THE UNIVERSITY OF MANITOBA

CYTOCHROMES OF *NITROBACTER*

BY

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To my parents and friends

ABSTRACT

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ABSTRACT

Optimal growth conditions were established for *Nitrobacter agilis* freshly isolated from soil. Oxygen tension below 10% or above 15% resulted in abnormal cell ultrastructure and reduced growth. High concentration of nitrite and nitrate also affected the growth and cell structure.

Optimally grown (2 g wet cell/ 10 l) cultures provided for the isolation and purification of three *c*-type cytochromes, cytochrome *a*-type oxidase, a copper-iron electron transfer protein, cytochrome *b*, ubiquinone, nitrite: cytochrome *c* reductase, NADH: cytochrome *c* oxido-reductase and a purple pigment.

Two of the three *c*-type cytochromes, cytochrome *c*-553 and *c*-550 had typical absorption spectra of cytochrome *c* and molecular weights of 11,500 and 12,500 respectively. Cytochrome *c*-553 was characteristically partially reduced when isolated and was not oxidized by ferricyanide. The completely reduced form displayed absorption peaks at 410, 523, and 553 nm. Cytochrome *c*-550 was similar to that purified previously by Ketchum *et al.* (1969) with an oxidized absorption peak at 411 nm and reduced peaks at 416, 521 and 550 nm. The amino acid compositions of the two cytochromes were different from each other. The N-terminal sequence of cytochrome *c*-550 showed a clear homology with the corresponding sequence portions of four *c*-type cytochromes from other sources, including horse heart.

The third cytochrome was an anomalous cytochrome of *c'*-type.

It had an oxidized absorption peak at 402 nm and bands at 525, 560 and 620 nm. The reduced form showed absorption maxima at 419, 523, 549 and 554 nm and a shoulder at 430 nm. The absorption maxima were markedly affected by pH. Sodium hydroxide at 0.1 N converted the anomalous spectra of cytochrome *c*-549, 554 to those of a typical cytochrome *c*. It had a molecular weight of 90,000 as estimated by gel filtration but appeared as a single polypeptide of 46,000 daltons by SDS polyacrylamide gel electrophoresis. Amino acid composition showed a high content of alanine. Cytochrome *c*-549, 554 was found to be highly autoxidizable. The ferrocytochrome reacted with CO and NO_2^- whereas both ferri- and ferrocytochrome combined with CN^-.

Cytochrome oxidase solubilized and purified from electron transport particles exhibited absorption spectra with maxima at 420 and 600 nm when oxidized and at 443 and 606 nm when reduced. The purified enzyme migrated as a single band during gel electrophoresis but appeared as three bands with molecular weights of 37,000, 25,000 and 13,000 in the presence of SDS and urea. Cytochrome *a*-type oxidase reacted rapidly with *Nitrobacter* cytochrome *c*-550 and horse heart cytochrome *c*. The enzyme activity was pH dependent and was inhibited by cyanide, azide and diethyl dithiocarbamate.

The copper-iron electron transfer protein similarly purified had a yellow-green color and absorbance maxima at 410 and 630 nm. The color disappeared upon reduction. It did not contain a methionine residue and had a molecular weight of 5,500 as judged by gel

filtration. The purified protein moved as a single band during electrophoresis. The copper-iron protein contained 0.34 g atom of copper and 0.60 g atom of iron per mole of protein and about 3% of phospholipid.

The isolated and partially purified cytochrome *b* was autoxidizable, reacted with CO and in the oxidized form had an absorption maximum at 405 nm; the reduced form maxima were at 432, 530 and 560 nm. Molecular weight estimated by gel filtration was 37,000. Fluorescence spectra indicated a flavin-like component present in cytochrome *b* preparations.

Absorption spectra, TLC, IR and PMR studies of the ubiquinone purified from whole cells suggest that it is probably coenzyme Q_{10}.

Nitrite: cytochrome *c* reductase had a yellow color and catalyzed the reduction of cytochrome *c* with nitrite. It had a Km value of 30 mM for nitrite and its activity was inhibited by nitrate. The enzyme was unstable and lost its activity overnight at $-20^{o}C$. It contained adenine nucleotide, possibly FAD. The yellow pigment was bleached when reduced as was the color of enzyme preparations.

NADH: Cytochrome *c* oxido-reductase had a red color and catalyzed the reduction of cytochrome *c* in the presence of NADH. Its enzyme activity was inhibited by nitrate but not by nitrite. Both the enzyme preparation and the red pigment extracted from it exhibited similar absorption spectra with a 480 nm peak. The red color disappeared upon reduction.

The purple pigment had a weak activity of NH_2OH: cytochrome *c* reductase. The pigment was decolorized upon reduction and at a pH below 6.5.

The study reported in this thesis may be considered as a further positive step towards the understanding of the biochemistry of nitrite oxidation, an important process of the nitrogen cycle. Not only have some new redox components of the *Nitrobacter* respiratory chain been demonstrated but the basis for further exploration of the energetics of this organism has also been extended.

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ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BIS	N,N'-methylene bisacrylamide
BSA	bovine serum albumin
^{13}CMR	carbon-13 magnetic resonance
CM	carboxymethyl
cyt.*c*	cytochrome *c*
DEAE	diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
ETP	electron transport particle(s)
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GSH	reduced glutathione
IR	infrared
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
PMR	proton magnetic resonance
Q_9	coenzyme Q_9
Q_{10}	coenzyme Q_{10}
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet

INTRODUCTION

CHAPTER 1

INTRODUCTION

Inorganic nitrogen compounds exist in nature in several oxidation states from NH_3(-3) to HNO_3(+5). Whereas the conversion of its most oxidized form, nitrate, into its most reduced form, ammonia, is carried out by a wide variety of microorganisms and plants, the biological oxidation of reduced inorganic compounds is carried out predominantly by species of the nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*. The former oxidizes ammonia to nitrite and the latter nitrite to nitrate.

Nitrobacter was first isolated and named by Winogradsky (1890) and is a chemoautotrophic bacterium that derives energy from the oxidation of nitrite (17.8 Kcal/mol) and assimilates CO_2 by using the Calvin cycle (Aleem 1965).

The era of biochemical studies of *Nitrobacter* essentially begins with the report that cytochromes are involved in nitrite oxidation (Lees and Simpson 1957). Aleem and Nason (1959) confirmed that nitrite oxidizing activity of *Nitrobacter agilis* resides in cytochrome-containing red particles. The subsequent work on the mechanism of nitrite oxidation by autotrophic bacteria over the years has formed the basis for several extensive reviews (Lees 1960, 1962, Nicholas 1963, Peck 1968, Wallace and Nicholas 1969, Aleem 1970, Kelly 1971, Suzuki 1974, Schlegel 1975, Aleem 1978).

Most of the studies to elucidate the mechanism of electron transfer (from nitrite to molecular O_2) and oxidative phosphorylation have been limited to those with whole cells, partially purified

fractions, and cell-free extracts or so-called electron transport particles (Straat and Nason 1965, Van Gool and Laudelout 1966, Kiesow 1967, Aleem 1968, Sewell and Aleem 1969, O'Kelley *et al.* 1972, Ingledew *et al.* 1974, Cobley 1976a,b). Exceptions include the report by Ketchum *et al* (1969) where they purified a *c*-type cytochrome and that by Yamanaka *et al.* (1979) where they isolated the terminal oxidase system (cytochrome *a*-type oxidase). More recently cytochrome *b* has also been isolated as a cytochrome *b* and *c* complex (Chaudhry *et al.* 1980). Very little is known about the rest of the components of the respiratory chain of *Nitrobacter*.

In the present study attempts were made to isolate several redox systems of *Nitrobacter agilis* and to study some of their physicochemical properties. I have succeeded in isolating and identifying some of the so-far unknown or poorly understood redox components of *Nitrobacter*, for example *c'*-type cytochrome. It is hoped that these findings will provide a renewed interest and stimulus for workers to attempt a further elucidation of nitrite oxidation mechanism by *Nitrobacter*.

HISTORY

CHAPTER 2

HISTORY

2.1 INTRODUCTION

In the second half of the nineteenth century breakthroughs occurred in many areas of Science, particularly in the developing science of Microbiology. In the year 1862, for example, Pasteur first proposed that the oxidation of ammonia to nitrate might be a biological process performed by soil microorganisms (cited in Nelson 1931). It took another thirty years, however, to demonstrate that the biological oxidation of ammonia involved two steps, the oxidation of NH_4^+ to NO_2^-, and the oxidation of NO_2^- to NO_3^- (Schloesing and Muntz 1877, Warington 1891). At the same time, Winogradsky (1890) reported the isolation of bacteria responsible for the process of nitrification and called them *Nitrosomonas* and *Nitrobacter*, the former converting ammonia to nitrite and the latter oxidizing nitrite to nitrate (Lees 1955).

The classical studies of Winogradsky (1890) and Winogradsky and Omeliansky (1899) on the physiology of nitrifying bacteria led to the concept of obligate autotrophy. Members of the genus *Nitrobacter* fulfil their energy requirement for the fixation of carbon dioxide by the oxidation of nitrite, an inorganic substrate (Winogradsky 1922) and thus they are also called chemoautotrophs or chemolithotrophs (Lees 1955, Kelly 1971).

There have been three general approaches used in attempts to understand this mechanism of respiration or oxidation of substrate. First, classical respirometric studies with intact cells; secondly,

isolation and characterization of individual components of the respiratory and oxidative system, and finally the reconstitution and examination of the system after reassembling of the individual components. The purpose of this dissertation was to apply the second approach to further facilitate our understanding of nitrite oxidation by *Nitrobacter*. Since most of the studies so far were carried out with intact cells, crude extracts or partially purified preparations, my review of the literature begins with a general survey of these studies on the mechanism of nitrite oxidation by *Nitrobacter*, followed by a discussion of the individual components, particularly cytochromes of the respiratory chain of these bacteria.

2.2 MECHANISM OF NITRITE OXIDATION

The classical studies by Meyerhof (1916, 1917a,b) on the respiratory process of nitrifying bacteria led to the belief that the substrate oxidation by chemoautotrophs was a process very similar to the cell respiration of heterotrophic organisms. An important discovery in this respect was made by Lees and Simpson (1957) when they observed that the addition of nitrite to a cell suspension of *Nitrobacter* resulted in appearance of three reduced cytochrome bands at 521-25, 551 and 589 nm. They implicated the role of this cytochrome in nitrite oxidation by *Nitrobacter* by postulating the following scheme:

$$Fe^{3+}cyt.551 + NO_2^- \rightarrow Fe^{2+}cyt.551 + NO_2.$$

$$Fe^{2+}cyt.551 + NO_2. + \tfrac{1}{2}O_2 \rightarrow Fe^{3+}cyt.551 + NO_3^-$$

The symbol $NO_2.$ in this scheme was proposed to represent some compound, free or bound to a carrier at the oxidation level of the nitrite radical.

Soon after, nitrite oxidation was demonstrated in a cell-free particulate fraction from *Nitrobacter* (Aleem and Alexander 1958). Aleem and Nason (1960) subsequently reported that the oxidation of nitrite was catalyzed by the cytochrome-electron transport particle termed "nitrite oxidase" and the process involved an enzymatic transfer of electrons from nitrite to molecular oxygen mediated by *c* and a_1-type cytochrome as shown below:

$$NO_2^- \xrightarrow[\text{reductase}]{NO_2^- \text{ cyt.}c} \text{cyt.}c \rightarrow \text{cyt.}a_1 \xrightarrow[\text{oxidase}]{\text{cytochrome}} O_2$$

They further found that nitrite oxidation was coupled to the synthesis of ATP, with a P/O ratio 0.2. A similar value for the P/O ratio was also observed by other workers (Fischer and Laudelout 1965) who considered that these low values were consistent with the molar growth yield of the organism.

While discussing the problem of nitrite oxidation, Lees (1962) pointed out that the redox potential of nitrite/nitrate couple ($E_{m,7}$ + 0.42V) was appreciably higher than that of the cytochrome *c* of *Nitrobacter*, which had an $E_{m,7.5}$ of +0.25V (Butt and Lees 1958). He therefore, suggested that to oxidize nitrite, *Nitrobacter* might have to modify it in some way, perhaps by synthesizing some such compound as adenyl nitrite, so as to lower the redox potential of nitrite to one more compatible with reduction of cytochrome *c*. Although this idea did not agree with the kinetic studies of Kiesow discussed below (Kiesow 1963, 1964, 1967), it nevertheless changed the pattern of future thinking on the mechanism of nitrite oxidation.

Spectrophotometric studies conducted with *Nitrobacter* particles showed that the reduction of cytochrome a_1 by nitrite could be

achieved both aerobically and anaerobically, whereas cytochrome *c* was reduced only aerobically. Addition of ATP however, resulted in the reduction of cytochrome *c* under anaerobic conditions as well (Kiesow 1967). These observations led to a conclusion that the reduction of cytochrome *c* requires energy but that of cytochrome a_1 does not. According to Aleem (1968), 8.3 kcal of energy is required to bridge the gap between cytochrome *c* and NO_3^-/NO_2^- system. Van Gool and Laudelout (1967) reported that the activation energy for the reduction of cytochrome *c* by nitrite is 15 kcal. These findings were further substantiated by uncoupler and inhibitor studies. It was proposed that cytochrome a_1 was the site of entry of nitrite in the *Nitrobacter* electron transport chain and the reduction of cytochrome *c* involved an energy-dependent reversal of electron transfer from cytochrome a_1 (Aleem 1968).

On the other hand a direct reduction of cytochrome *c* by nitrite in the presence of a nitrite-cytochrome *c* reductase was reported (O'Kelley *et al.* 1970) and the experiments supporting the energy-dependent reduction of cytochrome *c* and reversal of electron transfer flow from a_1 (Aleem 1968) were criticized on the basis of lack of sufficient control experiments (O'Kelley *et al.* 1970). They suggested that the entry site of nitrite is not at cytochrome a_1 level but at or prior to cytochrome *c*. The same workers also reported that nitrite showed higher affinity for enzyme (nitrite-cytochrome *c*-reductase) at a lower pH, suggesting HNO_2 rather than NO_2^- as the substrate for the nitrite oxidase system. In *Nitrosomonas*, ammonia also showed greater affinity at higher pH, suggesting NH_3 rather than

NH_4^+ as the substrate for oxidation (Suzuki 1974). Aleem (1970), however, postulated a hydrated form of nitrite ($NO_2^-.H_2O$) as the substrate of nitrite oxidation.

Redox potential studies at various pH values (Ingledew *et al.* 1974) showed that pH had no effect on the E_m of *Nitrobacter* cytochrome *c*, *a* and a_3, but the redox potentials of cytochrome a_1 components (two types of cytochrome a_1 had been identified by Straat and Nason 1965) became more positive with decreasing pH. Aleem (1978) has not only reported a similar pH dependent redox potential change for cytochrome a_1, but he further observed an analogous change in the midpoint potential of nitrite/nitrate in the presence of *Nitrobacter* cell-free extracts. The E_m of nitrite became more positive from pH 9.0 (E_m= 328 mV) to pH 6.8 (E_m = 414 mV). He therefore concluded that the reduction of cytochrome a_1 by nitrite posed no thermodynamic barrier, whereas that of cytochrome *c* did contrary to the findings of O'Kelley *et al.* (1970).

Recent studies suggested that the rate of nitrite oxidation by electron transport particles was directly related to the value of electrical potential of the membrane, $\Delta\psi$, but the NADH oxidation could be stimulated by the conditions which reduced both $\Delta\psi$ and ΔpH components of the proton motive force (Cobley 1976a).

A satisfactory mechanism of nitrite oxidation has to explain the following observations:

1. ATP induced changes in the mid-point potential of cytochrome *c* (Ingledew and Chappell 1975). 2. pH dependent redox potential of cytochrome a_1 (Ingledew *et al.* 1974) and NO_2^- (Aleem 1978), 3. the effect of

temperature on the reduction of cytochromes *c* and *a* (Van Gool and Landelout 1967) and 4. the energy independent reduction of cytochrome *c* (O'Kelley 1970).

Nitrite oxidation supplies both energy as ATP and reducing power as NADH for the chemosynthetic bacterium, *Nitrobacter*. Because of the redox potentials of NO_3^-/NO_2^- couple and NAD^+/NADH couple ($E_{m,7}$ - 0.32 V), the direct reduction of NAD^+ by nitrite is thermodynamically impossible unless energy is provided.

The first experimental evidence for NAD^+ reduction coupled to nitrite oxidation was offered by Kiesow (1963). He found that the NAD^+ reduction by nitrite in *Nitrobacter* cell-free preparations was inhibited by an uncoupler, 2,4 dinitrophenol (Kiesow 1964). Similar observations were reported independently by Aleem *et al.* (1963) and it was proposed that the reduction process of NAD^+ in *Nitrobacter* was energy-dependent, involving the reversed electron flow from reduced cytochrome *c*. The amount of energy required for the reduction of NAD^+ by nitrite was 2 ATP according to Kiesow (1967) and 4-6 ATP according to Sewell and Aleem (1969).

NADH oxidation by cell-free extracts of *Nitrobacter* was coupled to ATP synthesis aerobically, or anaerobically in the presence of nitrate (Kiesow 1964). A membrane fraction from *Nitrobacter*, so-called nitrite oxidase particles, oxidized NADH with a P/O ratio of 2.0 (Aleem 1968). On the basis of inhibitor effects on the oxidation of NADH, it was concluded that the electron transport chain of the chemosynthetic bacteria, *Nitrobacter* was analogous to the mitochondrial system (Aleem 1968). Justification for such a proposal requires the

isolation and identification of individual components involved in NADH oxidation.

2.3 CYTOCHROMES

Cytochromes are defined as haemoproteins whose principal biological function is electron and/or hydrogen transport by virtue of a reversible valency change of their haem iron (Commission Enzyme on the International Union of Biochemistry 1961). Our knowledge about cytochromes developed from the preliminary observations of MacMunn (1886) during his spectroscopic studies of mammalian tissue. His discovery of a colored pigment different from haemoglobin or myoglobin was criticized by the fellow workers (Hoppe-Seyler 1890, Levy 1889) and was overlooked until 1925, when the skillful observations of Keilin (1925) confirmed MacMunn's original findings. He found that the new compound was intimately related to cellular respiration and thus named it cytochrome. He also revealed that the four banded absorption spectrum of the "cytochrome" was in fact due to different compounds which he called cytochromes *a*, *b* and *c*. Initially, cytochromes were believed to be present only in aerobic organisms. Their later discovery in anaerobic organisms (Ishimoto and Koyama 1954, Kamen and Vernon 1954, Vernon and Kamen 1954) made it clear that cytochromes function in almost all types of organisms.

Cytochromes of *Nitrobacter* were first reported by Lees and Simpson (1957).

2.3.1 *a*-type cytochromes: Cytochromes of *a*-type as well as *c*-type were first implicated in nitrite oxidation of *Nitrobacter* (Lees and Simpson 1957). Later observations confirmed these results (Zavarzin

1958a,b, Silver 1961, Aleem and Nason 1959). Studies on the low temperature spectra and CO binding properties of intact cells as well as cell-free extracts of *Nitrobacter* indicated the presence of at least two *a*-type cytochromes with absorption maxima at 594 nm (cytochrome a_1) and 609 nm (cytochrome *a*)(Van Gool and Laudelout 1966). Similar observations were earlier reported in a more detailed study by Straat and Nason (1965). Their solubilized nitrate reductase from *Nitrobacter agilis* has an absorption peak at 589 nm indicative of cytochrome a_1 which was split into two peaks, 583 and 587 nm, at liquid nitrogen temperature. The component at 587 nm was involved in nitrate reduction, but no function could be attributed to the 583 nm component. This partially purified nitrate reductase contained cytochrome *a* also and had a cytochrome oxidase activity.

The redox potential analysis of various cytochromes in cell-free extracts indicated the presence of at least five cytochromes in *Nitrobacter agilis*. They were cytochrome *c* ($E_{m,7}$+ 274 mV), cytochrome *a* (E_{m7} + 240 mV), cytochrome a_3 (E_{m7} + 400 mV) and two components of cytochrome a_1 (E_{m7} + 352 mV and E_{m7} + 100 mV) (Sewell *et al.* 1972). Similar findings were independently reported by Cobley (1973) (cited in Aleem 1978).

More recently the *a*-type cytochrome oxidase system of *Nitrobacter agilis* was purified by DEAE-cellulose chromatography (Yamanaka *et al.* 1979). The purified enzyme had two subunits with molecular weights of 40,000 and 27,000. The enzyme oxidized rapidly the reduced cytochrome *c*.

2.3.2 Cytochrome *b*: Little is known about cytochrome *b* of *Nitrobacter*. Despite their exhaustive spectrophotometeric as well as inhibitor studies Van Gool and Laudelout (1966) could not detect cytochrome *b* in *Nitrobacter*. The enzyme preparations of nitrate reductase solubilized from *Nitrobacter agilis*, however, was thought to contain a *b*-type cytochrome (Straat and Nason 1965). These workers compared their nitrate reductase with the so-called respiratory type nitrate reductase from *E. coli*, which was reported to possess cytochrome b_1 (Iida and Yamasaki 1960, Itagaki *et al.* 1961).

The oxidation of NADH was inhibited by rotenone, antimycin and 2-n-heptyl-4-hydroxyquinoline N-oxide (HOQNO) implicating the participation of flavoproteins and possibly a cytochrome of *b*-type (Aleem 1968). Nitrite-reduced absorption spectra of cell-free extracts of *Nitrobacter agilis* suggested the presence of a cytochrome *b* (Aleem 1968). However, Wallace and Nicholas (1969) concluded that cytochrome *b* was not involved in nitrite oxidation.

Chaudhry *et al.* (1980) have isolated recently a cytochrome *b* and *c* complex from the soluble fraction of *Nitrobacter* cells and thus established the presence of *b*-type cytochrome in *Nitrobacter*.

2.3.3 Cytochrome *c*: A *c*-type cytochrome in *Nitrobacter* was discovered by Lees and Simpson (1957) and later characterized by Butt and Lees (1958). Although the isoelectric point of this cytochrome, pH 6.5 was different from that of mammalian cytochrome *c*, pH 10.65 (Tint and Reiss 1950), it was similar however to the latter on the basis of its redox potential and spectral properties (Butt and Lees 1958). Absorption spectra (Aleem and Nason 1959, Aleem 1968) and redox

potential (Sewell *et al.* 1972, Cobley 1973, Ingledew *et al.* 1974) for the *Nitrobacter* cytochrome *c* reported later support the original finding. Purified cytochrome *c* from *Nitrobacter* also exhibited similar spectral properties as well as redox potential (Ketchum *et al.* 1969).

Although only one *c*-type cytochrome was believed to be present in this bacterium, the absorption spectra reported in literature for cytochrome *c* reflect some doubts. For example the α peak at 552 nm observed by Zavarzin (1958) and Kiesow (1967) and 554 nm by Van Gool and Laudelout (1966) are different from 550 nm in the original reports (Lees and Simpson 1957, Ketchum *et al.*1969) and might be due to different types of cytochrome *c*. Moreover, a recent report of an electron transport component in the electron transport particles of *Nitrobacter* with an absorption maximum at 552.5 nm and a low redox potential (Cobley 1976b) is another indication of more than one *c*-type cytochrome in *Nitrobacter*.

2.3.4 Other electron transport components: Nitrite oxidation was found to be strongly inhibited by quinacrine but this inhibition could be reversed by flavin-adenine dinucleotide (FAD) (Van Gool and Laudelout 1966). In spite of a high redox potential of NO_2^-/NO_3^- couple these workers implicated flavin in nitrite oxidation by *Nitrobacter*. Such a possibility, however, was subsequently eliminated in view of the fact that the reduction of flavin by nitrite catalyzed by cell-free preparations from *Nitrobacter* was shown to be energy-dependent (Sewell and Aleem 1969).

The oxidation of NADH by cell-free extracts of *Nitrobacter* can be inhibited by rotenone and HOQNO. These inhibitors blocked the

electron flow between flavoproteins and the cytochrome system (Aleem 1968). Similar observations were recorded in the case of NAD^+ reduction by nitrite with the same inhibitors (Sewell and Aleem 1969). It was therefore proposed that flavoproteins are involved in NADH oxidation (Aleem 1968) as well as in the reduction of NAD^+ (Sewell and Aleem 1969). The former investigator also suggested the presence of ubiquinone in *Nitrobacter* as well.

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

Bovine serum albumin (Fraction V), horse heart cytochrome *c* (type III), 2-mercaptoethanol, ATP, NAD^+, NADH, NADPH, FMN, riboflavin ubiquinone (Q_{10}), Tris (Trizma Base ^{R}), urea, Tween-80, Triton X-100, Triton X-114, cholic acid (sodium salt), sodium deoxycholate L-ascorbate, sodium borohydride, sodium dithionite, SDS Molecular Weight Marker Kit were obtained from Sigma Chemical Co. St. Louis, Missouri. Superoxide dismutase was purchased from Miles Laboratories Inc. Kahkakee, Illinois. Ammonium sulfate ultrapure was supplied by Schwarz/Mann, Orangeburg, New York. Griess-Ilosvay reagents, GSH, EDTA, sodium azide, potassium cyanide and glass beads (100 mesh) were from B.D.H. Chemicals, England. Acrylamide, BIS, TEMED, SDS, ammonium persulfate, bromophenol blue, and Coommasie Blue were obtained from J.T. Baker, Phillipburg, New Jersey. Guaiacol was purchased from Eastman Kodak Co. Rochester, N.Y. Various types of Sephadex gel, octyl Sepharose CL-4B, DEAE-Sephadex, CM-Sephadex, Calibration Kits for molecular weight determination by gel filtration and prepacked G-25 columns were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE-32), CM-cellulose (CM-52) were from Whatman, Maidstone, Kent, England and diethyl dithiocarbamate and dialysing tubings from Fisher Scientific Co. Pittsburg, Pa. (USA). Various gases used in this study were obtained from Union Carbide Co., Canada. All other chemicals and reagents used were of reagent

grade and available commercially.

3.2 ORGANISM AND GROWTH CONDITIONS

Nitrobacter agilis was isolated by a method similar to that reported by Lees and Simpson (1957). After ensuring the purity of the enrichment culture (Gould and Lees 1960) and proper identification of the strain (Nelson 1931), the culture was maintained by successive transfers in fresh medium and used for further inoculations. Mass cultivation of the organism was performed in several 20 l carboys each containing 16 l sterilized medium (Cobley 1976a) at 28°C in the dark. Nitrite concentration was gradually increased (Lees and Simpson 1957) such that the highest application used was 65 mgions/1 NO_2^-. Oxygen requirements of the growing organism in each carboy were met by supplying air through two glass spargers. Sterilization of the air was ensured by allowing air to pass through a sterilized air filter before entering the spargers. The pH of the growing culture was maintained at pH 7.8 by a Radiometer pH Stat.

Actively growing cells (about to complete oxidation of the final aliquot of added nitrite judged by Griess-Ilosvay reagents) were harvested in a Sharples centrifuge (with a flow rate of 15 l h^{-1}). The cell paste was washed twice with 10 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 1mM GSH, 5 mM $MgCl_2$ and 300 mM sucrose. The cells were then suspended in the same buffer (1 g cells/ 5 ml, wet w/v) and stored at 0°C no longer than one week.

3.3 CONTINUOUS CULTURE PROCEDURE

Continuous cultures were grown by Chemostat (Baker 1968) in an arrangement similar to that photographically shown by Buchanan (1979) with minor modifications. A one liter Pyrex culture vessel with its outer surface painted black, able to contain 500 ml of working medium was used. The removable top of the vessel provided for the following: thermometer, pH electrode, oxygen electrode, titrant inlet, medium inlet, gas inlet and sampling tubing and an outlet on the side of the vessel.

The medium used for continuous cultures was the same as that for batch cultures with a dilution rate of 0.015 h^{-1}, which was maintained by a precision peristaltic pump (Watson-Marlow Ltd., England). The composition and flow rate of the mixture of gases was controlled by flow-meters (Manostat Corporation, New York). The oxygen levels of the cultures were measured by a sterile Pb/Ag galvanic membrane electrode (L.H. Engineering Co. Ltd., Bells Hills; Stoke Poges, Bucks, England) attached to an oxygen meter (Western Biological, Sherborne, Dorset) equipped with a Rustrak Chart recorder. The oxygen meter was standardized for 0% and 20% O_2 with nitrogen gas and with air only passing into the culture vessel respectively. The pH of the cultures was maintained by a Radiometer II titrator at pH 7.8 using a Radiometer combined glass electrode.

The temperature of the culture was automatically maintained at 28°C by an I.R. lamp controlled by a thermistor immersed in the culture.

The culture vessel assembly with the exception of the pH electrode was autoclaved at 15 psi, (120°C) for 45 min, prior to inoculation. The pH electrode was sterilized by immersion in 2-propanol for 30 minutes after its standardization with buffer. The continuous cultures were started as batch cultures.

3.4 PREPARATION OF ELECTRON TRANSPORT PARTICLES (ETP) AND SUPERNATANT FRACTION (F_0).

All the procedures used for the isolation and purification of enzymes were performed at 0-4°C.

Nitrobacter cell suspension was homogenised (in Bronwill "MSK" Mechanical Cell Homogenizer, Bronwill Scientific, Rochester, N.Y.) at 0°C by a method similar to that reported by Kiesow (1964) and Cobley (1976a) except that the homogenizer treatment consisted of eight 15 second pulses separated by 10 second intervals. The cell homogenate was centrifuged at 15,000 g for 15 min to remove unbroken cells. The supernatant obtained was further centrifuged at 144,000 g for 4 h. The Pellet (ETP) was used for the isolation of cytochrome oxidase and a copper-iron electron transfer protein, whereas the supernatant (F_0) was utilized for the isolation of *c*-type cytochromes, cytochrome *b* and other enzymes reported in this study.

3.5 ISOLATION OF *c*-TYPE CYTOCHROMES.

The supernatant fraction (F_0) was immediately applied to a Whatman DE-32 (DEAE-cellulose prepared according to the instructions of manufacturer) column (2.5 x 40 cm), previously equilibrated with

the buffer used for cell washing. The column was washed with the same buffer until the effluent became colorless. The red fractions thus obtained were pooled (F_I), and used for the isolation of cytochrome *c*-550, *c*'-type cytochrome and purple pigments; cytochrome *c*-553, cytochrome *b* and other enzymes were adsorbed on the DEAE-cellulose column.

3.6 PURIFICATION OF CYTOCHROME *c*-553

To isolate cytochrome *c*-553, the DEAE-cellulose column was first washed with 20 mM potassium phosphate, pH 7.8 and then with 50 mM potassium phosphate, pH 7.5. Cytochrome *c*-553 was eluted with 10 mM potassium phosphate pH 7.5. To the combined red fractions of eluate containing cytochrome *c*-553 ammonium sulfate was added to a concentration of 40% saturation. After centrifugation at 15,000 g for 10 min, the pellet was discarded and the ammonium sulfate concentration was raised to 80% of saturation in the supernatant. After an hour the supernatant was again centrifuged as above and the pellet containing cytochrome *c*-553 was dissolved in a minimum amount of 5 mM Tris-HCl, pH 8.5. This fraction was dialyzed overnight against 5 mM Tris-HCl, pH 8.5.

The dialyzed fraction was then applied to another DEAE-cellulose column (1 x 6 cm) preequilibrated with Tris buffer. The column was washed with 50 mM Tris-HCl, pH 7.5 and cytochrome *c*-553 was eluted with the same buffer to which 50 mM KCl had been added. Colored fractions were pooled and further purified and concentrated by ultrafiltration (Millipore Immersible CX, single

use Ultrafiltration Unit - NMWL 10,000).

The concentrated fraction of cytochrome *c*-553 was applied to a Sephadex G-100 column (2.5 x 40 cm) in 50 mM Tris-HCl buffer (pH 7.5) and eluted with the same buffer. Cytochrome *c*-553 fractions were again combined, concentrated as above and passed through a second Sephadex G-100 column in 50 mM Tris-HCl and 100 mM KCl (pH 7.5).

Cytochrome *c*-553 thus obtained was used for further studies.

3.7 PURIFICATION OF CYTOCHROME *c*-550

The fraction F_1 from the DEAE-cellulose column (see above) was dialyzed overnight against 5 mM potassium phosphate, pH 6.5 and loaded on a CM-cellulose (CM-52) column (4 x 6 cm) equilibrated with 5 mM potassium phosphate buffer, pH 6.5. Cytochrome *c*-550 was adsorbed on top of the column, while the fraction eluted with the same buffer contained cytochrome $c(F_2)$. The CM-cellulose column was then washed extensively with 10 mM potassium phosphate, pH 7.5. When the concentration of buffer was increased to 20 mM phosphate, the adsorbed cytochrome *c*-550 started moving down slowly. When the red band reached the middle of the column, the cytochrome *c*-550 was eluted as a single dark red band with 30 mM phosphate buffer, pH 7.8. The cytochrome *c*-550 containing fractions were diluted three fold with distilled water and adjusted to pH 6.5 with dil. HCl. This combined fraction was then loaded on a CM-Sephadex column (2 x 6 cm). The column was extensively washed with 20 mM phosphate, pH 7.5. With the increase in buffer concentration

to 50 mM phosphate, a red band of cytochrome *c*-550 started moving down the column; however, the elution rate was quite slow. When the red band was in the middle of the column, it was finally eluted with 100 mM phosphate, pH 7.5. This fraction was applied to a Sephadex G-100 column (2.5 x 80 cm) preequilibrated and eluted with 100 mM phosphate, pH 7.5. The cytochrome *c*-containing red colored fractions eluted from G-100 were concentrated by ammonium sulfate precipitation. The fraction obtained between 45-95% saturation of ammonium sulfate was desalted by passing through a Sephadex G-25 column in 50 mM Tris-HCl, pH 7.5 with 100 mM KCl. Cytochrome *c*-550 fraction was finally passed through a Sephadex G-75 column (2.5 x 40 cm) in 50 mM Tris-HCl containing 100 mM KCl and used as a purified fraction.

3.8 PURIFICATION OF A *c'*-TYPE CYTOCHROME

The fraction that passed through the CM-cellulose column (F_2) was adjusted to pH 8.5 with a dilute alkali and applied to a DEAE-Sephadex column (2.5 x 6 cm) previously equilibrated with 10 mM potassium phosphate, pH 8.5. Cytochrome *c'*-type hemoprotein was adsorbed on the DEAE-Sephadex. The column was washed with 50 mM phosphate, pH 7.8 and a yellow brown band of *c'*-type cytochrome was eluted with 100 mM potassium phosphate buffer (pH 7.0). The yellow brown fraction gave a spectrum with a flat-top peak at 549-555 nm. The fraction was treated with an ammonium sulfate concentration of 40% saturation then centrifuged at 15,000 g for 10 min. The supernatant was adjusted to 90% saturation of ammonium

sulfate and recentrifuged. The pellet obtained contained *c*-type cytochrome and was suspended and dialyzed against 50 mM Tris-HCl, pH 7.5. The dialyzed fraction was passed through a Sephadex G-150 column (2.5 x 40 cm) in Tris-HCl, and eluted with the same buffer. The fractions displaying 402 nm absorption were combined and concentrated by ultrafiltration as reported in case of cytochrome *c*-553.

The concentrated cytochrome fraction was finally passed through a Sephadex G-100 column (2.5 x 40 cm) in 50 mM Tris-HCl, pH 7.5 containing 100 mM KCl. The *c*-type cytochrome obtained from the eluate of this column was used as purified preparations.

3.9 PURIFICATION OF CYTOCHROME OXIDASE

Electron transport particles (ETP) obtained after 144,000 g centrifugation of cell homogenate were suspended at a concentration of 40 mg/ml in 100 mM Tris-SO_4, pH 7.8 containing 1 mM EDTA and 1.5% sodium cholate (detergent buffer). The suspension was subjected to sonic disintegration for 10 min at 10 kc (Sonic Oscillator, model DF101 Raytheon Manufacturing Co., Waltham, Mass., USA) and allowed to stand for one hour while being stirred slowly. Most of the non-specific protein was precipitated out with ammonium sulfate (40% saturation). After centrifugation at 15,000 g for 15 min, the concentration of ammonium sulfate in the supernatant (cytochrome oxidase fraction) was increased to 70% of saturation. The precipitate obtained after centrifugation (as above) was homogenized in three volumes of the detergent buffer containing 10%

saturation of ammonium sulfate and 0.1 M KCl then centrifuged at 40,000 g for 30 min. The resulting supernatant was applied to an octyl-Sepharose column (2.5 x 12.5 cm) equilibrated with detergent buffer containing 20% saturation of ammonium sulfate and 0.1 M KCl; the column was eluted at a flow rate of 2-3 ml per min with the same buffer mixture. The cytochrome oxidase and a low molecular weight electron carrier protein (Copper-Iron protein) were bound to the column, whereas most other pigments were eluted.

The column was washed with detergent buffer in which cholate concentration was increased to 5% and 5% saturation of ammonium sulfate was also included until the effluent of the column appeared colorless. The elution buffer was then changed to 0.1 M Tris-SO_4, pH 7.8 containing 1 mM EDTA, 0.1 M KCl and 0.25% Tween 80. The inclusion of Tween 80 was found necessary to assure complete removal of cholate from the column since cholate-containing cytochrome oxidase was found to lack activity.

Cytochrome oxidase was finally eluted with 50 mM Tris-SO_4, pH 7.8 containing 1% Triton X-114 and 1 mM EDTA with the reduced flow rate of 0.5 ml/min. A sharp green band moving slowly in the column was collected, concentrated and further purified by repeated ultrafiltration (the same system as used for *c*-type cytochrome).

3.10 ISOLATION OF THE COPPER-IRON ELECTRON TRANSFER PROTEIN.

Preparation of the homogenate of electron transport particles and its subsequent fractionation with ammonium sulfate were performed as reported for the isolation of cytochrome oxidase.

The ammonium sulfate precipitate pellet (40-70% saturation) was homogenized with three volumes of 0.1 M Tris-SO_4, pH 7.8 containing 1.5% cholate (w/v), 1 mM EDTA (detergent buffer) and ammonium sulfate (15% saturation). The suspension was gently stirred for an hour then centrifuged at 40,000 g for 30 min. The supernatant was applied to an octyl-Sepharose CL-4B column (2.5 x 12.5 cm) preequilibrated with the detergent buffer plus 20% saturation of ammonium sulfate and 0.1 M KCl. The column was washed until the effluent was colorless with the same buffer as used for equilibrating the column except that concentration of cholate was increased to 2% and ammonium sulfate decreased to 5% of saturation. Now, cytochrome oxidase and copper-iron protein with a small amount of contaminating cytochrome *c* were the only colored pigments left in the column.

The column was finally eluted with a buffer containing 0.1 M Tris-SO_4, pH 7.8, 1 mM EDTA and 5% cholate. This colored pigment (yellow-green) was next treated with ammonium sulfate (50% saturation) to precipitate most of the detergent. After centrifugation at 15,000 g for 10 min the ammonium sulfate concentration of the supernatant was raised to 80% saturation. The pellet obtained upon centrifugation was resuspended in 50 mM Tris-HCl, pH 7.8 containing 2 mM EDTA. This fraction was then repeatedly passed through a G-25 column equilibrated with 50 mM Tris-HCl, pH 7.8 plus 1 mM EDTA in order to desalt the copper-iron protein.

The fraction containing copper-iron protein was finally

passed through a Sephadex G-100 or G-75 column in 50 mM Tris-HCl and 0.1 M KCl (pH 7.5). A yellow colored fraction thus obtained was characterized for its physico-chemical properties.

3.11 ISOLATION OF CYTOCHROME *b*

The supernatant of 144,000 g (F_0) was applied to a DEAE-cellulose column (as above). The column was washed with 50 mM potassium phosphate, pH 7.5 containing 1 mM GSH until the effluent appeared colorless. Elution buffer was then changed to 0.15 M potassium phosphate (pH 7.5) and the column was washed with 1½ bed volumes of buffer. Cytochrome *b*-containing fractions (light yellow) were finally eluted with 0.2 M potassium phosphate and 0.1 M KCl (pH 7.5). The yellow fractions were combined and subjected to ammonium sulfate fractionation. The pellet obtained between 50 and 90% saturation of ammonium sulfate was suspended in 50 mM Tris-HCl, 100 mM KCl buffer (pH 7.5) and passed through a Sephadex G-25 column preequilibrated with the same buffer. The cytochrome *b*-containing fraction was finally passed through a Sephadex G-100 column (2.5 x 40 cm) in the same buffer. Cytochrome *b* was recovered from fractions with 405 nm absorption. After concentration by ultrafiltration and dialysis (as described for cytochrome *c*-553), the fraction was used for further studies.

3.12 ISOLATION OF NITRITE: CYTOCHROME *c* REDUCTASE

The fraction F_0 was applied to a DEAE-cellulose column as described above. The column was washed with 50 mM potassium phosphate, pH 7.0 until the effluent appeared colorless. The

elution buffer was then changed to 0.1 M potassium phosphate, pH 7.0 containing 0.2 M KCl. A yellow band moved down the column and was collected separately. This fraction had the activity of nitrite: cytochrome *c* reductase.

3.12.1 Isolation of yellow pigment

The yellow fraction containing nitrite: cytochrome *c* reductase activity was used for the isolation of yellow pigment as follows. The yellow fractions were pooled and ammonium sulfate was added to a concentration of 90% saturation. The mixture was centrifuged at 15,000 g for 10 min. The supernatant had almost all of the yellow pigment. It was then lyophilized, dissolved in water, and desalted in a Sephadex G-25 column. After repeating the above steps for several times, the spectrophotometric properties of yellow pigment were investigated.

3.13 ISOLATION OF NADH: CYTOCHROME *c* OXIDO-REDUCTASE

The DEAE-cellulose column after loading with F_0 was first washed with 20 mM potassium phosphate, pH 7.8 and then eluted with a linear gradient of potassium phosphate buffer (20 mM to 100 mM, pH 7.8). NADH cytochrome *c* oxido-reductase activity was found in the red fractions. These fractions were combined and passed through a Sephadex G-25 column in 5 mM potassium phosphate, pH 8.0. The desalted fraction was applied to a small DEAE-cellulose column (2 x 6 cm) previously equilibrated with 10 mM potassium phosphate, pH 8.0. The fractions which passed through this column contained red pigment and were precipitated with 100%

saturation of ammonium sulfate. After centrifugation at 15,000 g for 10 min, the supernatant was discarded and the pellet suspended in two volumes of 20 mM potassium phosphate, pH 7.8. Ammonium sulfate was added to this fraction to a concentration of 50% saturation and it was centrifuged as above. The pellet obtained contained red pigment and was suspended in 20 mM potassium phosphate, pH 7.8. After passing through a Sephadex G-25, the red fraction was used for the study of NADH cytochrome *c* oxidoreductase activity.

The red pigment from the above fraction was extracted with chloroform. After evaporating the chloroform, the red pigment was resuspended in ethanol for spectral studies.

3.14 ISOLATION OF PURPLE PIGMENT

An impure fraction of cytochrome *c*-550 (F_{1A}) was dialyzed overnight against 5 mM potassium phosphate buffer, pH 6.0. The dialyzed fraction was then applied to a DEAE-cellulose column (2 x 6 cm) in 5 mM potassium phosphate, pH 6.0. The column was first washed with 10 mM (pH 7.5) followed by 15 mM (pH 7.8) potassium phosphate. When all the cytochrome *c*-550 was washed away from the column, the CM-cellulose in the column appeared purple in color. This purple pigment was eluted with 20 mM potassium phosphate, pH 8.5 as a single band.

3.15 ISOLATION OF UBIQUINONE

The wet pellet of washed *Nitrobacter* cells was suspended in cold acetone (-20°C) containing 0.2% $MgCl_2$ (25 mg cell/ml). The suspension was stirred slowly overnight at 0°C in dark and centrifuged at 10,000 g for 10 min. The supernatant was saved and pellet was twice extracted with cold acetone as above. Acetone extracts were combined and dried in a rotary evaporator.

The dried material was dissolved in a minimum amount of acetone and applied on silica gel plates (20 x 20 cm glass plates coated with 2 mm thick silica gel, Macherey, Nagel Co. Germany). The plates were then developed in diethyl ether-petroleum ether (3:2 v/v). The yellow colored UV-absorbing band following the solvent front was eluted in acetone. After concentrating, it was applied to a second silica gel plate. This time the plates were developed in a chloroform-heptane mixture (1:3 v/v). There appeared six bands including one major band of ubiquinone. The three yellow colored UV absorbing bands were eluted separately in ethanol. The ubiquinone-containing fraction (major band) was identified by its characteristic UV absorption (275 nm). This fraction was concentrated and once again applied to a silica gel plate. The plate was twice developed in chloroform-heptane (1:9 v/v) and finally in benzene. The yellow colored band with absorption maxima at 275 nm was extracted in ethanol. Purity of the fraction was tested by comparing Rf values with known ubiquinone (Q_{10}) on a silica analytical TLC plage (Polygram ^{R} Sil G/UV254 developed

in either chloroform-heptane (4:1 v/v) or benzene.

3.16 PROTEIN DETERMINATION

Protein contents were determined according to Lowry *et al.* (1951). Bovine serum albumin was used as the standard.

3.17 PHOSPHOLIPID DETERMINATION

Phospholipid contents were estimated according to Raheja *et al.* (1973). Phosphatidylcholine was used as a standard and the color intensity was recorded by spectrophotometer at 710 nm, 30 min after the addition of chromogenic solution.

3.18 ESTIMATION OF METAL IONS

Both Cu and Fe were determined using a Varian AA5 atomic absorption spectrophotometer. Samples were either digested with perchloric acid after ashing with H_2SO_4 and HNO_3 or undigested samples were atomized in a carbon rod (Varian model-90) with corrected deuterium background.

3.19 DETERMINATION OF OXIDATION-REDUCTION POTENTIAL

The midpoint redox potential at pH 7.0 ($E_{m,7}$) of cytochrome *c*-550 was determined by titration with a $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox system according to the method of Davenport and Hill (1952) under aerobic conditions.

The experiments for the estimation of midpoint redox potential at pH 7.0 ($E_{m,7}$) of the *c'*-type cytochrome were carried out anaerobically using ferri-ferro-oxalate system (Hill 1954) in a 1 cm

light path cuvette with rounded neck. A sufficient quantity of cytochrome (≃10 nmole/ml) was suspended in 0.5 M potassium oxalate, 0.1 M potassium phosphate buffer at pH 7.0 and 1 mM ferric iron to give a final volume of 2 ml. The cuvette containing this mixture was then sealed with a Suba-seal rubber stopper and bubbled with argon gas through a needle for half an hour to ensure complete removal of oxygen. Then the argon inlet needle was pulled up just above the surface of cytochrome mixture in the cuvette and pressure of argon gas flow was maintained at 4 lbs/inch with a pressure guage. The mixture was then titrated with argon-saturated 0.5 M ferrous iron from a gas-tight microsyringe. The contents of the cuvette were gently stirred to achieve proper mixing after each addition of ferrous iron. Absorption spectra were measured five minutes after each addition of ferrous iron. The standard redox potential of the cytochrome was calculated from the standard equation (Hill 1954).

3.20 MOLECULAR WEIGHT DETERMINATION

Molecular weights of the purified fractions were determined by gel filtration on Sephadex G-100 or G-75 column using a method similar to that reported by Andrew (1964). In most cases this step was included in the final purification of different proteins reported in this study. Before or after running the unknown samples the columns were standardized using proteins from Pharmacia molecular weight estimation kit.

3.21 POLYACRYLAMIDE GEL ELECTROPHORESIS

(i) Polyacrylamide gel electrophoreses under non-dissociating conditions using 8-10% gel concentration were performed according to Davis (1964) with an exception in staining and destaining of the gel. After electrophoresis, the gels were fixed overnight in 50% TCA (w/v) and stained in 0.1% Coomassie Blue in 50% TCA for two hours. Destaining was carried out by diffusion in a solution of 10% acetic acid (v/v) and 20% ethanol (v/v).

(ii) SDS polyacrylamide gel electrophoreses were performed by the method of Laemmli (1970) with minor modification in gel staining (as used above). Gels were stained for two hours, four hours, and overnight for cytochrome *c*-550 and *c*-553, copper-iron electron transfer protein and *c*′-type cytochrome respectively.

(iii) SDS + Urea polyacrylamide gel electrophoreses were carried out by following the procedure of Downer *et al*. (1976) with some modifications. 6 M urea in 10% acrylamide was used. The protein samples were suspended in 62.5 mM Tris-HCl, pH 6.8 containing 2.0% SDS, 5% 2-mercaptoethanol and 6 M urea and were dissociated by incubating for two min in a boiling water bath prior to electrophoresis. After electrophoresis the gels were fixed in 50% TCA overnight and stained in 0.1% Coomassie Blue in TCA for a further 12 hours. Destaining was performed as stated above.

Molecular weights both in case of SDS and SDS + urea were estimated by running a parallel gel with standard proteins.

Densitometric traces of stained gels were carried out in a Densicord, Model 542, Photovolt Co., N.Y. using a blue filter No. 485.

3.22 ISOELECTRIC FOCUSING

Isoelectric focusing was done by the method of O'Farrell (1975) using carrier ampholytes. Ampholines with pH ranges 5-8 and 3.5-10 (4:1), 5-8, 3.5-10 and 2-11 (2:2:1) and 5-7 and 3.5-10 (4:1) were used for cytochrome *c*-553, *c*-550 and *c'*-type cytochrome respectively. The final concentration of ampholine used in each case was 2%. Isoelectric points were estimated by comparing a stained gel after isoelectric focusing with the pH gradient developed during isoelectric focusing in a blank gel run parallel to the sample gel. Alternatively the pH of the visible colored spot of the cytochrome in the gel was directly measured using a surface pH electrode (Ingold Electrodes Inc. Lexington, Mass. U.S.A.) attached to a pH meter.

3.23 AMINO ACID ANALYSES

Amino acid analyses were performed with a Technicon NC-2P single column amino acid analyzer. The samples were hydrolyzed for 20 h in vacuo at 110°C in constant boiling HCl (Moore and Stein, 1963). Cysteine was determined as cysteic acid after performic acid oxidation (Hirs 1956).

3.24 AUTOMATIC SEQUENCING

An attempt was made to determine a partial amino acid sequence starting at the N-terminal end of cytochrome *c*-550 by automatic sequencing as follows.

Edman degradations were performed automatically in a Beckman 890C Sequencer by standard methods (Niall, 1973). The programme

used was Beckman Catalogue No. 072172C, with 1 M Quadrol as buffer. The butyl chloride contained 0.1% ethylmercaptan or 15 mg/L Cleland's reagent to protect the thiazolinones of serine and threonine. Butyl chloride extracts were divided into two roughly equal proportions. One-half was reduced to dryness with a stream of nitrogen, heated 10 min at 80°C in 1 M HCl to convert the thiazolinones to phenylthiohydantoins, and the aqueous acid extracted twice with 0.7 ml proportions of ethyl acetate. The ethyl acetate extracts were again taken to dryness, and the residues examined for phenylthiohydantoins by gas chromatography (Pisano and Bronzert, 1972) and thin layer chromatography on Whatman silica gel plates, type LK6DF, using the system of Jeppsson and Sjöquist (Jeppsson and Sjöquist 1967). The other half of each butyl chloride extract was taken to dryness in a hydrolysis tube, covered with 1.0 ml of a freshly prepared solution of $SnCl_2$ (1 mg/ml) in constant boiling HCl, and the tube evacuated and sealed. After the hydrolysis of thiazolinones for 4.0 h at 150°C, the tubes were opened and dried *in vacuo* over solid NaOH. The residues, which contained free amino acids derived from the thiozolinones, were subjected to amino acid analysis. Most amino acids are recovered in good yield, but serine is converted into alanine in low yield, tryptophan to glycine and alanine in low yield, and threonine quantitatively to α=aminobutyric acid (Mendez and Lai 1975).

A known amount of the phenylthiohydantoin of norleucine (about 50 nmole) was added to each tube of the Sequencer fraction collector before beginning a run, and the recovery of norleucine in the amino

acid analyzer was used to calculate the yield of the amino acid obtained at each cycle of the Edman degradation.

All chemicals used in the Sequencer were Sequencer grade, from Beckman. The standard phenylthiohydantoins were from Pierce.

3.25 PREPARATION OF PYRIDINE HEMOCHROME

Cytochrome preparations were extracted with acid acetone (Rieske 1967). The residue fractions were used for heme *c* determination. The acid acetone extracts were dried on a rotary evaporator at 20°C and were used for the determination of heme *a* or/and *b*. In each case the fractions were diluted in a mixture containing equal volume of pyridine and 0.1 N NaOH and were reduced with 2 mg dithionite per ml in order to obtain the pyridine ferrohemochrome.

3.26 SPECTROPHOTOMETERY

Absorption spectra were measured in a Shimadzu MPS-50L or a Unicam SP700 spectrophotometer at room temperature using a cell with 1 cm light path. A Gilford Spectrophotometer was also routinely used during the course of protein purifications for the estimation of color intensity or protein contents.

3.27 FLUORIMETERY

Fluoresence spectra were recorded with an Aminco-Bowman Spectrofluorimeter, equipped with an OmniScribe$_{TM}$ recorder (Houston Instrument, Austin, Texas, USA).

3.28 MASS SPECTROSCOPY

Mass spectral studies were performed in a Finnigan 1015RF Quadrupole Mass Spectrophotometer. The electron energy used was 70 ev.

3.29 IR SPECTROPHOTOMETRY

Infrared spectra were measured on Unicam SP1000 Infrared Spectrophotometer. The samples were prepared in "Nujol".

3.30 NMR STUDIES

Proton spectra were obtained on a Bruker WH-90DS spectrometer (90.02 MHZ), equipped with a Nicolet-1180 Computer, the NTC-FT-1180 software package and quadrature phase detection.

Samples were prepared by twice freeze drying from D_2O. The dried samples were re-suspended in D_2O and examined in 5 mm outer diameter tubes.

3.31 NITRITE OXIDIZING ACTIVITY

The nitrite oxidation was routinely assayed by measurement of oxygen consumption in a thermostated vessel (25°C) equipped with a Teflon-covered Clark Oxygen Electrode (Gilson oxygraph, G.M.E., Wisconsin, U.S.A.) with an appropriate polarization circuitry. The reaction mixture contained, unless otherwise mentioned, 10 mM potassium phosphate, pH 7.8; 5 mM $MgCl_2$; cells or ETP and distilled water to make up final volume of 1.5 ml. The reaction was started by the addition of nitrite solution (final concentration, 5 mM) in microliter quantity by a Hamilton microsyringe. The volumes of

cells or ETP used for assay were 60 µl (40 mg/ml). The activity was expressed in nmoles O_2 consumed per minute under the above standard conditions.

3.32 CYTOCHROME OXIDASE ACTIVITY

The enzyme activity was measured spectrophotometrically at room temperature in a cuvette of 1 cm light path, as the rate of decrease in absorbance at 550 nm using reduced cytochrome *c* (horse heart or *Nitrobacter*). The reaction mixture contained 50 mM Tris-SO_4, 20 µM cytochrome *c*, 0.5% Tween 80 and enzyme with appropriate pH. The reaction was initiated with the addition of enzyme. The initial rate of oxidation of cytochrome *c* (nmoles min^{-1}) was measured as the activity of cytochrome oxidase.

3.33 NITRITE: CYTOCHROME *c* REDUCTASE ACTIVITY

The enzyme activity was measured by following the rate of reduction of cytochrome *c* (horse heart) at 550 nm in a spectrophotometer at 25°C. The assay system in a cuvette of 1 cm light path contained 50 mM potassium phosphate, pH 7.5, 30 µM oxidized cyt. *c*, nitrite, and enzyme. The reaction was started with the addition of enzyme. The initial rate of reduction of cytochrome *c* (nmol min^{-1}) was measured as the activity of enzyme.

3.34 NADH: CYTOCHROME *c* OXIDOREDUCTASE ACTIVITY

The enzyme activity was determined by the following the rate of reduction of cytochrome *c* (horse heart) at 550 nm or NADH oxidation at 340 nm in a spectrophotometer using a cuvette of 1 cm light path

at 25°C. The assay mixture contained 50 mM potassium phosphate (pH 7.8), 20 µM oxidized cytochrome *c*, 0.1 mM NADH and enzyme.

3.35 NH_2OH: CYTOCHROME *c* REDUCTASE ACTIVITY

The enzyme activity was measured spectrophotometrically at room temperature in a cuvette of 1 cm light path, as the rate of increase in absorbance at 550 nm using oxidized cytochrome *c* (horse heart). The assay system contained 50 mM potassium phosphate, pH 7.8, (or otherwise mentioned), 2 mM NH_2OH, 20 µM cytochrome *c* and enzyme. The reaction was started with the addition of enzyme.

3.36 PEROXIDASE ASSAY

The assay for peroxidase activity (George 1953) was performed spectrophotometrically in a cuvette of 1 cm light path, as the increase in absorbance at 470 nm using guaiacol as electron donor.

The assay system contained 50 mM potassium phosphate (pH 7.5), 100 µM guaiacol, enzyme; the reaction was started by the addition of H_2O_2 (0.4 µM).

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

The study of cytochromes of *Nitrobacter* is organized in this chapter into four parts. Part I provides a brief description of the organism used for the study of *Nitrobacter* cytochromes and some of the observations obtained during the growth of the organism. Part II reports on the isolation, purification and some properties of *c*-type cytochromes. Part III deals with purification and some physico-chemical properties of cytochrome oxidase and a copper-iron electron transfer protein. Part IV reports the isolation of cytochrome *b* and ubiquinone as well as some oxido-reductases.

PART I

GENERAL PROPERTIES OF THE ORGANISM

4.2 PART I GENERAL PROPERTIES OF THE ORGANISM

4.2.1 Results

4.2.1.1 Isolation and Identification

Nitrobacter was isolated from garden soil by an enrichment technique as described in Materials and Methods. The isolated nitrite-oxidizing bacterium was found to be motile, which suggested that the organism was *Nitrobacter agilis* rather than *Nitrobacter winogradskyi* (Nelson 1931). Electron micrographs of the pear shaped nitrifying bacteria do not show the flagellum (Figure 1A). The flagellum of *Nitrobacter* is known to be fragile and is easily lost by the ordinary preparative manipulation of samples (Pan 1971).

In order to grow normal cells and determine the optimum growth conditions, various parameters were examined in detail. Some of the important observations will be discussed below.

4.2.1.2 Effect of O_2 on the Growth and Structure of the Organism

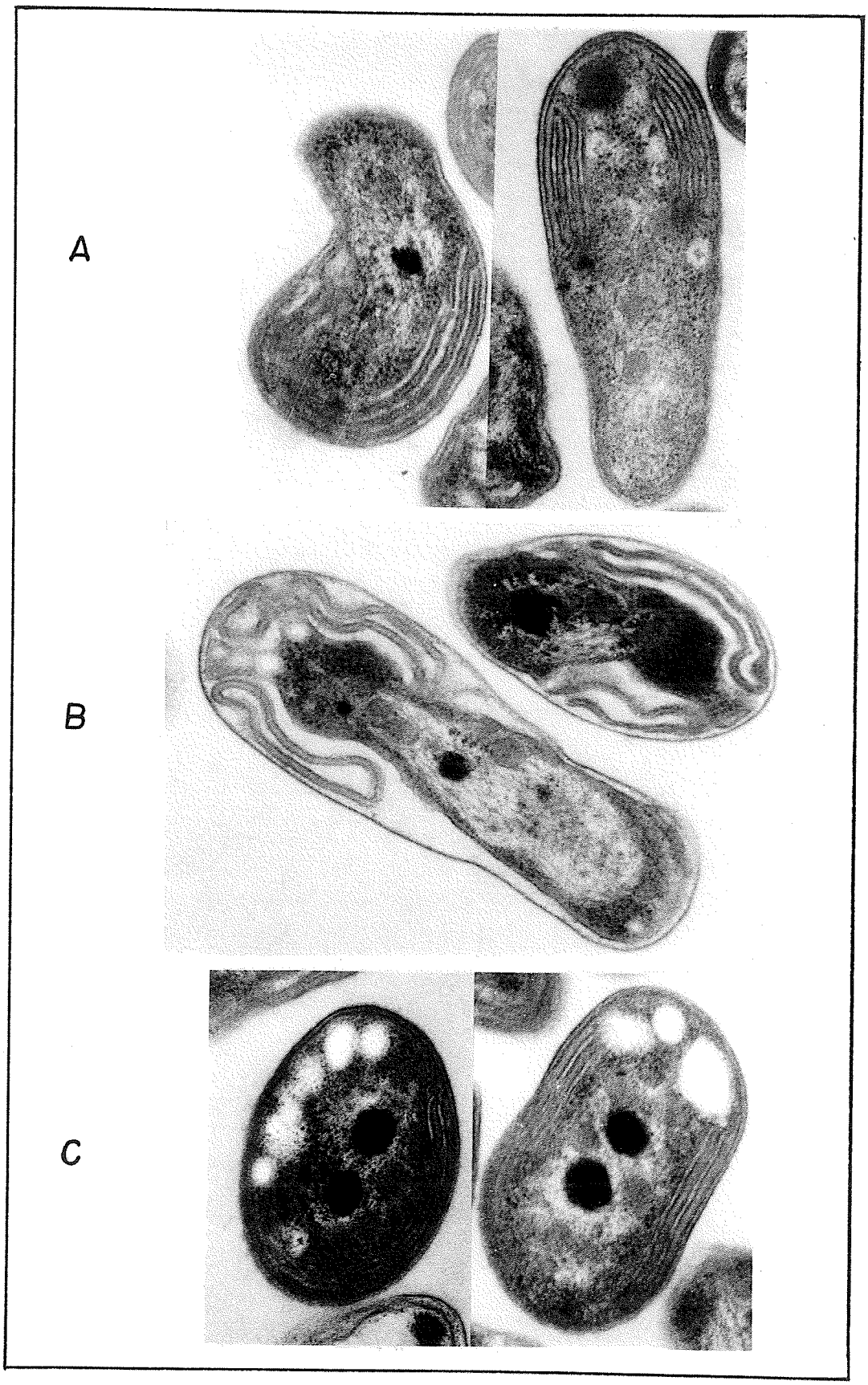
The experiments conducted in continuous culture under controlled conditions revealed that *Nitrobacter* grew poorly below 10% O_2 and above 15% O_2. The range of O_2 concentration for optimum growth was 10-15%. Electron micrographs of *Nitrobacter* cells growing at three different levels of O_2 (Figure 1) indicate considerable structural variations. Cells at 10% O_2 had the normal pear shape appearance with a characteristic lamellar array of membranes located peripherally at the swollen end of the cell (Figure 1A). In contrast, the structure of cells grown at 5% O_2, appeared

Figure 1. Electron micrographs of *Nitrobacter agilis* grown in continuous culture under different levels of O_2 tension. Final magnification, x 50,000 (approx.).

A: Cells grown in 10% oxygen.

B: Cells grown in 5% oxygen.

C: Cells grown in 20% oxygen.


A
B
C

different and cell membranes seemed to have shrunk losing their integrity (Figure 1B). On the other hand cells grown with 20% oxygen contained circular structures which did not take up the stains (Figure 1C).

4.2.1.3 Effect of NO_2^- on the Growth and Structure of the Organism

Nitrite at high concentrations has been reported to inhibit its own oxidation by *Nitrobacter* (Lees 1955).

The optimal growth of the nitrifying bacteria under the conditions mentioned in Materials and Methods was obtained with a 65 mgions/l final concentration of NO_2^-. Higher levels of nitrite caused reduction in growth rate. When cultures growing at the optimal rate were transferred to a medium with high nitrite (>85 mM), the nitrite-oxidizing activity of cells was progressively decreased with increasing periods of incubation.

Photomicrographs of cells incubated in high nitrite-containing medium indicated a loss of membrane layers within the cells (Figure 2A).

4.2.1.4 Effect of NO_3^- on the growth and structure of the organism

Nitrate also inhibits the oxidation of nitrite by *Nitrobacter* (Lees 1955).

When nitrate concentrations were increased (>85 mM) in cultures growing at the optimal growth rate, the nitrite oxidation as well as the growth rate were decreased. The effect of nitrate was observed to be small compared with nitrite. The structure of such

Figure 2. Electron micrographs of *Nitrobacter agilis* incubated in high concentrations of nitrite and nitrate. Final magnification, x 50,000 (approx.).

A: Cells incubated in 85 mM nitrite.

B: Cells incubated in 85 mM nitrate.

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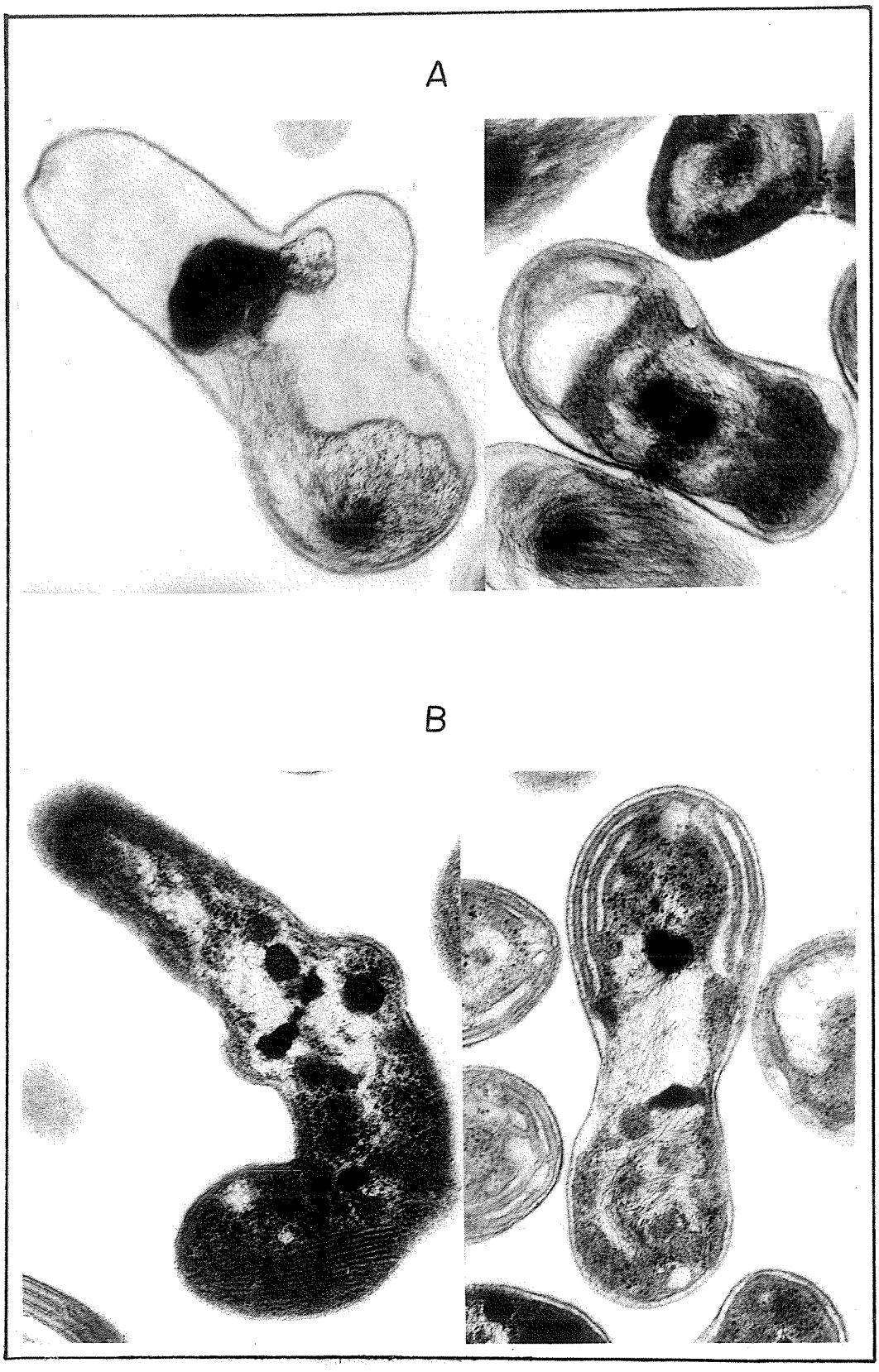

A
B

cells (Figure 2B) also looked more normal than that of the cells incubated in 85 mM nitrite although the shape was somewhat distorted.

4.2.1.5 Nitrite-oxidizing activity of cells and ETP

Actively growing cultures yielded about 2.0 g cells (wet weight) per 10 l of culture upon harvest. The nitrite-oxidizing activities of cells and ETP are shown in Table 1. The activities of cells and ETP were measured polarographically under the standard assay condition as mentioned in Materials and Methods. Approximately 15% of the activity present in the cells was recovered in ETP.

Table 1. Nitrite oxidizing activity of *Nitrobacter* cells, and electron transport particles (ETP)

Material	Wet Weight (mg)	Total Activity (nmole O_2 consumed/min)	Specific Activity (nmole O_2 consumed/min/mg)
Cells (washed)	1,000	29,000	29
ETP[a]	435	4,350	10

The assay procedure was as described in Materials and Methods.

[a]Amount of ETP obtained from 1 g of cells (see the Materials and Methods for details).

4.2.2 DISCUSSION

The nitrite-oxidizing bacterium isolated here oxidized nitrite rapidly under standard growth conditions. The activities of cells and of ETP obtained from these cells are shown in Table 1.

The isolated organism may be regarded as *Nitrobacter agilis* on the basis of its characteristic morphology and ultrastructure (Figure 1A) as well as motility (Nelson 1931, Murray and Watson 1965, Pan 1971).

The cell structure of *Nitrobacter* was studied in detail for autotrophically and heterotrophically grown cells by Murray and Watson (1965) and Pope *et al.* (1969). The effects of O_2, NO_2^- and NO_3^-, however, have not been reported so far.

The electron microscopic studies of *Nitrobacter* cells grown under different levels of O_2 showed extensive disorganization of membrane structures at 5% O_2 (Figure 1B) and the accumulation of electron transparent circular bodies arranged peripherally at 20% O_2 (Figure 1C). Similar transparent bodies were found in heterotrophically grown *Nitrobacter* cells, although they were present throughout the cell and were considered to be poly-β-hydroxybutyrate (Pope *et al.* 1969). In the present work, however, the PHB content of cells did not increase when grown at 20% O_2 (results not shown).

A nitrogen fixing bacterium, *Azotobacter chroococcum* has also been reported to develop peripheral vesicles similar to electron transparent circular bodies when grown in chemostat continuous

culture under high tension of oxygen. The appearance of these vesicles was related to the increased respiratory index (R.I.) at high oxygen tension (Hine and Lees 1976).

Loss of the nitrite-oxidizing ability of cells during incubation in a medium with high NO_2^- concentration was accompanied by disorganization of the lamellar array of membranes, which are believed to be the site of nitrite-oxidizing system (Bock 1976, Murray and Watson 1965, Wallace and Nicholas 1968). Nitrite seems to have a poisonous effect on the cytoplasmic structures of the cell.

Cells incubated in a high nitrate concentration medium, on the other hand resembled normal cells. Nitrate did not seem to affect the intracellular contents.

Optimization of growth conditions based upon these observations provided *Nitrobacter* cells in sufficient amounts for use in the study of cytochromes of *Nitrobacter* described in the next three sections.

PART II

c-TYPE CYTOCHROMES OF *NITROBACTER*

4.3 PART II *c*-TYPE CYTOCHROMES OF *NITROBACTER*

4.3.1 Results

4.3.1.1 Purification of cytochromes

A summary of purification procedures of the three *c*-type cytochromes is shown in Scheme (Figure 3).

In several preparations the isolation of cytochrome *c*-550 and *c'*-type cytochrome was always consistent by the procedures described in Materials and Methods. Isolation of cytochrome *c*-553, however, was found to be more difficult. In fact, the buffer used for washing the first DEAE-cellulose column was found to be very critical in removing other proteins which interfered in its isolation.

Cytochrome *c*-550 was always present in larger amounts than other cytochromes in the 144,000 g supernatant and its purification was the easiest. Results of many preparation trials gave average quantity ratios of 20:3:1 for cytochrome *c*-550:cytochrome *c'*: cytochrome *c*-553 (Table 2).

In view of difficulties in obtaining *Nitrobacter* cells in large quantities, the procedure used for the isolation of these cytochromes was devised in order to utilize the cell materials fully for the isolation of other enzymes and proteins as well. For example, cytochrome *b* and some oxido-reductases were isolated from the fraction adsorbed on the first DEAE-cellulose column, and ETP obtained from cell homogenate were used for the isolation of cytochrome oxidase and a copper-iron electron transfer protein.

Figure 3. Purification scheme for *c*-type cytochromes of *Nitrobacter agilis*.

Purification of *c*-type cytochromes

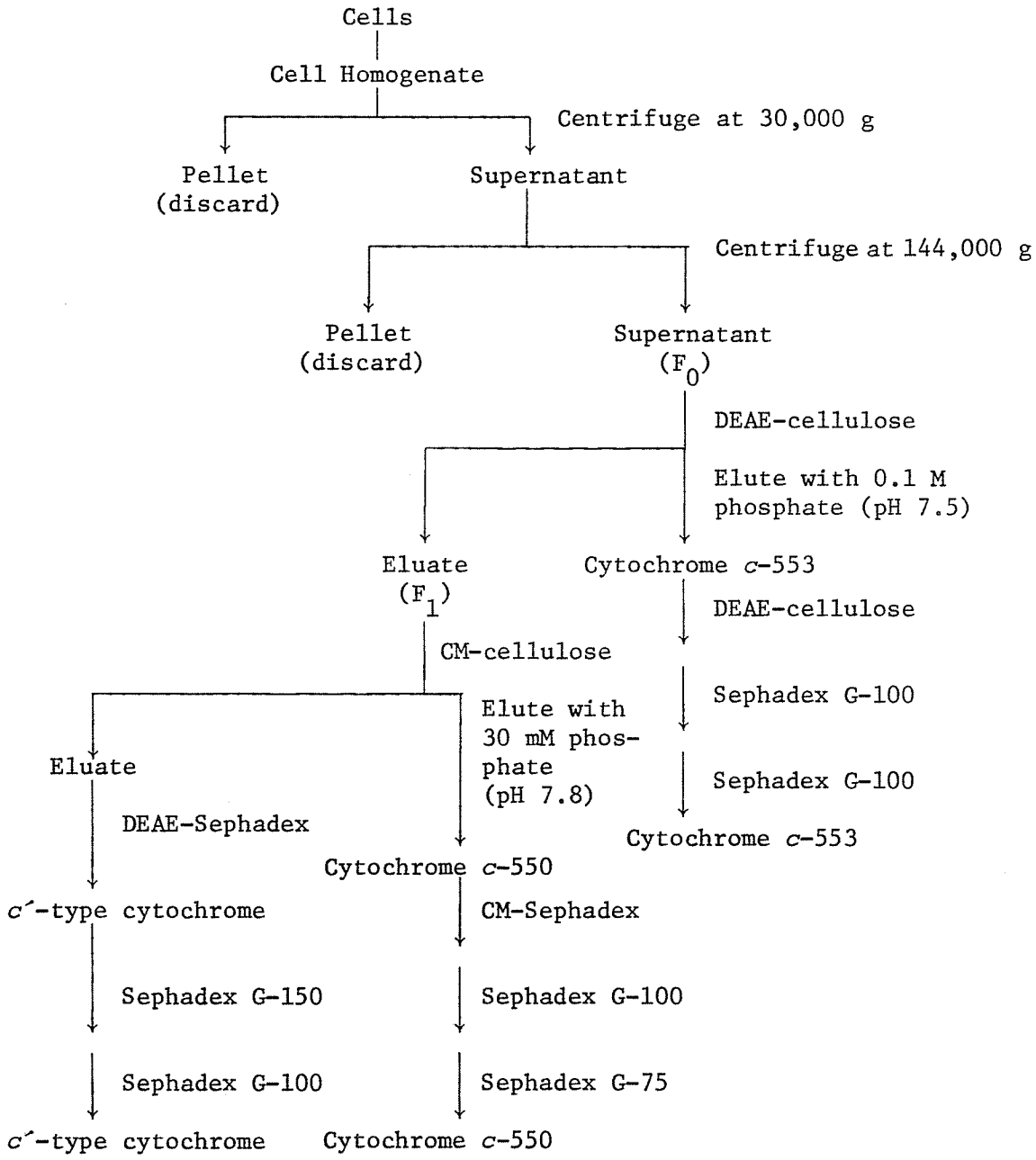


Table 2. Distribution of *c*-type cytochromes in *Nitrobacter agilis* fractions.

Total cytochrome *c*[a] in:	%
Cell homogenate	100
Unbroken cell	15
ETP	60
Supernatant (F_0)	25

[a]Amount of cytochrome *c* present in each fraction was estimated from absorption at 550 nm.

Ratio in F_0	cyt.*c*-550	:	cyt.*c*-549, 554	:	cyt.*c*-553
	20	:	3	:	1

4.3.1.2 Properties of Cytochrome *c*-553

Absorption spectra. The isolated cytochrome *c*-553 was found to be partially reduced (Figure 4) with absorption maxima at 410, 524, and 553 nm. The completely reduced form of cytochrome *c*-553 showed a shift of the Soret band to 419 nm, whereas the α and β bands remained at the same wavelengths, although they were more intense. Ferricyanide could not oxidize the partially reduced cytochrome *c*-553. Pyridine hemochrome prepared from cytochrome *c*-553 gave a spectrum typical of heme *c*. The absorption maxima of reduced cytochrome *c*-553 were similar to those reported by Van Gool and Laudelout (1966) with their crude cell-free extracts.

Molecular weight. A molecular weight of 24,000 was obtained for cytochrome *c*-553 preparations by gel filtration on Sephadex G-100 (Figure 5). Cytochrome *c*-553 moved as a single band during polyacrylamide gel electrophoresis under non-dissociating conditions. SDS-polyacrylamide gel electrophoresis performed on a purified preparation showed a molecular weight of 11,500 (Figure 6). This indicates that cytochrome *c*-553 was a dimer.

Isoelectric point. The isolated cytochrome *c*-553 was subjected to isoelectric focusing using a mixture of carrier ampholytes with pH ranges 5-8 and 3.5-10 in the ratio 4:1. A sharp red band in the isoelectrically focused gels corresponded to a protein band upon staining with Coomassie Blue. A pI value of 6.8 was measured for the red band of cytochrome *c*-553.

Figure 4. Absorption spectra of cytochrome *c*-553 in 50 mM potassium phosphate pH 7.8.

---- oxidized

—— reduced

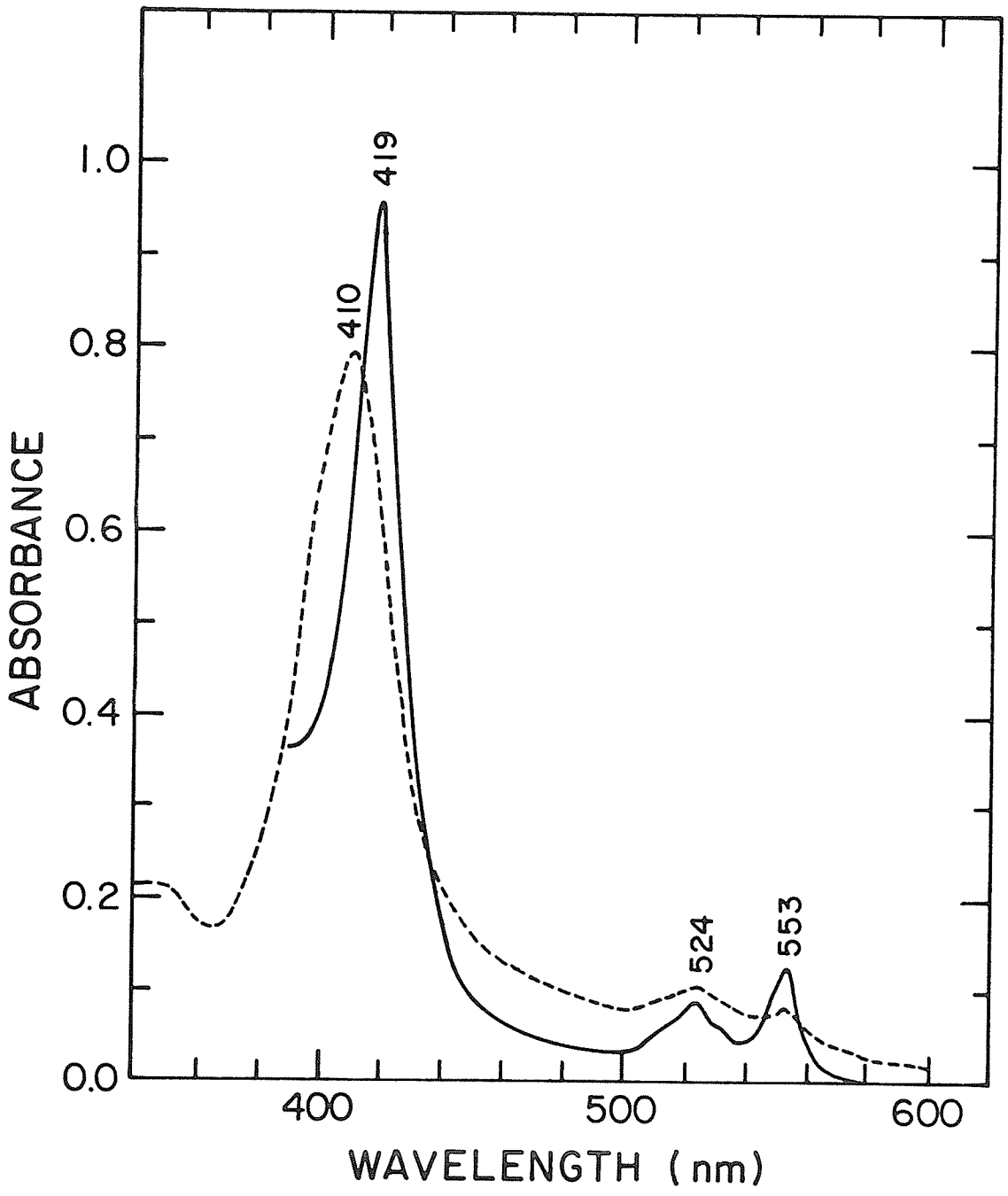

1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE
419
410
524
553
400
500
600
WAVELENGTH (nm)

Figure 5. Molecular weight estimation of cytochrome *c*-553 by Sephadex G-100 in 50 mM Tris-HCl, pH 7.5 containing 100 mM KCl. The marker proteins used were bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000), Chymotrypsinogen A (mol. wt. 25,000), ribonuclease A (mol. wt. 13,700) and cytochrome *c* (mol. wt. 12,384).

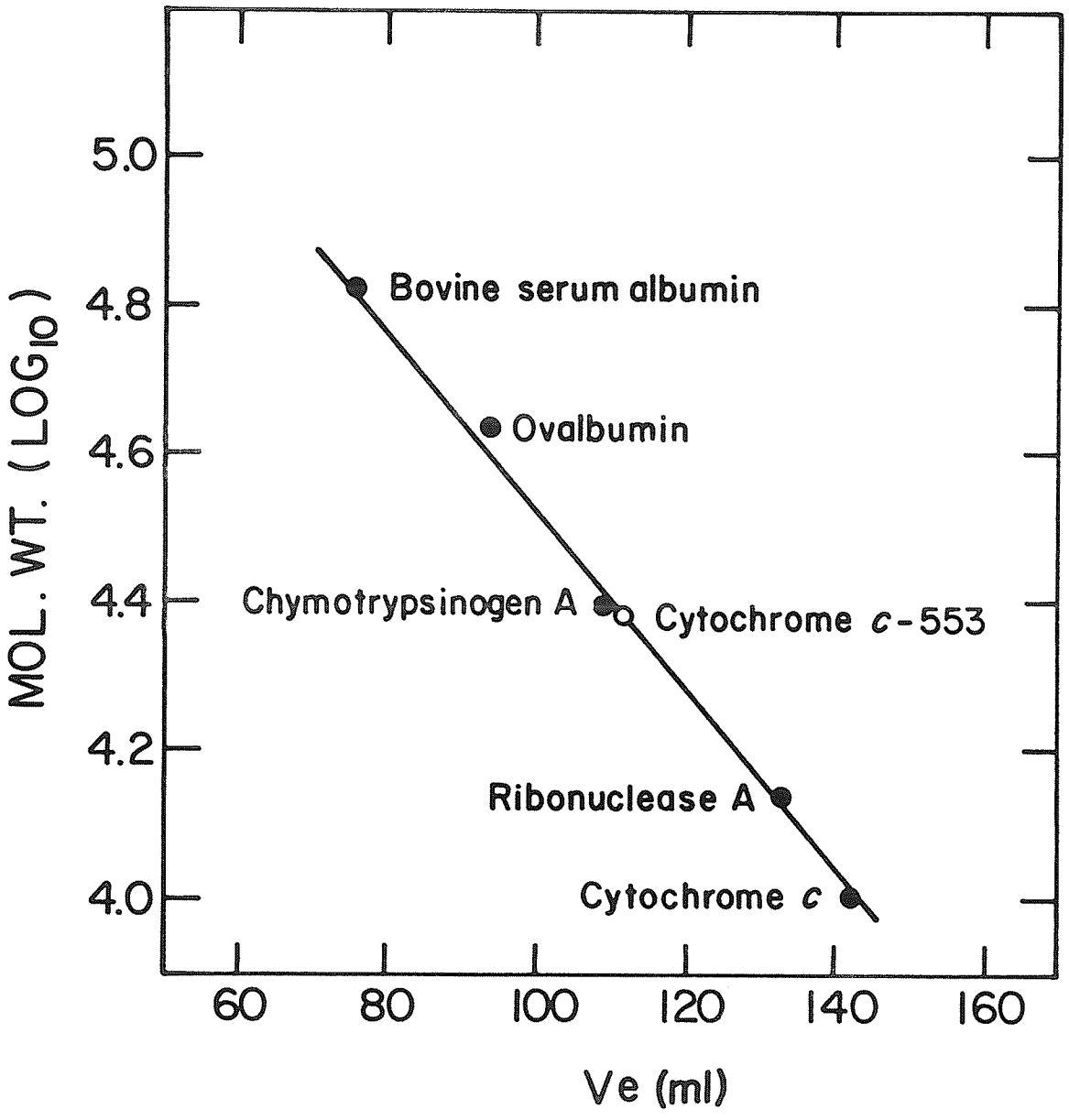
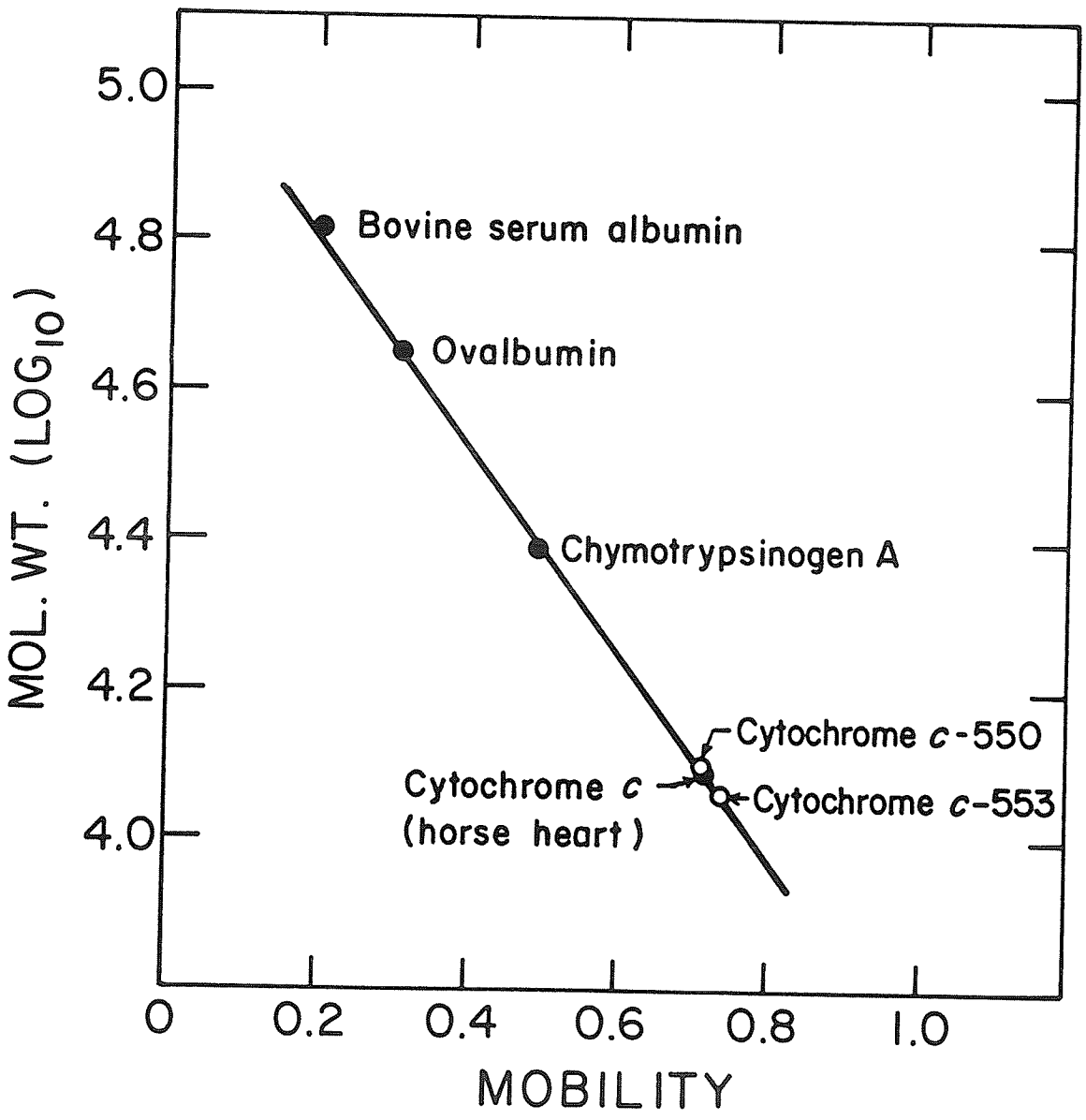

5.0
4.8
4.6
4.4
4.2
4.0
MOL. WT. (LOG10)
Bovine serum albumin
Ovalbumin
Chymotrypsinogen A
Cytochrome c-553
Ribonuclease A
Cytochrome c
60
80
100
120
140
160
Ve (ml)

Figure 6. Molecular weight estimation of cytochromes *c*-553 and *c*-550 by polyacrylamide gel electrophoresis in the presence of 0.1% SDS. The concentration of the acrylamide was 12.5%. Their molecular weights were found to be 11,500 and 12,500 respectively. The proteins used as markers to determine the molecular weights of the cytochromes were: bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000), chymotrypsinogen A (mol. wt. 25,000) and horse heart cytochrome *c* (mol. wt. 12,384).


5.0
4.8
4.6
4.4
4.2
4.0
MOL. WT. (LOG10)
Bovine serum albumin
Ovalbumin
Chymotrypsinogen A
Cytochrome c-550
Cytochrome c
(horse heart)
Cytochrome c-553
0
0.2
0.4
0.6
0.8
1.0
MOBILITY

Amino acid composition. Results of amino acid compositions are given in Table 3, which differentiate cytochrome *c*-553 from the other two *c*-type cytochromes isolated from *Nitrobacter*. This cytochrome has higher amounts of leucine, methionine and arginine residues compared with cytochrome *c*-550. Lysine, which is generally found in large amounts in *c*-type cytochromes, accounted for only 7 residues per molecule in cytochrome *c*-553. Cytochrome c_2's from many species of *Rhodospirillum* and *Rhodopseudomonas* have been isolated with a similar total number of residues. According to the classification of Dickerson (1980) cytochrome *c*-553 may be placed in the so-called "M" (medium-sized) group of *c*-type cytochromes although the other examples contain between 10 and 17 lysines per molecule.

4.3.1.3 Properties of Cytochrome *c*-550

Absorption spectra. The oxidized form of cytochrome *c*-550 showed an absorption maximum at 411 nm, and the reduced form maxima at 416, 521 and 550 nm (Figure 7). These spectra were similar to those reported earlier for a purified cytochrome *c* from *Nitrobacter* (Ketchum *et al.* 1969). The spectrum taken from the reduced pyridine hemochrome of cytochrome *c*-550 preparations gave a typical *c*-type heme spectrum with the absorption peaks at 415, 521 and 550 nm. From the absorbance at the α-peak of the pyridine ferrohemochrome, the εmM at the α-peak of cytochrome *c*-550 was determined to be 30.0, assuming the εmM at the α-peak of pyridine ferrohemochrome of heme *c* to be 29.1 (Drabkin 1942).

Table 3. Amino acid composition of cytochrome *c*-550, *c*-553, and *c*-549, 554 isolated from *Nitrobacter agilis*.

Amino acid	No. of residue per mole of cytochrome *c*-550	*c*-553	*c*-549, 554
Aspartic acid	14	12	48
Threonine	5	8	29
Serine	7	7	26
Glutamic acid	13	10	45
Proline	7	7	26
Glycine	15	10	41
Alanine	15	13	59
Valine	7	8	21
Methionine	1	2	3
Cysteine	2	-	9
Isoleucine	4	3	13
Leucine	7	11	37
Tyrosine	2	2	7
Phenylalanine	4	3	18
Histidine	3	2	5
Lysine	13	7	25
Arginine	2	4	20
Total residues	121	109	432
	12,500[a]	11,500[a]	46,000[a]
	12,500[b]	24,000[b]	90,000[b]

[a]Molecular weight estimated by SDS polyacrylamide gel electrophoresis.

[b]Molecular weight estimated by gel filtration.

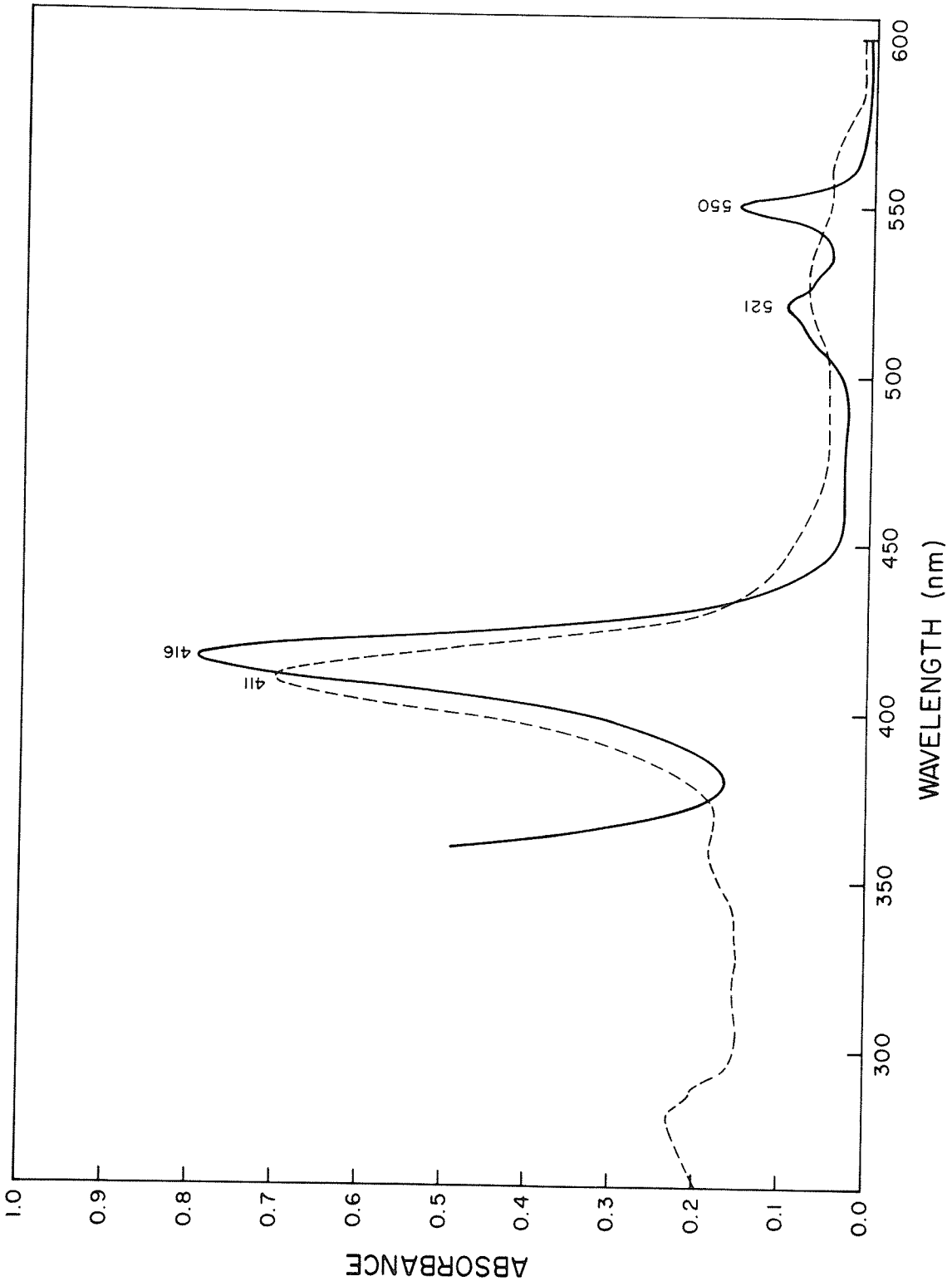
To determine cysteine samples were digested with performic acid prior to hydrolysis. Tryptophan estimations were not made.

Figure 7. Absorption spectra of cytochrome *c*-550 in 50 mM potassium phosphate, pH 7.8.

---- oxidized

—— reduced with $Na_2S_2O_4$

(The concentration of the cytochrome used was 4.4 μM.)


ABSORBANCE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
300
350
400
450
500
550
600
WAVELENGTH (nm)
411
416
521
550

Molecular weight. Gel filtration on Sephadex G-75 yielded a molecular weight of 12,500 for cytochrome *c*-550 (Figure 8). Cytochrome *c*-550 preparations moved as a single band during polyacrylamide gel electrophoresis both under nondissociating conditions and in the presence of SDS. The results obtained by gel electrophoresis in the presence of SDS gave the same molecular weight as that obtained by the gel filtration method (Figure 6), indicating that this molecule is a monomer.

Isoelectric point. Isoelectric focusing of cytochrome *c*-550 was carried out by standard procedure using carrier ampholytes of pH ranges 5-8, 3.5-10 and 2-11 in the ratio 2:2:1. The isoelectric point determined was 8.51. Isoelectric focusing of horse heart cytochrome *c* performed by the same procedure gave a pI value of 10.55.

Oxidation-reduction potential. The midpoint redox potential ($E_{m,7}$) of cytochrome *c*-550 was measured by titration with ferricyanide/ferrocyanide redox system under aerobic conditions. The $E_{m,7}$ value obtained was 0.271 (Figure 9) similar to that reported previously (Butt and Lees 1958, Ketchum *et al.* 1969, Sewell *et al.* 1972, Cobley 1973).

Amino acid composition. The purified preparations of cytochrome *c*-550 were subjected to amino acid analyses. The results reported in Table 3 indicate that *Nitrobacter* cytochrome *c*-550 is more closely related to the so-called "L" group of *c*-type cytochromes (Dickerson 1980). It is also apparent from the data that the

Figure 8. Molecular weight estimation of cytochrome *c*-550 by Sephadex G-75 in 50 mM Tris-HCl, pH 7.5 containing 100 mM KCl. The standard proteins used were bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000) chymotrypsinogen A (mol. wt. 25,000) ribonuclease A (mol. wt. 13,700) and horse heart cytochrome *c* (mol. wt. 12,384).

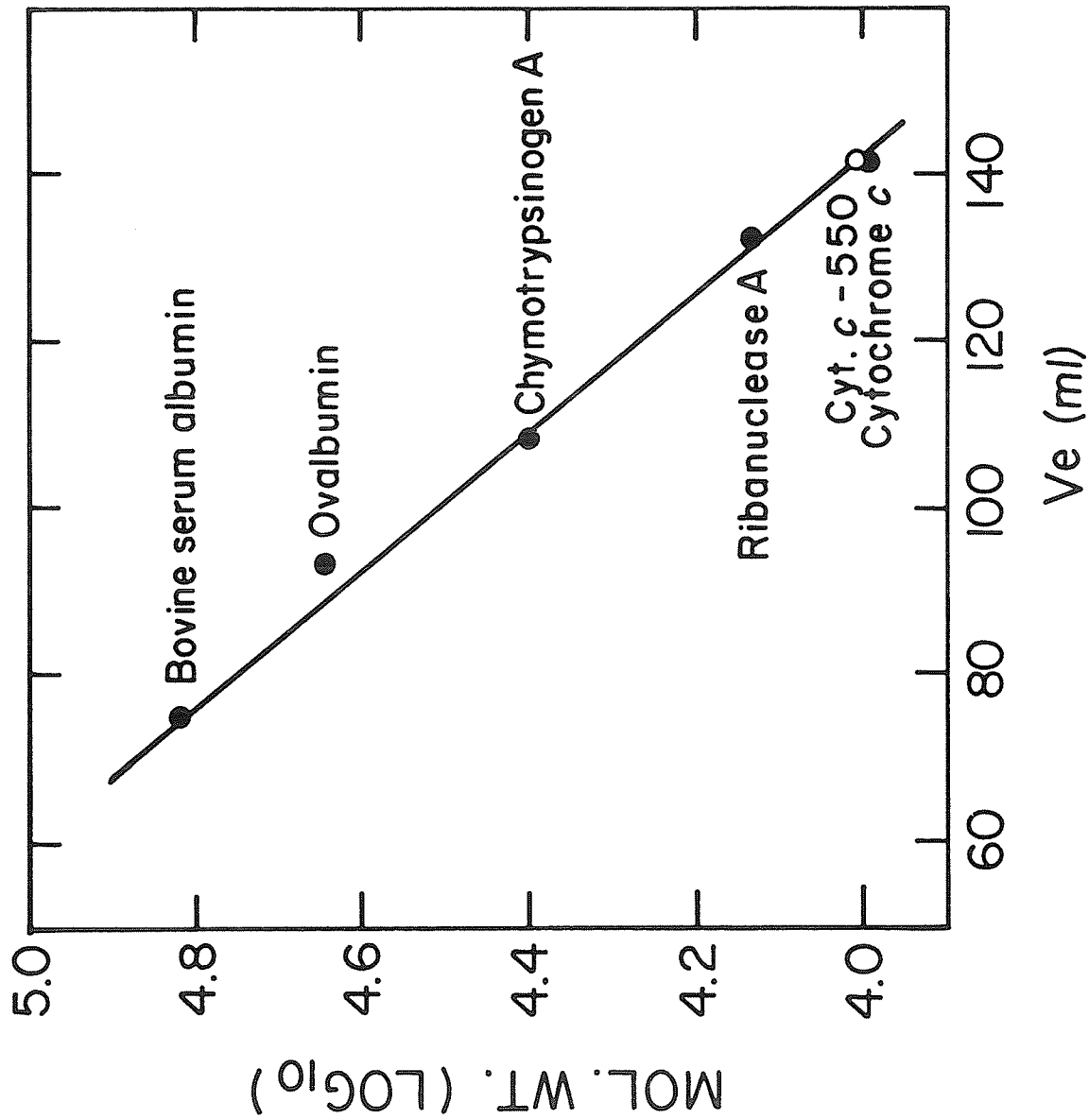
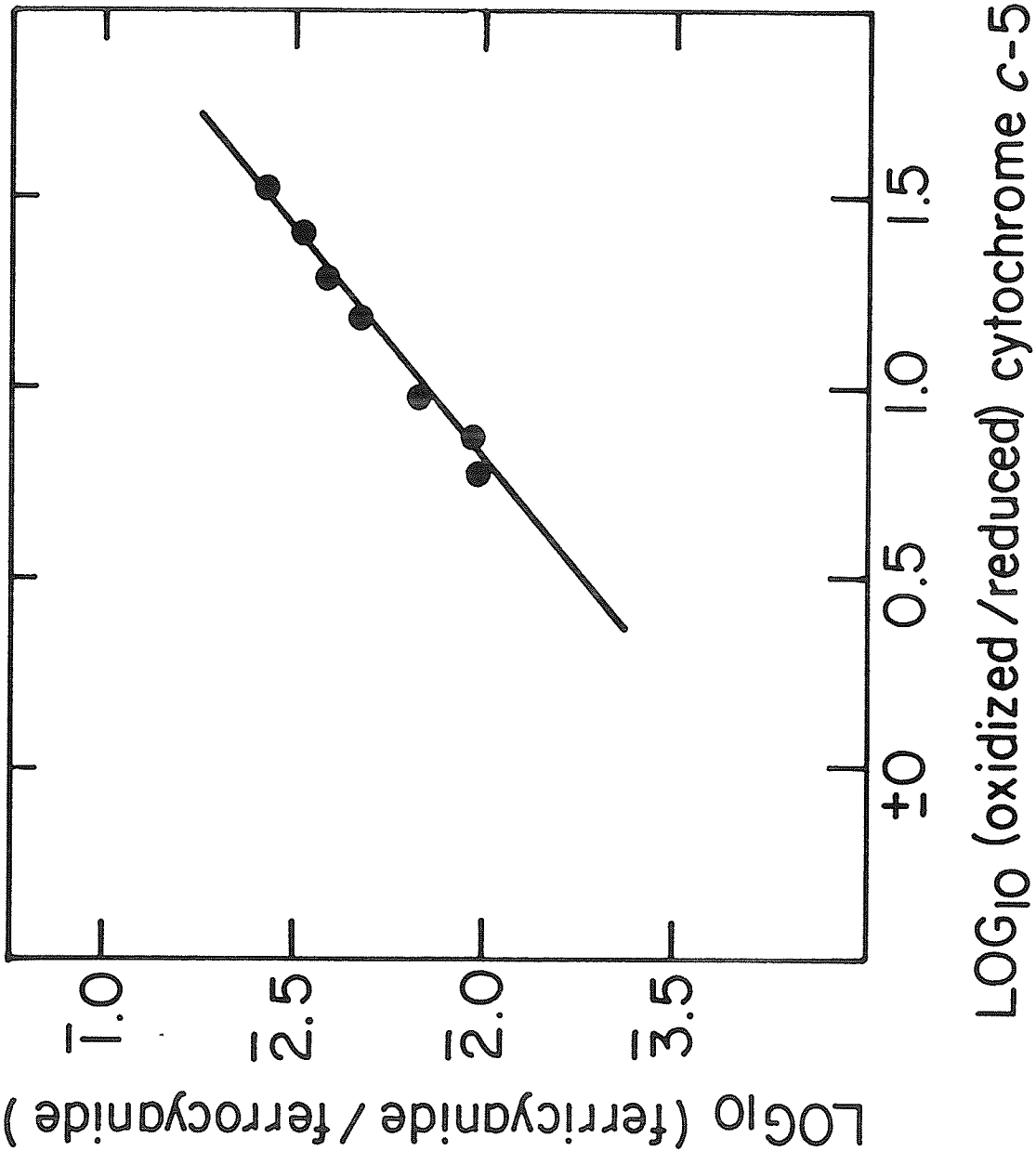

MOL. WT. (LOG_{10})
5.0
4.8
4.6
4.4
4.2
4.0
60
80
100
120
140
Ve (ml)
Bovine serum albumin
Ovalbumin
Chymotrypsinogen A
Ribanuclease A
Cyt. c - 550
Cytochrome c

Figure 9. Determination of redox potential ($E_{m,7}$) of cytochrome *c*-550 as described in Materials and Methods.


LOG10 (ferricyanide / ferrocyanide)
1.0
1.5
2.0
2.5
3.0
3.5
0.0
0.5
1.0
1.5
LOG10 (oxidized / reduced) cytochrome c-550

number of residues of lysine is similar to the numbers in typical *c*-type cytochromes (Dayhoff 1978).

Amino acid sequence. The N-terminal sequence of *Nitrobacter* cytochrome *c*-550 is shown in Table 4, along with the corresponding positions of the sequences of the *c*-type cytochromes from a few selected organisms. The alignments are discussed below.

The identification of serines at position 8 and 9 are tentative, and are based on low recoveries of alanine (45% of expected values) after back hydrolysis to the free amino acids (see ref. Mendez and Lai 1975). The free acid forms were assigned at position 2 and 4 from the thin layer method. Not enough sample was available for the thin layer method at position 11, and so the position is assigned as asX. No residue could be identified at positions 13 or 16; by analogy to other *c*-type cytochromes, these are probably the two cysteines to which the heme is attached (Table 4). Because so little sample was available, we did not attempt to remove the heme and to derivatize cysteine for easier identification, before running the protein in the sequencer. The evidence for histidine at position 17, and for the other residues, was unequivocal.

The initial coupling in the Sequencer run was 36.0% and the repetitive yield 89.8%, based on the recoveries of the residues at positions 1, 5, 6, 10 and 15.

Table 4. Comparison of N-terminal amino acid sequences of four *c*-type cytochromes with that of *Nitrobacter* cytochrome *c*-550.

Horse heart cyt. *c*	gly-asp-val-glu-lys-gly-lys-lys-ile-phe-val-gln-lys-			-cys-ala-gln-cys-his-thr-val-
Paracoccus denitrificans cyt. *c*-550	asn-glu-gly-asp-ala-ala-lys-gly-glu-lys-glu-phe-asn-		-lys-	-cys-lys-ala-cys-his-met-ile-
Nitrobacter agilis cyt. *c*-550	gly-asp-val-glu-ala-gly-lys-(ser)-(ser)-phe-asx-		-lys-	- x -lys-ala- x -his- x - x -
Pseudomonas denitrificans cyt. *c*-551		ser-thr-gly-glu-glu-leu-phe-lys-ala-lys-ala-		cys-val-ala-cys-his-ser-val-
Monochrysis lutheri cyt. c_6	gly-asp-ile-ala-asn-gly-glu-gln-val-phe-thr-gly-asn-			-cys-ala-ala-cys-his-ser-

4.3.1.4 Properties of *c'*-type cytochrome: cytochrome *c*-549, 554

Molecular weight. The purified preparations of cytochrome *c*-549, 554 yielded a molecular weight of 90,000 by gel filtration on Sephadex G-100 (Figure 10). The homogeneity of preparations was confirmed by polyacrylamide electrophoresis under non-dissociating conditions. A single red-colored band on the gel corresponded to the single protein band upon staining with Coomassie Blue. It was, however, observed that the protein moved as a rather broad band in the gel upon electrophoresis. When electrophoresis was performed in the presence of SDS, the cytochrome also migrated as a single band. Two minor protein bands, very close to each other, and much smaller than the main band, appeared in some preparations. These were regarded as impurities.

The molecular weight of the cytochrome estimated by SDS-gel electrophoresis was 46,000 (Figure 11). This result indicates that the molecular weight obtained by gel filtration was that of the dimer, so that cytochrome *c*-549, 554 had two polypeptide chains of the same size. A similar high molecular weight cytochrome *c*-552, 558 from *Ps. stutzeri* has been reported (Kodama and Mori 1969).

Heme content. The pyridine hemochrome prepared from cytochrome *c*-549, 554 indicated that it contained two hemes (*c*-type) per molecule assuming the molecular weight of 46,000 (as estimated by SDS electrophoresis). The εmM at the α-peak of pyridine hemochrome was estimated to be about 20 per heme. Similar low values of εmM

Figure 10. Molecular weight estimation of cytochrome *c*-549, 554 by Sephadex G-100 in 50 mM Tris-HCl, pH 7.5 containing 100 mM KCl. The protein markers used were bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000), chymotrypsinogen A (mol. wt. 25,000) and cytochrome *c* (mol. wt. 12,384).

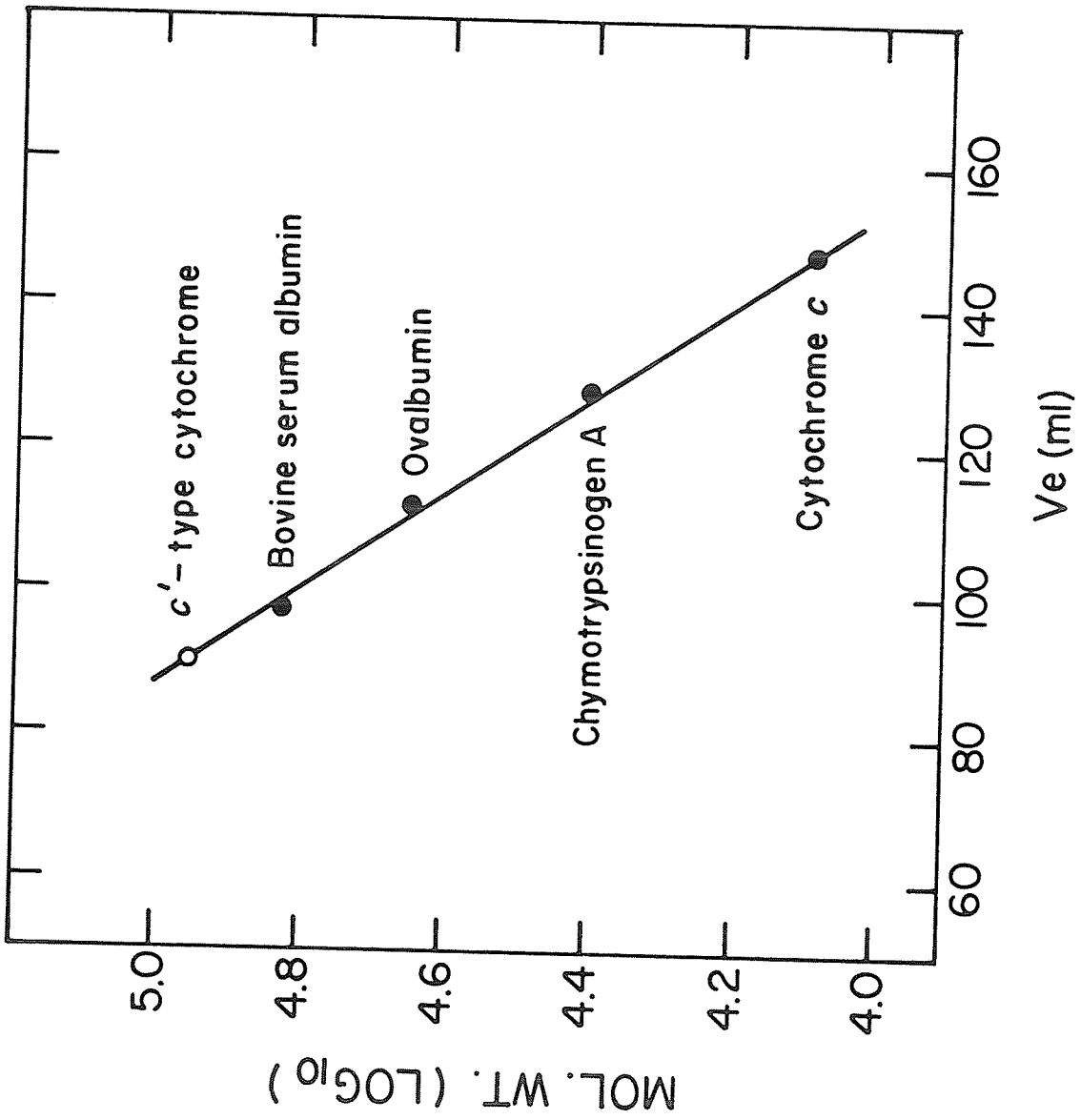
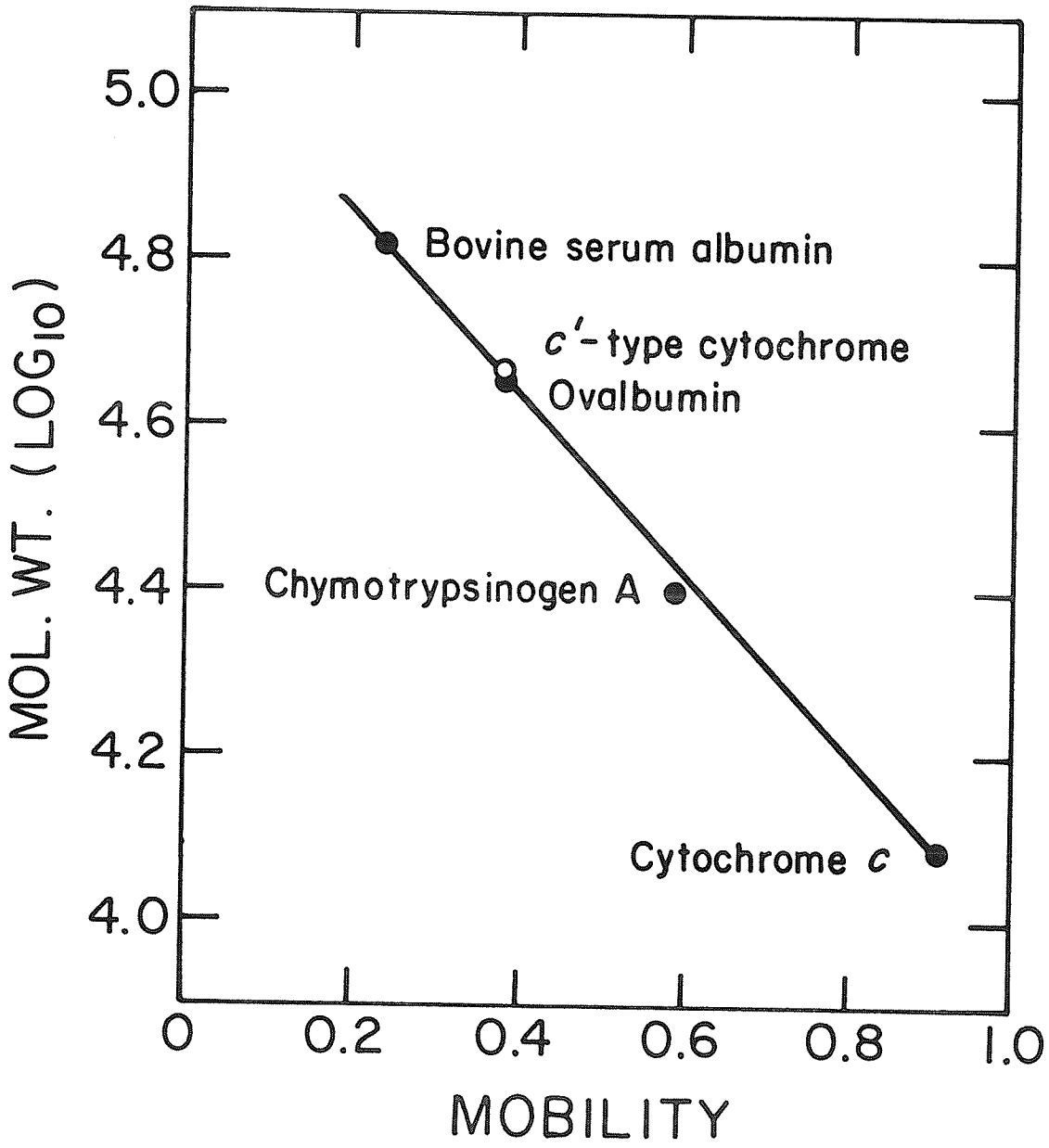

MOL. WT. (LOG10)
5.0
4.8
4.6
4.4
4.2
4.0
60
80
100
120
140
160
Ve (ml)
c'-type cytochrome
Bovine serum albumin
Ovalbumin
Chymotrypsinogen A
Cytochrome c

Figure 11. Molecular weight determination of cytochrome *c*-549, 554 by polyacrylamide gel electrophoresis in the presence of 0.1% SDS. The concentration of the acrylamide was 10%. Its molecular weight was found to be 46,000. The protein standards used to determine the molecular weight were: bovine serum albumin (mol. wt. 67,000), ovalbumin (mol. wt. 45,000), chymotrypsingen A (mol. wt. 25,000), and cytochrome *c* (mol. wt. 12,384).


5.0
4.8
4.6
4.4
4.2
4.0
MOL. WT. (LOG10)
Bovine serum albumin
c'-type cytochrome
Ovalbumin
Chymotrypsinogen A
Cytochrome c
0
0.2
0.4
0.6
0.8
1.0
MOBILITY

for heme *c* of *c'*-type cytochromes have been reported (see Iwasaki and Shidara 1969 and also Lemberg and Barret 1973).

Isoelectric focussing. Isoelectric focusing of cytochrome *c*-549, 554 was performed as described in the Materials and Methods, using carrier ampholytes of pH range 5-7 and 3.5-10 in a ratio 4:1. The isoelectric point was determined to be pH 5.6, which is similar to the values reported for *c'*-type cytochromes (Handerson and Nankiville 1966). It also agrees with the high content of acidic amino acid residues of the cytochrome (Table 3).

Amino acid composition. The purified preparations of cytochrome *c*-549, 554 were subjected to amino acid analysis. Table 3 shows the amino acid composition of the cytochrome. The high proportion of alanine (i.e. 59 residues) is consistent with the amino acid composition of other *c'*-type cytochromes (Dus and Kamen 1963, Cusanovich *et al.* 1970). The high content of aromatic amino acids in cytochrome *c*-549, 554 also agrees with the report for cytochrome *c'* (Dus and Sletten 1968).

Oxidation-reduction potential. Cytochrome *c*-549, 554 could not be reduced with ascorbate, sodium borohydride, or potassium ferrocyanide under aerobic conditions. When reduced with dithionite, the reduction was slow and a flat peak appeared at the α region. However, at a slow spectrophotometer scanning speed this flat peak was resolved into two separate peaks at 549 and 554 nm.

When midpoint redox potential ($E_{m,7}$) determinations were

carried out with ferri-ferro-oxalate redox couple as described in the Materials and Methods, only the peak at 553∿554 nm was reduced The Em,7 of this peak was estimated to be -0.006 V (Figure 12). It is likely that $E_{m,7}$ for the 549 nm peak is even more negative.

Absorption spectra. The oxidized form of cytochrome *c*-549, 554 had an absorption maximum at 402 nm and bands at 525, 560 and 620 nm (Figure 13). The Soret peak is at an unusually low wavelength compared to most *c*-type cytochromes. Figure 14 shows a spectrum of the reduced form with absorption peaks at 419, 523, 549 and 554 nm. The Soret region also has a shoulder at 430 nm. Cytochrome *c*-554 purified from another nitrifying bacterium, *Nitrosomonas europea* (Yamanaka and Shinra 1974) has also been reported to show a shoulder at 430 nm, but only a single peak in the α region, unlike this *Nitrobacter c*-type cytochrome with its double peaks. A variant cytochrome *cc'* has, however, been reported to exhibit a peak at 423 nm and a prominent shoulder at 430 nm (Horio and Kamen 1961) similar to the cytochrome *c*-549, 554. The isolated cytochrome was extracted with acid acetone. No *b* or *a*-type pyridine hemochrome could be detected in the acid acetone extract. The pyridine ferrohemochrome prepared from the residue possessed a heme *c* spectrum with absorption peaks at 415, 521 and 551 nm. Therefore, the cytochrome *c*-549, 554 may be regarded as a *c'*-type cytochrome.

Figure 12. Determination of redox potential ($E_{m,7}$) of *c*′-type cytochrome as described in Materials and Methods.

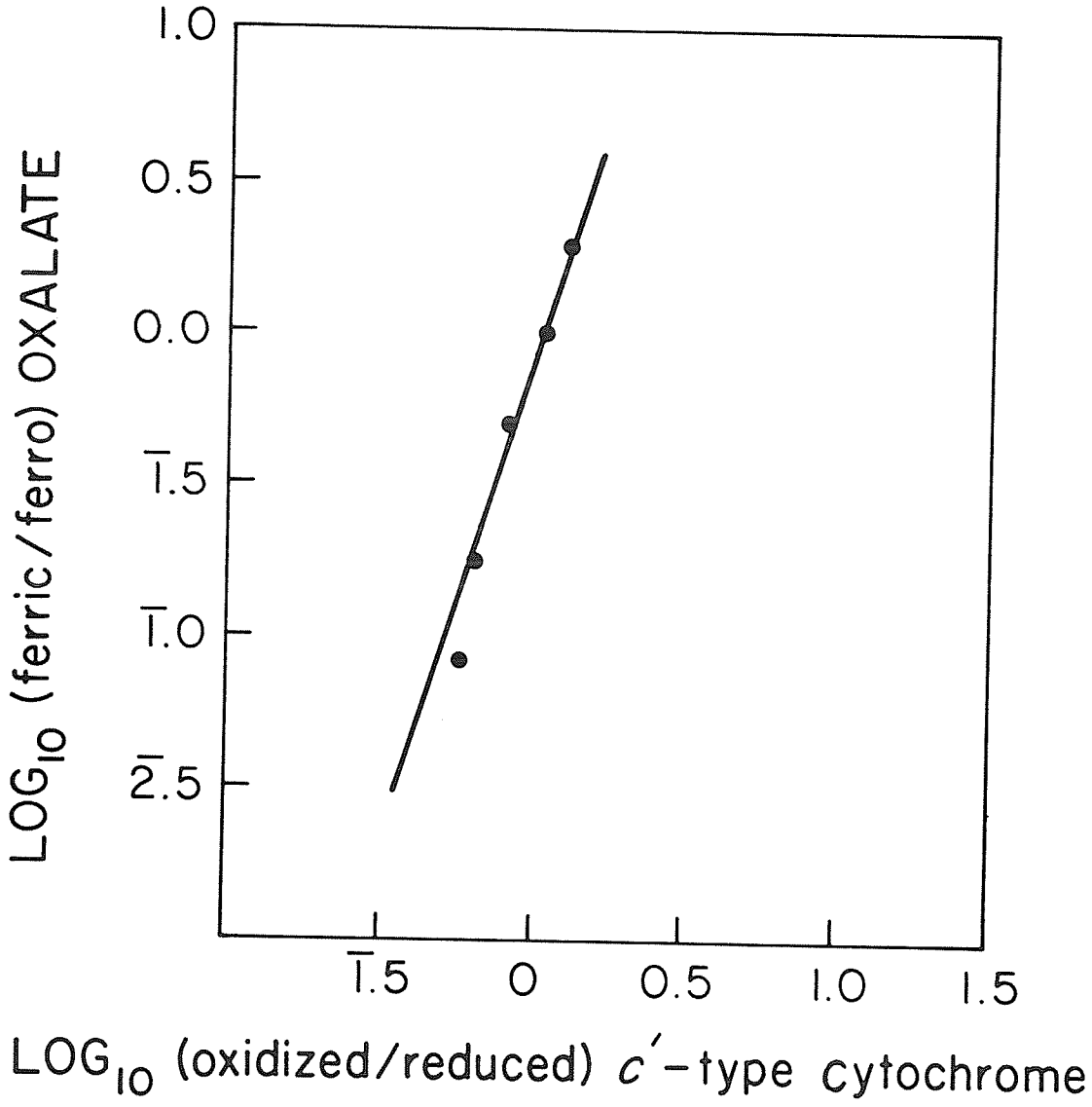

1.0
0.5
0.0
$\bar{1}$.5
$\bar{1}$.0
$\bar{2}$.5
LOG$_{10}$ (ferric/ferro) OXALATE
$\bar{1}$.5
0
0.5
1.0
1.5
LOG$_{10}$ (oxidized/reduced) c'-type cytochrome

Figure 13. Oxidized absorption spectrum of cytochrome *c*-549, 554 in 50 mM potassium phosphate, pH 7.5.

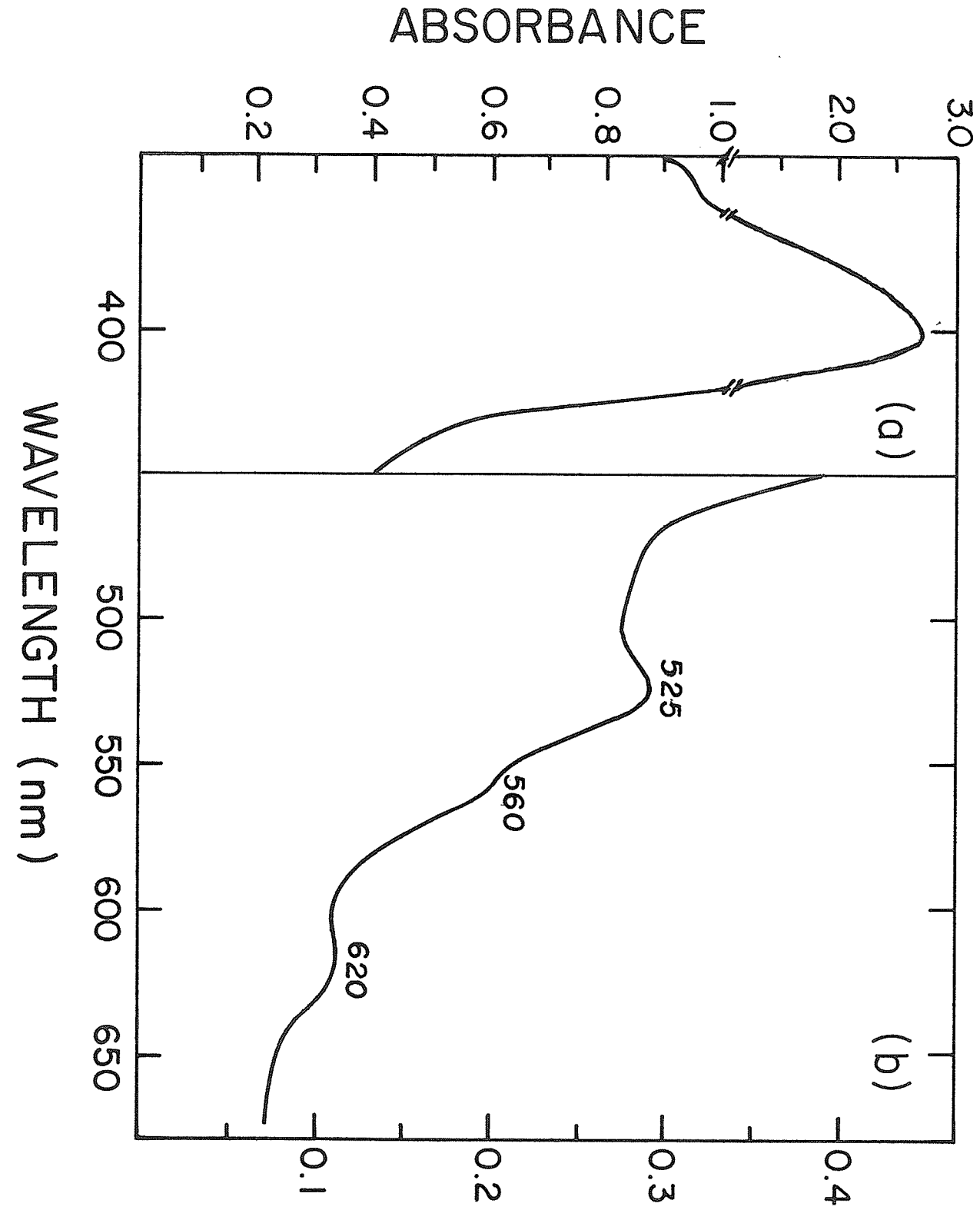
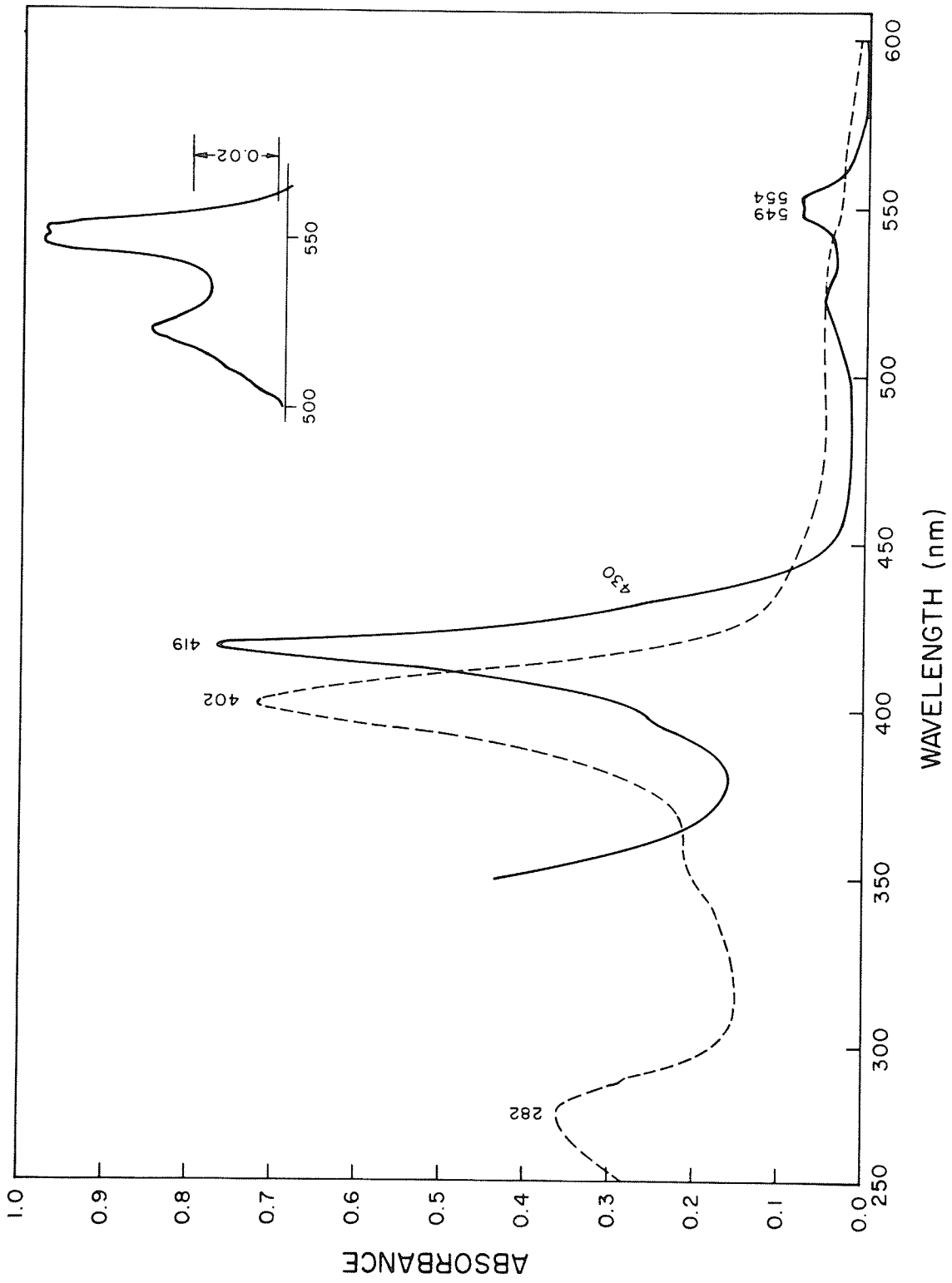

ABSORBANCE
0.2
0.4
0.6
0.8
1.0
2.0
3.0
(a)
400
500
550
600
650
WAVELENGTH (nm)
525
560
620
(b)
0.1
0.2
0.3
0.4

Figure 14. Absorption spectra of cytochrome *c*-549, 554 in 50 mM potassium phosphate, pH 7.5.

---- oxidized

—— reduced with $Na_2S_2O_4$

(The concentration of the cytochrome used was 3.9 μM.)


ABSORBANCE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
250
300
350
400
450
500
550
600
WAVELENGTH (nm)
282
402
419
430
549
554
0.02
500
550

Effect of O_2. Cytochrome *c*-549, 554 was highly autoxidizable since the ferrocytochrome was partially oxidized immediately upon mixing with air (Figure 17). Moreover, the reduction of cytochrome *c*-549, 554 was slow even under anaerobic conditions. Contrary to this, the *c* -type cytochromes, although autoxidizable, have been reported to be reduced rapidly by $Na_2S_2O_4$ (Ambler *et al*. 1979).

Effect of pH. Figure 15 shows the reduced spectra of cytochrome *c*-549, 554 in buffers of different pH. It is apparent that the cytochrome was reduced better in higher pH buffers. At pH 6.0 there was no obvious reduced α, β or γ peak, rather the spectrum was greatly distorted. These pH effects were reversible. The Soret region spectra of the oxidized cytochrome were also affected by pH (results not shown). These results are in close agreement with those reported for *cc'* or *c'*-type cytochromes (Kodama and Mori 1969, Imai *et al*. 1969, Horio and Kamen 1961). In these cytochromes there was no apparent difference in the absorption spectra of the ferrocytochrome from pH 7.0 to pH 10.0. The spectrum of *Nitrobacter* cytochrome, however, remained constant between pH 7.5 and 10.0.

The absorption spectra of the cytochrome *c*-549, 554 in 0.1 N NaOH is shown in Figure 16. The oxidized form showed a typical *c*-type ferrihemochrome spectrum. Reduction with sodium dithionite resulted in a *c*-type ferrohemochrome spectrum with a sharp increase in absorption at the Soret as well as α and β bands.

Figure 15. Absorption spectra of reduced cytochrome c-549, 554 at different pH's.

The spectra of reduced form at pH 7.5 and 10.0 were identical.

— ·—— . — pH 6.0

— ·· — ·· — pH 6.5

---------- pH 7.0

———————— pH 7.5

(The concentration of the cytochrome used was 1.9 μM.)

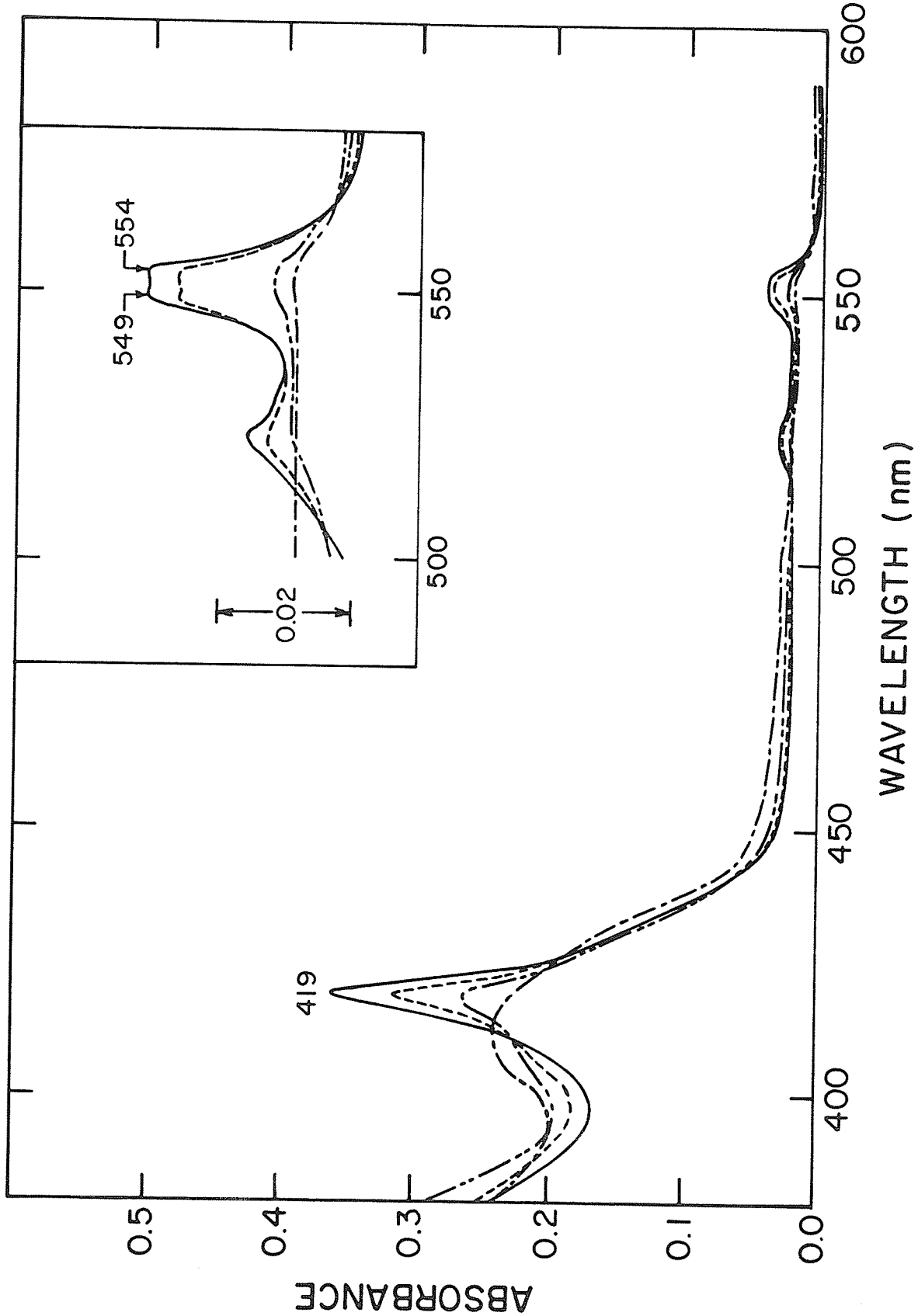
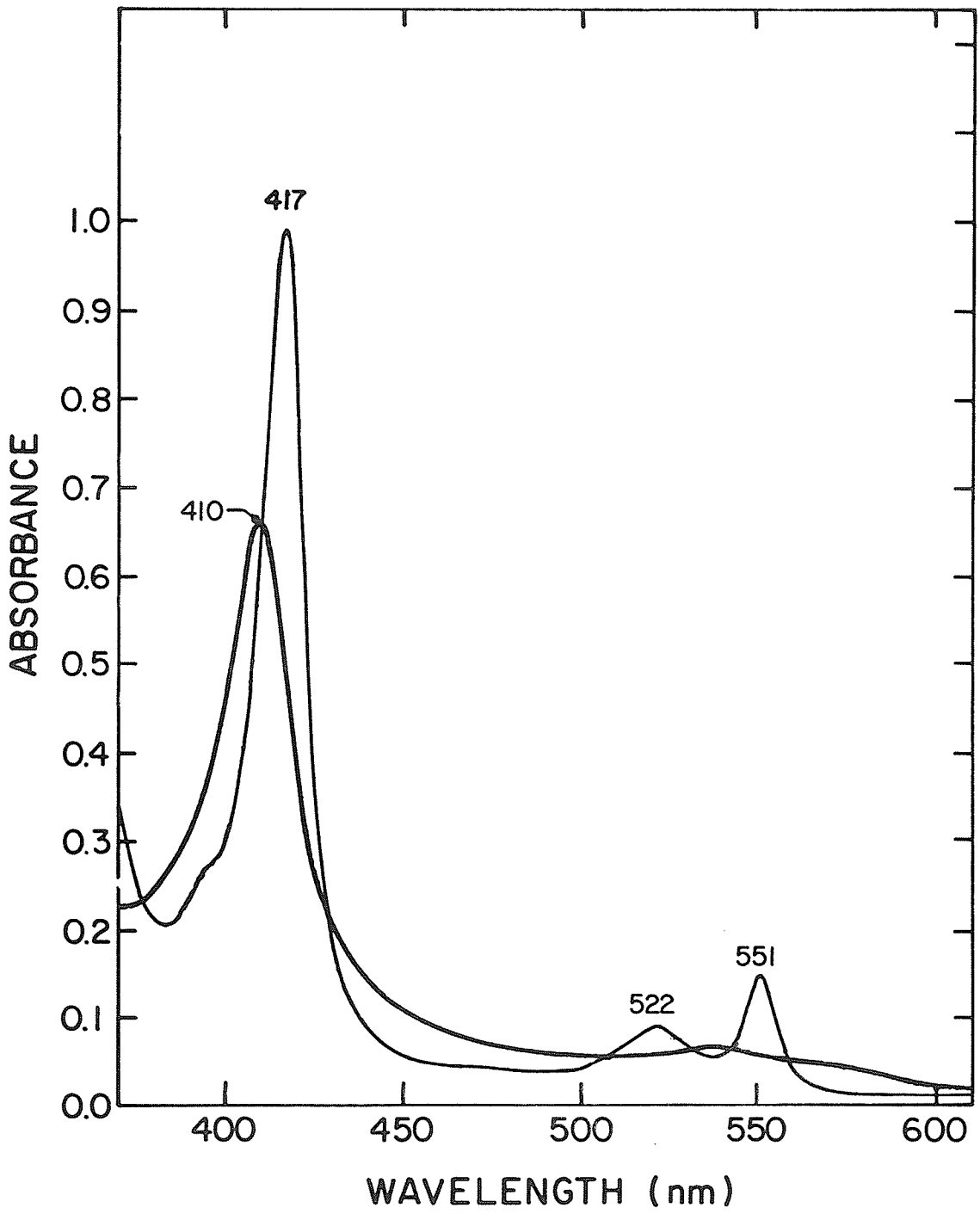

ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
400
450
500
550
600
WAVELENGTH (nm)
419
549
554
0.02
500
550

Figure 16. Absorption spectra of cytochrome *c*-549, 554 in 0.1 N NaOH.

═══ oxidized

—— reduced with $Na_2S_2O_4$

(The concentration of the cytochrome used was 3.75 μM).


417
410
522
551
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
400
450
500
550
600
WAVELENGTH (nm)

Reaction with CO. Cytochrome *c*-549, 554 was reduced with $Na_2S_2O_4$ in a Thunberg cuvette, the side arm of which had been plugged with a rubber stopper. To the evacuated cuvette CO was introduced through the side arm and the cuvette was shaken thoroughly to mix the contents of cuvette with the CO. The reduced α peak(s) as well as a shoulder at 430 nm were abolished and the Soret band was shifted from 419 to 412.5 nm. The spectrum of CO-ferrocytochrome *c*-549, 554 is presented in Figure 17. The CO-ferrocytochrome was found to be unaffected by aeration unlike the ferrocytochrome as mentioned earlier.

Reaction with cyanide. Potassium cyanide reacted with ferricytochrome *c*-549, 554 in that the 402 nm band shifted to 408 nm and the absorption intensity was also decreased (Figure 18). The effect of CN^- on the reduced form of cytochrome *c*-549, 554 was also investigated anaerobically as described for CO experiments. The CN-ferrocytochrome spectrum taken after 20 min of treatment with KCN (10 mM) showed an increase in the absorption in the α region at 553 nm but not at 549 nm (Figure 17). There was an absorption increase also at the α region, and the peak was shifted to 524 nm. The 430 nm shoulder was abolished as the case of CO-ferrocytochrome compound. The Soret peak was slightly shifted to 419.5 nm with increased absorption. It is interesting to note that the CN^- compound clearly differentiates the two hemes of ferrocytochrome *c*-549, 554 indicating that it is probably the one with absorption at 554 nm which binds to CN^-, leading to an

Figure 17. Effect of carbon monoxide on the reduced spectrum of cytochrome *c*-549, 554 in 50 mM potassium phosphate, pH 7.5 (see the results and discussion).

---------- ferrocytochrome

— · — · — ferrocytochrome mixed with air

———————— CO-ferrocytochrome

..... CO-ferrocytochrome mixed with air

(The concentration of the cytochrome used was 2.67 μM.)

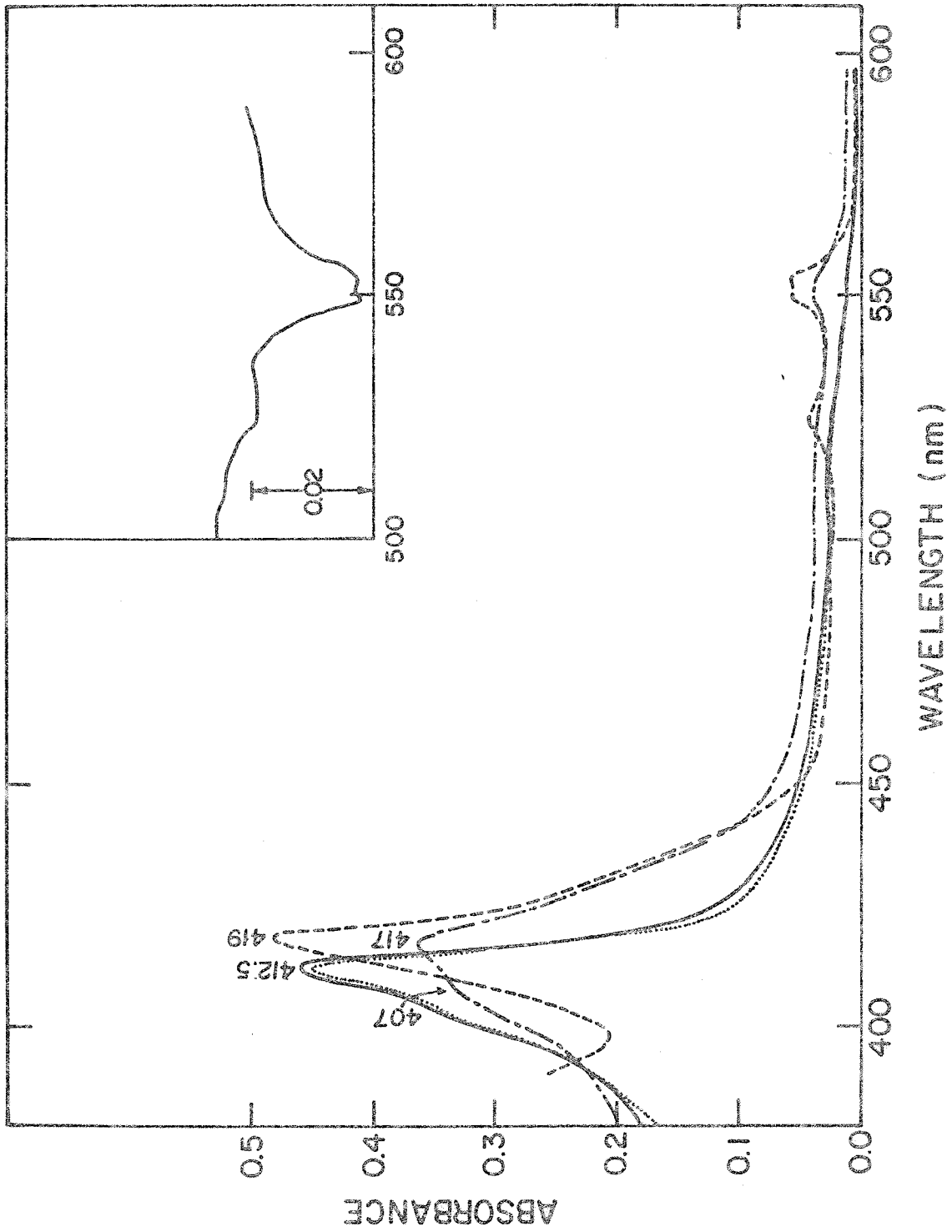

ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
WAVELENGTH (nm)
400
450
500
550
600
407
412.5
417
419
0.02

Figure 18. Effect of cyanide on the oxidized and reduced spectra of cytochrome *c*-549, 554 in 50 mM potassium phosphate, pH 7.5.

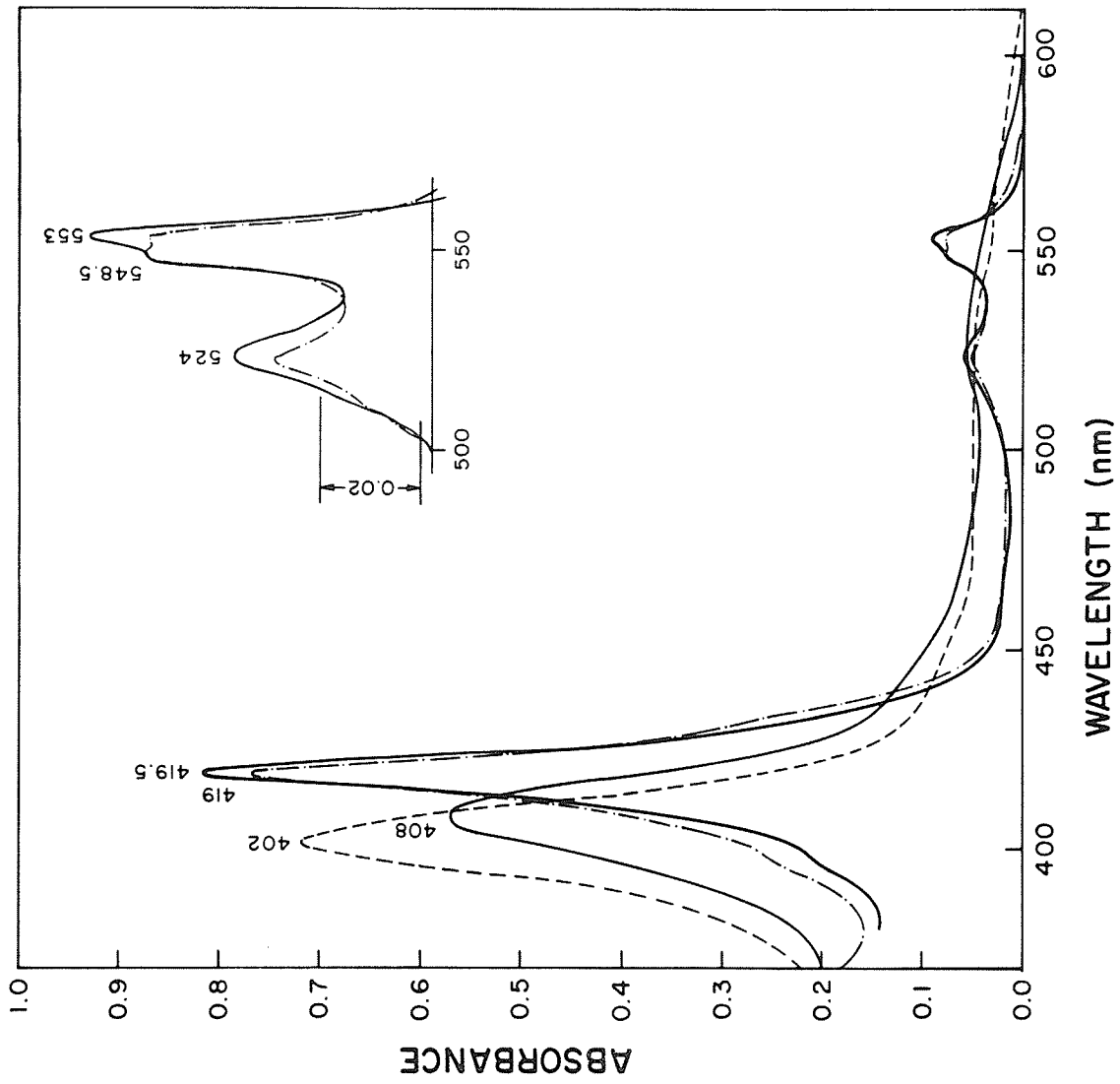
---------- ferricytochrome

———————— CN^--ferricytochrome

— · — · — ferrocytochrome

═══════ CN^--ferrocytochrome

(The concentration of the cytochrome used was 3.9 μM.)


ABSORBANCE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
400
450
500
550
600
WAVELENGTH (nm)
419
419.5
408
402
524
548.5
553
0.02

increased absorption at 553 nm. Cytochromes of *c*-type are, however, not known to form CN^- compounds (Iwasaki and Shidara 1969, Kodama and Mori 1969).

Reaction with Nitrite and Nitrate. Although cytochrome *c*-549, 554 was highly autoxidizable, it did not oxidize reduced cytochrome *c* (horse heart), or NADH or NADPH. It is unlikely to be an oxidase because of its low redox potential.

Attempts were made to determine the physiological reductant of cytochrome *c*-549, 554, and compounds involved in nitrogen metabolism such as NO_2^-, NH_2OH and NH_4^+ were tested. None of these compounds could reduce the cytochrome aerobically or anaerobically. Nitrite (3-6 mM), however, was found to react with the ferrocytochrome, in that the shoulder at 430 nm was abolished with some decrease in absorption at the α, β and γ regions; when air was admitted the γ peak was split into two peaks at 410 and 417 nm (Figure 19). The absorption spectrum of NO_2^--ferrocytochrome was affected by air, but only in the Soret region. The spectrum of the ferrocytochrome, treated with air in the presence of NO_2^-, was different from that obtained when the NO_2^- was omitted (Figure 17).

Although most of the *c'*-type cytochromes form NO-ferrocytochrome compounds (Lemberg and Barrett 1973), there is no report in which nitrite was found to react with *c'*-type cytochromes (Iwasaki and Shidara 1969, Cusanovich *et al.* 1970, Yamanada and Okunuki 1974).

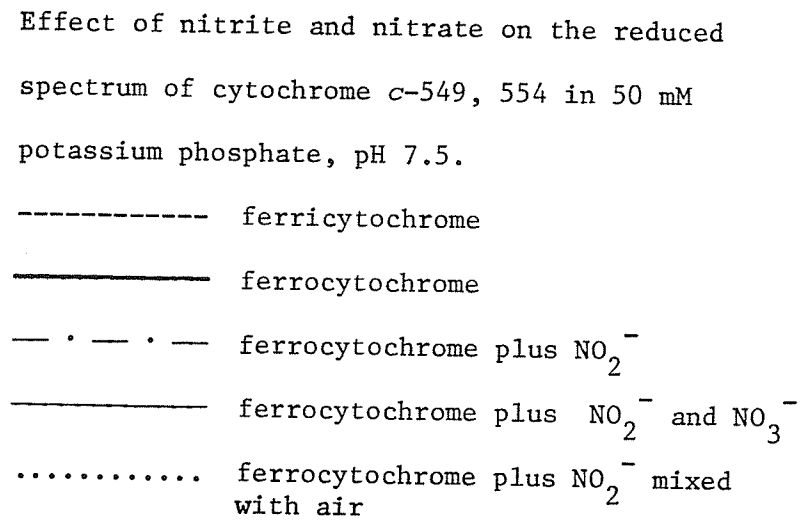


Figure 19. Effect of nitrite and nitrate on the reduced spectrum of cytochrome *c*-549, 554 in 50 mM potassium phosphate, pH 7.5.

---------- ferricytochrome

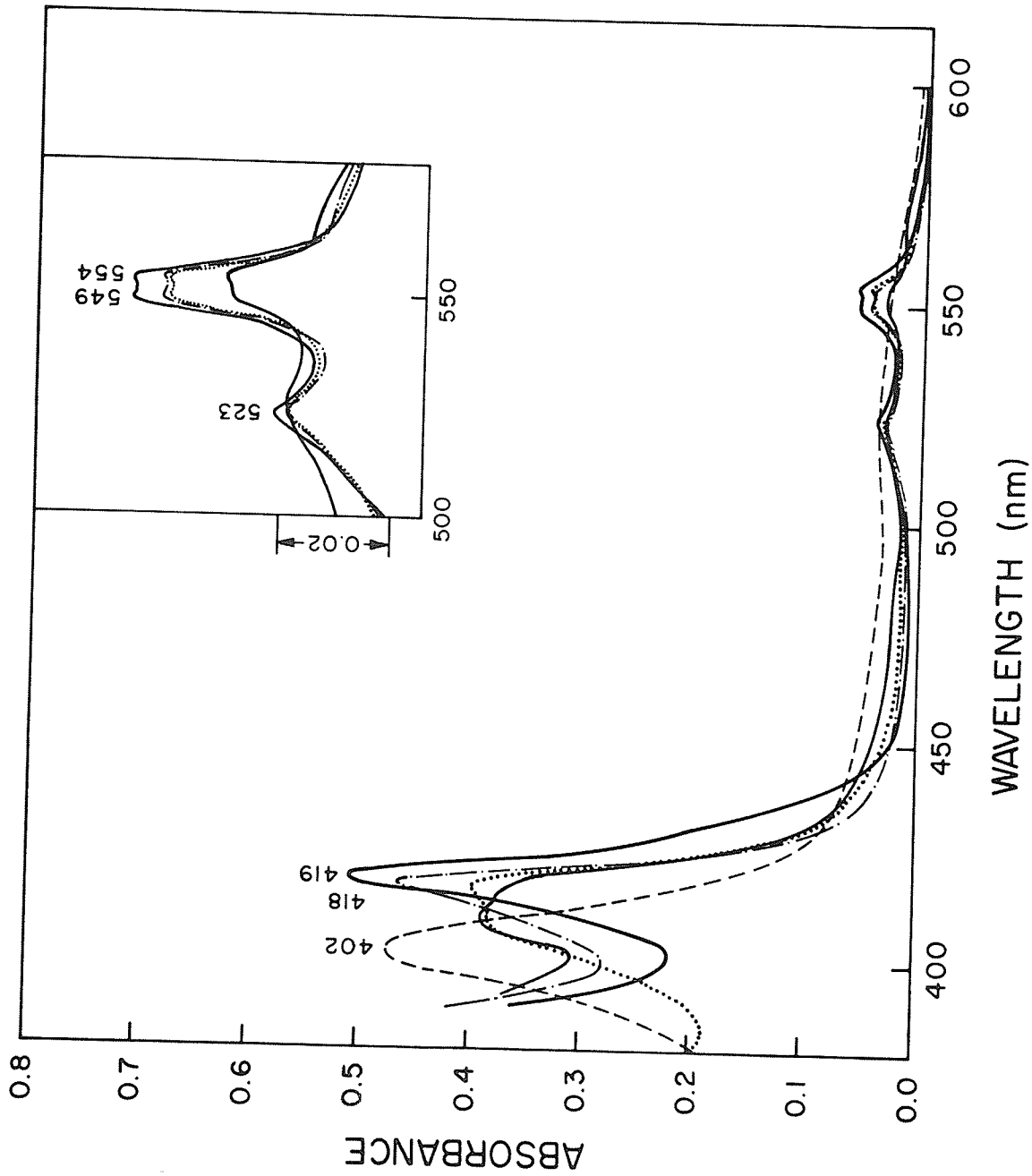
———————— ferrocytochrome

— · — · — ferrocytochrome plus NO_2^-

———————— ferrocytochrome plus NO_2^- and NO_3^-

..... ferrocytochrome plus NO_2^- mixed with air

(The concentration of the cytochrome used was 2.67 μM.)


ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
400
450
500
550
600
WAVELENGTH (nm)
419
418
402
523
549
554
0.02
500
550

Nitrate alone did not have any effect upon the oxidized or reduced form of cytochrome *c*-549, 554. When NO_3^- (1-2 mM) was applied aerobically to the NO_2^--treated ferrocytochrome, however, a further change in its absorption spectrum was observed. The absorption in the α and β regions was further decreased and the 417-418 nm peak changed to a shoulder (Figure 19). It seems that nitrate can react with the NO_2^--ferrocytochrome compound, but not with the ferri or ferrocytochrome itself.

Effect of other reagents. Various other treatments known to convert cytochrome *c'* spectrum to a typical cytochrome *c* spectrum were examined.

Ethyl or propyl alcohol at 50% shifted the γ peak of cytochrome *c*-549, 554 to a higher wavelength although the protein precipitated in a few minutes. This cytochrome *c*-549, 554 is thus less soluble in alcohol, like the one reported by Yamanaka and Imai (1972), but unlike other *cc'* or *c'*-type cytochromes (Imai *et al.* 1969).

The Soret peak of ferricytochrome *c*-549, 554 was shifted to 408 nm from 402 nm after 3 h in SDS (1%) at room temperature. A similar effect of detergents has been reported on some other *c'*-type cytochromes (Yamanaka and Imai 1972, Imai *et al.* 1969). Sodium salts of benzoate as well as salicylate, up to a concentration of 1.5 M, did not have any effect on either the oxidized or the reduced spectrum of cytochrome *c*-549, 554.

4.3.2 Discussion

Ever since cytochrome *c* was first demonstrated to be a component of the respiratory chain of the autotrophic bacterium, *Nitrobacter* (Lees and Simpson 1957) its role in nitrite oxidation has remained a matter of controversy (Lees 1962, Kiesow 1967, O'Kelley *et al*. 1970, Aleem 1970, 1978). In spite of extensive literature available on the electron transport components, only one type of cytochrome *c* has been reported to be present in *Nitrobacter*. However, a critical examination of the spectrophotometric evidence reported for cytochrome *c* in these papers (see Table 5) shows differences in absorption maxima, which are quite unlikely if only one type of cytochrome *c* has been reported to be present in *Nitrobacter*. These discrepancies in the data reported in the literature prompted us to reinvestigate the cytochrome *c* of this bacterium. In consequence of this investigation we have succeeded in isolating three *c*-type cytochromes from *Nitrobacter agilis* cells. Some properties of these cytochromes are discussed below.

In our investigation the spectral properties of reduced cytochrome *c*-553 (Figure 4) were similar to those observed by Van Gool and Laudelout (1966) and its isoelectric point (pH 6.8) was close to that reported by Butt and Lees (1958). Cytochrome

Table 5. Comparison of absorption maxima of *Nitrobacter* c-type cytochrome.

	Reduced			Oxidized			Materials Used
	α	β	γ	γ	$E_{m,7}$	PI	
Lees & Simpson	551	520	-	-	-	-	Cell suspensions
Butt & Lees	550	521	416	-	0.25 (pH 7.5)	6.5	Extracts
Zavarzin	552	-	-	-	-	-	Cell suspensions
Aleem & Nason	550	520	415	-	-	-	Extracts
Van Gool & Laudelout	554	523	419	-	-	-	Cell suspensions & Extracts
	552.5	523	416	411	-	-	Soluble fractions
Ketchum *et al.*	550	521	417	411	0.282	-	Purified cytochrome
Sewell *et al.*	-	-	-	-	0.274	-	Extracts
Ingledew *et al.*	-	-	-	-	0.270	-	ET Particles
Cobley	-	-	-	-	0.270	-	ET Particles
	552.5	-	-	-	-0.110	-	ET Particles
This report	550	521	416	411	0.271	8.51	Purified Cytochromes
	553	524	419	410	-	6.80	
	549-554	523	419	402	-0.006	5.60	

c-553 was reduced by ascorbate, ferrocyanide, sodium borohydride and dithionite, but NH_4^+, NO_2^-, NADH or NADPH had no effect. The observation that the isolated cytochrome *c*-553 (partially reduced) could not be oxidized by ferricyanide indicates that it has a high redox potential. Its possible role in nitrite oxidation should be interesting to study.

Cytochrome *c*-550, exhibited absorption spectra with peaks at 411 nm for the oxidized form and at 416, 521 and 550 nm for the reduced form and so it is similar to that purified by Ketchum *et al.* (1969). The molecular weight of cytochrome *c*-550, 12,500, was close to that of horse heart cytochrome *c* as judged by gel filtration and polyacrylamide gel electrophoresis. These cytochromes were different, however, in their pI values and amino acid compositions (Table 3). The isoelectric point of cytochrome *c*-550 was also different from the pI value reported earlier for cytochrome *c* of *Nitrobacter* (Butt and Lees). In this earlier work, isoelectric point determinations were carried out using a crude extract and a red band with a pI value of 6.5 was regarded as cytochrome *c*. In view of the present findings, the red band might have been cytochrome *c*-553 purified here, which had a pI value 6.8.

The N-terminal sequence of cytochrome *c*-550 showed a clear homology with the corresponding portions of the sequences of four other *c*-type cytochromes (Table 4): one a eukaryotic cytochrome *c* (horse heart); two bacterial cytochromes *c* of denitrifying organisms (*P. denitrificans* and *Ps. denitrificans*) and another also algal cytochrome *c* of photosynthetic organism (*M. lutheri*). The sequence

information and the alignments for these four cytochromes *c* are those as found elsewhere (Dayhoff 1978). The N-terminal region of this group of proteins is strongly conserved, and *Nitrobacter* cytochrome *c*-550 is homologous with all four of the sequences shown at positions 1, 2, 6, 10, 12 and 17 (and presumably 13 and 16, where the heme cysteines are expected to be, by analogy with the other sequences).

The *c*-type cytochromes of non-photosynthetic bacteria may be placed in two evolutionary groups on the basis of sequence data: one which contains *c*-550 and the c_2 cytochromes derived from a number of *Rhodospirillum* and *Rhodopseudomonas* species; and another which contains *c*-551 and a few miscellaneous cytochromes c_2 (Dayhoff 1978). The N-terminal sequence of *Nitrobacter* cytochrome *c*-550 resembles the first group more closely than the second, since it has only one residue between the constant residues phe-10 and lys-12 (*Nitrobacter* numbering), whereas all other groups of *c*-type cytochrome have two residues; and in having four amino acids between lys-12 and his 17, where cytochromes *c*-551 have five. The great length of *Nitrobacter* cytochrome chain (approximately 121 amino acids, see Table 3) is also consistent with its placement in the first group, since it is only in that group that *c*-type cytochromes as long as 130 amino acids are found (Dayhoff 1978).

The two cytochromes discussed above were typical *c*-type cytochromes. The finding of a third cytochrome *c*-549, 554 presented a novel instance of an anomalous cytochrome *c* in *Nitrobacter*.

A variant cytochrome, first discovered in *Rhodospirillium rubrum* (Elsden *et al.* 1953) and subsequently in other species of photosynthetic bacteria (Kamen *et al.* 1963, deKlerk *et al.* 1965, Dus *et al.* 1967), was designated RHP (Vernon and Kamen 1954). Later it became apparent that RHP was essentially an anomalous cytochrome *c*. Consequently the terms cytochrome *cc'* or *c'* were coined to designate members of a subclass of cytochrome *c*, having two hemes or one heme per mole of protein respectively (see Lemberg and Barrett 1973, Kamen 1963, Kamen and Horio 1970). Although some denitrifying bacteria (Iwasaki and Shidara 1969, Iwasaki and Matsubara 1971, Kodama and Shidara 1969), as well as a nitrogen fixing bacterium (Yamanaka and Imai 1972), have also been reported to contain *cc'*-type cytochromes, the isolation of an example from *Nitrobacter* here is so far unique in chemoautotrophic bacteria. The oxidized form of cytochrome *c*-549, 554 had an absorption band at 402 nm and so resembled most *c'* or *cc'*-type ferricytochromes which also possess a similar absorption maximum of wavelengths close to 400 nm (Lemberg and Barrett 1973, Yamanaka and Okunuki 1974). The reduced form of cytochrome *c*-549, 554 had double peaks of the same intensity at the α region. Another characteristic feature of the reduced spectrum was that the absorption ratio for α and γ peaks was larger than that of typical *c*-type cytochromes. Cytochrome *c*-549, 554 was also found to be highly autoxidizable.

Gel filtration on Sephadex G-100 indicated a molecular weight of 90,000 for the purified cytochrome *c*-549, 554. This value was

much higher than those reported for cytochrome *c′* of photosynthetic bacteria (Dus *et al.* (1967) but was similar to or even smaller than those of cytochromes *cc′* (Kodama and Mori 1969, Yamanaka and Imai 1972).

The SDS polyacrylamide gel electrophoresis gave a value of 46,000 daltons for the molecular weight of cytochrome *c*-549, 554. This suggested that the cytochrome was a dimer. The heme content determined on the basis of pyridine hemochrome prepared from cytochrome *c*-549, 554 showed two hemes per molecule of 46,000. The concept of the cytochrome *c*-549, 554 being a diheme cytochrome agrees with its double peak absorption spectrum in the α region and a shoulder at 430 nm in addition to γ peak at 419 nm. These observations, together with those discussed below, suggested that cytochrome *c*-549, 554 was similar to a *cc′*-type cytochrome isolated from a denitrifying bacterium (Kodama and Mori 1969). Recent studies, however, question such a concept of diheme cytochrome and claim that the cytochrome *cc′* is in fact a dimer consisting of two polypeptides of equal size, each containing a single heme (Cusanovich 1971, Kennel *et al.* 1972, Ambler *et al.* 1979). It has been proposed that the proper terminology for this class of proteins (cytochrome *cc′*) is cytochrome *c′*, (Kennel *et al.* 1972, Ambler *et al.* 1979). Therefore, cytochrome *c*-549, 554 in keeping with this proposal should be considered as a *c′*-type cytochrome.

Cytochrome *c*-549, 554 could not be dissociated beyond the apparent polypeptide size of 46,000 with SDS, which dissociates most other proteins into subunits. The possibility that this

diheme cytochrome of 46,000 daltons consists of smaller subunits is not entirely unlikely, in view of the reported strong association between monomers of *c'*-type cytochromes (Cusanovich 1971, Ambler *et al.* 1979). It is also possible that the molecular weight of 90,000 obtained by gel filtration may not be correct if cytochrome *c*-549, 554 behaves unlike normal proteins but similar to cytochrome *c'* from *Chromatium vinosum* (Kennel *et al.* 1972) which behaves anomalously during molecular weight determinations.

Cytochrome *c*-549, 554 was found to have a low redox potential (Figure 12) and an acidic isoelectric point (pH 5.6); these are properties of most *c'*-type cytochromes (Lemberg and Barrett 1972).

The spectrophotometric response of cytochrome *c*-549, 554 toward a variety of ligands known to react with the accessible heme group was also investigated and the results are summarized in Table 6. The reaction of this cytochrome with CO (Figure 17) is in close agreement with the results reported for *c*-type anomalous cytochromes (Kodama and Mori 1969, Taniguchi and Kamen 1963, Iwasaki 1960, Yamanaka and Imai 1972). The *c'*-type cytochromes have not been reported to combine with CN^-, although both oxidized and reduced forms of cytochrome *c*-549, 554 reacted with potassium cyanide (Figure 18). The reaction with NO_2^- may be a unique property of cytochrome *c*-549, 554 that is possibly related to the mechanism of nitrite oxidation by *Nitrobacter*.

Compounds such as SDS, salicylate and benzoate are known to react with hemoglobin and myoglobin, changing their spectra (Kaziro and Tsushima 1961). These compounds were also reported as

Table 6. Millimolar Extinction Coefficient of *c*′-type cytochrome, cytochrome *c*-549, 554.

State of *c*′-type cytochrome	λnm	Em M^{-1} $cm^{-1}/2$ heme calculated from molecular weight.[b]	Em M^{-1} $cm^{-1}/2$ heme calculated from Pyridine hemochrome
Ferricytochrome *c*′	402	180.0	
	530	16.6	
	620	6.0	
Ferrocytochrome *c*′	419	192.0	
	523	13.0	
	549	19.8	39.5[a]
	554	19.5	
CO-ferrocytochrome *c*′	412.5	179.5	
	524	7.7	
CN^--ferricytochrome *c*′	408.5	151.0	
	530	16.9	
	540	18.0	
CN^--ferrocytochrome *c*′	419.5	204.0	
	524	15.0	
	549	19.7	
	554	22.8	
NO_2^--ferrocytochrome *c*′	418	175.0	
	524	13.0	
	549	18.0	
	554	18.0	
Ferricytochrome *c*′ in 0.1 N NaOH	410	176.0	
Ferrocytochrome *c*′ in 0.1 N NaOH	417	264.0	
	522	24.0	
	551	39.0	

[a] $\lambda nm = 551$

[b] Extinction values are average of many samples.

able to modify the absorption spectra of *c'*-type cytochromes (Iwasaki and Shidara 1969, Yamanaka and Imai 1972). Similar studies with cytochrome *c*-549, 554 showed that only SDS induces the spectral conversion of the ferricytochrome.

In the presence of alcohol, ketones and phenols, the anomalous oxidized spectra of *c'*-type cytochromes have been reported to be converted into typical spectra of ferricytochrome *c*. This conversion property is thought to be a function of their hydrophobicity (Imai *et al*. 1969, Lemberg and Barrett 1973). Ethanol and propanol induced the same change in cytochrome *c*-549, 554, but the protein was less soluble in alcohol, like the purified *c'*-type cytochrome from *Azotobacter vinelandii* (Yamanaka and Imai 1972). The spectral behaviour of the cytochrome *c*-549, 554 in buffers of varying pH (Figure 15 and Figure 16) agrees with that reported for other *c'*-type cytochromes (Horio and Kamen 1961, Imai *et al*. 1969).

In the present study, the physiological role of cytochrome *c*-549, 554 remains to be elucidated. Despite intensive investigations over an extended period, the function of other *c'*-type cytochromes is not clear either. It has been suggested, however, that the *c'*-type cytochrome may be involved in the metabolism of nitrogen compounds (Yamanaka and Okunuki 1974), in view of its isolation from denitrifying and nitrogen fixing bacteria (Komada and Mori 1969, Ambler 1973, Yamanada and Imai 1972). Its discovery in *Nitrobacter* not only supports such an hypothesis but also provides a unique example of diversity found in cytochromes of chemoautotrophic bacteria.

PART III

CYTOCHROME OXIDASE AND A COPPER-IRON ELECTRON TRANSFER PROTEIN

4.4 PART III CYTOCHROME OXIDASE AND A COPPER-IRON ELECTRON TRANSFER PROTEIN

4.4.1 Results

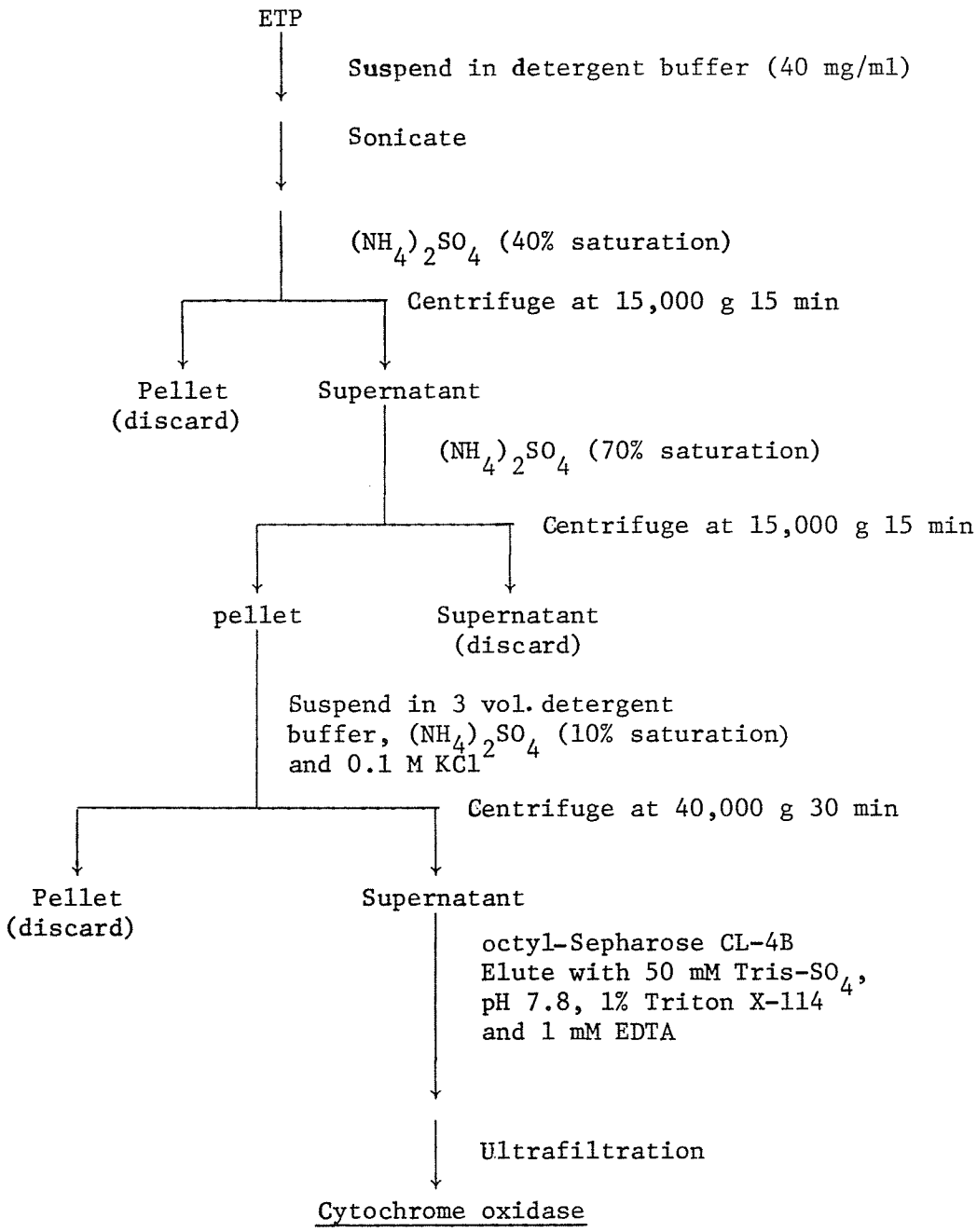
4.4.1.1 Purification of cytochrome oxidase

Purification of hydrophobic enzymes such as cytochrome oxidase is difficult mainly because their solubilization from membrane materials must be followed by intricate isolation procedures. Conventional column chromatography techniques have not been found to be very useful for the isolation of hydrophobic protein in most cases. Study of such enzymes has become easier with the advent of new methods of purification, particularly affinity chromatography. The method used for the isolation of cytochrome oxidase as well as the copper-iron protein is also one developed recently and is based on the selective reversible binding properties of octyl-Sepharose CL-4B toward hydrophobic proteins (Hjerten *et al*. 1974). A summary of the scheme used for the isolation of cytochrome oxidase is given in Figure 20.

With the exception of two important considerations, the rest of the purification procedure was quite simple. First, a proper salt concentration in the cytochrome oxidase preparation before loading onto octyl-Sepharose column was found to be very important, otherwise, the enzyme adsorption on the octyl-Sepharose either did not occur or did so very weakly. The second important consideration was the washing of the column where inclusion of Tween 80 in washing buffer not only helped to eliminate the other protein and cholate, but also desorbed the cytochrome oxidase to a certain extent. It was noted, however, that 0.25% Tween 80 did not cause

Figure 20. Purification scheme for cytochrome oxidase of *Nitrobacter agilis*.

Purification of cytochrome oxidase



much loss of the enzyme.

Repeated ultrafiltration as the final step of purification was found to be also essential in order to eliminate a small molecular weight protein present in the cytochrome oxidase preparation eluted from the octyl-Sepharose column.

4.4.1.2 Properties of Cytochrome Oxidase

Absorption spectra. The enzyme isolated exhibited the characteristic absorption spectrum of cytochrome *a*-type oxidase reported from prokaryotic as well as from eukaryotic systems (Yamanaka and Fukumori 1977, Horie and Morrison 1963, Vanneste 1966). Figure 21 shows that the oxidized form had an α band at 600 nm and another band at 420 nm (Soret band). When reduced with dithionite these bands shifted to 606 nm and 443 nm respectively. The purified preparations of cytochrome oxidase were extracted with acid acetone. Only heme *a* could be detected from the acid acetone extractions by forming the pyridine derivatives of hemochrome (Horie and Morrison 1963).

Figure 22 shows the reduced minus oxidized difference spectra of cytochrome oxidase. It was evident that the enzyme reduced slowly even in the presence of excess dithionite. An immediate scan after the dithionite treatment gave a partially reduced spectrum of cytochrome oxidase with an α band at 604 nm, which shifted to 606 nm upon complete reduction. Similar partially reduced form could also be obtained by aerating the reduced cytochrome oxidase.

Figure 21. Absorption spectra of cytochrome oxidase in 50 mM Tris-SO_4, pH 7.8 containing 0.5% Tween 80.

.... oxidized

—— reduced with $Na_2S_2O_4$

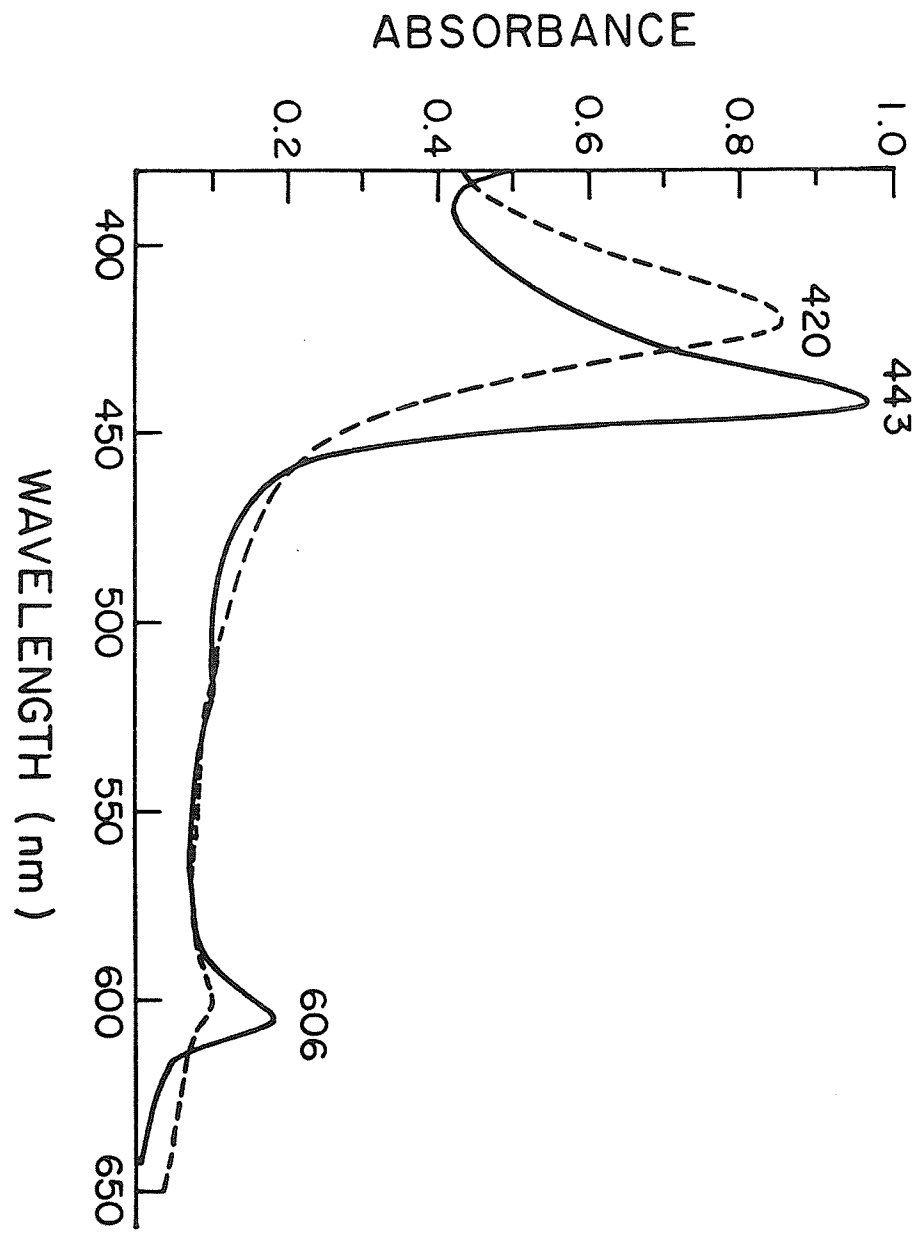
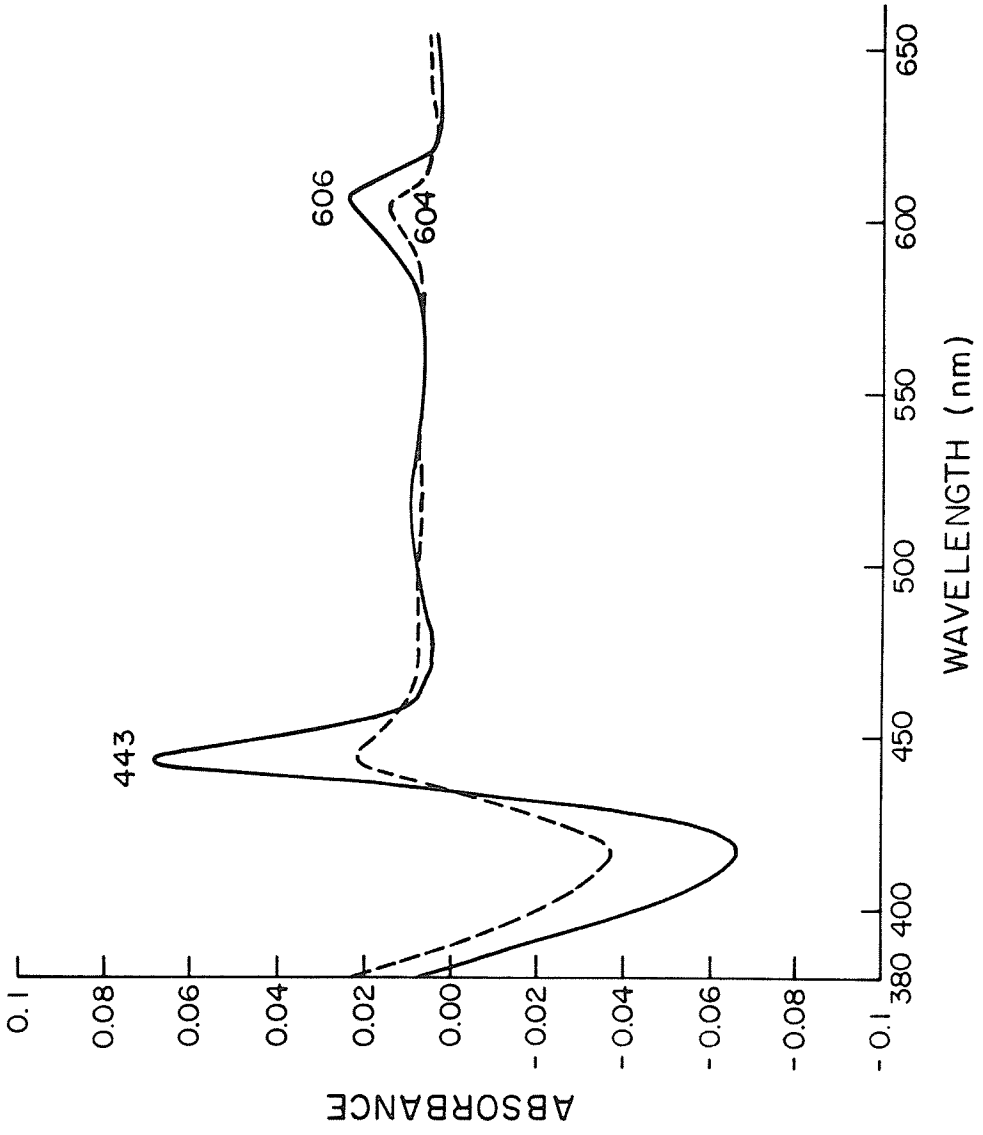

ABSORBANCE
0.2
0.4
0.6
0.8
1.0
400
450
500
550
600
650
WAVELENGTH (nm)
420
443
606

Figure 22. Reduced minus oxidized difference spectra of cytochrome oxidase in 5 mM Tris-SO_4, pH 7.8 containing 0.5% Tween 80.

---- spectrum taken immediately after the addition of $Na_2S_2O_4$

—— spectrum taken after complete reduction of cytochrome oxidase


443
606
604
0.1
0.08
0.06
0.04
0.02
0.00
- 0.02
- 0.04
- 0.06
- 0.08
- 0.1
380
400
450
500
550
600
650
ABSORBANCE
WAVELENGTH (nm)

Molecular weight. Analyses by polyacrylamide gel electrophoresis performed with the purified preparation of cytochrome oxidase under non-dissociating conditions gave a single band of protein upon staining the gel with Coomassie Blue. When electrophoreses were performed in the presence of sodium dodecyl sulfate and 6 M urea, the enzyme preparations dissociate into two larger and one smaller bands (Figure 23) with molecular weights around 37,000, 25,000 and 13,000 respectively (Figure 24).

Enzymatic activity and Stability The purified enzyme preparations in 50 mM Tris-SO_4 buffer, pH 7.8 containing 1% Triton X-114 were not stable; therefore, enzyme preparations were either passed through a Sephadex G-25 column or the concentration of Triton X-114 was reduced by ultrafiltration. The addition of 0.5% Tween 80 to the purified fractions helped the activity to persist overnight when stored at 0-5°C without any loss of activity but not when frozen. An impure fraction containing copper-iron protein, however, was relatively more stable provided the cholate was removed by ammonium sulfate precipitation followed by dialysis. The amount of phospholipid content in the enzyme preparation varied between 3% and 5%. No correlation, however, could be drawn between the amount of phospholipid and stability or activity of enzyme preparations.

Effect of pH. The enzyme activity of cytochrome oxidase was measured as mentioned in the Materials and Methods, and was found to be pH-dependent. The effect of pH on the reactivity of cytochrome oxidase with cytochrome *c* is shown in Figure 25. Interestingly

Figure 23. Densitometric traces of polyacrylamide gel of purified cytochrome oxidase. A blue filter No. 485 was used for gel scanning in a Densicord, Model 542, Photovolt Co., N.Y. Electrophoresis was carried out under dissociating conditions and the gel was stained in Coomassie Blue as described in Materials and Methods.

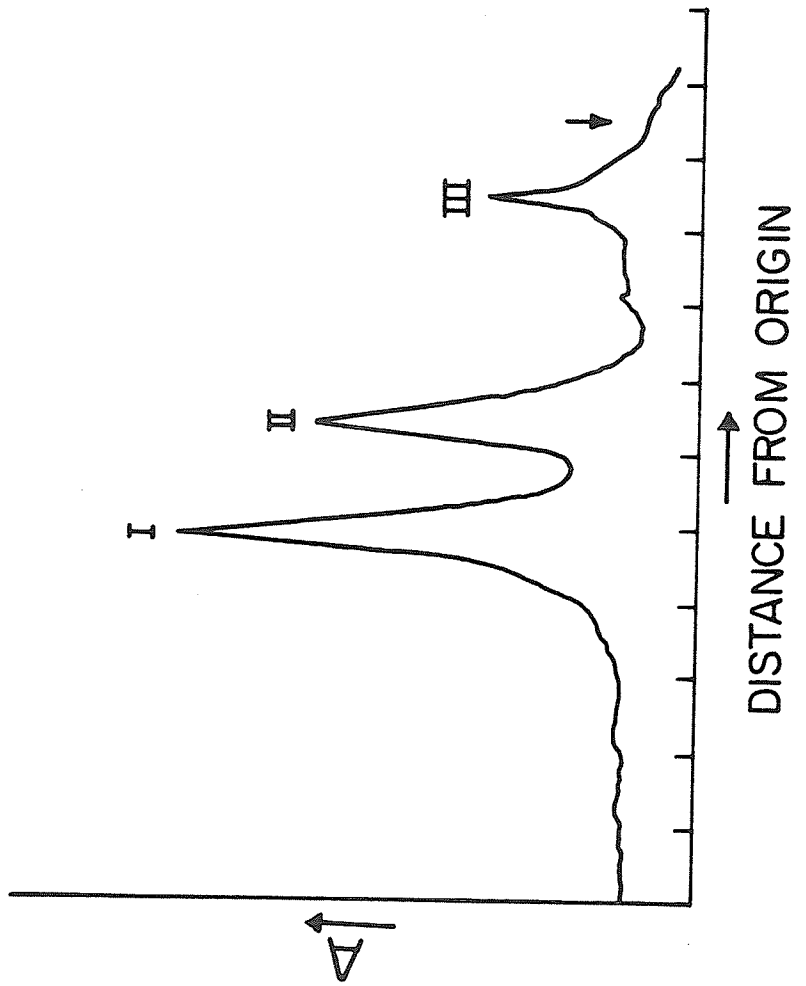

I
II
III
DISTANCE FROM ORIGIN
A

Figure 24. Molecular weight estimations of subunits of the cytochrome oxidase by polyacrylamide gel electrophoresis in the presence of 0.1% SDS and 6 M urea. The concentration of acrylamide was 10%. Their molecular weights were found to be 37,000, 25,000 and 13,000. The proteins used as markers to determine the molecular weights of the subunits were: bovine serum albumin (mol. wt. 67,000, ovalbumin (mol. wt. 45,000), chymotrypsinogen A (mol. wt. 25,000), lyzozyme (mol. wt. 14,300), and horse heart cytochrome *c* (mol. wt. 12,384).

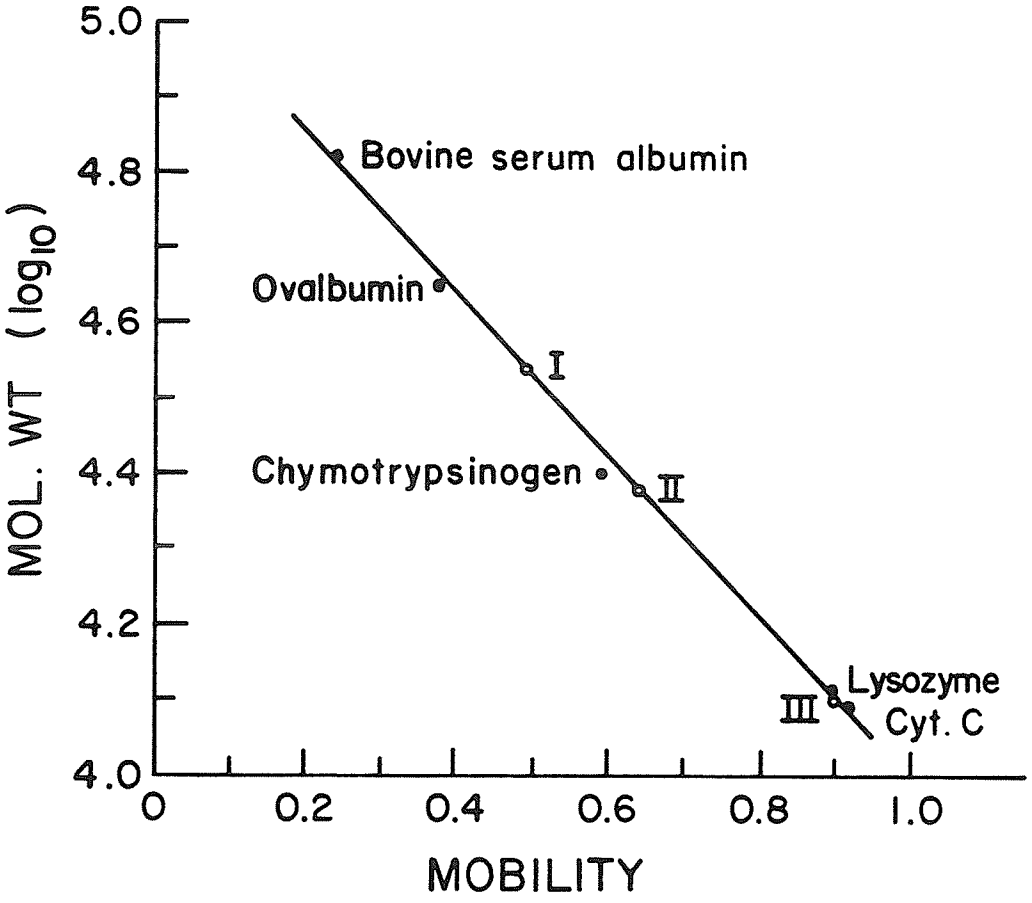
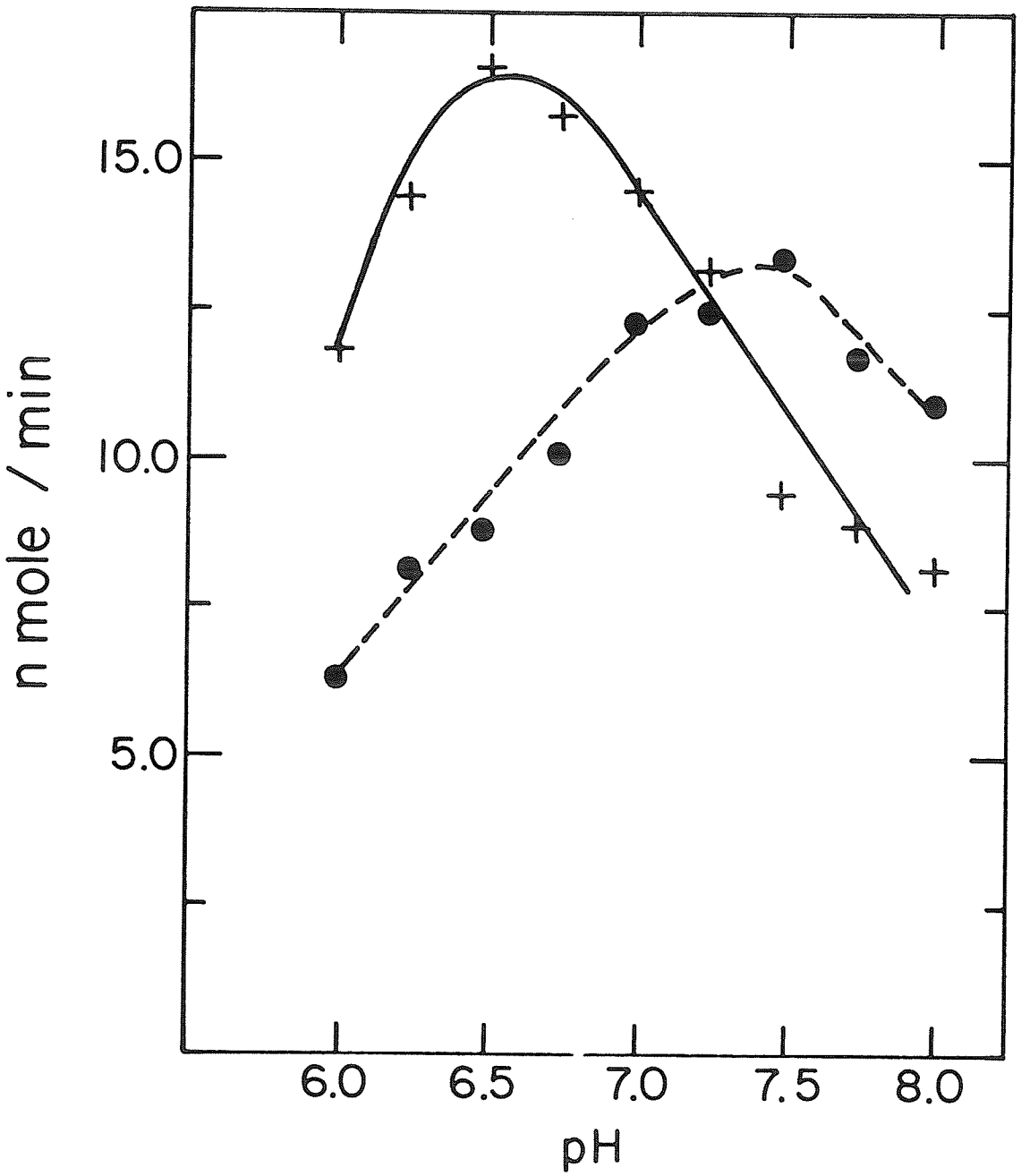

5.0
4.8
4.6
4.4
4.2
4.0
MOL. WT (log_{10})
Bovine serum albumin
Ovalbumin
I
Chymotrypsinogen
II
III
Lysozyme
Cyt. C
0
0.2
0.4
0.6
0.8
1.0
MOBILITY

Figure 25. Effect of pH on the activity of cytochrome oxidase. Oxidation rate of reduced cytochrome *c* was determined in a spectrophotometer at 550 nm described in Materials and Methods. The system contained 50 mM Tris-SO_4 buffer with appropriate pH, 0.5% Tween 80, 20 µM cytochrome *c* and enzyme.

---- *Nitrobacter* cytochrome *c*.

—— Horse heart cytochrome *c*.


15.0
10.0
5.0
n mole / min
6.0
6.5
7.0
7.5
8.0
pH

enough the enzyme was more effective with the native cytochrome *c* than with the horse heart cytochrome *c* below pH 7.2. The pH optima of cytochrome oxidase were pH 6.5 and 7.5 for *Nitrobacter* and horse heart cytochrome *c* respectively.

Effect of inhibitors. Table 7 shows inhibition of the horse heart cytochrome *c* oxidation by cytochrome oxidase in the presence of potassium cyanide, azide and diethyl dithiocarbamate. Addition of 1 mM $FeCl_3$ or 1 mM $CuSO_4$ in the reaction mixture did not restore the enzymatic activity in the presence of cyanide or diethyl dithiocarbamate. Sodium azide completely inhibited the activity of cytochrome oxidase at a 0.1 mM concentration.

4.4.1.3 Purification of Copper-Iron Electron Transfer Protein.

Preparation of ETP homogenate by sonication in detergent buffer was essentially the same as that in case of cytochrome *c* oxidase and is described in Materials and Methods. A brief summary of procedures used for the isolation of copper-iron electron transfer protein is shown in Figure 26. The fraction containing copper-iron protein was suspended in the same buffer as that used for cytochrome oxidase except that potassium chloride was excluded and ammonium sulfate concentration was raised to 15% of saturation. Under these conditions copper-iron protein was completely adsorbed on top. In various attempts a complete elimination of contaminating proteins could not be achieved even by excessive washings of the column, particularly, a small amount of cytochrome *c* was always present. It could, however, be removed by the subsequent Sephadex

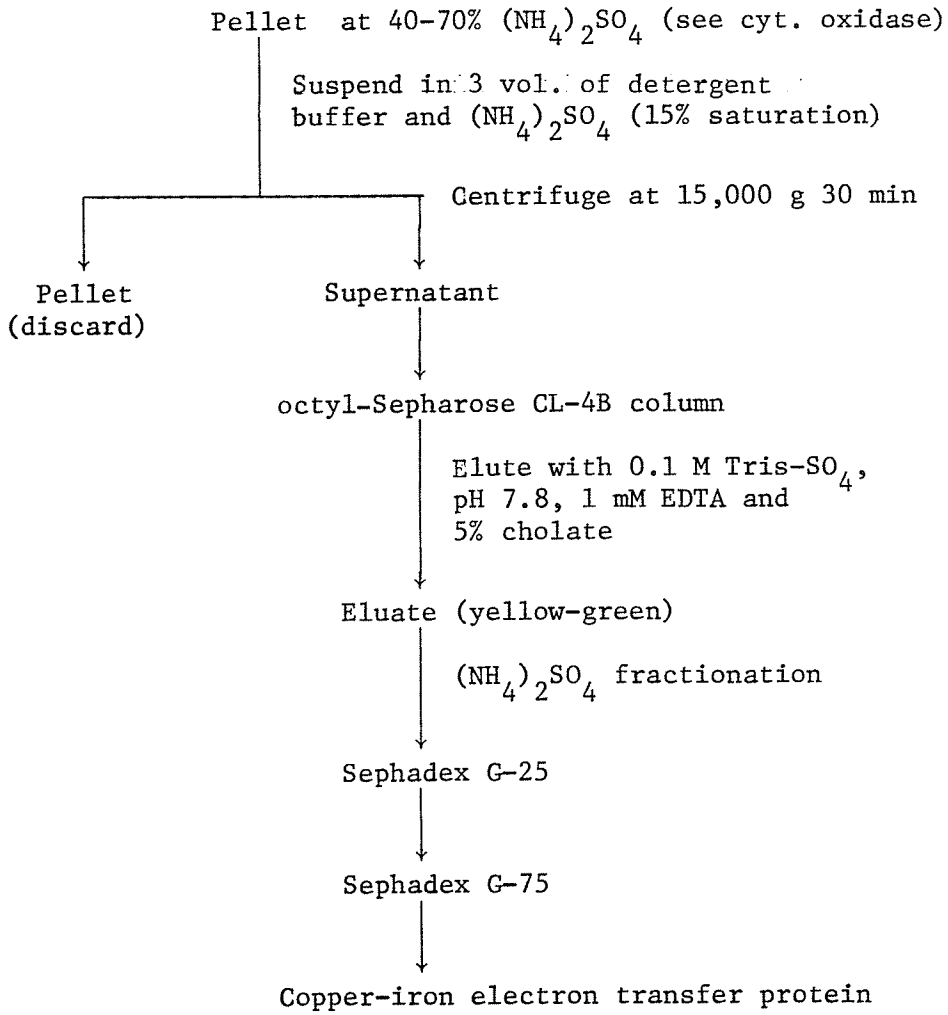
Table 7. Effect of inhibitors on cytochrome oxidase activity

Additions	Concentration mM	Enzyme Activity nmole/min	% Inhibition
None	-	12.5	-
KCN	0.1	1.3	90
	0.01	5.9	47
NaN_3	0.1	0	100
	0.01	3.4	73
Diethyl dithiocarbamate	0.05	3.1	76

Note: The enzymatic activity was determined by the standard procedure using reduced horse heart cytochrome *c*.

Figure 26. Purification scheme for copper-iron electron transfer protein from *Nitrobacter agilis*.

Purification of copper-iron electron transfer protein



G-75 column chromatography.

The elution profile of Sephadex G-75 column is presented in Figure 27. As is shown here, the bulk of the contaminating protein is eliminated in the void volume and in the early fractions of the eluted column. The contaminating cytochrome *c* also leaves the column earlier than the yellow-green band of copper-iron protein. This cytochrome appeared to be cytochrome *c*-550.

The octyl-Sepharose column eluted fractions containing copper-iron protein were also applied to a Sephadex G-100 column. It was observed, however, that the purification achieved was not as good as with Sephadex G-75. In view of its behaviour as a low molecular weight molecule on Sephadex G-75 or G-100 (see discussion), the impure fraction of copper-iron protein was also applied on Sephadex G-50 in an attempt to get more accurate molecular weight determination of the protein. Unfortunately the protein was irreversibly adsorbed on the top few cm of the column and could not be eluted even with concentrated buffer solutions.

4.4.1.4 Properties of the Copper-Iron Electron Transfer Protein

Absorption spectra. Figure 28 shows the oxidized and reduced absolute spectra of an impure fraction containing the copper-iron protein as well as cytochrome oxidase and cytochrome *c*.

The oxidized spectrum of the purified fraction of copper-iron protein has the absorption maxima at 410 and 630 nm with shoulders at 326, 460 and 495 nm (Figure 29). Upon reduction with dithionite, the 630 nm peak was abolished and 410 nm absorption was decreased

Figure 27. Purification of copper-iron electron transfer protein in Sephadex G-75. For details see Materials and Methods.

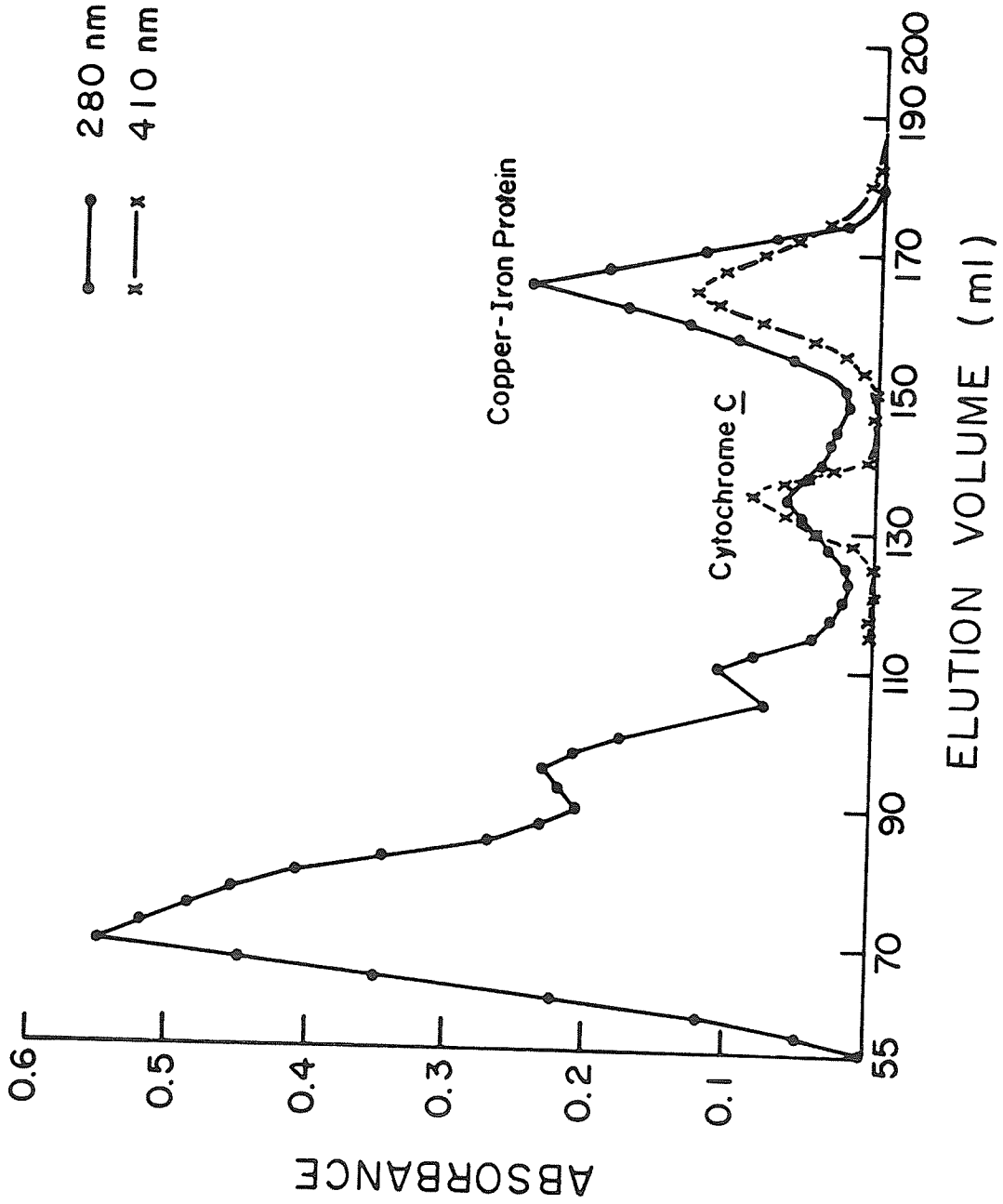

280 nm
410 nm
Copper-Iron Protein
Cytochrome C
ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
55
70
90
110
130
150
170
190 200
ELUTION VOLUME (ml)

Figure 28. Absorption spectra of copper-iron electron transfer protein containing cytochrome *c* and cytochrome oxidase in 50 mM Tris-HCl, pH 7.8.

---- oxidized

—— reduced with $Na_2S_2O_4$

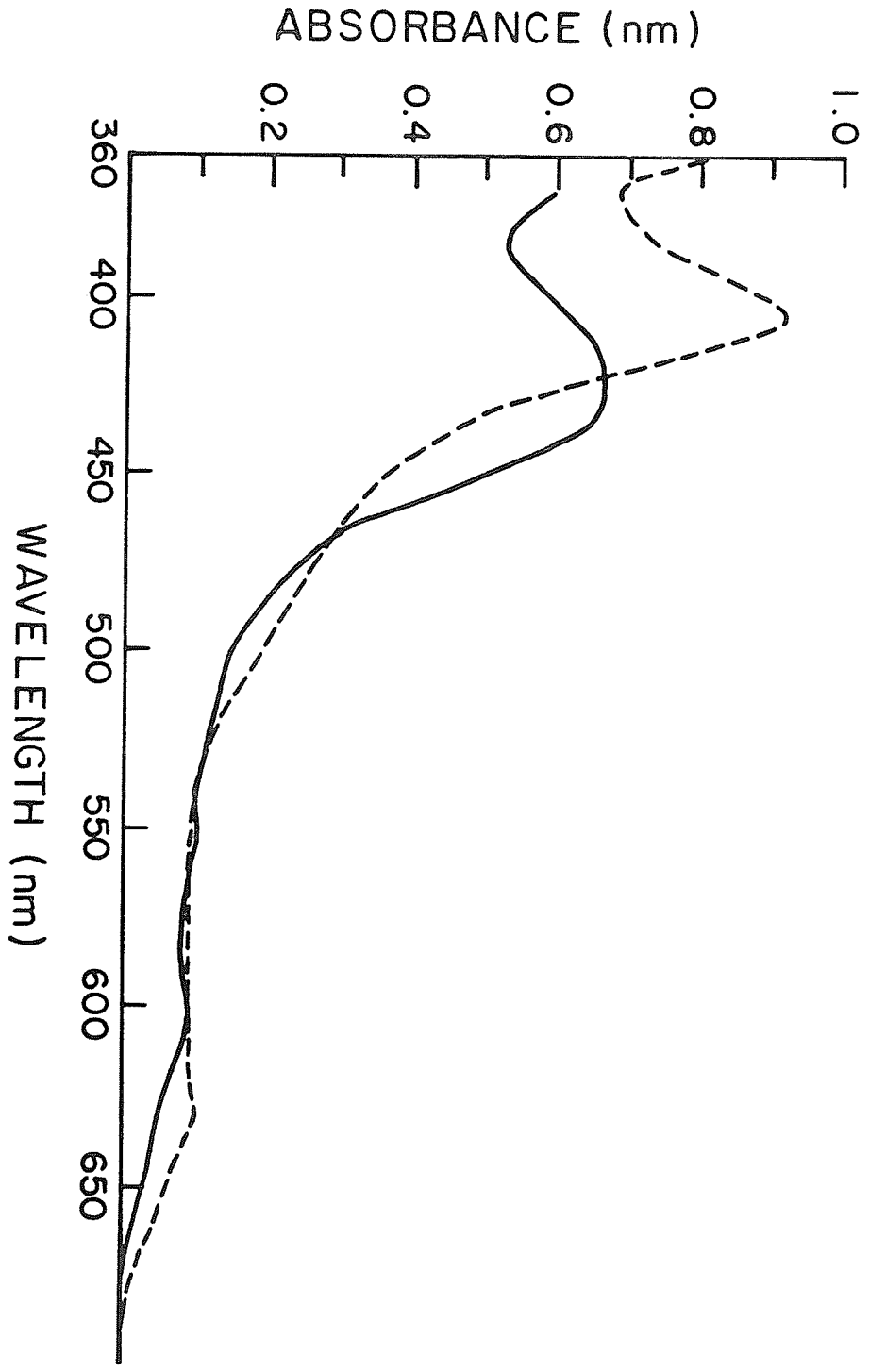
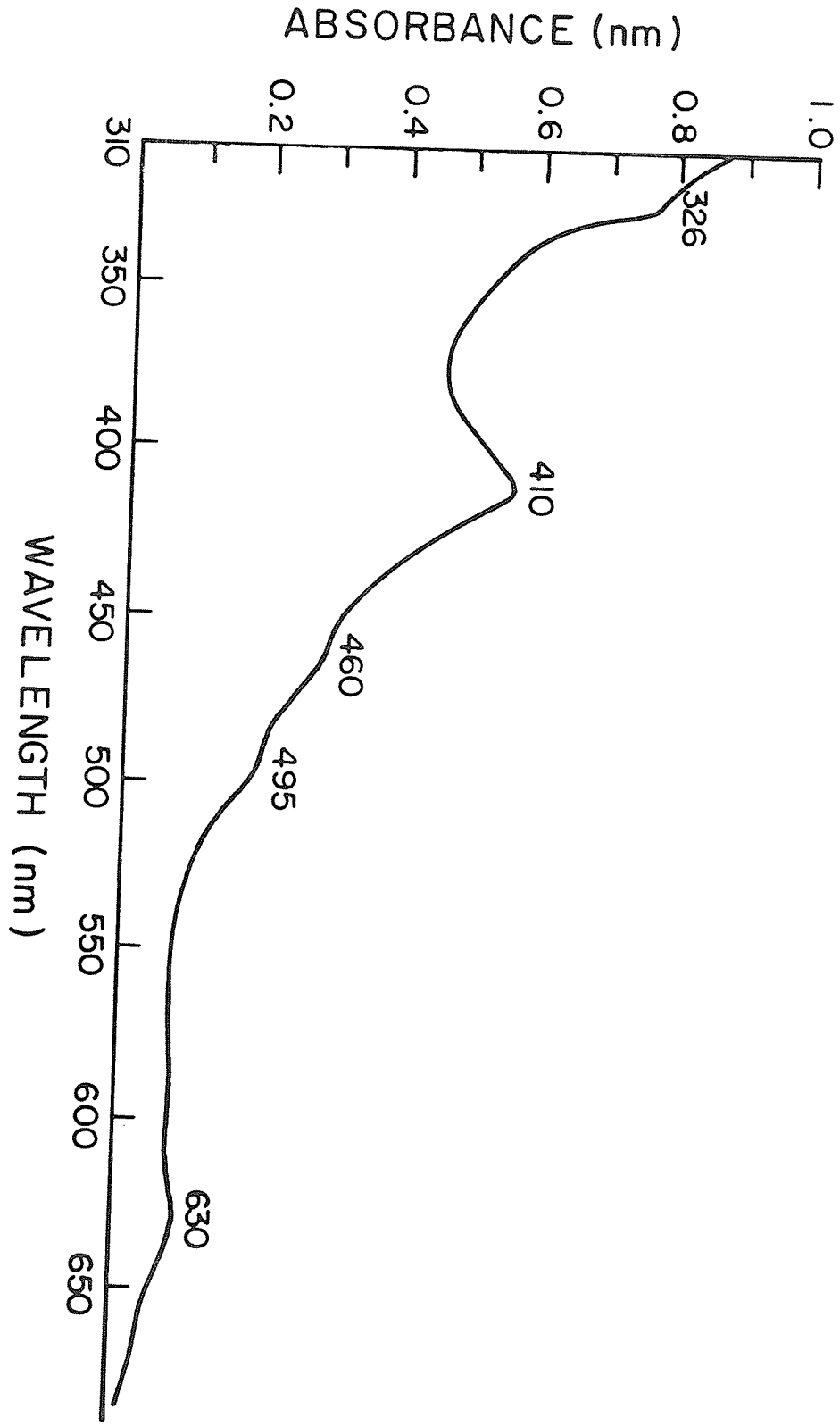

ABSORBANCE (nm)
0.2
0.4
0.6
0.8
1.0
360
400
450
500
550
600
650
WAVELENGTH (nm)

Figure 29. Absorption spectrum of purified copper-iron electron transfer protein in 50 mM Tris-HCl, pH 7.8 containing 1 mM EDTA.


ABSORBANCE (nm)
0.2
0.4
0.6
0.8
1.0
310
350
400
450
500
550
600
650
WAVELENGTH (nm)
326
410
460
495
630

to a great extent (Figure 30). Visual observations of the sample cuvette also confirmed the bleaching of yellow-green color of the protein immediately after the addition of dithionite. The reduced pigment was not oxidized after depletion of dithionite even upon excessive aeration.

In an attempt to find out the physiological role of the copper-iron protein, the oxidized form of the protein was treated with compounds such as NH_4^+, NH_2OH, NO_2^-, NADH and NADPH, but no effect by these compounds was observed under aerobic conditions. Similarly, the reduced form of the copper-iron protein also did not react with these compounds.

Molecular weight. The molecular weight of the purified copper-iron protein was estimated by gel filtration on a Sephadex G-100 or G-75 column (Andrew 1964). The buffer used for column elution was 50 mM Tris-HCl, pH 7.5 containing 100 mM potassium chloride. Standard proteins used for comparison were ribonuclease A, chymotrypsinogen A, ovalbumin and bovine serum albumin. The results of the determination are shown in Figure 31 and the molecular weight of copper-iron electron transfer protein was determined to be 4,000 and 5,500 with Sephadex G-100 and G-75 respectively. The molecular weight determination of copper-iron protein could not be accomplished by Sephadex G-50 because of its irreversible adsorption on the gel as reported earlier.

Figure 31 shows the molecular weight determination by Sephadex G-75. Molecular weight of the copper-iron protein was

Figure 30. Absorption spectra of copper-iron electron transfer protein in 50 mM Tris-HCl, pH 7.8 containing 1 mM EDTA.

---- oxidized

——— reduced

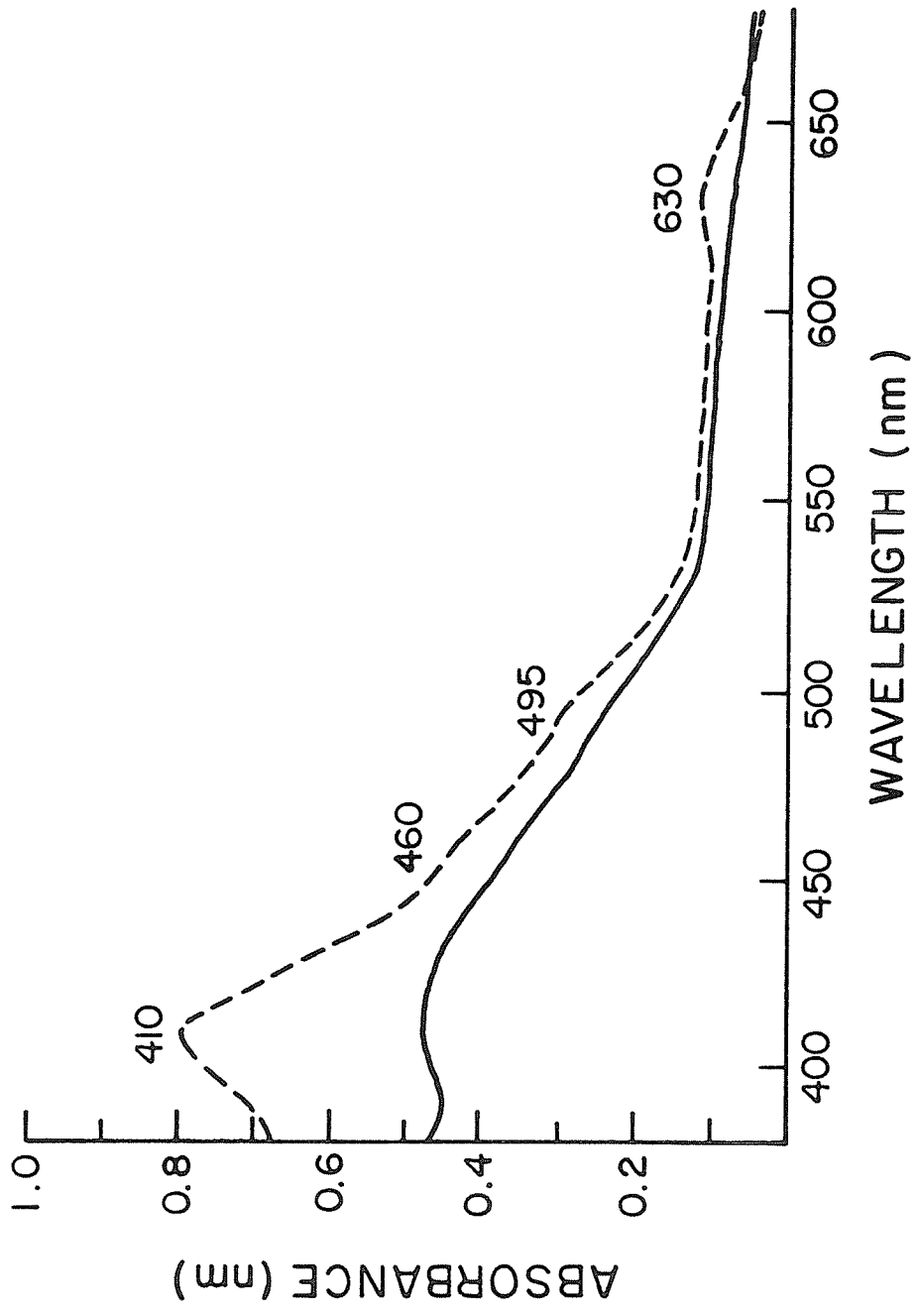
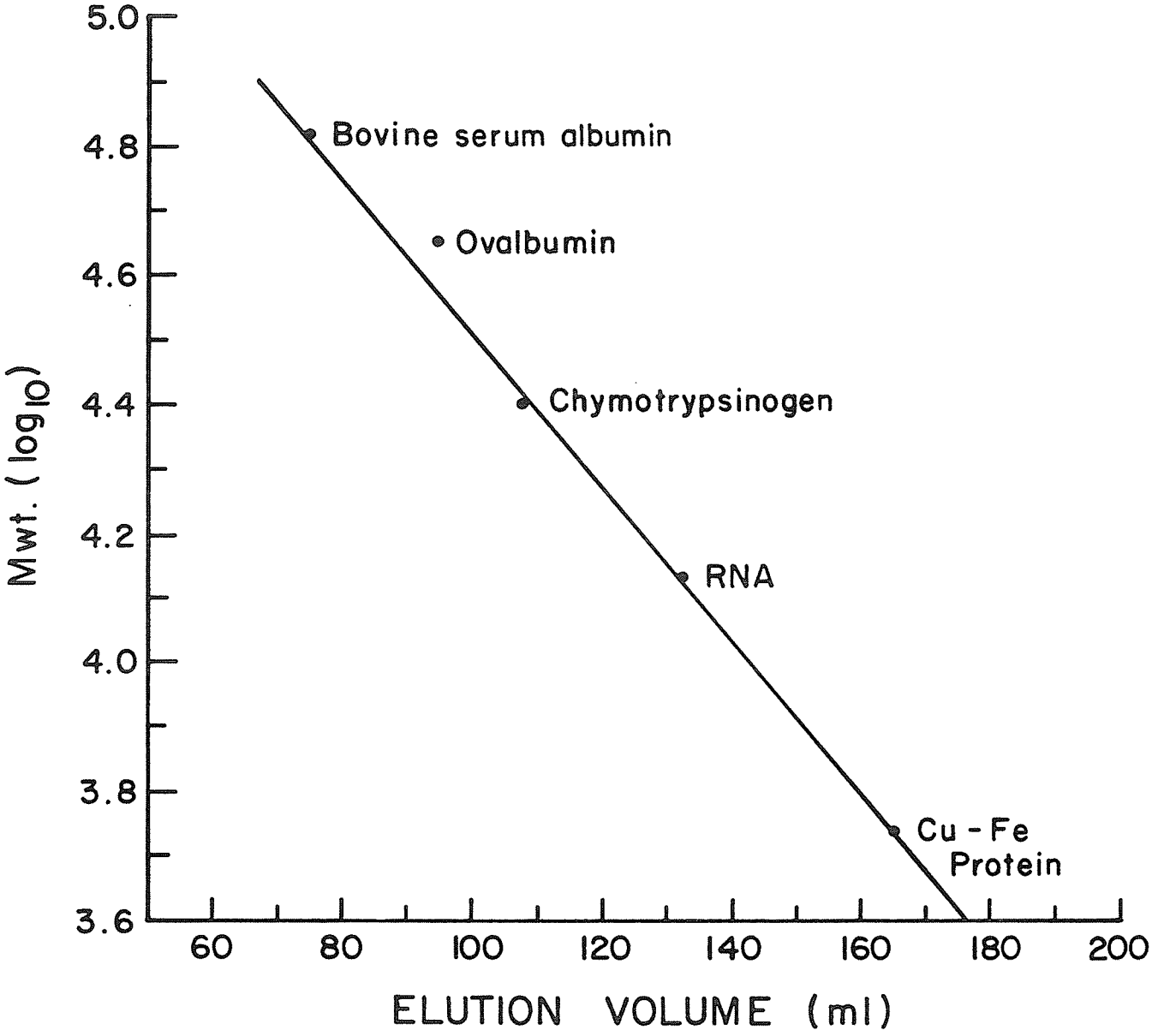

ABSORBANCE (nm)
1.0
0.8
0.6
0.4
0.2
400
450
500
550
600
650
WAVELENGTH (nm)
410
460
495
630

Figure 31. Molecular weight estimation of copper-iron electron transfer protein by Sephadex G-75 in 50 mM Tris-HCl, pH 7.5 containing 0.1 M KCl. The marker proteins used were bovine serum albumin (mol. wt. 67,000), chymotrypsinogen A (mol. wt. 25,000), ovalbumin (mol. wt. 45,000) and ribonuclease A (mol. wt. 13,700).


5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
Mwt. (log10)
Bovine serum albumin
Ovalbumin
Chymotrypsinogen
RNA
Cu - Fe
Protein
60
80
100
120
140
160
180
200
ELUTION VOLUME (ml)

estimated by extrapolating the standard curve.

This copper-iron protein is much smaller than the usual Cu or Fe containing proteins (Hardy and Burns 1973).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoreses of purified preparations of Cu-Fe protein were performed under non-dissociating conditions (Davis 1964) using 12.5% acrylamide gel. Electrophoreses were carried out for 1½-2 h at 3 mA/gel and fixed, stained and destained as described in the Materials and Methods. The copper-iron protein appeared to migrate as a single band as judged by the only band obtained upon staining the electrophoresed gel by Coomassie Blue. This indicates that the isolated protein preparations were pure.

Amino acid composition. Amino acid analyses were performed on the purified Cu-Fe protein preparations according to the standard method. Table 8 shows the various amino acids and their number of residues present per mole of copper-iron protein. Methionine was the only amino acid found to be missing in the polypeptide of this pigment. The minimum molecular weight calculated on the basis of amino acid composition gave a value of 5,243 with a total of 47 amino acid residues (excluding tryptophan and cysteine; they were not analyzed).

Metal content. The preparations of copper-iron protein were analyzed for their Cu and Fe content as earlier described in Material and Methods. The results of these analyses consistently gave low values of these metals in the pure protein preparations. The average amount of copper and iron estimated from several samples

Table 8. Amino acid composition of the copper-iron protein from *Nitrobacter agilis*.

Amino acid	No. of residues per mole of protein		
Aspartic acid	4		
Threonine	3		
Serine	3		
Glutamic acid	4		
Proline	3		
Glycine	5		
Alanine	5		
Valine	2		
Methionine	0		
Isoleucine	2		
Leucine	4		
Tyrosine	1		
Phenylalanine	2		
Histidine	1		
Lysine	6		
Arginine	2		
Total residues	47		
Phospholipid	3%		
Copper	0.34		
Iron	0.60		
Molecular weight	5243[a]	5500[b]	4000[c]

NOTE: Cysteine and tryptophan were not determined.

[a]Minimum molecular weight on the basis of amino acid analysis.

[b]Molecular weight determined by G-75 gel filtration.

[c]Molecular weight determined by G-100 gel filtration.

was 3.7 µg and 6 µg per mg of protein respectively. Assuming a molecular weight of 5,500 (Sephadex G-75), the protein has 0.34 g atom of Cu and 0.60 g atom of iron per mole of protein (Table 8). The amount of Cu determined in impure preparations was significantly higher than in purified protein as reported above. Perhaps the high concentration of ammonium sulfate used in the procedure resulted in the loss of copper. A decrease in copper content was found to be related with the treatments of ammonium sulfate during the isolation of plastocyanin (Katoh *et al*. 1962, Milne and Wells 1970). A similar problem was encountered by the use of high concentration of ammonium sulfate while isolating rusticyanin (Cox and Boxer 1978).

Phospholipid content. There were many observations which led to the suspicion that the copper-iron protein contains a lipid moiety as well; for example, partial precipitation of the copper-iron protein when treated with sodium hydroxide during protein determination. Furthermore, when hydrolyzed samples were resuspended in water for amino acid analyses, there appeared a very thin but noticeable lipid layer on top of the aqueous layer. Accordingly, the copper-iron protein was analyzed for phospholipid by the method of Raheja *et al*. (1973). The results of these determinations however, indicated the presence of only 30 µg phospholipid per mg of protein (i.e. 3%) as is shown in Table 8.

4.4.2 Discussion

4.4.2.1 Cytochrome Oxidase

Cytochrome oxidase, an enzyme tightly bound to the cell membrane, acts as a final electron transfer mechanism to O_2. This terminal enzyme of the respiratory chain (cytochrome *a*-type oxidase) has been purified and studied in detail from many eukaryotic and prokaryotic organisms (King *et al.* 1979, Frey *et al.* 1978, Phan and Mahler 1976, Schatz and Mason 1974, Erickson *et al.* 1972, Yamanaka and Fukumori 1977).

The initial studies of Lees and Simpson (1957) followed by others (Aleem and Nason 1959, Straat and Nason 1965, Van Gool and Landelout 1966, Kiesow 1967) proposed the involvement of *a*-type cytochromes in nitrite oxidation by *Nitrobacter*. Aleem (1968) also supported the implication of cytochrome a_1 and a_3 in nitrite oxidation. The study of the photochemical action spectrum by Hill and Taylor (1969) led them to suggest that cytochrome a_1 but not cytochrome a_3 was the cytochrome oxidase in *Nitrobacter*. Cytochrome a_1 has also been reported to act as oxidase in a closely related organism, *Nitrosomonas* (Erickson *et al.* 1972). With the latter exception, all these studies were conducted with whole cells or cell-free extracts of *Nitrobacter* and no attempt was made to isolate the cytochrome oxidase. A more detailed study of cytochrome in *Nitrobacter* showed the presence of *a* and a_3 as well as two a_1 cytochromes in the nitrifying bacteria, (Sewell *et al.* 1972); the precise role of each one of these cytochromes, however, remained

obscure.

In our preliminary efforts to isolate cytochrome oxidase as well as other *a*-type cytochromes, we were able to separate the two components of cytochrome a_1 but they were always complexed with either cytochrome *c*, *b* or both. The isolations were attempted by solubilizing the whole cells with detergents such as Triton X-100, deoxycholate and cholate followed by DEAE-cellulose column chromatography. Unfortunately, these investigations had to be discontinued at an early stage because of time constraints and poor yields. Nevertheless, such studies might help to determine the much discussed yet obscure role of cytochrome a_1 (O'Kelley *et al.* 1970, Aleem 1978). Recently Yamanaka *et al.* (1979) have purified cytochrome oxidase from two bacterial species including a species of *Nitrobacter* by following a method similar to that described above.

In this investigation cytochrome oxidase was isolated from ETP (which were obtained from cells) by a hydrophobic interaction chromatographic technique coupled with ammonium sulfate fractionation (Figure 20). The procedure followed here was similar to that used for the isolation of other hydrophobic proteins (Ozawa *et al.* 1980, Seligy 1978, Smyth *et al.* 1978). Some of the difficulties encountered in attempts to eliminate impurities (Rosen 1978) were overcome by including an ultrafiltration step in the purification procedure.

Figure 21 shows the absorption spectra of purified cytochrome oxidase. It has the appearance of a typical cytochrome *a*-type

oxidase (Yamanaka and Fukumori 1977, Hochli and Hackenbrock 1978, Rascati and Parsons 1979, Vanneste 1966, Horie and Morrison 1963). The oxidized form of the enzyme showed an absorption maxima at 420 and 600 nm and the reduced form at 443 and 606 nm. A similar reduced spectrum has been reported for cytochrome oxidase isolated from this bacterium by DEAE-cellulose column chromatography (Yamanaka *et al.* 1979).

The purified cytochrome oxidase reacted with *Nitrobacter* as well as with horse heart ferrocytochrome *c*. However, the pH optimum for reaction with cytochrome *c* was different for both sources. The reactivity of cytochrome oxidase with horse heart cytochrome *c* is in agreement with the results of Yamanaka *et al.* (1979), although they did not report the effect of pH on the enzyme activity. Difference in pH for the optimal activity of enzyme with *Nitrobacter* and horse heart cytochrome *c* may be related to slight variations in the amino acid composition of the two cytochromes as discussed earlier.

The enzyme activity was inhibited by standard cytochrome oxidase inhibitors (Table 7). Effects of cyanide and azide were similar to those reported for the nitrite oxidase activity of ETP (Aleem 1978).

Cytochrome *a*-type oxidase is a lipoprotein. The amount of bound lipid in purified mitochondrial cytochrome oxidase varied from 1-2% (Mason *et al.* 1973) to 20% (Kuboyama *et al.* 1972) and the amount recovered appears to be dependent on the nature of the detergent used for the solubilization and purification of the enzyme.

The phospholipid content of cytochrome oxidase from *Nitrobacter* was relatively low. The activity of the enzyme was not stimulated by the addition of phosphatidylcholine and phosphatidylethanolamine in the reaction mixture, although cytochrome oxidase from beef-heart purified by a similar method had been reported to be activated three fold by the addition of phosphatidylcholine (Rosen 1978).

Mitochondrial cytochrome *a*-type oxidase is a multiunit enzyme (Phan and Mahler 1976, Poyton and Schatz 1975, Rubin and Tzagoloff 1973), whereas the bacterial cytochrome *a*-type oxidase has been described as consisting of only two subunits (Yamanaka *et al.* 1979). The enzyme purified here moved as a single band under non-dissociating conditions during polyacrylamide gel electrophoresis. When electrophoresis was performed in the presence of SDS and 6 M urea, the enzyme preparations appeared as three protein bands upon staining the gel with Coomassie Blue (Figure 23). These bands corresponded to the molecular weight of 37,000, 25,000 and 13,000 (Figure 24). This indicates that the enzyme purified here consisted of three subunits, a finding that differs from the earlier report of cytochrome oxidase containing only two subunits (Yamanaka *et al.* 1979).

There are at least two possible reasons for this discrepancy in the results. First, that the low molecular weight band was an impurity which migrated also along with the enzyme under the non-dissociating conditions during electrophoresis. Second, and most likely is that the procedure used by Yamanaka *et al.* for electrophoresis was different from the one used here. There are reports

which state that the number of subunits resolved from mitochondrial cytochrome oxidase varies from 5 to 7 and depends upon the procedure used for the polyacrylamide gel electrophoresis (Downer *et al*. 1976).

The subunits of cytochrome *a*-type oxidases are compared in Table 9. Three subunits of *Nitrobacter* cytochrome oxidase appear to resemble the three major subunits of the mitochondrial enzyme. These findings are interesting in view of the earlier reports that the polypeptide chains for the three larger subunits of cytochrome oxidase are synthesized on the mitochondrial ribosomes whereas smaller subunits are synthesized on the cytoplasmic ribosomes (Mason and Schatz 1973, Schatz ane Mason 1974). This also suggests the close evolutionary relationship of *Nitrobacter* with *mitochondria* and is in agreement with the endosymbiotic hypothesis of Margulis (1970).

4.4.2.2 Copper-Iron Electron Transfer Protein

Various types of non-heme iron or copper containing electron transfer proteins have been isolated from a variety of organisms (Hardy and Burns 1973, Holm and Ibers, 1977, Cox and Boxer 1978, Milne and Wells 1970). A similar yellow-green pigment named as copper-iron protein was isolated from ETP-like cytochrome oxidase. The properties of this pigment are discussed here.

The copper-iron protein preparations applied to Sephadex column as a part of final step of its purification or in order to determine its molecular weight indicated differential behaviour was bleached

Table 9. Comparison of the molecular weights of the subunits between *Nitrobacter agilis* and mitochondrial cytochrome oxidases determined by SDS plus urea polyacrylamide gel electrophoresis.

Subunit	Beef heart cytochrome oxidase[b]	Rat liver cytochrome oxidase[b]	*Nitrobacter* cytochrome oxidase[a]
I	35,300	34,000	37,000
II	25,200	26,800	25,000
III	21,000 16,200	23,700 17,000	- 13,000
IV	12,100	12,500	-
V	6,700	9,500	-
VI	3,400	3,600	-

[a]Polyacrylamide gel electrophoresis was carried out with 10% acrylamide in the presence of SDS and 6 M urea by a procedure described in the Materials and Methods.

[b]The result of beef heart and rat liver oxidase obtained with 12.5% acrylamide in the presence of SDS and 8 M urea from the work of Höchli and Hackenbrock (1978).

with different types of Sephadex. The molecular weight of protein estimated in Sephadex-G-100 and G-75 was 4,000 and 5,500 respectively (Figure 31). Where Sephadex G-50 was used the protein was irreversibly adsorbed on the gel, possibly due to separation of small amounts of cholate from the protein making the latter insoluble. Such a property may be attributed to the hydrophobic nature of the protein. This observation was also supported by the results that showed the protein contained 3% phospholipid (Table 8).

Metal ion analyses showed that the protein possess both copper and iron. The Cu and Fe estimated on the basis of a molecular weight of 5,500 are quite low (Table 8). It is possible that the molecular weight of copper-iron protein estimated by gel filtration may not be true (because of its behavior in Sephadex as discussed above). On the basis of copper estimations alone, it should have a molecular weight around 15,000 assuming at least 1 g atom Cu per mole of protein. Such a molecular size would be similar to that of other copper containing proteins, rusticyanin (Cox and Boxer 1978) and plastocyanin (Milne and Wells 1970).

On the other hand, the minimum molecular weight obtained from the amino acid analysis gave a value of 5243 close to that estimated by gel filtration in Sephadex G-75 (Table 8). An observation that the copper-iron protein preparations were dialysable also agrees with these results.

The spectral studies showed that copper-iron protein has absorption maxima at 410 and 630 nm with shoulders at 326, 460 and 495 nm (Figure 29). The yellow-green color of the protein was bleached

upon reduction with dithionite. The spectrum of the oxidized form of the protein has some features similar to a molybdenum-iron-sulfur protein isolated from *Desulfovibrio africanus* (Hatchikian and Bruschi 1979), although the latter was a high molecular weight protein and the absorption spectrum was not affected by dithionite. An absorption band around 630 nm is not the characteristic of other copper containing proteins either (Cox and Boxer 1978, Milne and Wells 1970).

The amino acid analyses performed on the protein preparations revealed that it lacks methionine residues (Table 8), whereas x-ray crystallographic studies showed that the Cu in plastocyanin, a Cu containing protein, is coordinated to His 48, Cys 114, His 119, and Met 124 (Coleman *et al* 1978), indicating an important role of methionine in the orientation of the protein molecule. These observations as well as the presence of iron suggest that it may be a new protein which contains both copper and iron.

Attempts to determine the biological function of copper-iron protein were not successful. It is, however, likely that it may be some way associated with cytochrome oxidase, since the impure fractions of cytochrome oxidase containing copper-iron protein were more stable (as discussed above).

Further studies are required to investigate the structure and physiological role of this protein in the chemoautotrophic bacterium, *Nitrobacter*.

4.5 PART IV ISOLATION OF CYTOCHROME *b*, UBIQUINONE AND SOME OXIDO-REDUCTASES

4.5.1 Results

4.5.1.1 Cytochrome *b*

Unlike the situation with cytochrome *c* very few soluble preparations of *b*-type cytochromes have been obtained. Their investigation has, therefore, been largely restricted to particulate complexes, to associations with oxidases, with other cytochromes, or a variety of reductases. Little is known about cytochrome *b* in *Nitrobacter*. Throughout the course of this investigation several attempts were made to isolate the cytochrome *b*. The results of these studies are presented in this section.

4.5.1.2 Isolation of Cytochrome *b*

Two approaches were attempted to solubilize the cytochromes of *Nitrobacter* and then separate each component of the respiratory chain.

First, whole cells or ETP were solubilized by detergents such as Triton X-100 and deoxycholate or cholate followed by ammonium sulfate fractionation and DEAE-cellulose (or otherwise mentioned) column chromatography. In the second method the cytochromes were released from cells by homogenization with glass beads and the soluble fraction (F_0) was used as a source of starting material which led to the isolation of various cytochromes of *Nitrobacter*.

When whole cells were sonicated with detergents, the ensuing DEAE-cellulose chromatography did not lead to separation of the cytochromes. The treatment, however, was found to be useful

PART IV

ISOLATION OF CYTOCHROME *b*, UBIQUINONE AND SOME OXIDO-REDUCTASES

in the spectrophotometric demonstration of cytochrome *b* and *a*-type cytochromes in various complexes separated in DEAE-cellulose. An oxidized minus reduced difference spectrum of one such complex is shown in Figure 32. The complex had cytochromes *a*, *b*, and *c* with the absorption maxima at 592, 560 and 553 nm respectively. Similarly there were fractions which contained cytochrome *a* with absorption peaks at 587 and 583 nm (not shown). In view of their inseparable association, the method was considered to be unsatisfactory for the isolation of cytochrome *b*.

Cytochrome *b* was, therefore, isolated from F_0 by DEAE-cellulose chromatography as previously described. Initially cytochrome *b* was observed in fractions eluted with 0.2 M potassium phosphate, pH 7.8 (Chaudhry *et al.* 1980). These fractions, however, also contained a *c*-type cytochrome. Subsequent investigations made it clear that the inclusion of GSH in the washing buffer of DEAE-cellulose column could remove most of the cytochrome *c*. The slow elution of cytochrome *b* from the column was improved by using 0.2 M potassium phosphate, pH 7.5 and 0.1 M KCl. Ammonium sulfate fractionation removed most of the other contaminating proteins to give a 50% recovery of cytochrome *b*. Sephadex G-100 chromatography resulted in 10 fold purification, although the yield was poor (17%). Ultrafiltration gave a further 1.5 fold purification.

4.5.1.3 Properties of Cytochrome *b*.

Absorption spectra. Absorption spectra of a cytochrome *b* fraction containing some cytochrome *c* also are shown in Figure 33 and 34. Both the reduced absolute and difference spectra had two absorption peaks at 550 and 560 nm. The absorption

Figure 32. Reduced minus oxidized difference spectrum of cytochromes *a*, *b*, and *c* complex. The sample in 50 mM potassium phosphate pH 7.8 was reduced with $Na_2S_2O_4$.

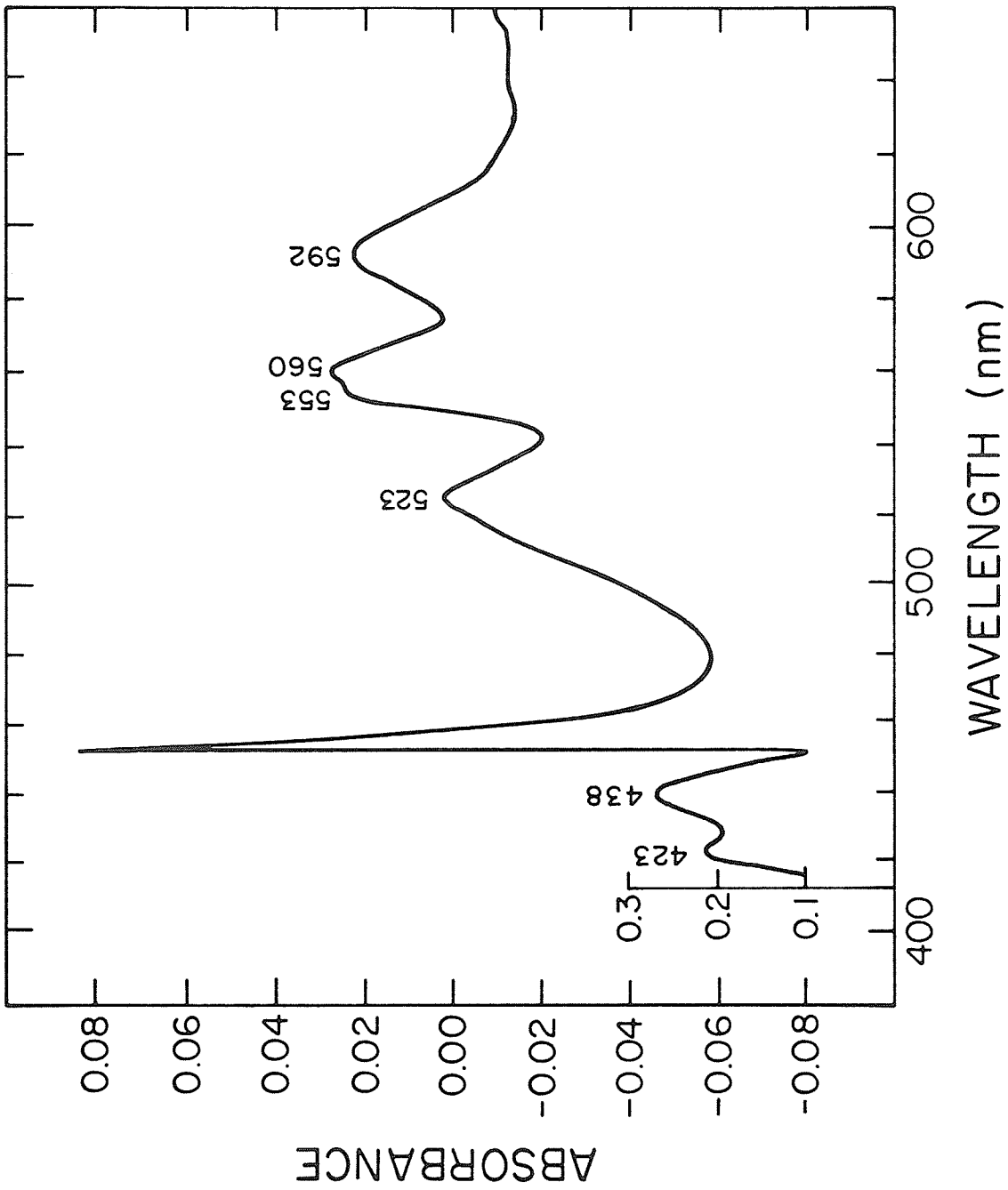

ABSORBANCE
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
0.3
0.2
0.1
400
500
600
WAVELENGTH (nm)
438
423
592
560
553
523

Figure 33. Absorption spectra of cytochromes *b* and *c* complex in 0.1 M potassium phosphate, pH 7.8.

---- oxidized

—— reduced with $Na_2S_2O_4$

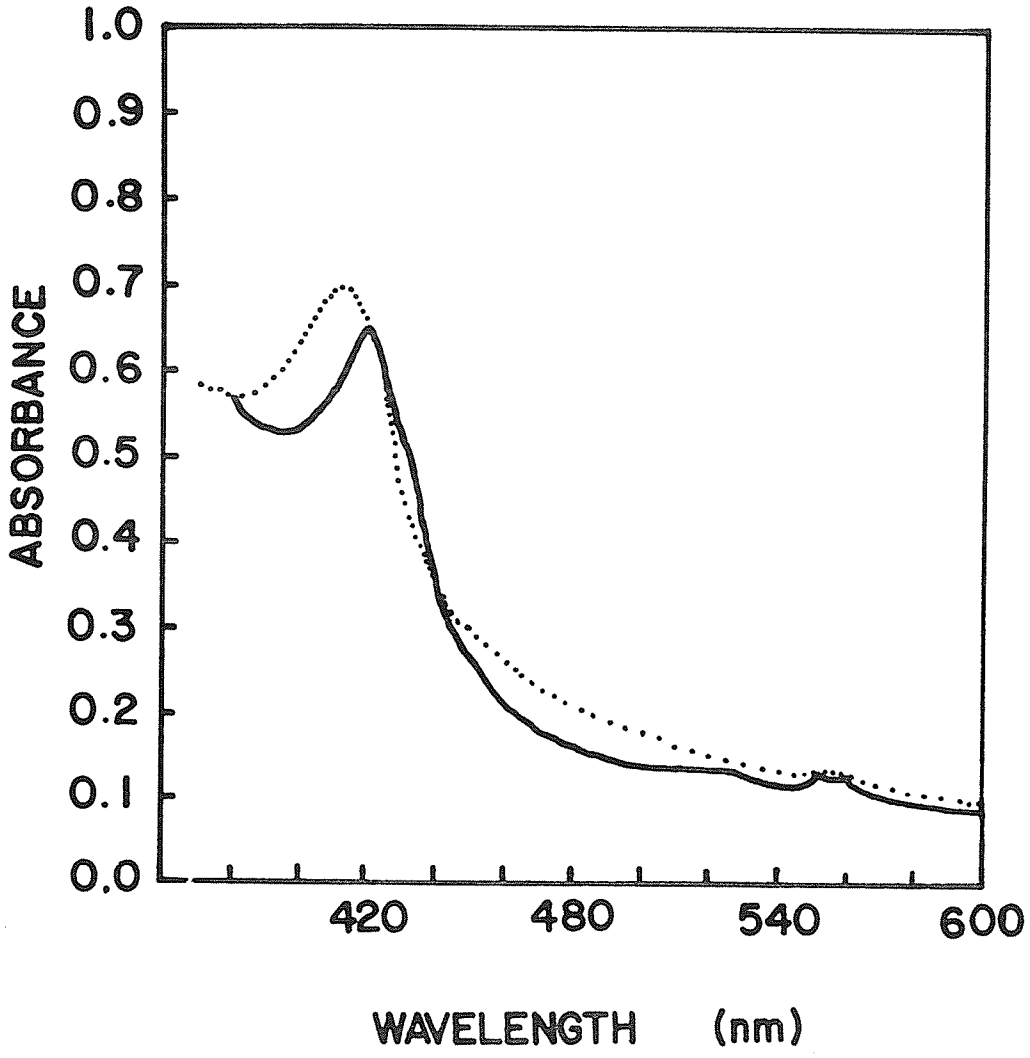
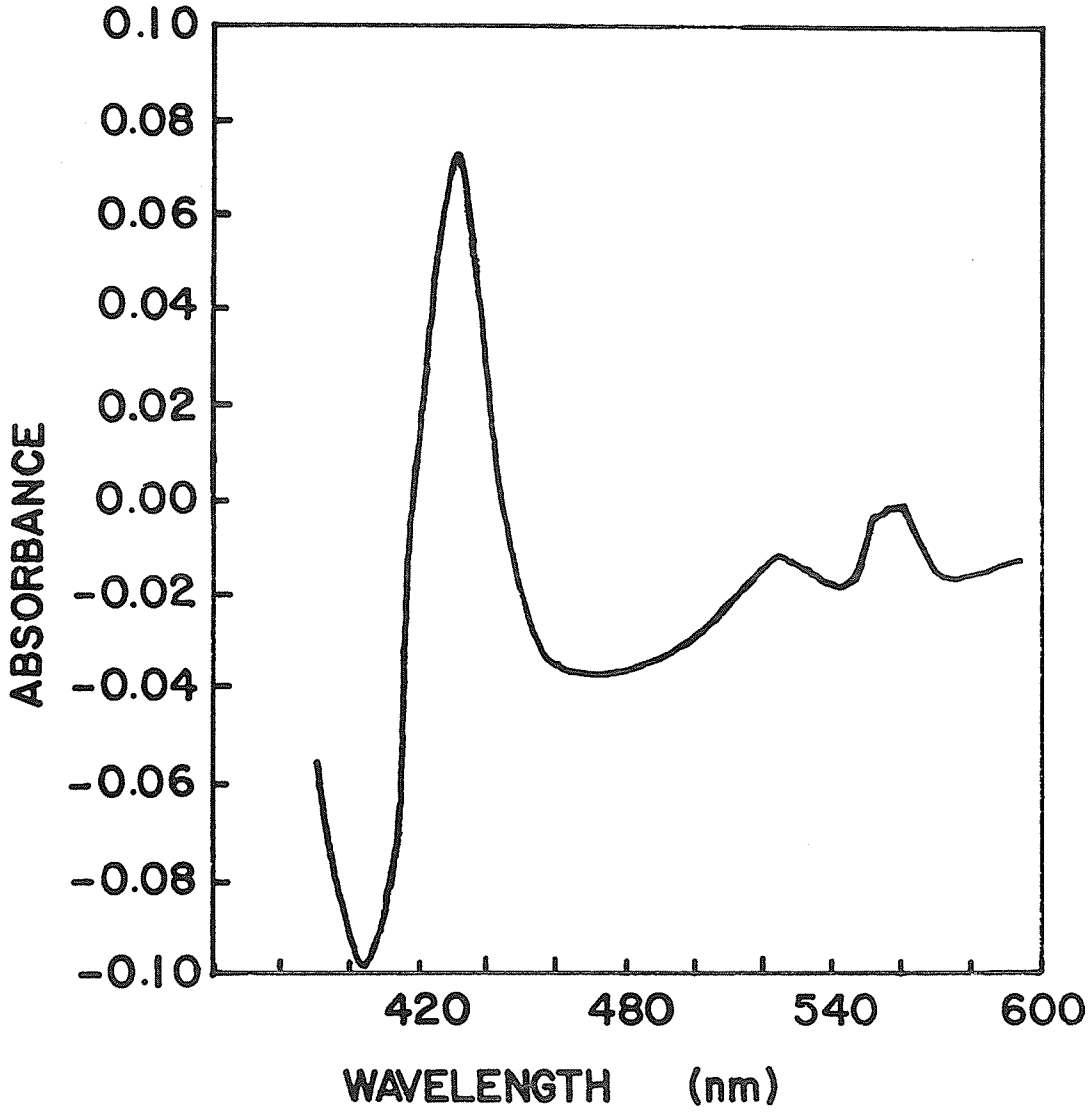

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
420
480
540
600
WAVELENGTH (nm)

Figure 34. Difference spectra of cytochromes *b* and *c* complex in 0.1 M potassium phosphate, pH 7.8. The sample cuvette was reduced with $Na_2S_2O_4$. The reference cuvette contained an identical sample, without added reducing agent.


0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
ABSORBANCE
420
480
540
600
WAVELENGTH (nm)

maxima at 560 nm in the α-region and at 430 nm in the Soret region indicated the presence of cytochrome *b*.

Cytochrome *b* preparations finally isolated by the procedure as reported in Materials and Methods did not contain cytochrome *c*. The oxidized spectrum had an absorption maximum at 405 nm with a shoulder at 378 nm (Figure 35). The reduced form showed three absorption peaks at 432, 530 and 560 nm. These spectra, however, are not of a typical cytochrome *b*. It suggests a possibility of either an impurity or some prosthetic group such as FMN or FAD attaching to the cytochrome *b*. All the heme in cytochrome *b* preparations was extracted with acid acetone, and was identified as heme *b* by the formation of pyridine derivative of hemochrome (Figure 36).

The fluorescence emission spectra of cytochrome *b* preparations with 450 nm excitation showed a single broad peak at 520 nm. A similar spectrum was obtained with a standard sample of FMN under the same conditions. Furthermore the UV spectrum of cytochrome *b* had a shoulder at 260 nm, an indication for the possible presence of a nucleotide in the sample. It is, therefore, tentatively suggested that our cytochrome *b* preparation also contained FMN or FAD, although no attempt was made to extract and identify this nucleotide from these samples.

Effect of CO. Reduced cytochrome *b* was found to be slowly oxidized in air and in the reduced form it also reacted with CO slowly. The absorption spectrum of CO-ferrocytochrome minus ferrocytochrome, Figure 37, had an absorption peak at 420 nm and a trough at 437 nm.

Figure 35. Absorption spectra of cytochrome *b* in 50 mM potassium phosphate, pH 7.8.

----- oxidized

——— reduced with $Na_2S_2O_4$

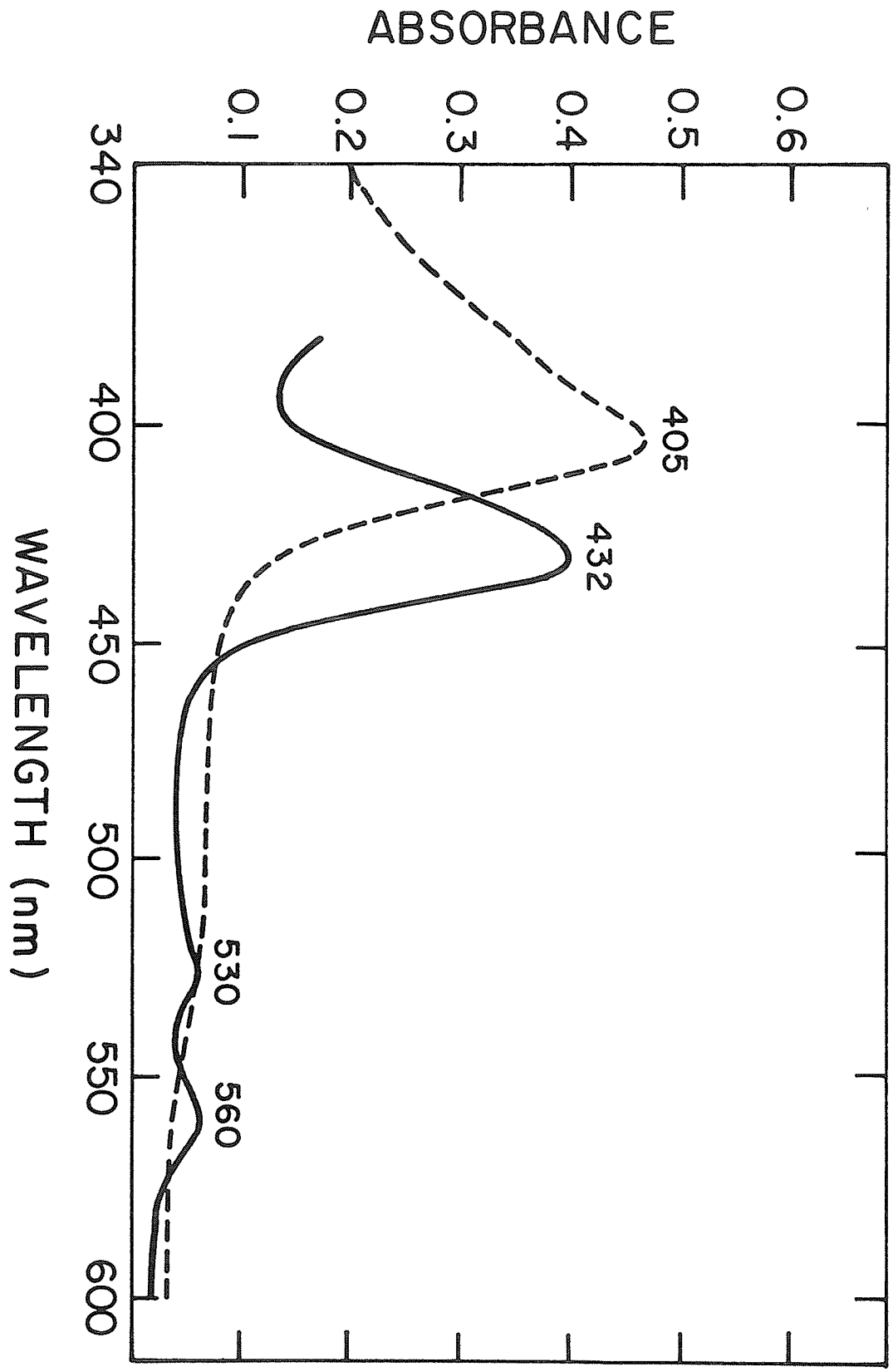

ABSORBANCE
0.1
0.2
0.3
0.4
0.5
0.6
340
400
450
500
550
600
WAVELENGTH (nm)
405
432
530
560

Figure 36. Absorption spectra of pyridine hemochrome *b* prepared from cytochrome *b* containing fractions.

---- ferrihemochrome *b*

—— ferrohemochrome *b*

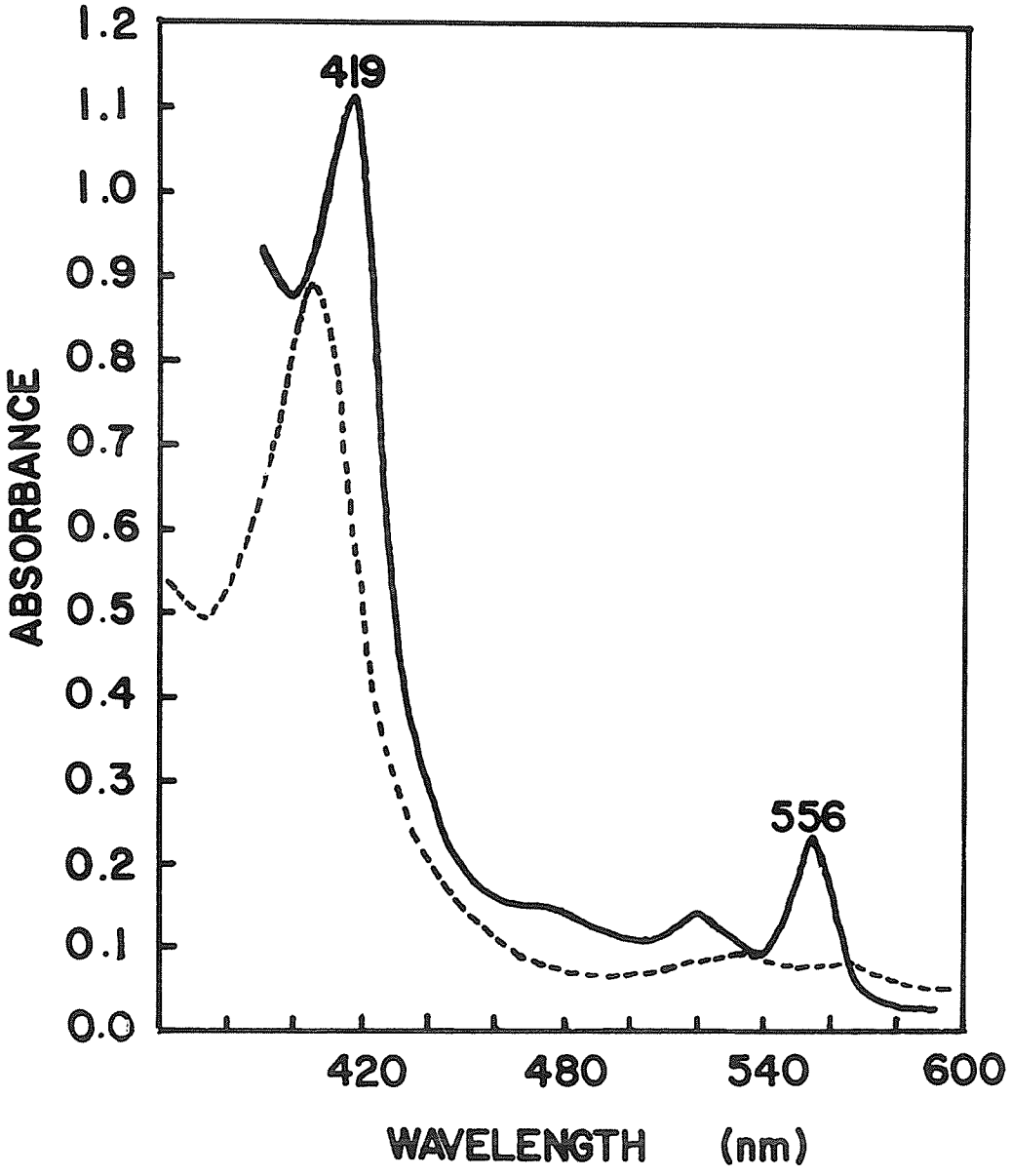
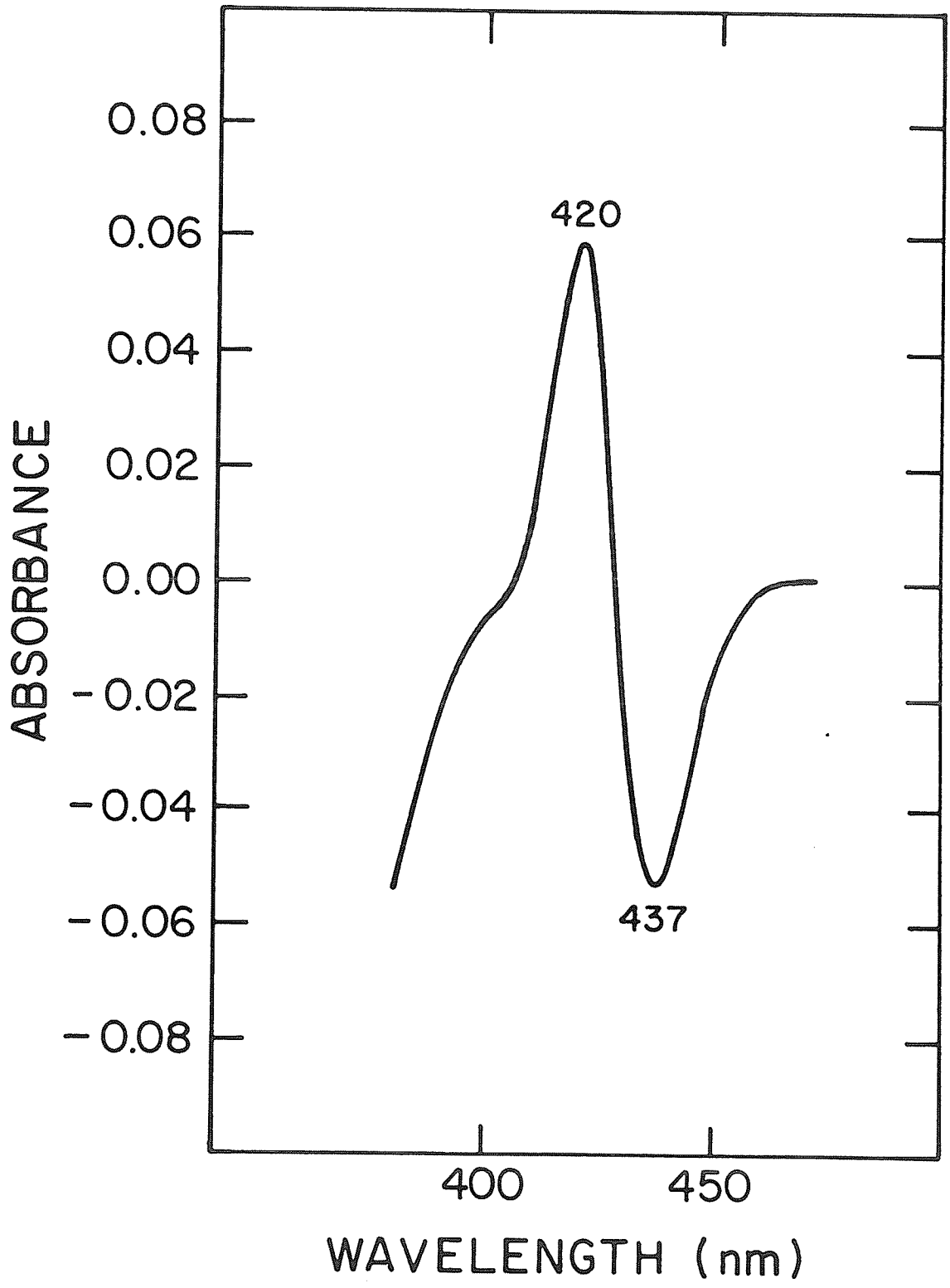

1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
419
556
420
480
540
600
WAVELENGTH (nm)

Figure 37. Effect of carbon monoxide on cytochrome *b* CO-ferrocytochrome minus ferrocytochrome (Soret region).


0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
ABSORBANCE
420
437
400
450
WAVELENGTH (nm)

Peroxidase activity. Impure fractions of cytochrome *b* containing cytochrome *c* demonstrated an appreciable amount of peroxidase. The fractions considered to have only cytochrome *b* and possibly FAD present still had a weak peroxidase activity. Complete elimination of peroxidase may be achieved by further purification of cytochrome *b*.

Other properties. Cytochrome *b* was autoxidizable and KCN at 10 mM did not inhibit the autoxidation. Cytochrome *b* was not reduced by ferrocyanide and only a partial reduction was achieved with ascorbate. Neither NADH, formate, succinate nor glucose could reduce the cytochrome *b*. Nitrite, which seemed to change the reduced spectrum of an impure preparation did not affect the spectrum of cytochrome *b* free from other cytochromes.

The molecular weight estimated from the gel filtration on Sephadex G-100 (which was included in purification procedures) gave a value of 37,000.

Polyacrylamide gel electrophoreses performed under non-denaturing conditions showed that cytochrome *b* preparations migrated as one major and two minor and faint bands. The minor bands were presumed to be contaminating proteins.

4.5.1.4 Ubiquinone

Purification. Ubiquinone purified by the method described elsewhere was tested for its purity. Rf values obtained on silica TLC plate were close to that of known Q_{10} in chloroform - heptane (4:1 v/v) as well as in benzene. Evidence for purity of the preparation

is also provided by the following data.

Absorption spectra and Properties of Ubiquinone. Preparations of *Nitrobacter* ubiquinone had a characteristic absorption maximum at 275 nm (Figure 38). A similar absorption spectrum was obtained for a known Q_{10}. Addition of a few crystals of borohydride resulted in loss of color and the absorption peak shifted to 292 nm. These results are in agreement with those of Lester *et al.* (1958).

Mass spectroscopy is usually a good tool for structural elucidation of small molecules. Mass spectrographs obtained for the purified ubiquinone did not contribute much information, since the only peaks obtained were at m/e 195 and 235. These values are far lower than the values for Q_{10}.

The I.R. spectrum showed two peaks in the region of 1630-1650 cm^{-1} (absorption due to a carbonyl group) corresponding to IR absorption of a 2,3-dimethoxybenzoquinone (Shunk *et al.* 1958).

In the NMR spectrum a band integrated for many protons was observed at δ 7.8 to 8.1. This value is in close agreement with the standard NMR spectrum of ubiquinone (Lester *et al.* 1958).

The melting point of the unknown preparations was found to be 43.5°C, whereas using a similar method the m.p. of known Q_{10} was 49.0°C.

4.5.1.5 Nitrite: Cytochrome *c* Reductase

Isolation. Attempts to isolate nitrite: cytochrome *c* reductase were prompted by the preliminary observations that the 144,000 g supernatant (F_0) could reduce cytochrome *c* in the presence of nitrite

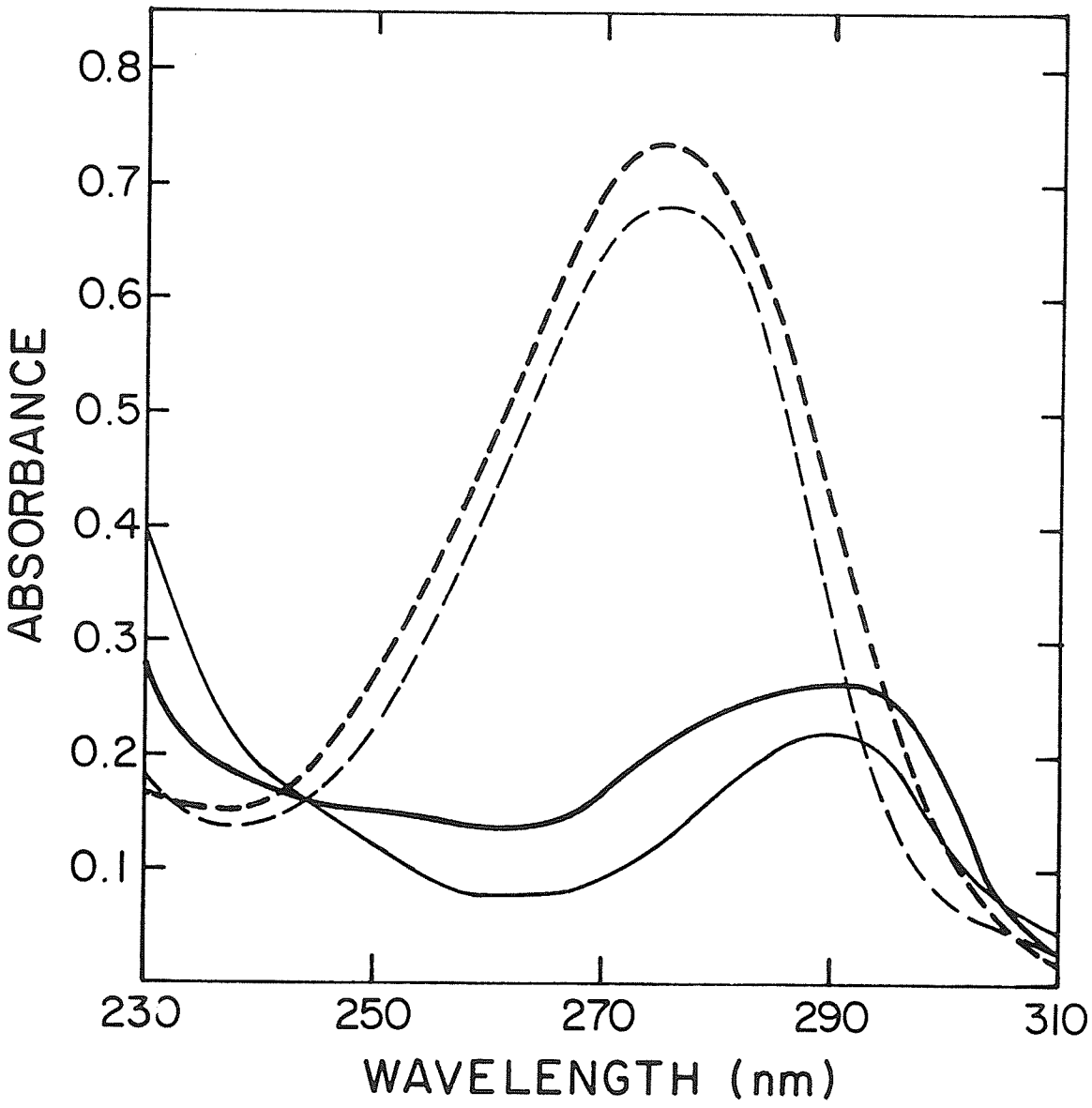
Figure 38. Absorption spectra of ubiquinone in ethanol.

----- oxidized spectrum of Q_{10}

▬▬▬ oxidized spectrum of *Nitrobacter* coenzyme Q

——— reduced spectrum of Q_{10}

▬▬▬ reduced spectrum of *Nitrobacter* coenzyme Q


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
230
250
270
290
310
WAVELENGTH (nm)

under anaerobic conditions. Such an activity was not, however, detected under aerobic conditions.

The isolated enzyme preparation contained a yellow pigment, a small amount of cytochrome *c* and possibly cytochrome a_1. Further purification of enzyme resulted in the loss of activity.

The yellow pigment was resolved from the enzyme fractions by ammonium sulfate precipitation of proteins when the pigment fractions were passed through the Sephadex in order to remove salt, the pigment behaved as a low molecular weight compound.

Absorption spectra. The enzyme preparation had an oxidized absorption spectrum with twin peaks at 352 and 360 nm and a broad shoulder around 410 nm (Figure 39). Addition of dithionite resulted in the loss of color and a small cytochrome *c* peak appeared at 415 nm. The enzyme preparation also had a peak at 260 nm (Figure 39).

The yellow pigment isolated from the enzyme preparation displayed a sharp peak at 260 nm (not shown). In the visible region the oxidized form had a similar absorption spectrum to that of the enzyme preparation except that the peaks were not as pronounced. As in case of the enzyme preparation, the reduced pigment lost its color, but did not have the cytochrome *c* peak (Figure 39).

PMR spectrum of the pigment had a band at δ 8.2-8.4 indicative of an aromatic compound. Because of interference by a small amount of ammonium sulfate present, the PMR spectrum did not prove to be of much help. In the IR spectrum, a band in the region of 1600-1630 cm^{-1} was indicative of an aromatic ring with a carbon double bond.

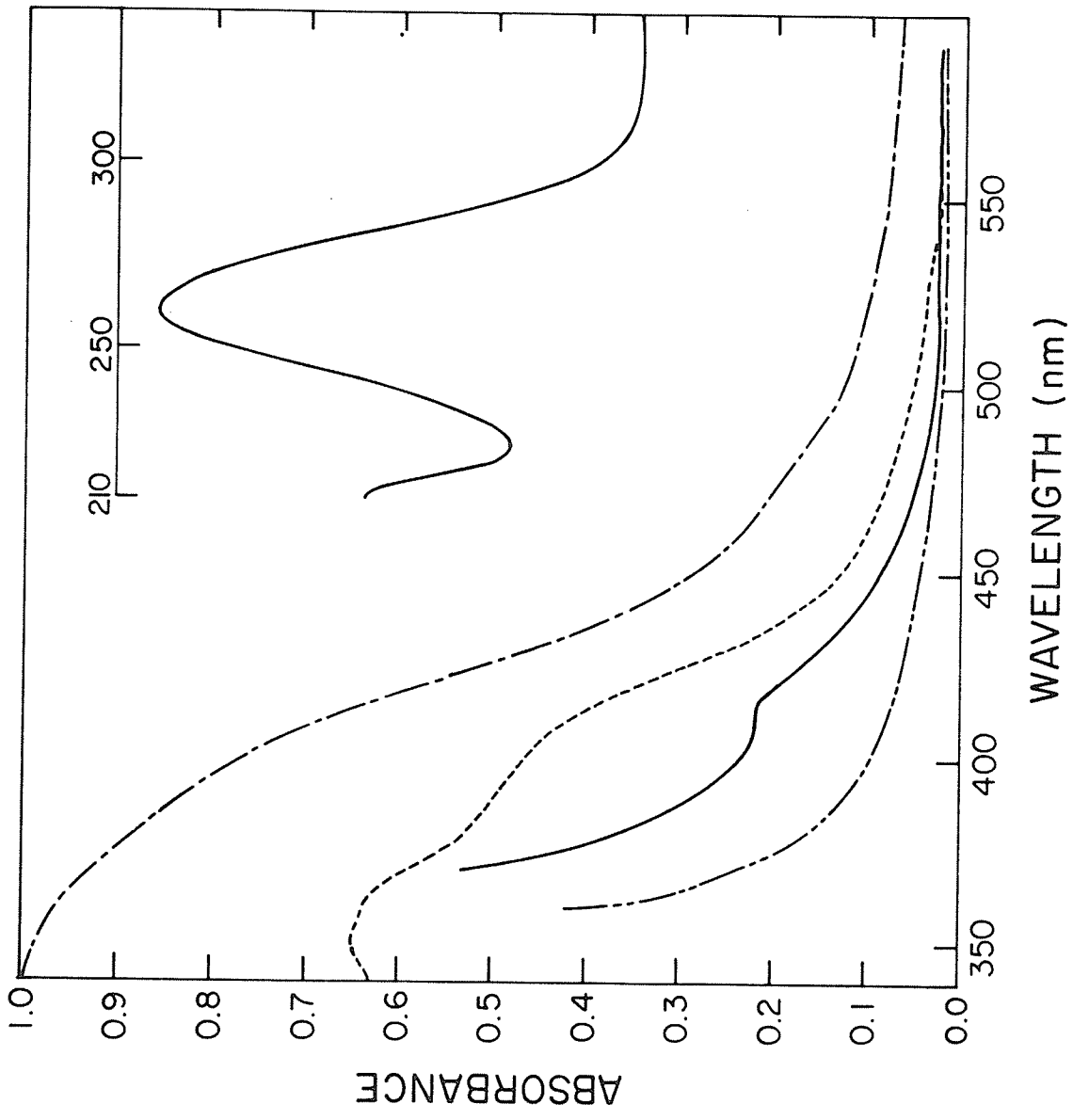
Figure 39. Absorption spectra of nitrite: cytochrome *c* reductase and yellow pigment in 50 mM potassium phosphate, pH 7.5. Inserted is UV spectrum of nitrite: cytochrome *c* reductase.

---------- oxidized spectrum of nitrite: cytochrome *c* reductase

———————— reduced spectrum of nitrite: cytochrome *c* reductase (reduced with $Na_2S_2O_4$)

—— · —— · —— oxidized spectrum of yellow pigment

—— ·· —— ·· —— reduced spectrum of yellow pigment (reduced with $Na_2S_2O_4$)


ABSORBANCE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
350
400
450
500
550
WAVELENGTH (nm)
210
250
300

The fluorescence emission spectrum (excitation at 365 nm) may be seen in Figure 40a. A similar spectrum has been reported for Lumichrome (Koziot, 1971). Figure 40b shows the excitation spectra of riboflavin and the unknown pigment. These spectra appeared to be similar, although not identical.

Enzyme activity. The yellow fractions had the activity of nitrite: cytochrome *c* reductase under aerobic and anaerobic conditions. The enzyme activity measured at different levels of nitrite is shown in Figure 41. Activity was markedly dependent upon the amounts of nitrite and cytochrome *c* in the reaction mixture. A double reciprocal nitrite vs. enzyme activity plot gave a Km value of 30 mM for nitrite.

The enzyme activity was found to be inhibited by nitrate. An amount of 0.1 mM nitrate caused a 50% inhibition in the presence of 20 mM NO_2^-. Since the enzyme reaction is not affected by the absence of oxygen or addition of superoxide dismutase, the reduction of cytochrome *c* is unlikely to be mediated by superoxide ion. It was suspected that the enzyme preparation had a flavin-like compound and the effect of FMN, FAD and riboflavin on the enzyme activity was therefore examined. None of these compounds, however, stimulated activity of the enzyme. The yellow pigment separated from the enzyme fraction did not show any enzyme activity. The enzyme activity was unstable; the preparations lost their activity completely upon overnight storage at $-20^{\circ}C$. Even at $4^{\circ}C$ it lost half the activity overnight. Bovine serum albumin up to an amount of 5 mg/ml could not stabilize or increase the

Figure 40. Fluorescence spectra of yellow pigment separated from nitrite cytochrome *c* reductase. The spectra recorded in 50 mM potassium phosphate, pH 7.8.

(a) Emission spectrum of yellow pigment with 365 nm excitation.

(b) Upper line: Excitation spectrum of yellow pigment at 500 nm emission.

Lower line: Excitation spectrum of riboflavin at 500 nm emission.

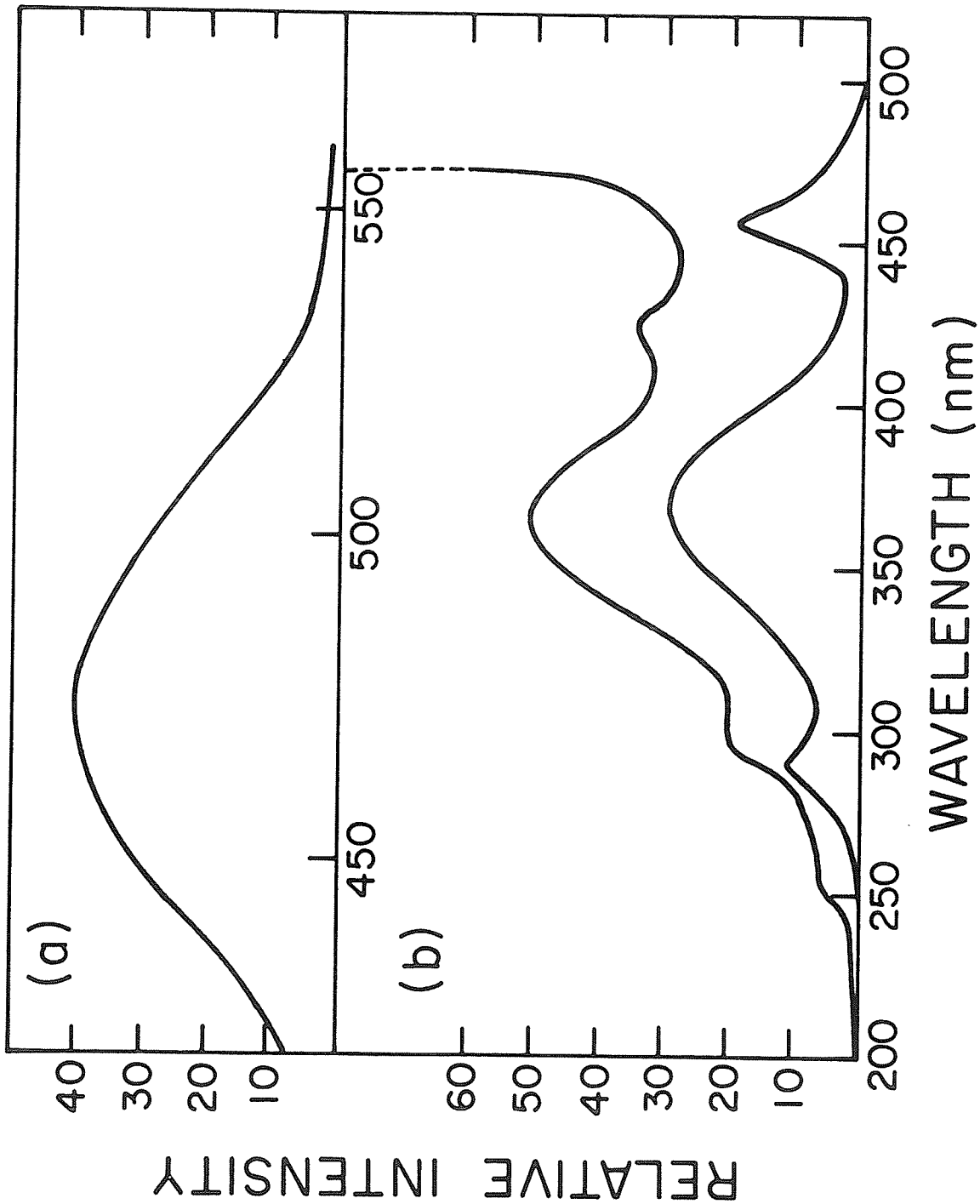
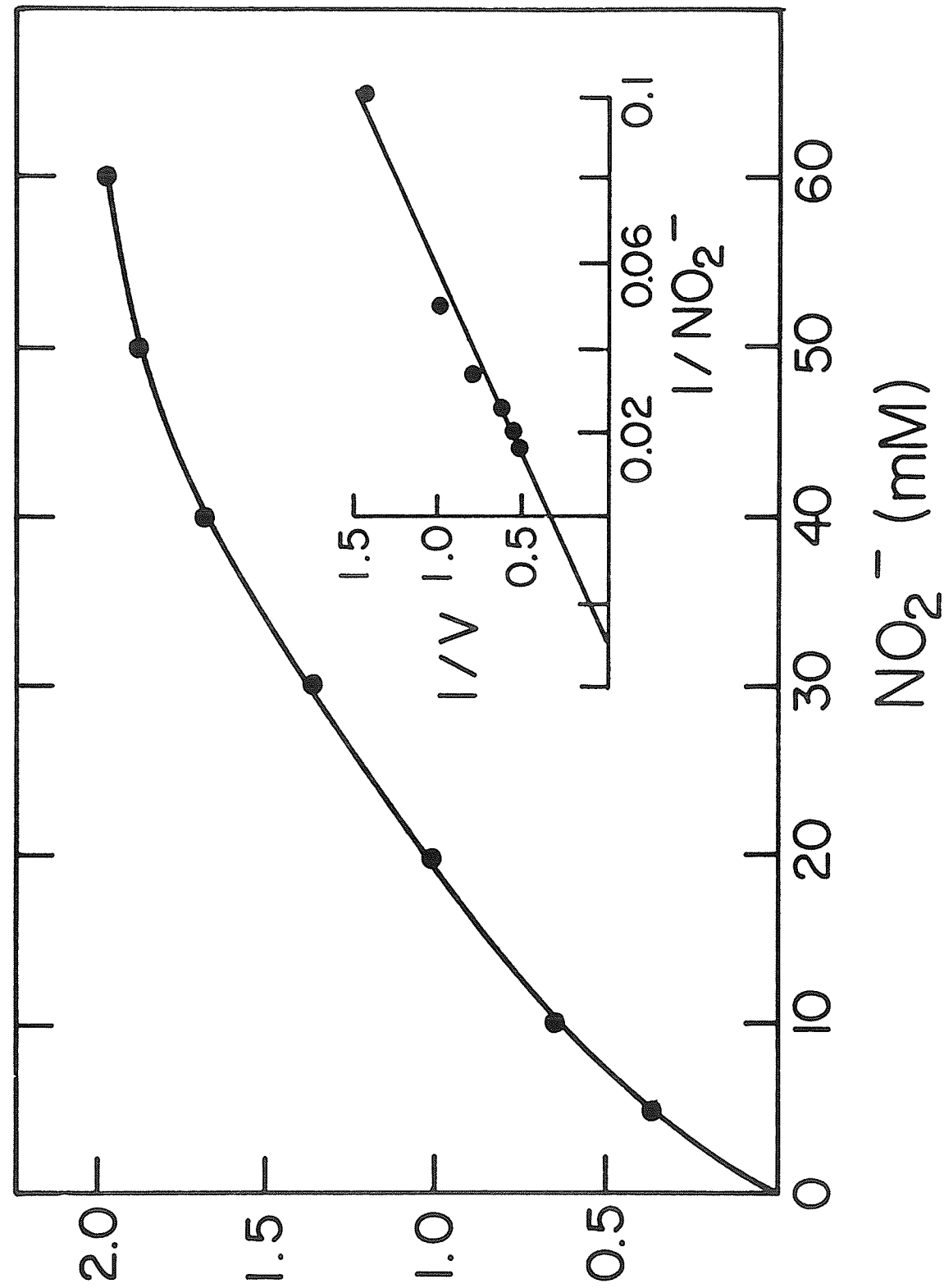

RELATIVE INTENSITY
WAVELENGTH (nm)
(a)
(b)
40
30
20
10
60
50
40
30
20
10
450
500
550
200
250
300
350
400
450
500

Figure 41. Effect of nitrite concentration on the nitrite: cytochrome *c* reductase.
Enzyme activity was measured according to the standard assay as described in Materials and Methods.
Inserted is the double reciprocal plot of the rate vs. nitrite concentrations.


Reduction of cyt. c (nmole / min)
2.0
1.5
1.0
0.5
0
10
20
30
40
50
60
NO_2^- (mM)
1.5
1.0
0.5
1 / V
0.02
0.06
0.1
1 / NO_2^-

enzyme activity.

4.5.1.5 NADH: Cytochrome *c* Oxido-reductase

Absorption spectra. The fraction having NADH: cytochrome *c* oxido-reductase activity contained a red pigment and showed an oxidized absorption spectrum with a sharp peak at 284 nm (Figure 42a) and a broad peak at 480 nm (Figure 42b). The reduced form lacked any absorption maximum in the visible region due to bleaching of the red pigment. Once reduced with dithionite, the pigment was not oxidized until left for overnight when a partial reoxidation was noticed. The fractions with enzyme activity were also decolorized by ascorbate and borohydride.

Interestingly, the red pigment was extractable with organic solvent. The absorption spectrum of the extracted pigment was similar to that of the enzyme fraction (Figure 42c). It had the same absorption maximum at 480 nm, though a little sharper, which was abolished upon reduction.

Enzyme activity. The isolated red preparations were examined for various enzyme activities. These preparations catalyzed the reduction of cytochrome *c* in the presence of NADH under aerobic as well as anaerobic conditions. An average specific activity measured was 1.25 μmole of cytochrome *c* reduced min^{-1} mg^{-1}. These fractions could not, however, oxidize NADPH. The red pigment extracted from the enzyme preparation was inactive in catalyzing the oxidation of NADH or the reduction of cytochrome *c*. Nitrate but not nitrite was found to inhibit the enzyme activity. An amount

Figure 42. Absorption spectra of NADH: cytochrome *c* oxidoreductase.

a) UV spectrum in 20 mM potassium phosphate, pH 7.8.

b) Visible spectra in 20 mM potassium phosphate. pH 7.8.

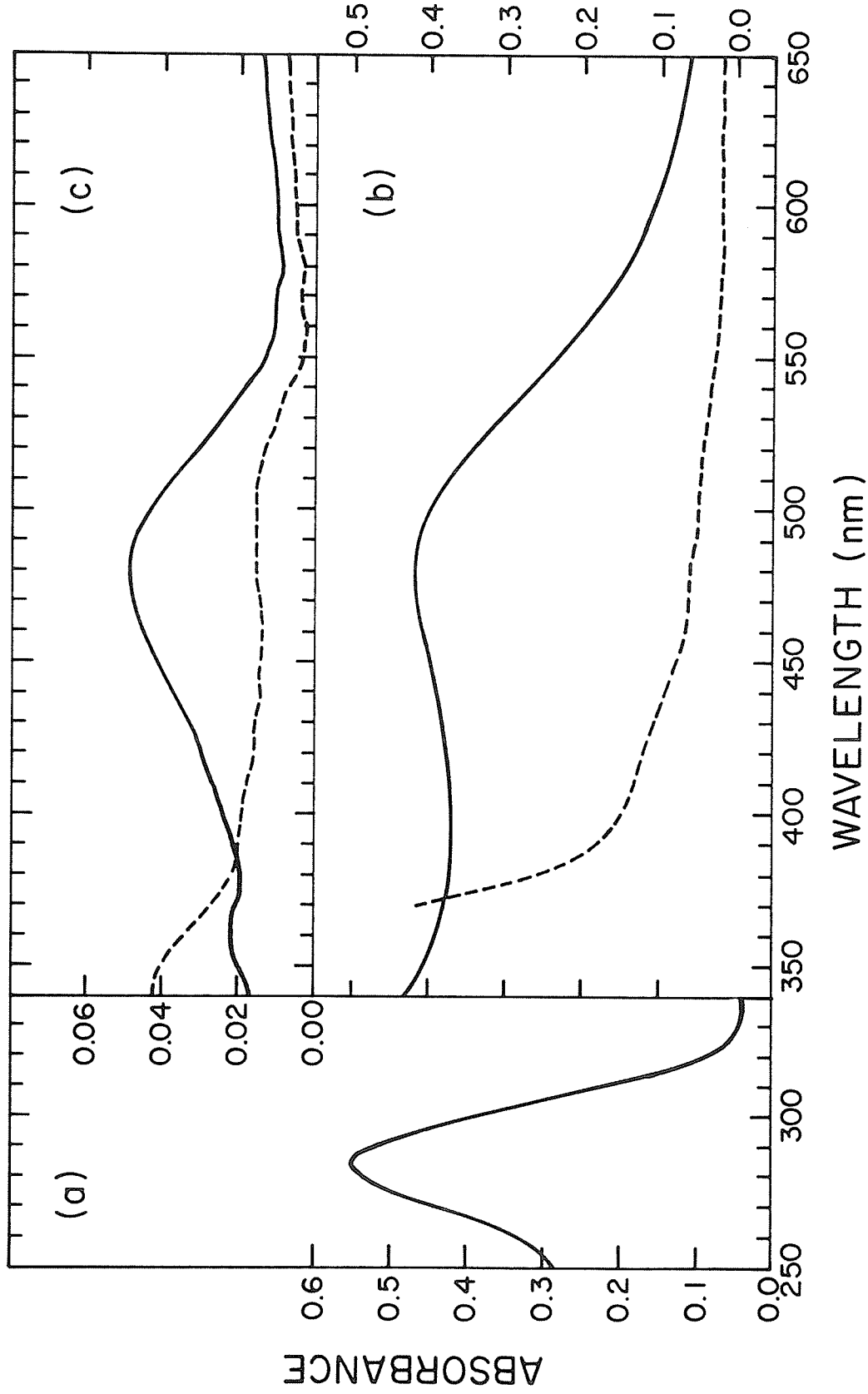
—— oxidized

---- reduced with $Na_2S_2O_4$

c) Visible spectra of red pigment in ethanol.

—— oxidized

---- reduced with $Na_2S_2O_4$


(a)
(b)
(c)
ABSORBANCE
WAVELENGTH (nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.00
0.02
0.04
0.06
250
300
350
400
450
500
550
600
650

of 0.5 mM nitrate inhibited 50% of enzyme activity under the standard assay conditions. Whereas nitrite up to 5 mM had no suppressive effect on enzyme activity.

4.5.1.6 Purple pigment

Absorption spectra. The absorption spectra of the purple pigment in 50 mM potassium phosphate, pH 7.8 are presented in Figure 43a,b. The oxidized form exhibited absorption maxima at 275, 540 and 580 nm. The reduced form showed a general decrease in absorption with the disappearance of peaks at 540 and 580 nm. The pigment could be reduced by dithionite, borohydride and slowly by ascorbate but not by NH_2OH.

Effect of pH. The effect of pH on the purple pigment was investigated in view of its sudden appearance in the CM-cellulose column upon change in eluting buffer from acidic to basic pH. The pigment was bleached below pH 6.5. This change in color, however, was reversible when the pH was raised back to above neutrality. The pigment color was stable under alkaline conditions up to pH 10.0.

Enzyme activity. The purple fractions were also investigated for possible enzymatic activities. These fractions appeared to exhibit NH_2OH: cytochrome *c* reductase activity. This activity was found to be correlated with pH of the buffer. The enzyme preparation did not show the activity below pH 6.0. Maximum activity was obtained in buffer of pH 8.0. Neither nitrite nor nitrate inhibited the enzyme activity.

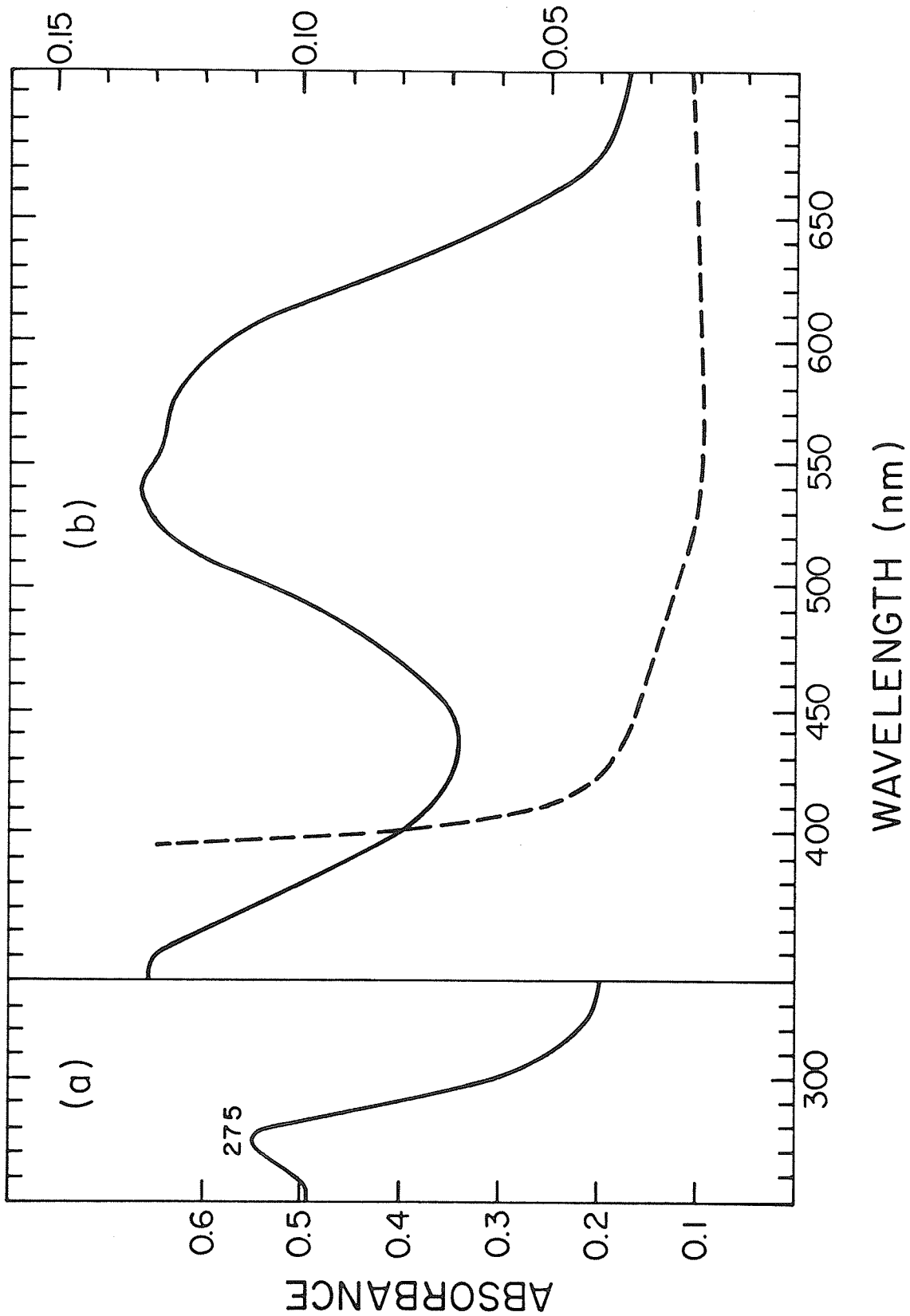
Figure 43. Absorption spectra of purple pigment in 50 mM potassium phosphate, pH 7.8.

a) UV spectrum

b) Visible spectra

—— oxidized

---- reduced with $Na_2S_2O_4$


(a)
(b)
275
0.6
0.5
0.4
0.3
0.2
0.1
0.15
0.10
0.05
300
400
450
500
550
600
650
ABSORBANCE
WAVELENGTH (nm)

4.5.2 DISCUSSION

4.5.2.1 Cytochrome *b*

Nitrobacter has been reported to possess several types of cytochromes (Aleem 1978). With the exception of cytochromes *c*, the other cytochromes are tightly bound to the membranes.

The solubilization of membrane bound cytochromes have been achieved in other organisms either by detergents or by proteolytic enzyme digestion and sonic disintegration (Lemberg and Barrett 1973). In some cases *b*-type cytochromes have also been extracted by simple salt solutions (Yamanaka and Okunuki 1974).

Solubilization of the *Nitrobacter* membranes by detergent did not prove to be satisfactory for the isolation of individual cytochromes. The detergent-solubilized fractions were eluted from a DEAE-cellulose column as complexes of cytochromes *a* and *b*, *b* and *c*, or *a*, *b*, and *c* (Figure 32).

In an earlier report (Chaudhry *et al.* 1980) cytochrome *b* preparations obtained from a cell homogenate also contained cytochrome *c* (Figure 33). The purification was further improved by including ammonium sulfate fractionation and ultrafiltration in the isolation procedures. As a result preparations of cytochrome *b* free from cytochrome *c* were obtained. The pyridine derivative formed from these preparations showed only the reduced spectrum of pyridine hemochrome *b* in alkaline solution (Orlando and Horio 1961). However, the examination of absorption spectra precludes the possibility of stating that these preparations were free from other pigments as well (Figure 35). Fluorimetric studies indicate the presence of

an FMN or FAD like compound in the preparation of cytochrome *b*. Yeast lactate dehydrogenase (cytochrome b_2) has been reported to possess protoheme as well as FMN (Appleby and Morton 1959). Gel electrophoresis in the presence of SDS split lactate dehydrogenase into two polypeptide chains; one has a molecular weight of 36,000 and the other a molecular weight of 21,000. The heme is thought to be bound to the former polypeptide (Lederer and Simon 1971). A molecular weight of 37,000 estimated for the isolated cytochrome *b*, is similar to the larger subunit of yeast lactate dehydrogenase, but no enzyme activity could be assigned to the *Nitrobacter* cytochrome.

The *b*-type cytochromes, which are rapidly autoxidizable and react with CO, e.g. cytochrome *a*, function as cytochrome oxidase (Chance 1953). There are other *b*-type cytochromes which react sluggishly with oxygen or with CO (Kamen and Horio 1970). The isolated *Nitrobacter* cytochrome seems to belong to the latter type of cytochrome *b*, since it was only slowly autoxidizable and reacted with CO sluggishly (Figure 37). In addition it did not oxidize reduced horse heart cytochrome *c*.

Autoxidation of this cytochrome was found to be unaffected by KCN. Cytochromes *b* with similar properties have been isolated which are autoxidizable (Jackson and Lawton 1959) and which bind CO (Inoue and Kubo 1965).

A weak peroxidase activity suggested the presence of contaminating proteins in the preparation and this proved to be supported by the results of the gel electrophoresis.

The physiological role of isolated cytochrome *b* remains to be resolved, although on the basis of inhibitor studies the involvement of *b*-type cytochrome in the oxidation or reduction of pyridine nucleotides in *Nitrobacter* has been hypothesized (Aleem 1968). Further investigation is necessary to purify cytochrome *b* free from other proteins and to determine whether the flavin component is an integral part of cytochrome *b* or is simply a contaminant.

4.5.2.2 Ubiquinone

An early observation that the red pigment of NADH: cytochrome *c* oxidoreductase could be extracted by organic solvents prompted the attempt to isolate it from whole cells. Such efforts were found to be futile as far as the red pigment was concerned; they led, however, to the purification of ubiquinone from *Nitrobacter*.

Ubiquinone which is also called coenzyme Q serves as a highly mobile electron carrier between the flavoproteins and the cytochromes of the respiratory chain of the mitochrondria. A similar function for ubiquinone in *Nitrobacter* has been proposed, primarily to keep in harmony with the mitochondrial system (Aleem 1970). Ubiquinones isolated from bacterial sources vary on the basis of length of their isoprenoid side chains (Lester *et al*. 1958). The coenzyme Q isolated from *Nitrobacter* and a known Q_{10} sample were similar on the basis of their Rf values on silica TLC plates using different solvents. PMR and IR spectra also supported evidence for the presence of ubiquinone in the preparation. PMR spectra, however, could not provide sufficient information with

respect to the length of polyisoprenoid chain. NMR studies remained restricted because of limited amounts of available samples. Otherwise the ^{13}CMR spectrum might have provided enough information about the polyisoprenoid chain. Mass spectra of unknown samples did not contribute any useful information in this respect either.

The melting point of ubiquinone depends upon the length of polyisoprenoid side chain, the shorter the chain, the lower will be the melting point. The melting point obtained for the unknown sample (43.5°C) was lower than that for the known Q_{10} (49.0°C). This result alone inferred that the unknown ubiquinone may be Q_9, one unit of isoprene smaller than Q_{10}. Ubiquinone from Torula yeast (Q_9) has been reported to have a similar m.p. of 45.2°C (Lester *et al.* 1958). It is also possible that the unknown sample contained impurities which lowered the melting point.

4.5.2.3 Nitrite: Cytochrome *c* Reductase

The activity of nitrite: cytochrome *c* reductase was first reported by O'Kelley *et al.* (1970) in particulate fractions from *Nitrobacter*. In explanation of the redox potential difference between NO_2^-/NO_3^- couple and cytochrome *c* (Lees 1962), an energy dependent reversal of electron flow from cytochrome a_1 to *c* has been proposed (Kiesow 1967 and Aleem 1968). Therefore, the discovery of an enzyme which could reduce cytochrome *c* in the presence of nitrite without any requirement for energy has been criticized (Aleem 1978).

During the present investigation a similar enzyme activity

was found under anaerobic conditions in the supernatant fraction (F_0). This activity was subsequently isolated by DEAE-cellulose chromatography. The isolated enzyme not only demonstrated the energy-independent reduction of cytochrome *c* in the presence of nitrite but it was equally effective under aerobic as well as anaerobic conditions. The enzyme preparations, however, were not very stable. Attempts to stabilize the enzyme by BSA were not successful. BSA is known to help the integrity of enzyme complex (Dular 1975). This enzyme which probably plays a key role in the mechanism of nitrite oxidation should attract the attention of workers.

The enzyme activity was directly correlated with the concentration of nitrite and cytochrome *c* at low levels. The enzyme was capable of catalyzing the reaction at as low nitrite and cytochrome *c* as 2 mM and 10 μM respectively. The enzyme activity was found to be linear for well over 3 minutes under standard assay conditions, whereas the enzyme reported by O'Kelley *et al.* (1970) rapidly leveled off its reaction after only 90 seconds. The Km for nitrite was estimated to be 30 mM (Figure 41) and enzyme activity was inhibited by nitrate. These latter results, however, are in agreement with those of O'Kelley *et al.* (1970).

Spectrophotometric studies showed the decolorization of enzyme fractions upon reduction (Figure 39). The enzyme preparations appeared to contain small amounts of cytochrome *c*, possibly cytochrome a_1 and largely yellow pigment. Attempts were therefore also made to find out the nature of the yellow pigment present in

the enzyme preparations.

The spectrum of the isolated pigment had the appearance of an adenine spectrum with an absorption peak at 260 nm. PMR and IR spectra also showed the presence of an aromatic substance in the yellow pigment preparations and the fluorescence spectra (Figure 40) appeared to show similarity with a flavin. Although this evidence suggests that the yellow pigment may be an FAD-like compound, more detailed information is needed to define its structure. Such information might also provide an insight into the highly labile nature of nitrite: cytochrome *c* reductase.

4.5.2.4 NADH: Cytochrome *c* Oxido-reductase

Wallace and Nicholas (1968) proposed that the reductase enzymes such as nitrite, nitrate and hydroxylamine reductases are located in the cytoplasm and are relatively more soluble. Nitrite oxidase, on the other hand, is located on the intracellular membranes of *Nitrobacter* (Murray and Watson 1965). NADH: cytochrome *c* oxido-reductase was isolated from the soluble fraction and therefore may be present in the cytoplasm.

The enzyme preparations rapidly reduced cytochrome *c* in the presence of NADH. The average specific activity of the enzyme was found to be 1.25 μmole of cytochrome *c* reduced per min per mg. The enzyme activity was inhibited by nitrate but not by nitrite. The preparations of the enzyme seemed to be free from any cytochromes (Figure 42b), whereas Aleem (1968) has suggested the involvement of flavoprotein as well as cytochromes in the NADH

oxidation. Red pigment, the only pigment present in these preparations, was extractable by organic solvent and gave a similar absorption spectrum as that of the enzyme preparations (Figure 42c). The extracted pigment, however, lacked enzyme activity. The isolation of red pigment from whole cells could not be accomplished by extraction with organic solvents.

4.5.2.5 Purple pigment

The pigment color disappeared upon reduction (Figure 43) and in the buffer with a pH below 6.5. The effect of pH was reversible and pigment color reappeared in alkaline buffer.

The isolated purple pigment fractions had a weak activity of NH_2OH: cytochrome *c* reductase. The role of such an enzyme in *Nitrobacter* is not known. Further investigation of such an enzyme will be interesting.

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