN)_6]^{-3}$. Discrepancy in the level of cytochrome oxidation by O_2 gas and ferricyanide has been observed in other bacterial systems (Probst and Schlegel, 1976; Greenwood *et al.*, 1978; Barber *et al.*, 1978), with O_2 gas, and H_2O_2 not oxidizing the cytochromes as completely as did ferricyanide.

Qualitative difference spectra of the cell-free extracts reduced by dithionite and oxidized respectively by H_2O_2, O_2 gas, and compressed air are reproduced in Figure 25. In this instance, the Soret band for cytochrome b and c absorbed at 422nm. No Soret peak could be obtained with ferricyanide as oxidant due to strong absorption of the chemical itself below 510nm. Quantitative estimations of the respective spectra (Table XII) revealed that $(S_2O_4)^{-2}$ *versus* $[Fe(CN)_6]^{-3}$ consistently gave the highest possible difference recorded with a stable oxidant. When taken as 100%, *i.e.*, as the maximum oxidation value recorded by difference absorption spectrophotometry, and compared to oxidation levels achieved with H_2O_2 and O_2 gas, it was observed, that 80 to 85% of the cytochromes present in CFE were biologically oxidizable. The possibility that oxidation of endogenous substrate(s) could be responsible for the partial reduction of the cytochromes is not excluded.

Reduction of Cytochromes at Steady State

Physiological reduction of the cytochromes present in cell-free extracts was improved by deriving the extracts from starved stationary phase cells and by exposing the extracts to an oxidizing agent prior to substrate reduction. Although the endogenous reduction of the cytochromes could not be wiped out entirely, net reduction of the cytochromes by NADH, succinate, ascorbate and ascorbate/TMPD was definitely recorded. The series of graphs presented in Figure 26 illustrate clearly this point. The A,B,C,D series represent the reduction level of b-and c-type cytochromes by NADH

Figure 25. Dithionite-reduced *minus* oxidized difference absorption spectra of cell-free extracts of A. *haloplanktis*, using solid crystals of sodium dithionite as reducing agent and H_2O_2, O_2 gas, compressed air and $[Fe(CN)_6]^{-3}$ as oxidizing agents.

Difference spectra of cell-free extracts reduced with sodium dithionite in the sample cuvettes and oxidized with either H_2O_2 (1.0 mM), O_2 gas, compressed air or solid crystals of ferricyanide in the reference cuvettes. The spectra were recorded 10 minutes after exposure to the various oxidants at room temperature in cuvettes of 10 mm light path. The baseline was obtained by scanning the same concentration of untreated protein (11.4 mg/ml) present in the sample and the reference cuvettes. The vertical bars represent one tenth of the absorbancy units at which the spectra were recorded and the wavelengths are expressed in nanometers.

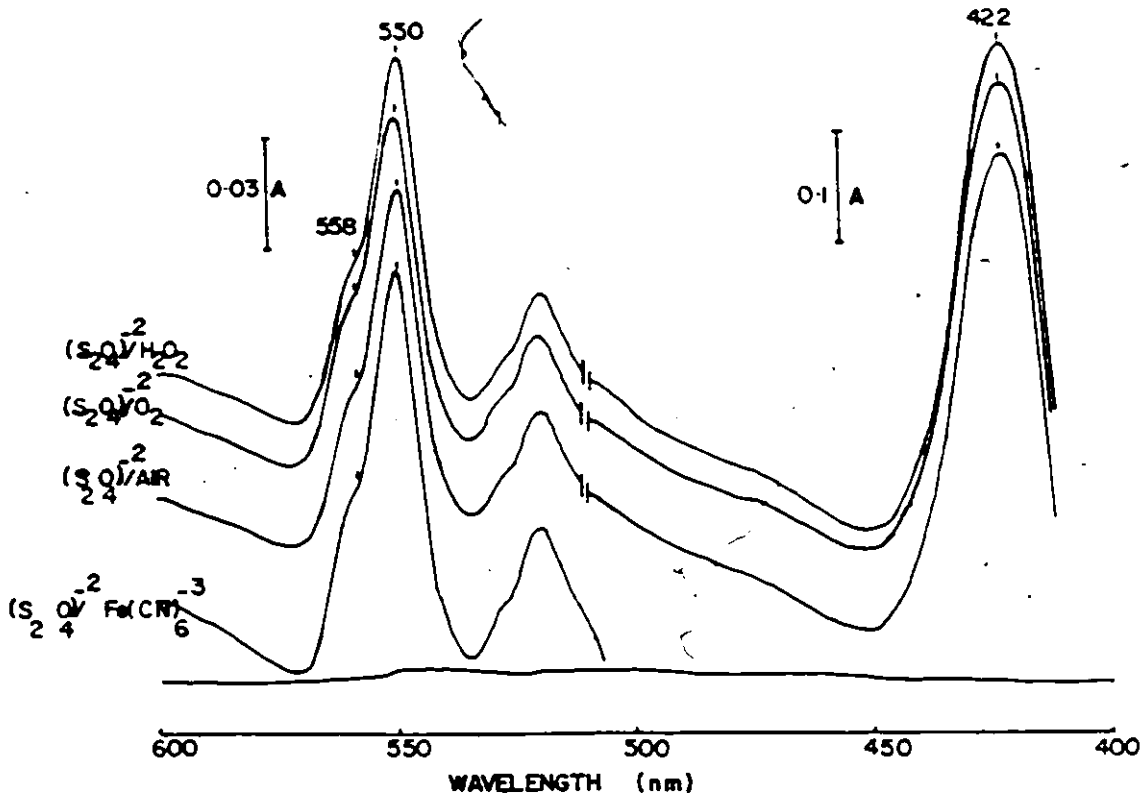

550
422
558
0·03 A
0·1 A
$(S_2O_4^{-2}/H_2O_2$
$(S_2O_4^{-2}/O_2$
$(S_2O_4^{-2}/AIR$
$(S_2O_4^{-2}$ $Fe(CN)_6^{-3}$
600
550
500
450
400
WAVELENGTH (nm)

TABLE XII. Steady state oxidation levels of *b*- and *c*-type cytochromes in cellular extracts of *A. haloplanktis* obtained with various oxidants and using $(S_2O_4)^{-2}$ as reductant, measured by difference absorption spectrophotometry.

TREATMENT*	Cytochrome content** (n moles/mg protein)								
	b-type cytochromes					*c*-type cytochromes			
	mean	S.D.†	C.V.††	% oxidation†††		mean	S.D.†	C.V.††	% oxidation†††
$(S_2O_4)^{-2}/[Fe(CN)_6]^{-3}$	.302	± .026	8.5	100		.628	± .072	11.4	100
$(S_2O_4)^{-2}/H_2O_2$	.264	± .026	9.7	87.4		.503	± .076	15.0	80.1
$(S_2O_4)^{-2}/O_2$	.261	± .028	10.6	86.4		.512	± .052	10.2	81.5

*sample cuvette/reference cuvette

**average values based on 11 separate determinations.

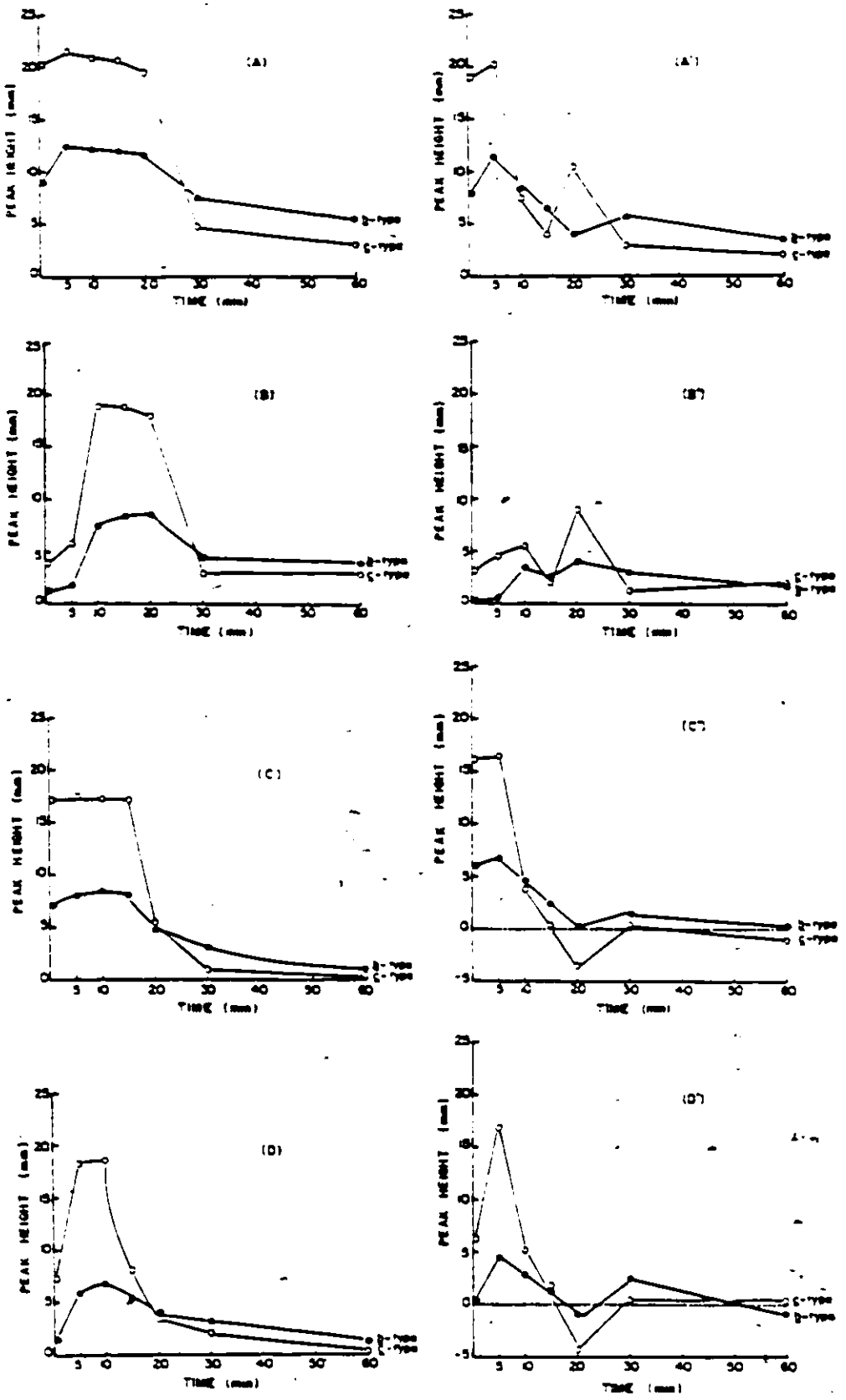
The figures given are mean values together with the standard deviation (†) of the mean and the variation coefficient (††). The percentages (†††) given refer to that percent of cytochromes oxidized compared to that quantity of the components oxidizable by ferricyanide as quantified in a dithionite-reduced *minus* ferricyanide-oxidized difference spectrum.

Wavelengths and extinction coefficients used in calculating cytochrome content:

b-type: 559 nm *minus* 571 nm, $\Delta\varepsilon_{mM}$: 17.5; *c*-type: 552 nm *minus* 534 nm, $\Delta\varepsilon_{mM}$: 17.3.

Figure 26. Levels of reduction of the cytochromes present in cell-free extracts of A. *haloplanktis* as measured by difference absorption spectrophotometry with time and with various reductants.

The cell-free extracts were derived from stationary phase cells of A. *haloplanktis* grown in complex medium and starved aseptically in complete salt solution. The cellular extracts were bubbled for 10 min with O_2 gas prior to treatment with the reductants. The substrate-reduced *minus* O_2-oxidized difference spectra were recorded at room temperature at predetermined time intervals and the peak heights measured in mm; the absorbancy scale at which the spectra were recorded was 0.3 and the protein concentration 14.0 mg/ml. In the A,B,C,D series, the cytochromes were reduced by residual endogenous reserves as well as by the following exogenous substrates: (A) NADH, 2.5 mM; (B) succinate, 10 mM; (C) ascorbate, 10 mM; (D) ascorbate/TMPD, 10+0.5 mM. In the A',B',C',D' series, the residual endogenous reduction was substracted and only the net exogenous substrate reduction is shown. The empty circles (o——o) represent the c-type cytochromes absorbing at 552 nm at room temperature and the closed circles (●——●) represent the *b*-type cytochromes absorbing at 559 nm at room temperature.


(A)
(A')
(B)
(B')
(C)
(C')
(D)
(D')
PEAK HEIGHT (mm)
TIME (min)
g-type
c-type

succinate, ascorbate and ascorbate/TMPD along with the endogenous reduction level of the cell-free extracts. The A',B',C',D' series represent the net reduction level of the cytochromes by the respective exogenous substrates. Within the first 5 minutes of exposure to NADH, ascorbate and ascorbate/TMPD (Figure 26, series A',C',D') net reduction of the extracts was recorded for the respective substrates. With succinate (Figure 26, series B') 15 to 20 minutes of exposure were required to achieve net reduction.

Quantitative estimates of cytochrome reduction by various substrates are detailed in Table XIII. Dithionite is the reductant used to detect total pigments as it permits the reduction of those whose natural substrates or reductases were lost during fractionation (Appleby, 1969a,b). NADH could fully reduce the *b*-and *c*-type cytochromes present in cell-free extracts when compared to dithionite *versus* O_2 treated samples. Succinate, ascorbate and ascorbate/TMPD reduced the cytochromes *b* to 70% the extent of that obtained with dithionite and NADH; the cytochromes *c* were reduced above 85% by all the substrates tested, NADH and ascorbate/TMPD having access to all the *c*-type cytochromes of the chain as evidenced by the very high percentage of reduction when compared to dithionite $(S_2O_4)^{-2}$ used as reductant (Table XIII).

The cytochromes present in whole cells were also substrate reducible. Figure 27 shows a series of difference spectra recorded at the temperature of liquid air (83°K) after reduction of the cytochromes by dithionite (trace *a*), ascorbate/TMPD (trace *b*), succinate (trace *c*), NADH (trace *d*) and remaining endogenous substrate(s) (trace *e*) of the starved cells. No *a*-type cytochromes were spectrophotometrically detectable in the 650 to 585 nm region of the visible range, while *b*-and *c*-type cytochromes absorbed at characteristic wavelengths, 558 and 556 nm for the *b*-types, 549 and 546 nm for the *c*-types respectively.

Cytochrome reduction by physiological and non-physiological

TABLE XIII. Steady state reduction levels of *b*-and c-type cytochromes in cellular extracts of A. *haloplantis* obtained with various reductants and using O_2 as the oxidant, measured by difference absorption spectrophotometry.

TREATMENT*	Cytochrome content*** (n moles/mg protein) *b*-type cytochromes				c-type cytochromes			
	mean	S.D.†	C.V.††	% reduction†††	mean	S.D.†	C.V.††	% reduction†††
$(S_2O_4)^{-2}/O_2$	.240	± .024	10.1	100	.463	± .053	12.4	100
NADH/O_2	.243	± .023	9.6	100	.463	± .059	12.6	100
Succinate/O_2	.174	± .037	21.6	72.5	.423	± .094	22.3	91.4
Ascorbate/O_2	.160	± .038	23.8	66.7	.398	± .057	14.3	86.0
Asc. + TMPD/O_2	.222	± .017	7.5	76.0	.549	± .064	11.7	100
ENDOGENOUS/O_2	.071	± .010	14.1	29.6	.207	± .032	15.4	44.7

*sample cuvette/reference cuvette

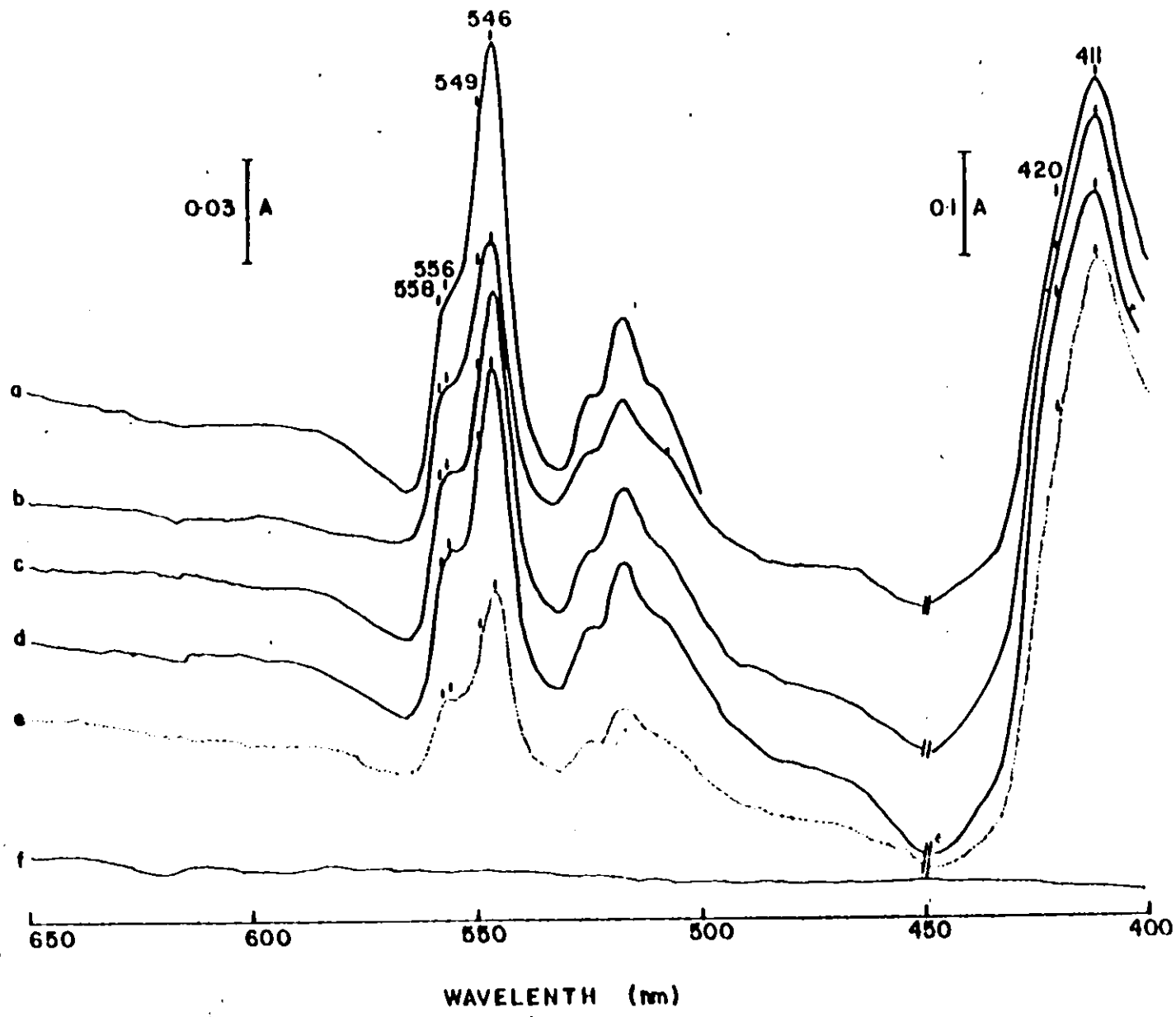
**average values based on at least 5 separate determinations.

The figures given are mean values together with the standard deviation (†) of the mean and the variation coefficient(††). The percentages (†††) given refer to that percent of cytochromes reduced compared to that quantity of the cytochromes reducible by dithionite as quantified in a dithionite-reduced *minus* O_2-oxidized difference spectrum.

Wavelength and extinction coefficients used in calculating cytochrome content:
b-type: 559 nm *minus* 571 nm, $\Delta\varepsilon$mM: 17.5; c-type: 552 nm *minus* 534 nm, $\Delta\varepsilon$mM: 17.3.
The concentration of substrates employed are as outlined in Materials and Methods.

Figure 27. Difference absorption spectra of intact cells of A. *haloplanktis* in the presence of various reductants.

Substrate-reduced *minus* O_2-oxidized difference spectra of whole cells of A. *haloplanktis*. The starved cell suspension was mixed (1:1) with sucrose (0.75 M, final concentration), bubbled with O_2 gas for 10 min and divided into the sample and the reference cuvette. The reductants used were from top to bottom: a) dithionite; b) ascorbate/TMPD, 10+0.5 mM; c) NADH, 2.5 mM; d) succinate, 10 mM; e) remaining endogenous substrate(s). The oxidant used in trace a was ferricyanide and O_2 gas for the following traces. The baseline (trace j) was obtained by scanning the same concentration of O_2-oxidized cells (19.5 mg protein/ml) present in the sample and the reference cuvettes. The spectra were recorded at 83°K in cuvettes of 2 mm light path. The vertical bars represent one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.


546
549
411
420
0·03 A
0·1 A
556
558
a
b
c
d
e
f
650
600
550
500
450
400
WAVELENTH (nm)

substrates was likewise observable at the membrane and periplasm level. Figure 28 presents low temperature difference spectra of the periplasmic fraction reduced by NADH and by ascorbate/TMPD and oxidized by ferricyanide. With either substrate, only one cytochrome species absorbed symmetrically at 546 nm. Residual endogenous reserves also reduced the periplasmic cytochrome absorbing at the same wavelength. Figure 29 presents low temperature difference spectra of the membrane fraction reduced by NADH, succinate and ascorbate/TMPD and oxidized by ferricyanide. Again remaining endogenous reserves reduced the same cytochromes at typical wavelengths. The presence of two cytochromes *b* absorbing at 558 and 556 nm and two cytochromes *c* absorbing at 547 and 544 nm in the membrane fraction were detected with the various reductants employed. As mentioned, endogenous levels of reduction were detected in both fractions and are represented by the dotted lines.

Cytochrome Reduction in the Presence of Cyanide

Detailed study of the electron transport chain requires the use of respiratory inhibitors. The spectrophotometric as well as the polarographic approach were both useful in elucidating the sequence of the respiratory components.

Careful studies were carried out with cyanide and other inhibitors in whole cells, cell-free extracts, membrane and periplasmic fractions. It soon became obvious that the endogenous metabolism of the marine bacterium was interfering with the study undertaken and was complicating the interpretation of the data. Meaningful results could only be obtained providing the endogenous respiration of the microorganism was eliminated or at least minimized. In a system which has not been submitted to any starvation method, the difference spectrum initially recorded between a reduced and an oxidized preparation fades away with time due to reduction of the contents of the reference cuvette by the endogenous

Figure 28. Substrate-reduced *minus* ferricyanide-oxidized difference spectra at low temperature of the periplasmic fraction of *A. halocplanktis* grown in complex medium.

The substrates used with the periplasmic fraction were from top to bottom: ascorbate/TMPD (10 mM + 0.5 mM), NADH (2.5 mM) and endogenous (dotted line). The substrates were added to the contentsof the sample cuvette, solid crystals of ferricyanide were added to the reference cuvette and the difference spectra recorded. The protein suspension was mixed (1:1) with sucrose (0.75 M, final concentration) prior to the treatment with the oxidant or the reductants. The baseline (bottom curve) was obtained by scanning the same concentration of $[Fe(CN)_6]^{-3}$-oxidized proteins (3.5 mg/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded; the wavelengths are expressed in nanometers.

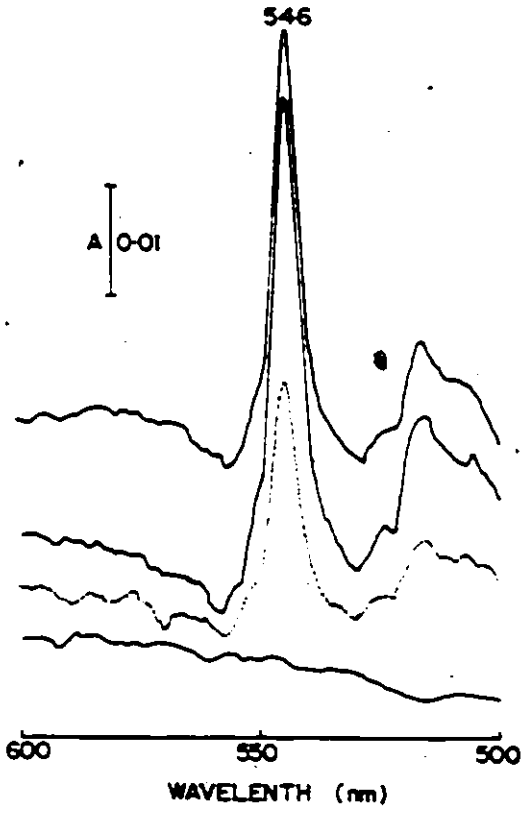
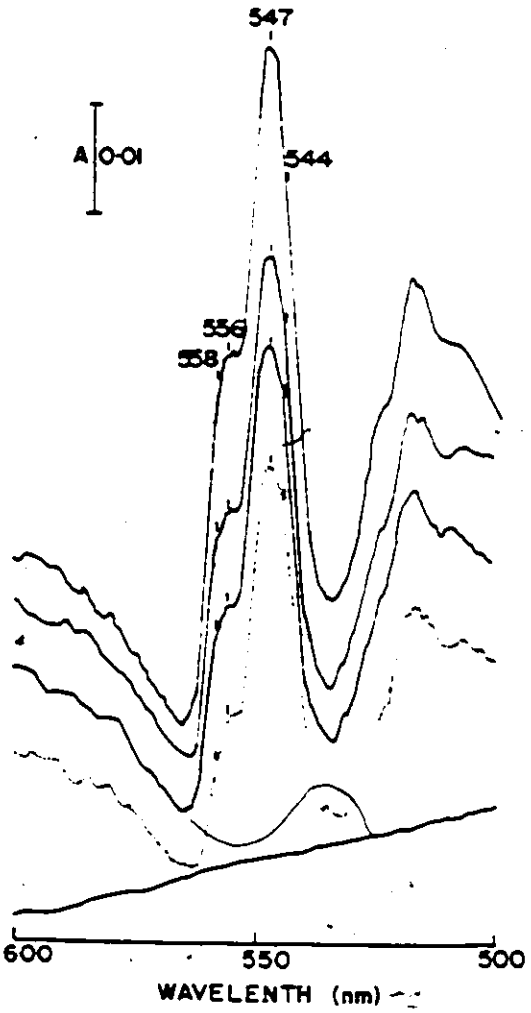

546
A 0·01
600
550
500
WAVELENTH (nm)

Figure 29. Substrate-reduced *minus* ferricyanide-oxidized difference spectra at low temperature of the membrane fraction of *A. haloplanktis* grown in complex medium.

The substrates used with the membrane fraction were from top to bottom: ascorbate/TMPD (10mM + 0.5 mM), NADH (2.5 mM) and succinate (10 mM). The substrates were added to the contents of the sample cuvette solid crystals of ferricyanide were added to the reference cuvette. The protein suspension was mixed (1:1) with sucrose (0.75 M, final concentration) prior to treatment with the oxidant or the reductants. The baseline (bottom curve) was obtained by scanning the same concentration of $[Fe(CN)_6]^{-3}$-oxidized proteins (10.5 mg/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded; the wavelengths are expressed in nanometers.

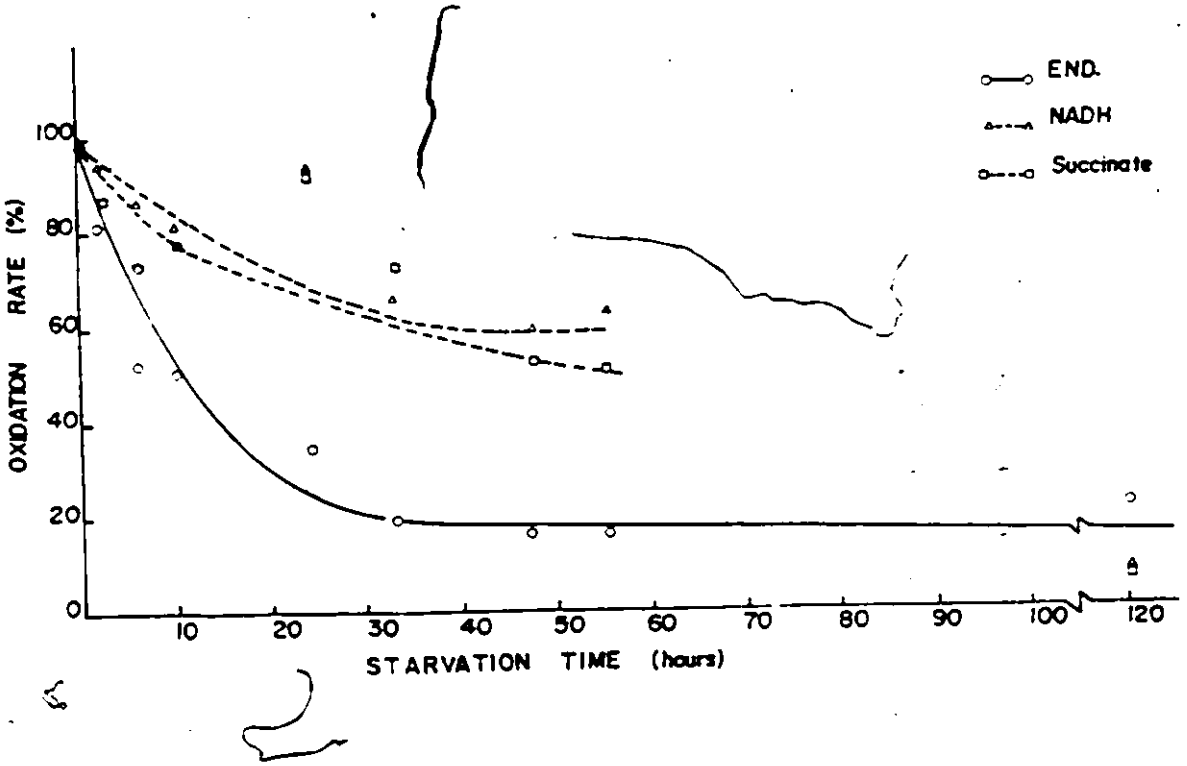

547
A 0·01
544
556
558
600
550
500
WAVELENTH (nm)

metabolism. However, starvation in a physiological solution for at least 24 hours gave consistent and reproducible difference spectra within one hour of manipulation. Starvation in salt solutions buffered either with Tris or Tris-PO_4 gave similar results, *i.e.*, lowering of the endogenous respiration by 65-70% after 24 hours of agitation at 25°C. The ideal starvation system is one which abolishes the endogenous respiration while leaving untouched the exogenous respiration under investigation. The starvation procedure which was found to be the most effective for A. *haloplanktis* cells was a prolonged starvation period in complete salt solution. Such procedure lowered the endogenous respiration by 80% within 48 hours of agitated incubation at 25°C, while allowing the cells to respire on exogenous substrates at rates above 50% of their initial value. Results are presented in Figure 30. Other bacteria also require extended starvation periods before depletion of endogenous reserves becomes obvious (MacKelvie *et al.*, 1968; Arcuri and Ehrlich, 1980).

The mechanism of inhibitory action of cyanide is still not fully characterized and understood. For instance, CN^- will react preferentially with the oxidized form of cytochrome *o* in *Azotobacter vinelandii* (Yang and Jurtshuk, 1978; Kauffman and Van Gelder, 1973), *Vitreoscilla* (Webster and Liu, 1974), or the cultured *Rhizobium japonicum* (Appleby, 1969b), but still will form a complex with

Figure 30. Capacity of A. *haloplanktis* cells to oxidize either endogenously or in the presence of NADH or succinate after different starvation times.

Alteromonas haloplanktis was grown in the routine complex medium to the stationary phase of growth. Stationary phase cells were harvested by centrifugation and resuspended in volumes of sterile salt solutions equal to the growth volumes. The salt solution used was made up of .3M NaCl, .05M $MgCl_2$, .01M KCl. Since the respiratory rates may vary (± 10%) between different sets of experiments, the data are presented in % oxidation, the 100% value being the oxidase activity of the washed/unstarved whole cell suspensions oxidizing endogenous (solid line) or exogenous (dotted line) substrates. The exogenous substrates were: NADH (Δ---Δ) 2.5 mM and succinate (□---□) 10 mM.


END.
NADH
Succinate
OXIDATION RATE (%)
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
120
STARVATION TIME (hours)

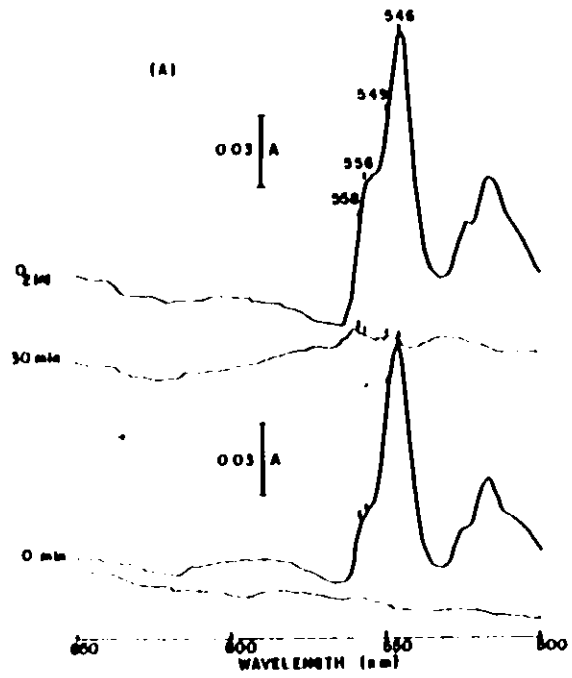
either the oxidized or the reduced form of cytochrome a_3 (Revsin *et al.*, 1970; Webster and Hackett, 1966). Likewise, both ferrous and ferric haemoproteins form cyanide complexes (Saunders *et al.*, 1964). Still in other microorganisms (Pudek and Bragg, 1974; Kauffman and Van Gelder, 1973; Pritchard and Asmundson, 1980), CN^- is known to react with an intermediate between the oxidized and reduced form of cytochrome *d* (a_2), forming a cyanocytochrome. Finally, CN^- is equally able to form stable complexes with several metals and react with keto and thiol groups (Knowles, 1976).

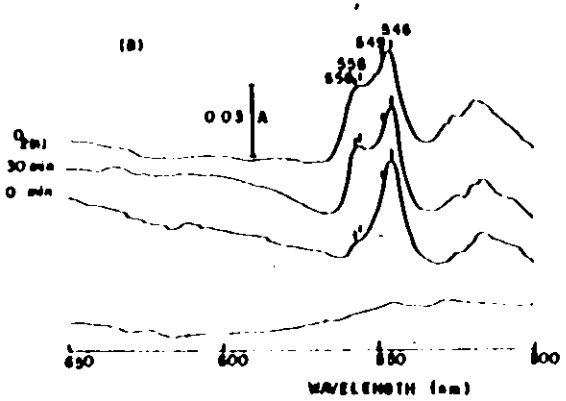
Recent studies on the active sites of hemoproteins revealed that unlike azide binding, cyanide binding causes structural changes in the neighborhood of the heme and is responsible for changes in the tertiary structure (Blumenthal and Kassner, 1979; Jones *et al.*, 1984). Seemingly, the formation and the stability of the CN^- complexes are related to factors such as the rate of electron flux through the respiratory chain (Pudek and Bragg, 1974), the affinity of a particular oxidase for the inhibitor (Jones, 1973) and the final concentration of cyanide used in the reaction mixture (Yang, 1978).

In an attempt to look into the action of cyanide in *A. haloplanktis*, careful spectrophotometric studies were carried out with this conventional third site inhibitor. As a consequence of the effect of the endogenous metabolism, particularly evident in whole cells, exposure of unstarved cells to CN^- for a certain lapse of time resulted in cancellation of the difference spectrum initially recorded between a CN^- treated and an untreated suspension. With cyanide in the sample cuvette, the cytochromes became quickly reduced with endogenous reserves (Figure 31A, 0 min). In 30 minutes the cytochromes were also completely reduced in the reference cuvette due to endogenous reserves and were reoxidized again when O_2 gas was bubbled in (Figure 31A, upper trace). The difference in rate of reduction in sample and reference cuvette can be ascribed

Figure 31. Development of difference spectra when the sample cuvette contained unstarved intact cells of *A. halo-planktis* plus 10 mM CN and the reference cuvette contained the cells oxidized with O_2.

Cyanide + endogenously-reduced *minus* O_2-oxidized difference spectra. The whole cell suspensions were mixed (1:1) with sucrose (0.75M , final concentration), bubbled with O_2 gas for 10 min and divided into the sample and the reference cuvette. Cyanide (10 mM) was then added to the contents of the sample cuvettes and the difference spectra were recorded. The reducing substrate(s) were the endogenous reserves in (A) and (B). The stationary phase cells were washed and left as is in (A) or further shaken for 48 hr at 25°C in sterile complete salt solution (.3M NaCl, .05M $MgCl_2$, .01M KCl) in (B). The numbers beside the curves indicate the time at which the scans were started after the addition of cyanide. After 30 min incubation at room temperature, O_2 gas was bubbled again into the reference cuvettes (upper traces). The difference spectra were recorded at 83°K using cuvettes of 2 mm light path. The baselines were obtained by scanning the same concentration of whole cells (21.0 mg protein/ml, in (A) 18.5 mg protein/ml in (B)) present in the sample and the reference cuvettes. The vertical bars represent one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.


(A)
546
545
556
558
0·03 A
30 min
0·03 A
0 min
600
650
WAVELENGTH (nm)


(B)
546
545
538
536
0·03 A
30 min
0 min
600
650
WAVELENGTH (nm)

to the inability of the cytochromes to be re-oxidized when CN^- is present. Following starvation, some of the endogenous reserves of the cell suspension have been exhausted. With CN^- in the sample cuvette (Figure 31B, 0 min) still enough reserves are present to reduce the c-type cytochromes immediately and the b-type after 30 minutes but not enough when competing O_2 is present to oxidize the cytochromes in the absence of CN^-. Variation of cytochrome absorption occurring within 10% of the original scan is considered normal (Pudek and Bragg, 1974; Downie and Cox, 1978). The stabilities of the CN^- complexes were checked in cell-free extracts the following way (Figure 32). Cyanide was added to an oxidized preparation and the reaction left to proceed for at least 30 min. The difference spectra developed as expected. When the O_2 gas was vigorously flushed into the CN^- treated sample cuvette, no change in peak height was observed: all the cytochromes liganded by CN^- remained reduced despite the addition of the oxidant. Further addition of $(S_2O_4)^{-2}$ to the sample cuvette (Figure 32, upper trace) reduced the unreduced cytochromes presumably not on the endogenous pathway sensitive to CN^-. The different rate of electron flow generated by the biological as opposed to the chemical reductant could also explain the difference in the reduction level. The peak heights in the visible region and the shift in the Soret band from 419 to 423 nm indicate that the majority of the non-endogenously reduced cytochromes in the presence of CN^- were of the b-type.

Similar experiments were carried out with the periplasmic fraction containing cytochrome c. Reduction occurring with time in the reference cuvette had been prevented by treating its contents with the chemical oxidant ferricyanide. Figure 33 presents a time course reaction of CN^- with the periplasmic fraction. With time cyanide allowed complete reduction of the cytochrome by endogenous material to such an extent that further addition of dithionite to the sample cuvette (upper trace) did not alter the difference

Figure 32. Stability of CN-difference spectra of cell-free extracts to oxidizing or reducing agents.

Cyanide + endogenously-reduced *minus* O_2-oxidized difference spectra. At first, the cell-free extracts were bubbled with O_2 gas for 10 min and divided into the sample and the reference cuvette. Cyanide (1.0 mM) was added to the contents of the sample cuvette and the difference spectra were recorded. The numbers beside the curves indicate the time in minutes at which the scans were started after the addition of CN^-. After 30 min of reaction at room temperature, O_2 gas was bubbled into the CN-treated (sample) cuvette and then solid crystals of dithionite were added to the same cuvette. The spectra were recorded at room temperature in cuvettes of 10 mm light path. The baseline was obtained by scanning the same concentration of untreated extracts (11.6 mg protein/ml) present in the sample and the reference cuvettes. The vertical bars represent one tenth of the absorbancy units at which the spectra were recorded and the wavelengths are expressed in nanometers.

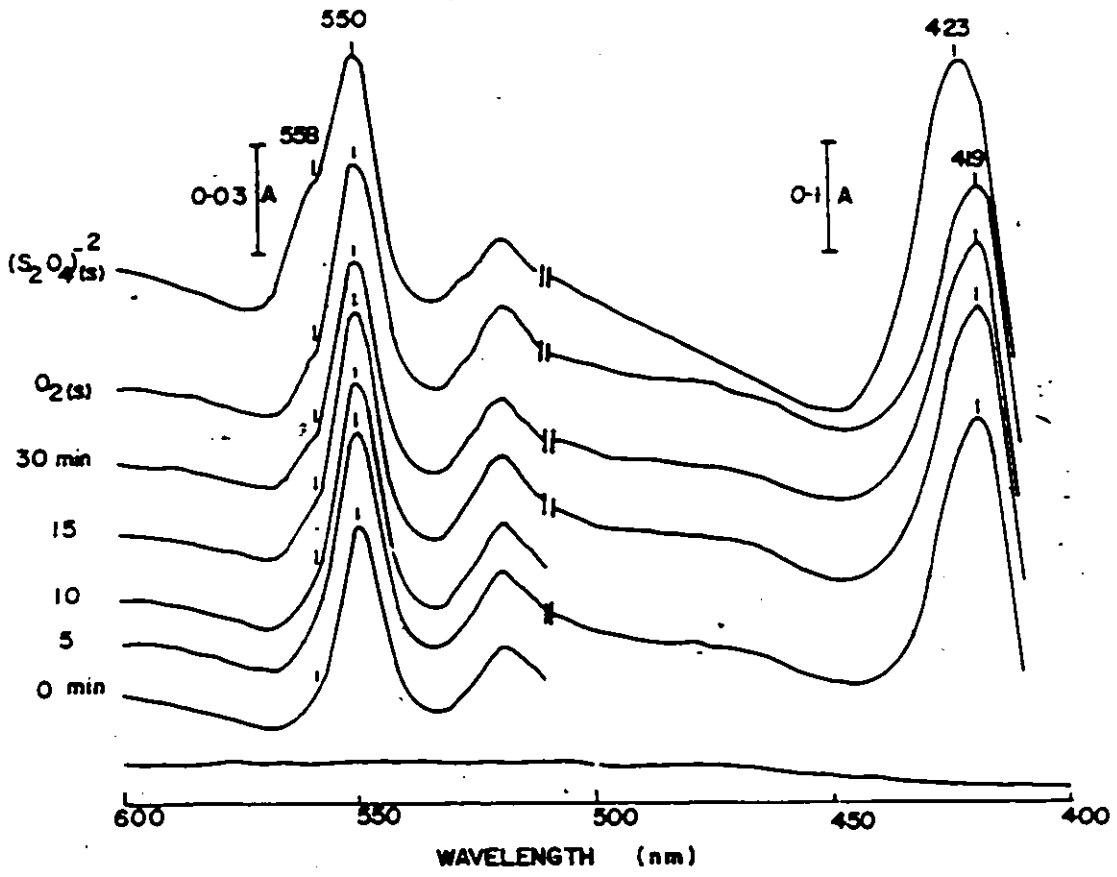
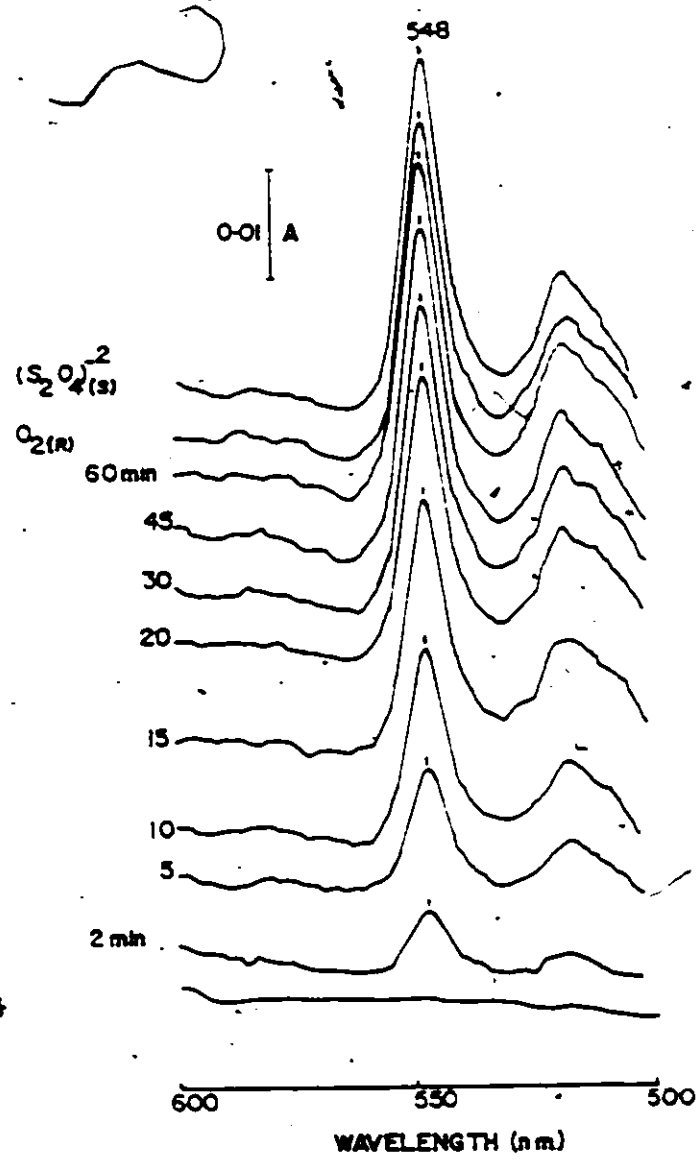

550
423
558
419
0·03 A
0·1 A
$(S_2O_4)^{-2}_{(s)}$
$O_{2(s)}$
30 min
15
10
5
0 min
600
550
500
450
400
WAVELENGTH (nm)

Figure 33. Spectral changes observed as a function of time after treatment of the periplasmic fraction of *A. halo-planktis* with CN^-. Ferricyanide was used as oxidant.

Cyanide + endogenously-reduced *minus* $[Fe(CN)_6]^{-3}$-oxidized difference spectra. Cyanide (1.0 mM) was added to the contentsof the sample cuvette, solid crystals of ferricyanide to the reference cuvette and the difference spectra were recorded. The numbers beside the curves indicate the time in minutes at which the scans were started after the addition of CN^-. After development of the difference spectra, the sample and the reference cuvettes were treated as specified beside the curves. The spectra were recorded at room temperature in cuvettes of 10 mm light path. The baseline was obtained by scanning the same concentration of $[Fe(CN)_6]^{-3}$-oxidized proteins (4.7 mg/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.


548
0·01 A
$(S_2O_4)^{-2}_{(s)}$
$O_{2(g)}$
60 min
45
30
20
15
10
5
2 min
600
550
500
WAVELENGTH (nm)

spectrum. Addition of O_2 gas to the reference cuvette did not alter either the difference spectrum as expected when ferricyanide is the oxidant and CN^- is absent.

It should be pointed out that autoreducible cytochromes c have been reported in *Pseudomonas* AM1 (O'Keeffe and Anthony, 1980). Autoreduction is defined by the authors as the reduction of cytochrome c occurring at alkaline pH in the absence of added reductant. The mechanism of autoreduction postulated involved intramolecular electron transfer. The final reaction was the same whether the enzyme preparation was treated initially with CN^- and subsequently with $(S_2O_4)^{-2}$ or *vice versa* (Figure 34); a baseline was regained following the addition of $[Fe(CN)_6]^{-3}$ to the sample cuvette (Figure 34, upper trace) indicating that the electron flow can be drained at the end of the chain by an artificial electron acceptor such as ferricyanide in the presence of CN^-.

The same experiments were carried out with the membrane fraction (Figures 35,36). As for the other cell fractions, the endogenous metabolism caused an increase in cytochrome reduction with time in the presence of cyanide (Figure 35). Upon addition of dithionite (Figure 35, upper trace), the cytochromes not reduced by endogenous reductants, particularly the b-type cytochromes, became reduced by dithionite. The order of addition of cyanide, before or after dithionite, did not affect the final result: complete reduction of the cytochromes b and c (Figure 36). As with the periplasmic fraction, ferricyanide added in excess to the contents of the sample cuvette cancelled out the difference absorption spectrum (Figure 36, upper trace).

The sequence of absorption changes following the addition of cyanide to a membrane preparation oxidized by O_2 gas is presented in Figure 37. A reduced *minus* oxidized difference spectrum is developed as the cytochromes in the sample cuvette are being reduced by endogenous reductant(s). By 15 minutes, however, the

Figure 34. Room temperature difference spectra of the periplasmic fraction treated with CN^-, dithionite and ferricyanide.

The reducing substrates were the endogenous substrate(s) and/or sodium dithionite. The oxidant was ferricyanide in all the cases. The order of addition of the substrate and/or the inhibitor (CN, 1.0 mM) to the sample cuvette was as specified beside the curves. The spectra were recorded at room temperature in cuvettes of 10 mm light path. The baseline was obtained by scanning the same concentration of $[Fe(CN)_6]^{-3}$-oxidized proteins (4.7 mg/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were scanned and the wavelengths are expressed in nanometers.

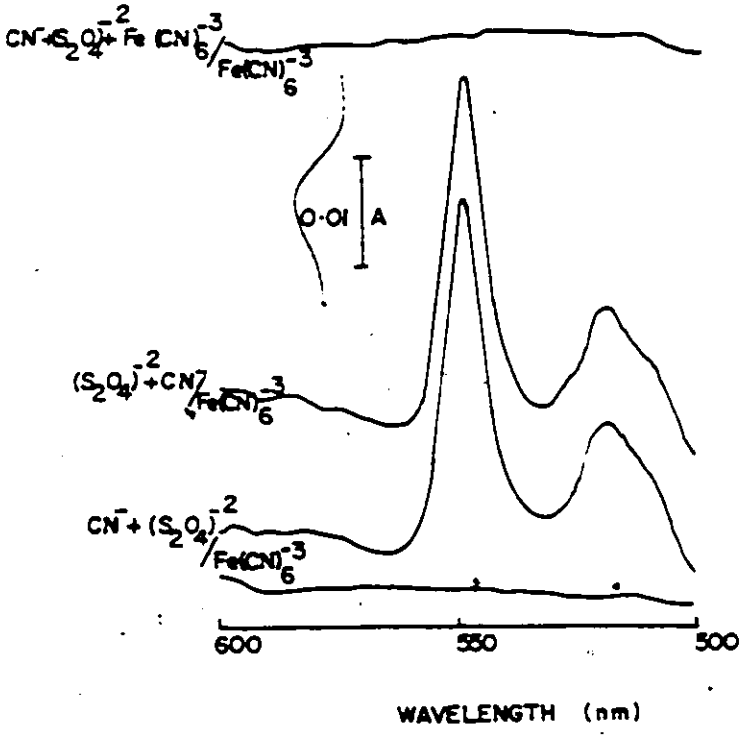

CN⁻+(S2O4)⁻²+Fe(CN)6⁻³/Fe(CN)6⁻³
0·01 A
(S2O4)⁻²+CN⁻/Fe(CN)6⁻³
CN⁻+(S2O4)⁻²/Fe(CN)6⁻³
600
550
500
WAVELENGTH (nm)

Figure 35. Spectral changes observed as a function of time after treatment of the membrane fraction of *A. halophanktis* with CN^-. Ferricyanide was used as oxidant.

Cyanide + endogenously-reduced *minus* $[Fe(CN)_6]^{-3}$-oxidized difference spectra. Cyanide (1.0 mM) was added to the contents of the sample cuvette, solid crystals of ferricyanide to the contents of the reference cuvette and the difference spectra were recorded. The numbers beside the curves indicate the time in minutes at which the scans were started after the addition of CN^-. After development of the difference spectra, the sample and the reference cuvettes were treated as specified beside the curves. The spectra were recorded at room temperature in cuvettes of 10 mm light path. The baseline was obtained by scanning the same concentration of $[Fe(CN)_6]^{-3}$-oxidized membranes (5.6 mg protein/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.

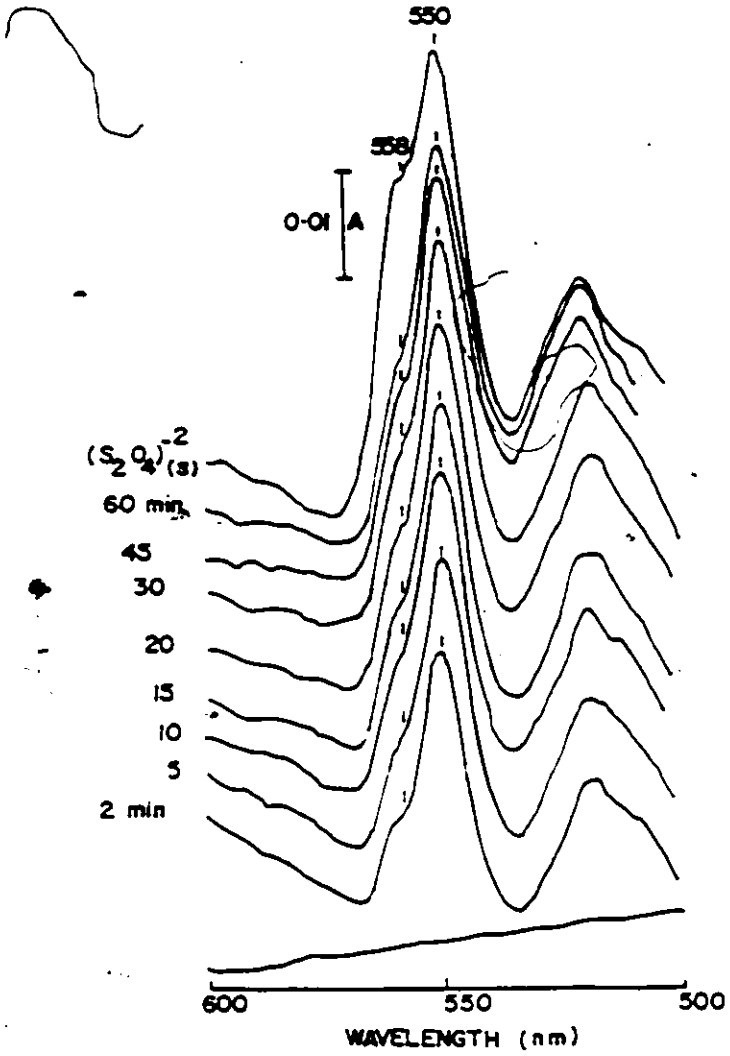

550
558
0·01 A
$(S_2O_8)^{-2}_{(s)}$
60 min
45
30
20
15
10
5
2 min
600
550
500
WAVELENGTH (nm)

Figure 36. Room temperature difference spectra of the membrane fraction treated with CN^-, dithionite and ferricyanide.

The reducing substrates were the endogenous substrate(s) and/or sodium dithionite. The oxidant was ferricyanide in all the cases. The order of addition of the substrate and/or the inhibitor (CN^-, 1.0 mM) to the sample cuvette was as specified beside the curves. The baseline was obtained by scanning the same concentration of $[Fe(CN)_6]^{-3}$-oxidized membranes (5.6 mg protein/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.

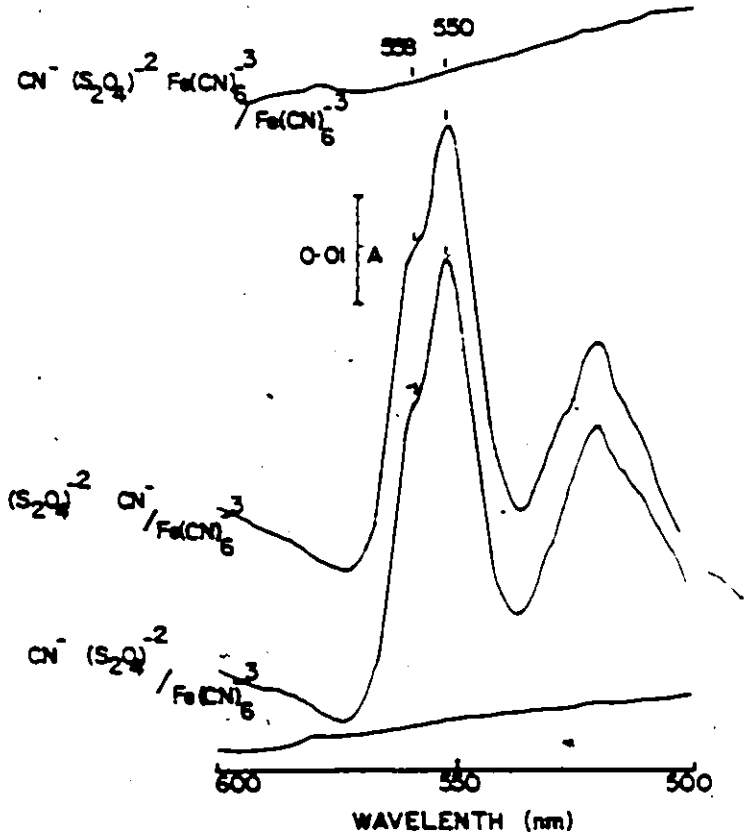
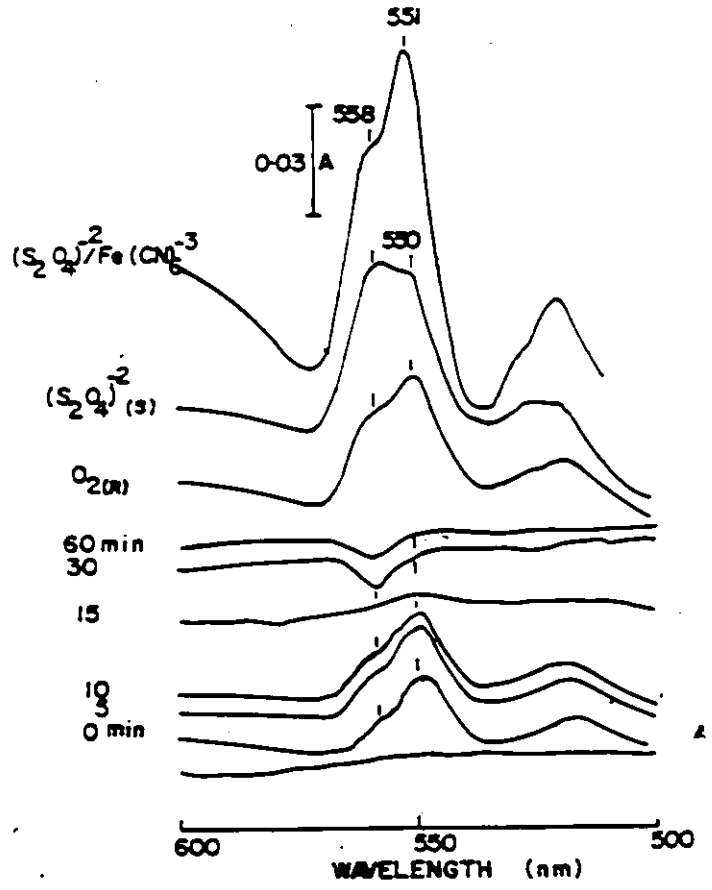

550
358
$CN^- (S_2O_4)^{-2} Fe(CN)_6^{-3}$
$Fe(CN)_6^{-3}$
0·01 A
$(S_2O_4)^{-2} CN^- / Fe(CN)_6^{-3}$
$CN^- (S_2O_4)^{-2} / Fe(CN)_6^{-3}$
600
550
500
WAVELENTH (nm)

Figure 37. Spectral changes observed as a function of time after treatment of the membrane fraction of A. *haloplanktis* with CN^-. O_2 gas was used as oxidant.

Cyanide + endogenously-reduced *minus* O_2-oxidized difference spectra. The membrane fraction was bubbled with O_2 gas for 10 min and divided into the sample and the reference cuvettes. Cyanide (1.0 mM) was added to the contentsof the sample cuvette and the difference spectra were recorded. The numbers beside the curves indicate the time in minutes at which the scans were started after the addition of CN^-. After development of the difference spectra, the sample and the reference cuvettes were treated as specified beside the curves. The spectra were recorded at room temperature in cuvettes of 10 mm light path. The baseline was obtained by scanning the same concentration of untreated membranes (16.0 mg protein/ml) present in the sample and the reference cuvettes. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.


551
558
0·03 A
550
$(S_2O_4)^{-2}/Fe(CN)_6^{-3}$
$(S_2O_4)^{-2}$ (3)
O_2 (0)
60 min
30
15
10
5
0 min
600
550
500
WAVELENGTH (nm)

cytochromes in the reference cuvette have also become endogenously reduced to the point where there is essentially no difference spectrum. At 30 and 60 minutes, the reduced + CN^- *minus* reduced spectrum appeared as a pseudo-oxidized *minus* reduced spectrum (trough at 558 nm), demonstrating that the reduced form of cytochrome *o* (a *b*-type cytochrome) can possibly bind CN^-. Addition of dithionite to the sample cuvette completely reduced the cytochromes. Addition of ferricyanide to the reference cuvette completed the oxidation initiated by O_2 gas.

Cytochrome Reduction in the Presence of Other Inhibitors

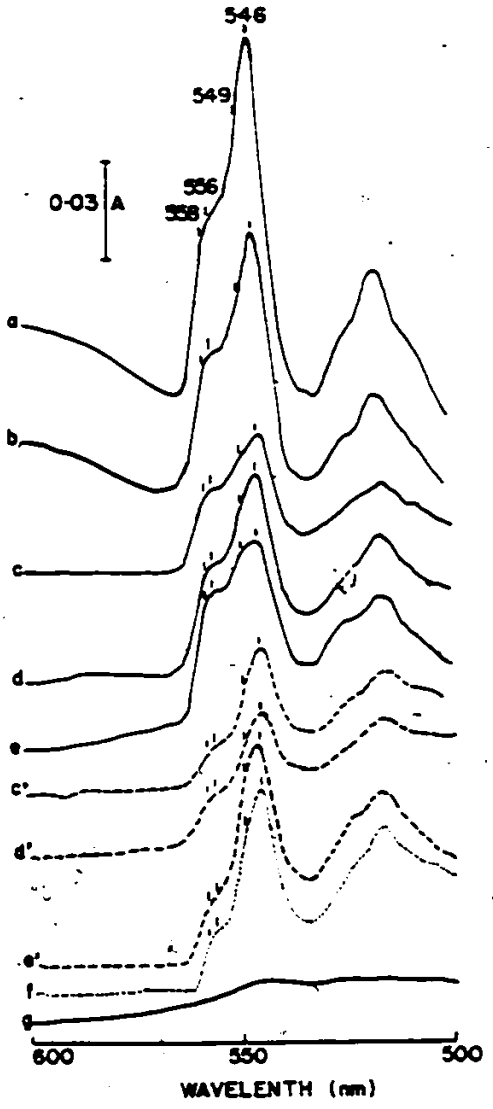
To further characterize the respiratory chain of A. *haloplanktis*, a series of respiratory inhibitors were tested by means of difference absorption spectrophotometry at the level of whole cells, cell-free extracts, periplasmic and membrane fractions.

The conventional site one inhibitors, rotenone and amytal and, to some extent, atebrine were looked at spectrophotometrically in the presence of NADH and remaining endogenous substrate(s) of starved whole cells (Figure 38). At steady state, the cytochromes present in whole cells were reduced by NADH (Figure 38, trace *b*) when compared to the dithionite-reduced *minus* ferricyanide-oxidized difference spectrum (Figure 38, trace *a*). Rotenone (trace *c*), atebrine (trace *d*) and amytal (trace *e*) prevented cytochrome reduction by NADH and endogenous substrate(s) to various degrees. Among the three inhibitors tested, rotenone was the most effective causing approximately 50% inhibition. These spectra could indicate that a portion of the electron flow driven by the oxidation of NADH reached the final electron acceptor via a route insensitive to the first site inhibitors.

Specific inhibition of succinate dehydrogenase by thenoyltrifluoroacetone, (TTFA), could be demonstrated spectrophotometrically in whole cells. Indeed, the series of difference spectra

Figure 38. Low temperature difference spectra showing the effect of the first site respiratory inhibitors on the cytochrome system of complex medium grown cells reduced by NADH and remaining endogenous substrate(s).

The starved cell suspension was mixed (1:1) with sucrose (0.75 M , final concentration), bubbled with O_2 gas for 10 min and divided into the sample and the reference cuvettes. The following inhibitors were incubated for 3 min with the contents of the sample cuvettes prior to substrate reduction: rotenone (1.0 mM), atebrine (0.5 mM) and amytal (1.0 mM). The concentration of NADH was 2.5 mM.
Trace *a*: dithionite-reduced *minus* ferricyanide-oxidized difference spectrum;
trace *b*: NADH-reduced *minus* O_2-oxidized;
trace *c*: Rotenone-inhibited/NADH-reduced *minus* O_2-oxidized;
trace *d*: Atebrine-inhibited/NADH-reduced *minus* O_2-oxidized;
trace *e*: Amytal-inhibited/NADH-reduced *minus* O_2-oxidized;
trace *c'*: Rotenone-inhibited/endogenously-reduced *minus* O_2-oxidized;
trace *d'*: Atebrine-inhibited/endogenously-reduced *minus* O_2-oxidized;
trace *e'*: Amytal-inhibited/endogenously-reduced *minus* O_2-oxidized;
trace *g*: endogenously-reduced *minus* O_2-oxidized difference spectrum. The baseline (trace *g*) was obtained by scanning the same concentration of O_2-oxidized whole cells (22.5 mg protein/ml) present in the sample and the reference cuvettes. The spectra were recorded at 83°K in cuvettes of 2 mm light path. The vertical bars represent one tenth of the absorbancy units at which the spectra were recorded and the wavelengths are expressed in nanometers.


546
549
556
558
0·03 A
a
b
c
d
e
c'
d'
e'
f
g
600
550
500
WAVELENTH (nm)

presented in Figure 39 showed that TTFA did prevent cytochrome reduction when succinate was serving as the electron donor (trace c), whereas reduction was not impeded in the absence of the inhibitor (trace b). Mainly, the c-type cytochromes reduced by remaining endogenous substrate(s) (trace d) were inhibited by TTFA (trace c').

When inhibition with the conventional second site inhibitors antimycin A and 2-heptyl 4-hydroxyquinoline (HOQNO) were looked at spectrophotometrically, the following results were obtained with aseptically starved cells (Figure 40). As noticed earlier, the physiological oxidation and reduction of the cytochromes by O_2 gas and NADH (Figure 40A, trace b) was of less numerical importance than the chemical oxidation and reduction of the cytochromes by ferricyanide and dithionite respectively (Figure 40, trace a). Nevertheless, the inhibitory action of antimycin A (Figure 40A, trace e) and HOQNO (Figure 40A, trace d) of NADH-reduced cytochromes was obvious in as much as only reduction of the cytochromes b was allowed in the presence of the inhibitors, the pronounced trough in the 550nm region was evidenced for the oxidation of the c-type cytochromes. As expected, the cytochromes located on the oxygen side of the inhibitory block were readily oxidized. When the peak heights were taken into consideration, it was also noticed that a certain proportion of the b-type cytochromes were not reduced to the extent of the uninhibited control (Figure 40A, trace b) indicating partial resistance of the b cytochromes toward the second site inhibitors on the NADH pathway. With succinate-reduced whole cells, very different sensitivity patterns developed with antimycin A and HOQNO (Figure 40B). Nearly complete reduction of b-and c-type cytochromes was observed in the presence of either inhibitor, indicating that antimycin A and HOQNO would not act between cytochrome b and c on the succinate pathway, although a portion of the cytochromes c was partially oxidized when the peak heights (Figure 40B, traces c and d) were compared to the peak heights of the uninhibited control (Figure 40B, trace b). This

Figure 39. Low temperature difference absorption spectra showing the effect of the respiratory inhibitor TTFA on the cytochrome system of complex medium grown cells reduced by succinate and remaining endogenous substrate(s).

The starved cell suspension was mixed (1:1) with sucrose (0.75 M, final concentration) bubbled with O_2 gas for 10 min and divided into the sample and the reference cuvette. The inhibitor thenoyltrifluoroacetone TTFA (1.0 mM) was incubated for 3 min with the contents of the sample cuvette prior to substrate reduction. The concentration of succinate used was 10 mM.
Trace *a*: dithionite-reduced *minus* ferricyanide-oxidized difference spectrum;
trace *b*: succinate-reduced *minus* O_2-oxidized;
trace c: TTFA-inhibited/succinate-reduced *minus* O_2-oxidized;
trace c': TTFA-inhibited/endogenously-reduced *minus* O_2-oxidized;
trace *d*: endogenously-reduced *minus* O_2-oxidized difference spectrum. The baseline (trace e) was obtained by scanning the same concentration of O_2-oxidized whole cells (18.5 mg protein/ml) present in the sample and the reference cuvettes. The spectra were recorded at 83°K in cuvettes of 2 mm light path. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.

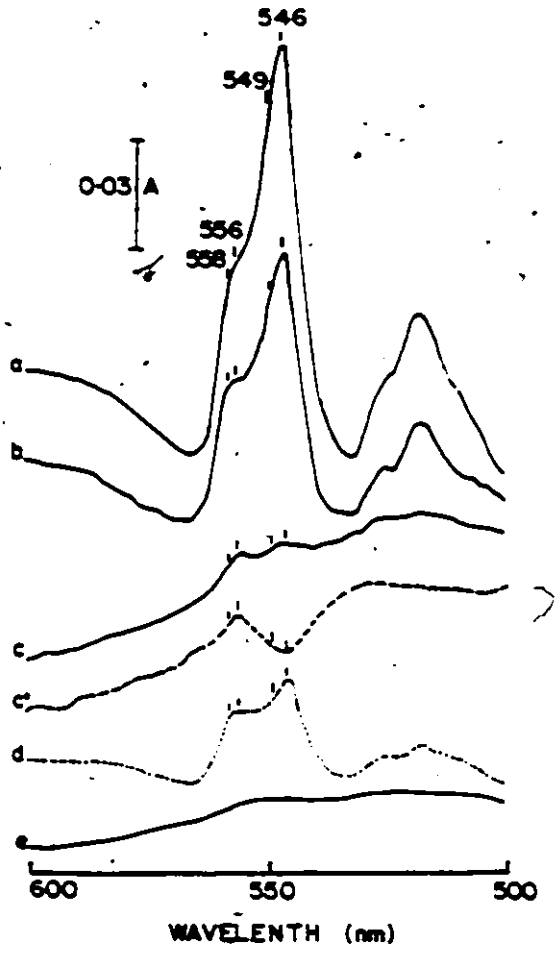
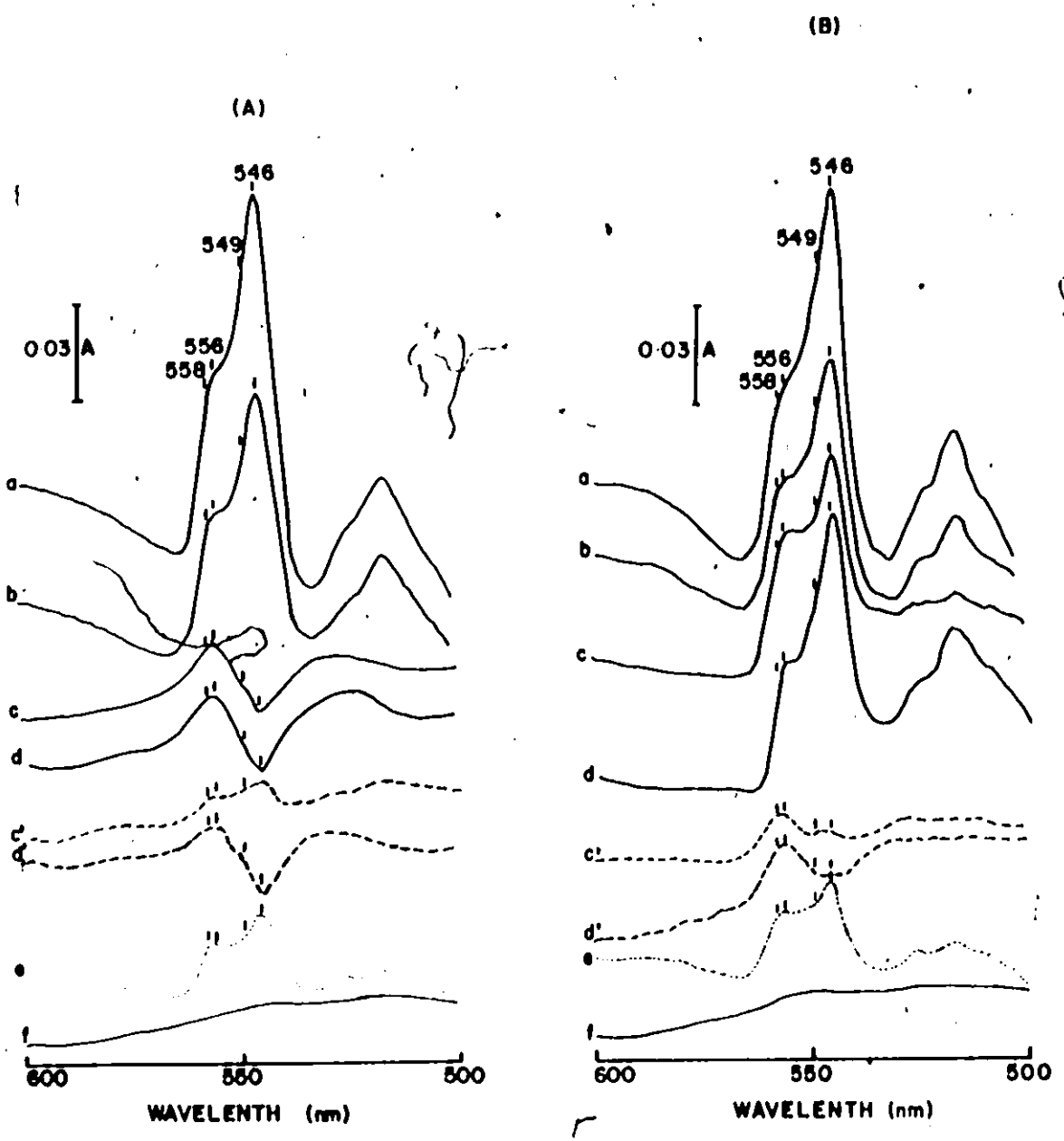

546
549
0·03 A
556
558
a
b
c
c'
d
e
600
550
500
WAVELENTH (nm)

Figure 40. Low temperature difference absorption spectra showing the effect of the second site respiratory inhibitors on the cytochrome system of complex medium grown cells reduced by NADH and remaining endogenous substrate(s) in (A) and by succinate and remaining endogenous substrate(s) in (B).

The starved cell suspension was mixed (1:1) with sucrose (0.75M, final concentration), bubbled with O_2 gas for 10 min and divided into the sample and the reference cuvette. The inhibitors antimycin A (1.0 mM) and HOQNO (0.5 mM) were incubated for 3 min with the contentsof the sample cuvettes prior to substrate reduction. The concentrations of substrates used were: NADH (2.5 mM) and succinate (10 mM). Series of difference spectra presented in section (A):
Trace *a*: dithionite-reduced *minus* ferricyanide-oxidized;
trace *b*: NADH-reduced *minus* O_2-oxidized;
trace *c*: Antimycin A-inhibited/NADH-reduced *minus* O_2-oxidized;
trace *d*: HOQNO-inhibited/NADH-reduced *minus* O_2-oxidized.
trace *c'*: Antimycin A-inhibited/endogenously-reduced *minus* O_2-oxidized;
trace *d'*: HOQNO-inhibited/endogenously-reduced *minus* O_2-oxidized;
trace *e*: endogenously-reduced *minus* O_2-oxidized. The baseline (trace *j*) was obtained by scanning the same concentration of O_2-oxidized whole cells (18.5 mg protein/ml) present in the sample and the reference cuvettes. The spectra were recorded at 83°K in cuvettes of 2 mm light path. The vertical bar represents one tenth of the absorbancy unit at which the spectra were recorded and the wavelengths are expressed in nanometers.

Series of difference spectra presented in section (B):
trace *a*: dithionite-reduced *minus* ferricyanide-oxidized;
trace *b*: succinate-reduced *minus* O_2-oxidized;
trace *c*: Antimycin A-inhibited/succinate-reduced *minus* O_2-oxidized;
trace *d*: HOQNO-inhibited/succinate-reduced *minus* O_2-oxidized;
trace *c'*: Antimycin A-inhibited/endogenously-reduced *minus* O_2-oxidized;
trace *d'*: HOQNO-inhibited/endogenously-reduced *minus* O_2-oxidized;
trace *e*: endogenously-reduced *minus* O_2-oxidized. The baseline (trace *j*) was as specified in section (A) and the protein concentration was adjusted to 18.5 mg/ml.


(A)
546
549
0·03 A
556
558
a
b
c
d
c'
d'
e
f
600
550
500
WAVELENTH (nm)
(B)
546
549
0·03 A
556
558
a
b
c
d
c'
d'
e
f
600
550
500
WAVELENTH (nm)

could also be caused by blockage of reduction of the cytochrome c by remaining endogenous reserves in the presence of the inhibitors (Figure 40B, trace c' and d'). It is known that respiratory systems expressing different sensitivities toward cyanide often express different sensitivities toward antimycin A (Degn *et al.*, 1978).

Several attempts were made to resolve spectrophotometrically the electron transport chain of A. *haloplanktis* at the membrane and periplasm levels but the poor resolution of the difference spectra allowed us only to conclude that total inhibition of cytochrome reduction using rotenone, amytal, TTFA, antimycin A and HOQNO at various concentrations was not observed, although with dehydrogenase-specific inhibitors, flavoprotein troughs were leveled off and thus indicated some inhibition of the flavoproteins *per se*. Such results suggested that even at the reduced end of the chain, electron flow may bypass specific flavoproteins to further reduce the cytochromes by an alternative route which could possibly be identified as the periplasmic route. The difficulties encountered in resolving the chain at the sub-cellular level stemmed mainly from the fact that : (*i*) physiological substrate reduction of bacterial cytochromes in isolated membranes was of lower extent than in cell-free extracts or in whole cells; (*ii*) the possibility always remained that part of the membrane-bound enzymes became disoriented or were solubilized (to a certain degree) during the fractionation procedure and thus unable to respond to membrane-specific inhibitors; (*iii*) the sub-cellular electron carriers are by nature very reactive and their redox states were more easily disturbed by unspecific effects of solvents or other chemicals present in the reaction mixture. Furthermore, extensively washed membrane particles derived from starved cells revealed that indeed endogenous substrate(s) could not entirely be washed free from the preparations.

B. POLAROGRAPHIC STUDY OF THE ELECTRON TRANSPORT CHAIN

The spectrophotometric data obtained from the interaction of cellular and sub-cellular fractions with specific respiratory inhibitors and substrates were substantiated with polarographic data. This aspect of the study was concentrated on whole cells since they represent the most physiological entity that respires oxygen.

The polarographic study was carried out with the third site inhibitors, cyanide and azide, in combination with the substrates NADH, succinate and ascorbate/TMPD. Table XIV presents the 50% and 90% inhibitory concentrations for the respective oxidases in the presence of cyanide and azide. Among the substrates tested, ascorbate/TMPD was the most sensitive to cyanide with a median inhibitory dose in the vicinity of 1 uM ($I_{0.5}$ = .0015 mM) as well as to azide ($I_{0.5}$ = .045 mM); succinate oxidase was 100 times less sensitive to cyanide ($I_{0.5}$ = .120 mM) and very resistant to azide ($I_{0.5}$ > 100mM). NADH oxidase was even less sensitive to cyanide ($I_{0.5}$ = .450mM) but comparatively more sensitive to azide ($I_{0.5}$ = 10mM) than succinate oxidase ($I_{0.5}$ > 100mM). These concentrations were derived from the inhibitory curves plotting the percentages of oxidase activities *versus* various concentrations of the third site inhibitors for NADH, succinate and ascorbate/TMPD (Figures 41A to 43A). Plots of the reciprocal of fractional inhibition against the reciprocal of inhibitor concentration that intercept the ordinate at 1.0 indicate that total inhibition may be effected at infinite concentrations of the inhibitors (Niven *et al.*, 1975). In the same line of thought, a straight line intercepting the abcissa would indicate sensitivity to the inhibitor at a definite concentration and a straight line intercepting the ordinate would signify resistance to the inhibitor. Degrees of resistance would vary depending on the slope of the straight line and on its point of intercept with the ordinate.

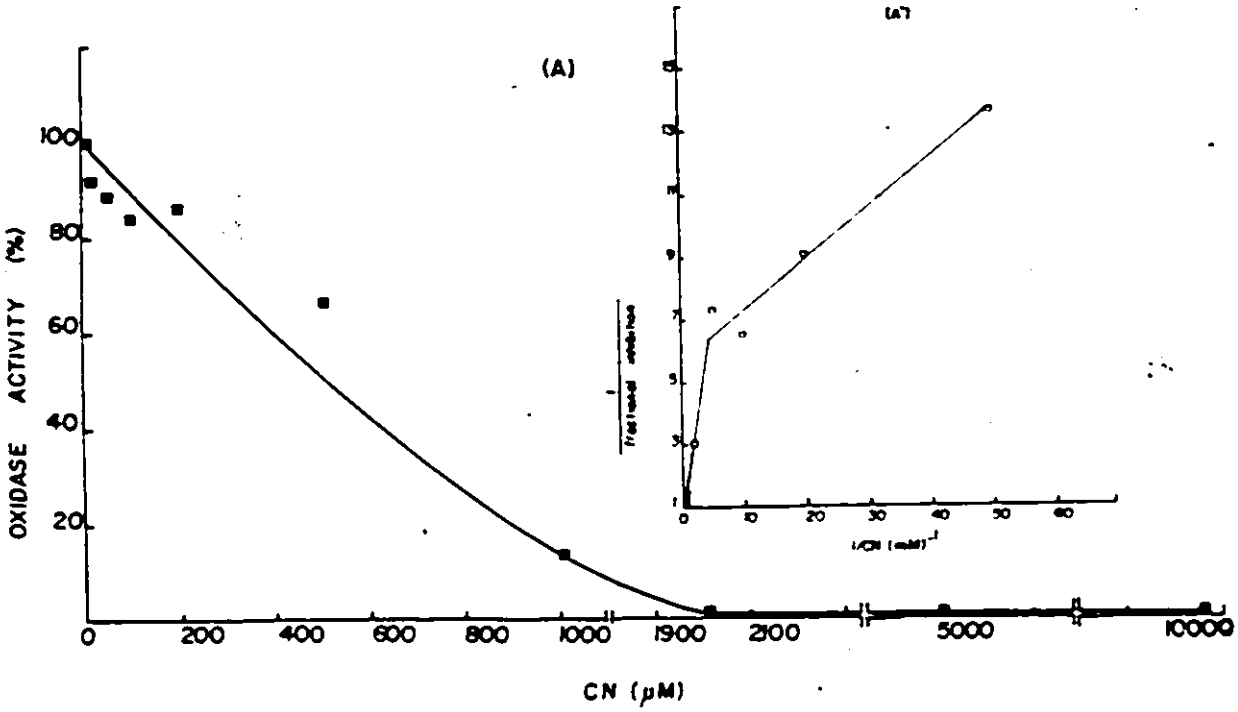
TABLE XIV. Concentrations of cyanide and azide required for 50% ($I_{0.5}$) and 90% ($I_{0.9}$) inhibition of NADH, succinate and ascorbate/TMPD oxidase activities of intact cells of *Alteromonas haloplanktis* grown in complex medium.

	Inhibitors			
	Cyanide		Azide	
Substrate	Conc. for an inhibition of		Conc. for an inhibition of	
	$I_{(0.5)}$	$I_{(0.9)}$	$I_{(0.5)}$	$I_{(0.9)}$
NADH	.450 mM	.900 mM	10 mM	40 mM
succinate	.120 mM	> 10 mM	>100 mM	>100 mM
ascorbate/TMPD	.0015 mM	.045 mM	≤.001 mM	4 mM

The values presented in this table are based on 3 separate experiments for each substrate with no less than 9 determinations each.

Figure 41. The effect of various concentrations of cyanide and azide on intact cells of A. *halopolaritis* oxidizing NADH.

The complex medium grown cells were harvested at the stationary phase, washed twice in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution and starved for 48 hr at 25°C, 200 rpm, in a volume of sterile salt solution equal to the growth volume. The starved cells were harvested by centrifugation and resuspended in an appropriate volume of complete salt (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1 mM, pH 7.4) to a concentration of 19.5 mg protein/ml in (A) and 21.8 mg protein/ml in (B). The whole cell proteins (0.65 mg in (A), 0.72 mg in (B) were preincubated for 3 min with various concentrations of cyanide (0.01 to 10 mM in (A) and azide (0.01 to 100 mM in (B)). The reactions were started by the addition of 2.5 mM NADH. Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C. Cyanide and azide inhibitions are expressed as the percentages of NADH oxidase activity in the presence of various concentrations of cyanide or azide in (A) and (B) respectively. In (A') and (B'), the double reciprocal plots of 1/fractional inhibition *versus* 1/inhibitor $(mM)^{-1}$ are inserted in the main figures. The slopes of the lines and the intercepts with the axes were calculated by linear regression.


(A)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
200
400
600
800
1000
1900
2100
5000
10000
CN (μM)

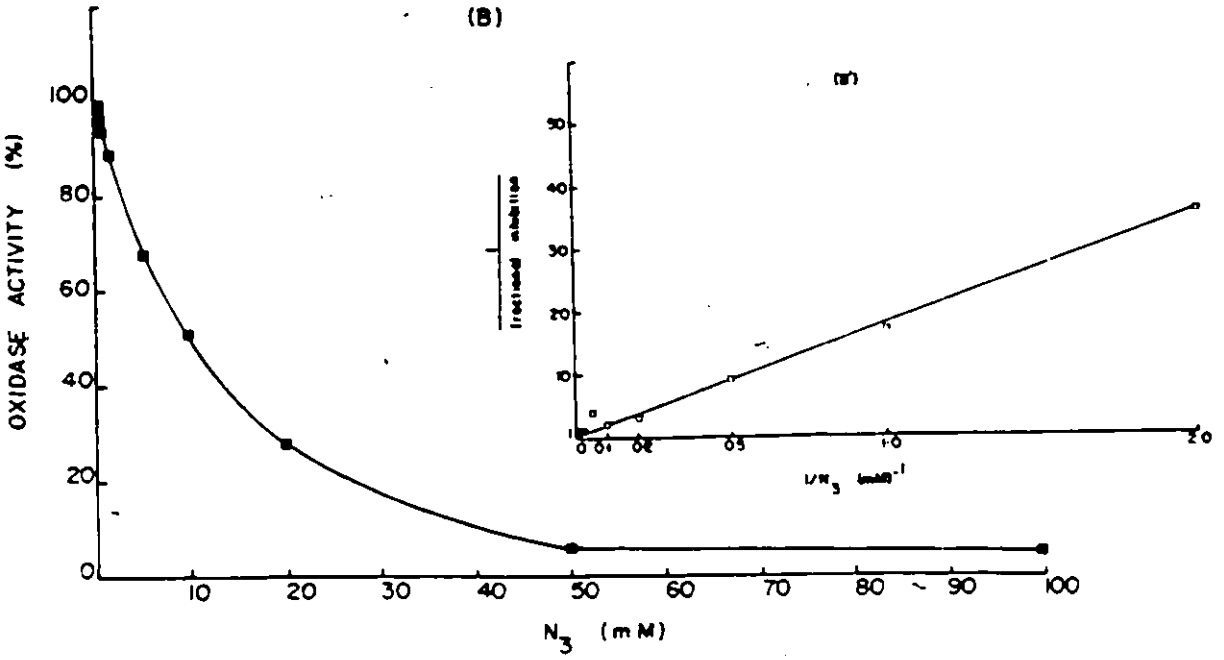
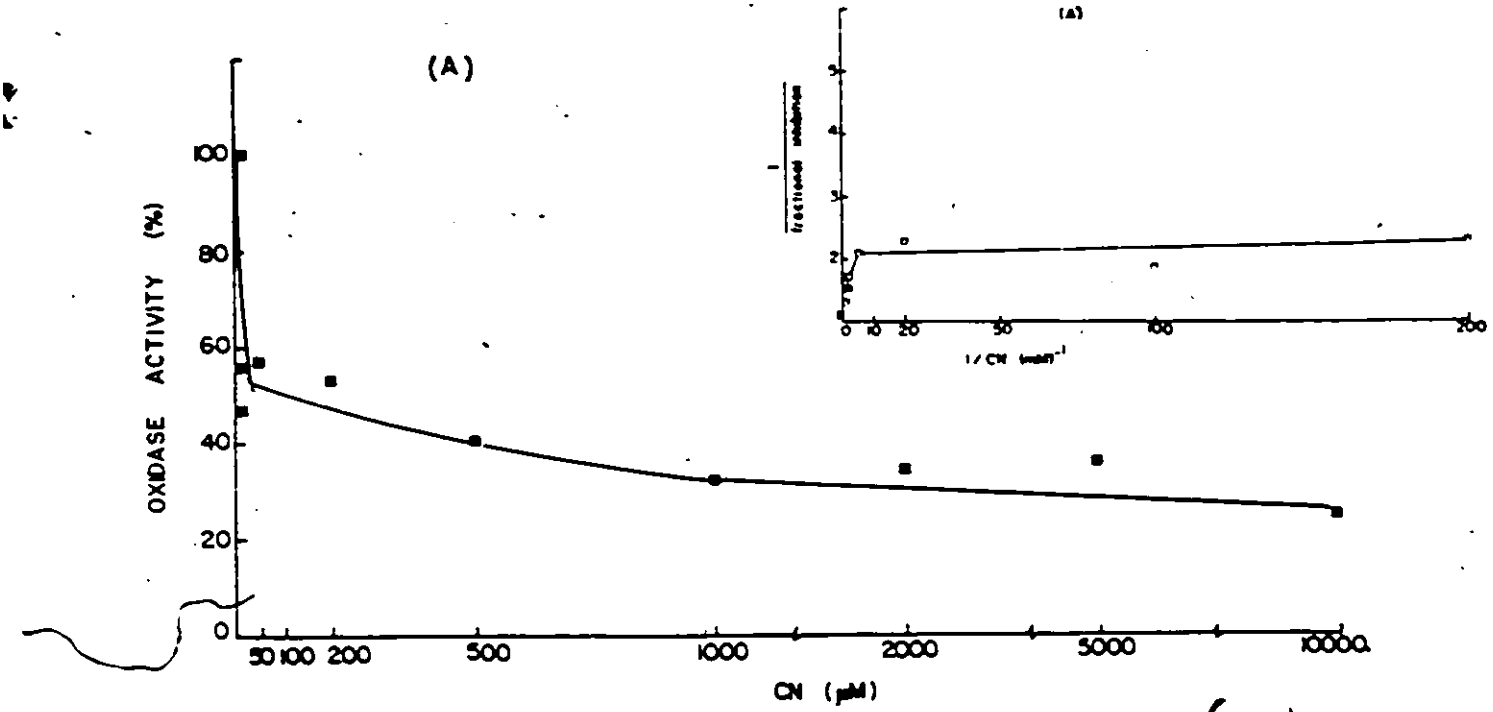

(B)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
N3 (mM)

Figure 42. The effect of various concentrations of cyanide and azide on intact cells of *A. halophilus* oxidizing succinate.

The complex medium grown cells were harvested at the stationary phase, washed twice in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution and starved for 48 hr at 25°C, 200 rpm, in a volume of sterile salt solution equal to the growth volume. The starved cells were harvested by centrifugation and resuspended in an appropriate volume of complete salt (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1mM, pH 7.4) to a protein concentration of 22.5 mg/ml in (A) and 20.0 mg/ml in (B). The whole cell proteins (0.75 mg in (A) and 0.50 mg in (B)) were pre-incubated for 3 min with various concentrations of cyanide (0.01 to 10 mM in (A) and azide (0.5 to 100 mM in (B)). The reactions were started by the addition of 10 mM succinate. Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C. Cyanide and azide inhibitions are expressed as the percentages of succinate oxidase activity in the presence of various concentrations of cyanide or azide in (A) and (B) respectively. In (A') and (B'), the double reciprocal plots of 1/fractional inhibition *versus* 1/inhibitor $(mM)^{-1}$ are inserted in the main figure. The slopes of the lines and the intercepts with the axes were calculated by linear regression.


(A)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
50 100 200 500 1000 2000 5000 10000
CN (μM)
0 10 20 50 100 200

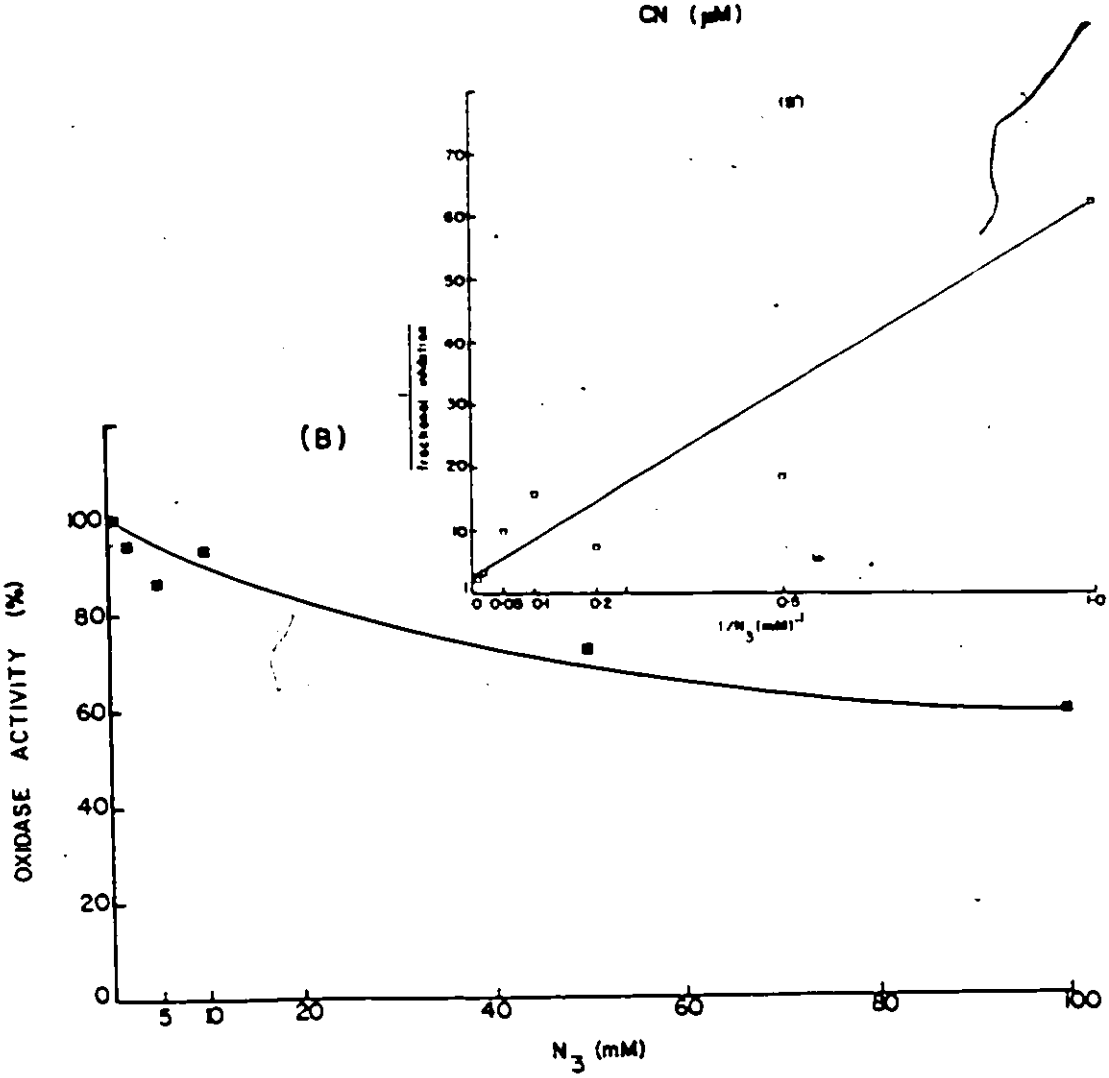
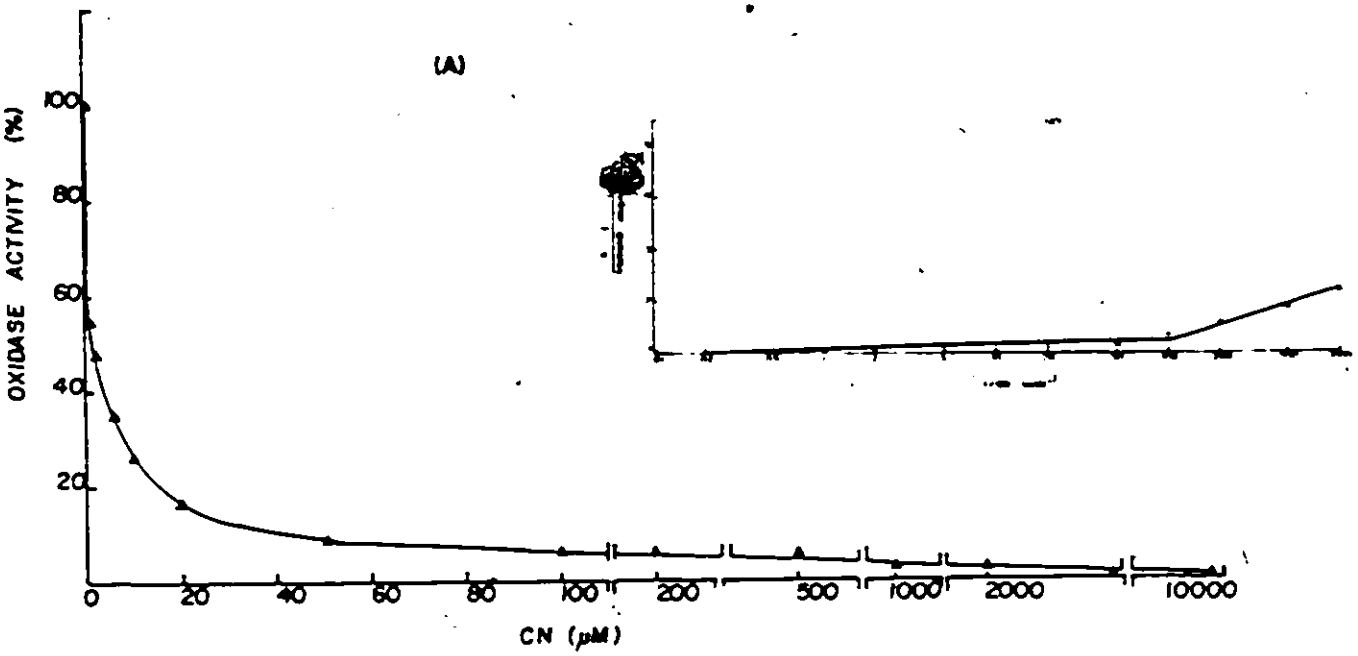
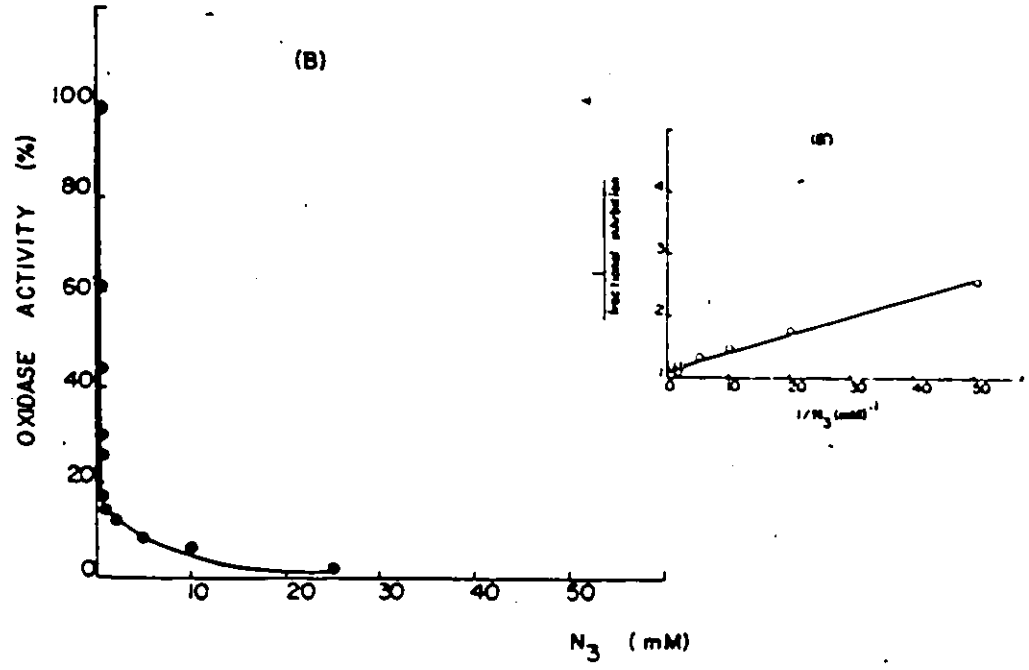

(B)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
5 10 20 40 60 80 100
N3 (mM)
0 0·08 0·1 0·2 0·5 1·0

Figure 43. The effect of various concentrations of cyanide and azide on intact cells of *A. haloplanktis* oxidizing ascorbate/TMPD.

The complex medium grown cells were harvested at the stationary phase, washed twice in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution and starved for 48 hr at 25°C, 200 rpm, in a volume of sterile salt solution equal to the growth volume. The starved cells were harvested by centrifugation and resuspended in an appropriate volume of complete salt (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1 mM, pH 7.4) to a protein concentration of 19.0 mg/ml in (A) and 20.0 mg/ml in (B). The whole cell proteins (0.63 mg in (A) and 0.66 mg in (B)) were pre-incubated for 3 min with various concentrations of cyanide (0.001 to 10 mM in (A) and azide (0.01 to 25 mM in (B)). The reactions were started by the addition of 10 + 0.5 mM ascorbate/TMPD. Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C, and corrected for the autoxidation of the dye TMPD. Cyanide and azide inhibitions are expressed as the percentages of ascorbate/TMPD oxidase activity in the presence of various concentrations of cyanide or azide in (A) and (B) respectively. In (A') and (B'), the double reciprocal plots of 1/fractional inhibited *versus* 1/inhibitor $(mM)^{-1}$ are inserted in the main figures. The slopes of the lines and the intercepts with the axes were calculated by linear regression.


(A)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
20
40
60
80
100
200
500
1000
2000
10000
CN (μM)


(B)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
10
20
30
40
50
N3 (mM)

The double reciprocal plots of the inhibition curves for NADH, succinate and ascorbate/TMPD are shown in the inserts of Figures 41 to 43. They suggest that NADH system (Figure 41A') was made up of two oxidases: one NADH oxidase sensitive to cyanide, inhibited at 2.0 mM (intercepting the ordinate at 0.64) and another NADH oxidase resistant to cyanide (intercepting the ordinate at 5.8). In fact, a recent study on intact cells of the marine bacterium revealed that *Alteromonas haloplanktis* oxidizes NADH both exogenously and endogenously by processes distinguishable from one another for their requirements for alkali metal ions (Khanna *et al.*, 1984). The results are explained in terms of two sites for oxidation of NADH, one on the outside and one on the inside surface of the cell cytoplasmic membrane (Khanna *et al.*, 1984). NADH oxidase would be sensitive to azide at infinite concentration (Figure 41B').

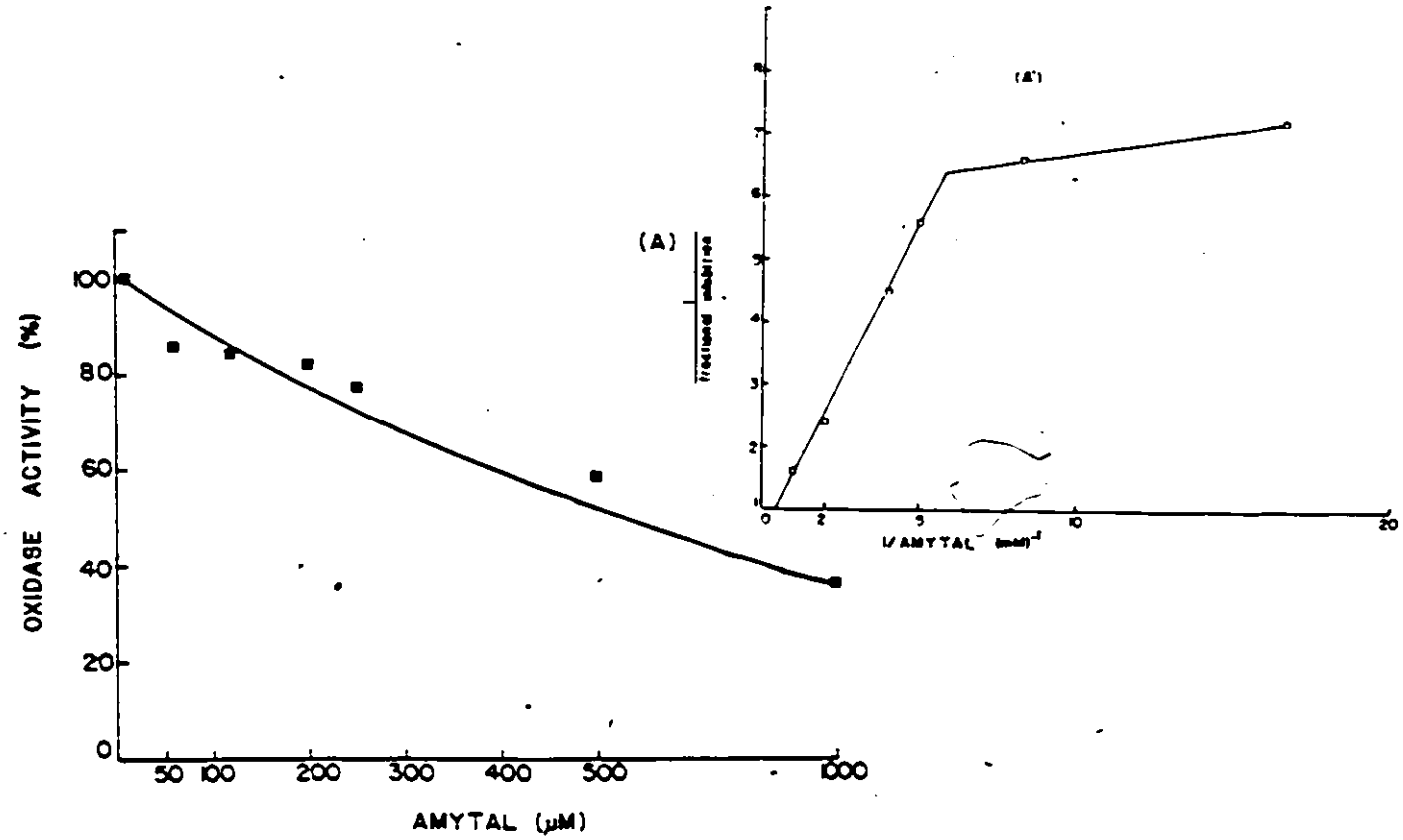
As far as succinate is concerned, the double reciprocal plots (Figure 42A', B') show that the oxidase system would be resistant to both cyanide and azide. Ascorbate/TMPD oxidase, on the other hand, would be very sensitive to cyanide ($I_{0.5}$ = 1.5 µM) and to azide ($I_{0.5}$ = 1µM) (Figure 43A,B respectively), being fully inhibited at low and high concentrations according to the double reciprocal plot (Figure 43A'), but resistant to azide (Figure 43B'), the straight line intercepting the ordinate at 1.1.

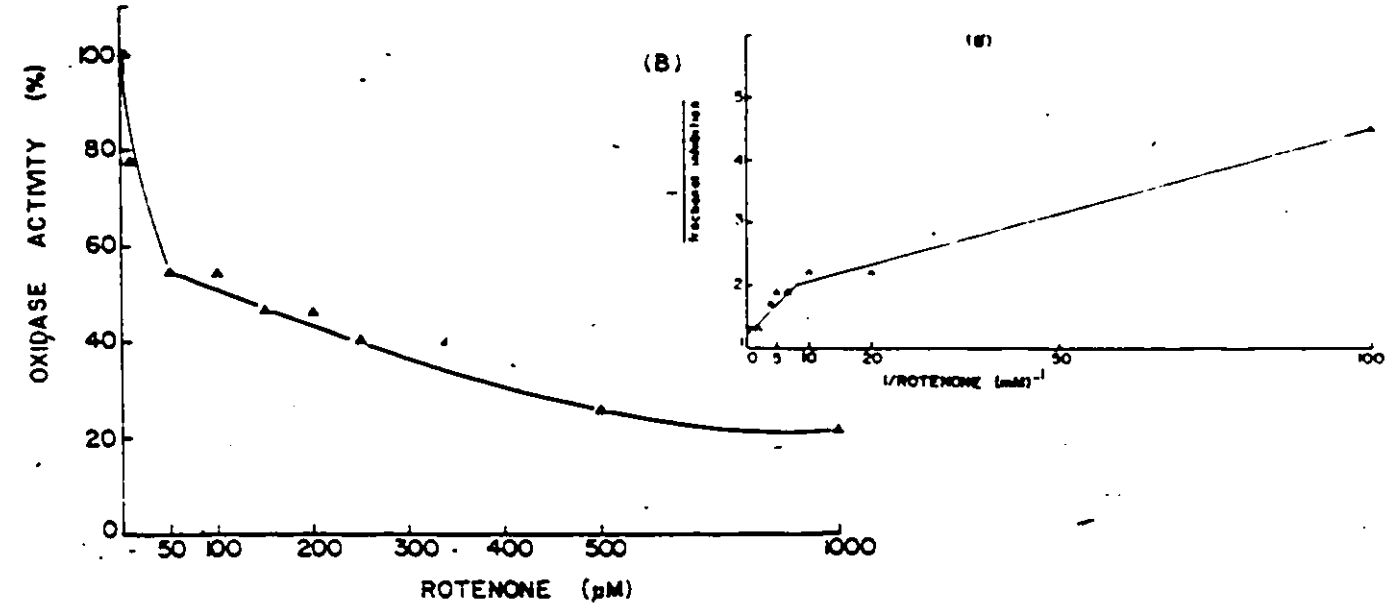
In summary, it appears that two pathways would be required for the oxidation of NADH, one pathway resistant to cyanide and one pathway sensitive to cyanide and azide. Succinate oxidation on the other hand, would be conveyed via two pathways resistant to cyanide and one pathway resistant to azide. Finally, ascorbate/TMPD would be oxidized via two pathways sensitive to cyanide and one pathway resistant to azide.

The results of the experiments carried out with starved cells and the NADH dehydrogenase specific inhibitors rotenone and amytal are presented in Figure 44A,B. According to the double reciprocal

Figure 44. The effect of various concentrations of amytal and rotenone on intact cells of A. *haloplanktis* oxidizing NADH.

The complex medium grown cells were harvested at the stationary phase, washed twice in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution and starved for 48 hr at 25°C, 200 rpm, in a volume of sterile salt solution equal to the growth volume. The starved cells were harvested by centrifugation and resuspended in an appropriate volume of complete salt (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1 mM, pH 7.4) to a concentration of 30.0 mg protein/ml. The whole cell proteins (0.50 mg) were preincubated for 3 min with various concentrations of (A) amytal and (B) rotenone (0 to 1,000 µM). The reactions were started by the addition of 2.5 mM NADH. Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C. Amytal and rotenone inhibitions are expressed as the percentages of NADH oxidase activity in the presence of various concentrations of amytal or rotenone in (A) and (B) respectively. In (A') and (B'), the double reciprocal plots of 1/fractional inhibition *versus* 1/inhibitor $(mM)^{-1}$ are inserted in the main figures. The slopes of the lines and the intercepts with the axes were calculated by linear regression.


(A)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
50
100
200
300
400
500
1000
AMYTAL (μM)
(A')
1/AMYTAL (mM)⁻¹
0
2
5
10
20


(B)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
50
100
200
300
400
500
1000
ROTENONE (μM)
(B')
1/ROTENONE (mM)⁻¹
0
5
10
20
50
100

plots (Figure 44A',B') one NADH oxidase is resistant to amytal (intercepting the ordinate at 6.0), the other sensitive at 2.0 mM concentration (intercepting the ordinate at 0.5). With respect to rotenone (Figure 44B') the NADH oxidases are both resistant to the inhibitor (intercepting the ordinate at 1.8 and 1.2 respectively).

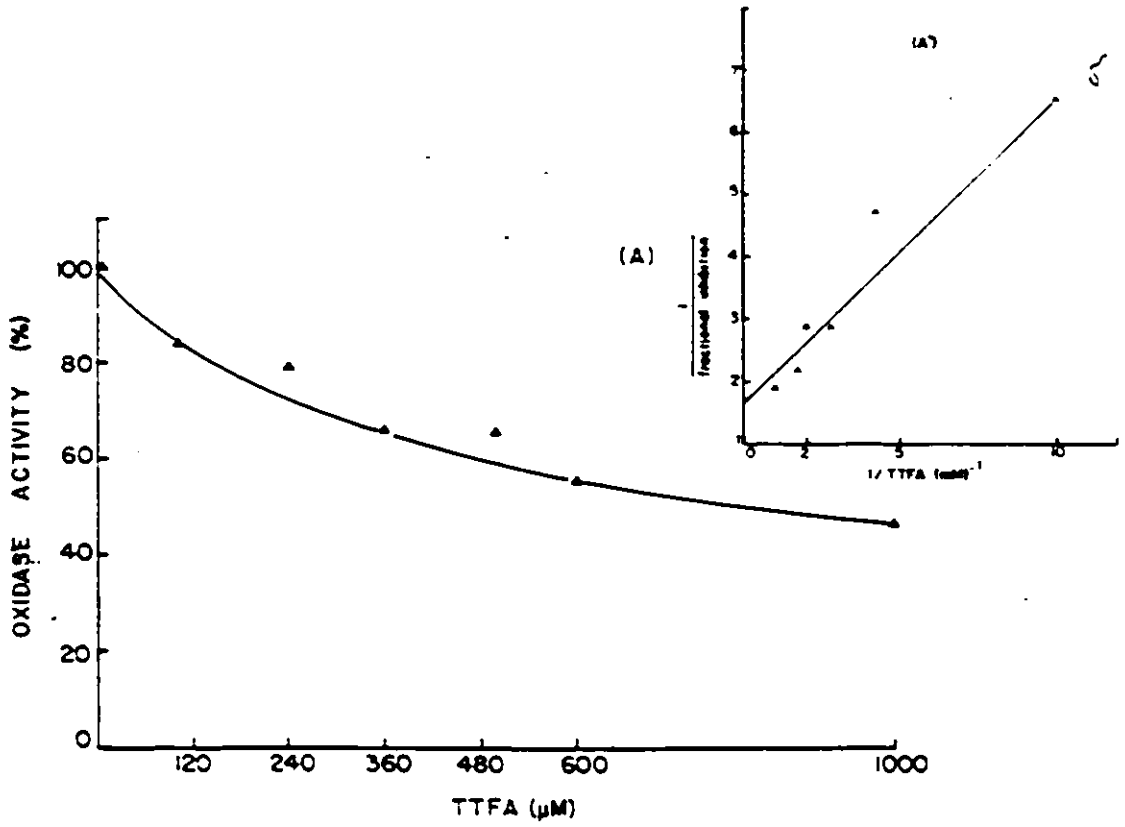
The specific inhibitor of succinate dehydrogenase, TTFA, was tested in starved cells grown in complex and chemically defined media (Figure 45). More than 800 μM of TTFA were required for 50% inhibition of complex medium grown cells oxidizing succinate ($I_{0.5}$ = 825 μM) whereas a five times less concentration ($I_{0.5}$ = 165 μM) was sufficient to inhibit 50% oxidation of succinate by cells grown in succinate chemically defined medium. The respective double reciprocal plots (Figure 45A',B') revealed that the cells grown in complex medium are resistant to the inhibitor (slope intercepting the ordinate at 1.7) whereas the cells grown in succinate chemically defined medium are sensitive to the inhibitor at concentration around 500 μM (slope intercepting the ordinate at 0.4).

With respect to antimycin A, a conventional inhibitor of the second site of oxidative phosphorylation, NADH oxidase was more sensitive to the inhibitor ($I_{0.5}$ = 460 μM) than succinate oxidase ($I_{0.5}$> 1.0 mM), (Figure 46A,B). The double reciprocal plots of both oxidase systems (Figure 46A',B') strongly suggest that an antimycin A sensitive and an antimycin A resistant pathways are operative in the respiratory chain of A. *haloplanktis*.

For HOQNO inhibition (Figure 47A,B), succinate oxidase was more sensitive ($I_{0.5}$ = 10 μM) than NADH oxidase ($I_{0.5}$ = 250 μM). When the double reciprocal plots (Figure 47A',B') were taken into consideration, the resistance of succinate oxidase toward HOQNO was less pronounced (slope intercepting the ordinate at 1.3) than the resistance of NADH oxidase (slope intercepting the ordinate at 42.7), when the slopes

Figure 45. The effect of various concentrations of TTFA on intact cells of A. *haloplanktis* oxidizing succinate.

The complex medium grown cells in (A) and the succinate chemically defined medium grown cells in (B) were harvested at the stationary phase, washed in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution and starved for 48 hr at 25°C, 200 rpm, in a volume of sterile salt solution equal to the growth volume. The starved cells were harvested by centrifugation and resuspended in an appropriate volume of complete salt (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1 mM, pH 7.4) to a protein concentration of 20.5 mg/ml in (A) and 15.0 in (B). The whole cell proteins (0.50 mg) were preincubated for 3 min with various concentrations of TTFA (0 to 1000 µM). The reactions were started by the addition of 10 mM succinate. Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C. TTFA (thenoyl-trifluoroacetone) inhibitions are expressed as the percentages of succinate oxidase activity in the presence of various concentrations of the inhibitor with complex medium grown cells in (A) and succinate chemically defined medium grown cells in (B) respectively. In (A') and (B'), the double reciprocal plots of 1/fractional inhibition *versus* 1/inhibitor $(mM)^{-1}$ are inserted in the main figures. The slopes of the lines and the intercepts with the axes were calculated by linear regression.


(A)
OXIDASE ACTIVITY (%)
100
80
60
40
20
0
120
240
360
480
600
1000
TTFA (μM)
1/ TTFA (mM)⁻¹

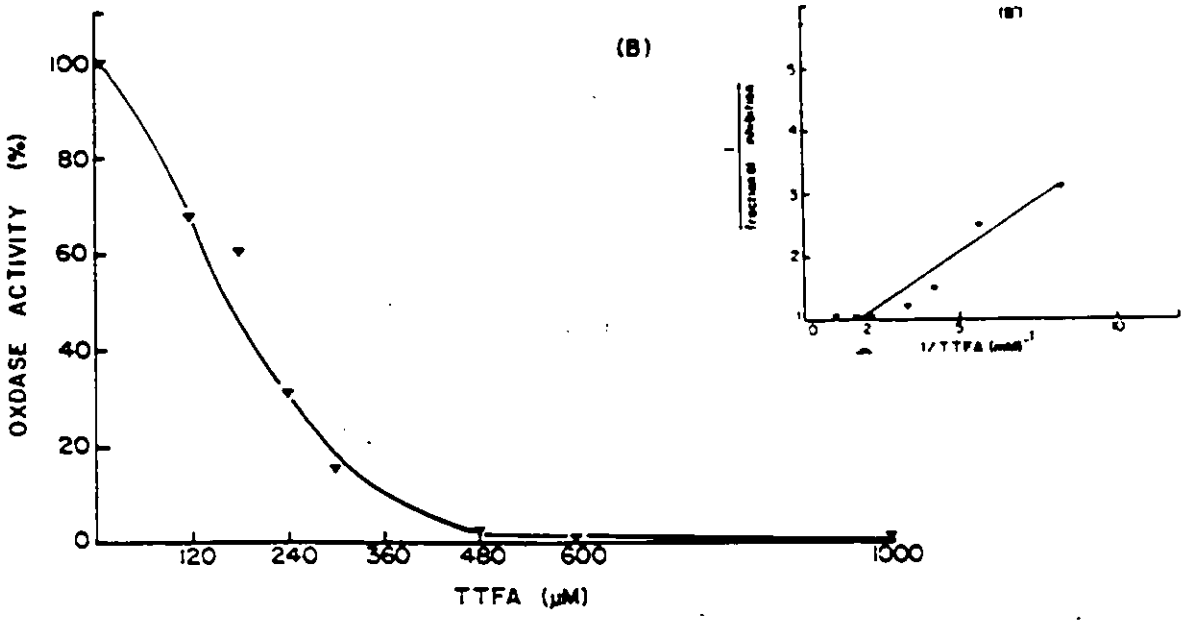

(B)
OXDASE ACTIVITY (%)
100
80
60
40
20
0
120
240
360
480
600
1000
TTFA (μM)

Figure 46. The effect of various concentrations of antimycin A on intact cells of *A. halophilus* oxidizing NADH and succinate.

The complex medium grown cells were harvested at the stationary phase, washed twice in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution and starved for 48 hr at 25°C, 200 rpm, in a volume of sterile salt solution equal to the growth volume. The starved cells were harvested by centrifugation and resuspended in an appropriate volume of complete salt (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1 mM, pH 7.4) to a concentration of 21.7 mg protein/ml in (A) and (B). The whole cell proteins (0.50 mg) were preincubated for 3 min with various concentrations of antimycin A (0 to 1000 μM). The reactions were started by the addition of 2.5 mM NADH in (A) and 10 mM succinate in (B). Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C. Antimycin A inhibitions are expressed as the percentages of NADH oxidase activity in (A) and succinate oxidase activity in (B) in the presence of various concentrations of the inhibitor. In (A') and (B') the double reciprocal plots of 1/fractional inhibition versus 1/inhibitor $(mM)^{-1}$ are inserted in the main figures. The slopes of the lines and the intercepts with the axes were calculated by linear regression.

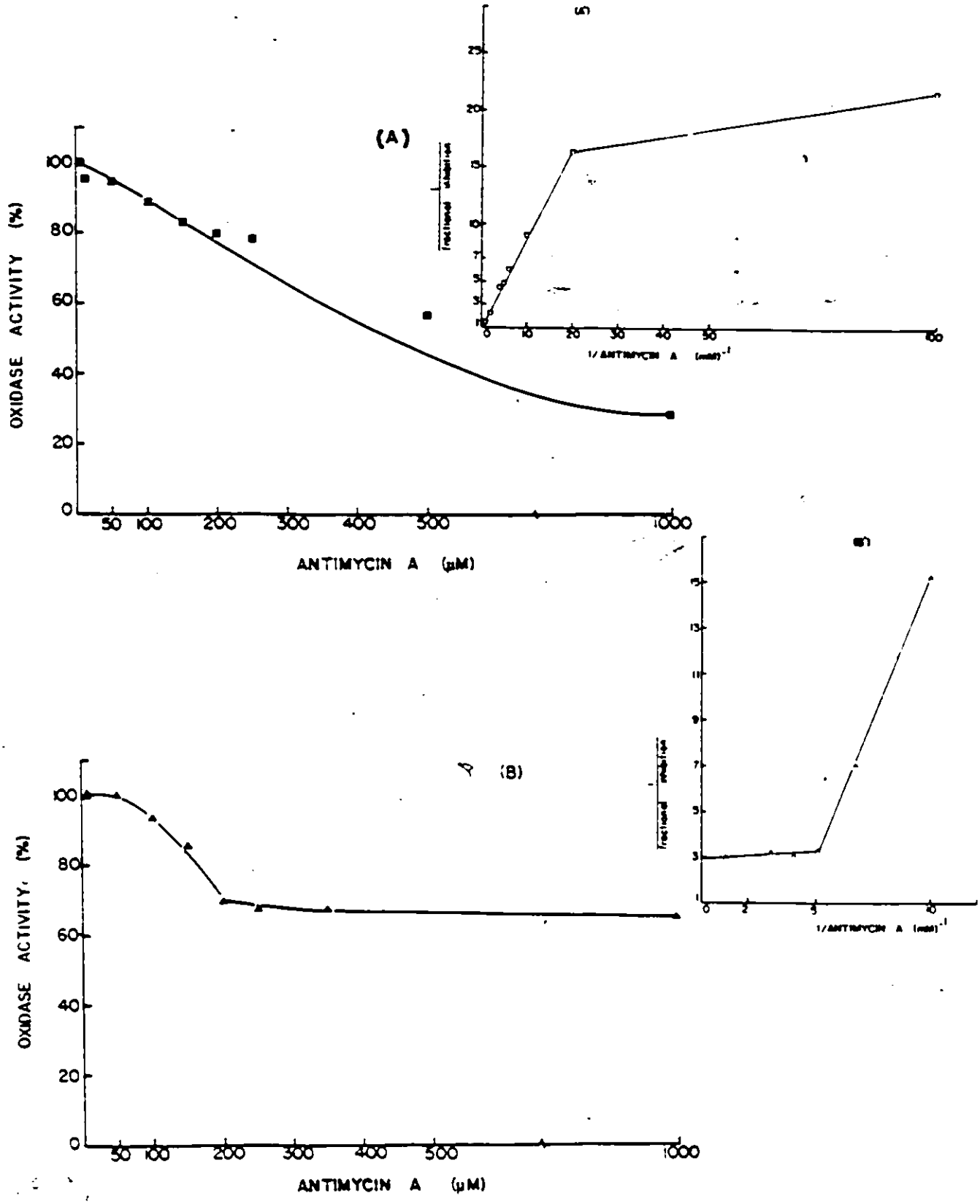
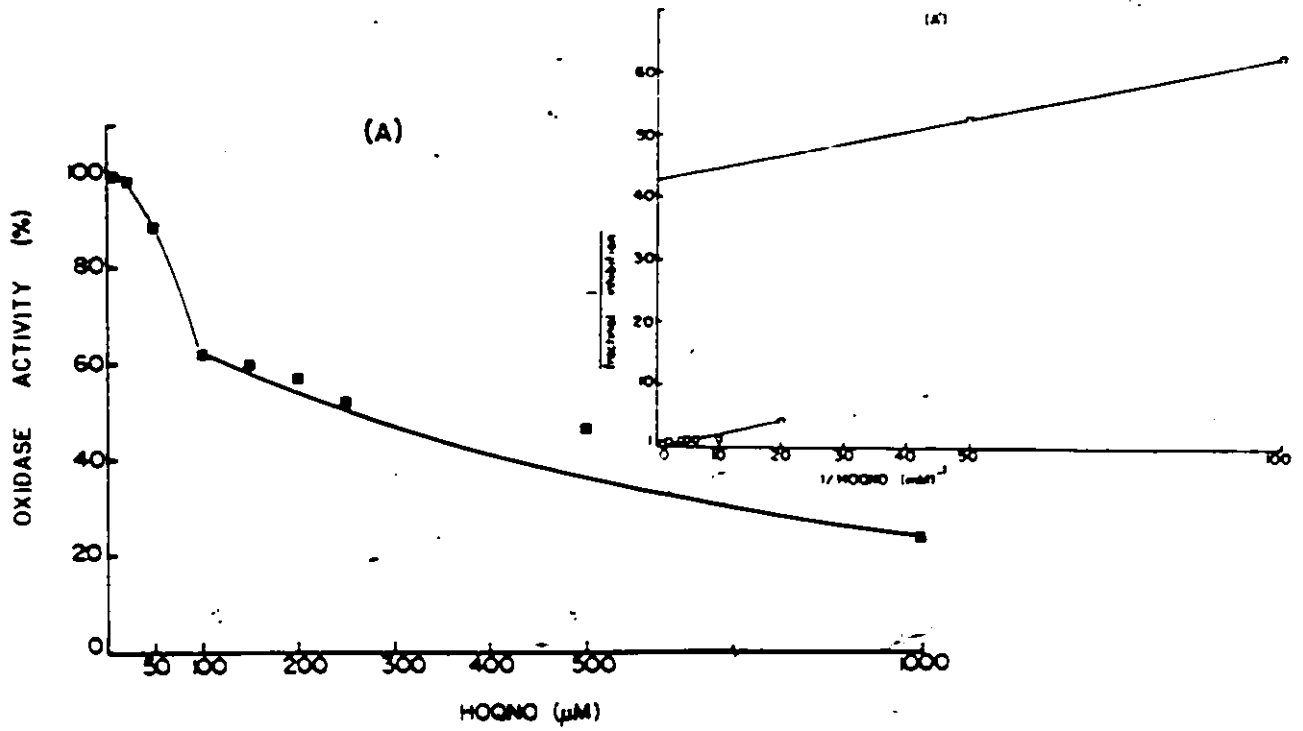
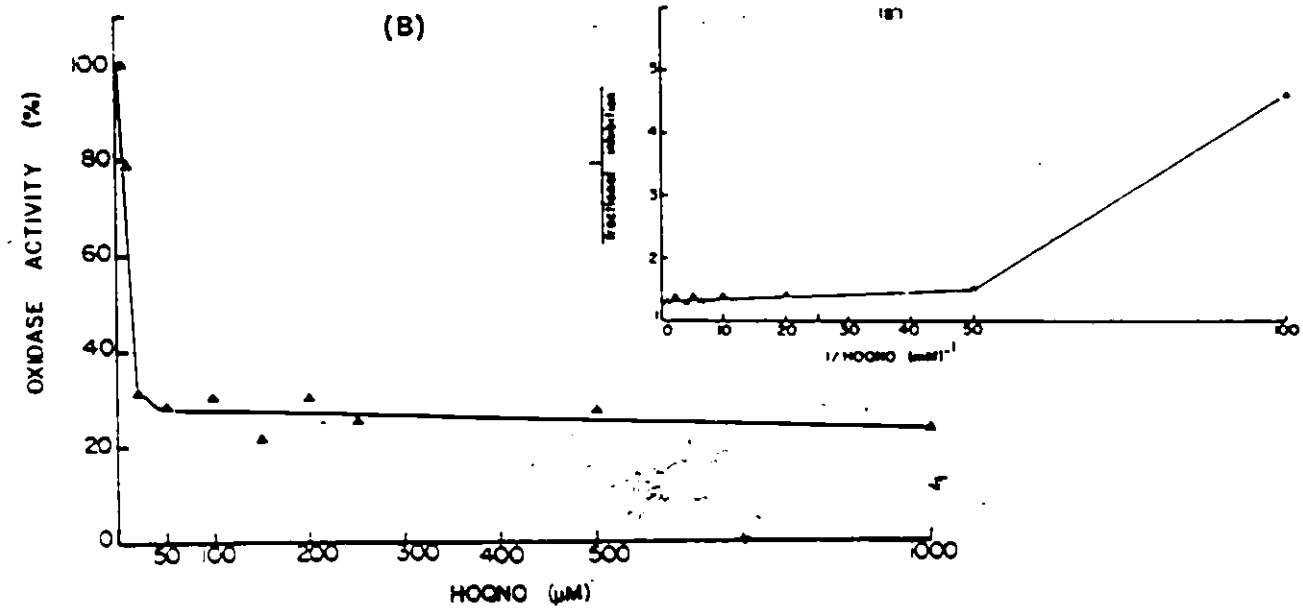

(A)
OXIDASE ACTIVITY (%)
ANTIMYCIN A (μM)
(B)
OXIDASE ACTIVITY (%)
ANTIMYCIN A (μM)

Figure 47. The effect of various concentrations of HOQNO on intact cells of *A. haloplanktis* oxidizing NADH and succinate.

The complex medium-grown cells were harvested at the stationary phase, washed twice in complete salt (.3M NaCl, .05M $MgCl_2$, .01M KCl) solution and starved for 48 hr at 25°C, 200 rpm, in a volume of sterile salt solution equal to the growth volume. The starved cells were harvested by centrifugation and resuspended in an appropriate volume of complete salt (.3M NaCl, .05M $MgSO_4$, .01M KCl) Tris/PO_4 (50 + 1 mM, pH 7.4) to a protein concentration of 23.5 mg/ml in (A) and 21.7 mg/ml in (B). The whole cell proteins (0.50 mg) were pre-incubated for 3 min with various concentrations of HOQNO (0 to 1000 μM). The reactions were started by the addition of 2.5 mM NADH in (A) and 10 mM succinate in (B). Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C. HOQNO inhibitions are expressed as the percentages of NADH oxidase activity in (A) and succinate oxidase activity in (B) in the presence of various concentrations of the inhibitor. In (A') and (B'), the double reciprocal plots of 1/fractional inhibition *versus* 1/inhibitor $(mM)^{-1}$ are inserted in the main figures. The slopes of the lines and the intercepts with the axes were calculated by linear regression.


(A)
OXIDASE ACTIVITY (%)
HOQNO (μM)
1/ HOQNO


(B)
OXIDASE ACTIVITY (%)
HOQNO (μM)

and the points of intercept to the ordinates were calculated. Also both succinate and NADH oxidase have an oxidase sensitive to HOQNO (second set of slopes, Figure 47A',B'), succinate oxidase being more sensitive than NADH oxidase. The results suggest that HOQNO and antimycin A act either at the same site (conventional second site of oxidative phosphorylation, between cytochrome b and c) or at a different site with antimycin A having a further site of action on the pathway of NADH oxidation, possibly before the entry of succinate in the respiratory chain.

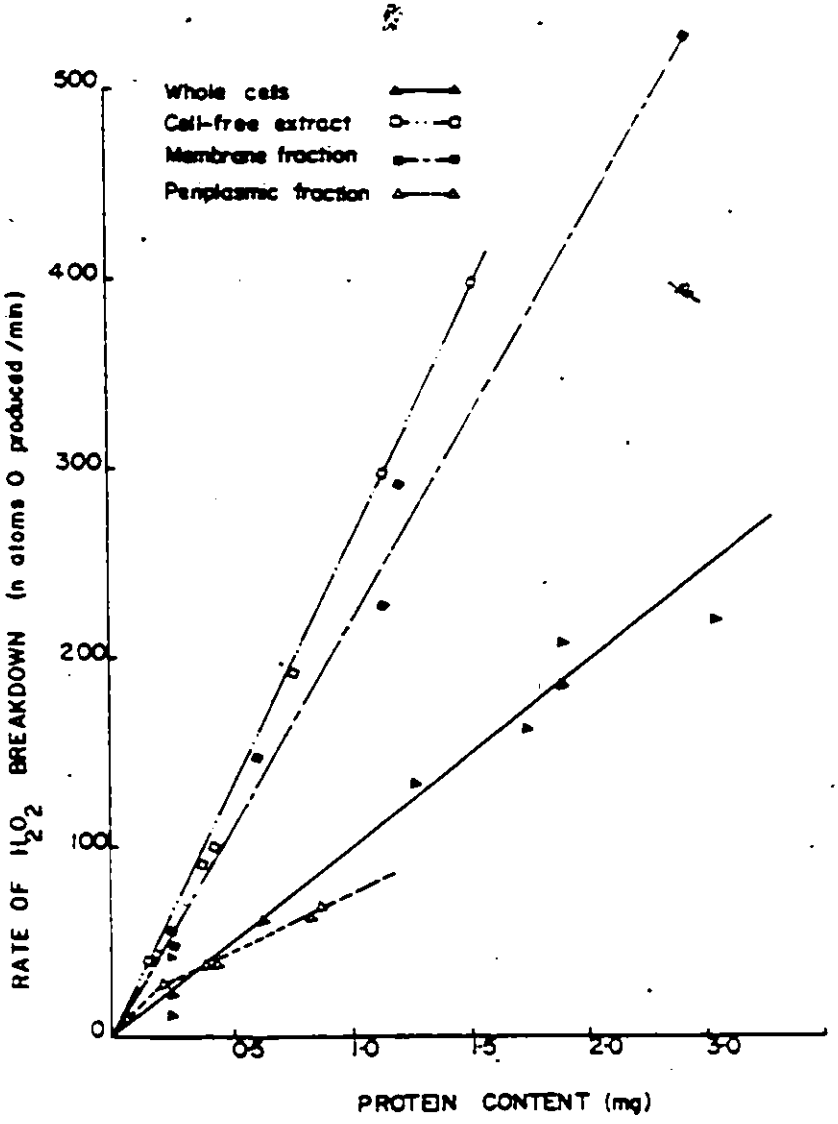
C. TERMINAL SEGMENT OF THE ELECTRON TRANSPORT CHAIN

In addition, O_2 electrode experiments using H_2O_2 and various cell fractions were carried out to shed light on the terminal segment of the electron transfer pathway(s). Also in view of the fact that a proportion of the electron transport chain is resistant to known respiratory inhibitors and that inhibitor-resistant pathway(s) are often associated with the production of H_2O_2 (Webster, 1975; Ainsworth *et al.*, 1980a,b), the following experiment was considered necessary to assess the presence or the absence of the enzyme catalase in the marine bacterium. Catalases along with peroxidases form a group of enzymes called hydroperoxidases responsible for the breakdown of peroxides. Depending on the substrate used to assay the enzyme and depending on the end product of the reaction, it will be a catalase or a peroxidase. A known function of hydroperoxidases is to supply additional oxygen to microorganisms (Mahler and Cordes, 1971; Ibrahim and Schlegel, 1980). The results presented in Figure 48 were consistent with the presence of a catalase enzyme predominantly active in whole cells, cell-free extracts and its membrane fraction. The source of enzyme was membrane-bound and hence the relatively low content found in the periplasmic fraction. The peroxidase enzyme has not been assayed due to the lack of specificity of most hydrogen donors used for quantitative determination (Saunders *et al.*, 1964). However, it was ascertained that the enzyme present in the periplasmic fraction was sensitive to azide and thus contrasted with the azide-insensitive cytochrome c peroxidase present in *Pseudomonas fluorescens* (Lenhoff and Kaplan, 1956).

Insights on the possible electron carriers terminating the respiratory chain and thus acting as oxidoreductases came from results obtained by differenceabsorption spectrophotometry using

Figure 48. Catalase activity in whole cells, cell-free extracts, membrane and periplasmic fractionsof A. *haloplanktis* grown in complex medium.

Cells of A. *haloplanktis* were grown in complex medium, harvested at the stationary phase, washed, resuspended in appropriate volumes of buffered salt solution and fractionated into cell-free extracts, membrane and periplasmic fractions. The catalase activity was estimated polarographically with a Clark oxygen electrode covered with a teflon membrane and maintained at the constant temperature of 25°C. During the breakdown of hydrogen peroxide by the enzyme catalase, according to the relationship: $H_2O_2 \longrightarrow 1/2\ O_2 + H_2O$, oxygen is evolved. O_2 evolution was initiated by the addition of H_2O_2 (0.58 mM) to the suspension of whole cells, cell-free extracts, membrane and periplasmic fractions. The initial rates of H_2O_2 breakdown are expressed in n atoms O produced/min.

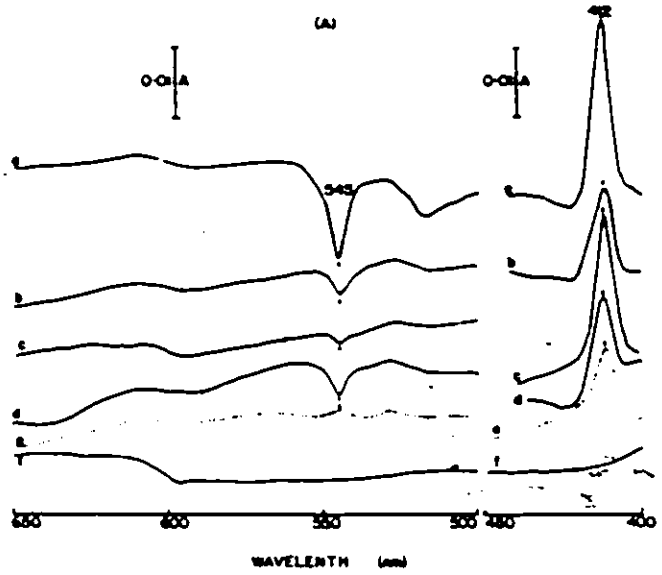

Whole cells
Cell-free extract
Membrane fraction
Periplasmic fraction
500
400
300
200
100
0
RATE OF H_2O_2 BREAKDOWN (in atoms O produced /min)
0·5
1·0
1·5
2·0
3·0
PROTEIN CONTENT (mg)

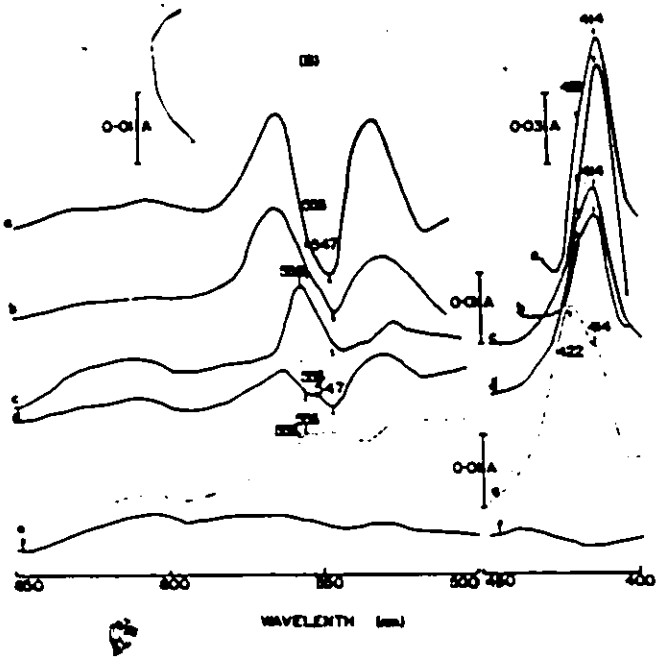
cellular fractions in combination with reducing substrates and carbon monoxide. Figure 49 illusrates CO-difference spectra recorded with the periplasmic fraction and the membrane fraction utilizing physiological and non-physiological sources of electrons. In the periplasmic fraction (Figure 49A), the trough at 545 nm in the visible region and the Soret peak at 412 nm are indicative that carbon monoxide binds the periplasmic c when reduced with dithionite (trace *a*), ascorbate/TMPD (trace *b*), succinate (trace c), NADH (trace *d*), but not when reduced with remaining endogenous substrate(s) (trace e) exhibiting a reduced peak at 545 nm. There is no evidence of CO-binding in the 585 to 650 nm region of the spectrum due to *a*-type cytochrome. In the membrane fraction (Figure 49B), visible trough at 556 and 547 nm and Soret peaks at 419 and 414 nm reveal the presence of CO-binding *b*(*o*)-and c-type cytochromes respectively when reduced with dithionite (trace *a*), ascorbate/TMPD (trace *b*), and NADH (trace *d*). In the presence of succinate (trace c), there is no obvious pseudo-oxidation of cytochrome *b* (*o*) at 556 nm but there is pseudo-oxidation of cytochrome c_{CO} at approximately 547 nm with a definite Soret peak at 414 nm. As with the periplasmic fraction, the remaining endogenous substrate(s) in the membrane fraction (trace e) do not allow binding of carbon monoxide and the membrane-bound cytochromes *b* absorbing at 558 and 556 nm are reduced in the visible region along with the Soret peak at 422 nm. The Soret peak at 414 nm accounts for the reduced c-type cytochromes. There is no evidence of CO-binding in the 585 to 650 nm region due to *a*-type cytochrome. Thus three CO-binding cytochromes are present in *A. haloplanktis*: one associated with the periplasmic cytochrome c and two others associated with a *b* (*o*) cytochrome and a c cytochrome bound to the membrane.

The other attempts made to further characterize the periplasmic and the membrane-bound cytochromes of the marine bacterium

Figure 49. Substrate-reduced + CO *minus* substrate-reduced difference absorption spectra of (A) the periplasmic and (B) the membrane fraction of A. *haloplanktis* using various reductants.

Starved cells of A. *haloplanktis* were washed, resuspended in buffered salt solution and fractionated into membrane and periplasmic fractions. The fractions were mixed (1:1) with sucrose (0.75 M, final concentration) and reduced with the following substrates in (A) and (B): *a*] dithionite; *b*] ascorbate/TMPD (10+0.5 mM); *c*] succinate (10 mM); *d*] NADH (2.5 mM); *e*] remaining endogenous substrate(s). Carbon monoxide was bubbled for 3 min into the samples which were incubated at room temperature in darkness for an additional 10 min. The baseline (traces *f*) were obtained by scanning the same concentration of protein (A) 7.7 mg/ml, (B) 20.0 mg/ml, present in the sample and the reference cuvettes. The difference spectra were recorded at 83°K in cuvettes of 2 mm light path. The vertical bars represent one tenth of the absorbancy units at which the spectra were recorded and the wavelengths are expressed in nanometers.


(A)
0·01 A
0·01 A
545
492
a
b
c
d
e
f
600
550
500
480
400
WAVELENTH (nm)


(B)
0·01 A
0·03 A
0·01 A
0·01 A
494
484
422
a
b
c
d
e
f
650
600
550
500
480
400
WAVELENTH (nm)

were: (*i*) testing the sensitivity of the redox carriers toward the terminal oxidase inhibitors, cyanide and azide, and (*ii*) scanning nitric oxide (NO) difference spectra to clarify the nature of the cytochrome(s) involved in the reaction.

A common substrate for the periplasmic and the membrane-bound cytochromes is ascorbate/TMPD. Results of the inhibitory effect of CN^- and N_3^- on ascorbate/TMPD oxidase activity in both fractions are presented in Figures 50 and 51. Although the inhibitory curves of ascorbate/TMPD oxidase *versus* various concentrations of cyanide and azide in combination with the periplasmic fraction (Figure 50A,B) indicate that the oxidase is quite sensitive to both inhibitors, cyanide ($I_{0.5}$ = 2 µM) and azide ($I_{0.5}$ = 20 µM), still some resistance is encountered as indicated by the reciprocal curves (Figure 50A',B') with straight lines intercepting the ordinate above the value of 1.1. The slope of the cyanide curve being much less pronounced than the slope of the azide curve indicates that the periplasmic fraction is more sensitive to cyanide than to azide (Jones, 1973).

The inhibitory median doses for inhibiting the membrane-bound ascorbate/TMPD oxidase by cyanide and azide are ($I_{0.5}$ = 1.0 µM) and ($I_{0.5}$ = 20 µM), respectively (Figure 51A,B). The double reciprocal plots for both inhibitors (Figure 51A',B') intercept the ordinate at the origin, confirming the sensitivity of the oxidase for the inhibitors.

Thus the substrate ascorbate/TMPD is oxidized by a cyanide and azide-sensitive membrane-bound oxidase and a cyanide-insensitive periplasmic oxidase. In several bacterial systems, the oxidation of this non-physiological substrate is linked to *o*-and *c*-type cytochromes binding carbon monoxide and readily inhibited by low concentrations of cyanide and azide (King and Drews, 1976; Jurtshuk and Yang, 1980; Matsushita *et al.*, 1982; Carver and Jones, 1983).

Figure 50. The effect of various concentrations of cyanide and azide on the periplasmic fraction of *A. haloplanktis* oxidizing ascorbate/TMPD.

The periplasmic fraction was derived from stationary phase cells grown in complex medium. The protein content of the fraction was 7.5 mg/ml. The periplasmic proteins (0.50 mg) were pre-incubated for 3 min with various concentrations of cyanide (0.5 to 100 µM in (A) or azide (5 to 1000 µM in (B)). The reactions were started by the addition of 10 + 0.5 mM ascorbate/TMPD. Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C and corrected for autooxidation of the dye TMPD. Cyanide and azide inhibitions are expressed as the percentages of ascorbate/TMPD oxidase activity in the presence of various concentrations of cyanide or azide in (A) and (B) respectively. In (A') and (B'), the double reciprocal plots of 1/fractional inhibition *versus* 1/inhibitor $(\mu M)^{-1}$ are inserted in the main figures. The slopes of the lines and the intercepts with the axes were calculated by linear regression.

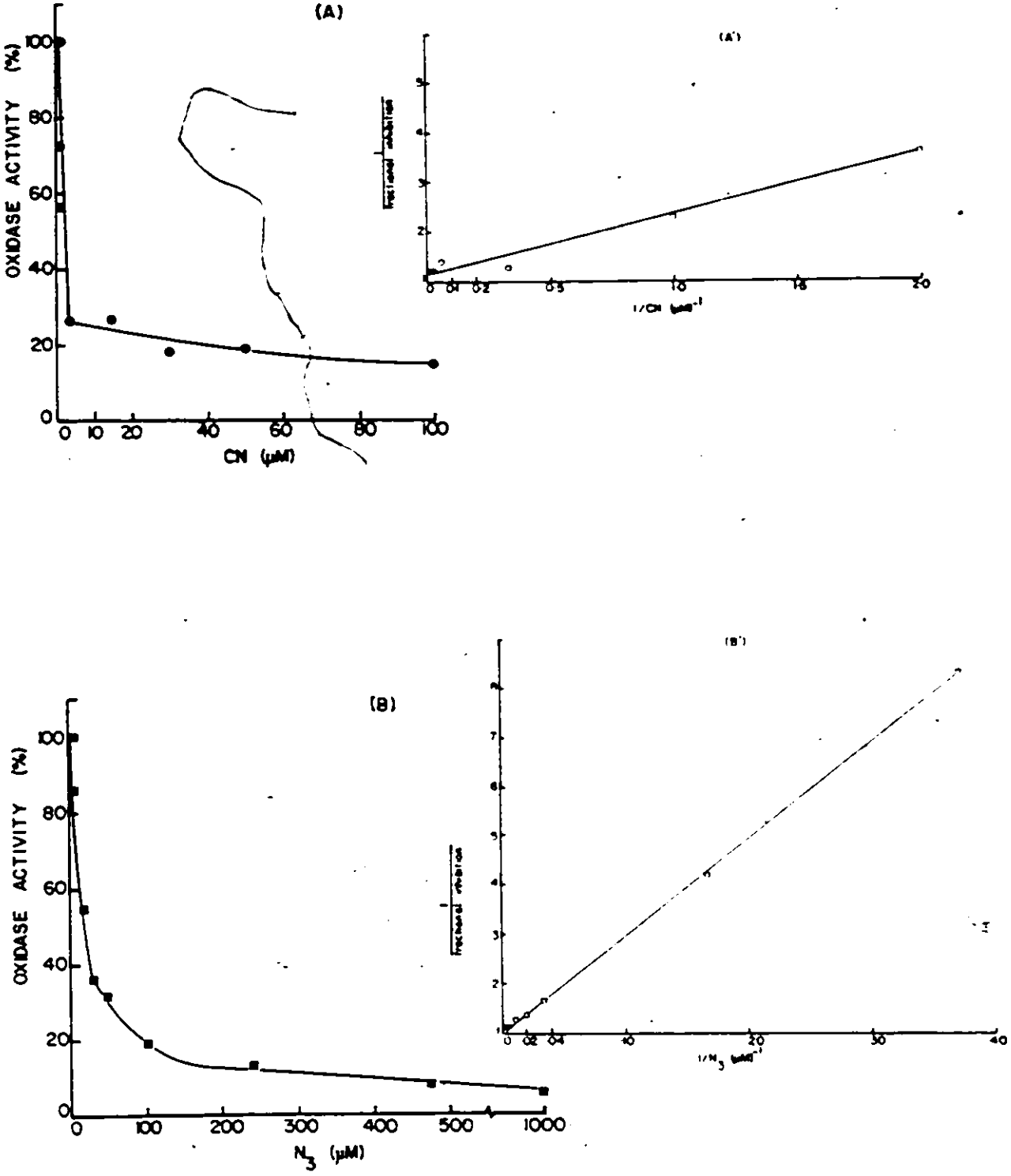
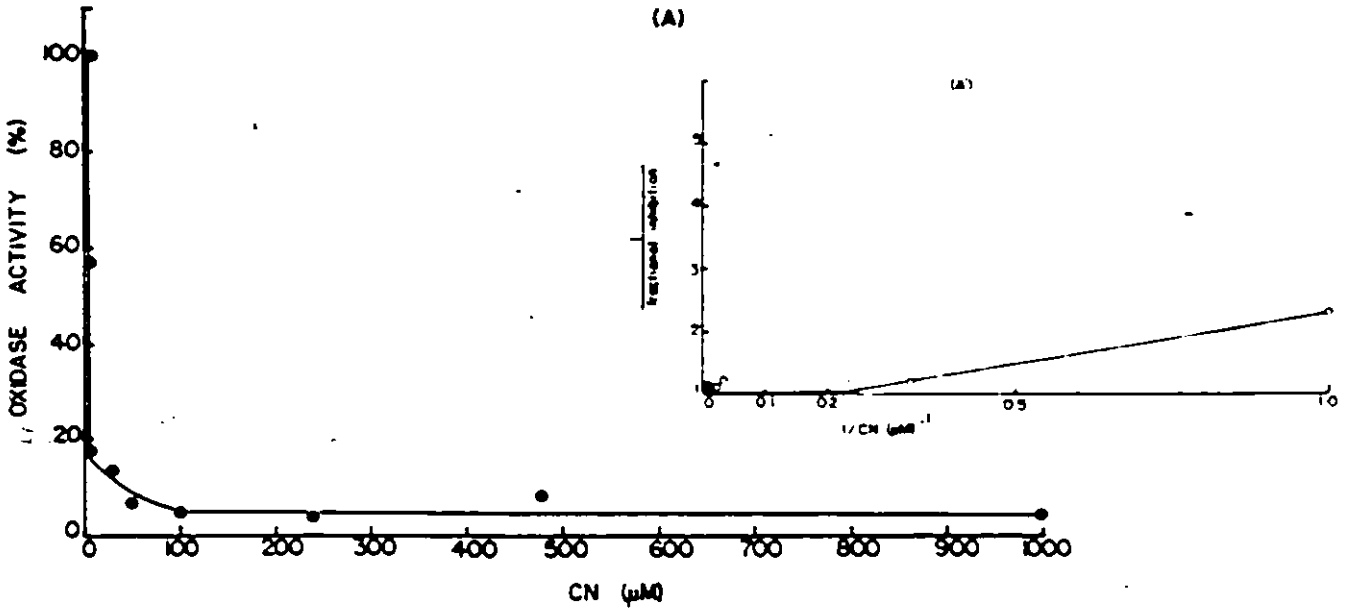
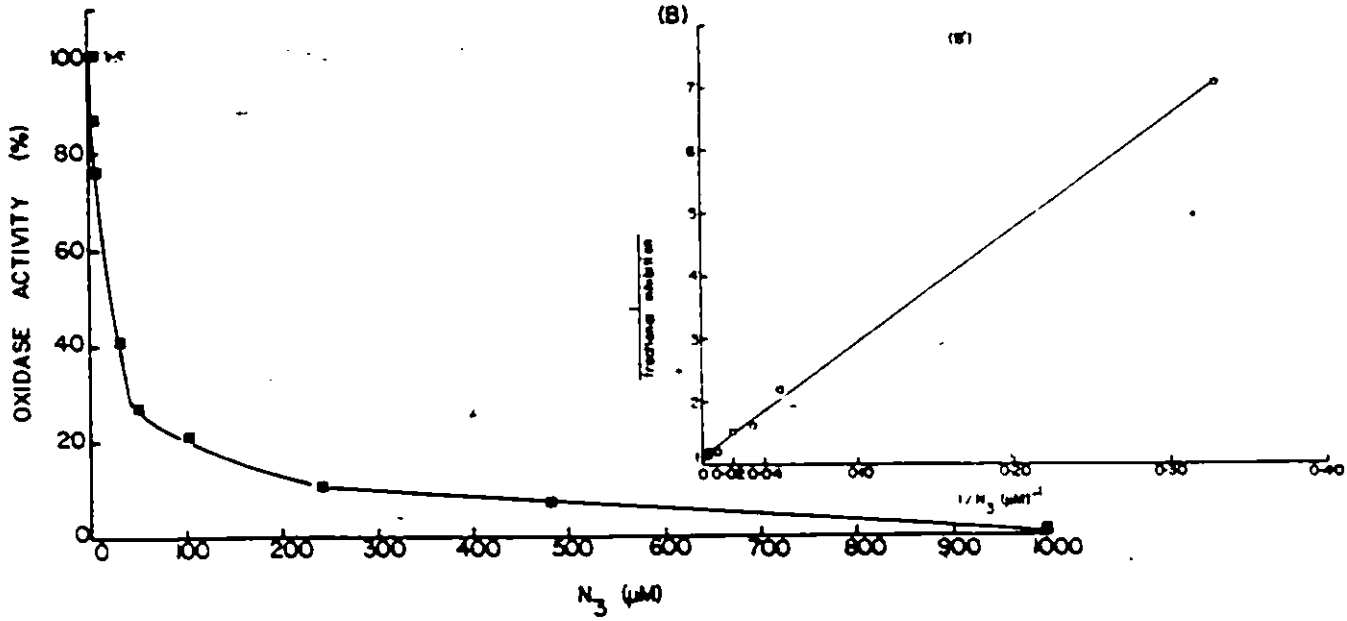

(A)
OXIDASE ACTIVITY (%)
CN (μM)
(A')
1/CN (μM⁻¹)
(B)
OXIDASE ACTIVITY (%)
N3 (μM)
(B')
1/N3 (μM⁻¹)

Figure 51. The effect of various concentrations of cyanide and azide on the membrane fraction of A. *haloplanktis* oxidizing ascorbate/TMPD.

The membrane fraction was derived from stationary phase cells grown in complex medium. The protein content of the fraction was 22.0 mg/ml. The membrane proteins (0.50 mg) were pre-incubated for 3 min with various concentrations of cyanide (0.5 to 1000 µM in (A) or azide (5 to 1000 µM in (B)). The reactions were started by the addition of 10 + 0.5 mM ascorbate/TMPD. Initial rates of oxygen consumption were measured for 10 min in an O_2 electrode cell at 25°C and corrected for autooxidation of the dye TMPD. Cyanide and azide inhibitions are expressed as the percentages of ascorbate/TMPD oxidase activity in the presence of various concentrations of cyanide or azide in (A) and (B) respectively. In (A') and (B'), the double reciprocal plots of 1/fractional inhibition *versus* 1/inhibitor $(\mu M)^{-1}$ are inserted in the main figures. The slopes of the lines and the intercepts with the axes were calculated by linear regression.


(A)
OXIDASE ACTIVITY (%)
CN (μM)
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800
900
1000


(B)
OXIDASE ACTIVITY (%)
N3 (μM)
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800
900
1000

Difference absorption spectra using nitric oxide, NO, with the oxidized and reduced form of the periplasmic and membrane-bound cytochromes of A. *haloplanktis* were scanned in an attempt to further characterize the nature of the cytochromes involved.

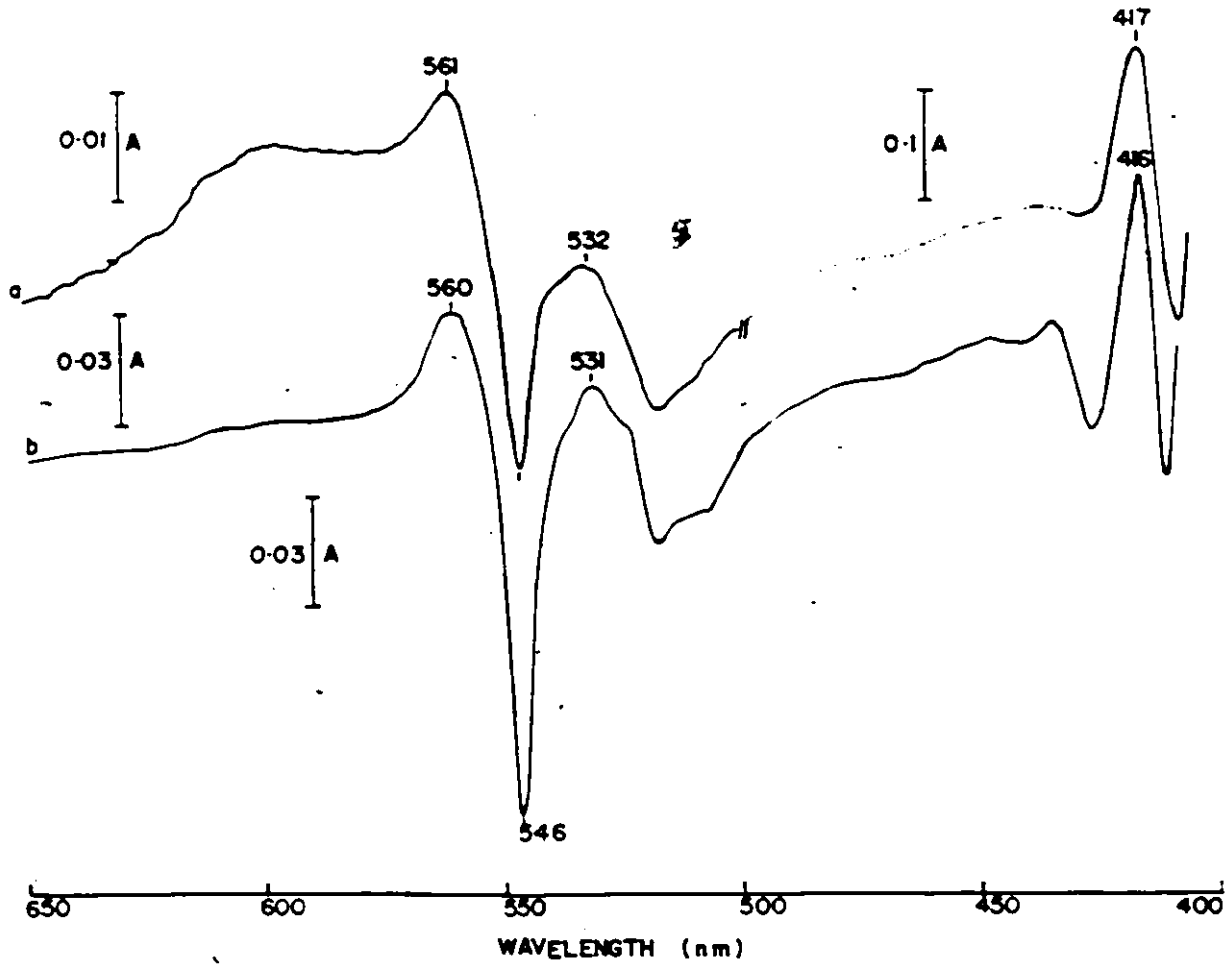
The *Rhodospirillum* haem protein (RHP) of the purple photosynthetic bacteria *Rhodospirillum rubrum* was proposed by Taniguchi and Kamen (1963) as the prototype of a new class of haemoprotein called cytochromoids (cytochrome c variant or cytochrome c'). The unique characteristic of this haem protein was the ability of its oxidized and reduced form to bind nitric oxide.

Earlier work by Bartsch and Kamen (1958) had been presented implicating the RHP in an oxidase system. Cox *et al.*, (1971) concluded from a study done with *Pseudomonas perfectomarinus* that the c-type cytochromes involved in nitrite reduction were all present in the NO complex form. Both haems of *Pseudomonas aeruginosa* cytochrome oxidase *cd* (nitrite reductase) react with NO in the oxidized and reduced states and are intermediates in the mechanism of nitrite reduction (Shimada and Orii, 1976). The c cytochromes appear to be "periplasmic", freely soluble in phototrophic bacteria, and comprise the largest and most widespread class of bacterial cytochromes known, yet the functional roles of these cytochromes have eluded definition (Meyer and Kamen, 1982). Recently, a soluble c-type cytochrome present in *Neisseria meningitidis* has been classified as cytochrome c' on the basis of its reactivity with nitric oxide (Yu *et al.*, 1979). No functional role could be attributed to the c' cytochrome.

When nitric oxide, NO, difference spectra were scanned with the ferric and the ferrous forms of the periplasmic (Figure 52) and

Figure 52. Nitric oxide (NO) difference absorption spectra of the periplasmic fraction of A. *haloplanktis* (a) oxidized with hydrogen peroxide and (b) reduced with sodium dithionite.

Cells of A. *haloplanktis* were grown in complex medium to the stationary phase. Stationary phase cells were harvested by centrifugation, washed, resuspended in buffered salt solution and fractionated into the membrane and the periplasmic fractions. The periplasmic fraction was mixed (1:1) with sucrose (0.75M final concentration). In (a) the fraction was oxidized with H_2O_2 (1 drop of 1:100 30% solution) and divided into the sample and the reference cuvettes. NO gas was then bubbled for 3 min through the contents of the sample cuvette and the reaction allowed to proceed for 10 min at room temperature before the difference spectrum was scanned at 83°K in cuvettes of 2mm light path. In (b) the fraction was reduced with solid crystals of dithionite and divided into the sample and the reference cuvettes. NO gas was then bubbled for 3 min through the contents of the sample cuvette and the reaction allowed to proceed for 10 min at room temperature before the difference spectrum was scanned at low temperature. The protein content of the fraction in (a) and (b) was 4.5 mg/ml. The vertical bars represent one tenth of the absorbancy units at which the spectra were recorded; the wavelengths are expressed in nanometers.

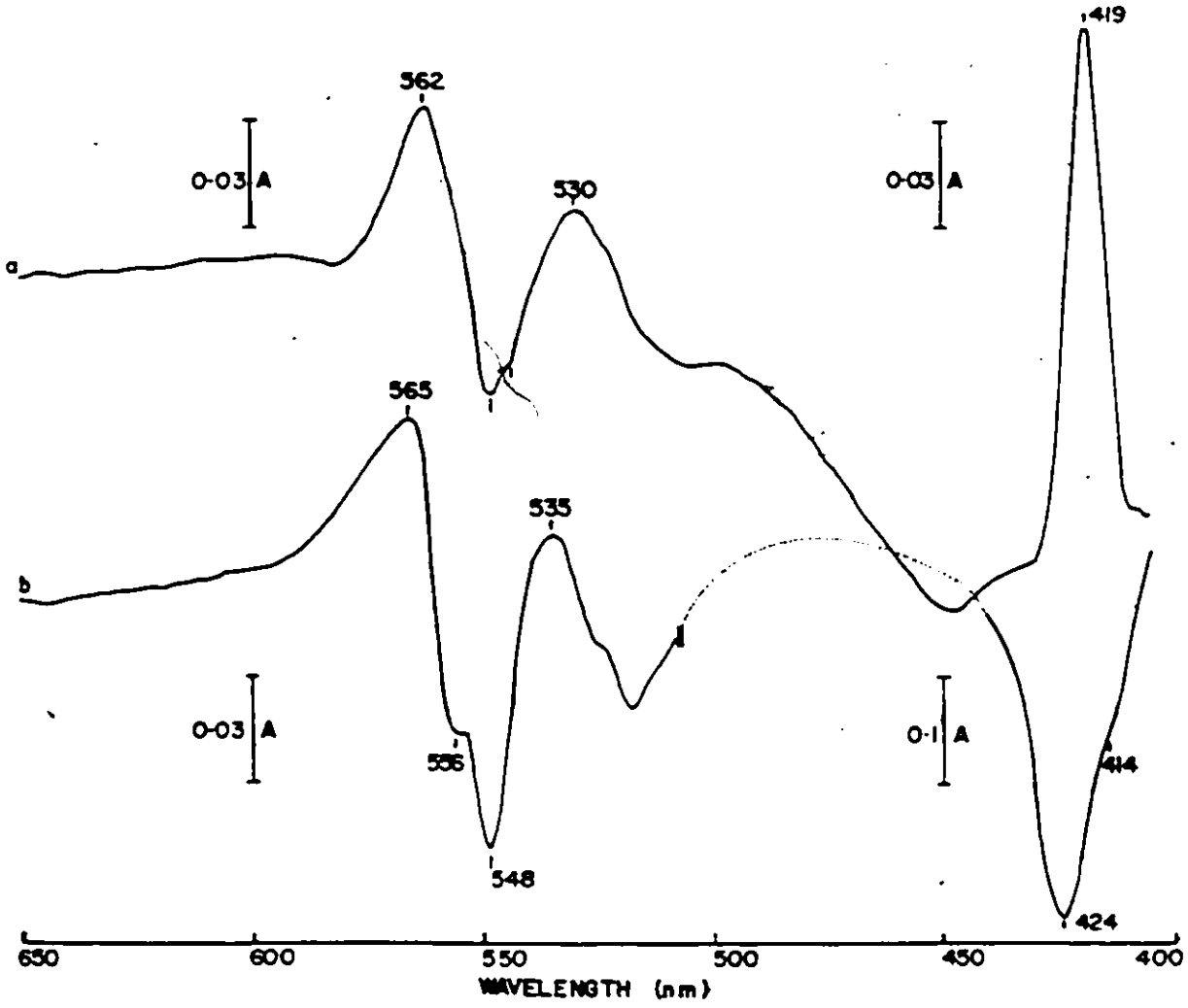

561
417
0·01 A
0·1 A
416
532
a
560
0·03 A
531
b
0·03 A
546
650
600
550
500
450
400
WAVELENGTH (nm)

the membrane fraction (Figure 53) of A. *haloplanktis*, the α, β and γ absorption maxima of the periplasmic ferri-NO complex absorbed at 561, 532 and 417 nm respectively (Figure 52, trace a); the ferro-NO complex absorbed at 560, 531 and 416 nm respectively (Figure 52, trace b). These absorption maxima are in good agreement with the absorption maxima published for the oxidized RHP-NO complex (α_{563}, β_{530}, γ_{417}) and the reduced RHP-NO complex (α_{560}, β_{530}, γ_{417}) (Taniguchi and Kamen, 1963). Thus, on spectral basis, the periplasmic cytochrome c_{549} of the marine bacterium could be classified as a cytochrome c'.

As far as the membrane-bound cytochromes were concerned, only the oxidized form of the cytochromes reacted in a characteristic fashion with nitric oxide (Figure 53, trace a) with absorption maxima absorbing at 562, 530 and 419 nm; the reduced form of cytochromes *o* and c bound NO (Figure 53, trace b) with shifted peaks at 565 and 535 nm but the Soret troughs at 424 and 414 nm appeared anomalous when compared to the Soret region of the oxidized + NO *minus* oxidized difference spectrum (Figure 53, trace a).

Figure 53. Nitric oxide (NO) difference absorption spectra of the membrane fraction of A. *haloplanktis* [a] oxidized with hydrogen peroxide and [b] reduced with sodium dithionite.

Cells of A. *haloplanktis* were grown in complex medium to the stationary phase. Stationary phase cells were harvested by centrifugation, washed, resuspended in buffered salt solution and fractionated into the membrane and the periplasmic fractions. The membrane fraction was mixed (1:1) with sucrose (0.75M final concentration). In [a], the fraction was oxidized with H_2O_2 (1 drop of 1:100 30% solution) and divided into the sample and the reference cuvettes. NO gas was then bubbled for 3 min through the contents of the sample cuvette and the reaction allowed to proceed for 10 min at room temperature before the difference spectrum was scanned at 83°K in cuvettes of 2mm light path. In [b], the fraction was reduced with solid crystals of dithionite and divided into the sample and the reference cuvettes. NO gas was then bubbled for 3 min through the contents of the sample cuvette and the reaction allowed to proceed for 10 min at room temperature before the difference spectrum was scanned at low temperature. The protein content of the fraction in [a] and [b] was 11.0 mg/ml. The vertical bars represent one tenth of the absorbancy units at which the spectra were recorded; the wavelengths are expressed in nanometers.


a
b
562
530
419
565
535
556
548
424
414
0·03 A
0·03 A
0·03 A
0·1 A
650
600
550
500
450
400
WAVELENGTH (nm)

4. CONCLUSION

The dynamic aspects of the participation of cytochromes in the process of carrying reducing equivalents along the respiratory chain cannot be rendered by the sole means of polarography. Spectrophotometry is also required to identify and follow the behavior of the redox carriers. Indeed, only through spectrophotometry can the cytochrome segment of the respiratory chain be looked at in detail, with the possibility of focussing on any one component in particular. The spectrophotometric technique using difference absorbance with and without respiratory substrates and inhibitors has been used successfully in mitochrondrial (Chance and Williams, 1955d, 1956; Haddock and Garland, 1971; Fuyu *et al.*, 1980), as well as in bacterial systems (Arima and Oka, 1965; Appleby, 1969a,b; Revsin *et al.*, 1970; Ishaque *et al.*, 1971; Kauffman and Van Gelder, 1973; Yu and De Voe, 1980).

The same principles were applied to the study of the electron transport chain of *Alteromonas haloplanktis*. Throughout the course of the spectrophotometric study, it was found that biological and chemical oxidation of the enzyme preparations was a prerequisite for better resolution of the cytochromes present in the marine bacterium. It was observed for instance, that if the degree of oxidation achieved with ferricyanide was taken as 100%, only 80% or so of the cytochromes of *A. haloplanktis* were physiologically oxidizable by oxidants such as air, hydrogen peroxide or oxygen gas.

Chemical and biological cytochrome reduction could be demonstrated spectrophometrically in whole cells, cell-free extracts, membrane and periplasmic fractions. NADH was proven to reduce all the cytochromes involved in the respiratory chain of *A. haloplanktis;* succinate was confined to the membrane-containing segment(s), although a portion of the electron flow could split at the end of the chain to reach the CO-binding cytochrome

c located in the periplasmic space. The artificial electron donors ascorbate and ascorbate/TMPD readily reduced all the c-type cytochromes as well as a good proportion of the b-types. The redox potentials of succinate (-31 mV) and ascorbate (+80 mV) diverge by some 110 mV, therefore the comparable levels of cytochrome reduction achieved by both substrates can only be explained in terms of partial reversed electron flow. The standard redox potentials of the reductants (NADH, -320 mV; succinate, -31 mV; ascorbate, +80 mV, at 25°C, pH 7.0 (Mahler and Cordes, 1971)) and of the oxidants (ferricyanide, +380 mV; O_2 gas, +820 mV) defined the points of entry and exit of the electrons in the respiratory chain of the marine bacterium.

A detailed study of the chain using respiratory inhibitors permitted further resolution of the cytochrome sequence. In *Alteromonas haloplanktis*, the effect of cyanide on the cytochrome segment of the respiratory chain was investigated in whole cells, cell-free extracts, periplasmic and membrane fractions. Spectrophotometrically, not too many definite conclusions could be drawn due to the ubiquitous presence of residual endogenous material in all the enzyme fractions. However, it could be shown that cyanide in the presence of exogenous and residual endogenous substrates blocked the flow of reducing equivalents at the oxidized end of the chain causing reduced *minus* oxidized difference spectra to develop. Re-oxidation of the CN-treated samples by O_2 gas did not alter the difference spectra previously recorded, demonstrating the inability of the cytochromes to be re-oxidized when cyanide was present. On the other hand, re-oxidation of the CN-treated samples by ferricyanide did cancel the difference spectra, indicating that the flow of electrons can be drained at the end of the chain by an artificial electron acceptor. No *a*-type cytochromes were spectrophotometrically involved in the reaction with

the terminal oxidase inhibitor. In the membrane fraction, the reduced + CN *minus* reduced difference spectrum developed with time as a pseudo-oxidized *minus* reduced spectrum, demonstrating that the reduced form of cytochrome *o* (a *b*-type cytochrome) possibly binds cyanide. Finally, in all the fractions tested, cyanide reacted more readily with the *c*-type than the *b*-type cytochromes, indicating that the *c*-type cytochromes are clustered at the oxidized end of the chain where the effect lies of the respiratory inhibitor.

In the presence of NADH dehydrogenase specific inhibitors, amytal and rotenone, the difference spectra scanned with starved whole cells could only show that rotenone was preventing in approximately 50% the reduction of cytochromes. Possibly, the endogenous material as well as portion of the exogenous NADH dehydrogenase were insensitive to the conventional site one inhibitors.

In the presence of succinate dehydrogenase specific inhibitor, TTFA, more than 90% of the cytochrome reduction was abolished; some insensitivity toward the inhibitor was encountered with the remaining endogenous substrate(s).

When the conventional site two inhibitors, antimycin A and HOQNO, were looked at spectrophotometrically, the results showed that the flow of electrons generated by the oxidation of NADH but not by succinate was blocked between cytochromes *b* and cytochromes *c* as evidenced by the reduced absorption peaks of the cytochromes *b* and the oxidized absorption troughs of the cytochromes *c*. The cytochromes reduced by the residual endogenous material present in the fractions were insensitive to some degree to both inhibitors. All the experiments were conducted with stationary phase cells since the full complement of the respiratory proteins are synthesized in quantities sufficient enough to be detected

spectrophotometrically at that stage of growth in A. *haloplanktis*. However, it must be kept in mind that exponential cells are generally more sensitive to respiratory inhibitors (Pudek and Bragg, 1974).

Polarographic measurements using the same substrates and inhibitors substantiated the spectrophotometric data and enabled us to further characterize the electron transport chain of *Alteromonas haloplanktis*. The protein concentration used in polarographic assays was 1/20 the protein concentration used in spectrophotometric assays and thus the eventual remaining endogenous material of starved cells was not interfering with the measurements of exogenous substrate oxidation and respiratory inhibitor effects.

The inhibitory curves of NADH, succinate and ascorbate/TMPD oxidase activities in the presence of cyanide and azide revealed that the median inhibitory doses for the inhibition of the respective oxidases by cyanide were: $I_{0.5\ (CN/NADH)} = .450$ mM; $I_{0.5\ (CN/succinate)} = .120$ mM; $I_{0.5\ (CN/ascorbate + TMPD)} = .0015$ mM and for azide: $I_{0.5\ (N_3/NADH)} = 10$ mM; $I_{0.5\ (N_3/succinate)} > 100$ mM; $I_{0.5\ (N_3/ascorbate+TMPD)} \leq .001$ mM), suggesting the existence of up to three oxidases: one oxidase very sensitive to cyanide and azide mediating the oxidation of ascorbate/TMPD and two others (or one common oxidase) relatively insensitive to cyanide and azide, to accommodate the oxidation of NADH and succinate. The possibility that NADH could also channel electrons via the inhibitor sensitive oxidase is not excluded, on the contrary. The double reciprocal plots of these inhibitory curves revealed that NADH oxidase system is made up of two oxidases: one NADH oxidase sensitive to cyanide, inhibited at a concentration of 2.0 mM

and another NADH oxidase resistant to cyanide. In the case of succinate oxidase, the double reciprocal plots showed that the oxidase system would be resistant to both cyanide and azide.

The results of the experiments carried out with the NADH dehydrogenase specific inhibitors, rotenone and amytal, proved again that rotenone was more effective than amytal, preventing 50% of the electron flow driven by the oxidation of NADH at concentration of 100 µM whereas 500 µM amytal was required for the same inhibition.

The specific inhibitor of succinate dehydrogenase, TTFA, was five times more effective on cells grown in succinate chemically defined medium than on cells grown in complex medium. It is expected that the cells of the former medium synthesized succinate dehydrogenase in larger quantities than the cells of the latter medium which was not supplemented with succinate.

With respect to antimycin A and HOQNO inhibition, the results obtained stated that NADH oxidase was more sensitive to antimycin A than to HOQNO, when the oxidase activity was tested at various concentrations of the respective inhibitors. Succinate oxidase on the other hand, was more sensitive to HOQNO than to antimycin A, although at infinite concentrations of both inhibitors there was resistance expressed as revealed by the double reciprocal plots of the inhibitory curves. The results were reconciled by suggesting that HOQNO and antimycin A act either at the same site (conventional second site, between cytochromes *b* and *c*) or at different sites with an additional site for antimycin A inhibition prior to the entry of succinate in the respiratory chain. With the two substrates, the flow of electrons resistant to antimycin A and HOQNO would have to be mediated via another route than the membrane-bound one and the periplasmic route is suggested.

Insights on the possible electron carriers terminating the respiratory chain and thus acting as oxidoreductases came from results obtained by difference absorption spectrophotometry using cellular fractions in combination with reducing substrates and carbon monoxide. The experimental data focussed on three CO-binding cytochromes present in *A. haloplanktis*: one associated with the periplasmic cytochrome c and two others associated with a b (*o*) cytochrome and a c cytochrome bound to the membrane. No *a*-type cytochromes were involved in the CO-binding reactions.

By virtue of the ability of cytochrome $b_{556.5}$ to bind CO, it is believed that this cytochrome could be identified as a cytochrome (*o*) oxidase. Cytochrome *o* is the most widely distributed of bacterial cytochrome oxidases based on the identification of a CO-binding *b*-type cytochrome in CO difference spectra (Poole, 1983b). Potentiometric analyses suggest that cytochrome *o* contributes to a band at 556 nm (Reid and Ingledew, 1979).

Although no c-type cytochromes have been to date ascribed an oxidase function *per se* in bacterial respiratory systems, it is speculated that in *Alteromonas haloplanktis* the c-type cytochromes (periplasmic and/or membrane-bound) are involved to some extent in the reduction of molecular oxygen. In *Azotobacter vinelandii* (Yang, 1978; Jurtshuk and Yang, 1980), *Pseudomonas aeruginosa* (Matsushita *et al.*, 1982), *Rhodopseudomonas palustris* (King and Drews, 1976), and *Methylophilus methylotrophus* (Carver and Jones, 1983), cytochromes c are part of an enzyme complex with cytochrome *o* as the major CO-sensitive terminal oxidase.

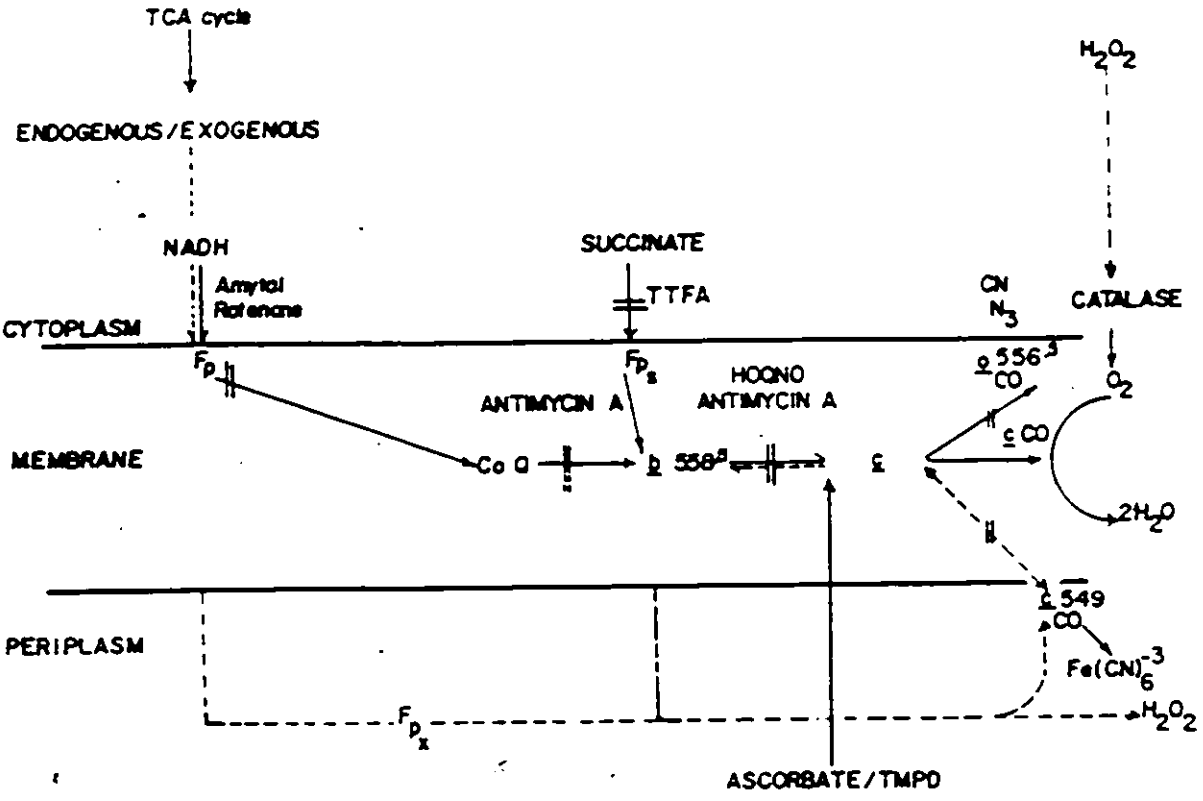
Finally, based on the spectrophotometric and polarographic data presented, the electron transport pathways (membrane-bound and periplasmic) outlined in Figure 54 are proposed to reconcile the experimental data with the reality of the cell envelope of the Gram negative bacterium.

The electrons driven by the oxidation of NADH, succinate and ascorbate/TMPD would be carried through a major pathway, membrane-bound (solid line), sensitive to the conventional inhibitors rotenone, amytal, TTFA, antimycin A, HOQNO, cyanide and azide. In addition, a minor pathway, periplasmic (dotted line), would account for the inhibitor-insensitive portion of the chain in addition to carry reducing equivalents oxidized on the outside surface of the cytoplasmic membrane. Dithionite *minus* O_2 and dithionite *minus* H_2O_2 difference spectra of the periplasmic fraction (Figure 24) showed the presence of flavoprotein (absorption minima at 450 nm) and thus such unspecific electron acceptor (identified here as F_{Px}) would transfer the electrons from the flavin region to the terminal portion of the chain via cytochrome c_{549}. It is also believed that in the endogenous electron transport chain and in the inhibitor-insensitive pathway, the electrons may be accepted directly by O_2 from the flavin system or transferred directly from cytochrome $b_{558.5}$ to O_2 via the periplasmic route (Figure 54, vertical dotted lines).

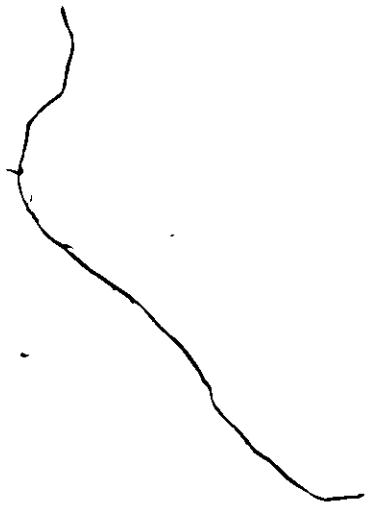
Interestingly enough, very useful functions are fulfilled by alternative oxidases. In yeast, for instance, CN-resistant respiration provides an alternative pathway for the oxidation of reduced pyridine nucleotides in the presence of high concentration of catabolite repressors (Ainsworth *et al.*, 1980a,b). Inhibitor-resistant respiration also develops in species which are

Figure 54. Proposed scheme for the membrane-bound and the periplasmic routes of electron flow in *Alteromonas haloplanktis*.

$b_{558.5}$, $c_{556.5}$, c_{CO}, c_{549}: cytochromes; CoQ: coenzyme Q; F_p, F_{ps}: membrane-bound flavoproteins; F_{px}: Periplasmic flavin-linked oxidoreductase(s) unspecific electron acceptor(s) which could transfer the electrons from the flavin region to cytochrome c. Reductants: endogenous reserves, NADH, succinate, ascorbate/TMPD. Oxidants: oxygen, hydrogen peroxide, ferricyanide, carbon monoxide. Respiratory inhibitors: amytal, rotenone, thenoyl-trifluoroacetone (TTFA), 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO), antimycin A, cyanide (CN), azide (N_3). The solid line represents the major pathway, the membrane-bound pathway; the dotted line represents the minor pathway, the periplasmic pathway of electron flow. The reader is referred to the text for discussion.


TCA cycle
ENDOGENOUS/EXOGENOUS
NADH
Amytal
Rotenone
SUCCINATE
TTFA
CN
N_3
CATALASE
H_2O_2
CYTOPLASM
F_p
F_{p_s}
ANTIMYCIN A
HOQNO
ANTIMYCIN A
MEMBRANE
Co Q
b 558
c
$\underline{a}$ 556
CO
$\underline{c}$ CO
O_2
$2H_2O$
PERIPLASM
$\underline{a}_3$ 549
CO
$Fe(CN)_6^{-3}$
F_{p_x}
H_2O_2
ASCORBATE/TMPD

subjected to physiological changes such as transfer from anaerobic to aerobic environments, aging, stress (Henry and Nyns, 1975), and change in temperature (Shepherd *et al.*, 1978). Seemingly, alternate oxidases are synthesized to counteract a depression in the level of cytochrome oxidase or other respiratory components (Henry and Nyns, 1975). They are synthesized in response to increased concentrations of cyanide in the environment or may result from the spatial organization of the respiratory system across the cytoplasmic membrane (Knowles, 1976). In several instances, flavoprotein oxidases were involved in the inhibitor-insensitive pathway (Bonartseva *et al.*, 1973; Ainsworth *et al.*, 1980a,b), and hydrogen peroxide was the end product of the oxidase systems (de Vries *et al.*, 1978; Pritchard and Asmundson, 1980; Ainsworth *et al.*, 1980a,b).



BIBLIOGRAPHY

Ackrell, B.A.C., Jones, C.W.
1971a The respiratory system of *Azotobacter vinelandii*. I. Properties of phosphorylating respiratory membranes. Eur. J. Biochem., 20: 22-28.

Ackrell, B.A.C., Jones, C.W.
1971b The respiratory system of *Azotobacter vinelandii*. II. Oxygen effects. Eur. J. Biochem., 20: 29-37.

Ainsworth, P.J., Ball, A.J.S., Tustanoff, E.R.
1980a Cyanide-resistant respiration in yeast. I. Isolation of a cyanide insensitive NAD(P)H oxidoreductase. Arch. Biochem. Biophys., 202: 172-186.

Ainsworth, P.J., Ball, A.J.S., Tustanoff, E.R.
1980b Cyanide-Resistant Respiration in Yeast. II. Characterization of a Cyanide-insensitive NAD(P)H oxidoreductase. Arch. Biochem. Biophys., 202: 187-200.

Alefounder, P.R., Ferguson, S.J.
1980 The location of dissimilatory nitrite reductase and the control of dissimilatory nitrate reductase by oxygen in *Paracoccus denitrificans*. Biochem. J., 192: 231-240.

Alefounder, P.R., Ferguson, S.J.
1981 A periplasmic location for methanol dehydrogenase from *Paracoccus denitrificans*. Implications for proton pumping by cytochrome aa_3. Biochem. Biophys. Res. Commun., 98: 778-784.

Alexander, M.
1956 Localization of enzymes in the microbial cell. Bacteriol. Rev., 20: 67-93.

Almassy, R.J., Dickerson, R.E.
1978 *Pseudomonas* cytochrome c_{551} at 2Å resolution: enlargement of the cytochrome c family. Proc. Natl. Acad. Sci. USA, 75: 2674-2678.

Anthony, C.
1975 The microbial metabolism of C_1 compounds. The cytochromes of *Pseudomonas* AM1. Biochem. J., 146: 289-298.

Appleby, C.A.
1969a Electron transport systems of *Rhizobium japonicum*. I. Haemoprotein P-450, other CO-reactive pigments, cytochromes and oxidases in bacteroids from N_2-fixing root nodules. Biochim. Biophys. Acta, 172: 71-87.

Appleby, C.A.
1969b Electron transport systems of *Rhizobium japonicum*. II. *Rhizobium* hemoglobin, cytochromes and oxidases in free-living (cultured) cells. Biochim. Biophys. Acta, 172: 88-105.

Arcuri, E.J., Ehrlich, H.L.
1979 Cytochrome involvement in Mn(II) oxidation by two marine bacteria. Appl. Env. Microbiol., 37: 916-923.

Arcuri, E.J., Ehrlich, H.L.
1980 A comparison of the cytochrome complements of seven strains of marine manganese-oxidizing bacteria. Zeit. für allgemeine Mikrobiologie, 20: 583-586.

Arima, K., Oka, T.
1965 Cyanide resistance in *Achromobacter*. I. Induced formation of cytochrome a_2 and role in cyanide-resistant respiration. J. Bacteriol., 90: 734-743.

Azzi, A., Casey, R.P.
1979 Molecular aspects of cytochrome c oxidase: structure and dynamics. Mol. Cell. Biochem., 28: 169-184.

Azzone, G.F., Petronilli, V., Zoratti, M.
1984 "Cross-talk" between redox and ATP-driven H^+ pumps. Biochem. Soc. Trans., 12: 414-416.

Barber, D., Parr, S.R., Greenwood, C.
1978 The oxidation of *Pseudomonas* cytochrome c_{551} oxidase by potassium ferricyanide. Biochem. J., 173: 681-690.

Barber, J.
1984 Further evidences for the common ancestry of cytochrome b-c complex. Trends Biochem. Sci., 9: 209-211.

Bartsch, R.G., Kamen, M.D.
1958 On the new hemeprotein of facultative photoheterotrophs. J. Biol. Chem., 230: 41-63.

Baumann, P., Baumann, L., Bowditch, R.D., Beaman, B.
1984 Taxonomy of *Alteromonas*: A. *nigrifaciens* sp. nov., nom. rev.; A. *macleodii*; and A. *haloplanktis*. Int. J. Syst. Bacteriol., 34: 145-149.

Beardmore-Gray, M., O'Keeffe, D.T., Anthony, C.
1983 The methanol:cytochrome c oxidoreductase activity of methylotrophs. J. Gen. Microbiol., 129: 923-933.

Bell, G.R., LeGall, J., Peck, H.D.
1974 Evidence for the periplasmic location of hydrogenase in *Desulfovibrio gigas*. J. Bacteriol., 120: 994-997.

Berger, E.A.
1973 Different mechanisms of energy coupling for the active transport of proline and glutamine in *Escherichia coli*. Proc. Natl.Acad. Sci., 70: 1514-1518.

Berger, E.A., Heppel, L.A.
1974 Different mechanisms of energy coupling for the shock-sensitive and shock-resistant amino-acid permeases of *Escherichia coli*. J. Biol. Chem., 249: 7747-7755.

Bergersen, F.J., Turner, G.L.
1979 Systems utilizing oxygenated leghaemoglobin and myoglobin as sources of free, dissolved O_2 at low concentrations for experiments with bacteria. Analytical Biochem., 96: 165-174.

Bergersen, F.J., Turner, G.L.
1980 Properties of terminal oxidase systems of bacteriods from root nodules of soybean and cowpea and of N_2-fixing bacteria grown in continuous culture. J. Gen. Microbiol., 118: 235-252.

Bernard, H., Probst, J., Schlegel, H.G.
1974 The cytochromes of some hydrogen bacteria. Arch. Microbiol., 95: 29-37.

Bhatti, A.R., DeVoe, I.W., Ingram, J.M.
1976 The release and characterization of some periplasmic-located enzymes of *Pseudomonas aeruginosa*. Can. J. Microbiol., 22: 1425-1429.

Bhatti, A.R., Ingram, J.M.
1982 The binding and secretion of alkaline phosphatase by *Pseudomonas aeruginosa*. FEMS Microbiol. Lett., 13: 353-356.

Blumenthal, D.C., Kassner, R.J.
1979 Azide binding to the cytochrome c ferric heme octapeptide. A model for anion binding to the active site of high spin ferric heme proteins. J. Biol. Chem., 254: 9617-9620.

Bonartseva, G.A., Taptykova, S.D., Vovob'eva, L.I., Krainova, O.A., Bryukacheva, N.L.
1973 The aerobic metabolism of propionic acid bacteria. Mikrobiologiya, 42: 765-771.

Boogerd, F.C., van Verseveld, H.W., Stouthamer, A.H.
1981 Respiration driven proton translocation with nitrite and nitrous oxide in *Paracoccus denitrificans*. Biochim. Biophys. Acta, 638: 181-191.

Booth, I.R., Kroll, R.G., Findlay, M.J., Stewart, L.M.D., Rowland, G.C.
1984 Properties and physiology of energy-coupled mutants of *Escherichia coli*. Biochem. Soc. Trans., 12: 409-411.

Boyer, P.D.
1965 In: Oxidases and related redox systems. King, T.E., Mason, H.S., Morrisons, M. (eds.) Vol. 2: 994-1008, John Wiley, New York.

Burnstein, C., Tiankova, L., Kepes, A.
1979 Respiratory control in *Escherichia coli*. Eur. J. Biochem., 94: 387-392.

Burton, S.M., Byrom, D., Carver, M.A., Jones, G.D.D.
1983 The oxidation of methylated amines by the methylotrophic bacterium *Methylophilus methylotrophus*. FEMS Microbiol. Lett., 17: 185-190.

Carr, L.D., Hiebert, R.D., Currie, W.D., Gregg, C.T.
1971 A stable, sensitive and inexpensive amplifier for oxygen electrode studies. Analytical Biochem., 41: 492-502.

Carver, M.A., Jones, C.W.
1983 The terminal respiratory chain of the methylotrophic bacterium *Methylophilus methylotrophus*. FEBS Lett., 155: 187-196.

Castor, L.N., Chance, B.
1959 Photochemical determinations of the oxidases of bacteria. J. Biol. Chem., 234: 1587-1592.

Chaix, P., Petit, J.F.
1956 Etude de différents spectres cytochromes de *Bacillus subtilis*. Biochim. Biophys. Acta, 22: 66-71.

Chance, B.
1977 Electron transfer: pathways, mechanisms and controls. Ann. Rev. Biochem., 46: 967-980.

Chance, B., Leigh, J.S., Jr.
1977 Oxygen intermediates and mixed valence states of cytochrome oxidase: Infrared absorption difference spectra of compounds A, B and C of cytochrome oxidase and oxygen. Proc. Natl. Acad. Sci., U.S.A., 74: 4777-4780.

Chance, B., Williams, G.R.
1955a Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. J. Biol. Chem., 217: 383-393.

Chance, B., Williams, G.R.
1955b Respiratory enzymes in oxidative phosphorylation. II. Difference spectra. J. Biol. Chem., 217: 395-407.

Chance, B., Williams, G.R.
1955c Respiratory enzymes in oxidative phosphorylation. III. The steady state. J. Biol. Chem., 217: 409-427.

Chance, B., Williams, G.R.
1955d Respiratory enzymes in oxidative phosphorylation. IV. The respiratory chain. J. Biol. Chem., 217: 429-438.

Chance, B., Williams, G.R.
1956 The respiratory chain and oxidative phosphorylation. Adv. Enzymol., 17: 65-134.

Chappell, J.B.
1964 The oxidation of citrate, isocitrate and cis-aconitate by isolated mitochondria. Biochem. J., 90: 225-237.

Clark-Walker, G.D., Rittenberg, B., Lascelles, J.
1967 Cytochrome synthesis and its regulation in *Spirillum itersonii*. J. Bacteriol., 94: 1648-1655.

Cole, J.A., Newman, B.M., White, P.
1980 Biochemical and genetic characterization of *nir* B mutants of *Escherichia coli* K12 pleiotropically defective in nitrite and sulphite reduction. J. Gen. Microbiol., 120: 475-483.

Cole, J.S., Aleem, M.I.H.
1973 Electron transport-linked compared with proton-induced ATP generation in *Thiobacillus novellus*. Proc. Natl. Acad. Sci., U.S.A., 70: 3571-3575.

Cox, C.D., Payne, W.J., Dervartanian, D.V.
1971 Electron paramagnetic resonance studies on the nature of hemoproteins. Biochim. Biophys. Acta, 253: 290-294.

Cox, G.B., Gibson, F.
1974 Studies on electron transport and energy-linked reactions using mutants of *Escherichia coli*. Biochim. Biophys. Acta, 346: 1-25.

Cox, J.C., Ingledew, W.J., Haddock, B.A., Lawford, H.G.
1978 The variable cytochrome content of *Paracoccus denitrificans* grown aerobically under different conditions. FEBS Lett., 93: 261-265.

Cross, A.R., Anthony, C.
1980 The purification and properties of the soluble cytochromes c of the obligate methylotroph *Methylosinus methylotrophus*. Biochem. J., 192: 421-427.

Cypionka, H., Meyer, O.
1982 Influence of carbon monoxide on growth and respiration of carboxydobacteria and other organisms. FEMS Microbiol. Lett., 15: 209-214.

Cypionka, H., Meyer, O.
1983 The cytochrome composition of carboxydotrophic bacteria. Arch. Microbiol., 135: 293-298.

Daniel, R.M.
1970 The electron transport system of *Acetobacter suboxydans* with particular reference to cytochrome *o*. Biochim. Biophys. Acta, 216: 328-341.

Degn, H., Lloyd, D., Hill, G.C.
1978 Functions of alternative terminal oxidases. FEBS 11th Meeting, Copenhagen, Vol. 49, Colloquium B6, Pergamon Press, Oxford and New York.

Degn, H., Wohlrab, H.
1971 Measurement of steady-state values of respiration rate and oxidation levels of respiratory pigments at low oxygen tensions. A new technique. Biochim. Biophys. Acta, 245: 347-355.

DeMaio, R.A., Webster, D.A., Chance, B.
1983 Spectral evidence for the existence of a second cytochrome *o* in whole cells of *Vitreoscilla*. J. Biol. Chem., 258: 13768-13771.

deVries, W., Donkers, C., Boellaard, M., Stouthamer, A.H.
1978 Oxygen metabolism by the anaerobic bacterium *Veillonella alcalescens*. Arch. Microbiol., 119: 167-174.

Dickerson, R.E.
1980 The cytochromes C: an exercise in scientific serendipity. The evolution of protein, structure and function. Academic Press, New York, pp. 173-202.

Dietrich, W.E., Jr., Biggins, J.
1971 Respiratory mechanisms in the Flexibacteriaceae: Terminal oxidase systems of *Saprospira grandis* and *Vitreoscilla* species. J. Bacteriol., 105: 1983-1089.

Downie, J.A., Cox, G.B.
1978 Sequence of *b* cytochromes relative to ubiquinone in the electron transport chain of *Escherichia coli*. J. Bacteriol., 133: 477-484.

Eilermann, L.J.M.
1970 Oxidative phosphorylation in *Azotobacter vinelandii*, as a fluorescent probe for the energized state. Biochim. Biophys. Acta, 216: 231-233.

ElKurdi, A.B., Leaver, J.L., Pettigrew, G.W.
1982 The c-type cytochromes of *Campylobacter sputorum* ssp. *mucosalis*. FEMS Microbiol., 14: 177-182.

Ensley, B.D., Jr., Finnerty, W.R.
1980 Influences of growth substrates and oxygen on the electron transport system in *Acinetobacter* sp HO1-N. J. Bacteriol., 142: 859-868.

Erecinska, M., Davis, J.S., Wilson, D.F.
1979 Regulation of respiration in *Paracoccus denitrificans*: The dependence on redox state of cytochrome c and (ATP)/(ADP) (P_i). Arch. Biochem. Biophys., 197: 463-469.

Faller, A.H., Gotz, F., Schleifer, K.H.
1980 Cytochrome patterns of staphylococci and micrococci and their taxonomic implications. Zbl. Bakt., I Abt. Orign. C1, 26-39.

Fein, J.E., MacLeod, R.A.
1975 Characterization of neutral amino acid transport in a marine pseudomonad. J. Bacteriol., 124: 1177-1190.

Ferguson, S.J., Parsonage, D.
1984 Analysis of relationships between the protonmotive force and rates and extents of ATP synthesis. Biochem. Soc. Trans., 12: 416-419.

Forsberg, C.W., Costerton, J.W., MacLeod, R.A.
1970a Separation and localization of cell wall layers of a gram-negative bacterium. J. Bacteriol., 104: 1338-1353.

Forsberg, C.W., Costerton, J.W., MacLeod, R.A.
1970b Quantification, chemical characteristics and ultrastructure of the three outer cell wall layers of a gram-negative bacterium. J. Bacteriol., 104: 1354-1368.

Franck, B.
1979 Key building blocks of natural product biosynthesis and their significance in chemistry and medicine. Angewadte Chemie, 18: 429-439.

Franck, B., Bock, W., Bringmann, G., Fels, G., Grubenbecher, F., Marsmann, M., Pietschmann, R., Schäpers, K., Spiegel, U., Steinkamp, R., Ufer, G., Wagner, C.
1980 Chemistry of prophyrin biosynthesis: results and applications. Int. J. Biochem., 12: 671-679.

Froud, S.J., Anthony, C.
1984 The purification and characterization of the *o*-type cytochrome oxidase from *Methylophilus methylotrophus*, and its reconstitution into a "methanol oxidase" electron transport chain. J. Gen. Microbiol., 130: 2201-2212.

Fujita, T.
1966 Studies on soluble cytochromes in Enterobacteriaceae, cytochrome b_{562} and c_{550}. J. Biochem. (Tokyo), 60: 329-334.

Fuyu, Y., Qingru, X., Wang, S
1980 Cyanide-insensitive respiration in corn mitochondria. I. Comparison between sensitivities of α-Ketoglutarate and succinate oxidations to cyanide. Scientia Sinica, 6: Vol. XXIII: 774-784.

Garrard, W.T.
1971 Selective release of proteins from *Spirillum itersonii* by Tris (hydroxymethyl) aminomethane and ethylenediaminetetraacetate. J. Bacteriol., 105: 93-100.

Gaul, D.F., Gray, G.O., Knaff, D.B.
1983 Isolation and solubilization of two soluble heme-c containing proteins from *Chromatium vinosum*. Biochim. Biophys. Acta, 723: 333-339.

Gauthier, D.K., Clark-Walker, G.D., Garrard, W.T., Jr., Lascelles, J.
1970 Nitrate reductase and soluble cytochrome c in *Spirillum itersonii*. J. Bacteriol., 102: 797-803.

Goldstein, D.B.
1968 A method for assay of catalase with the oxygen cathode. Analytical Biochem., 24: 431-437.

Gornall, A.G., Bardawill, C.S., David, M.M.
1949 Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem., 177: 751-763.

Gould, J.M.
1979 Respiration-linked proton transport charges in external pH and membrane energization in cells of *Escherichia coli*. J. Bacteriol., 138: 176-184.

Gow, J.A., DeVoe, I.W., MacLeod, R.A.
1973 Dissociation in a marine pseudomonad. Can. J. Microbiol., 19: 695-701.

Gray, G.O., Gaul, D.F., Knaff, D.B.
1983 Partial purification and characterization of two soluble c-type cytochromes from *Chromatium vinosum*. Arch. Biochem. Biophys., 222: 78-86.

Green, G.N., Gennis, R.B.
1983 Isolation and characterization of a mutant in *Escherichia coli* lacking the cytochrome *d* terminal oxidase. J. Bacteriol., 154: 1269-1275.

Green, G.N., Kranz, R.G., Lorence, R.M., Gennis, R.B.
1984 Identification of subunit I as the cytochrome b_{558} component of the cytochrome *d* terminal oxidase complex of *Escherichia coli*. J. Biol. Chem., 259: 7994-7999.

Greenberg, D.M.
1969a Carbon catabolism of amino acids. In: Metabolic Pathways, Vol. 3, pp. 96-189, D.M. Greenberg (ed.), Academic Press Inc., New York.

Greenberg, D.M.
1969b Biosynthesis of amino acids and related compounds. In: Metabolic Pathways, Vol. 3, pp. 238-315, D.M. Greenberg (ed.), Academic Press Inc., New York.

Greenwood, C., Barber, D., Parr, S.R., Antonini, E., Brunori, M., Colosimo, A.
1978 The reaction of *Pseudomonas aeruginosa* cytochrome c_{551} oxidase with oxygen. Biochem. J., 173: 11-19.

Hacket, N.R., Bragg, P.D.
1983 Membrane cytochromes of *Escherichia coli chl* mutants. J. Bacteriol., 154: 719-727.

Haddock, B.A., Garland, P.B.
1971 Effect of sulphate-limited growth on mitochondrial electron transfer and energy conservation between reduced nicotinamide adenine dinuleotide and the cytochromes in *Torulopsis utilis*. Biochem. J., 124: 155-170.

Haddock, B.A., Schairer, H.U.
1973 Electron-transport chains of *Escherichia coli*. Reconstitution of respiration in a ^{5}-aminolaevulinic acid-requiring mutant. Eur. J. Biochem., 35: 34-45.

Haddock, B.A., Downie, J.A., Carland, P.B.
1976 Kinetic characterization of the membrane-bound cytochromes of *Escherichia coli* grown under a variety of conditions using a stopped-flow dual wavelength spectrophotometer. Biochem. J., 154: 285-294.

Haddock, B.A., Jones, C.W.
1977 Bacterial respiration. Bacteriol. Rev., 41: 47-99.

Harrison, D.E.F.
1972 Physiological effects of dissolved oxygen tension and redox potential on growing populations of microorganisms. J. Appl. Chem. Biotechnol., 22: 417-440.

Harrison, D.E.F.
1976a The regulation of respiration rate in growing bacteria. Adv. Microbiol. Physiol., 14: 243-309.

Harrison, D.E.F.
1976b The oxygen metabolism of microorganisms. In: Patterns of Progress Microbiology, Meadowfield Press Ltd., Durham England.

Harrison, D.E.F., Pirt, S.J.
1967 Influence of dissolved oxygen concentration on respiration and glucose metabolism of *Klebsiella aerogenes* during growth. J. Gen. Microbiol., 46: 193-211.

Harrison, D.E.F., MacLennan, D.G., Pirt, S.J.
1969 Responses of bacteria to dissolved oxygen tension in growing cultures with glucose and hydrocarbon substrates. In: Fermentation Advances. D. Perlman (ed.), Academic Press, New York.

Hauska, G., Baccarini-Melandri, A.
1980 The dual role of cytochrome c_2 in the facultative photosynthetic bacterium *Rhodopseudomonas capsulata*. Monatshefte für Chemie, 111: 821-827.

Heinen, W.
1971 Inhibitors of electron transport and oxidative phosphorylation. In: Methods Microbiol., Norris, J.R. and Ribbons, D.W. (eds.), Academic Press, London, New York, Vol. 6A: 383-393.

Hempfling, W.P., Mainzer, S.E.
1975 Effects of varying the carbon source limiting growth on yield and maintenance characteristics of *Escherichia coli* in continuous culture. J. Bacteriol., 123: 1076-1087.

Henry, M.F., Nyns, E.J.
1975 Cyanide insensitive respiration. An alternative mitochondrial pathway. Sub-Cell. Biochem., 4: 1-65.

Henry, M.F., Vignais, P.M.
1979 Induction by cyanide of cytochrome *d* in the plasma membrane of *Paracoccus denitrificans*. FEBS Lett., 100: 41-46.

Heppel, L.A.
1967 Selective release of enzymes from bacteria. Science, 156: 1451-1455.

Heppel, L.A.
1971 The concept of periplasmic enzymes. pp. 223-247. In: Structure and Function of Biological Membranes, L.I. Rothfield (ed.), Academic Press Inc., New York.

Heppel, L.A.
1972 Studies on binding proteins, periplasmic enzymes and active transport in *Escherichia coli*. In: The Molecular basis of Biological transport, pp. 133-156, J.F. Woessner Jr. and F. Huying (eds.), Academic Press, New York.

Herrmann, K.M., Somerville, R.L. (eds.)
1983 Amino acids: Biosynthesis and genetic regulation. Addison-Wesley Publishing Company Inc., Reading, Massachusetts, USA.

Horio, T., Kamen, M.D.
1970 Bacterial cytochromes. II. Functional aspects. Ann. Rev. Microbiol., 24: 399-428.

Ibrahim, H., Schlegel, H.G.
1980 Oxygen supply to bacterial suspensions of high cell densities by hydrogen peroxide. Biotechnol. Bioengineer., XXII: 1877-1894.

Ingledew, W.J., Poole, R.K.
1984 The respiratory chain of *Escherichia coli*. Microbiol. Rev., 48: 222-271.

Ingram, J.M., Cheng, K.J., Costerton, J.W.
1973 Alkaline phosphatase of *Pseudomonas aeruginosa*: the mechanism of secretion and release of the enzyme from whole cells. Can. J. Microbiol., 19: 1407-1415.

Ishaque, M., Donawa, A., Aleem, M.I.H.
1971 Electron transport and coupled energy generation in *Pseudomonas saccharophila*. Can. J. Microbiol., 49: 1175-1182.

Ishaque, M.
1984 Cytochrome system in cultivated *Mycobacterium lepraemurium*. Cytobios. 39: 165-171.

Ishaque, M., Kato, L.
1974 The cytochrome system in *Mycobacterium lepraemurium*. Can. J. Microbiol., 20: 943-947.

Iwasaki, H., Shidara, S.
1969 Crystallization of cytochrome c_{553} in aerobically grown *Pseudomonas denitrificans*. J. Biochemistry, 66: 775-781.

Iwasaki, H., Matsubara, T.
1971 Cytochrome $c_{557(551)}$ and cytochrome *cd* of *Alcaligenes faecalis*. J. Biochem. (Tokyo), 69: 847-857.

Johnson, M.J.
1959 Oxygen supply in continuous cultures. In: Recent Progress in Microbiology, pp. 397-402. G. Tanewall (ed.), Stockholm, Almqvist and Wiksell, Upsala, Sweden.

Johnson, M.J.
1967 Aerobic microbial growth at low oxygen concentrations. J. Bacteriol., 94: 101-108.

Jones, C.W.
1973 The inhibition of *Azotobacter vinelandii* terminal oxidases by cyanide. FEBS Lett., 36: 347-350.

Jones, C.W.
1977 Aerobic respiratory systems in bacteria. Symp. Soc. Gen. Microbiol., 27: 23-61.

Jones, C.W., Brice, J.M., Downs, A.J., Drozd, J.W.
1975 Bacterial respiration-linked proton translocation and its relationship to respiratory-chain composition. Eur. J. Biochem., 52: 265-271.

Jones, C.W., Brice, J.M.,Edwards, C.
1978 Bacterial cytochrome oxidases and respiratory chain conservation. In: Functions of alternate terminal oxidases. H. Degn, D. Lloyd, G.C. Hill (eds.), Pergamon Press, Oxford.

Jones, C.W., Kingsbury, S.A., Dawson, M.J.
1982 The partial resolution and dye-mediated reconstitution of methanol oxidase activity in *Methylophilus methylotrophus*. FEMS Microbiol. Lett., 13: 195-200.

Jones, C.W., Meyer, D.J.
1976 The distribution of cytochromes in bacteria. In: Handbook of Microbiology, Laskin, A.I. and H.A. LeChevalier (eds.),Vol. II. Cleveland Rubber Company Press, Cleveland, Ohio.

Jones, C.W., Redfearn, E.R.
1966 Electron transport in *Azotobacter vinelandii*. Biochim. Biophys. Acta, 113: 467-481.

Jones, H.E.
1972 Cytochromes and other pigments of dissimilatory sulphate-reducing bacteria. Arch. Microbiol., 84: 207-224.

Jones, M.G., Bickar, D., Wilson, T., Brunori, M., Colosimo, A., Sarti, P.
1984 A re-examination of the reactions of cyanide with cytochrome C oxidase. Biochem. J., 220: 57-66.

Jurtshuk, P. Jr., McQuitty, D.N., Riley, W.H.
1979 Use of 3,3'-diaminobenzidine as a biochemical electron donor for studies on terminal cytochrome oxidase activity in *Azotobacter vinelandii*. Curr. Microbiol., 2: 349-354.

Jurtshuk, P. Jr., Mueller, T.J., Accord, W.C.
1975 Bacterial terminal oxidases. CRC Critical reviews in Microbiology, 3: 399-468.

Jurtshuk, P. Jr., Mueller, T.J., McQuitty, D.N., Riley, W.H.
1978 The cytochrome oxidase reaction in *Azotobacter vinelandii* and other bacteria. In: Function of alternate terminal oxidases. Degn, H., Lloyd, D. and Hill, G.C. (eds.), Proc. 11th FEBS meeting, Pergamon Press, Oxford, pp. 99-123.

Jurtshuk, P., Yang, T.S.
1980 Oxygen reactive hemoprotein components in bacterial respiratory systems. In: Diversity of bacterial respiratory systems (Knowles, C.J. ed.), Vol. 1, pp. 137-159, CRC Press, Cleveland, Ohio.

Kajie, S., Miki, K., Lin, E.C.C., Anraku, Y.
1984 Isolation of an *Escherichia coli* mutant defective in cytochrome biosynthesis. FEMS Microbiol. Lett., 24: 25-31.

Kasprzak, A.A., Steenkamp, D.J.
1983 Localization of the major dehydrogenases in two methylotrophs by radiochemical labeling. J. Bacteriol., 156: 348-353.

Kauffman, H.F., Van Gelder, B.F.
1973 The respiratory chain of *Azotobacter vinelandii*. II. The effect of cyanide on cytochrome *d*. Biochim. Biophys. Acta, 314: 276-283.

Keilin, D.
1925 On cytochrome, a respiratory pigment, common to animals, yeast and plants. Proc. Roy. Soc. B., 98: 312-339.

Kell, D.B., Hitchens, G.D.
1984 Proton-transfer pathways during bacterial electron-transport phosphorylation. Biochem. Soc. Trans., 12: 413-414.

Kenimer, E.A., Lapp, D.F.
1978 Effect of selected inhibitors on electron transport in *Neisseria gonorrhoae*. J. Bacteriol., 134: 537-547.

Khanna, G., DeVoe, L., Brown, L., Niven, D.F., MacLeod, R.A.
1984 Relationship between ion requirements for respiration and membrane transport in a marine bacterium. J. Bacteriol., 157: 59-63.

Kim, Y.M., Hageman, G.D.
1981 Electron transport system of an aerobic carbon-monoxide-oxidizing bacterium. J. Bacteriol., 148: 991-994.

King, M.T., Drews, G.
1976 Isolation and partial characterization of the cytochrome oxidase of *Rhodopseudomonas palustris*. Eur. J. Biochem., 68: 5-12.

Knobloch, K., Ishaque, M., Aleem, M.I.H.
1971 Oxidative phosphorylation in *Micrococcus denitrificans* under autotrophic growth conditions. Arch. Microbiol., 76: 114-123.

Knowles, C.J.
1976 Microorganisms and cyanide. Bacteriol. Rev., 40: 652-680.

Knowles, C.J.
1980a Diversity of bacterial respiratory systems, Vols. I and II, CRC Press Inc., Boca Raton, Florida.

Knowles, C.J.
1980b Heme-requiring bacterial respiratory systems. In: Diversity of bacterial respiratory systems, Vol. II, pp. 139-159. C.J. Knowles (ed.), CRC Press, Inc., Boca Raton, Florida.

Knowles, C.J., Calcott, P.H., MacLeod, R.A.
1974 Periplasmic CO-binding c-type cytochrome in a marine bacterium. FEBS Lett., 49: 78-83.

Kodoma, T.
1970 Effects of growth conditions on formation of cytochrome system of a denitrifying bacterium *Pseudomonas stutzeri* Plant. Cell. Physiol., 11: 231-239.

Koronen, T., Sarate, M., Ellfolk, N.
1975 The subunit structure of *Pseudomonas* cytochrome oxidase. Biochim. Biophys. Acta, 393: 48-54.

Krieg, N.R.
1976 Biology of the chemoheterotrophic *Spirilla*. Bacteriol. Rev., 40: 55-115.

Lanyi, J.K.
1968 Studies of the electron transport chain of extremely halophilic bacteria. 1. Spectrophotometric identification of the cytochromes of *Halobacterium cutirubrum*. Arch. Biochem. Biophys., 128: 716-724.

Lascelles, J.
1961 Synthesis of tetrapyrroles by microorganisms. Physiological Reviews, 41: 417-441.

Lascelles, J., Rittenbert, B., Clark-Walker, G.D.
1969 Growth and cytochrome synthesis in a hemin-requiring mutant of *Spirillum itersonii*. J. Bacteriol., 97: 455-456.

Lehninger, A.L., Brand, M.D., Reynafarje, B.
1975 Pathways and stoichiometry of H^+ and Ca^2 transport-coupled to mitochondrial electron transport. In: Electron transfer chains and oxidative phosphorylation. E. Quagliariello (ed.), pp. 329-334, North Holland Publishers, Amsterdam.

Lehninger, A.L.
1984 Proton translocation coupled to mitochondrial electron transport. Biochem. Soc. Trans., 12: 386-388.

Lemberg, R., Barret, J.
1973 Cytochromes. Academic Press Inc., (London).

Lenhoff, H.M., Kaplan, N.O.
1956 A cytochrome peroxidase from *Pseudomonas fluorescens*. J. Biol. Chem., 220: 967-982.

Lenhoff, H.M., Nicholas, D.J.D., Kaplan, N.O.
1956 Effects of oxygen, iron and molybdenum on routes of electron transfer in *Pseudomonas fluorescens*. J. Biol. Chem., 220: 983-995.

Lennette, E.H., Balows, A., Hausler, W.J., Truant, J.P. (eds.)
1980 Manual of Clinical microbiology. 3rd Edition, American Society of Microbiology, Washington, D.C., USA, pp. 989-990.

Lineweaver, H., Burk, D.
1934 Determination of enzyme dissociation constants. J. Amer. Chem. Soc., 56: 658-666.

Linton, J.D., Harrison, D.E.F., Bull, A.T.
1975 Molar growth yields, respiration and cytochrome patterns of *Beneckea natriegens* when grown at different medium dissolved oxygen tensions. J. Gen. Microbiol., 90: 237-246.

Linton, J.D., Bull, A.T., Harrison, D.E.F.
1977 Determination of the apparent K_m for oxygen of *Beneckea natriegens* using the respirograph technique. Arch. Microbiol., 114: 111-113.

Lipman, F.
1941 Metabolic generation and utilization of phosphate bond energy. Adv. Enzymology 1: 99-162.

Liu, M-Y., Liu, M-C., Payne, W.J., Peck, H.D., LeGall, J.
1983 Cytochrome components of denitrifying *Pseudomonas stutzeri*. Curr. Microbiol., 9: 89-92.

Lorence, R.M., Yoshida, T., Findling, K.L., Fee, J.A.
1981 Observations on the c-type cytochromes of the extreme thermophile *Thermus thermophilus*. HB8: cytochrome c_{552} is located in the periplasmic space. Biochem. Biophys. Res. Commun., 99: 591-599.

Ludwig, B.
1980 Heme aa_3-type cytochrome c oxidases from bacteria. Biochim. Biophys. Acta, 594: 177-189.

MacGregor, C.H., Bishop, C.W.
1977 Do cytochromes function as oxygen sensors in the regulation of nitrate reductase biosynthesis? J. Bacteriol., 131: 372-373.

MacKelvie, R.M., Campbell, J.J.R., Gronlund, A.R.
1968 Survival and intracellular changes of *Pseudomonas aeruginosa* during prolonged starvation. Can. J. Microbiol., 14: 639-645.

MacLeod, R.A.
1965 The question of the existence of specific marine bacteria. Bacteriol. Rev., 29: 9-23.

MacLeod, R.A., Hori, A.
1960 Nutrition and metabolism of marine bacteria. VIII. Tricarboxylic acid cycle enzymes in a marine bacterium and their response to inorganic salts. J. Bacteriol., 80: 464-471.

MacLeod, R.A., Hori, A., Fox, S.M.
1960 Nutrition and metabolism of marine bacteria. X. The glyoxylate cycle in a marine bacterium. Can. J. Microbiol., 6: 639-644.

MacLeod, R.A., Onofrey, E.
1957 Nutrition and metabolism of marine bacteria. III. The relation of sodium and potassium to growth. J. Cell. and Comp. Physiol., 50: 389-402.

MacLeod, R.A., Onofrey, E., Norris, M.E.
1954 Nutrition and metabolism of marine bacteria. I. Survey of nutritional requirements. J. Bacteriol., 68: 680-686.

Macy, J., Kulla, H., Gottschalk, G.
1976 H_2-dependent anaerobic growth of *Escherichia coli* on L-malate: succinate formation. J. Bacteriol., 125: 423-428.

Mahler, H.R., Cordes, E.H.
1971 Biological Chemistry, 2nd ed., Harper and Row Publishers, p. 642, New York.

Maloney, P.C., Wilson, T.H.
1975 ATP synthesis driven by a protonmotive force in *Streptococcus lactis*. J. Membrane Biol., 25: 285-310.

Maloney, P.C.
1977 Obligatory coupling between proton entry and the synthesis of adenosine 5'-triphosphate in *Streptococcus lactis*. J. Bacteriol., 132: 564-575.

Maloney, P.C.
1978 Coupling between H^+ entry and ATP formation in *Escherichia coli*. Biochem. Biophys. Res. Comm., 83: 1496-1501.

Maloney, P.C., Kashket, E.R., Wilson, T.H.
1974 A protonmotive force drives ATP synthesis in bacteria. Proc. Natl. Acad. Sci. USA, 71: 3896-3900.

Massey, L.K., Sokatch, J.R., Conrad, R.S.
1976 Branched-amino-acid catabolism in bacteria. Bacteriol. Rev., 40: 42-54.

Matsushita, K., Shinagawa, E., Adachi, O., Ameyama, M.
1982 *o*-type cytodhrome oxidase in the membrane of aerobically grown *Pseudomonas aeruginosa*. FEBS Lett., 139: 255-258.

McInerney, M.J., Holmes, K.S., Hoffman, P., DerVartanian, D.V.
1984 Respiratory mutants of *Azotobacter vinelandii* with elevated levels of cytochrome *d*. Eur. J. Biochem., 141: 447-452.

Meijer, E.M., van Verseveld, H.W., van der Beek, E.G., Stouthamer, A.H.
1977 Energy conservation during aerobic growth in *Paracoccus denitrificans*. Arch. Microbiol., 112:25-34.

Meijer, E.M., van der Zwaan, J.W., Stouthamer, A.H.
1979 Location of the proton consuming site in nitrite reduction and stoichiometry for proton pumping in anaerobically grown *Paracoccus denitrificans*. FEMS Microbiol. Lett., 5: 369-372.

Meyer, D.J., Jones, C.W.
1973a Distribution of cytochromes in bacteria: Relationship to general physiology: Int. J. Syst. Bacteriol., 23: 459-467.

Meyer, D.J., Jones, C.W.
1973b Oxidative phosphorylation in bacteria which contain different cytochrome oxidases. Eur. J. Biochem., 36: 144-151.

Meyer, D.J., Jones, C.W.
1973c Reactivity with oxygen of bacterial cytochromes a_1 aa_3 and *o*. FEBS Lett., 33: 101-105.

Meyer, T.E., Kamen, M.D.
1982 New perspectives on C-type cytochromes. In: Advances in Protein chemistry. Vol. 35, pp. 105-212. Anfinsen, C.B., Edsall, J.T., Richards, F.M. (eds.), Academic Press, (London) Ltd.

Miller, M.J., Gennis, R.B.
1983 The purification and characterization of the cytochrome terminal oxidase complex of the *Escherichia coli* aerobic respiratory pathway. J. Biol. Chem., 258: 9159-9165.

Mitchell, P.
1961a Approaches to the analysis of specific membrane transport. p. 581-603. In: T.W. Goodwin and D. Lindberg (eds.), Biological structure and function. Vol. 2, Academic Press, Inc., New York.

Mitchell, P.
1961b Coupling phosphorylation to electron hydrogen transfer by a chemi-osmotic type of mechanism. Nature 191: 144-148.

Mitchell, P.
1966 Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev., 41: 445-502.

Mitchell, P.
1976 Vectorial chemistry and the molecular mechanics of chemiosmotic coupling: Power transmission by proticity. Biochem. Soc. Trans., 4: 399-430.

Mitchell, P.
1980 Portonmotive cytochrome system in mitochondria. Ann. N.Y. Acad. Sci., 341: 564-584.

Mitra, S., Bersohn, R.
1980 Location of the heme groups in cytochrome cd_1 oxidase from *Pseudomonas aeruginosa*. Biochemistry, 19: 3200-3203.

Moriarty, D.J.W., Nicholas, J.D.
1969 Enzymic sulphide oxidation by *Thiobacillus concretivorus*. Biochim. Biophys. Acta, 184: 114-123.

Moss, F.
1952 The influence of oxygen tension on respiration and cytochrome a_2 formation of *Escherichia coli*. Aust. J. Exp. Biol. Med. Sci., 30: 531-540.

Moss, F.
1956 Adaptation of the cytochromes of *Aerobacter aerogenes* in response to environmental oxygen tension. Aust. J. Exp. Biol. Med. Sci., 34: 395-406.

Moyed, H.S., O'Kane, D.J.
1956 Enzymes and coenzymes of the pyruvate oxidase of *Proteus*. J. Biol. Chem., 218: 831-840.

Mueller, T.J., Jurtshuk, P. Jr.
1972 Solubilization of cytochrome oxidase from *Azotobacter vinelandii*. Fed. Proc., 31: 888 Abs.

Nagai, S., Nishizawa, Y., Onodera, M., Aiba, S.
1971 Effect of dissolved oxygen on growth yield and aldolase activity in chemostat culture of *Azotobacter vinelandii*. J. Gen. Microbiol., 66: 197-203.

Niven, D.F.
1984 The cytychrome complement of *Haemophilus parasuis*. Can. J. Microbiol., 30: 763-773.

Niven, D.F., Collins, P.A., Knowles, C.J.
1975 The respiratory system of *Chromobacterium violaceum* grown under conditions of high and low cyanide evolution. J. Gen. Microbiol., 90: 271-285.

Niven, D.F., Collins, P.A., Knowles, C.J.
1977 Adenylate energy charge during batch culture of *Beneckea natriegens*. J. Gen. Microbiol., 98: 95-108.

Niven, D.F., MacLeod, R.A.
1978 Sodium-ion proton antiport in a marine bacterium. J. Bacteriol., 134: 737-743.

Niven, D.F., MacLeod, R.A.
1980 Sodium ion-substrate symport in a marine bacterium. J. Bacteriol., 142: 603-607.

Oelze, J., Drews, G.
1972 Membranes of photosynthetic bacteria. Biochim. Biophys. Acta, 265: 209-239.

O'Keeffe, D.T., Anthony, C.
1980 The interaction between methanol dehydrogenase and the autoreducible cytochromes c of the facultative methylotroph *Pseudomonas* AM1. Biochem. J., 190: 481-484.

Orth, V., Chippaux, M., Pascal, M.C.
1980 A mutant defective in electron transfer to nitrate in *Escherichia coli* K12. J. Gen. Microbiol., 117: 257-262.

Pacá, J.
1976 Oxygen transfer rate respiration and yields in batch and chemostat cultures of *Klebsiella aerogenes*. Folia Microbiol., 21: 417-430.

Papa, S.
1976 Proton translocation reactions in the respiratory chains. Biochim. Biophys. Acta, 456: 39-84.

Papa, S.
1982 Molecular mechanism of proton translocation by the cytochrome system and the ATPase of mitochondria. Role of proteins. J. Bioenerg. Biomembr., 14: 69-86.

Papa, S., Izzo, G., Guerrieri, F.
1982 On the inhibition of the b-c_1 segment on the mitochondrial respiratory chain by quinone analogues and hydroxyquinoline derivatives. FEBS Lett., 145: 93-98.

Papa, S., Guerrieri, F., Izzo, G.
1983 The mechanism of proton translocation by the cytochrome system of mitochondria. Biochem. J., 216: 259-272.

Parr, S.R., Barber, D., Greenwood, C., Phillips, B.W., Melling, J.
1976 A purification procedure for the soluble cytochrome oxidase and some other respiratory proteins from *Pseudomonas aeruginosa*. Biochem. J., 157: 423-430.

Pasteur, L.
1861 Animacules infusoires vivant sans gaz oxygène libre et déterminant les fermentations. Comptes rendus hebdomadaires des séances de l'Académie des sciences, 52: 344-347.

Peschek, G.A., Schmetterer, G., Wagesreiter, H.
1982 Oxidation of exogenous c-type cytochromes by intact spheroplasts of *Anacystis nidulans*. Arch. Microbiol., 133: 222-224.

Peterson, J.A.
1970 Cytochrome content of two pseudomonad containing mixed-function oxidase systems. J. Bacteriol., 103: 714-721.

Pirt, S.J.
1975 Principles of microbe and cell cultivation. Hulsted Press, John Wiley and Sons, New York, 1st ed.

Poole, R.K.
1983a The oxygen reactions of bacterial cytochrome oxidases. Trends Biochem. Sci., 7: 32-34.

Poole, R.K.
1983b Bacterial cytochrome oxidases. A structurally and functionally diverse group of electron-transfer proteins. Biochim. Biophys. Acta, 726: 205-243.

Poole, R.K., Blum, H., Scott, R.I., Cottinge, A., Ohnishi, T.
1980 The orientation of cytochromes in membrane multilayers prepared from aerobically grown *Escherichia coli* K12. J. Gen. Microbiol., 119: 145-154.

Poole, R.K., Salmon, I., Chance, B.
1983 The reaction with oxygen of cytochrome oxidase (cytochrome *d*) in *Escherichia coli* K12: optical studies of intermediate species and cytochrome *b* oxidation at sub-zero temperatures. J. Gen. Microbiol., 129: 1345-1355.

Poole, R.K., Waring, A.J., Chance, B.
1979 Evidence for a functional oxygen-bound intermediate in the reaction of *Escherichia coli* cytochrome *o* with oxygen. FEBS Lett., 101: 56-58.

Preiss, J., Handler, P.
1958 Biosynthesis of diphosphopyridine nucleotide. J. Biol. Chem., 233: 488-492.

Pritchard, G.G., Asmundson, R.V.
1980 Aerobic electron transport in *Propionibacterium shermanii*. Effects of cyanide. Arch. Microbiol., 126: 167-175.

Probst, I., Schlegel, H.G.
1976 Respiratory components and oxidase activities in *Alcaligenes eutrophus*. Biochim. Biophys. Acta, 440: 412-428.

Pudek, M.R., Bragg, P.D.
1974 Inhibition by cyanide of the respiratory chain oxidases of *Escherichia coli*. Arch. Biochem. Biophys., 164: 682-693.

Quilter, J.A., Jones, C.W.
1984 The organization of methanol dehydrogenase and c-type cytochromes on the respiratory membrane of *Methylophilus methylotrophus*. FEBS Lett., 174: 167-172

Reichelt, J.L., Baumann, P.
1973 Change of the name *Alteromonas marinopraesens* (Zobell and Upham) Baumann et al. to *Alteromonas holoplanktis* (Zobell and Upham) comb. nov. and assignment of strain ATCC 23821 (*Pseudomonas enalia*) and strain c-Al of DeVoe and Oginsky to this species. Int. J. Syst. Bacteriol., 23: 438-441.

Reid, G.A., Ingledew, W.J.
1979 Characterization and phenotypic control in the cytochrome content of *Escherichia coli*. Biochem. J., 182: 465-472.

Reid, G.A., Ingledew, W.J.
1980 The purification of a respiratory oxidase complex from *Escherichia coli*. FEBS Lett., 109: 1-4.

Revsin, B., Marquez, E.D., Brodie, A.F.
1970 Cytochromes from *Mycobacterium phlei*. I. Isolation and spectral properties of a mixture of cytochromes ($a + a_3$) (o). Arch. Biochem. Biophys., 139: 114-120.

Rice, G.W., Hempfling, W.P.
1978 Oxygen-limited continuous culture and respiratory energy conservation in *Escherichia coli*. J. Bacteriol., 134: 115-124.

Richmond, M.H., MaaLøe, O.
1962 The rate of growth of *Salmonella typhimurium* with individual carbon sources related to glucose metabolism or to the Krebs cycle. J. Gen. Microbiol., 27: 285-297.

Rodwell, V.W.
1969 Carbon catabolism of amino acids. In: Metabolic Pathways. Vol. 3, pp. 191-236, D.M. Greenberg (ed.), Academic Press Inc., New York.

Rørth, M., Jensen P.K.
1967 Determination of catalase activity by means of the Clark oxygen electrode. Biochim. Biophys. Acta, 139: 171-173.

Rosenberg, M., Friedberg, I.
1984 Respiratory control in *Micrococcus lysodeikticus*. J. Bioenerg. Biomembr., 16: 61-68.

Rosenberg, R.F., Kogut, M.
1958 The influence of growth rate and aeration on the respiratory and cytochrome system of a fluorescent Pseudomonad grown in continuous culture. J. Gen. Microbiol., 19: 228-243.

Sadler, M.H., Johnson, E.J.
1972 A comparison of the NADH oxidase electron transport systems of two obligately chemiolithotrophic bacteria. Biochim. Biophys. Acta, 283: 167-179.

Sands, D.C., Gleason, F.H., Hildebrand, D.C.
1967 Cytochromes of *Pseudomonas syringae*. J. Bacteriol., 94: 1785-1786.

Sapshead, L.M., Wimpenny, J.W.T.
1972 The influence of oxygen and nitrate on the formation of the cytochrome pigments of the aerobic and anaerobic respiratory chain of *Micrococcus denitrificans*. Biochim. Biophys. Acta, 267: 388-397.

Sasaki, T., Motikawa, Y., Kikuchi, G.
1970 Occurrence of both *a*-type and c-type cytochromes as the functional terminal oxidase in *Rhodopseudomonas sphaeroides*. Biochim. Biophys. Acta, 197: 284-291.

Saunders, B.C., Holmes-Siedle, A.G., Stark, B.P.
1964 The Spectra and function of the hemoproteins. In: Peroxidase, pp. 112-124, B.C. Saunders, A.G. Holmes-Siedle, B.P. Stark (eds.), Butterworths, London.

Sawhney, V., Nicholas, D.J.D.
1978 Sulphide-linked nitrite reductase from *Thiobacillus denitrificans* with cytochrome oxidase activity. J. Gen. Microbiol., 106: 119-128.

Scholes, P.B., Smith, L.
1968 Composition and properties of the membrane-bound respiratory chain system of *Micrococcus denitrificans*. Biochim. Biophys. Acta, 153: 363-373.

Sedgwick, E.G., MacLeod, R.A.
1980 Energy coupling to K+ transport in a marine bacterium. Can. J. Microbiol., 58: 1206-1214.

Shepherd, M.G., Chin, C.M., Sullivan, P.A.
1978 The alternate respiratory pathway in *Candida albicans*. Arch. Microbiol., 116: 61-67.

Shidara, S.
1980 Components of the cytochrome system of *Alcaligenes sp.* N.C.I.B. 11015, with special reference to particulate bound c-type cytochromes. J. Biochem., 87: 1177-1184.

Shimada, H., Orii, Y.
1976 Oxidation-reduction behavior of the heme c and heme *d* moieties of *Pseudomonas aeruginosa* nitrite reductase and the formation of an oxygenated intermediate of heme *d*. J. Biochem. (Tokyo) 80: 135-140.

Shipp, W.S.
1972 Cytochromes of *Escherichia coli*. Arch. Biochem. Biophys. 150: 459-472.

Singh, A.P., Bragg, P.D.
1979 ATP synthesis driven by a pH gradient imposed across the cell membranes of lipoic and unsaturated fatty acid auxotrophs of *Escherichia coli*. FEBS Lett., 98: 21-24.

Slater, E.C.
1953 Mechanism of phosphorylation in the respiratory chain. Nature 172: 975-978.

Smith, L.
1954 Bacterial cytochromes. Bacteriol. Rev., 18: 106-130.

Smith, L., White, D.C.
1962 Structure of the respiratory chain as indicated by studies with *Haemophilus parainfluenzae*. J. Bacteriol., 83: 851-859.

Smith, L.
1968 The respiratory chain of bacteria. In: T.P. Singer (ed.) Biological Oxidations. Interscience Publishers, New York, pp. 55-122.

Smith, L.
1978 Bacterial cytochromes and their spectral characterization. Methods Enzymol., 54 Biomembranes (Part D) Biological oxidations. pp. 202-212.

Smith, L., White, D.C., Sinclair, P., Chance, B.
1970 Rapid reactions of cytochromes of *Haemophilus parainfluenzae* on addition of substrates or oxygen. J. Biol. Chem., 245: 5096-5100.

Sone, N., Hinkle, P.C.
1982 Proton transport by cytochrome c oxidase from the thermophilic bacterium PS3 reconstituted in liposomes. J. Biol. Chem., 257: 12600-12604.

Sone, N., Kagawa, Y., Orii, Y.
1983 Carbon-monoxide-binding in the respiratory chain of the thermophilic bacterium PS3 grown with sufficient and limited aeration. J. Biochem., 93: 1329-1336.

Sone, N., Naqui, A., Kumar, C., Chance, B.
1984 Reaction of caa_3 type terminal cytochrome oxidase from the thermophilic bacterium PS3 with oxygen and carbon monoxide at low temperature. Biochem. J., 221: 529-533.

Sprott, G.D., Drozdowski, J.P., Martin, E.L., MacLeod, R.A.
1975 Kinetics of Na^+-dependent amino acid transport using cells and membrane vesicles of a marine pseudomonad. Can. J. Microbiol., 21: 43-50.

Sprott, G.D., MacLeod, R.A.
1974 Nature of the specificity of alcohol coupling to L-alanine transport into isolated membrane vesicles of a marine pseudomonad. J. Bacteriol., 117: 1043-1054.

Stanier, R.Y., Palleroni, N.J., Doudoroff, M.
1966 The aerobic Pseudomonads: A taxonomic survey. J. Gen. Microbiol., 43: 159-171.

Steel, R.G.D., Torrie, J.H.
1960 Principles and procedures of STATISTICS with special reference to the biological sciences. McGraw-Hill Book Company, Inc., New York, Toronto, London.

Stouthamer, A.H., Bettenhaussen, C.W.
1976 Energetic aspects of anaerobic growth of *Aerobacter aerogenes* in complex medium. Arch. Microbiol., 111: 21-23.

Sweet, W.J., Peterson, J.A.
1978 Changes in cytochrome content and electron transport patterns in *Pseudomonas putida* as a function of growth phase. J. Bacteriol., 133: 217-225.

Taniguchi, S., Kamen, M.C.
1963 On the anomalous interactions of ligands with *Rhodospirillum* haem protein. Biochim. Biophys. Acta, 74: 438-455.

Then, J., Trüper, H.G.
1983 Sulfide oxidation in *Ectothiorhodospira abdelmalekii*. Evidence for the catalytic role of cytochrome c_{551}. Arch. Microbiol., 135: 254-258.

Thompson, J., MacLeod, R.A.
1974a Specific electron donor-energized transport of α-aminoisobutyric acid and K^+ into cells of a marine Pseudomonad. J. Bacteriol., 117: 1055-1064.

Thompson, J., MacLeod, R.A.
1974b Potassium transport and the relationship between intracellular potassium concentration and amino acid uptake by cells of a marine pseudomonad. J. Bacteriol., 120: 598-603.

Tonge, G.M., Knowles, C.J., Harrison, D.E.F., Higgins, I.J.
1974 Metabolism of one-carbon compounds: cytochromes of methane and methanol-utilizing bacteria. FEBS Lett., 44: 106-110.

Tonge, G.M., Harrison, D.E.F., Knowles, C.J., Higgins, I.J.
1975 Properties and partial purification of the methane-oxidizing enzyme system from *Methylosinus trichosporium*. FEBS Lett., 58: 293-299.

Tonge, G.M., Harrison, D.E.F., Higgins, I.J.
1977 Purification and properties of the methane mono-oxygenase enzyme system from *Methylosinus trichosporium* OB3b. Biochem. J., 161: 333-344.

Tomlinson, N., MacLeod, R.A.
1957 Nutrition and metabolism of marine bacteria. IV. The participation of Na^+, K^+ and Mg^{++} salts in the oxidation of exogenous substrates by a marine bacterium. Can. J. Microbiol., 3: 627-638.

Tsuchiya, T.
1977 Adenosine 5'-triphosphate synthesis driven by a protonmotive force in membrane vesicles of *Escherichia coli*. J. Bacteriol., 129: 763-769.

van Verseveld, H.W.
1979 Influence of environmental factors on the efficiency of energy conservation in *Paracoccus denitrificans*. Ph.D. Thesis, University of Amsterdam, Netherlands.

van Versevels, H.W., Stouthamer, A.H.
1978 Electron-transport chain and coupled oxidative phosphorylation in methanol-grown *Paracoccus denitrificans*. Arch. Microbiol., 118: 13-20.

van Verseveld, H.W., Braster, M., Boogerd, F.C., Chance, B., Stouthamer, A.H.
1983 Energetic aspects of growth of *Paracoccus denitrificans*: oxygen limitation and shift from anaerobic nitrate-limitation to aerobic succinate-limitation. Arch. Microbiol., 135: 229-236.

Van Wielink, J.E., Reijnders, W.N.M., Oltman, L.F., Stouthamer, A.H.
1983 The characterization of the membrane-bound b- and c-type cytochromes of differently grown *Escherichia coli* cells by means of coupled potentiometric analysis and spectrum deconvolution. FEMS Microbiol. Lett., 18: 167-172.

Vignais, P.M., Henry, M.-F., Sim, E., Kell, D.B.
1981 The electron transport system and hydrogenase of *Paracoccus denitrificans*. In: Sanadi, D.R., (ed.) Current topics in Bioenergetics, Vol. 12. Academic Press, New York, London, Toronto, Sydney, San Francisco, pp. 115-196.

Ward, J.A., Hunter, C.N., Jones, O.T.G.
1983 Changes in the cytochrome composition of *Rhodopseudomonas sphaeroides* grown aerobically, photosynthetically and on dimethyl sulphoxide. Biochem. J., 212: 783-790.

Watanabe, H., Kamita, Y., Nakamura, T., Takimoto, A., Yamanaka, T.
1979 The terminal oxidase of *Photobacterium phosphoreum*. A novel cytochrome. Biochim. Biophys. Acta, 547: 70-78.

Webster, D.A.
1975 The formation of hydrogen peroxide during the oxidation of reduced nicotinamide adenine dinucleotide by cytochrome *o* from *Vitreoscilla*. J. Biol. Chem., 250: 4955-4958.

Webster, D.A., Hackett, D.P.
1966 Respiratory chain of colorless algae. II. Cyanophyta. Plant Physiol., 41: 599-605.

Webster, D.A., Liu, C.Y.
1974 Reduced nicotinamide adenine dinucleotide cytochrome reductase associated with cytochrome *o* purified from *Vitreoscilla*. J. Biol. Chem., 249: 4257-4260.

Webster, D.A., Orii, Y.
1977 Oxygenated cytochrome *o*: an active intermediate observed in whole cells of *Vitreoscilla*. J. Biol. Chem., 252: 1834-1836.

Webster, D.A., Orii, Y.
1978 Physiological role of oxygenated cytochrome *o*: Observations on whole cell suspensions of *Vitreoscilla*. J. Bacteriol., 135: 62-67.

Weston, J.A., Knowles, C.J.
1973 A soluble CO-binding c-type cytochrome from the marine bacterium *Beneckea natriegens*. Biochim. Biophys. Acta, 305: 11-18.

Weston, J.A., Knowleds, C.J.
1974 The respiratory system of the marine bacterium *Beneckea natriegens*. I. Cytochrome composition. Biochim. Biophys. Acta, 333: 228-236.

Weston, J.A., Collins, P.A., Knowles, C.J.
1974 The respiratory system of the marine bacterium *Beneckea natriegens*. II. Terminal branching of respiration to oxygen and resistance to inhibition to cyanide. Biochim. Biophys. Acta, 368: 148-157.

White, D.C.
1962 Cytochrome and catalase patterns during growth of *Haemophilus parainfluenzae*. J. Bacteriol., 83: 851-859.

White, D.C.
1963a Factors affecting the affinity for oxygen of cytochrome oxidases in *Haemophilus parainfluenzae*. J. Biol. Chem., 238: 3757-3761.

White, D.C.
1963b Respiratory systems in the hemin-requiring *Haemophilus* species. J. Bacteriol., 85: 84-96.

White, D.C.
1964 Differential synthesis of five primary electron transport dehydrogenases in *Haemophilus parainfluenzae*. J. Biol. Chem., 239: 2055-2060.

White, D.C., Sinclair, P.R.
1971 Branched electron-transport systems in bacteria. Adv. Microbiol. Physiol., 5: 173-211.

Williams, R.J.P.
1961 Possible functions of chains of catalysts. J. Theor. Biol., 1: 1-13.

Williams, R.J.P.
1978 The history and hypothesis concerning ATP-formation by energized protons. FEBS Lett., 85: 9-19.

Willison, J.C., John, P.
1979 Mutants of *Paracoccus denitrificans* deficient in c-type cytochromes. J. Gen. Microbiol., 115: 443-450.

Wood, P.M.
1978a Periplasmic location of the terminal reductase in nitrate respiration. FEBS Lett., 92: 214-218.

Wood, P.M.
1978b A chemiosmotic model for sulphate respiration. FEBS Lett., 95: 12-19.

Woodrow, G., Schatz, G.
1979 The role of oxygen in the biosynthesis of cytochrome c oxidase of yeast mitochondria. J. Biol. Chem., 254: 6088-6093.

Yagi, T., Maruyama, K.
1971 Purification and properties of cytochrome c_3 of *Desulfovibrio vulgaris*, Miyazaki. Biochim. Biophys. Acta, 243: 214-224.

Yamamoto, I., Ishimoto, M.
1978 Hydrogen-dependent growth of *Escherichia coli* in anaerobic respiration and the presence of hydrogenases with different functions. J. Biochem., 84: 673-679.

Yamanaka, T., Fukumori, Y., Tanaka, Y.
1984 A relationship between prokaryote and eukaryote observed in *Nitrobacter agilis* cytochrome aa_3 and c. Origins Life, 14: 739-747.

Yanaka, Y., Fukumori, Y., Yamanaka, T.
1983 Purification of cytochrome a_1c_1 from *Nitrobacter agilis* and characterization of nitrite oxidation system of the bacterium. Arch. Microbiol., 135: 265-271.

Yang, T.Y.
1978 Purification and characterization of cytochrome *o* from *Azotobacter vinelandii*. Biochim. Biophys. Acta, 502: 543-548.

Yang, T.Y.
1984 Spectral studies on cytochrome *d* of *Azotobacter vinelandii*. Curr. Microbiol., 10: 309-312.

Yang, T.Y., Jurtshuk, P. Jr.
1978 Studies on the red oxidase (cytochrome *o*) of *Azotobacter vinelandii*. Biochem. Biophys. Res. Commun., 81: 1032-1039.

Yu, E.K.C., DeVoe, I.W., Gilchrist, J.E.
1979 A soluble CO-and NO-binding c-type cytochrome in *Neisseria meningitidis*. Curr. Microbiol., 2: 201-206.

Yu, E.K.C., DeVoe, I.W.
1980 Terminal branching of the respiratory electron transport chain in *Neisseria meningitidis*. J. Bacteriol., 142: 879-887.

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