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STUDIES ON CYTOCHROME C OXIDASE:
1- A Proposed Alternative Electron Transfer Pathway at Low pH
2- Interaction Term of Different pH, Ionic Strength, and Tween-80

Zahra Rassi

A Thesis
in
The Department
of
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ABSTRACT

Studies on Cytochrome c Oxidase:

1. A Proposed Alternative Electron Transfer Pathway at Low pH
2. Interaction Term of Enzymic Activity at Different pH, Ionic Strength, and Tween-80

Zahra Rassi, Ph. D.
Concordia University, 1988

Cytochrome c oxidase is the terminal acceptor in the mitochondrial electron transport chain. The details of the electron transfer mechanism are not fully known, but it is believed that the primary electron acceptor is cytochrome a which then transfers electron to the oxygen via Cu_A and cytochrome a3. Cytochrome a3 is also the binding site for oxygen and inhibitors.

The activity and spectral properties of cytochrome c oxidase are pH dependent. The object of this work was to investigate the stoichiometry of binding of CO and CN in the pH range 7.8 to 5 and to draw conclusions concerning possible alternate electron transport pathways within the oxidase. One such pathway might be electron transfer from cytochrome a /Cu_A to O_2 as well as from cytochrome a3/Cu_B to O_2. For such a pathway, the expected stoichiometry at the pH optimum for activity would be two ligands bound per aa3, that is, one bound to cytochrome a3 and the other to cytochrome a.

Over the entire pH range tested, one carbon monoxide bound to a functional unit of enzyme and the integrity of the enzyme was

maintained. In contrast, the apparent stoichiometry of cyanide binding to partially reduced enzyme increased substantially at pHs below 6.7. Several factors may cause an apparent increase in cyanide binding; these are discussed in the text.

The study has a second part concerning the effect of pH, ionic strength, and Tween-80 on the activity of low-lipid cytochrome c oxidase. The idea behind the study was to derive an equation with interaction terms which could be used to predict the enzymic activity of the enzyme under different conditions.

Under all conditions tested, a pH optimum was apparent and the location of this optimum varied as a function of ionic strength and Tween-80. The activity was found to increase with increasing ionic strength; the Michaelis-Menten constant also increased with ionic strength. A complete mathematical description of the enzyme was not achieved.

DEDICATION

To the untold causalities suffered by both sides during the Iraq-Iran War

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I would like to thank my neices N. Neseh and A. Rassi who shared with me a period which was emotionally most difficult, at a time when my country was undergoing severe political upheaval. This was the period when I was doing the research leading to this thesis, and without their support, I would not have brought it to a close.

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I most gratefully appreciate the guidance from Dr. J.A. Kornblatt throughout the course of this research and preparation of this thesis.

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A	Absorption
ATP	Adenosine 5-Triphosphate
cyt. c (Fe)3+	Ferricytochrome c
cyt. c(Fe)2+	Ferrocytochrome c
cyt. c ox.	Cytochrome C Oxidase
cyt. aa3	Cytochrome C Oxidase
cyt. a	Cytochrome a
cyt. a3	Cytochrome a3
CO	Carbon Monoxide
CN	Cyanide
DPG	Diphosphatidylglycerol
EDTA	Ethylenediaminetetraacetic Acid
EPR	Electron Paramagnetic Resonance
EXAFS	Extended X-ray Absorption Fine Structure
ε	Extinction Coefficient
I	Ionic Strength
KCN	Potassium Cyanide
Km	Michalis-Menten Constant
MAmax	Molecular Activity Maximum
MCD	Magnetic Circular Dichroism
NIR	Near Infrared
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipid
RR	Resonance Raman
TMPD	Tetramethyl-p-phenylenediamine
UV	Ultra-Violet
VIS	Visible
λ	Wavelength

1

INTRODUCTION

Cytochrome c oxidase (EC.1.9.3.1) consumes most of the dioxygen absorbed by aerobic organisms. This reaction, which the enzyme catalyzes, transfers electrons from cytochrome c to dioxygen. It provides most of the free energy needed for the life processes of aerobic organisms by coupling electron transport to the synthesis of ATP according to the following equation:

Equation [1]

$$4\ \text{cyt.c}\ (Fe)^{2+} + O_2 + 4\ H^+(m) \xrightarrow{\text{cyt. c.ox.}} 4\ \text{cyt. c}\ (Fe)^{+3} + 2\ H_2O + \text{Energy}$$

m = mitochondria

Because of the vital role of oxidative phosphorylation, cytochrome c oxidase has a wide biological distribution. It is present in all animals, plants, aerobic yeasts, and in some bacteria. Furthermore, in all cases, it is firmly associated with a membrane, either the inner membrane of mitochondria, the respiratory organelle of the eukaryotes, or in the membrane of the bacterial cell.

Most authors maintain, as was first proposed by Mitchell in 1961 (1), that ATP synthesis in oxidative phosphorylation is the result of an electrochemical potential across the membrane. This potential is generated

by coupling electron transfer to proton translocation in the respiratory chain (62,101).

It has been shown that cytochrome c oxidase is directly involved in the generation of a membrane potential (2,101). The nature of the coupling between the reaction of the equation [1] and the formation of the proton gradient at present remains unknown. It is agreed by mostly everyone that cytochrome c oxidase functions as an active proton pump (8) transferring protons from the inner to the outer mitochondrial space (2,6-8). Several groups have reported that conformational changes during the catalytic cycle may be the cause of the proton pump (10,12,30,190).

The generation of the electrochemical potential across the membrane requires a change in equation [1] since it does not completely describe the reaction catalyzed by cytochrome c oxidase

$$4\ \text{cyt. c}(Fe^{2+}) + O_2 + 8\ H^+(m) \longrightarrow 4\ \text{cyt c}(Fe^{3+}) + 2H_2O + 4\ H^+(c) + \text{energy}$$

m = mitochondria
c = cytosol

The main interest of many investigators working on heme proteins has been to elucidate the mechanism of electron transfer and dioxygen activation. Although numerous studies over the past 50 years have provided considerable knowledge about this complex molecule, it is thought that this enzyme will require more years of extensive research for a complete understanding. Many aspects of dioxygen activation and the

energy production mechanism are still not completely understood; these include the specific features of the unique bimetallic centers and the elucidation of the electron transfer mechanism.

This study is a very small contribution to the cytochrome c oxidase world and consists of two parts. The first part of the study addresses the question of a possible alternate electron transfer pathway. The second part investigates the interaction of pH, ionic strength and detergent on the activity of the enzyme.

2

LITERATURE REVIEW

2. 1. A BRIEF HISTORICAL SURVEY

As early as 1886, MacMunn reported the four-band spectrum of histo or myohaematin while he was investigating the spectral absorption by a large variety of organisms and tissues (5). A resulting controversy with Hoppe-Seyler, however, raised doubts as to the validity of some of the discoveries by MacMunn. Following their work, Koenig in 1894 started pioneering studies on the photochemical action spectra of visual receptors. In 1924 Warburg (4) followed Koenig 's technique when he studied the photochemical action spectrum of the yeast respiration system. From his studies on the catalysis of the oxidation of cysteine by iron-charcoal, Warburg developed a model for cellular respiration. He concluded that oxygen activation was the all-important process in cellular respiration. He also concluded that an iron-containing enzyme, the "respiratory enzyme", or what he named, "Atmungsferment", is solely responsible for the transport of the oxidizing equivalents of oxygen to the substrate. Nevertheless, an opposing view was taken by Thunberg who had detected a large variety of dehydrogenases in tissues (5). Moreover, Thunberg's view was supported by Wieland who used palladium-hydrogen as a "model" of tissue respiration. Wieland believed that substrate-specific hydrogen activations were characteristics of all biological oxidation processes. The

"dehydrogenation" theory lasted more than two decades and several dehydrogenases which do not utilize oxygen were isolated.

The controversy regarding the respective roles and importance of hydrogen and oxygen activation faded into the background when, following his initial observation, Keilin demonstrated (3) that the four-banded cytochrome spectrum observed in 1886 was in fact the spectrum of the ferrous or reduced forms of three distinct cytochromes a , b , and c . Keilin obtained a soluble preparation of cytochrome c from baker's yeast. Between 1937-1938 he and Hartree (3), showed that the indophenol oxidase activity of a particulate tissue preparation was simply the result of a non-enzymic reduction of cytochrome c by dimethyl- phenylenediamine, with the reduced heme protein being oxidized by indophenol oxidase in the presence of oxygen. Having established the nature of the final step of tissue respiration, they renamed the enzyme "cytochrome c oxidase" since its only function appeared to be the oxidation of cytochrome c . There was little doubt concerning the physiological importance of this system since Haas had already found that the rate of oxygen uptake was identical with that of cytochrome c reduction in a number of tissues (6). This demonstrated that nearly all of the oxidizing equivalents of oxygen were transmitted by the cytochrome c oxidase system.

In 1939 Keilin and Hartree found that in the presence of carbon monoxide, cytochrome a showed up as two spectroscopic components. They further demonstrated that the new cytochrome, cytochrome a3 , was the substance responsible for the photochemical action spectrum introduced by

Warburg. Cytochrome a3 was thus identified as the respiratory enzyme reacting directly with oxygen, and the system was considered to be composed of three entities, cytochrome c , cytochrome a , and cytochrome a3 , which were reacting consecutively.

2. 2. STRUCTURAL ASPECTS OF CYTOCHROME C OXIDASE

Cytochrome c oxidase (EC.1.9.3.1) is one of the most important enzymes in nature. In eukaryotes the enzyme is located in the mitochondrial inner membrane, whereas in prokaryotes it is found in the plasma membrane. In both cases, it is inserted into the lipid bilayer and released only by treatment with a detergent. Electron microscopy and image reconstruction studies (17-19,24,83) have shown that two-dimensional crystals of cytochrome c oxidase can be isolated. Two different crystal forms were obtained. In one form, the enzyme is a dimer inserted across the lipid bilayer of flattened vesicles (17,21,25). In the second crystal form, the enzyme is monomeric and arranged in detergent-rich sheets with no membrane bilayer present (18,19). Comparison of the structures of the two forms shows excellent agreement in the major features of the protein (19,21,111).

The enzyme is a Y-shaped protein (18,19,25,117), [Fig. 2.1] and there is a large domain extending 5.5 nm from what would be the outer or cytoplasmic (C domain) surface of the mitochondrial inner membrane. The matrix (M) domain is larger at the membrane's surface where it is

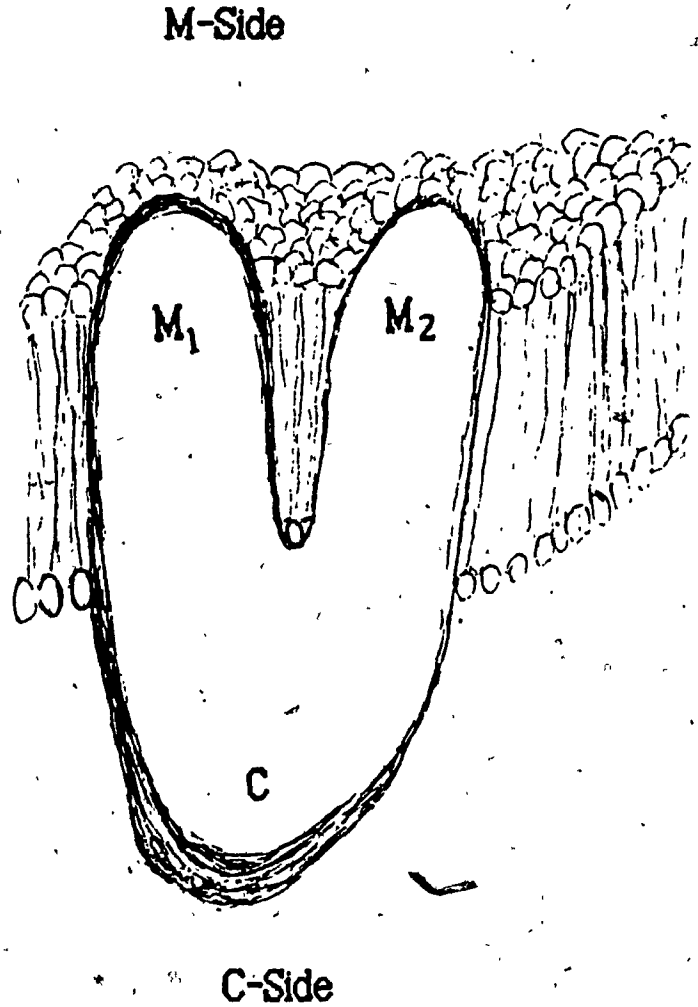


Fig. 2. 1. The Y-shaped monomer structure of cyt. c ox. inserted into the lipid bilayer. C_o, M_1 and M_2 label the cytoplasmic and two matrix domain, respectively.

separated into two distinct sub-domains of about equal size (M1 and M2). The C domain terminates in a smaller sub-domain. The two matrix domains (M1 and M2) become separated closer to the outer surface of the mitochodrial inner membrane and each spans the lipid bilayer, extending almost to 2 nm from the matrix (M) side of the inner membrane (19,24).

Cytochrome c oxidase is composed of a variable number of polypeptide subunits (16,23,33,48,82); this seems to correlate with the evolutionary stage of the organism. In prokaryotes the enzyme contains 2-3 subunits (39,203,204), in unicellular eukaryotes 7-9 (81) and in higher eukaryotes a monomer form has 12-13 different polypeptides (15-19, 23, 27,29,48,159) which occur in stoichiometric amounts. The structure of beef heart cytochrome c oxidase has recently been reinvestigated (82). This study shows that beef heart cytochrome c oxidase contains about 12 different polypeptides and 1793 amino acids.

On the extraction of the non-covalently bound heme from the protein, only heme a is found (11,75). Typical features of heme a are the carbonyl group in position 8 and the long isopropenoid chain in position 2 of the porphyrin ring (11,41). Heme iron may be further liganded by two (fifth and sixth) coordination bonds in the axial direction perpendicular to the plane of the ring (11,75,148) [Fig. 2.2]. In addition to iron, the protein also contains two (1,1,20, 30) or three (209) moles copper per functional unit. It has been reported that the purified enzyme from beef heart also contains tightly bound zinc and magnesium (207,138). Two heme moieties and two copper atoms constitute the four redox-active prosthetic groups. The

functional features of the additional copper, zinc and magnesium atoms remain to be investigated.

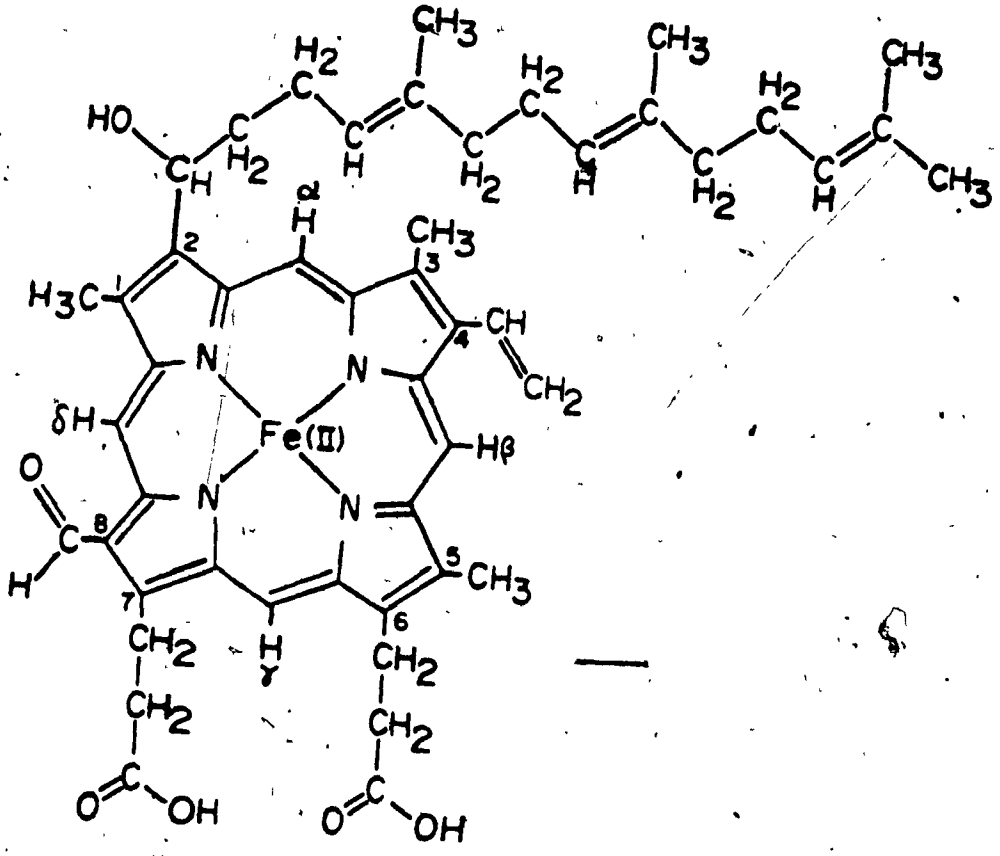


Fig. 2.2. Structural Formula of Heme a

2.2.1. SUBUNIT STRUCTURE OF THE ENZYME

The synthesis of cytochrome c oxidase requires both the mitochondrial and nuclear genome (23,25,27,48). Sub-units I, II, III, are encoded on mitochondrial-DNA and made on mitochondrial ribosomes (26,27). The smaller subunits are all encoded on nuclear DNA and

Table. 2. 1.

	I	II	III	IV	V	VIa	VIb	VIc	VII	VIIIa	VIIb	VIIIc
Asp	16	10	4	8	9	4	2	3	6	0	1	2
Asn	19	5	6	4	4	4	4	1	5	2	1	3
Thr	38	17	24	5	5	7	7	2	5	1	4	4
Ser	30	22	19	12	4	5	5	4	5	3	5	2
Glu	9	5	8	13	1	8	2	4	4	3	1	3
Gln	6	6	7	3	11	4	0	1	5	1	1	2
Pro	28	13	12	6	7	7	7	1	4	3	4	1
Gly	47	8	21	5	6	9	9	4	4	4	2	6
Ala	40	8	14	13	8	8	7	11	6	3	6	3
Val	38	12	16	11	8	6	2	3	4	2	2	3
Met	34	16	11	4	1	1	0	4	1	2	0	1
Ile	37	18	14	5	7	5	2	2	4	2	2	1
Leu	59	34	31	11	11	7	8	5	2	5	6	9
Tyr	19	11	11	7	4	2	2	4	4	1	2	3
Phe	42	6	24	7	3	1	6	8	4	6	2	2
Lys	9	6	3	16	7	6	3	8	6	4	5	5
His	17	7	17	4	3	4	7	1	1	2	1	2
Arg	8	6	5	5	7	4	7	7	7	2	0	2
Cys	1	2	2	0	1	4	1	0	4	0	0	2
Trp	17	5	12	6	2	2	3	0	4	1	1	0
	514	227	261	147	109	98	84	73	85	47	46	56
	1	1	1	1	1	1	1	1	1	1	2	1

Amino acid composition and stoichiometry of the protein components of Bovine Heart cyt. c ox. complex (Ref.82).

Table 2.2.

Polypeptide	Molecular weight	Synthesis site
I	56993	Mit.
II	26049	Mit.
III	29918	Mit
IV	17153	Cyt.
V	1 2436	Cyt.
VIa	10668	Cyt.
VIb	9419	Cyt.
VIc	8480	Cyt.
VII	10063	Cyt.
VIIIa	5441	Cyt.
VIIIb	4962	Cyt.
VIIIc	6243	Cyt.

Mit. = mitochondria Cyt.= cytosol

Exact molecular weights and synthesis site of protein components of Bovine Heart cyt. c ox. (Ref. 82).

transported into the inner membrane from the cytoplasmic side (26-28). The subunit structure of eukaryotic cytochrome c oxidase is still under debate. Table 2.1 lists the amino acid composition as well as the stoichiometry of the protein components of beef heart cytochrome c oxidase polypeptides. The protein sequences of a few mitochondrial subunits of cytochrome c oxidase from bacterial sources (39,203,204), yeast (26,46,81), shark (205), rat liver (107,188), bovine heart (29,33,82) and human placenta (23,206) have been determined.

The amino acid sequences of all the subunits of beef heart enzyme have been completed (82,207). On the basis of amino acid sequence, a monomer molecular weight of 200000 was calculated. Table. 2.2 lists the apparent molecular weights and the synthesis site of each subunits. However, the exact molecular weight and the number of subunits remain unclear.

2.2.2. THE ARRANGEMENT OF SUBUNITS IN THE ENZYME

A number of investigators have proposed (13,29-33,48) an arrangement of subunits in the membrane for cytochrome c oxidase on the basis of cross-linking and chemical-labelling experiments. Considering the number of difficulties involved in the different techniques, the results are good and agree with one another. Some subunits are located in the aqueous C-domain (29,36) these include II, III, possibly V,VI and I. Subunits IV, possibly III; VII are located in the M-domain. Subunits I, II, VII, and IV to

some extent are in contact with the phosphplipid bilayer. Subunits III and VII span the membrane (33,36). [Fig. 2.3] shows a schematic presentation of nearest-neighbour relationships of cytochrome c oxidase polymers (18).

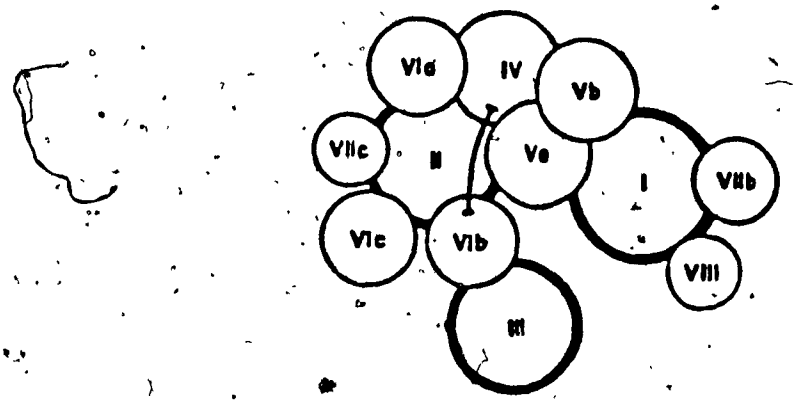


Fig. 2. 3. Schematic presentation of nearest-neighbour relationship of cyt. c ox. polymers based on the cross-linked products obtained with DTB and DSP (Ref.16).

2.2.3. THE LOCATION OF HEMES, COPPERS, AND CYTOCHROME C BINDING SITE

Attempts have been made to identify the location of metal centers and the cytochrome c binding site (34,35,39,203). Evidence that the prosthetic groups of cytochrome c oxidase are located in subunit I and II was obtained from studies of the prokaryotic enzyme (36,38,114). Bacterial enzymes generally have 2-3 subunits with two hemes and two coppers.

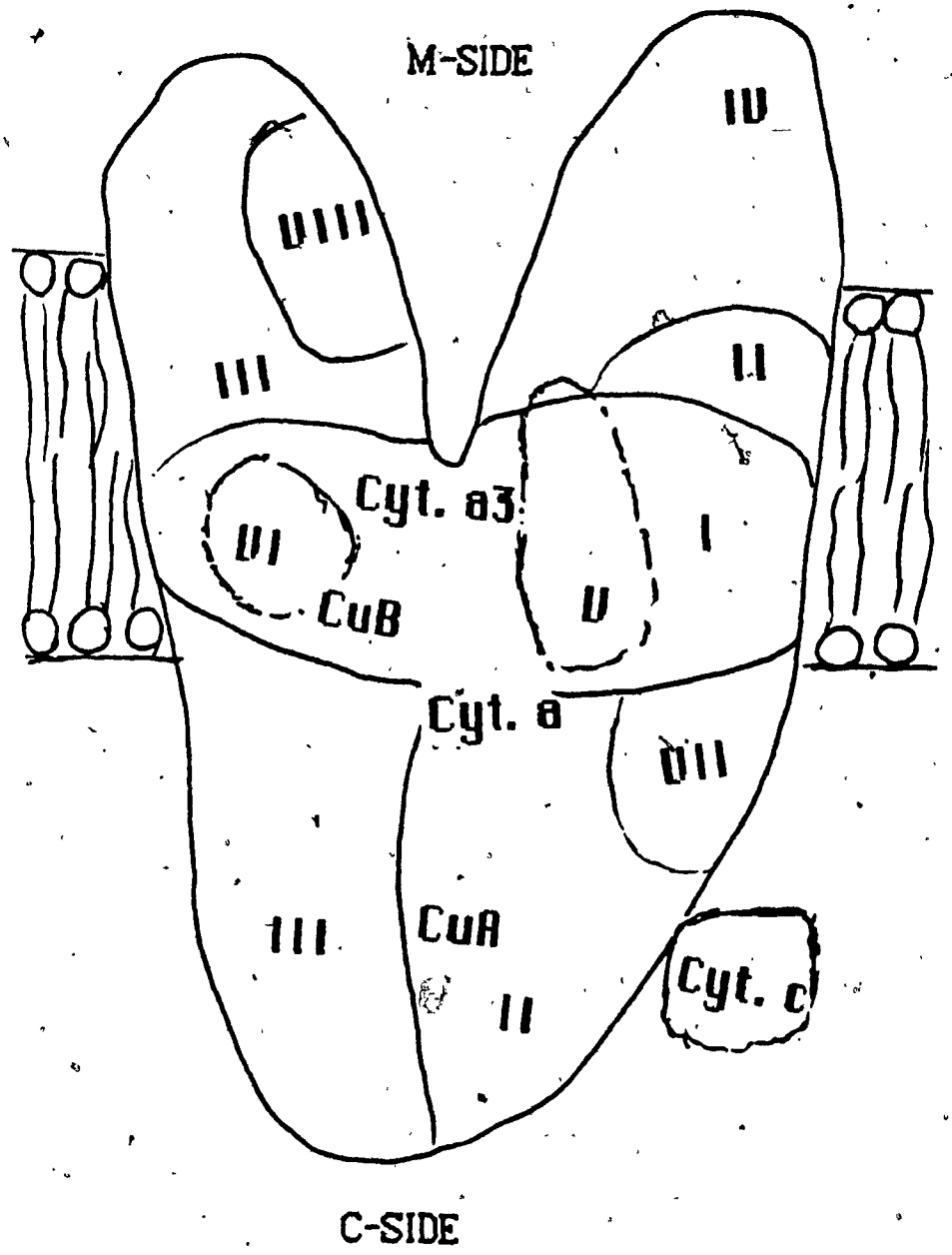


Fig.2.4. Schematic presentation of metal centers and cytochrome c binding site location.

The spectral properties of these enzymes are identical with those of the eukaryotes (39, 203). Direct study of the eukaryotic enzyme is difficult because conditions required to dissociate subunits disturb the redox centers. Indirect analysis suggests that subunits I, II and IV may jointly accommodate the four metal centers (34,37,79,207).

The interaction of cytochrome c with cytochrome c oxidase occurs at the cytosolic side of the mitochondrial membrane. Isolated cytochrome c oxidase at low ionic strength contains two cytochrome c binding sites (127, 128 131). Most studies (19,29,36) suggest that a high affinity binding site involving subunit II and subunit III may be required in proton translocation (36,181,189). Fig. 2.4. shows a schematic presentation of metal centers and cytochrme c binding site.

2. 2. 4. ROLE OF PHOSPHOLIPIDS

A phospholipid-rich preparation of cytochrome c oxidase contains as much as 50-100 moles of phospholipid per mole of enzyme (31); this enzyme is highly active in the absence of added lipids or detergent (208). The majority of lipids are phosphatidylethanolamine (PE), phosphatidyl-choline (PC), and cardiolipin (CL) or diphosphatidyglycerol (DPG) (45). It is possible to prepare virtually lipid-free cytochrome c oxidase (31,43). It has been shown that depletion of phospholipid to less than 1 to 2 mole of lipid per mole of protein does not interfere with the formation of 1:1 complex of cytochrome c oxidase with cytochrome c (43). However lipid depletion does

interfere with electron transfer within the oxidase molecule (43,45,46,113). Many investigators (40,44,45) believe that cytochrome c oxidase has an absolute requirement for phospholipids.

It has been reported that subunit I is the site of tight binding for cardiolipin. Since subunits I and II may both contain heme a , it is likely that cardiolipin has some role in electron transfer from heme to heme within the molecule (43).

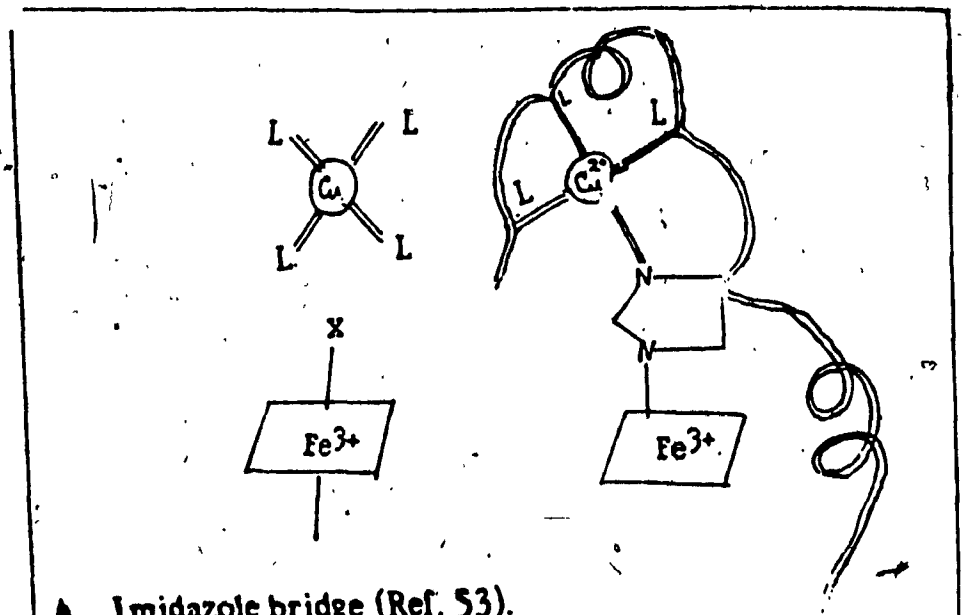
2. 2.5. CHARACTERISTICS OF METAL CENTERS

There are two distinct heme components in the cytochrome c oxidase, one low-spin and low potential, the other high-spin and high potential (20,9,41,75,76,78). This conclusion is based on studies which show that only part of the heme in oxidized cytochrome c oxidase is EPR-detectable (20,41, 78). The copper of cytochrome c oxidase is also only partly EPR-detectable in the oxidized state (8,51,60,108,169). The low-spin EPR-detectable iron in the fully oxidized enzyme is associated with the EPR-detectable copper and the two constitute the cytochrome a Cu_A (also known as CuD or CuA) center. The high-spin EPR-undetectable iron is paired with an EPR-undetectable copper; the two define the cytochrome a3 Cu_B (also known as CuU or CuB) center (41,42,72,76). The absence of an EPR signal for both heme a3 and Cu_B was proposed to be due to, either the magnetic association of the iron with the copper or to the association of a3 with the iron of another molecule of heme a3 (49,52,101). Raman spectroscopy (49) revealed that cytochrome

a3 is six-coordinate and low-spin in the resting, oxidized enzyme (49,60), and probably five-coordinate in the pulsed enzyme and five-coordinate and high-spin in the reduced state. Cytochrome a is six-coordinate and low-spin in both states (49,62,72).

2. 2. 6. MAGNETIC INTERACTION BETWEEN METAL CENTERS

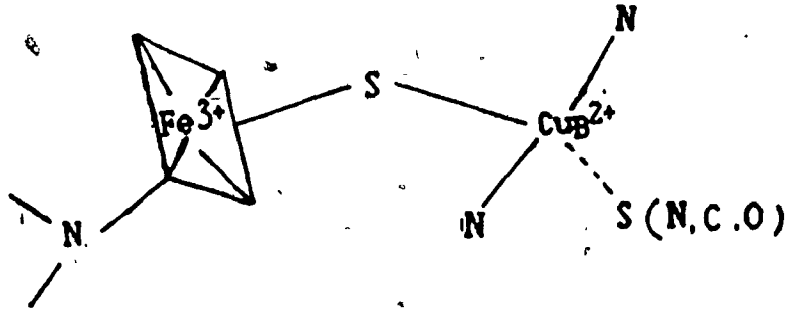
The magnetic interaction between hemes a and a3,or heme-copper may be the explanation for the EPR-silence of half of the enzyme (20,51, 53,54,60,76,100,115). A distance of 1.5 nm between cytochrome a and Cu_A has been suggested (51,76,81,94) which implies that the distance between Cu_A and cytochrome a3:Cu_B must be larger than 1.5 nm. The distance between cytochrome a3 and Cu_B in the oxidized enzyme is less than 0.5 nm (55,63,81,118,201). Because of the close proximity of cytochrome a3 and Cu_B, a strong magnetic interaction between them is expected (20,57,63,92, 153) and the two centers may be bridged by a common ligand. This ligand mediates the strong magnetic interaction between cytochrome a3 and Cu_B. The ligand has been suggested to be the imidazole of a histidine (53), the sulphur of a cysteine (52) or an oxygen atom (57,61) [Fig.2.5. A, B, C]. Most recent studies failed to show an imidazole bridge (102,103), but there is no hard evidence for the other two ligands either. Many studies have shown that in the reduced cytochrome c oxidase the bridge between cytochrome a3 and Cu_B disappears (118,201).



A. Imidazole bridge (Ref. 53).

$$Fe^{3+} - O - Cu_B^{2+}$$

B. Oxide bridge (Ref. 54).



C. Sulphur bridge (Ref. 52).

Fig. 2.5. Schematic presentation of the bridge between cyt. a3 and Cu_B

2.3. MECHANISM OF DIOXYGEN REDUCTION

At the present time, we lack a complete understanding of the mechanism of Equation [1]. Chemically, dioxygen can be reduced to water by the addition of four electrons providing that the conditions are right. The midpoint oxidation-reduction potential for the addition of the first electron is only -330 mV (58), which is indicative of a poor oxidizing agent. The three subsequent steps, however, have Em values of +680, +720, and +1380 mV, respectively (197).

Cytochrome c oxidase contains two hemes and two coppers and can accept four electrons which cycle between the fully oxidized and reduced steps (49,52). For the reduction of O_2 in four one-equivalent steps of electron transfer, there may be a thermodynamic barrier for the first step (93). Alternatively, dioxygen may be reduced in two two-equivalent steps, (62,78,79). Most studies (62,64,65,70,79,95,118,193) suggest that the first event is the combination of oxygen with the enzyme. The transfer of the two first electrons may be simultaneous, or occur rapidly beyond the detectibility of present spectroscopic techniques. This is supported by the fact that cytochrome a_3 and its associated copper (Cu_B) are positioned within 0.5 nm of one another (55,136,155). Low temperature studies (57,64,70) of the mechanism of dioxygen reduction by the enzyme suggest that in the first step of the reaction an oxy-compound is formed.

scheme [1]

$Fe^{2+}\ Cu_A^{1+}\quad Fe^{2+}Cu_B^{1+} + O_2$

↓

$[Fe^{2+}\ Cu_A^{1+}\ Fe^{2+}\ Cu_B^{1+}] : O_2$

compound A or oxygenated compound

↓

$Fe^{2+}Cu_A^{1+}\quad Fe^{3+}Cu_B^{1+}:O_2^{1-}$

compound B_1 or intermediate [I]

↓

$Fe^{2+}Cu_A^{1+}\quad Fe^{4+}Cu_B^{1+}: O_2^{2-}$

compound B2 or intermediate [II]

↓

$Fe^{2+}Cu_A^{1+}\quad Fe^{3+}Cu_B^{2+}O_2^{2-} + 4\,H^+$

compound B3 or intermediate [III]

↓

$Fe^{3+}\ Cu_A^{2+}\quad Fe^{3+}Cu_B^{2+} + 2\,H_2O$

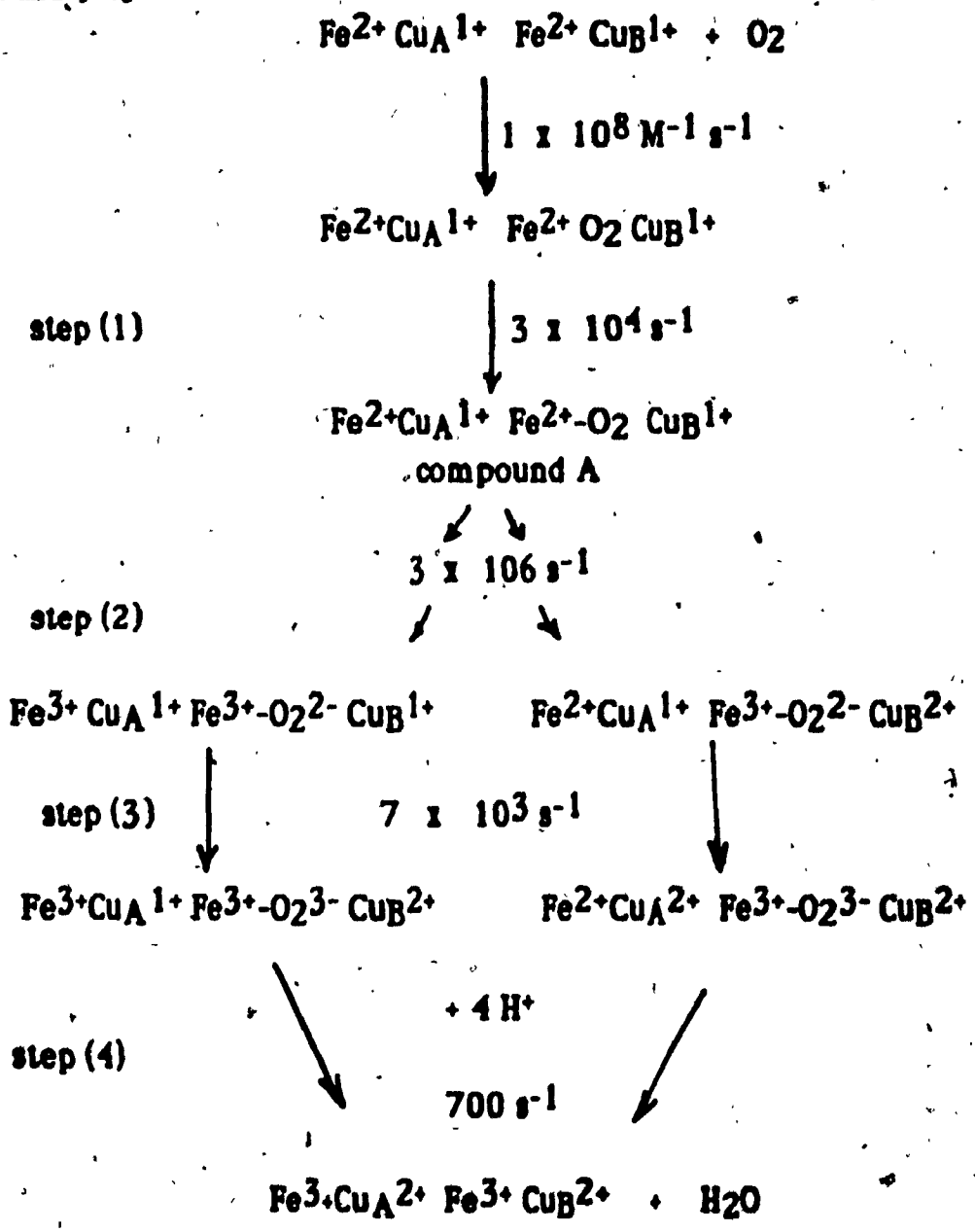
Resting or oxidized cyt. c ox.

The spectral properties of this complex are similar to those of the fully reduced enzyme. Following this step, electron transfer from cytochrome a3 and Cu_B occur and a peroxy compound is produced. The peroxy compounds serve as effective electron acceptors from cytochrome a and Cu_A. This is shown in the scheme [I]. Cytochrome a3 in the intermediate [II] has been suggested (57,62,64) is in the ferryl oxidation state.

Scheme [1] does not explain the relative absorbtion changes observed during fast and slow phases. Some investigators (64,150) suggest that along with cytochrome a3 some cytochrome a is oxidized in the first step of electron transfer. This proposal requires a branch in the electron transfer pathway of the fully reduced enzyme with oxygen. The branched mechanism (150) of oxygen reduction is shown in [scheme III].

EXAFS studies (118) suggest that cytochrome c oxidase may have two functions, it can act as an oxidase or peroxidase. In the oxidase function, a bridge between heme a3 and Cu_B is broken and a bridged peroxy complex (compound B) is formed. When it acts as a peroxidase, the enzyme functions in a side catalytic cycle. However, the oxygen activation schemes presented above are controversial and the details of the electron transfer mechanism remain unknown.

scheme [II]


$Fe^{2+}\ Cu_A^{1+}\ Fe^{2+}\ Cu_B^{1+} + O_2$
$1 \times 10^8\ M^{-1}\ s^{-1}$
$Fe^{2+} Cu_A^{1+}\ Fe^{2+}\ O_2\ Cu_B^{1+}$
step (1)
$3 \times 10^4\ s^{-1}$
$Fe^{2+} Cu_A^{1+}\ Fe^{2+}\text{-}O_2\ Cu_B^{1+}$
compound A
$3 \times 106\ s^{-1}$
step (2)
$Fe^{3+}\ Cu_A^{1+}\ Fe^{3+}\text{-}O_2^{2-}\ Cu_B^{1+}$
$Fe^{2+} Cu_A^{1+}\ Fe^{3+}\text{-}O_2^{2-}\ Cu_B^{2+}$
step (3)
$7 \times 10^3\ s^{-1}$
$Fe^{3+} Cu_A^{1+}\ Fe^{3+}\text{-}O_2^{3-}\ Cu_B^{2+}$
$Fe^{2+} Cu_A^{2+}\ Fe^{3+}\text{-}O_2^{3-}\ Cu_B^{2+}$
$+ 4\ H^+$
step (4)
$700\ s^{-1}$
$Fe^{3+} Cu_A^{2+}\ Fe^{3+}\ Cu_B^{2+} + H_2O$

2. 3. 2. TRANSIENT FORMS OF THE CYTOCHROME c OXIDASE

oxygenated, pulsed and "mixed-valence" states

Fast re-oxidation of cytochrome c oxidase produces a form of the enzyme which was originally called the "oxygenated" form (59) or "pulsed" (60,61). This oxygenated compound is characterized by a red-shifted Soret band near 428 nm and a higher extinction of the band near 600 nm, relative to the "resting" enzyme. The pulsed compound is not red-shifted in the Soret but shows enhanced (over the oxygenated) absorption in the visible. This compound has a higher reactivity than resting enzyme towards cytochrome c oxidation and ligand binding (61). Since its discovery many other investigators (52,53,55,60, 62,79,102) have reported similar compounds of the oxygen reaction with the enzyme. Studies of magnetic circular dichroism (MCD) and Resonance Raman (RR) spectroscopy (57,64,65) revealed three "oxygenated" derivatives of cytochrome c oxidase. Different nomemclatures by different investigators for these derivatives have been used including compound C and resting H_2O_2. Recent studies (52,55,60,61,79,102) show that the oxidation state of the metal centers in these oxygenated derivatives are not the same. EXAFS studies (118) revealed that the structure of the a_3-Cu_B center is different compared to that of the resting enzyme. The following structure has been suggested (61) for "pulsed" cytochrome c oxidase:

$$a^{3+}, Cu_A^{2+}; a_3^{2+}, Cu_B^{1+}, O_2^{2-}$$

It has been reported that resting cytochrome c oxidase reacts with peroxide (161) and produces $\underline{a}^{3+}\underline{a}_3^{3+}$-$H_2O_2$. This compound may be responsible for the Soret band at 427 nm. The peroxy adduct decays to the pulsed enzyme .

2. 4. LIGAND BINDING TO CYTOCHROME C OXIDASE

The binuclear $\underline{a}_3$ Cu_B metal center of cytochrome c oxidase is remarkably effective in reducing oxygen to water. It releases a minimum number of free radical intermediates on the one hand, and promotes a maximum efficiency of energy conservation on the other hand. The steps of this reaction are difficult to study due to the presence of multiple hemes and copper atoms in the enzyme, and because the absorption bands overlap. Also EPR only detects one heme and one copper due to spin coupling of the other heme and copper centers.

The reaction of cytochrome c oxidase with different ligands such as carbon monoxide (CO), cyanide (CN^-) azide (N_3), and nitroxide (NO), have received considerable attention. Ligand binding studies permit the investigation of the binding of a molecule of similar nature and size as dioxygen with some flexibility in controlling the stages of reaction. It is clear that the study of the reactions of different ligands can provide useful information concerning the active site of cytochrome c oxidase.

Some ligands react with the oxidized form, some with reduced cytochrome c oxidase, and others react with both. Since the binding aspects of carbon monoxide and cyanide are the object of this study, only those experiments directly related to these ligands will be considered below.

2. 4. 1. BINDING OF CARBON MONOXIDE TO CYTOCHROME C OXIDASE

Carbon monoxide has long been recognized as an inhibitor of cytochrome c oxidase. It binds to a3 Fe[II] and allows spectral resolution of cytochrome a and cytochrome a3. This reaction involves a high-spin to low-spin transition of cytochrome a3 (9,51). The reaction of carbon monoxide with the enzyme is considered to be similar to the reaction of oxygen with the enzyme (66,87,91,92,165). The optical spectrum shows a small decrease of the visible band at 605 nm plus a shift to 590 nm. A distinct change in the Soret is found (see Fig.3. 2).

The CO-inhibited form of cytochrome c oxidase is photolabile; the action spectrum follows a heme pattern. It is likely that CO is bound to the heme of cytochrome c oxidase. Since CO competes with oxygen for the O_2 binding site, CO probably binds to heme a3. Both CO and O_2 seem to be in a non-polar environment, well isolated from the external solvent (92,97). The heme pocket is large enough to accommodate the heme iron of cytochrome a3, the Cu_B, and a CO molecule which can move reversibly from Fe to Cu depending on light, temperature, and redox state conditions (104). Kinetic studies (69,70) of photo-dissociation and reassociation of CO at low

temperature (69,70) also show that the ligand pocket or CO binding site is positioned near the heme iron and has the capacity for only one ligand.

Carbon monoxide in the absence of oxygen donates electrons to the resting enzyme and forms a mixed valence cytochrome c oxidase liganded to CO (195). Studies with pulsed cytochrome c oxidase revealed that CO binding occurs when both a3 and Cu_B are reduced (99,161,195). Carbon monoxide has been established as one of the most useful probes in studying the structure of the oxygen-binding site (57,70,104,157,158,161).

2.4.2. BINDING OF CYANIDE TO CYTOCHROME C OXIDASE

Oxidized cytochrome c oxidase in the "resting" state is virtually non-reactive with most ligands such as cyanide (73,162) in spite of the fact that cyanide is a well known poison of the enzyme. However, cyanide reacts rapidly with a partially reduced form of the enzyme (71,74,90,106, 162, 169). The reaction of cytochrome c oxidase with the oxidized enzyme is very slow (68,106,162) and causes a spectral change which is consistent with a high to low-spin transition. This complex does not show any EPR signal (162), but MCD spectroscopy can identify the generation of a low-spin heme a3-HCN (69,119,169). In the presence of a reducing agent a g=3.58 resonance is generated, which is typical of a low-spin cyanide complex (54,106,163, 168,169).

For years many investigators have tried to explore the mechanism of cyanide binding, but, because of the complex behavior of the ligand towards

the enzyme, this problem is still under investigation. It has been suggested (63) that a low-spin binuclear complex a3.CN (S=1/2)-CuB(S=1/2) is formed. The antiferromagnetic coupling between cytochrome a3.CN and CuB yields a diamagnetic ground state. An imidazolate nitrogen(53,63), cysteine sulphur (52) or an oxygen ligand (54,60,66,170) has been proposed to serve as a bridge between cytochrome a3 and CuB. In the partially reduced enzyme this bridge breaks and an open cyanide binding site is formed (63,106,170,169).

2.5. SPECTROSCOPIC PROPERTIES OF CYTOCHROME C OXIDASE

2.5.1. UV-VIS and NIR SPECTRUM

Significant steps forward in our knowledge of cytochrome c oxidase were made by the application of spectroscopic methods. Because of the intense color of the enzyme and availability of the relevant instrumentation, visible spectroscopy has been the most widely used technique. The two heme and the two copper metal centers in cytochrome c oxidase are structurally different. The different electronic configurations cause different optical spectra. Cytochromes a and a3 have strong visible (VIS) and near-ultra violet absorption. The early studies indicated that cytochrome a contributes between 72% and 80% to the total reduced minus oxidized absorption band at 604 nm and approximately 50% at 444 nm (3,96). Recent studies (119) support the early results and postulate a strong interaction between the two hemes which modulates their redox potentials

(101). The contribution of copper to the Soret and VIS regions (400 nm to 700 nm) is very small or non existent (96). A single absorption band at 830 nm in the resting enzyme is present (42,154). Optical transition spectra of oxidized and reduced cytochrome c oxidase are presented in Fig. 2. 6.

2. 5. 2. EPR

In the "resting" oxidized state of cytochrome c oxidase, one of the hemes and one copper center are EPR detectable. The absence of EPR signals from two of the centers led to suggestions that they are interacting as an antiferomagnetically coupled pair (20). The EPR detectable heme is essentially identical with the component identified spectrophotometrically as ferric cytochrome a (9). The EPR detectable copper is closely related to the 830 nm chromophore (6,7,154).

A major problem with all available techniques (such as UV-VIS, NIR, MCD, RR, NMR, EPR, MS,EXAFS ...) is that they assume that the four metal centers act as independent centers in taking up electrons or binding to ligands. We must therefore be cautious in weighing spectroscopic data.

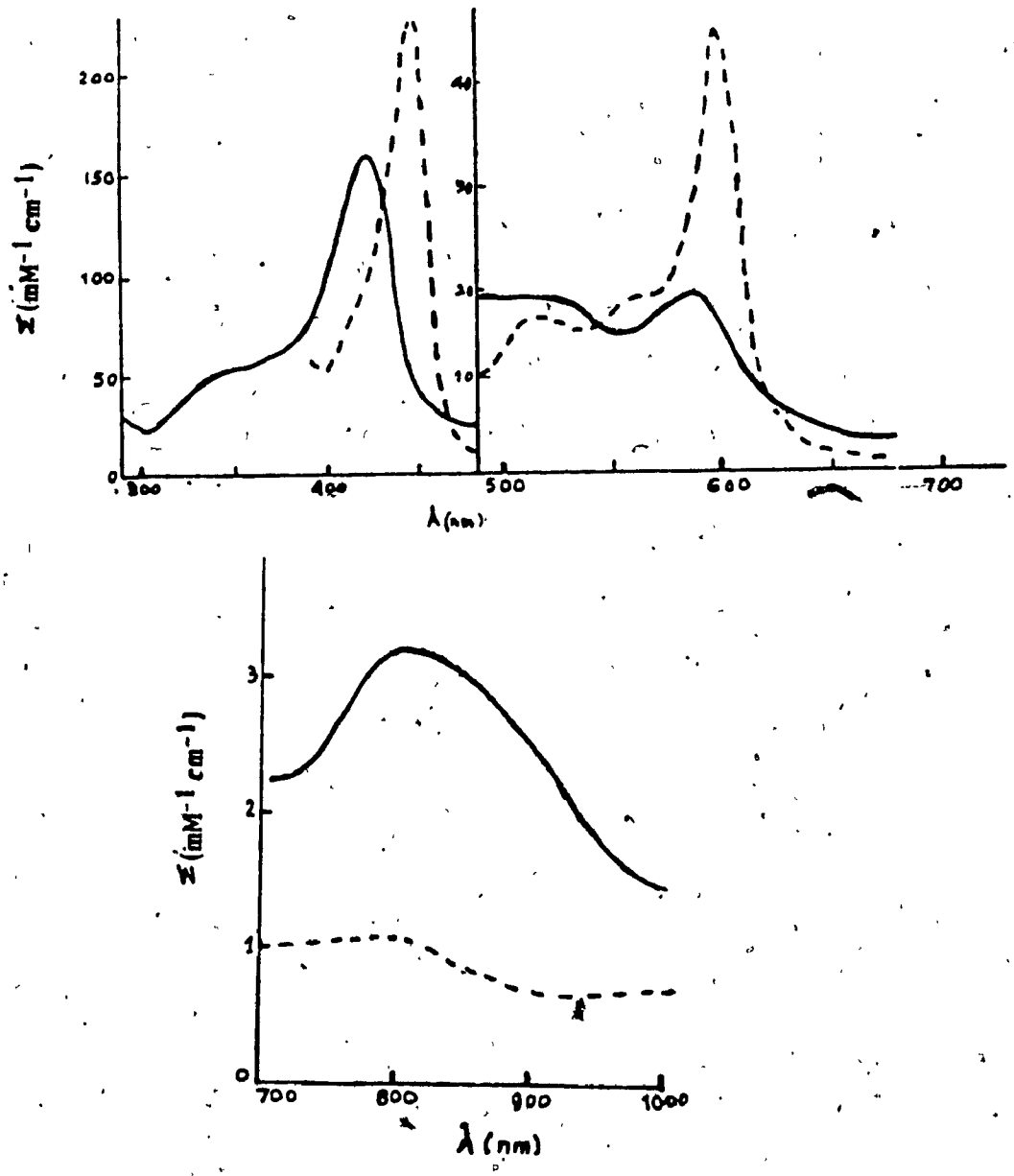


Fig. 2. 6. UV-VIS and NIR absorption spectra of oxidized ——— and reduced - - - - - - cyt. c ox. from 300 nm to 1000 nm range (Ref. 112).

3

ENZYME PREPARATION AND METHODOLOGY

3.1. ENZYME PREPARATION AND PURIFICATION

Beef heart-cytochrome c oxidase was prepared by the modified method (84) of Yonetani (85) as explained below.

Day 1: In each preparation, 2-3 beef hearts were obtained from the slaughterhouse within a few minutes of the animals being killed. The hearts were sliced into small pieces and placed in crushed ice. All the steps of the preparation were carried out in the cold room at 4°C. Slices of beef heart were cut into smaller pieces and trimmed completely of fat and connective tissue. The better the trimming of the fat at this stage, the less work was required at the later stages. The pieces of lean meat (about 2.6 Kg) were ground in a precooled meatgrinder and washed as follows:

The minced meat was washed three times with deionized water mixed with crushed ice. Subsequently, the washed mince was incubated with 10 volumes of 0.02 M sodium phosphate dibasic (HNa_2PO_4) for about 1 hour. This was followed by washing it twice with distilled H_2O (at least 10 times the volumes of H_2O each time). Each washing was then followed by squeezing the liquid from the mince using a cheesecloth.

The mince was squeezed and divided into 300 g portions and kept in plastic bags at 4°C until the next day.

Day 2: Each portion of mince (300 g) was mixed with 150 ml of 0.2 M potasium phosphate buffer, pH 7.4 and about 150 g of crushed ice. The mixtures were homogenized in a 1 litre Waring blender and finally diluted to 10 litre with distilled water (dilution depends on the thickness of the homogenate and its volume). The homogenate was centrifuged for 20 minutes at 800 x g. The supernatant was then carefully separated from the loosely packed pellet (great care is required not to mix the loosely packed pellet with the supernatant). The collected supernatants were pooled together and saved. The precipitates (pellets) were homogenized with 1.5 to 2 litre of 0.02 M phosphate buffer, pH 7.4, for three minutes and centrifuged for 20 minutes at 800 x g. The pellets were discarded. The pH of the pooled supernatant was lowered to 5.6 very slowly with 1 M acetic acid. The acidified mixture was immediately centrifuged at 800 x g for 10 minutes. The precipitate was washed with 10 volumes of distilled water and centrifuged at 800 x g for 10 minutes. Subsequently, the precipitate was suspended in 400 ml of 0.2 M phosphate, pH 7.4. The pH of the suspension was adjusted to 7.4 with 3 M amonium hydroxide (NH_4OH) and the volume to 1 liter with distilled water. At this stage a greenish brown color can be identified. To the homogenate 10% cholate was added (25 ml 10% cholate per 100 ml of homogenate). Next, ammonium sulfate (22 g per 100 ml of homogenate) was added to bring the solution to 30% saturation (ammonium

sulfate must be added very slowly). The pH was kept at 7.4, (to adjust the pH, ammonium hydroxide 3M or acetic acid 1M was used). The homogenate was left to stand for 1 hour and the mixture was then centrifuged at 10,000 x g for 10 minutes. The brown supernatant was brought to 50% ammonium sulfate saturation, and immediately centrifuged at 10,000 x g for 10 minutes. The precipitate was dissolved in 225 ml of 0.1 M potassium phosphate buffer, pH 7.4 containing 2% cholate. The solution was brought to 25% ammonium sulfate saturation, (33.3 ml of saturated ammonium sulfate per 100 ml of homogenate) and the pH was maintained at 7.4 with ammonium hydroxide (3M). This mixture was finally left overnight at 0°C. During the standing period most of the cytochrome $\underline{c}_1$ and cytochrome $\underline{b}$ precipitated. If, after this step the enzyme still showed high cytochrome $\underline{c}_1$ or cytochrome $\underline{b}$ concentrations, the last step was repeated.

Day 3: The mixture was centrifuged at 10,000 x g for 10 minutes and the precipitate was discarded. The deep brownish-red supernatant was brought to 35% ammonium sulfate saturation with saturated ammonium sulfate (15.4 ml of saturated ammonium sulfate per 100 ml of enzyme), and centrifuged for 10 minutes at 10,000 x g. The brown precipitate was dissolved in the smallest volume possible (75-100 ml) of phosphate buffer containing 2% cholate. This solution was fractionated several times with saturated ammonium sulfate solution, between 26% and 33% until a pure enzyme was obtained following the purity test below.

3.1. 1. PURITY TEST

After each ammonium sulfate fractionation, a very small portion of the 26% to 35% precipitate was dissolved in 0.1 M phosphate buffer pH 7.4, containing 1% Tween-80. The transparent solution obtained was examined for purity spectrophotometrically. The spectrum of the oxidized enzyme was recorded between 260 nm to 630 nm. The solution was then reduced by adding a few grains of dithionite and, after 30 minutes, the spectrum recorded again. The purity criteria are as follows (85).

I- Complete removal of cytochrome b and cytochrome c can be identified by removal of the absorption bands at 560 nm and 552 nm in the reduced state of the enzyme.

II- Removal of modified cytochrome c oxidase which is associated with a shoulder at around 422 nm in the reduced state of the enzyme.

III- A ratio of more than 1.25 for A 445 nm (reduced) to A 422 nm (oxidized).

IV- A ratio of less than 2.5 for A 280 nm (oxidized) to A 445 nm (reduced).

After the purity test was satisfied, the enzyme was suspended in Tween-80 containing phosphate buffer. The concentration of the enzyme was determined on the basis of an extinction coefficient (reduced minus oxidized) of 11 mM^{-1} cm^{-1} at 605 nm (85). The enzyme concentration was between 300 to 450 uM in different preparations. The purified enzyme was clear and transparent when dissoved in buffer.

At this stage the enzyme was divided into aliquots of approximately 1 ml placed in small vials and stored at -20°C for several months. All concentrations will be reported in terms of heme a and should be halved in order to determine the concentration of a functional unit of the enzyme (13). The protein concentration was determined by the method of Lowry et al. (142), using bovine serum albumin as the standard.

3. 1. 2. FINAL STEP OF PURIFICATION

The frozen preparation of cytochrome c oxidase has traces of cholate and some denatured cytochrome c oxidase. The cholate lowers the activity of the enzyme with its substrate cytochrome c. The cholate can be removed by dialysis or passage of the enzyme through a molecular sieving column. The later technique was chosen in this work because of a better resolution. Sepharose Cl-6B is macroporous and has a good capacity for molecules up to $1x10^6$ in molecular weight. The flow properties, stability of bed volume and insensitivity to changes in ionic strength (I) and pH make it a good choice for this study.

A column 3.8 cm x 100 cm and with about 100 mg protein capacity was used. The column was equilibrated with the required buffer. The fractions with the highest absorbance at 418 nm were chosen for assays. During this final step of purification all traces of cholate and denatured cytochrome c oxidase are removed from the enzyme, and Tween-80 is substituted for cholate. Following this last step of purification, the enzyme

remained stable for at least 48 hours at pH 5 to 7.8 at 0° to 4°C. The elimination of this final step of purification (passage through the column) causes the enzyme to be less stable especially at pH lower than 6.5.

3. 2. PREPARATION OF LOW LIPID CONTENT CYTOCHROME C OXIDASE

All the steps for the enzyme isolation were followed according to Yonetani (85) and Kornblatt (84). The final ammonium sulfate fractionation between 27% to 33% was repeated several times after the purity test was passed. The phospholipid (PL) content was determined by measuring the inorganic phosphate and assuming an average PL molecular weight of 775 (143). Ammonium sulfate fractionation was carried out until PL content dropped to 7 to 10 mole of PL per heme a. No further qualitative or quantitative test of individual phospholipids was carried out.

A final purification (84) by gel filtration (sepharose Cl-6B gel) was performed prior to activity assays. The column was equilibrated with the buffer subsequently used for assays, (different buffers were used each time with different ionic strength, pH and Tween-80 contents).

3. 3. BUFFERS

All ligand binding assays were performed in a complex buffer consisting of 25 mM phosphate, 25 mM phthalate, 5 mM tris, 5 mM EDTA, 25 mM NaCl and 0.5% Tween-80, titrated to the required pH with 2 M NaOH

or 2 M HCl. Isolated experiments were performed using a buffer consisting of 10 mM tris-HCl, 10 mM EDTA, 50 mM NaCl, 1 mM NaF, and 0.5% Tween-80 which yielded the same results as the more complex buffer.

The activity of cytochrome c oxidase over the pH range 5 to 8 was determined in phosphate buffer by preparing buffers containing various amounts of Tween-80 and different ionic strengths as follows:

A series of phosphate buffers with ionic strengths (I) in the range of 5 mM to 100 mM (in the order of 5, 10, 15, 20, 25, 30,40,50 100 mM) were prepared. The content of Tween-80 of each buffer were 0% to 0.5% (in order of 0%, 0.05%, 0.1% 0.2% 0.3%, 0.5%). The pH of each buffer was in the range of 5 to 8 (in order of 5, 5.5, 6, 6.5, 7, 7.5, 8).

Ionic strength (I) of the potassium phosphate buffers, in pH range of 5 to 8 were calculated using the following formula (145);

$$I = \frac{K_w}{(H^+)} + \frac{\frac{K_{a1}}{(H^+)}\left(1+\frac{3K_{a2}}{(H^+)}\left(1+\frac{2K_{a3}}{(H^+)}\right)\right)}{1+\frac{K_{a1}}{(H^+)}\left(1+\frac{K_{a2}}{(H^+)}\left(1+\frac{K_{a3}}{(H^+)}\right)\right)} \times P_i$$

P_i = Total phosphate concentration
$pK_{a1} = 2.12$
$pK_{a2} = 7.21$
$pK_{a3} = 12.67$
$pK_w = 14.0$

The pH of all solutions was determined at 20° C with a Corning model 12 expanded-scale pH meter, using a Fisher model 13-639-90 combination electrode. The electrode was calibrated at pH 7.00 and 4.00 using a Fisher standard solution. Over the pH range 5,0 to 8.0 the electrode deviated from a known standard at pH 5.00 by less than 0.02 units.

Aminco DW-2 and Cary 2290 spectrophotometers thermostated at 20° C were used. All spectra of carbon monoxide and cyanide binding were carried out with the DW-2 and kinetics studies were performed with the Cary 2290.

All kinetics data were treated by linear regression.

3.4. CARBON MONOXIDE GENERATOR

A home-made carbon monoxide generator was used to prepare a saturated carbon monoxide solution in distilled water, [Fig. 3.1]. Carbon monoxide is generated upon the addition of formic acid to concentrated sulphuric acid. It was washed by passing through calcium hydroxide and led to distilled water. Distilled water saturated with carbon monoxide was tightly sealed and aliquots of this solution were added with a Hamilton syringe (1 to 30 ul). The concentration of carbon monoxide in such a solution at room temperature is 0.0027 g per 100 ml of solution (86) or about 1 mM per liter. This solution was used as stock and prepared fresh before the assays.

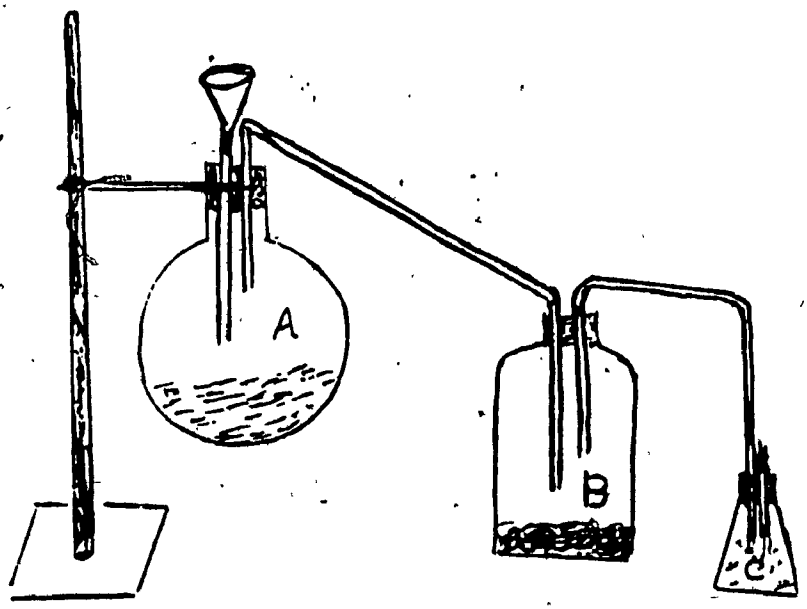


Fig. 3. 1. Schematic presentation of CO generator;

A contains concentrated sulfuric acid.

B contains calcium hydroxide.

C contains distilled water.

Upon addition of formic acid to sulfuric acid, CO is generated. CO is passed through container B to absorb the acid impurities; then enters C and is dissolved in distilled water and produces saturated CO solution. This saturated CO solution which contains 0.0027 g / 100 ml water (86) was used as stock solution.

3. 5. PREPARATION OF STANDARD POTASSIUM CYANIDE SOLUTION

KCN solutions were freshly prepared every day using solid potassium cyanide (Sigma). This solution was standardized by titration against standard silver nitrate (144). The silver nitrate itself was standardized with standard KCl (Sigma).

3.6. PREPARATION OF REDUCED CYTOCHROME c

1-2 mg cytochrome c Type III (90-100% pure, Sigma) was dissolved in 1 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA saturated with nitrogen. The solution was reduced by adding a minimal amount of solid dithionite to produce an orange color. The reduced solution was passed through a column (10 x 200 mm) of sephadex G-25 (fine grain beads), which had previously been equilibrated with the same buffer saturated with nitrogen.

The column was kept under minimal hydrostatic pressure to obtain a slow flow rate. This results in a maximal separation of reduced cytochrome c from exess dithionite and oxidation products (85). The concentration of reduced cytochrome c in the elutate was determined spectrophotometrically using the following extinction coefficients (85):

$\varepsilon 550$ nm (reduced) = 27.7 $mM^{-1}cm^{-1}$

$d\varepsilon 550$ nm (reduced minus oxidized) = 18.5 mM^{-1} cm^{-1}

The eluted reduced cytochrome c should have a ratio of 12 or more for the absorbance at 550 nm (reduced cyt.c) to absorbance at 565 nm (oxidized). The reduced cytochrome c is unstable in the air and in the light; it was bubbled with nitrogen gas and kept in the ice and dark. This solution is stable for 24 hours.

All the chemicals used were bought from the Sigma or Canlab chemical companies and were of high quality.

3.7. ASSAYS

3.7.1. CARBON MONOXIDE (CO) BINDING ASSAYS

Each cuvette, in a series of ten, contained 3 ml of enzyme. The spectrum of the oxidized enzyme was recorded in the range of 380 nm to 630 nm. The enzyme was reduced with a few grains of dithionite or TMPD plus ascorbate at final concentrations of 0.33 mM and 3.3 mM respectively. TMPD and ascorbate were not effective reductants at low pH, thus dithionite was used. After 30 minutes., the spectrum was recorded. When no further change in the spectrum was monitored, indicated amounts of CO-saturated solution were added. The spectrum was recorded for each solution (see Fig.3.2). The ratio of CO bound to heme a was calculated as explained below.

As CO bound to the enzyme increases, the absorbance at 445 nm decreases. At saturation, when all cytochrome a3 is ligated to CO, there is no further decrease in the 445 nm band [Fig.3.2]. To calculate the number of CO

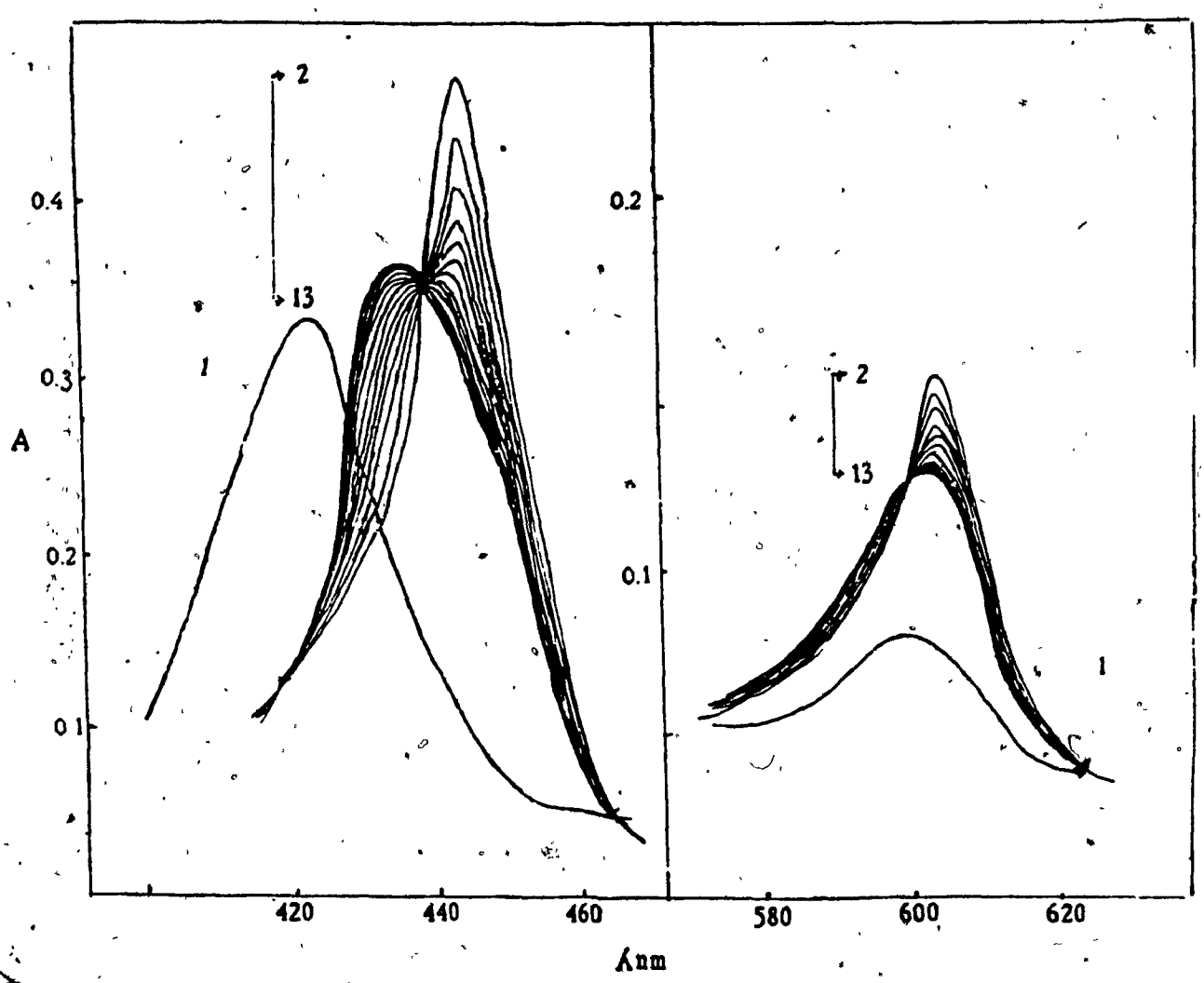
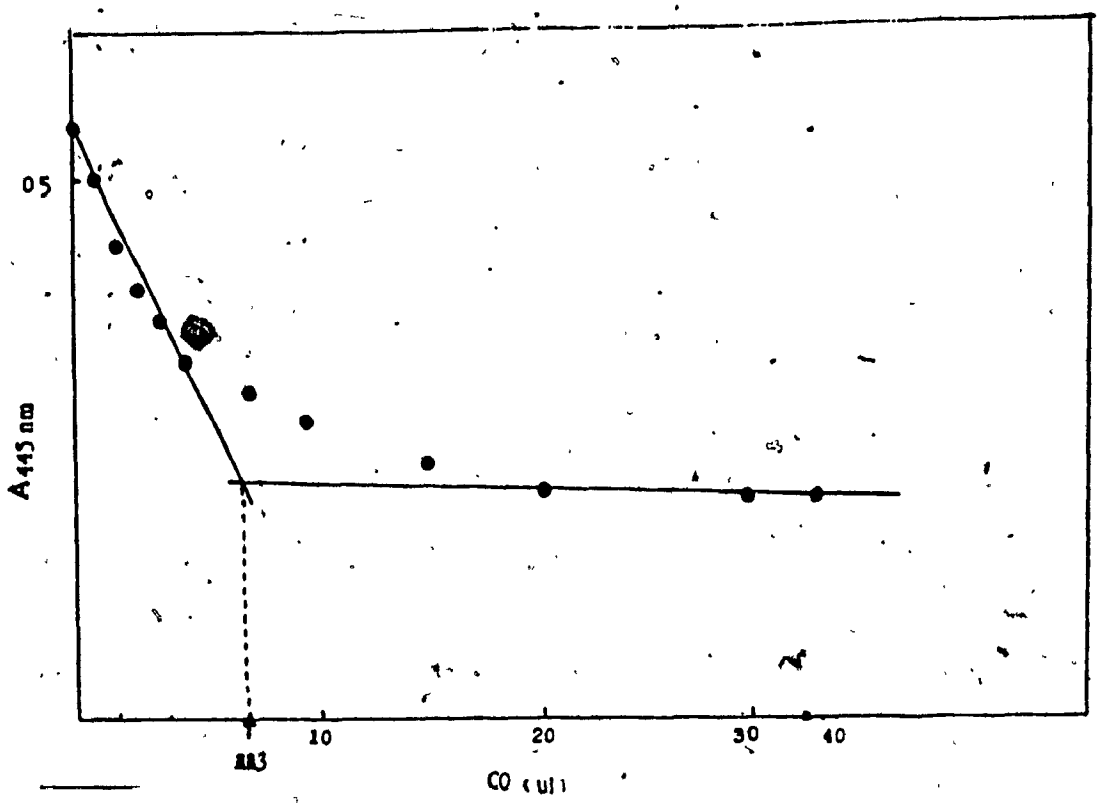

2
13
1
0.4
0.3
0.2
0.1
A
420
440
460
0.2
0.1
2
13
1
580
600
620
λnm

Fig. 3. 2. THE VARIATION OF THE SPECTRUM OF THE REDUCED CYTOCHROME C OXIDASE AS A FUNCTION CO BINDING.

3 ml of 2.5 uM heme aa3 (5 uM heme a) in a solution containing 10 mM tris, 5 mM EDTA, 50 mM NACL, 1 mM NaF, and 0.5% (V/V) of Tween-80 at pH 7.0 and 15°C. The spectrum (1) was recorded and then the enzyme was reduced with a few grains of dithionite. After complete reduction, when no further change occurred in the spectrum of reduced cytochrome c oxidase, 0, 1, 2, 3, 4, 6, 8, 10, 15, 20, 30, 40 ul of 1 mM CO stock solution were added (# 2 to 13) and the spectrum was recorded. When the enzyme was saturated with CO and no further change in the absorption spectrum was monitored, the addition of CO was stopped. Likewise, binding of CO to aa3 over the pH range 5 to 7.8 was studied.



Heme a = 5μM
aa3 = 2.5 μM
Total volume (V) = 3 ml = 10^3 μl
CO volume at satration (v) = 7 μl
CO concentration = 1 mM = 10^3 μM

$$\frac{v\ (\mu l) \times [CO]}{V(ul) \times aa3\ (\mu M)} \rightarrow \frac{7 \times 10^3}{3 \times 10^3 \times 2.5} = 1.68\ CO\ /\ aa3$$

Fig. 3. 3. CALCULATION OF THE NUMBER OF CO MOLE (S) BOUND PER FUNCTIONAL UNIT OF ENZYME.

Absorbances at 445 nm of cyt. aa3 (5 uM heme a) from Fig. 3. 2. were plotted vs the indicated amounts of CO solution associated with the absorbance change at 445 nm. The point of intersection of the two asymptotes was extrapolated to the CO concentration axis. This point shows the amount of CO bound per indicated amount of cyt. aa3. The amount of CO and CN^- bound per functional unit of cyt. aa3 was calculated in the same way as for all other pHs.

bound per functional unit of the enzyme, the absorbance changes versus carbon monoxide concentrations were plotted as shown in [Fig.3.3]. The intersection of the two lines yields the amount of CO bound to enzyme.

The assays generally were repeated five times for each pH. Fig.3. 2 represents the assay at pH 7.0. Similar results were obtained for other assays over the pH range 5 to 7.8. The concentrations of enzyme were 4 to 8 uM heme a.

3. 7. 2. CYANIDE BINDING ASSAYS

A series of cuvettes was set up, each containing 3 ml of enzyme . To each cuvette, different aliquots (0-30 µl) of 1 mM KCN were added. After less than 1 minute the enzyme was reduced with dithionite and layered with mineral oil. The spectrum of each sample was recorded; in Fig.3.4 , spectrum # 1 represents the fully reduced enzyme in the absence of cyanide and spectra # 2 to 15 are those of the enzyme when 1 to 50 ul of 1 mM KCN solution were added. As the concentration of CN^- increases, the absorbance at 445 nm decreases. At saturation (with all cytochrome a3 ligated to CN^-) the addition of as much as 50 µl cyanide (1 mM solution) did not change the absorbance at 445 nm. The ratio of cyanide bound to the enzyme was calculated, as was done for CO. These runs were done at 27° different pH's in the 5.2 to 7.8 range and repeated several times.

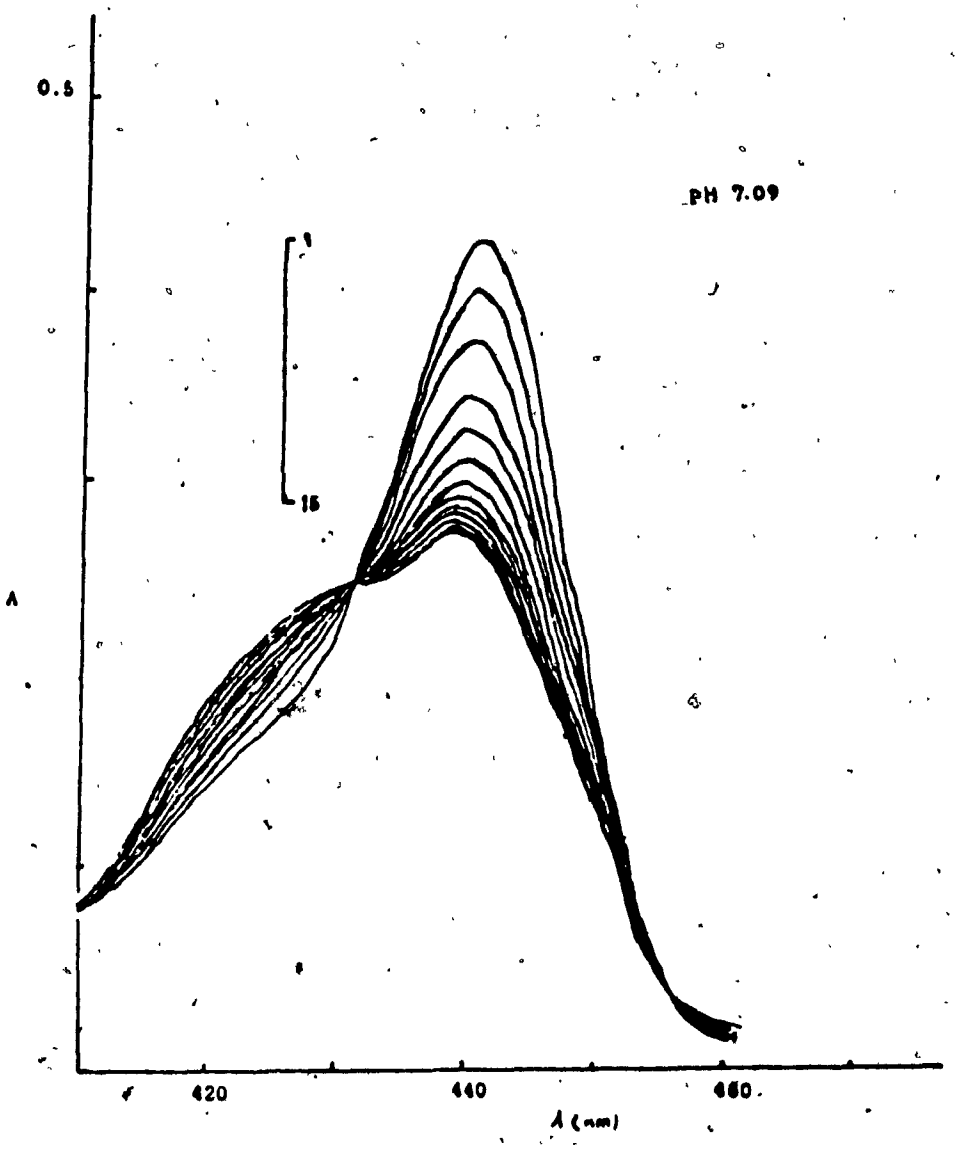


Fig. 3. 4. THE VARIATION OF THE SPECTRUM OF THE PARTIALLY REDUCED CYTOCHROME c OXIDASE AS A FUNCTION OF CN^- BINDING.

The spectra of solutions of 4.4 uM heme a in 10 mM tris, 5 mM EDTA, 50 mM NaCl, 1 mM NaF and 0.5% Tween-80 at pH 7.09 and 15°C were recorded as following;

* 1 shows the spectrum of cyt. c ox. after the addition of a few grains of dithionite and complete reduction.
* 2 to 15 show the spectra of the enzyme obtained upon titration with 1, 2, 3, 4, 5, 6, 8, 10, 15. 20, 25, 30, 40, 50, ul of 1 mM KCN followed by reduction with dithionite.

3. 7. 3. ACTIVITY ASSAYS

Activity assays were performed using Yonetani's method (85) as follows:

A set of six or seven cuvettes contained 2 ml buffer (controlled for pH, ionic strength and Tween-80) plus cytochrome c at concentrations between 8 uM and 35 uM. The reaction was then initiated by adding 5 ul of 0.5 uM cytochrome c oxidase to the cuvette. Cytochrome c oxidation was monitored by following the decrease of absorbance at 550 nm during the initial 60 seconds of the reaction. The molecular activity (MA) or turnover number (TN) of the enzyme was calculated as follows;

$$MA = \frac{OD_{550nm} \times V_{\text{total of the reaction mixture}}}{E_{550nm} \times t \times [\text{cyt. c.ox.}] \times V_{\text{cyt.c.ox.}}}$$

Total V = 2000 µl
E = 0.0185 $\mu M^{-1}cm^{-1}$
Enzyme concentration [cyt.c.ox.] = 0.5 µM
reaction time (t) = 60 second
Enzyme volume (cyt.c.ox.) = 5 µl

Molecular activities at different cytochrome c concentrations were calculated; MA_{max} (molecular activity maximum) and K_M (Michalis-Menten constant) were obtained from double reciprocal plots (Linweaver-burk) [Fig. 3.5].

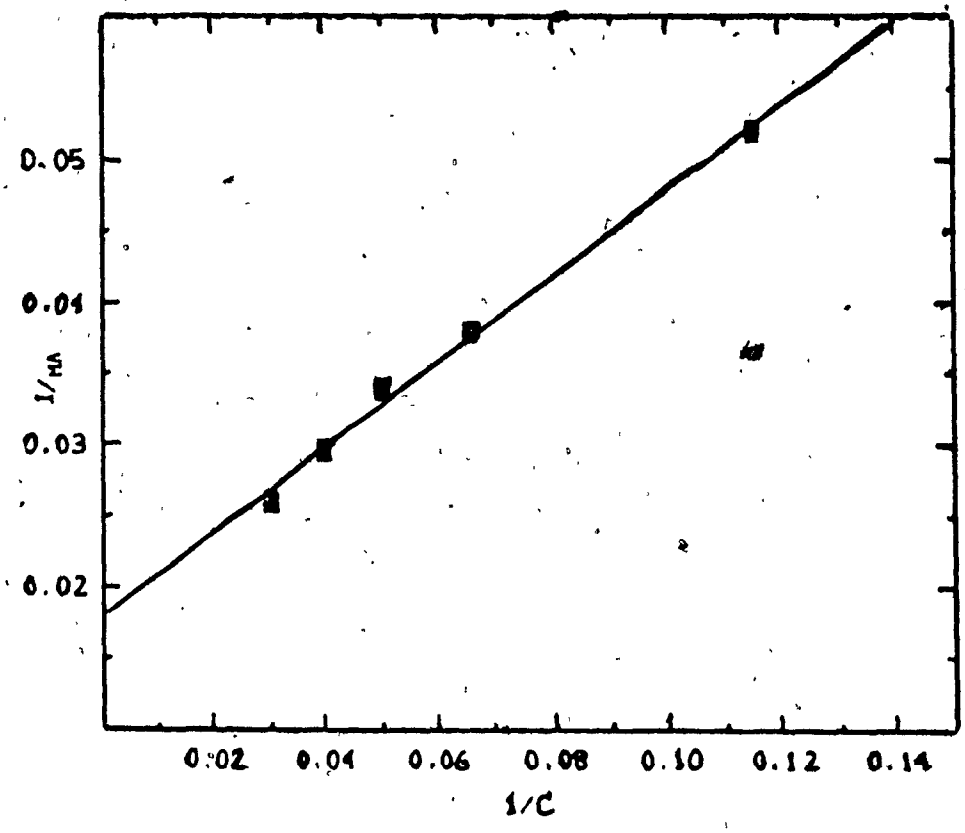


Fig. 3. 5. LINEWEAVER-BURK PLOT FOR CYTOCHROME c OXIDASE ACTIVITY. The rate of reduced cytochrome c oxidation by cytochrome c oxidase was followed spectrophotometrically at 550 nm. The concentration of cytochrome c oxidase was 1.25 nM. The range of cytochrome c tested was over 5 uM to 40 uM. A phosphate buffer with ionic strength of 20 mM containing 0.1% Tween-80 at pH 6.5 was used. Temperature was kept at 20°C. Molecular activities (MA) or turn-over numbers (TN) were obtained using formula of Yonetani (85) and molecular activity maximum (MA_{max}) and Michaelis- Menten constant (K_m) were obtained by regression analysis. Data with more than 99% correlation coeffient were used to calculate MA_{max} and K_m. In all other conditions of pH, ionic strength and Tween-80 concentration, MA_{max} and K_m were obtained by the same procedure.

4

THE EFFECTS OF pH ON THE STOICHIOMETRIC BINDING OF CO AND CN^- TO CYTOCHROME c OXIDASE

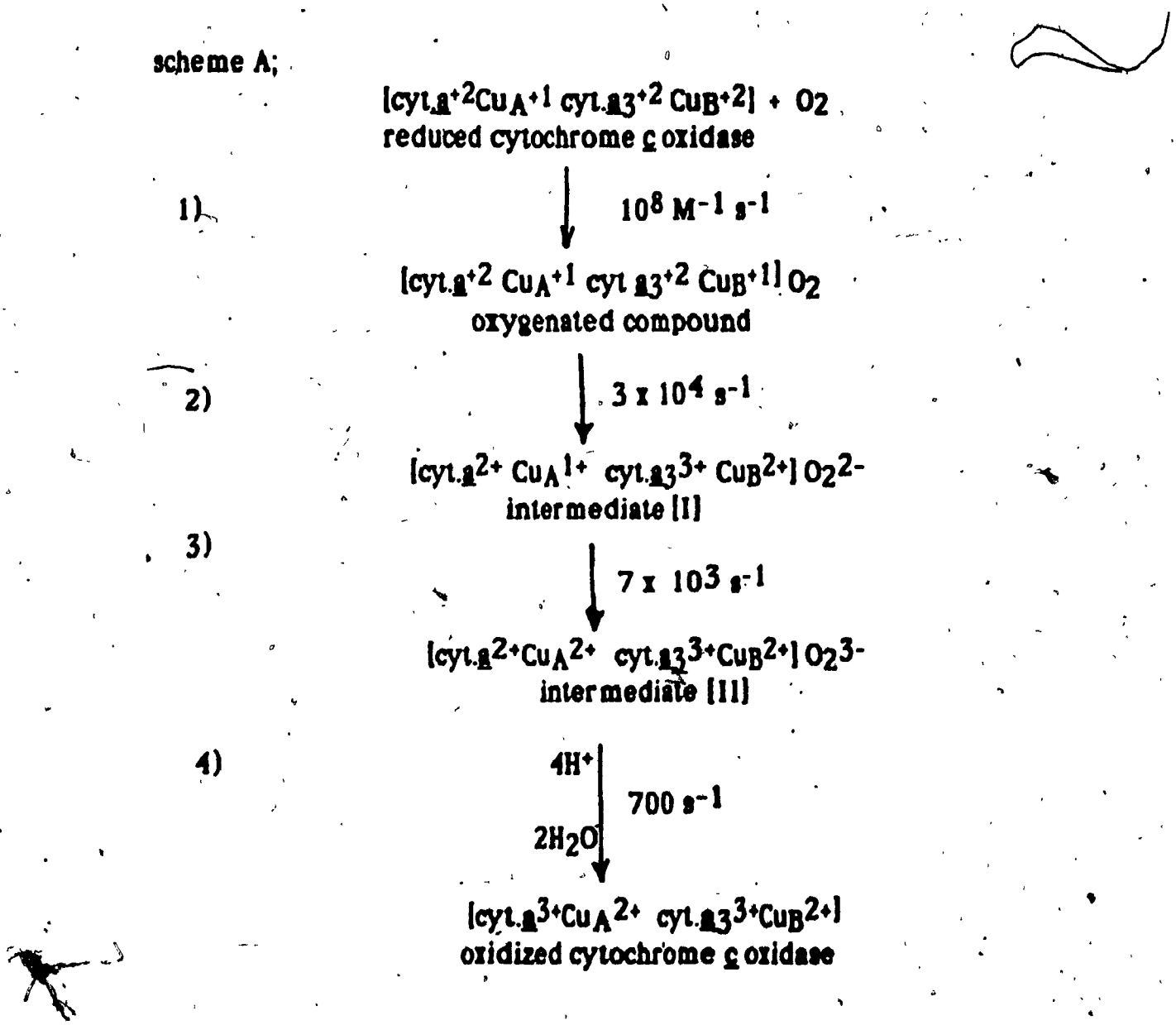
4.1. INTRODUCTION

The electron transfer pathway by which oxygen is reduced to water by catalytic reaction of cytochrome c oxidase is still uncertain. Determination of the sequence in which each component of the enzyme reacts is very difficult. This is because the time separation of the reactions is very small and the intermediates involved cannot be identified with certainty with the available techniques. Introduction of flash-photolysis (95) opened a new approach in the study of the oxygen reaction with the enzyme. In this technique, the photolabile CO-bound enzyme is mixed with oxygen in the dark, then an intense flash is applied. The enzyme dissociates from CO and rapidly reacts with 02. Studies of the oxygen reaction at low temperature using flash-flow and EPR techniques (57, 64, 70,79) led to the discovery of oxygen reaction intermediate compounds with the enzyme. Moreover, it was discovered that the structures of these intermediates are similar to the oxygenated compounds described earlier. The following electron transfer pathway is a generally accepted [scheme A] (57,62,64,70, 79, 150,157):

The first step of the reaction of reduced cytochrome c oxidase with oxygen is combination of the enzyme with molecular oxygen (oxygenated compound). Then a rapid two-electron transfer from the a_3-Cu_B site to

dioxygen (intermediate I) occurs. This step is followed by slower transfer

scheme A;

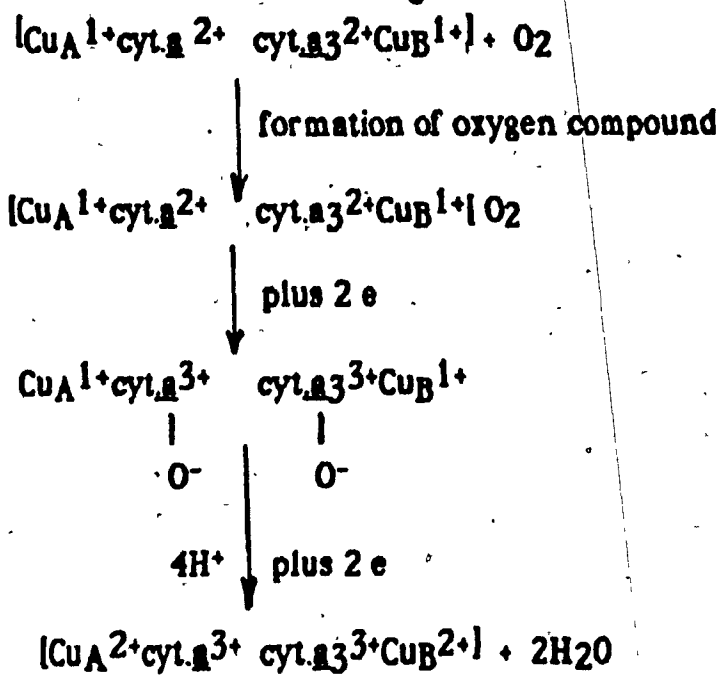


of one electron from the Cu_A site (or cytochrome a site) (intermediate II). The last step involves transfer of the fourth electron, yielding fully oxidized cytochrome c oxidase. There are still many details which remain unclear. The static spectral behavior of the reaction of the reduced enzyme with oxygen does not completely follow the kinetic data. For example, there is no spectroscopic evidence for the first step of the oxygen reaction (oxygenated compound) as well as the electron transfer at the Cu_B site. Moreover, the optical properties and activity of cytochrome c oxidase are pH-dependent (12,128, 129,131, 134,145, 175,184) and this is not explained by the above mentioned electron transfer pathway.

The effect of pH on optical properties and activity of the enzyme could imply that an alternate pathway for dioxygen reduction proceeds at low pH compared to high. The effect of pH on the oxygen binding site was studied using a ligand binding technique. The stoichiometry of binding of CO and CN^- in the pH range of 5 to 7.8 was studied. The object of this work was to draw conclusions concerning a possible alternate electron transfer pathway which would involve both hemes during the first step of electron transfer [scheme B]:

For such a pathway to exist, the expected stoichiometry of binding at the pH optimum for activity would be two ligands bound per aa3, that is, one bound to cytochrome a3 and the other to cytochrome a.

Scheme B;



4.2. THE STOICHIOMETRY OF CO BINDING TO CYTOCHROME c OXIDASE

In the fully oxidized "resting" or fully reduced state differentiation of heme a and heme a3 by means of available techniques is difficult. At the present time, a complete three-dimensional structure of cytochrome c oxidase is not available. Ligand binding investigations are an interesting approach to study the oxygen binding site. The classical inhibitors of respiration, CO and CN^-, have been used (57,54,66,87-92,104,155,156) to investigate the different aspects of cytochrome c oxidase. These

molecules are similar to dioxygen. Their use permits, among other things, a differentiation between cytochrome a and cytochrome a3. In spite of the view of some investigators who maintain that the use of these inhibitors has not been very informative (93), recent studies (97,155-158,164) have proven otherwise.

CO combines with fully reduced, partially reduced or fully oxidized forms of the enzyme (92,155,157,158). The rate of reaction of CO with the enzyme depends on the oxidation state of the metal centers. The oxidized form of the enzyme reacts very slowly, whereas with the pulsed form of the enzyme the reaction is much faster (158). In both cases, in the absence of oxygen, a mixed valence ligated form is formed. Binding of CO to the fully reduced form of the enzyme is very fast. The association rate constant for CO binding to a3 is 8×10^4 M^{-1} s^{-1}; the dissociation rate constant of the complex is 0.02 s^{-1} (87,91,95). This leads to a dissociation constant (K_d) of 2.5×10^{-7} at 20°C; direct equilibrium determinations at 28°C gave a value of 4×10^{-7} M (87). This affinity is high enough to permit direct titration of cytochrome c oxidase with only small corrections necessary for the CO that remains in the solution.

Many studies have focussed on the stoichiometry of CO binding to the enzyme (87-92,96,129). Most of these studies show that one mole of CO binds to one mole of the enzyme. However, there are some contradictory reports (87-90). Generally, the stoichiometry of one mole of CO bound to one functional unit of enzyme has been accepted. Stoichiometric CO-binding studies have mainly been performed at neutral

or around neutral pHs. The effect of pH on cytochrome c oxidase has also been studied extensively (10, 12,51,61,100,128,129,131, 134, 145, 147, 175,184) and it is known that the activity of the enzyme is affected by pH. Whether this effect is due to a secondary electron transport pathway which would be expected to increase the stiochiometry of CO binding to the enzyme to two is not known and the object of this study was to answer this question.

4.2.1. RESULTS

The stoichiometric binding of CO over pH range of 5.2 to 7.8 was studied by titrating the fully reduced enzyme in the above pH range with saturated carbon monoxide solution. Fig.3.2 shows how the spectrum of the fully reduced cytochrome c oxidase changes as the protein is titrated with CO at pH 7.0. The absorption at 445 nm decreases as increasing amounts of CO solution are added. At the saturation point no further decrease of absorption can be identified even with addition of large amounts of CO . The absorption decrease at 604 nm is very small. Titration of the enzyme with CO solution at all other pHs showed a similar pattern. Fig. 4.1 (A,B,C) presents titration spectra of the enzyme at pHs 5.2, 6.5, and 7.5 (since the decrease of the absorption at 604 nm is very small and not as informative, only the Soret region is shown). As is evident from these spectra, only a single component varies reversibly as the concentration of CO is increased. The isobestic points associated with

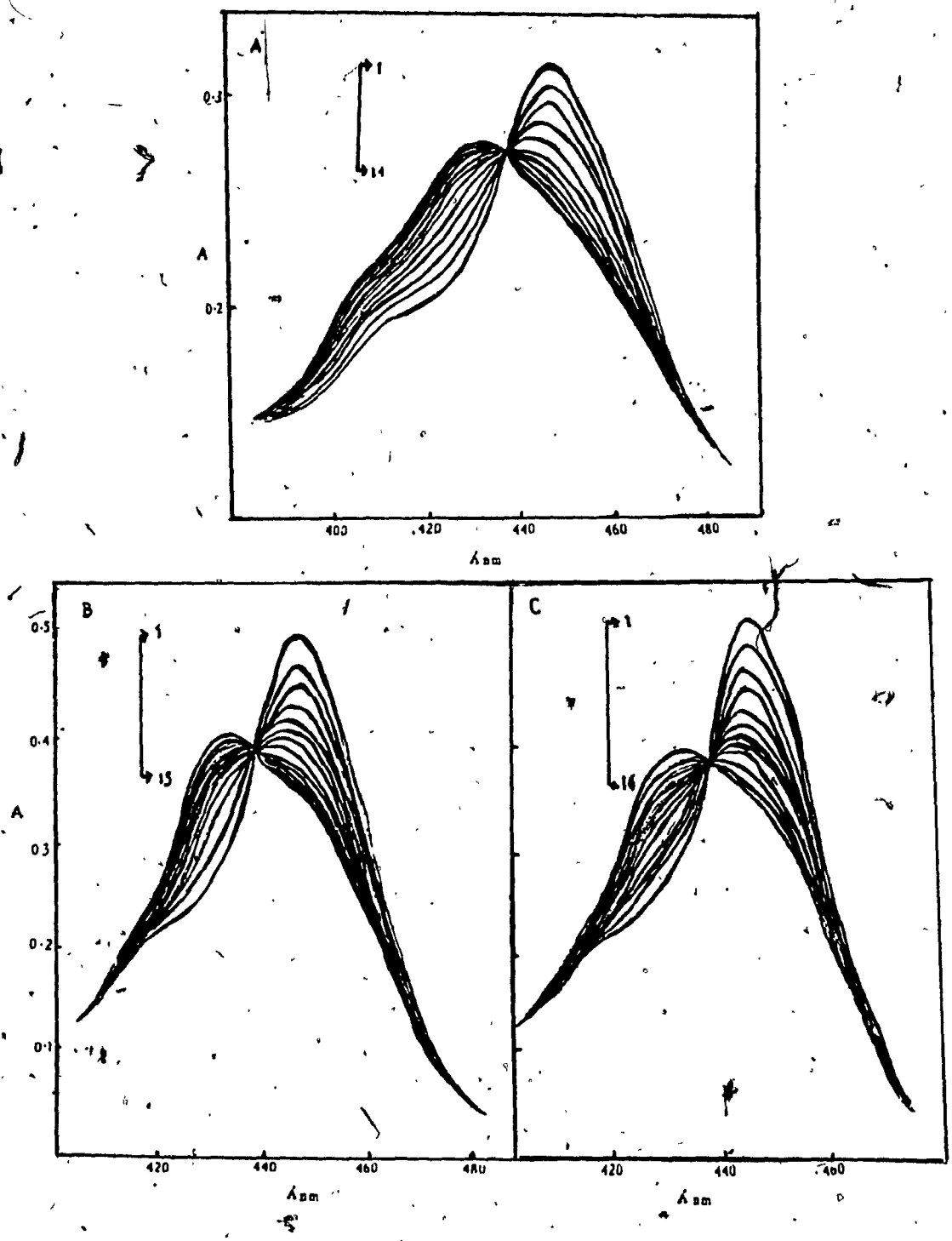

A
1
14
0·3
A
0·2
400
420
440
460
480
λ nm
B
1
15
0·5
0·4
A
0·3
0·2
0·1
420
440
460
480
λ nm
C
1
16
420
440
460
λ nm

Fig. 4.1.

Fig. 4. 1. TITRATION SPECTRA OF FULLY REDUCED CYTOCHROME C OXIDASE AT pH 5.2, 6.3, AND 7.2 WITH CO

A shows the Soret region spectra of a 1.55 uM cyt. aa3 solution at pH 5.5 titrated with 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, 30, 40 (* 2 to 14) ul of 1 mM CO solution.

B shows the Soret region spectra of a 2.5 uM cyt. aa3 at pH 6.3 titrated with 1,2,3,4, 5, 6, 7, 8, 10, 15, 20, 30, 40, 50, (* 2 to 15) ul of 1 mM CO solution.

C shows the Soret region of a 2.5 uM cyt. aa3 solution at pH 7.20 titrated with 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 20, 25, 30, 40, 50 (*2 to 16) ul of 1 mM CO solution.

All titrations were carried out in the same buffer as in Fig. 3.2, 15°C and dithionite was used as reductant.

the titrations show that there is no irreversible structural change caused by CO binding.

The ratio of CO bound to aa3 was obtained by plotting the OD (optical density) change at 445 nm versus CO concentration. The intersection of two asymptotes corresponds to the moles of CO bound/mole of cytochrome c oxidase as explained in the legend of Fig.3.3. [Table.4.1] shows the stoichiometry of CO binding over the pH range 5 to 7.8. This ratio varies between 0.88 to 1.3, the deviation from one is partly due to experimental errors and partly due to the pH effect on the protein. This will be discussed further in the interpretation of results. The ratios are the average of at least five trials; at every pH at least two trials had ratios very close to one.

Fig 4.2

A plot of the number of CO bound per functional unit of enzyme vs pH

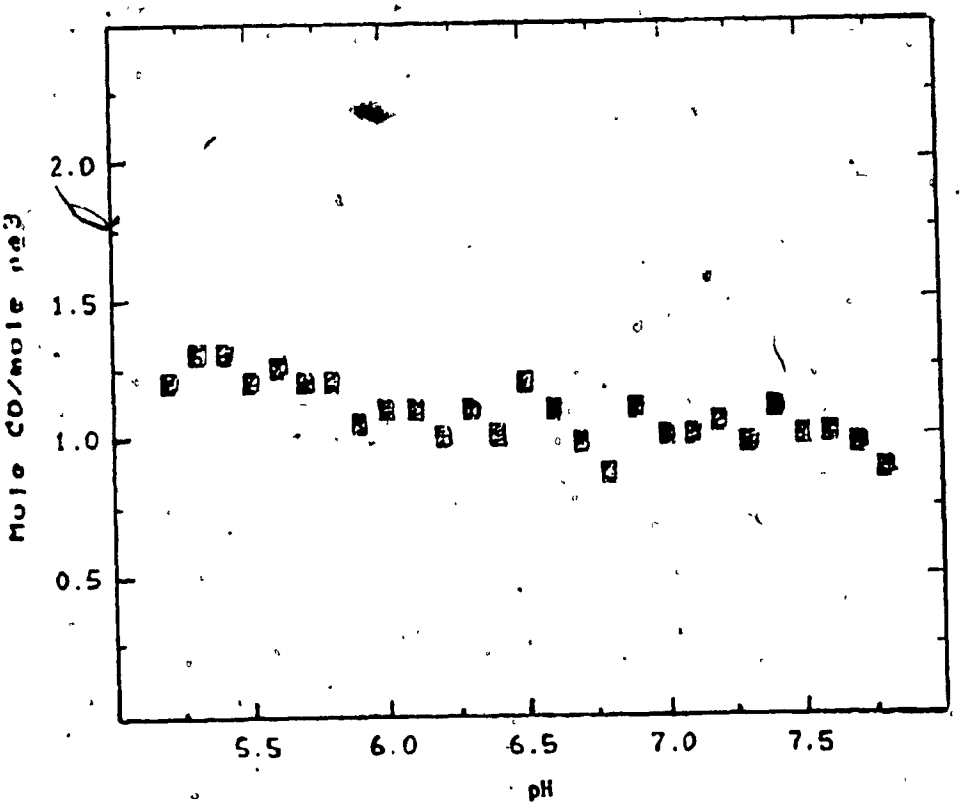


Table 4.1. The ratio of mole (s) of CO and CN bound to per functional unit of cytochrome c oxidase at different pH

pH	mole CO / unit cyt.c ox.	mole CN^- / unit cyt.c ox.
7.8	0.88	1.0
7.7	0.94	0.98
7.6	1.1	0.98
7.5	0.98	1.0
7.4	1.09	0.98
7.3	0.97	0.89
7.2	0.97	1.1
7.1	1.0	1.0
7.0	1.0	0.99
6.9	1.12	1.1
6.8	0.97	1.1
6.7	1.0	1.2
6.6	0.98	1.3
6.5	1.0	1.3
6.4	1.0	1.4
6.3	1.0	1.6
6.2	1.1	2.1
6.1	1.1	2.4
6.0	1.1	2.8
5.9	1.0	3.5
5.8	1.1	4.2
5.7	1.2	4.8
5.6	1.2	5.4
5.5	1.1	5.9
5.4	1.2	7.8
5.3	1.3	8.33
5.2	1.3	10.2

Fig.4.2 shows a plot of CO bound per cytochrome c oxidase (functional unit), as function of pH. It is evident from this graph that the stoichiometry of CO binding to the enzyme is not affected by pH.

The results show that over a pH range of 5.2 to 7.8 one mole of CO binds to one mole of aa3. The stoichiometry of one for CO binding to cytochrome c oxidase (at neutral pH) has been reported by other investigators (69,85,87,90,91,95,98,104,129). This study shows that the same stoichiometry was maintained over a broad range of pH.

4. 2. 2. INTERPRETATION OF RESULTS

The investigation of the stoichiometric binding of CO over the pH range 5.2-7.8 revealed that neither the ratio of CO binding nor the pattern of spectral behavior of the enzyme varies with pH. Clearly, the integrity of the CO binding site is maintained over this pH range. The component of the enzyme that binds CO has been identified as heme a3 (3,6,129) and perhaps the Cu_B center (88,92) which is associated with heme a3. The maintenance of the pattern of the spectral changes upon CO binding at different pH indicates that the same component of the enzyme is involved over the entire pH range. This is evident from Fig.4.1(A,B,C) which shows the absorption spectra of the enzyme at pH 5.2, 6.5 and 7.5 titrated with CO. The unchanged isobestic points indicate that the structure of the enzyme is maintained. Thus, the other components of the enzyme, heme a and the, Cu_A center exclude CO.

These results allow us to reject the proposed secondary electron pathway outlined under scheme [B] in the introduction since it requires binding of more than one CO per functional unit of enzyme. Although the results do not constitute evidence for scheme A, they certainly are consistent with it. The results also show that the CO-binding site is highly protected from pH effects. This is supported by other studies (66,69) which have shown that the CO- binding site or pocket is hydrophobic. It has been suggested (88,92) that the ligand moves into a pocket consisting mainly of lipid with a highly ordered environment (88,92). A significant and important outcome of this study is that it shows that the pH effect does not disturb the CO-binding site. There is however, a pH effect on the mid-point potentials of the hemes (8,50,119) and on the activity (10,13,61,80,145, 185) of the enzyme in binding cytochrome c. Therefore it is clear that pH mainly affects the protein moiety of the enzyme. Whatever this effect is, it does not affect the stoichiometry of CO binding to the a3/Cu_B center nor does it convert the a/Cu_A center into a species capable of binding to CO.

4.3. THE STOICHIOMETRY OF CN^- BINDING TO CYTOCHROME c OXIDASE

Cyanide is a well known respiratory poison. In spite of its popularity, in spite of many studies over the past 50 years, the mechanism of its reaction with cytochrome c oxidase is still not well

understood. The binding of cyanide to the enzyme in the "resting" form or fully oxidized state is very slow (73,74). This is surprising, because heme a3 binds the ligand only in the oxidized state. Binding of cyanide to the fully oxidized enzyme causes a red shift in the Soret band of the oxidized enzyme (73,74,183). It also leads to a high-spin to low-spin transition of heme a3 which is not EPR detectable (63), but it can be identified with MCD spectroscopy (60,100,105). The rate of cyanide binding to a fully oxidized or fully reduced enzyme is too slow to account for the rate of enzymic inhibition (74).

Under turnover conditions where part of the enzyme is reduced, the reaction of the enzyme with cyanide is very fast (72,74,162,164). This results in the appearance of a new EPR signal which is absent in the oxidized state of the enzyme (60,169). It was suggested (74) that a partially reduced enzyme is the species capable of binding to cyanide. The rate of inhibition in this oxidation state is 10^5 times faster than to the fully oxidized state of the enzyme (164). The magnetic properties of the a3/Cu_B binuclear site in the binding of cyanide to different oxidation states of the enzyme are still not totally clear. A number of recent studies have revealed that cyanide may bind as a bridge between two metal centers in the partially reduced state and to Cu_B in the oxidized state (105,106,120, 201). The slow reactivity of the "resting" enzyme towards cyanide is due to a bridged ligand between heme a3 and Cu_B (52,53,54,179,201). In the partially reduced enzyme this bridge is replaced by a stronger axial ligand (120,160).

The stoichiometry of cyanide binding to the enzyme was studied at elevated pH to investigate the effect of pH on a partially reduced enzyme where the a_3/Cu_B center is in the oxidized state and heme a and Cu_A are in the reduced state. A stoichiometry of one mole of cyanide per mole of cytochrome c oxidase at neutral pH has been reported by other investigators (74,162,168). The effect of pH on the partially reduced form of the enzyme is important because this species is an intermediate of the oxygen reaction at the first step of electron transport (57,64). In intact mitochondria the reaction of cyanide with the enzyme is pH-dependent (14) and an apparent pK of 6.9 has been reported. This reactivity of the enzyme with cyanide at acidic pH and the location of the pH optimum at acidic pH (129,134,135, 147, 148) may be related.

4. 3. 1. RESULTS

The stoichiometry of cyanide binding to cytochrome c oxidase was studied by titrating the partially reduced enzyme in the pH range of 5.2 to 7.8 with cyanide solution. Fig.3. 4 shows the absorption spectra of the partially reduced enzyme with cyanide at pH 7.1. As the amount of cyanide increased, the absorption band at 445 nm decreased. This decrease is limited and at saturation, further addition of cyanide does not cause any further decrease of the absorption band at 445 nm. Similar absorption spectra were obtained over the pH range of 7.8 to 5.5. Fig.4.3 shows the titration spectra of cytochrome c oxidase at pH 7.5 and 5.8

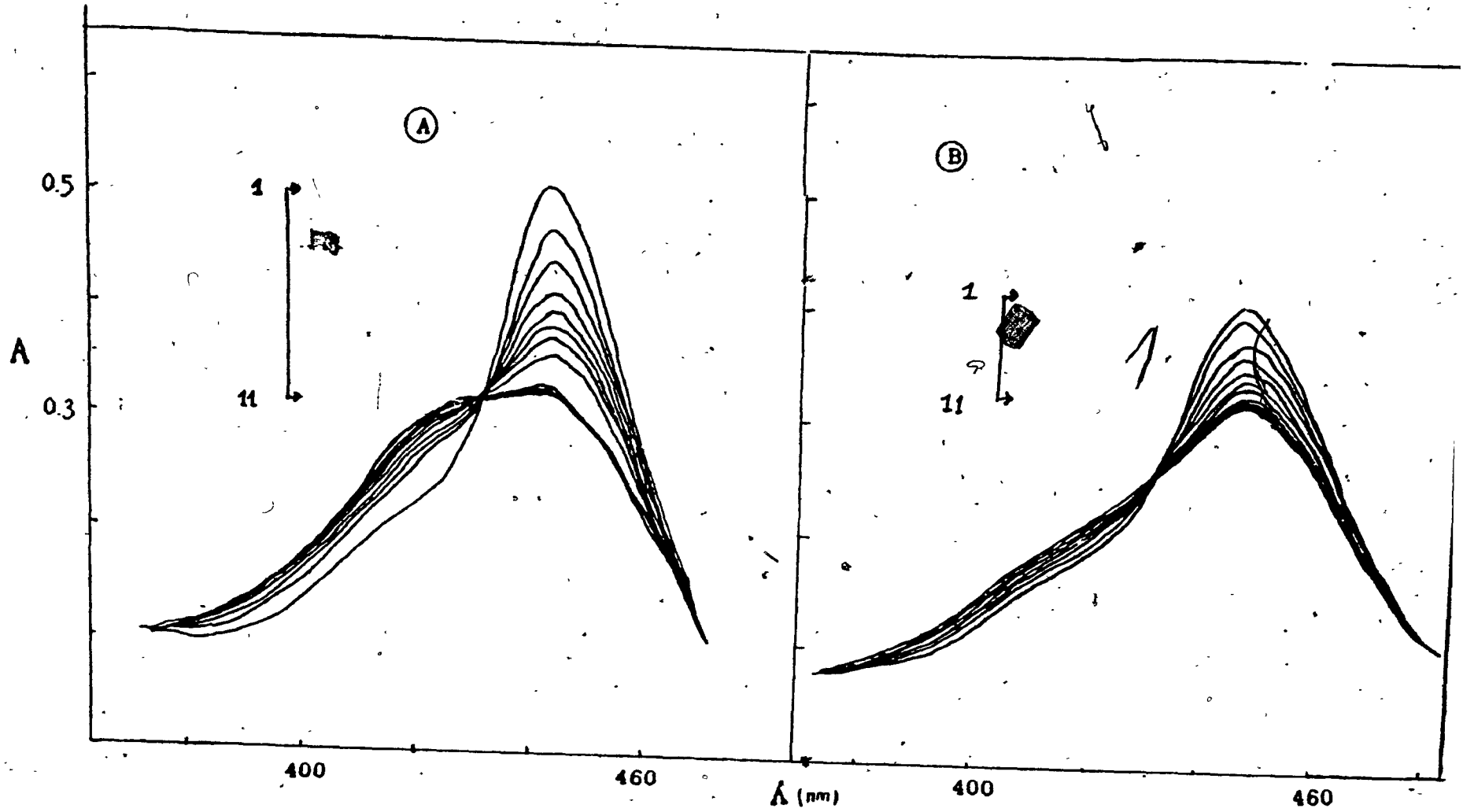

A
0.5
0.3
1
11
400
460
λ (nm)
B
1
11
400
460

Fig. 4. 3. SPECTRAL CHANGES OF CYTOCHROME C OXIDASE AS A FUNCTION OF CYANIDE BINDING AT pH 7.5 AND pH 5.5

A shows the titration spectra of a 2.5 uM cyt. aa3 (5uM heme a) at pH 7.5.

* 1 spectrum of fully reduced enzyme with 0 KCN
* 2 to 11 spectra were obtained when 1, 2, 3, 4, 5, 6, 8, 10, 15, 20 ul of 1 mM KCN solution were added to 3 ml enzyme solutions and then were reduced with dithionite.

with dithionite (final CN^- concentration: 6.66 uM).

B shows the titration spectra of 2.5 uM cyt. aa3 (5 uM heme a) at pH 5.5.

* 1 spectrum of fully reduced enzyme with 0 KCN
* 2 to 11 spectra were obtained when 1, 2, 3, 4, 5, 6, 8, 10, 15, 20 ul of 50 mM KCN solution were added to 3 ml enzyme and then were reduced with dithionite (final CN^- concentration 0.33 mM).

All titrations were carried out in the same conditions as Fig. 3.4.

with cyanide. The overall patterns of the spectral changes due to increasing amount of cyanide were similar; the stoichiometry of cyanide binding to the enzyme increased as the pH was decreased. Table.4.1 shows the number of cyanide bound per functional unit of enzyme from pH 5.2 to 7.8.

The apparent increase in the number of cyanides bound to the enzyme at low pH may be partly due to the evaporation of HCN. Although the pH of the cyanide solution was adjusted to 7.0 and the enzyme solution was layered with mineral oil, some HCN may have evaporated before equilibrium was obtained. The small change observed in the A445 nm at low pH may be in part the result of the above phenomenon.

The reported K_D for cytochrome c oxidase-CN varies from 90 nM to 700 uM (94,135,162,164,166); the K_a of HCN is 7.2×10^{-10} (167). Theoretically, at low pH, the HCN concentration in a solution is higher and CN^- ion concentration is lower than expected (Appendix 1). It has been demonstrated (210) that weak acids such as HCN may bind differently to different heme proteins. A dissociated form or CN^- binds metmyoglobin, whereas the acid form binds catalase. If CN^- is a species which binds to cytochrome c oxidase, an insufficient amount of CN^- in the solution would lead to an increase in the "apparent" $[CN^-]/aa_3$ ratio with decreasing pH. If HCN is the species that binds, the stoichiometry should decrease with increasing pH since the amount of HCN present in solution decreases. If both species, CN^- and HCN can bind, then the analysis becomes quite complex if the pocket pH is different from that of the solution.

The results of this study suggest that CN^- is the species which binds the enzyme. For determination of the stoichiometry, it is important to know the exact concentration of CN^- present in the solution and the number of CN^- bound to the enzyme. Several quantitative methods were tried: a cyanide electrode method, a polarometric titration, removal of bound CN^- with silver nitrite and titration of CN^- heme aa3 with methemoglobin. All methods failed to determine the actual concentration of the cyanide either after it was added to the enzyme solution, or after it was bound to the enzyme. On the basis of the results of this study, one CN^- binds a functional unit of enzyme at pH above 6.7. At lower pH, the stoichiometry increases but the extent of this increase could not be accurately determined.

Fig.4.4 shows the titration of resting cytochrome c oxidase at pH 5.5 with cyanide. Cyanide caused a red shift in the Soret band spectrum of the protein at 418 nm. At saturation it shifted by about 10 nm to 428 nm. It is important to note that the spectrum of the oxidized protein with cyanide is not pH-dependent. The cytochrome c oxidase-CN spectrum is the same at pH 5.5 and 7.8. The number of cyanides apparently bound per functional unit and causing this shift (10 nm) was 19. This ratio was calculated at pH 5.5 on the basis of the absorption shift from 418 nm to 428 nm. Binding of more than one cyanide per functional unit of enzyme has been seen by other studies (162).

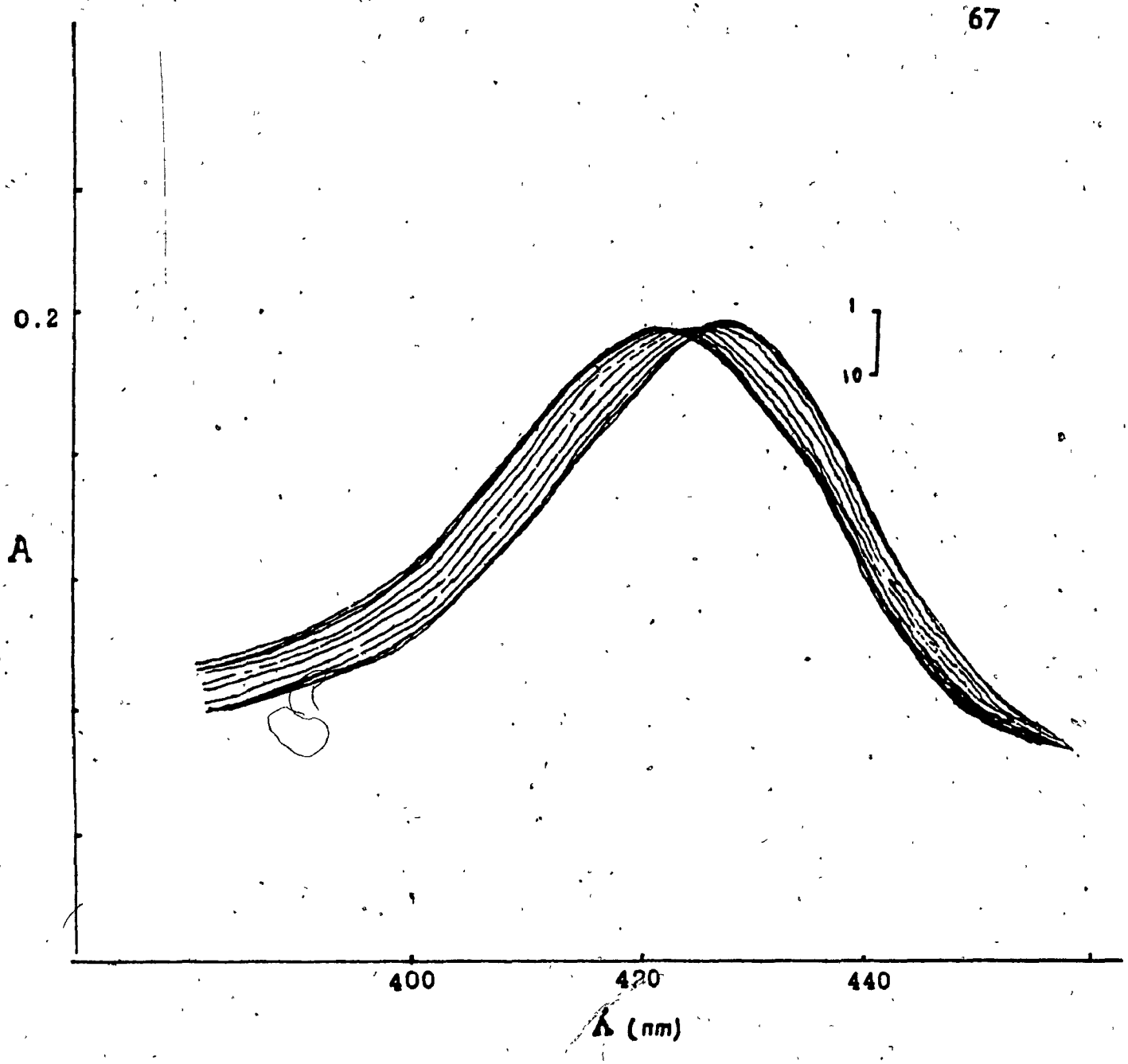


Fig. 4. 4. TITRATION SPECTRA OF OXIDIZED CYTOCHROME C OXIDASE WITH CYANIDE AT pH 5.5

The titration spectra of 1.5 uM oxidized cyt. aa3 in a tris solution as Fig 3. 4. at pH 5.50 with 0, 1, 2, 5, 10, 15, 20, 30, 40, 50 (#1 to 10) ul of 50 mM KCN solution.

4.3.2. INTERPRETATION OF RESULTS

The stoichiometry of one cyanide per functional unit of partially reduced cytochrome c oxidase obtained over the pH range 7.8 to 6.7 agrees with that reported by other investigators at neutral pH (74,162,168). This indicates that only the a_3/Cu_B center binds cyanide and the a/Cu_A moiety is not affected by pH and does not bind cyanide in this pH range. At a pH below 6.7 the "apparent" stoichiometry of cyanide binding to partially reduced enzyme increased but the integrity of the enzyme was maintained. This is evident from the spectrum of the fully inhibited enzyme at high and low pH presented in Fig.4.3. The pattern of spectral changes due to the addition of cyanide at high and low pH are identical. This suggests that the structure of the metal centers ligated to cyanide at low and high pH are the same. The "apparent" increase in stoichiometry at low pH does not seem to be due to the binding of cyanide to heme a. This is supported by the resolution of the spectrum of the enzyme in the Soret region into two components due to the cyanide binding to one of the hemes. It is possible that cyanide binds to copper atoms and increases the stoichiometry but does not interfere with the spectrum of the enzyme. Alternatively, this increase of stoichiometry may be due to one of the following causes.

a) The partially reduced state of the enzyme may be affected by pH. It has been reported (14) that cyanide binding to intact mitochondrial

cytochrome c oxidase is strongly pH-dependent. Mitochondrial enzyme has been reported (119) to be in a partially reduced or mixed valence state of oxidation. The results of this study suggest that the binding of cyanide to the isolated enzyme in the partially reduced state is pH- dependent. Other studies (12,134,137,184) show that electron transfer reactions are affected by pH. It has also been reported (64,78) that both the oxygenated and the pulsed cytochrome c oxidase are more reactive in the oxidation of cytochrome c. The spectrum of oxidized enzyme ligated to cyanide is similar to that of the oxygenated enzyme and shifts to 428 nm [Fig.4.4]. Thus, the high reactivity of the enzyme with cyanide may be due to the structure of the a_3/Cu_B center. This structural difference could result from either a conformational change or formation of a dimeric or monomeric species or some other change which would increase the binding affinity of the enzyme for CN^-. Although, the precision of this study at low pH is questionable, it constitutes enough evidence to believe that the stoichiometry of cyanide binding increases as pH decreases. The complex behavior of cyanide binding to the enzyme and the increase of stoichiometric binding under different conditions has been seen in other studies (74,162,164).

b) The role of Cu[redacted]gand binding as well as CN^- binding to the enzyme is not clear. Some investigators ((63,105,106,120,201)

believe CN^- binds as a bridging ligand between the cytochrome a3 and Cu_B metal centers. Cytochrome c oxidases prepared by different methods show different affinity in binding to CN^- (179). The authors suggest (179) the difference is due to the absence or presence of the bridge between the two metal centers. Cytochrome c oxidase is different from other heme proteins in that it has two copper atoms at its active site. The presence of copper makes this enzyme more complicated than the other heme proteins. Monitoring the involvement of Cu_B in the reactions of cytochrome c oxidase is difficult because it does not contribute in the absorption bands in the Soret, visible or NIR regions. However, the participation of Cu_B in the reactions of cytochrome c oxidase and the ability to bind exogenous ligands is established (79,120,153,201). The binding of cyanide to Cu_B could result in a conformational change at the ligand binding site or may be the cause of the high-spin to low-spin change of heme a3. The spin transition due to cyanide binding has been identified by MCD spectroscopy (100, 105). The oxidation state and the electronic environment of Cu_B may be affected by PH and result in the increase of affinity of CuB in binding to CN^- at different pH. A recent study (209) reports that there are more than two copper atoms associated with the enzyme structure. It is likely that the peculiar interaction of cyanide with different oxidation states of the enzyme is due to multiple binding sites.

c) The effect of pH on the concentration of CN^- present in the enzyme solution and capable of binding to active site causes uncertainty in the interpretation of the results of this study. As shown in Appendix 1, the decrease of one unit in pH results in a 10 fold decrease in CN^- present in solution. If CN^- is the species that binds to the active site of the enzyme, the drastic increase of the stoichiometry is due to a low amount of CN^- present in solution. It is desirable to find a technique capable of determining the concentration of CN^- in a low pH enzyme solution or the amount bound to the enzyme. Techniques used in this study for the determination of CN^- failed, either because of harsh basic or acidic conditions which caused denaturation of the enzyme or because of interference from other ions present in the enzyme solution. Equilibrium dialysis with ^{14}CN could provide the necessary sensitivity and internal controls.

On the whole the results of the cyanide binding studies show that at pH above 6.7 one mole of cyanide binds to a functional unit of the enzyme in a partially reduced oxidation state. This indicates that the electronic configuration of the active site in this range does not change as a result of varying pH. It is also clear that the cyanide binding site in different oxidation states shows different affinities for cyanide. Other studies (164) have shown that a partially reduced form of cytochrome c oxidase features an open cyanide binding site. At pH below 6.7, the oxidation state required for cyanide binding is pH-sensitive but, because of difficulties in the determination of the exact amount of CN^- bound to the

enzyme, this study fails to report accurately on stoichiometric binding.

4. 4. CONCLUSION

The spectrophotometric study of the stoichiometric binding of carbon monoxide and cyanide with cytochrome c oxidase revealed that the spectral behavior of the component of the enzyme which binds these ligands does not change qualitatively over pH range 7.8 to 5.2. In spite of the difference in the oxidation states of the a3/CuB center (in the reduced state in binding to carbon monoxide and in oxidized state in binding to cyanide) the titration spectra of CO-aa3 and CN-aa3 showed similar patterns. This is evident from the spectra shown in Fig.3.2 and Fig.3.4. The stoichiometry of one CO per functional unit of the enzyme obtained over entire pH range indicates that the metal centers are not affected by pH in the fully reduced oxidation state. Similar conclusions can be drawn in the binding of cyanide at pH's above 6.7. At pH below 6.7, the spectral behavior of the enzyme due to cyanide binding did not change. This indicates that the increase of stoichiometry obtained at lower pH is not due to the binding of cyanide to heme. I believe that CN^- is the species that binds to the active site and that the increase of stoichiometry is partly due to an insufficient amount of CN^- present in solution. Fig.4.5. A presents the spectrum of the enzyme fully inhibited with carbon monoxide [CO-aa3] and Fig.4.5. B shows the spectrum of the enzyme fully inhibited with cyanide [CN- aa3]. It is known that absorption peak of

reduced cytochrome a is at 444 nm and that of oxidized at 426 nm; absorption peak of oxidized cytochrome a3 is at 414 nm and its reduced form absorbs at 442 nm (96,115). In [A] the absorption maximum at about 428 nm [a] is due to cytochrome a_3^{2+}-CO and in [B] it belongs to cytochrome a_3^{3+}-CN, whereas, the absorption maximum at about 440 nm [b] in [A] and in [B] belongs to cytochrome a. In cytochrome c oxidase-CO complex [A], the apparent oxidation states of the metal centers are reduced. This spectrum, is probably similar to that of the first oxygenated compound produced in the reaction of oxygen with the enzyme. In the CN-enzyme complex [B], heme a3/Cu_B centers are oxidized and heme a/Cu_A centers are reduced. It is evident that the contribution of cytochrome a in [A], is less and in [B] is more than 50%. This difference in optical densities of the two complexes may be due to the different oxidation states of metal centers. Alternatively, the spectrum of the CO-complex may represent the early stage of bridge formation between cytochrome a3 and Cu_B. The CN-complex may represent the breakage of this bridge. It is clear that the spectral behavior of cytochrome a and a3 are not independent. The spectral properties of the enzyme varies as the interaction between heme a and a3 vary and the oxidation states of metal centers changes. It has been suggested (97) that the redox state of CuB strongly influences the absorption properties of one or both hemes.

In conclusion, this study shows that the integrity of cytochrome a3/Cu_B and cytochrome a/Cu_A is maintained over the pH range of 5.2 to 7.8. The dependence of enzymatic activity on pH is not due to the

conversion of the cytochrome a/Cu_A center to a species capable of binding ligands. This excludes the proposed alternative mechanism of electron transfer at low pH (Scheme B).

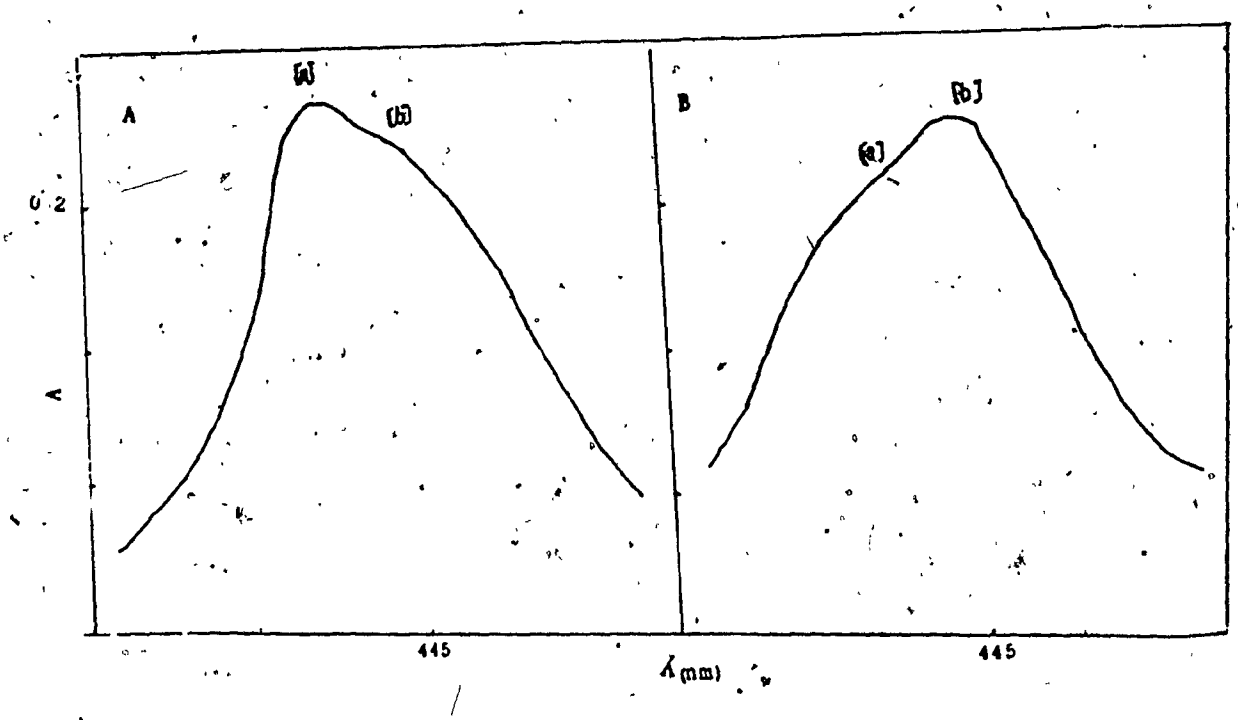


Fig. 4. 5. SPECTRA OF CYTOCHROME C OXIDASE SATURATED (fully inhibited) WITH CO AND CN^-

A shows the spectrum of 2.5 uM aa3 (5 uM heme a) at pH 7.0 saturated with CO

a shows the spectrum of $a3^{2+}$ – CO

b shows the spectrum of cyt. a^{2+}

B shows the spectrum of 2.5 uM aa3 (5 uM heme a) at pH 7.0 saturated with CN^-

a shows the spectrum of cyt. $a3^{3+}$ - CN

b shows the spectrum of cyt. a^{2+}

5

CORRELATION OF THE EFFECTS OF pH, IONIC STRENGTH, AND TWEEN-80 ON THE ENZYMIC ACTIVITY OF ISOLATED CYTOCHROME c OXIDASE

The study of the effect of pH on the stoichiometric binding of carbon monoxide and cyanide to cytochrome c oxidase over the pH range of 5.2 to 7.8 showed that the integrity of the metal centers was maintained. On the other hand, many studies have shown that the electron transfer activity of the enzyme is affected by pH, ionic strength, and detergent. Different conditions of pH, ionic strength, detergent as well as the method of preparation of the enzyme and the method of study of enzymic activity lead to different results and are in contradiction. Some investigators report an optimum pH (134,135,145,147-149) and ionic strength (135,147-149) while other studies show that the enzymic activity decreases (191) or increases (184,192) as ionic strength increases and it deceases as pH increases (145,175,184).

Under different conditions of ionic strength, phospholipid or detergent content, the enzyme may exist in different states of aggregation (109,110). The answer to the question of what is cause and what is effect and which part of the enzyme is responsible for these various results is not an easy task. This requires complete definition of the structure of the cytochrome c binding site and its surroundings. A complete study of the effects of pH, ionic strength, different anions, different detergents, temperature, and the interactions of these on each

other and with enzymic activity of the enzyme is needed. The objective of this part of the study was to investigate the effects of pH, ionic strength, and Tween-80 on the enzymic activity of the enzyme and to possibly derive an interaction term which can be used to predict the enzymic activity of the enzyme at different conditions of the above mentioned factors. The study of the effects of all factors and conditions used in different studies is very long and not only needs time but statistical expertise as well. This study does not claim to achieve this but rather presents preliminary results on the effect of pH, ionic strength and detergent on low lipid content cytochrome c oxidase.

5. 1. INTRODUCTION

Cytochrome c , a low molecular weight protein efficiently mediates electron transfer between two large mitochondrial proteins, cytochrome c reductase (c_1) and cytochrome c oxidase. They are the three components of the terminal segment of the mitochondrial electron transport chain. The interaction of reduced cytochrome c with cytochrome c oxidase has been the subject of many kinetic studies (129,131-135,145-149,191-194). When this reaction is studied over an extended range of substrate concentrations the Eadie-Hofstee plots are biphasic (35,124). Stopped-flow studies revealed that the second order rate constant for the initial reaction of cytochrome c with purified cytochrome c oxidase is very fast.

very fast. The rate constant (k_1) for the initial reaction varies from 10^6 M^{-1} s^{-1} in 100 mM phosphate buffer (35) to greater than 2×10^8 M^{-1} s^{-1} in 5 mM phosphate buffer (147,174,191). The oxidation of cytochrome c can be followed at 550 nm. The initial rate of electron transfer has been attributed to cytochrome a (35) because the absorption changes at 550 nm corresponds to absorption changes at 443 nm and 603 nm. Cu_A has been suggested to be the second electron acceptor (35). The initial rate of electron transfer is not affected by cyanide (149,165) and this implies that cytochrome a_3 is not involved. However, the exact contribution of hemes a, a_3 and copper atoms Cu_A, Cu_B to the kinetic processes still remains unclear.

Studies of the purified enzyme from beef heart with reduced cytochrome c at low ionic strength revealed (67,123,131,175,184) two binding sites for substrate per molecule of enzyme. These binding sites are not equivalent; one has high apparent affinity and low activity, whereas the other shows low apparent affinity and high activity (67,131). The high affinity cytochrome c interaction site on the enzyme has been proposed (178) to consist of carboxyl groups on subunit II of cytochrome c oxidase. This has been demonstrated (189) by cross-linking experiments with chemically modified cytochrome c derivatives. These molecules were bound to subunit II of the enzyme. Moreover, modification of carboxyl groups on subunit II strongly influences the high-affinity reactions (188). Our knowledge about the high affinity interaction site is limited. There is even less information available about

the low-affinity cytochrome c interaction site. A dimeric form of the enzyme (176,205) or cardiolipin (46,189) has been proposed to be involved in the low-affinity site. The existence of two binding sites is still a matter of controversy (22,).

It has been observed that the reaction rate of cytochrome c oxidase with cytochrome c , i.e. $\underline{c}^2 + \underline{a}^3\underline{a}_3{}^3 \longrightarrow \underline{c}^3 + \underline{a}^2\underline{a}_3{}^2$ is strongly influenced by ionic strength. Generally a decrease of the reaction rate with increasing of ionic strength has been reported (123,124,128,191). It has been suggested that the interaction between the two proteins is electrostatic in nature (174,199). Ionic strength affects the electrostatic interactions between the two proteins. At low ionic strength these interactions are very strong and at high ionic strength they are weakened (123,124, 128, 174). The area on the surface of cytochrome c which interacts strongly with cytochrome c oxidase has been shown (121,125,177, 196,198) to be located where a set of positively charged lysine residues are exposed. This is supported by inhibition of the enzyme with chemically modified cytochromes c at single lysyl residues (67,125,127). It has been suggested that the center of the charges is located near phenylalanine-82 (77,151,178). The driving force for the orientation of cytochrome c with respect to its partner cytochrome c oxidase is the large dipole moment that is associated with the sum of the total positive and negative charges on cytochrome c (121,128) and cytochrome c oxidase .

The effect of pH on the activity of cytochrome c oxidase is particularly important because protons are used as a substrate in the steady state oxidation of cytochrome c^{+2} to cytochrome c^{+3} by dioxygen:

$$4 \text{ cyt. } c^{+2} + 4 H^{+} + O_2 \xrightarrow{\text{cyt. c ox.}} 4 \text{ cyt. } c^{+3} + 2 H_2O$$

Furthermore, cytochrome c oxidase is involved in proton transport across the mitochondrial inner membrane (2,7,62,136,190) and, hence, acts as a proton pump. Proton release or uptake is affected by pH which results in a change of the redox state of the enzyme (10,51,61,100).

Cytochrome c oxidase is known to be at least partially surrounded by a hydrophobic environment in vivo (31,141). When the enzyme is isolated from the membrane, phospholipids or non-denaturing detergents are required to regulate electron transfer activity (43-46,140). Each detergent activates the lipid-depleted enzyme to a different extent (141). The nature of the detergent complex formed has not been characterized, but it is known that tightly bound phospholipids cannot be substituted with detergent (141). The ratio of lipid to protein is an important factor in the enzymic activity of the enzyme. Tween-80 is extensively used as a non-ionic, non-denaturing detergent to substitute the loosely bound lipid in cytochrome c oxidase. However, there is no study as to the amount of Tween-80 required.

The activity of cytochrome c oxidase in the reaction with its substrate cytochrome c may be affected by various factors. Since a complete three dimensional structure of the enzyme is not available, the rationalization of the contradictory results obtained by different investigators is not possible. But an overall study of the effect of the factors influencing the enzymic activity may help to provide a better understanding of the enzyme structure and of the mechanism of electron transport.

5.2. EXPERIMENTAL DETAILS

The enzyme was prepared as explained in sections 2.1. and 2.2. Enzyme Preparation and Methodology. The enzyme contained 7-10 mole lipid per mole of protein (functional unit). The quantitative and qualitative study of the individual lipids were not performed as this study was not designed for it. Other studies have shown that at least three different lipids, DPG, PE, and PC account for almost all of the phospholipid present (141).

The enzyme was incubated with the required buffer and then passed through a sepharose Cl-6B column, equilibrated with the same buffer. The enzyme was stable for 48 hours at 0°C. Gel filtration removes the cholate and substitutes it with Tween-80. Thus the eluted enzyme is

a protein-lipid/ Tween-80 complex. The assays were performed with phosphate buffers of 5 to 100 mM ionic strength. The pH of each buffer was varied over the range 5 to 8 and the amount of Tween-80 used in each buffer was between 0.002% to 0.5% (V/V).

52.1. THE EFFECT OF pH, IONIC STRENGTH AND TWEEN-80 ON THE ENZYMIC ACTIVITY OF LOW-LIPID CONTENT CYTOCHROME C OXIDASE.

Molecular activities or turn-over numbers of the enzyme were calculated as was described in 3.7.3. From reciprocal plots of molecular activities versus cytochrome c concentrations, molecular activity maxima (MA_{max}) were obtained. Fig. 5.1 shows a plot of molecular activity maxima versus pH at 40 mM ionic strength and at 0.05% and 0.1% Tween -80. Under all conditions examined the enzyme was first order. Fig. 5.1 A1,2 shows a typical decrease of absorption of reduced cytochrome c at 550 nm at pH 5,54 and 7.05. In Fig. 5.1 B 1,2 semilogarithmic plots of first order oxidation of indicated amounts of cytochrome c at pHs 5.54 and 7.05 are presented.

MA_{max} increased as ionic strength was increased from 5 mM to 30 mM as shown in Fig. 5. 2. No significant increase was observed when the ionic strength was further increased. This is surprising, although it may explain why some studies (184,191) suggest that the enzymic activity is

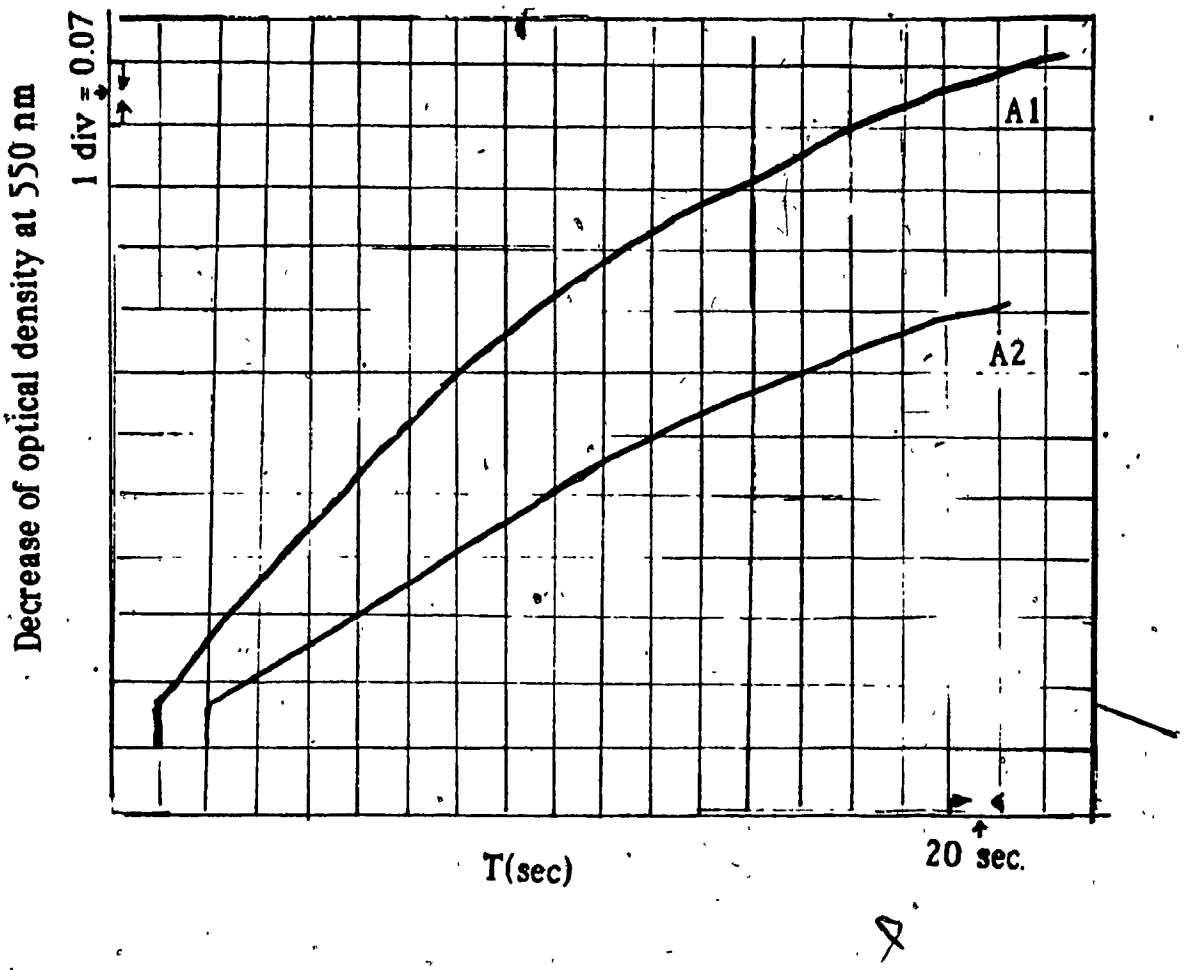


Fig 5.1.A. Traces of the decrease in optical density at 550 nm after the addition of oxidized cytochrome c oxidase (1.25 nM) to ferrocytochrome c (35 μM and 37 μM in 1 and 2 respectively) in 50 mM phosphate buffer at pH 5.54 (1) and pH 7.05 (2) at 20°C.

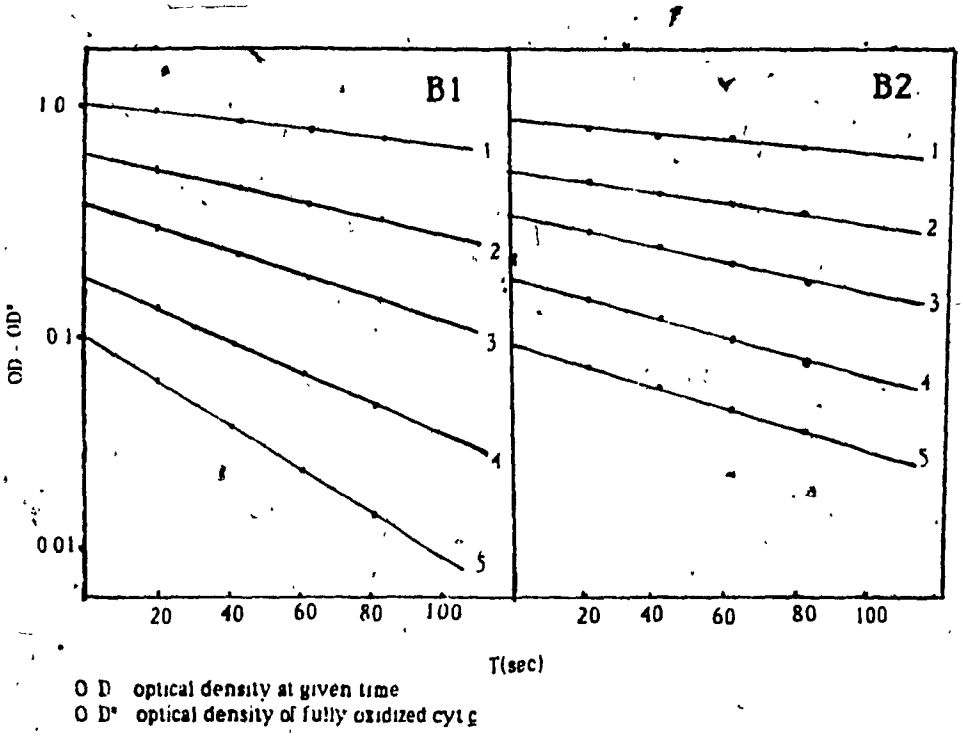


Fig. 5.1. B. Semilogarithmic plots of the oxidation of varing concentration of ferrocytochrome c, catlyzed by the same concentration of cytochrome c oxidase. The absorbance was followed at 550 nm, 50 mM phosphate at 0.1 % (v/v) Tween-80 at 20 °C at pH 5.54 (B1) and pH 7.05 (B2). Concentration of cytochrome c oxidase is 1.25 nM and that of ferrocytochrome c as follows;

B1 1 = 37 μM 2 = 29 μM 3 = 22 μM 4 = 16 μM 5 = 8 μM

B2 1 = 35 μM 2 = 27 μM 4 = 21 μM 4 = 14 μm 5 = 5 μM

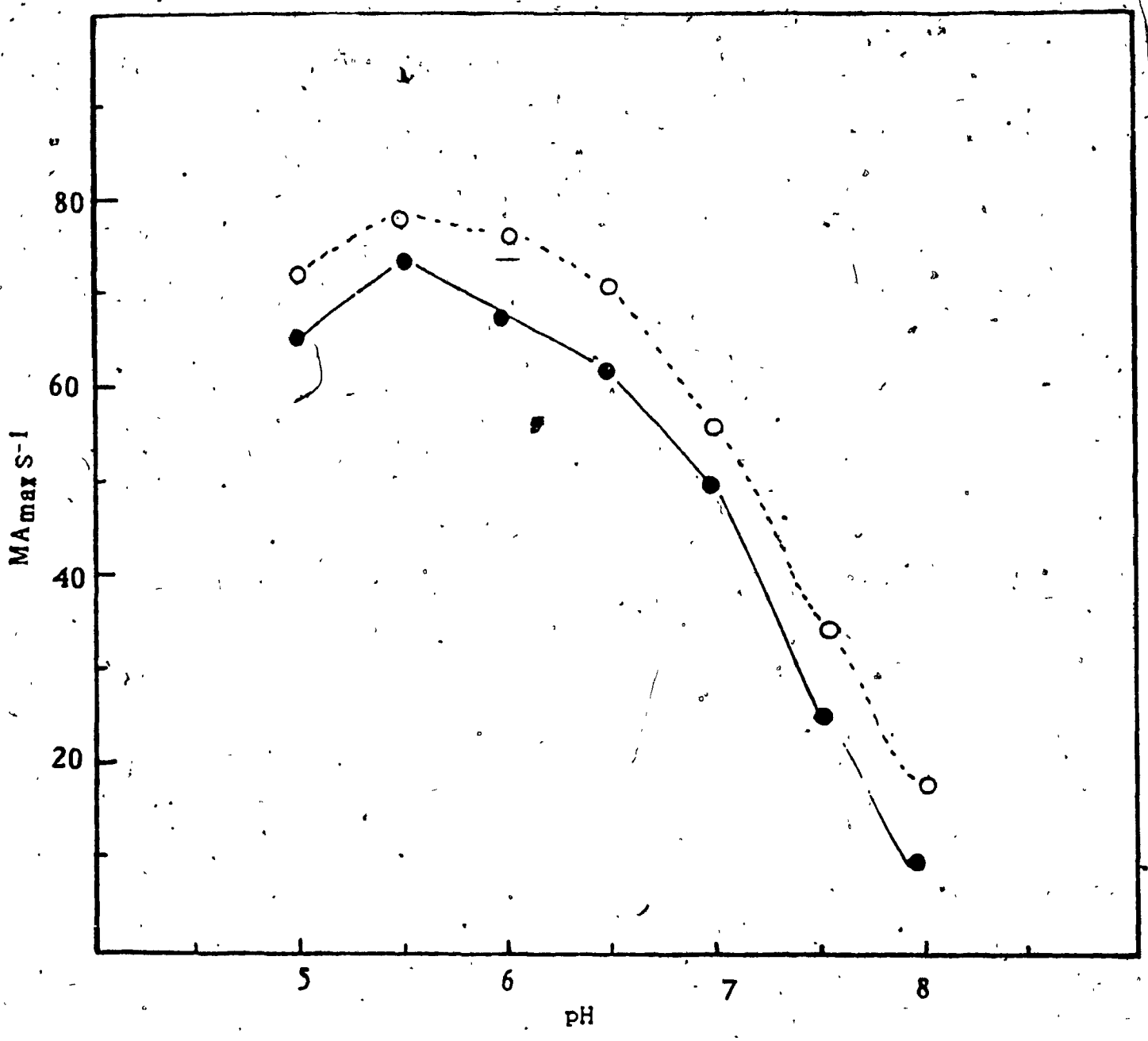


Fig. 5. 1. THE EFFECT OF pH AND TWEEN-80 ON MA_{max}

MAmax were obtained as described in Fig. 3. 5. MA_{max} are plotted vs pH. As it is evident a pH optimum is involved. A phosphate buffer with ionic strength of 40 mM and Tween-80 concentrations of 0.05% ●——● and 0.1% o——o were used. Assays were carried out at 20°C

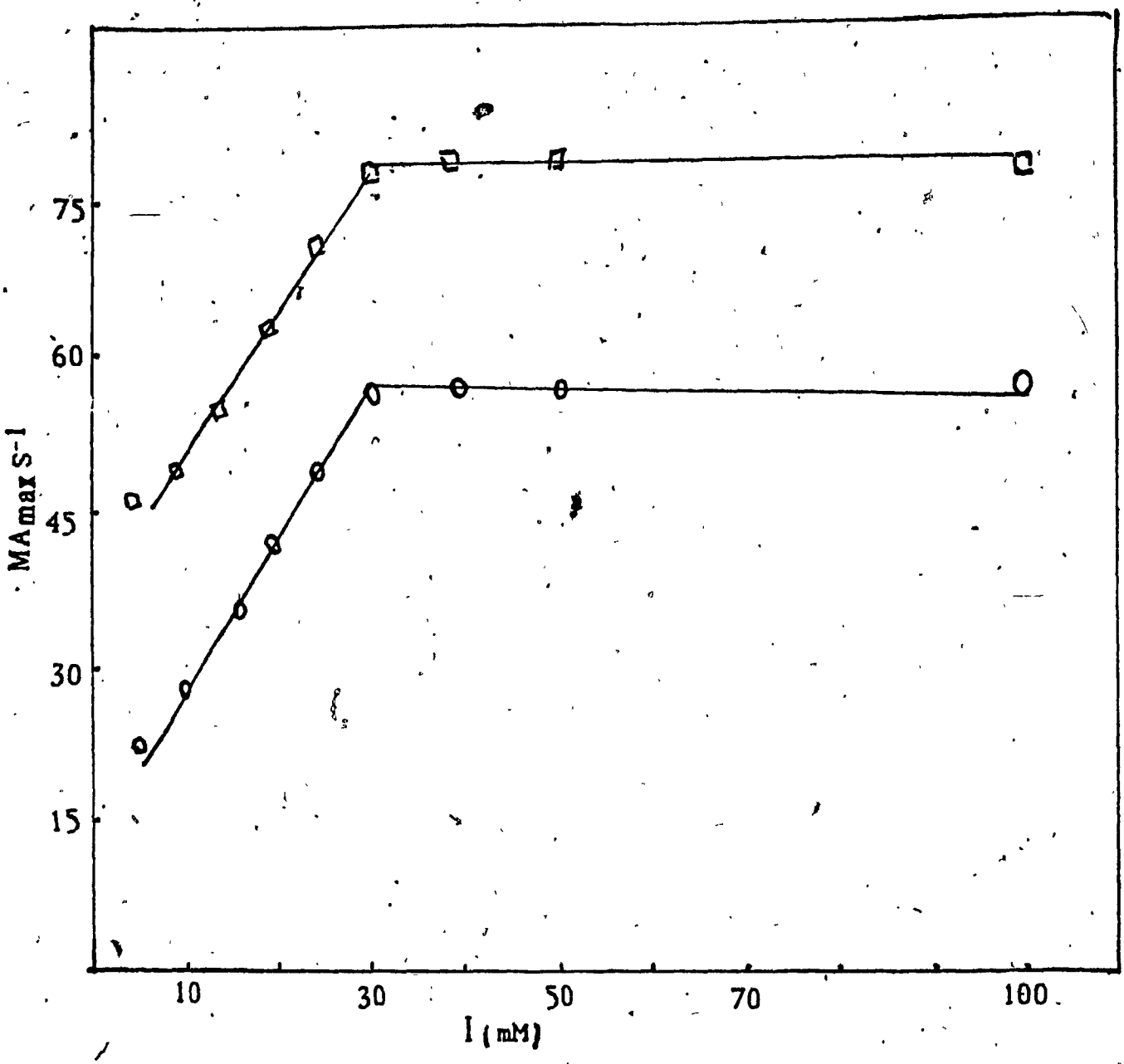


Fig. 5. 2. THE EFFECT OF IONIC STRENGTH ON MA_{max}

MA_{max} values at pH 5.5 □——□ and pH 7.5 ○——○ are plotted vs ionic strength. Tween-80 concentration 0.1%, cyt. c and cyt. c ox. concentrations were 5-40 μM and 1.25 nM respectively. All assays were carried out at 20°C.

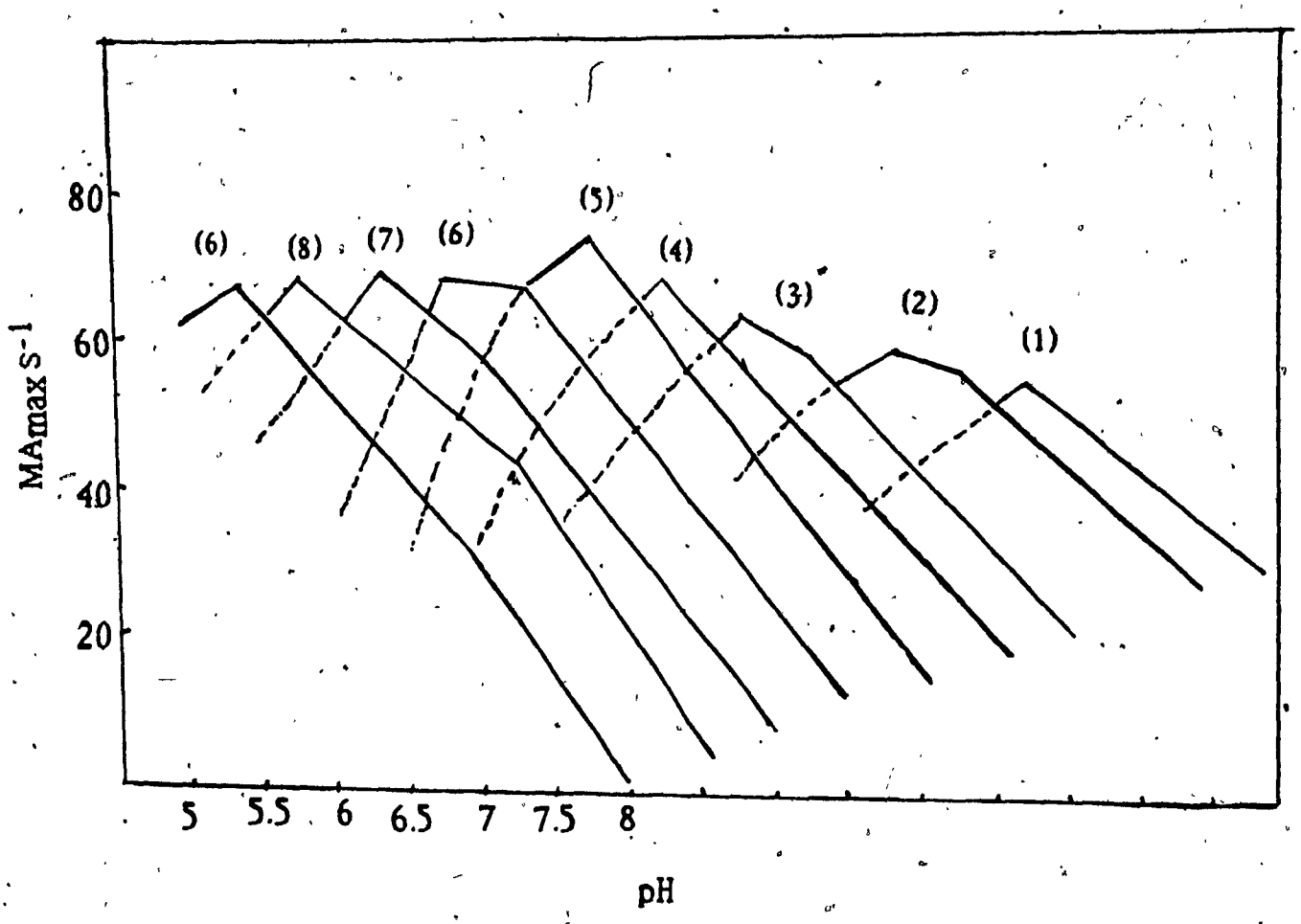


Fig. 5. 3. A DEMONSTRATION OF THE COMBINED EFFECT OF pII AND IONIC STRENGTH ON MA_{max}

MA_{max} are polotte over pH range of 5 to 8 at I = 5 mM (1), I = 10 mM (2), I= 15 mM (3), I = 20 mM (4), I = 25 mM (5), I = 30 mM (6), I = 40 mM (7), I = 50 mM (8), and I = 100 mM (9) to compare the complex behavior of the enzyme at different pH and ionic strength.

Table 5.1. The effect of Tween-80 on Km and MAmax at high and low ionic strength at pH 5.5 and pH 7.5

Tween-80%		0.002	0.05	0.1	0.2	0.3	0.5
I(mM)	pH						
5	5.5	32	26	21	18	15	11
	7.5	24	19	13	11	11	9
50	5.5	58	51	47	44	42	38
	7.5	45	41	39	36	35	33
Km (μM)							
5	5.5	31	35	43	45	45	45
	7.5	18	25	29	30	30	30
50	5.5	38	46	56	58	58	58
	7.5	30	33	38	38	38	38
MA_{max} (s^{-1})							

The values of MA_{max} and K_m were obtained as described in Fig. 3. 5. The assays were performed in phosphate buffers with the indicated ionic strength and Tween-80 concentrations. All other conditions were the same as in Fig.3.5. The kinetic parameters were calculated by regression analysis. Correlation coefficients were 0.99 or better.

Under all experimental conditions straight lines were obtained in the Lineweaver-Burk plots (a plot of 1/MA versus 1/cyt.c concentration). This suggests that only one cytochrome c binding site was kinetically apparent. The high affinity (low activity) generally has been seen at very low ionic strength and cytochrome c concentrations. The reason that the high affinity site is not seen in this work may be due to the high substrate concentrations used here (5 uM to 40 uM). Fig. 5. 3 shows how MAmax. is affected by pH and ionic strength. At low ionic strength, a distinct pH optimum for the enzyme was found, whereas at high ionic strength decreasing pH increased the activity.

Table 5.1 presents MA_{max} and K_m data at pHs 5.5, 7.5 and ionic strengths of 5 mM and 50 mM and at concentrations of Tween-80 ranging between 0.002% to 0.5% . MA_{max} at both pHs and ionic strengths increased by about 15% as the Tween-80 concentration was increased from 0.002% to 0.1%. Further increase of Tween-80 did not cause any significant increase in MA_{max}. A small but steady decrease in K_m was observed as the concentration of Tween-80 was increased from 0.002% to 0.5%. The same typical phenomenon was observed for all pHs and ionic strengths examined. The effect of Tween-80 was enhanced at lower pH as is evident from Table 5.1. In this table MA_{max} and K_m values at pH 5.5 and 7.5 are compared at two ionic strengths (5 mM and 50 mM). Generally, the enzymic activity was higher at pH 5.5 and lower at pH 7.5 regardless of the ionic strength and Tween-80 concentration.

Table. 5. 2. The effect of ionic strength (I) and Tween-80 on MAmax at pH 5.0 and pH 7.0.

Tween-80%	0.002		0.05		0.1		0.2		0.3		0.5	
I	pH											
(mM)	5	7	5	7	5	7	5	7	5	7	5	7
5	18	28	23	33	31	38	33	38	35	38	35	38
10	23	31	27	38	33	41	35	42	38	42	40	38
15	25	34	29	38	33	43	36	43	39	43	41	43
20	28	37	35	41	41	46	43	46	45	46	46	46
25	31	39	36	45	43	51	45	49	46	49	48	49
30	33	39	36	48	41	53	44	54	46	54	48	54
40	34	39	38	48	43	53	44	54	47	54	48	54
50	36	39	41	48	45	53	47	54	47	54	48	54
100	36	39	39	48	47	53	48	54	51	54	51	55

The values of MAmax were obtained as explained in Fig. 3. 5. The assays were performed in phosphate buffers with the indicated ionic strength (I) and Tween-80 concentrations at pH 5 and pH 7 at 20° C. Other conditions were the same as in Fig. 3. 5.

The effect of Tween-80 over the pH range of 5 to 8 is compared in Fig. 5. 1. ; Table. 5. 2. shows the combined effect of ionic strength and Tween-80 concentration on MA_{max} at pH 5 and 7. The results suggest that at low ionic strength and low pH, increasing Tween-80 increases the molecular activity. This increase was small but observable and apparent in all trials. However, as it is noticeable, increasing Tween-80 to more than 0.1% did not increase the activity. The results showed that the low-lipid sample has low activity and most of this activity is recovered at a very small amount of Tween-80 (0.002%). The increase of Tween-80 to 0.1% increased the activity by 15% to 20% and no further increase in activity was observed by increasing the Tween-80 to 1%.

5.2. 2. pH OPTIMUM AND THE EFFECT OF IONIC STRENGTH AND TWEEN-80 ON ITS LOCATION

A pH optimum was generally observed under the conditions tested in Fig.5.1. and Fig.5.4. The pH optimum seen here has been reported by many investigators (129,134, 135,147,148,151). However, other studies (145,184) observed a decrease of molecular activity with increasing pH. This work showed that this discrepancy is due to the experimental conditions. The location of this pH optimum was not constant; it varied as a function of ionic strength and Tween-80 concentrations. Fig. 5. 4. shows a plot of MA_{max} versus pH at 10 mM and 50 mM, 0.1% Tween-80 ; as is evident, at 5 mM ionic strength, the pH optimum is located around pH 6

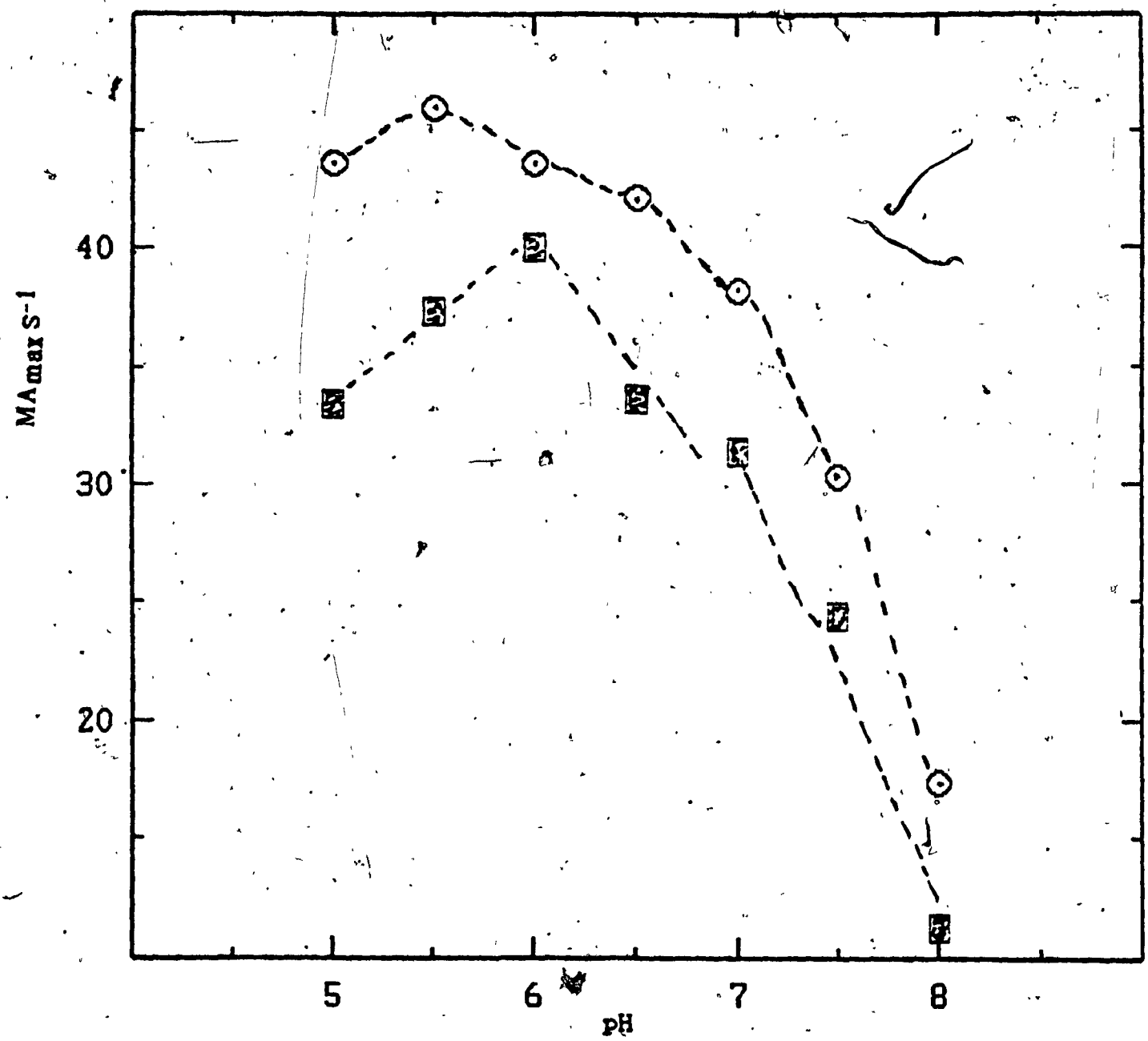


Fig. 5. 4. THE EFFECT OF IONIC STRENGTH ON pH OPTIMUM

MA_{max} obtained at I = 15 mM ⊡——⊡ and I = 50 mM ⊙——⊙ as discribed in Fig. 3. 5 are plotted vs pH to demonstrate the effect of I on pH optimum. The assays were carried out at 20 °C and 0,1% Tween-80.

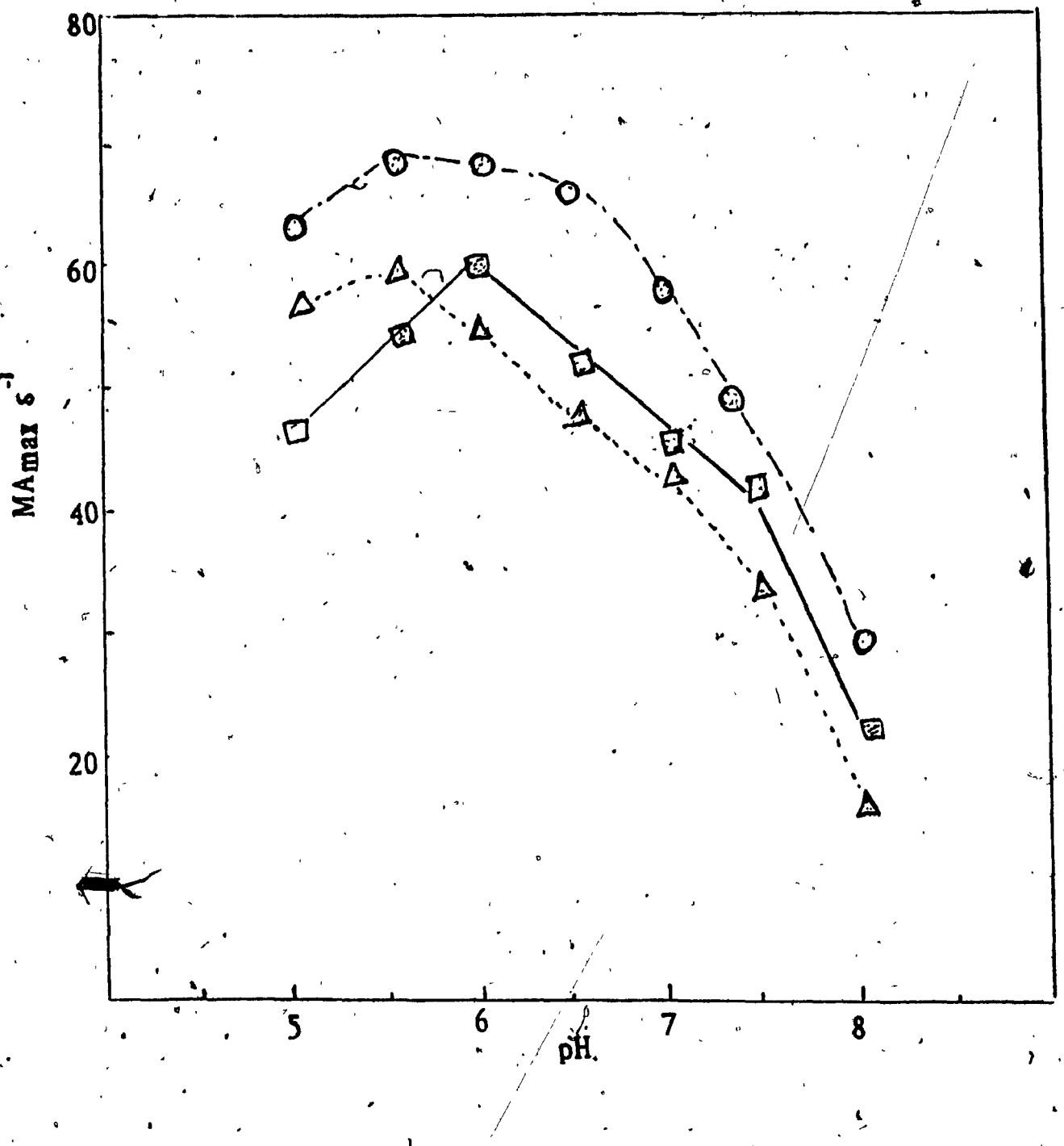


Fig. 5. 5. THE EFEECT OF IONIC STRENGTH ON MODIFICATION OF pH OPTIMUM

MA_{max} were obtained as described in Fig. 3. 5. in phosphate buffers of ▫——▫ I = 10 mM △——△ I = 50 mM ○——○ I = 100 mM and Tween-80 concentration 0.1%.

to 6.5 whereas at 50 mM ionic strength it is closer to pH 5.5. Generally, a pH optimum of about 5 to 5.5 was observed at ionic strengths above 30 mM while an optimum around 6 was seen at ionic strengths below 30 mM. Similar results have been reported by another study (134) which used Tween-20 and PL substitutes. At Tween-80 concentrations lower than 0.1 % at constant pH and ionic strength, the pH optimum is slightly lowered (Fig. 5. 1.) but, above 0.1% Tween-80 concentrations, no significant effect can be observed. The increase of ionic strength and Tween-80 resulted in the disappearance or modification of the pH optimum. Fig. 5. 5. shows the effect of increasing ionic strength. It is evident that as ionic strength increases from 15 to 100 mM, the pH optimum becomes less apparent. Fig.5. 3. compares the combined effects of pH and ionic strengths on MA_{max}. A more enhanced pH optimum can be observed at ionic strength lower than 50 mM in this graph.

5.2.3. THE EFFECTS OF pH, IONIC STRENGTH AND TWEEN-80 ON THE K_m OF LOW LIPID-CONTENT CYTOCHROME C OXIDASE

Km was affected by pH, ionic strength and Tween-80 concentrations. Fig. 5. 6. shows a plot of K_m versus pH; a decrease of K_m was observed as the pH was increased. In general, increasing Tween-80 caused a decrease in Km at all pHs and ionic strengths [Table. 5.1]. Table 5.3 presents the effect of ionic strength and Tween-80 on K_m under the same conditions as shown in Table 5.2. A gradual increase

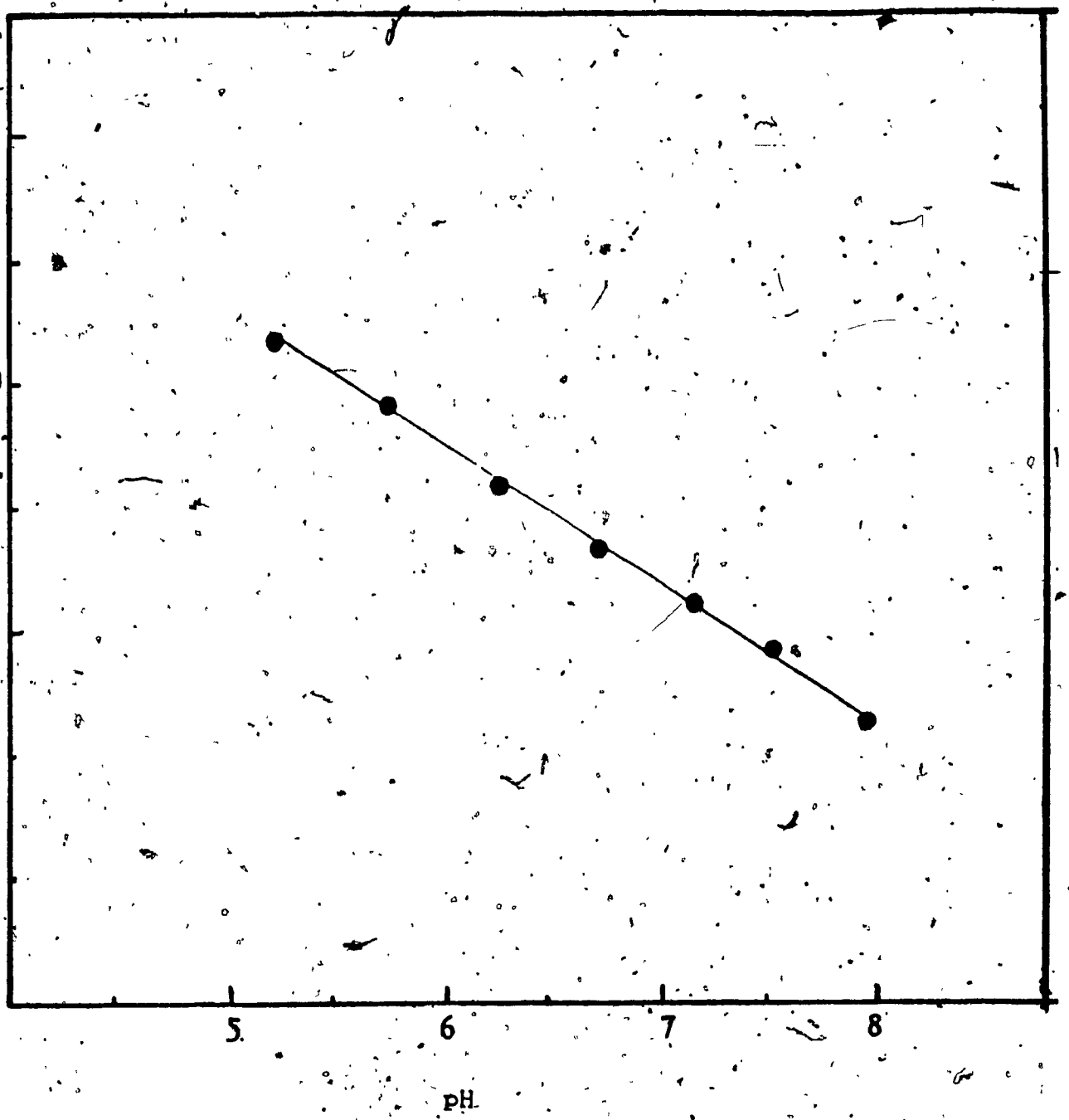


Fig. 5. 6. THE EFFECT OF pH ON K_m

K_m values were obtained as described in Fi. 3. 5. The assays were performed in phosphate buffers of I = 50 mM, 0.1% Tween-80 concentration over pH range 5 to 8 at 20°C.

in K_m was observed as ionic strength was increased. This agrees with other studies (128,145,184), but the Km's obtained here were greater than others have reported. This is due to the experimental conditions of this study. The K_m seen here is considered to represent the low affinity reaction. I believe that lipid depletion is the cause of low enzymic activity and the high K_m. Increasing Tween-80 slightly decreased K_m, although the decrease was small but reproducible.

Table 5. 3. The effect of varying ionic strength (I) and Tween-80 concentration on Km (uM) at pH 5 and pH 7.

Tween-80%	0.002		0.05		0.1		0.2		0.3		0.5	
I(mM)	pH											
	5	7	5	7	5	7	5	7	5	7	5	7
5	28	15	25	15	23	14	21	13	19	11	17	9
10	31	21	29	19	26	15	24	14	22	13	21	11
15	36	26	31	21	28	19	27	19	25	17	23	18
20	41	33	35	31	29	25	28	20	27	18	25	21
25	48	39	42	33	38	31	35	29	33	27	31	27
30	54	45	49	40	45	37	43	33	41	31	35	30
40	58	49	55	47	52	45	49	43	48	41	45	40
50	61	51	58	48	54	45	53	43	51	42	50	41
100	65	55	61	52	57	48	55	46	54	45	52	45

All conditions are the same as in Table 5. 2.

The net charge of cytochrome c oxidase is negative (121,139) and that of cytochrome c is positive (123,147,174)). The product of the interaction charges between them can be calculated using the extended form of Debye-Huckel equation providing Ka is known. K_m is a complicated combination of a large number of rate constants (195,208) but when in equation [5.2.] (page 102) $k_{-1} \gg k_2$ the equation simplifies to $K_m = K_D$. This simplification, as well as the equality $K_a = 1/K_D$ allows us to calculate $K_a(0)$ from a plot of K_a versus $I^{1/2} / (1+I^{1/2})$. Knowing $K_a(0)$, then Z_aZ_b, the product of the interaction charges on cytochrome c oxidase and cytochrome c at different pHs can be calculated. Assuming $K_m = K_D$ and appling equation 5. 1. I tested the assumption using the values of Km at pH 5 and 7 (table 5. 3.) as well as Kms at pH 6 (data not shown). As seen in Fig. 5.7. K_m at zero ionic strength at pH 5 extrapolated to about 10 μM, at pH 6 to about 5.5 μM and at pH 7 to 3.5 μM. Z_aZ_b, the product of interaction charges on the two proteins (cyt.c ox. and cyt.c) calculated from these graphs were lower than reported values (132,173).

$$\lg K_a = \lg K_{a(0)} + Z_aZ_b [(I^{1/2}) / (1 + I^{1/2}) \qquad \text{[Equation 5.1]}$$

In general the value of $K_D(0)$ found previously were lower than those found here; the values of ZaZb were greater than the values I determined. This indicates that the assumption Km = KD is not justified.

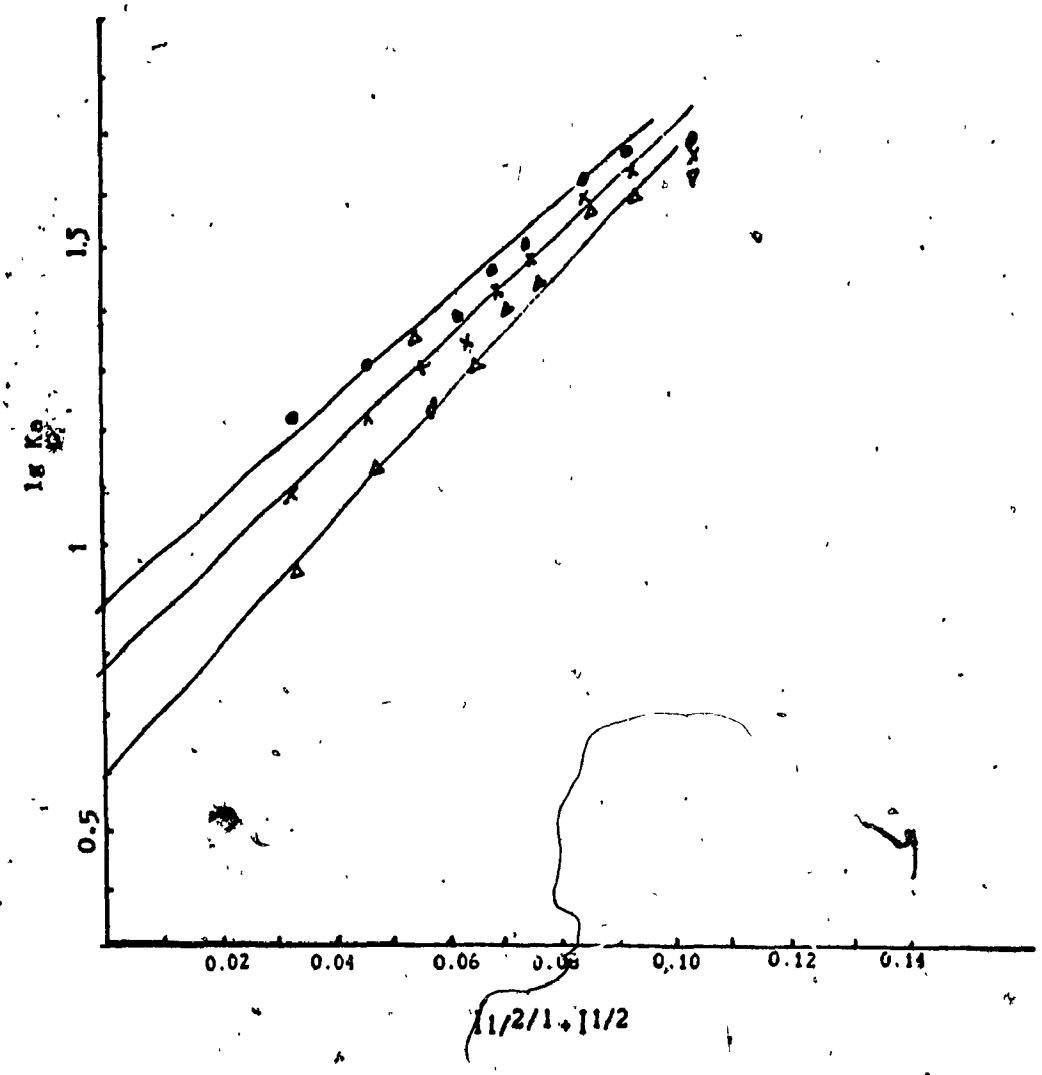


Fig. 5. 7. THE EFFECT OF pH ON $K_a(0)$

K_m values obtained as was explained in Fig. 3. 5. K_a were calculated from $1/K_D$ on the basis $K_D = K_m$. lg K_a vs $I^{1/2}/1 + I^{1/2}$ at pH 5 ●——● pH 6 ×——× and pH 7 △——△ are ploted and extrapolated to lg K_a axis to obtain $K_a(0)$.

5.3. INTERPRETATION OF RESULTS

The enzymic activity of a sample of cytochrome c oxidase with 7 to 10 mole phospholipid per functional unit of the enzyme was studied. The oxidation of reduced cytochrome c by the enzyme, under various conditions of pH, ionic strength and Tween-80 showed the correlation between these factors and enzymic activity to be far too complex for interaction terms to be determined statistically. But the study is still very useful in that it can prove helpful for the selection of experimental conditions. The effect of these factors on the enzymic activity of cytochrome c oxidase will be interpreted in detail; the correlation between them will be discussed.

5.3.1. THE INTERACTION OF CYTOCHROME C OXIDASE WITH CYTOCHROME C IS NOT SOLELY OF ELECTROSTATIC NATURE.

The oxidation of reduced cytochrome c catalyzed by cytochrome c oxidase showed first-order kinetics under all experimental conditions. This is in agreement with many studies (123,145,184) and also contrasts with others (129). Representative traces at pH 5.54 and pH 7.05 are shown in Fig. 5.1 A1,2. The semilogaritmic transformations shown in Fig. 5.1 B1,2 indicate the first order reaction kinetics. In a very recent paper by Gregory and Ferguson-Miller (211) the authors also studied the pH dependence of turnover number and K_m. They comment on the fact that in thier hands: the oxidase showed only first order kinetics, the oxidase

showed only a single low affinity site and, lastly, the spectrophotometric and oxygen electrode assays gave virtually the same results. It might have been expected that the two assays for oxidase activity would have given different results as was originally demonsterated by Smith and Conrad (212,213). This would most easily be seen at low ionic strength. Gregory and Ferguson-Miller obviate this problem by working only at high ionic strength, wheras I did not look to see whether the two assays went from agreement at high salt to no agreement at low salt.

The results demonstrated that, at constant pH and Tween-80 concentration, and at ionic strength below 30 mM, MA_{max} increased as the ionic strength was raised. However, at ionic strength above 30 mM the increase of MA_{max} was not significant. Km, on the other hand, increased as ionic strength was raised from 5 mM to 100 mM. This increase was small but reproducible.

The ionic strength effect on K_m shows that the interactions of the two molecules are the result of opposite charges. The net charge of cytochrome c is positive (121,139) and that of cytochrome c oxidase is negative (121,123,147, 174). It has been reported (171) that the electrostatic forces result in a very tight binding at zero ionic strength. This phenomenon which has been reported by a number of other investigators (188,191,199) is involved in the formation of a 1:1 complex of cytochrome c and cytochrome c oxidase. This complex is stable to chromatography and ultracentrifugation (127,131,132). This complex can be dissociated at high salt concentration. These studies have mainly been

carried out under conditions where the enzyme is highly active. My results agree with these studies in that, under favorable conditions, the interaction between the two molecules is largely electrostatic.

However, under all experimental conditions a pH optimum was observed. The location of this pH optimum varied as a function of ionic strength and Tween-80. At ionic strengths above 30 mM and 0.1-0.5 % Tween-80, it was less than 6 whereas below this ionic strength it is located around 6. The observed pH optimum in the enzymic activity and the effect of ionic strength on the pH optimum suggest that the electrostatic interactions between the two proteins are not the sole determining factor of enzymic activity.

Fig. 5.3. depicts the variation of the enzymic activity over pH range of 5 to 8 at ionic strengths of 5 to 100 mM. This graph is not a real tridimentional presentation, but, serves my purpose. At pH 8 the activity of the enzyme at 5 mM ionic strength is higher than that at100 mM. The optimum activity of the enzyme is much higher at 100 mM than at 5 mM. Similarly, the optimum activity at 5 mM was seen at pH 6.5 and that of 100 mM at pH 5.5 or lower. This is in contrast to other studies (145,184) which report an increase of MA_{max} upon increasing ionic strength and decreasing pH. The observed relatively high activity of the enzyme at pH > 6.5, at ionic strengths less than 30 mM is unexpected if electrostatic interactions alone control the enzymic activity. These observations suggest that conformational changes occur due to pH variation. The behaviour of these different conformers are different. At high ionic

strength, a fully protonated conformer is highly active. At low ionic strength, a form of enzyme which is not fully protonated must be responsible for the higher activity observed at very low ionic strength at higher pH. This suggests that the enzyme may remain active at basic pH. Conformational changes resulting from changed pH (185) have been suggested earlier and support this study. The results of this work indicate that different conformations show different behavior under different conditions of ionic strengths.

Other studies have shown that the enzymic activity either decreases (128,147, 184) or shows an optimum (134,135,145-149) as pH increases. Enzymic activity decreases by lipid depletion and increases when phospholids are added (140,141,152). These studies support the results of this work and indicate that the interactions between cytochrome c and cytochrome c oxidase are regulated by a number of chemical and physical phenomena.

K_m increased as ionic strength was raised [Table 5.3] and decreased as pH was increased [Fig.5.5]. Similar results have been seen by other investigators (128,134,145). It has been suggested (177) that a number of carboxyl groups located on subunit II donate negative charges at pH 6.2. On the other hand, the net charge on cytochrome c at pH 6.2 is +8 (121) and at this pH one or two of histidyl residues of this protein may be protonated. As the pH is lowered, the increase of positive charges on cytochrome c and decrease of negative charges on cytochrome c oxidase affect K_m.

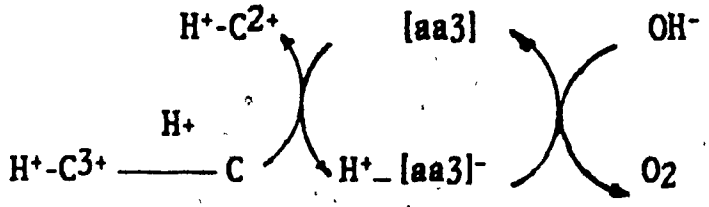
Under any experimental conditions, even at low ionic strength (5 mM), only one binding site was apparent. Since the concentrations of substrate used were high (5-40 uM) the existence of only one cytochrome c binding site cannot be confirmed. The identified Km belongs to the low affinity reaction site, but it was affected by pH and ionic strength. Other studies (47,184) found that only the high affinity reaction site was influenced by pH and ionic strength. It is likely that different conditions cause different aggregation and conformational states of the enzyme, which in turn affect the cytochrome c binding site.

In conclusion then, this work showed that the effect of pH, ionic strength and Tween-80 on cytochrome c - cytochrome c oxidase interactions are very complex. A single statistical formula can therefore not be derived. In the following, the effect of pH and Tween-80 are discussed.

5.3.2 EFFECT OF pH

The effect of PH on the enzymic activity of cytochrome c oxidase has been explained (7,61,147) in terms of the degree of protonation of acid / base groups participating in the catalytic functions of the enzyme. Proton release or uptake is induced by a change in the redox state of cytochrome a (51). This may cause a shift in pK_a (47,145) or result in a conformational change (185). The effect of pH in different stages of electron transport is then as follows: the transfer of an electron from

ferrocytochrome c to oxygen is accompanied by a stoichiometric proton transfer (80). The detail of this reaction is not known, but the reduction and protonation of oxygen to water may proceed via the following scheme:



After the intermolecular transfer of a proton, the reduction of molecular oxygen leads to the formation of a protonated oxygen (-OH^+). Further electrons and protons are transferred to complete the formation of water (61,145).

pH affects the interaction of cytochrome c oxidase with its substrate cytochrome c by changing the rate constants at each stage of reactions as follows:

$$\text{cyt. } a^{3+} + \text{cyt. } c^{2+} \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} \text{cyt.}a^{3+}\text{:cyt.}c^{2+} \xrightarrow{K_2} \text{cyt.}a^{2+}\text{:cyt.}c^{3+} \underset{K_{-3}}{\overset{K_3}{\rightleftharpoons}} \text{cyt } a^{2+}\ \text{cyt.}c^{3+}$$

[eqation 5. 2.]

This is evident from Z_aZ_b, the product of interaction charges calculated by assuming that $K_m = K_D$. Km is a function of K_1, K_2. K_3 and

K_{-1} and K_{-3} which may all be pH dependent (195,208). Z_aZ_b at different pH varies, but the values determined here are not representative of binding.

Z_aZ_b <1 indicates that more than one charged group is involved in the binding (134,176). These charged interactions are affected by ionic strength. Since the cytochrome c binding site is located on subunit II of the enzyme (114,176), at least one of these groups belongs to this subunit. Carboxyl and hydroxyl groups located near the cytochrome c binding site are affected by pH. Hydrogen bonds and salt bridges are influenced by pH and ionic strength. The orientation of the negative and positive charges on the two proteins can be affected by pH and ionic strength. It is known that pH may influence both spin state and conformation (8,62). The redox potential of the enzyme is also affected by pH (121). A combination of these effects regulates the activity and results in complex behavior of the enzyme under different conditions of pH and ionic strength. As shown above, there is always a pH optimum involved in the activity of the enzyme but it may not be seen under certain different conditions of ionic strength, pH, and detergent.

5.3.3. THE EFFECT OF TWEEN-80

The low enzymic activity observed was expected since the sample of enzyme used had 7 to 10 mole phospholipid per functional enzyme unit. It is well known that the enzyme isolated from the membrane using

a detergent such as cholate or deoxycholate is unreactive (43,140,141). The activity is partially restored if the enzyme is incubated in a non-ionic, non-denaturing detergent. One of the most common detergents used is Tween-80. It has been shown (45) that it can recover as much as 50% of the lost enzymic activity. The isolated enzymes with a ratio of 40 mole of phospholipid per functional enzyme unit are highly active (202). Decreasing this ratio to less than 1 or 2 mole of phospholipid per mole of enzyme impairs the enzymic activity (26). The enzymic activity can be restored only in presence of phospholipids. In most of the studies on cytochrome c oxidase, enzymes with 10-20 mole of phospholipid per mole of enzyme have been used in presence of a detergent. Many studies have focussed on the structural aspects of phospholipids and detergents. The quantity of detergent needed to provide the required fluidity for the enzyme reaction is an important matter but too much causes difficulties and too little may not be effective. In this study the effects of pH and ionic strength at different amounts of Tween-80 were studied.

The effect of Tween-80 on MA_{max} and K_m at pH 7 and 5 is shown in Table 5. 1. These two pHs were chosen because the enzyme showed similar MA_{max} at constant ionic strength (less than 40 mM) and Tween-80. The results indicate that increasing Tween-80 to 0.1% increases MA_{max}. The phospholipid content (7-10 mole per mole of enzyme) of the enzyme is high enough to prevent any structural disturbance. Tween-80 facilitates electron transfer or permits a better contact between the two proteins. This is supported by the steady decrease of K_m with

increasing Tween-80 concentration from 0.002% to 0.5%. Although this increase is not high enough to allow postulation that the apparent high and low affinity reactions are the result of one binding site reacting differently under different conditions, it still shows a significant effect of Tween-80 on the activity. Tween-80 is more effective at low ionic strength and low pH. It has been reported that the boundary layer of cytochrome c binding sites are occupied with phospholipids (141,187). DPG is required for high affinity reactions (187) whereas PC and PE or Tween-80 are responsible for low affinity reactions (46,187).

The low activity observed under all conditions is the result of insufficient amounts of phospholipid present in the enzyme. Since the increase of Tween-80 concentrations did not lead to a fully active enzyme (compare to an enzyme with 14-17 mole phospholipid/ mole of enzyme) it is concluded that a lipid tail of 18:1 such as is present in Tween-80 is not sufficient to activate the enzyme as was suggested by other studies (187). Alternatively, this low enzymic activity may be due to partial removal of tightly bound DPG which can not be substituted with Tween-80. At high ionic strength (40-100 mM) and pH (6.5-8) the results showed Tween-80 to be less effective. This implies that phospholipids are involved in the formation of a unique alignment of the charged groups around the cytochrome c binding site. At high ionic strength, the electrostatic interactions of the two proteins are low (188,191,199) and repulsion of charges present is high. Thus, the Tween-80 effect is masked. At high pH, the increase of negative charges on the enzyme may

disturb the hydrophobicity and the decrease of the positive charges of the substrate results in a decrease of the interactions between the two molecules, resulting in low enzymic activity. The aggregation state of the enzyme have also been suggested (43,141,151) to be affected by a decrease of the lipid content of the enzyme.

In conclusion, the results of this study indicate that the enzymic activity of a sample of enzyme with low lipid content can not be fully recovered by increasing the amount of Tween-80. The effect of Tween-80 varies as a function of pH and ionic strength.

5.4. CONCLUSION

The kinetic behaviour of a sample of cytochrome c oxidase with 7 to 10 mole phospholipid per functional enzyme unit was studied under experimental conditions of 5 to 100 mM ionic strength, pH 5 to 8 and 0.002% to 0.5% Tween-80. Results show that the interelationship between these factors is too complex to be represented by an interaction term. The electron transfer activity of the enzyme is affected by environmental factors as well as by the structural features of the enzyme. To understand the effects of the above mentioned factors on the enzymic activity of the enzyme, a detailed structural knowledge as well as detailed experimental studies of the effects of each factor on different samples of the enzyme are required. This is beyond the scope of this work but, the study still led to some useful results which are summarized as follows;

1› Under all conditions, the sample with 7-10 mole lipid per mole of enzyme showed lower enzymic activity when compared to a sample with 14-17 mole lipid per mole of enzyme.

2› A pH optimum is involved under different conditions of ionic strength and Tween-80. The location of this optimum varies and may not be seen under some conditions. As the ionic strength increases the pH optimum decreases. This optimum is lowered in a sample with high lipid content or in the presence of detergent. K_m increases as pH or ionic strength increases, but it may be lowered by increasing Tween-80.

3› Increasing ionic strength increases to some extent the activity of the enzyme sample with low lipid content. K_m increased as ionic strength increased. The results indicate that although the structural features of the enzyme are maintained, some modification of the enzyme has occurred. This may be due to the aggregation states of the enzyme or distortion of the orientation and alignment of charged groups near the cytochrome c binding site.

A CONCLUDING REMARK OF THIS STUDY

This, like other studies, raises more questions than it answers. One of these questions is as follows;

Stoichiometric studies of cyanide binding showed that the behaviour of the enzyme at low pH towards cyanide is different from that at high pH. Kinetic studies revealed that the molecular activity of the enzyme also varies as a function of pH. The question then, is there a link between them?

Although the experimental conditions of the two studies were different, the two data sets may be connected. I would like to think that, at low pH, the enzyme is in an excited state which results in higher activity. This excited state may occur as a result of conformational change, a spin change, a change in the orientation or charges of the ligands located near active sites or ligand binding sites, changes in the oxidation state of the metal centers or changes in the aggregation state of the enzyme. Whatever this change is, it effects the behaviour of the enzyme as exemplified by cyanide binding and molecular activity.

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APPENDIX [1]

cyt. c ox. + CN^- ⟶ cyt. c ox.- CN

$$Kco = \frac{[\text{cyt. c ox.- CN}]}{[\text{cyt. c ox.}]\,[CN^-]}\ 10^6 \text{ or more (Ref. 170)} \qquad (A)$$

co = complex

$$K_a = \frac{[H^+]\,[CN]}{[HCN]} = 7.2 \times 10^{-10} \qquad (B)$$

cyt. c ox. concentration = 5 uM in heme a aa_3 = 2.5 uM

Final cyanide concentration in the reaction tube = 10^{-5}

Total cyt. c ox. = [cyt. c ox.] + [cyt. c ox.-CN] = 2.5×10^{-6}

Total CN^- = [HCN] + $[CN^-]$ + [cyt. c ox.-CN] = 10^{-5}

Assuming a complete conversion of cytochrome c oxidase to cyt. c ox.- CN complex;

cyt. c ox.- CN = 2.5×10^{-6}

$$[HCN] = \frac{[H^+]\,[CN]}{7.2 \times 10^{-10}}$$

At pH 8 $[CN^-] = 2 \times 10^{-6}$, at pH 6 $[CN^-] = 2 \times 10^{-8}$ and at pH 5 $[CN^-] = 2 \times 10^{-9}$

Free cytochrome c oxidase can be calculated from equation (A)

at pH 8 cyt. c ox. = 10^{-6}

at pH 6 cyt. c ox. = 10^{-4}

at pH 5 cyt. c ox. = 10^{-3}

at the same KCN concentration as pH is lowered one unit, CN^- present in the solution decreases ten fold; whereas free cytochrome c oxidase increases ten fold. As it has been shown in acidic solutions, pH < 6 the $[CN^-]$ is nearly zero and no complex ion forms (167).