STRUCTURE-FUNCTION STUDIES ON CYTOCHROME *c* OXIDASE

PART I. AN INVESTIGATION INTO THE NATURE OF THE METAL SITES IN CYTOCHROME *c* OXIDASE USING X-RAY ABSORPTION SPECTROSCOPY

PART II. AN INVESTIGATION INTO THE LIPID FACTORS AFFECTING PROTEIN ACTIVITY AND RESPIRATORY CONTROL IN RECONSTITUTED CYTOCHROME *c* OXIDASE MEMBRANES

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Valerie Wailin Hu

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To my mother, Kam Lit, a very special friend--

with best wishes on her birthday;

and

to the two special David's

in my life,

my husband and my father

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ABSTRACT

Cytochrome c oxidase is a membrane-bound enzyme of the mitochondrial respiratory chain which is ultimately responsible for almost all of the oxygen consumption in aerobic organisms. In a rapid four-electron process, it reduces molecular oxygen to water and, at the same time, couples the energy derived from this exothermic reaction with energy conservation by driving ATP synthesis. The proper functioning of cytochrome oxidase in oxygen reduction and energy coupling requires not only phospholipids but also an intact, vectorially-oriented membrane. In the study described briefly below, two different aspects of the cytochrome oxidase problem have been examined: 1) the nature of the metal sites in the isolated molecule with regard to their participation in the oxidation/reduction process, and 2) the functional role of lipids in the activation of the protein.

Part I deals with an investigation into the nature of the metal sites in cytochrome c oxidase. For this purpose, X-ray absorption edge spectroscopy was employed to examine the oxidation states of the copper and iron centers in the protein under oxidized, fully-reduced, and partially-reduced conditions. In addition, the edge spectra of oxidized and reduced plastocyanin (a "blue copper" protein containing a single Cu ion) as well as of a number of model Cu compounds in various oxidation states were obtained to establish energy ranges for the several bound to bound transitions of Cu in the different oxidation states. A comparison of the fine structure detail in the absorption edge spectra of cytochrome oxidase with those of the models indicates that one of the two

copper ions in the oxidized protein is in the +1 oxidation state. Upon reduction of the protein with dithionite, the second copper becomes Cu(I). The Fe K-edge spectra of cytochrome c oxidase reveals a small shift ($\sim$ 2 eV) towards lower energies upon reduction. This is comparable with the shift observed for the reduction of the heme iron in cytochrome c [1]. Studies on the cyanide complexes of cytochrome oxidase indicate that 1) cyanide has no effect on the Cu K-edge spectrum of oxidized cytochrome oxidase, 2) the $1s \rightarrow 3d$ transition of the Fe edge of oxidized oxidase is enhanced by the presence of cyanide, and 3) both of the Cu ions are reduced in the partially-reduced cyanide complex.

Part II of this thesis is concerned with an investigation into the lipid factors affecting protein activity and respiratory control in reconstituted cytochrome oxidase membranes. Delipidated cytochrome c oxidase was reconstituted with various natural and synthetic phospholipids and several aspects of protein-lipid interactions were studied. In a well-defined series of model membranes, it was shown that cytochrome oxidase activity, monitored at a constant temperature, was sensitive to the physical state of the lipids, being higher when the lipids were in the liquid-crystalline phase. In addition, the temperature at which the reconstitution was allowed to take place was found to affect the manifestation of phase-dependent oxidase activity. A passive charge diffusion model has been proposed to explain the observed phenomena.

The effect of protein on the lipid phase transition has also been investigated using light scattering techniques. A heating/cooling hysteresis in the absorbance versus temperature curves has been attributed

to a reversible aggregation and dispersal of the proteins in the bilayer which is dependent on the physical state of the lipids.

Several lipid factors have been investigated with regard to their influence on the reconstitution of cytochrome oxidase membranes exhibiting respiratory control. It was found that high lipid/protein ratios and the presence of acidic polar head groups are conducive to the establishment of respiratory control as evidenced by increased rates of oxygen consumption in the presence of chemical uncouplers. In addition, the mode of vesicle preparation affected the responsiveness of the samples towards uncoupling agents.

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"Many of us have been led astray by experiments that were 'so easily done'... . If we could only look ahead and see how long the easy road is and where it will lead us!"

Efraim Racker
in
A New Look at Mechanisms
in Bioenergetics

PART I. AN INVESTIGATION INTO THE NATURE OF THE METAL SITES IN CYTOCHROME *c* OXIDASE USING X-RAY ABSORPTION SPECTROSCOPY

I. GENERAL INTRODUCTION

Situated at the terminal end of the mitochondrial respiratory chain, cytochrome c oxidase (ferrocytochrome c: oxygen oxidoreductase; EC 1.9.3.1) catalyzes the electron transfer between cytochrome c and molecular oxygen. The importance of this membrane-bound reaction lies in the fact that the ultimate oxidation of fuel molecules by molecular oxygen to CO_2 and H_2O increases the energy conserving efficiency of aerobic organisms over that of anaerobic organisms (lacking the respiratory enzymes) by a factor greater than 5. This is accomplished by the coupling of the ATP synthesis reaction to the highly exothermic redox reactions of the respiratory chain, the last of which is the reduction of oxygen by cytochrome c oxidase. Since cytochrome oxidase is uniquely suited for the direct reduction of oxygen, considerable effort has been directed at unraveling the molecular details of its structure and function.

Cytochrome c oxidase is a highly complex molecule of molecular weight ~200,000 consisting of two heme A's, two copper ions, and seven distinct polypeptides per heme A [1,2]. The molecular weights of the individual polypeptide subunits of cytochrome oxidase from yeast and beef heart mitochondria are presented in Table I. The similarity between the subunit molecular weights of the enzymes derived from these two phylogenetically distant species attests to the highly conservative nature of this protein. With the yeast enzyme, it has been shown that the three largest subunits are synthesized in the mitochondrion, whereas the remaining four subunits are cytoplasmically synthesized [3]. In

TABLE I. A Comparison of the Subunit Molecular Weights of Beef Heart and Yeast Cytochrome c Oxidase

Subunit	Beef Heart[a]	Yeast[b]
I	35,400	40,000
II	24,100	33,000
III	21,000	22,000
IV	16,800	14,500
V	12,400	12,700
VI	8,200	12,700
VII	4,400	4,600

[a]From Ref. 2

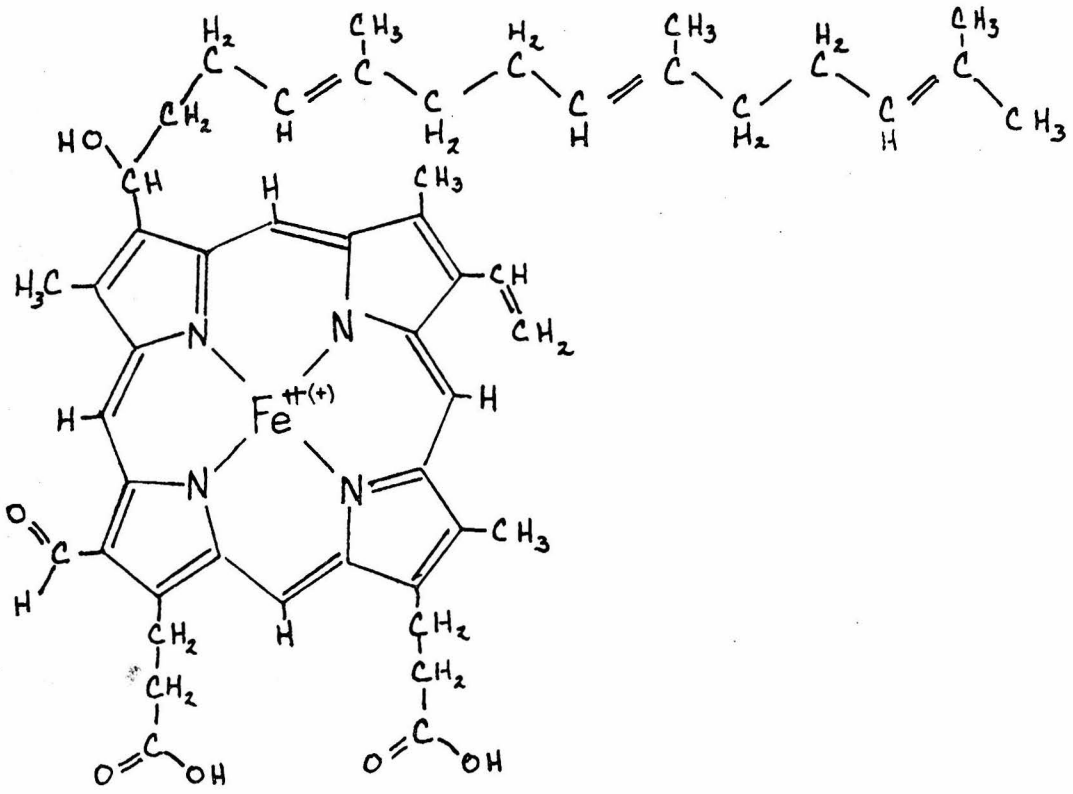
[b]From Ref. 4

addition, the mitochondrially synthesized subunits are larger, less acidic, and more hydrophobic than the cytoplasmic subunits [4]. Recently, Phan and Mahler reported the preparation of a catalytically active, water-soluble enzyme by removal of the three largest subunits of yeast cytochrome c oxidase [5]. The resulting four-subunit enzyme not only catalyzes the oxidation of ferrocytochrome c by molecular oxygen with kinetics similar to that of the seven-subunit enzyme, but also exhibits the same spectral properties as the whole protein. It was thus concluded that the catalytic sites are contained within this water-soluble fraction of the enzyme. However, since the three larger subunits appear to be essential for the biogenesis of the enzyme, it was suggested that the three larger subunits may be involved in the proper binding of the four-subunit enzyme to the membrane or, perhaps, in the energy coupling process.

In any case, it is clear that the membrane-associated cytochrome oxidase is a multimeric complex comprised of at least 14 polypeptide chains. The dimensions of this complex are roughly $50 \times 60 \times 80$ Å [6]. However, very little is known about the organization of the subunits in the complex. In addition, the positions of the hemes and coppers in the protein are as yet unidentified.

The structure of heme A is unusual in that it contains a long olefinic side chain attached to the C-2 carbon of the porphyrin ring (see Fig. 1)[7]. It has been suggested that this long side chain may play a direct role in the intramolecular shuttling of electrons between the metal centers of cytochrome oxidase [8]. Furthermore,

Fig. 1. The structure of heme A



unlike the heme in cytochrome c, it is noncovalently bound to the protein [9]. Other than the porphyrin nitrogens, the fifth and sixth ligands to the iron atoms in the oxidized (resting) protein are unknown, as are the ligands to the two copper atoms. It is known, however, that one of the heme irons is able to complex a variety of ligands, such as carbon monoxide and cyanide [9]. This ligand-binding heme is generally referred to as heme a_3. On the other hand, the two copper atoms are not known to bind to any ligands and are not easily removed from the intact protein by the usual complexing agents [8,10]. Thus, the enzyme has been fairly resistant to chemical dissection techniques.

In this respect, spectroscopic dissection has been a lot more successful. The optical absorption spectra of the protein in various oxidation states have been well characterized and confirm the existence of two distinct hemes in the presence of heme ligands [1,8]. However, the relative contributions of the heme components to the absorption bands as well as the copper contribution to the optical spectrum (in particular the 830 nm absorption band) are still matters of debate [11-13]. Electron paramagnetic resonance studies also provide evidence of the non-equivalence between the hemes of cytochrome oxidase, in that only half of the total heme iron in the protein is EPR-visible in the oxidized protein [14,15]. Similarly, a signal accounting for no more than half of the copper in the protein is also detected [14,16,17]. Since potentiometric titration of the oxidized protein indicates that it can accept four reducing equivalents [18], the EPR results are somewhat confusing if one assumes that all four metal centers must be

oxidized in the resting enzyme. One possible explanation for the apparent discrepancy between the EPR and the potentiometric titration data is that two of the metal centers (heme iron and copper) are antiferromagnetically coupled in the oxidized protein and are thus EPR-invisible.

Recent magnetic circular dichroism (MCD) and magnetic susceptibility data indicate that one of the hemes (heme a_3) is in the high-spin state in both the oxidized and reduced forms of the protein [19,20]. In addition, the magnetic susceptibility evidence strongly suggests that the high-spin heme is antiferromagnetically coupled to another paramagnetic species of spin 1/2 (a Cu(II) ion)in the oxidized state [20].

Taken together, these results would indicate that the two hemes in cytochrome c oxidase are indeed chemically, spectroscopically, and magnetically inequivalent, and that there appears to be a strong interaction between at least two of the metal centers in cytochrome oxidase. The distinction between the copper ions is less well defined. In point of fact, at least one of the copper atoms is spectroscopically invisible and no direct evidence has been obtained as to the oxidation states of the two copper atoms in the oxidized enzyme. In addition, the structure of both the copper and heme sites in the protein are unknown. It is clear, then, that these points must be resolved before the reaction mechanism for the catalytic electron transfer between the physiological reductant, ferrocytochrome c, and molecular oxygen can be fully developed.

Towards the resolution of these problems, x-ray absorption spectroscopy is proving to be a useful tool in structural studies of

metalloproteins [21]. In general, x-ray absorption spectroscopy involves excitation of the core electrons of an atom into the higher unfilled orbitals and, with increasing photon energy, into the continuum. As the electrons are excited to the higher bound states, the absorption coefficient rises steeply until the critical cutoff (Fermi edge) is reached, where the excited electron becomes unbound. At this point, the absorption coefficient drops suddenly and monotonically (see Fig. 2). This sawtooth function is called the absorption edge, and its energy is characteristic of the principal shell from which the electrons were excited, as well as the nature of the absorbing atom [22,23].

Upon expansion of the sawtooth spectrum, a set of closely spaced peaks, called the absorption edge fine structure (AEFS), can often be detected within an energy range of 30-40 eV just below the Fermi edge (see Fig. 3). These peaks correspond to the electronic transitions between the filled core levels and the unfilled outer orbitals. In turn this information may be related to the oxidation states and point symmetry of the absorbing atom by comparison with model compounds of known valence and structure [22,24].

In cases where the excited atoms are closely bound to other atoms, there is yet another feature to the absorption spectrum. This consists of the Kronig fine structure, or extended x-ray absorption fine structure (EXAFS) region extending several hundred eV above the Fermi edge where the slowly decreasing absorption coefficient exhibits an oscillatory behavior (see Fig. 4)[22]. This oscillation in the absorption coefficient is a result of the interference of the backscattered

Fig. 2. Variation of absorption coefficient as a function of photon energy in the vicinity of an absorption edge

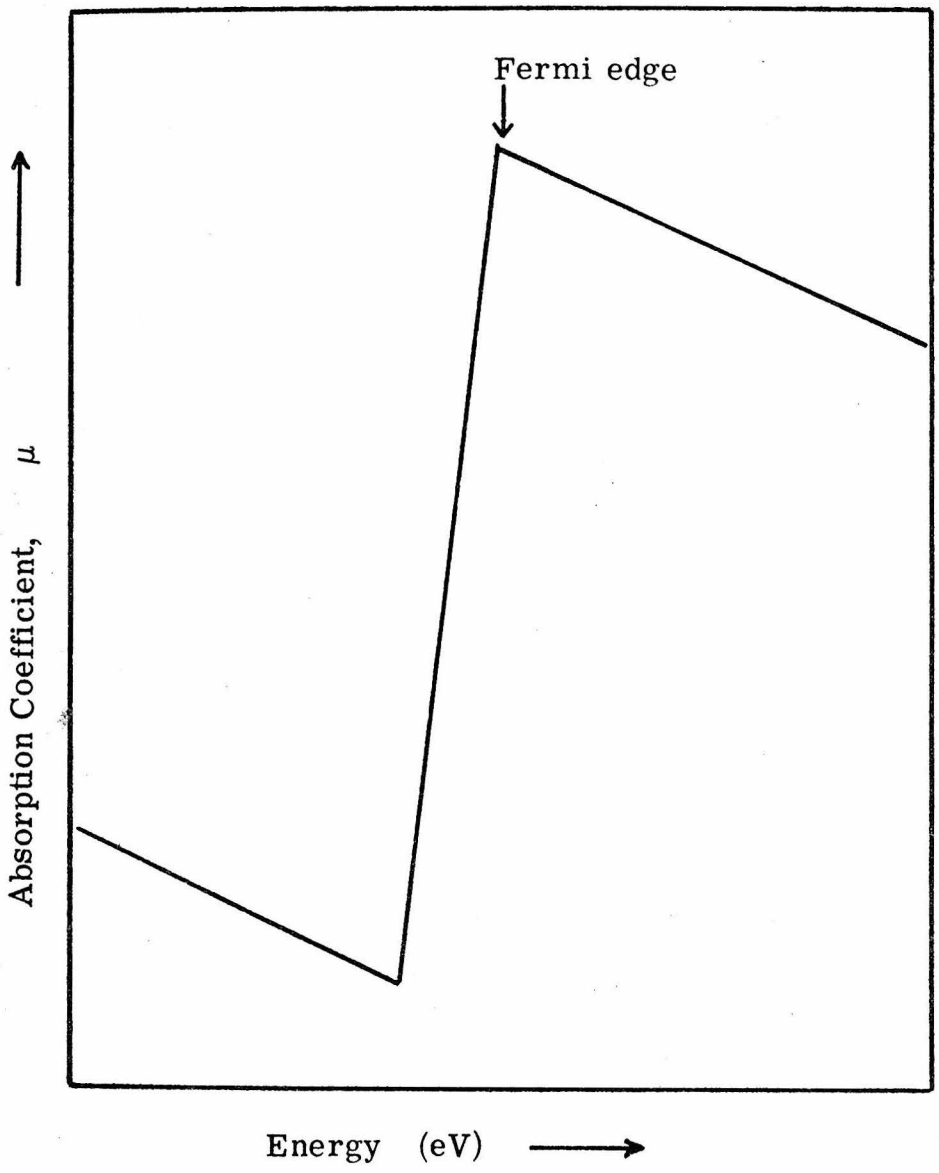

Fermi edge
Absorption Coefficient, μ
Energy (eV)

Fig. 3. An x-ray absorption spectrum showing absorption edge fine structure (AEFS)

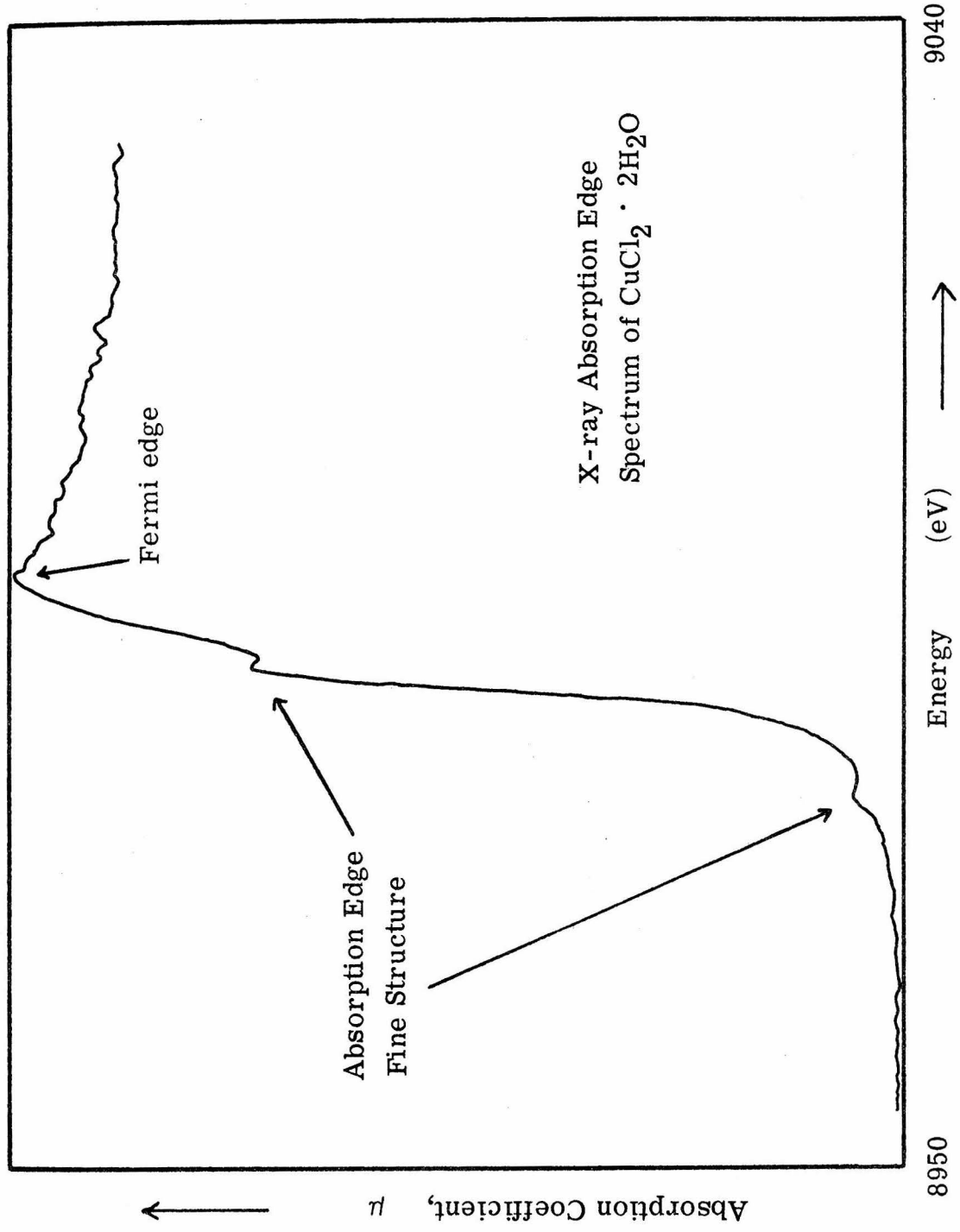
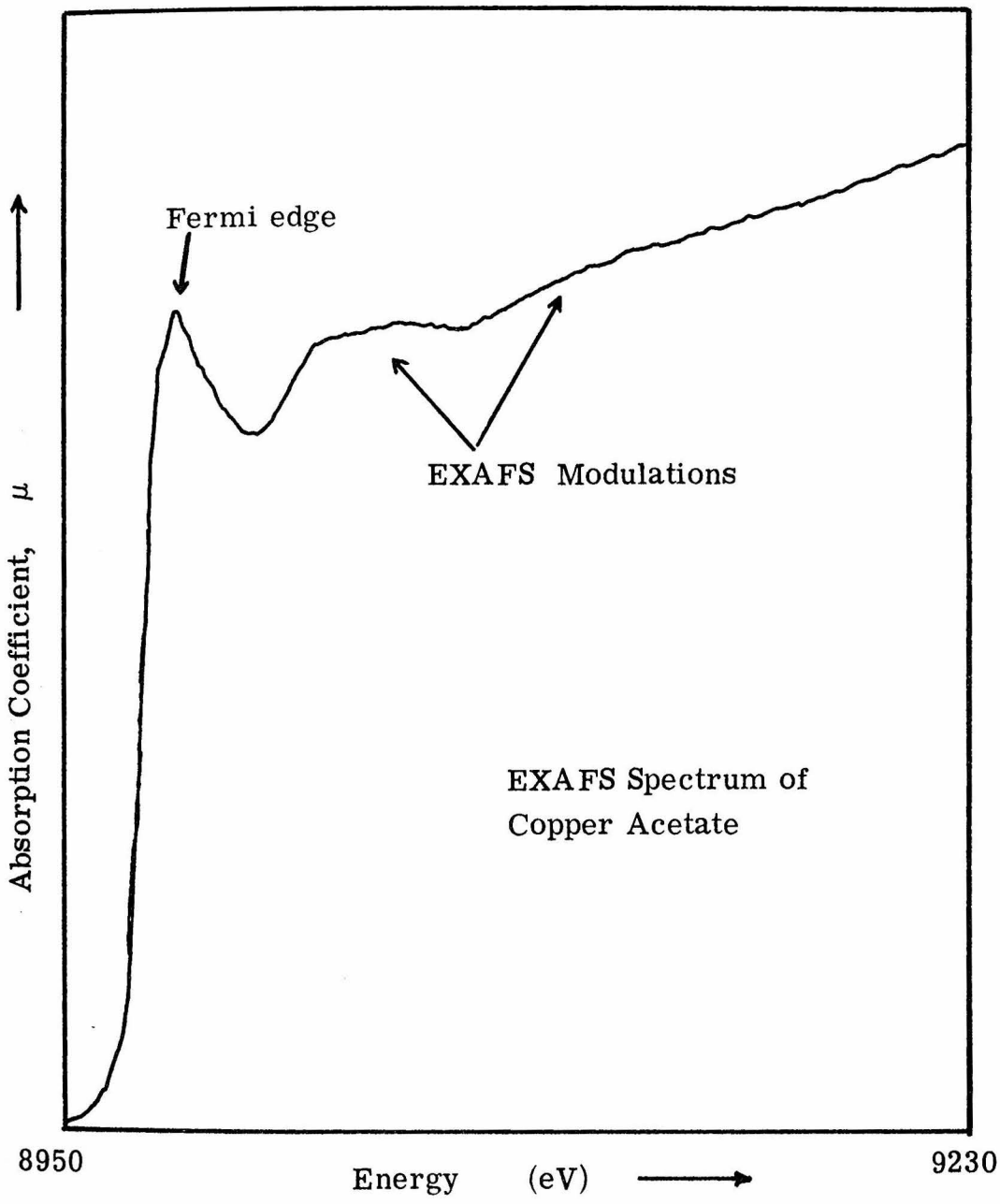

X-ray Absorption Edge
Spectrum of $CuCl_2 \cdot 2H_2O$
Fermi edge
Absorption Edge
Fine Structure
Absorption Coefficient, μ
Energy (eV)
8950
9040

Fig. 4. An x-ray absorption spectrum showing the extended x-ray absorption fine structure (EXAFS) region

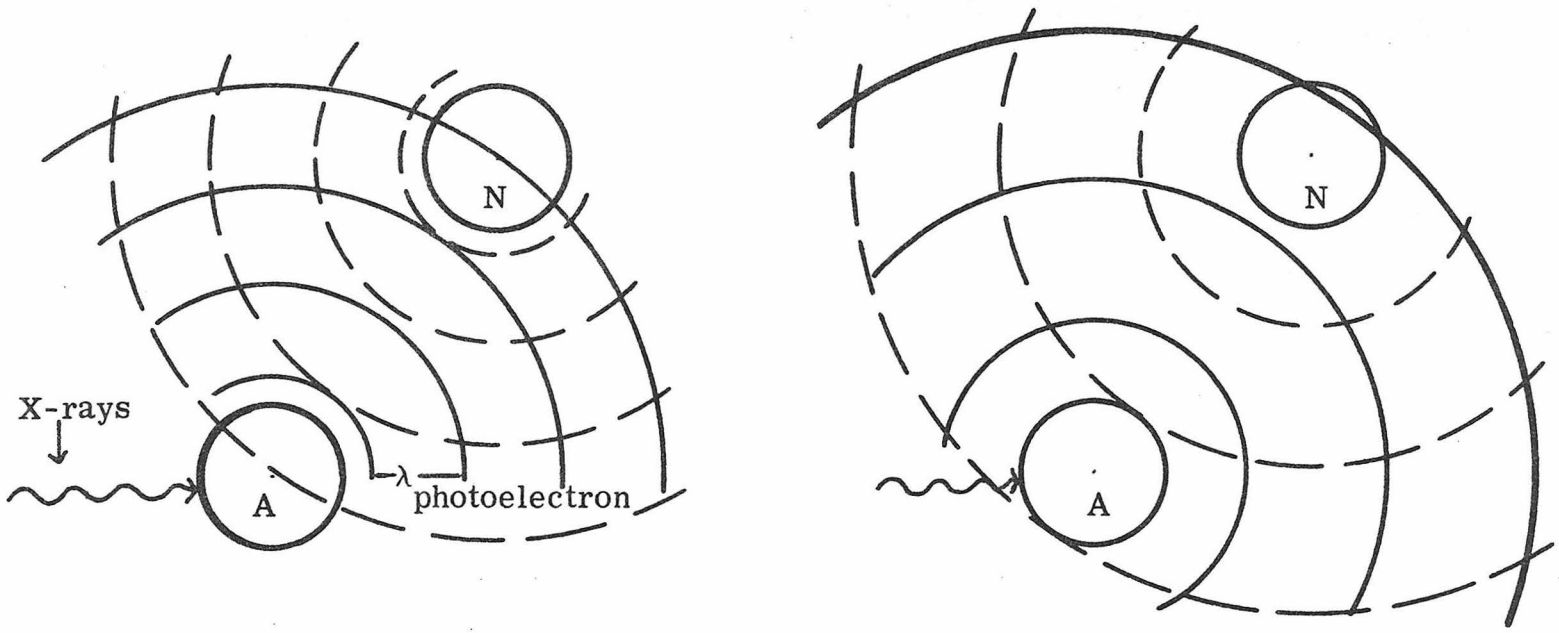

Fermi edge
EXAFS Modulations
Absorption Coefficient, μ
EXAFS Spectrum of
Copper Acetate
8950
Energy (eV)
9230

photoelectron wave with the outgoing photoelectron wave as the emitted photoelectrons are reflected off the neighboring atoms (see Fig. 5). The absorption coefficient reaches a maximum in this region when the outgoing and backscattered waves add constructively, and a minimum when they are 180° out of phase [25]. In principle, the frequency of the oscillations contains information about the interatomic distances between the absorber and the nearest neighbor atoms, while the amplitude of the oscillations is related to the nature of the surrounding atoms [26,27]. Thus, several types of information may be obtained from an x-ray absorption spectrum.

When applied to metalloproteins, x-ray absorption spectroscopy is potentially capable of providing information about the oxidation states and structure of the metal sites. In the past, such studies of biological macromolecules were limited by the low fluxes of conventional x-ray sources. However, with the introduction of high-flux synchrotron radiation sources, the feasibility of studying dilute biological samples has greatly increased. A dramatic example of the high sensitivity of currently available spectrometers has been the detection of Mn in the chloroplasts of a leaf, although the Mn concentration was on the order of 10-50 ppm. Other successful applications of x-ray absorption spectroscopy to biomolecules are illustrated by the following examples.

With regard to the AEFS region of the absorption spectrum, information can be obtained from both the position of the absorption edge and the fine structure details of the edge. For example, the edge shift between the oxidized and reduced forms of cytochrome c was found to be only 1 eV, a shift comparable to the shift observed between the covalent

Fig. 5. The mechanism of extended x-ray absorption fine structure The circles represent the crests of the photoelectron wave that is ejected from the excited atom and backscattered from the neighboring atom. In (a), the amplitudes of the emitted and reflected waves add constructively at the site of the absorbing atom giving rise to a maximum in the absorption probability. In (b), the emitted and backscattered waves add destructively at the absorbing atom, resulting in an absorption minimum.



A = Absorbing Atom

N = Neighboring Atom

(a) (b)

ferro- and ferrihexacyanides [28]. In contrast, the ionic divalent and trivalent iron fluorides show an edge shift of 5 eV. On the basis of these results, Shulman et al. discuss the covalency of the heme iron in cytochrome c. Another demonstration of the usefulness of edge positions involves the determination of the "coordination charge" of molybdenum (Mo) in the nitrogen-fixing enzyme, nitrogenase [29]. In a study of the absorption edge spectra of a number of model Mo compounds, Cramer et al. found a linear relationship between "coordination charge" and edge energy, the latter increasing by 10 eV for a charge increase of 5. By comparing the Mo-edge energy of nitrogenase to those of the model compounds, they estimated that the "coordination charge" of Mo in nitrogenase is 2.3 ± 0.3. Thus, edge positions can provide information about the overall charge density about the absorbing atom.

On the other hand, structural information may be derived from a thorough analysis of the absorption edge fine structure. For example, analysis of the AEFS of rubredoxin showed that the intensity of the $1s \rightarrow 3d$ transition of the iron-sulfur protein is 7 times greater than the corresponding transition in an octahedrally-coordinated iron-sulfur complex [28]. Since the iron in rubredoxin is known to be tetrahedrally coordinated to four sulfur ligands, the intensity increase of the $1s \rightarrow 3d$ transition is attributed to the increased p-character of the Fe-S bonds due to hybridization of the metal-(d) and ligand-(p) orbitals. Although the results obtained here are merely supportive of the already known structure, it is clear that similar analyses of the edge detail in the absorption spectra of metalloproteins of unknown metal coordination geometry can be informative.

In terms of structure determinations, the EXAFS region of the absorption spectra of metalloproteins is most applicable to problems involving metal-ligand distances. Using this technique, a number of investigators have determined the iron-sulfur distances in rubredoxin [30,31]. The bond lengths obtained were in agreement with the average distances obtained by crystal structure determinations. In addition, a change of $\sim$0.05Å in the bond lengths could be detected upon reduction of the protein. Similarly, the Fe-N bond distances in oxy- and carbonmonoxyhemoglobin were obtained and found to be constant within 0.02Å [32]. Furthermore, the EXAFS spectra of high and low affinity deoxyhemoglobin showed that the heme iron environments were the same to within 0.02Å. On this basis, the strain energy associated with the high and low affinity forms of hemoglobin has been estimated to be $\leq 4 \times 10^{-3}$ eV, a negligible fraction of the difference in the oxygen binding energy of the high and low affinity forms (0.15 eV). The implication of this finding is that the energies responsible for the increase in oxygen affinity are not localized on the heme. Thus, the EXAFS spectrum of a metalloprotein can yield important information about the structure of the metal site in the protein.

From the above examples, it is clear that the structural information obtained from x-ray absorption spectra is restricted to a very small portion of a biological macromolecule. Nevertheless, x-ray absorption spectroscopy employing synchrotron radiation has a number of special advantages. Since the broadband source is easily made tunable, a number of different metal centers may be conveniently studied. Also, samples

may be examined in the solid, liquid, or gaseous states. In this respect, x-ray absorption is complementary to x-ray diffraction techniques.

In the work presented below, we have applied the techniques of x-ray absorption spectroscopy to the study of the oxidation states of the metal atoms in cytochrome c oxidase. In Chapter II we analyze the absorption edge fine structure of the copper K-edge spectra of oxidized and reduced cytochrome c oxidase in relation to the AEFS of a large number of model copper compounds and determine the oxidation states of the copper atoms in the oxidized protein. The results obtained suggest the presence of Cu(I) in the oxidized protein and have certain implications regarding the mechanism of electron transfer to oxygen.

Chapter III deals with the oxidation states of the Cu atoms in a partially reduced cyanide complex of cytochrome c oxidase. In addition, the effect of cyanide on the iron K-edge spectrum of the oxidized protein is discussed.

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II. X-RAY ABSORPTION EDGE STUDIES ON OXIDIZED AND REDUCED CYTOCHROME c OXIDASE

1. Introduction

Cytochrome c oxidase is the membrane-bound enzyme involved in the terminal step of respiration, that is, the reduction of molecular oxygen to water. It is of central importance to all aerobic organisms, since the exothermic reaction of the enzyme with oxygen is coupled with energy conservation in the form of ATP, which is the "biological currency" for almost all energy-requiring biological reactions. In its functional form, the oxidase contains two heme irons and two copper ions, each of which appears to be distinct from the other, yet interacting. Electron paramagnetic resonance (EPR) studies on the oxidized protein suggest the presence of a very unusual Cu(II) and a low-spin FE(III) (1-4). The other Cu and heme iron atoms appear to be EPR-silent. One possible explanation for this is that the silent Cu and heme iron are antiferromagnetically coupled to each other. An alternate explanation would be the presence of Cu(I) or low-spin Cu(III) and Fe(II). The strongest argument against Cu(I) and Fe(II) in the fully oxidized state of the protein is provided by the results of coulometric titrations which show that the oxidase can be titrated reversibly with four equivalents of electrons generated under anaerobic conditions (5). This argument involves the assumption that the only redox-active centers in the protein are the metal ions. Upon reduction, the protein exhibits no EPR spectrum, implying full reduction of the coppers and heme irons to diamagnetic Cu(I) and Fe(II) (4,6).

Thus, the current picture of cytochrome oxidase is one in which the oxidized protein contains two ferric irons and two cupric coppers, and the reduced protein two ferrous irons and two cuprous coppers. Unfortunately, this picture is based mainly on indirect evidence and the question of the oxidation states of some of the metal centers (particularly of the coppers) in the oxidized protein must still be considered as unsettled at this time.

In an effort to resolve this question, we have applied the technique of X-ray absorption edge spectroscopy to examine the charge states of the Cu and Fe centers in cytochrome c oxidase. X-ray absorption spectroscopy in the neighborhood of the K-shell absorption edge of the metal can provide information about the charge density, degree of covalency, and the point symmetry of the metallic site. In the present work, we have used the intense broadband synchrotron radiation from the SPEAR storage ring at the Stanford Linear Accelerator Center. The advantage of this source is that the X-ray flux is high over a continuum of energies (down to the critical cutoff), thus permitting X-ray absorption spectroscopy to be done on relatively dilute samples (e.g., < 1 mM). Thus, this method provides a means of studying the previously elusive Cu atoms in cytochrome oxidase and a new probe into the electronic environments about the Fe atoms. In addition to the cytochrome oxidase spectra, we have also obtained edge spectra of another copper protein (plastocyanin) and of a number of model copper compounds. These spectra have been analyzed in terms of peak positions in the different oxidation states, and are used as a starting point in the analysis of the oxidase spectra.

2. Materials and Methods

Cytochrome c Oxidase. Bovine heart cytochrome c oxidase was the generous gift of Dr. Tsoo E. King and coworkers at the State University of New York at Albany. It was suspended in a 0.5% sodium cholate—50 mM sodium phosphate buffer, pH 7.4, at concentrations of either 0.5 mM or 1.25 mM in total heme A. The reduced protein was prepared by dissolving a 50-fold excess of solid sodium dithionite in a solution of the oxidized oxidase under nitrogen. About 200 µl of sample were required for each measurement. The samples were kept frozen in liquid nitrogen until spectroscopic measurements were made. The X-ray absorption measurements were performed at room temperature.

Plastocyanin. Bean plastocyanin (M. W. 10,800) at a concentration of 1 mM in 0.05 M ammonium acetate, pH 6.0, was the gift of Mr. David Dooley and Dr. Harry Gray. A participant in photosynthetic electron transport, plastocyanin contains one copper per functional unit and no other metal atoms. The reduced protein was prepared by adding 0.01 ml of 0.2 M nitrogen-flushed sodium dithionite solution to 0.2 ml of the oxidized protein under nitrogen. The blue color of the oxidized form immediately disappeared upon addition of the dithionite, indicating reduction of the copper center. The samples were kept frozen in liquid nitrogen until the measurements were made.

Model Compounds. CuCl, CuI, CuCN, $Cu(Me_2PIMI)CO$, $[Cu(HB(pz)_3)]_2$, and $CuCO(HB(pz)_3)$ were used as representatives of the +1 oxidation state of copper. Reagent grade CuCl, CuI and CuCN were used. The latter three

compounds were kindly donated by Dr. Robert Gagné and are shown in Figure 1.

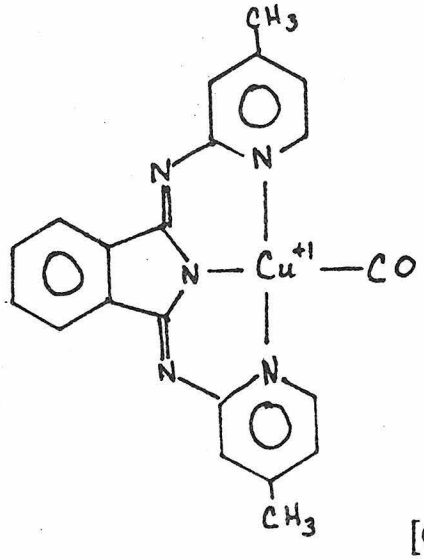
Of the Cu(II) compounds, $Cu_2(PAA)_2en$ and the binuclear Cu(II) complex were gifts from Dr. Gagné. $Cu(dien)(NO_3)_2$, $CuCl(HB(pz)_3)$, $Cu(tren)Im(PF_6)_2$, and Cs_2CuCl_4 were generously contributed by Dr. Harry Gray and coworkers. $CuCl_2 \cdot 2H_2O$ was commercially obtained. Data for $K_2Cu(bi)_2$, CuO, $Cu(II)(H_{-3}G_4)^{2-}$, and Cu(II)-periodate were kindly made available to us by Dr. William E. Blumberg, personal communication. The structures of some of the compounds are given in Figure 2.

Dr. Blumberg also contributed data on the Cu(III) compounds: Cu_2O_3, $KCu(bi)_2$, $Cu(III)(H_{-3}G_4)^-$, and Cu(III)-periodate. Figure 3 shows the structure of $KCu(bi)_2$ and $Cu(III)(H_{-3}G_4)^-$.

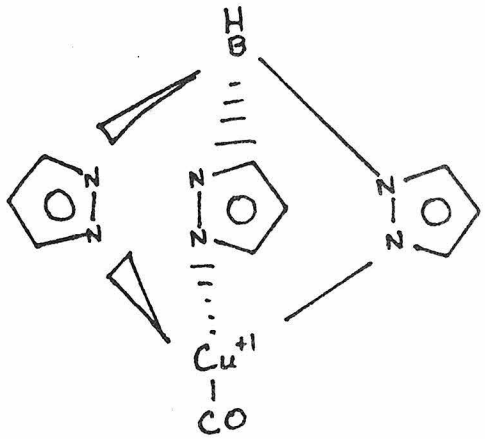
X-Ray Absorption Measurements. The X-ray absorption measurements were performed at the Stanford Synchrotron Radiation Project. The experimental apparatus is depicted in Figure 4. The collimated, broadband synchrotron radiation from the SPEAR storage ring passed through a channel-cut silicon crystal monochromator. The transmitted beam then passed through a 15 cm nitrogen-filled ion chamber and onto the sample (7). The Cu or Fe fluorescence Kα radiation from the target was detected by a nine-element array of NaI scintillation counters. Data were collected by accumulating counts from the NaI detectors into individual channels for a period of one second. A weighted sum of the counters, as well as the output of the voltage controlled oscillator, I_o, were stored in the computer. The monochromator was advanced by approximately 0.2 eV, and the cycle was repeated. For the model compounds, a single scan on the neat

Figure 1. Structures of several Cu(I) model compounds

$Cu(Me_2PIMI)CO$



$CuCO(HB(pz)_3)$



$[Cu(HB(pz)_3)]_2$

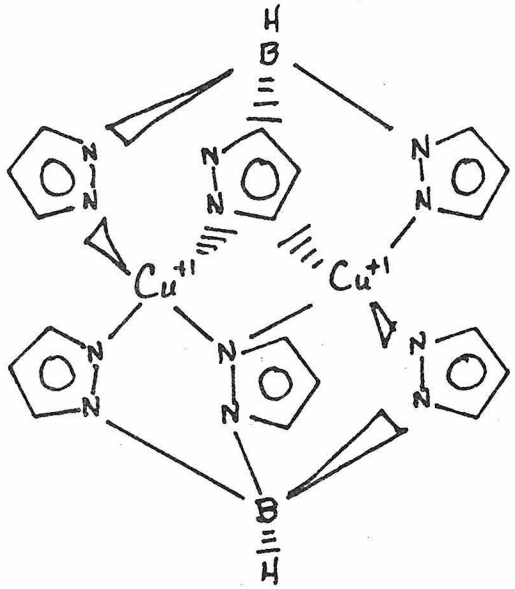
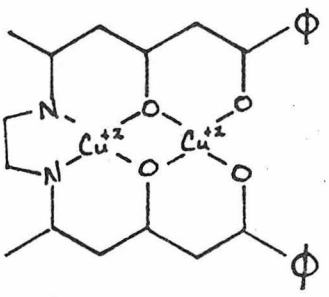
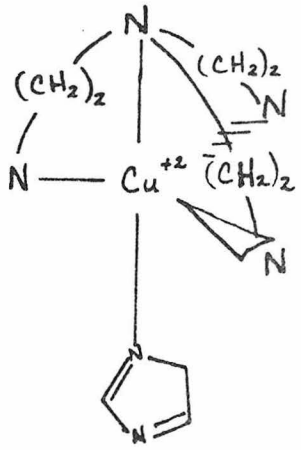


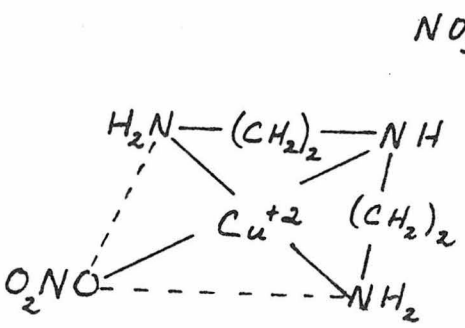
Figure 2. Structures of a number of Cu(II) model compounds



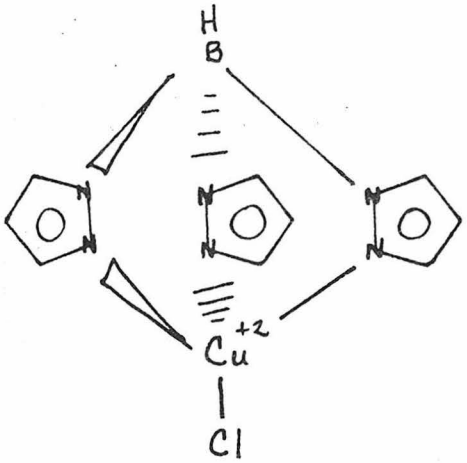
$Cu_2(PAA)_2en$



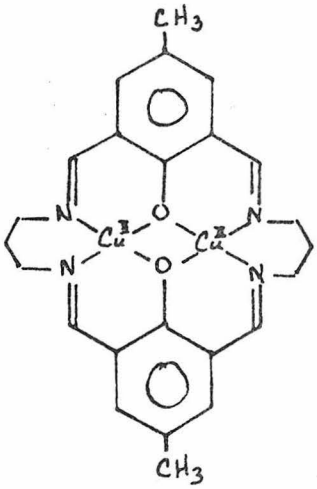
Cu(tren)Im



$Cu(dien)(NO_3)_2$



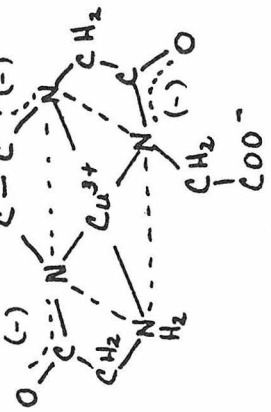
$CuCl(HB(pz)_3)$



Cu(II)-dimer

Figure 3. Structures of Cu(III) biuret and Cu(III) tetraglycine

$Cu(III)(H_{-3}G_4)^-$



$KCu(bi)_2$

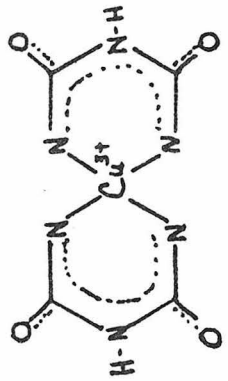
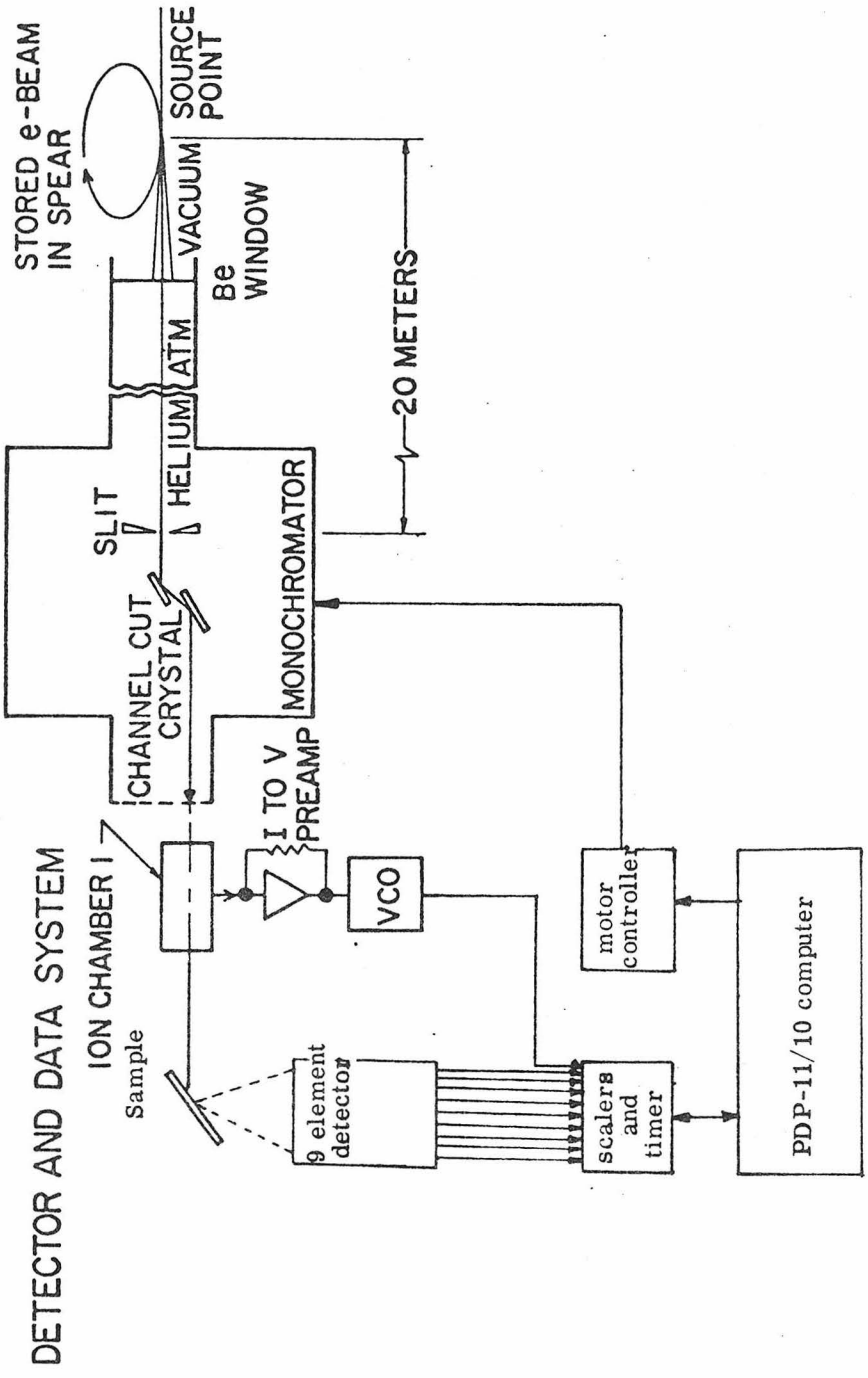


Figure 4. Schematic diagram of the experimental set-up


STORED e-BEAM IN SPEAR
SOURCE POINT
VACUUM
Be WINDOW
ATM
HELIUM
SLIT
CHANNEL CUT CRYSTAL
MONOCHROMATOR
20 METERS
DETECTOR AND DATA SYSTEM
ION CHAMBER I
Sample
I TO V PREAMP
VCO
9 element detector
motor controller
scalers and timer
PDP-11/10 computer

solid afforded sufficiently precise data. For the protein data, four or more individual spectra were recorded and then later summed.

Data Analysis. The transition metal X-ray fluorescence radiation emerging from the sample is proportional to the photoabsorption cross section (8). For dilute samples, there is also present an unavoidable background due to compton and elastic scattering, which has a very nearly linear dependence upon the energy over the 100 eV range studied. This linear background was removed by fitting a straight line to the data below the absorption edge and subtracting the fit from the data set.

Since the $1s \rightarrow 3d$ and $1s \rightarrow 4s$ transitions fall on the rapidly rising slope of the dominant $1s \rightarrow 4p$ transition, their positions and intensities are difficult to determine visually. Therefore, their positions and intensities were determined by subtracting from the data a smooth background curve representing the $1s \rightarrow 4p$ contribution to the absorption spectrum. The background function was taken to be a polynomial over a ±4.0 eV range centered about the apparent peak position, and the fitting criterion forced the polynomial to fit the data relatively well at the extremes of the 4 eV range. Thus the polynomial subtraction tends to effectively remove the $1s \rightarrow 4p$ spectral contribution with a minimum distortion of the signal. This assumption was tested by successively subtracting polynomials of order 1, 2, 3, and 4, and by varying the region of the polynomial subtraction. The transition energies were determined by fitting a parabola to the remaining data, and taking the energy (in eV) to be the center of the parabola. Finally, the transition strengths were calculated by integrating the data over a

±1.0 eV range about the calculated transition energy. The integral was normalized to the photoabsorption coefficient at 9020 eV, where the absorption coefficient is insensitive to the details of the bound-to-bound transitions but is somewhat modulated by the so-called extended fine structure (EXAFS). This modulation is typically 10% or less and is the chief uncertainty in this normalization procedure. The integration procedure was found to be sensitive to the limits of integration, but the relative strengths of corresponding transitions among different compounds were found to be independent of the integration limits to a precision of ±20%.

Optical Spectra. The optical spectra of the irradiated and unirradiated cytochrome oxidase samples were obtained on a Beckman Acta CIII UV-visible spectrophotometer using a specially constructed air-tight optical cell. The reduced oxidase was diluted appropriately with nitrogen-flushed buffer for the visible absorption measurements and was handled exclusively under nitrogen. The spectra obtained verified the state of reduction of the samples as compared with previously published spectra (9) and indicated that no changes occur in the heme chromophores upon prolonged exposure to X-rays.

Electron Paramagnetic Resonance Spectra. The EPR spectra of the irradiated and unirradiated oxidized samples of cytochrome oxidase were obtained on a Varian E-line EPR spectrometer operating at 6°K. The spectra demonstrated the EPR features usually indicative of oxidized cytochrome oxidase (1) and furthermore verified that no changes associated with the EPR-visible parametric species had occurred upon

irradiation.

Cytochrome Oxidase Activity Assays. The activity of the irradiated protein was compared to that of the unirradiated protein by measuring the rate of oxygen consumption in a reaction mixture containing the reconstituted oxidase and reducing substrates (cytochrome c and ascorbate). Cytochrome oxidase was reconstituted with asolectin (mixed soybean lipids) using the procedure described by Eytan et al. (10). The polarographic activity assay has been described elsewhere (11,12). The activities of both the unirradiated and irradiated protein were roughly 6 nmol O_2/nmol heme A/sec. Thus, x-irradiation was shown to have no deleterious effect on the activity of the protein.

3. Results

Model Compounds. The K-absorption edge spectral data of the Cu(I) compounds are presented in Table 1. The lowest energy absorption in all of the compounds except $[Cu(HB(pz)_3)]_2$ and $CuCO(HB(pz)_3)$ has been assigned to the forbidden $1s \rightarrow 4s$ transition. The next absorption is much stronger than the first and occurs between 2.7 and 6.2 volts to higher energy. It is attributed to the fully allowed $1s \rightarrow 4p$ transition. In Table 2, the energy separation between these assigned transitions for each compound is compared with the energy separation obtained from spectroscopic tables (13) for the corresponding excited states of the Z + 1 or, in this case, the Zn(II) ion. The validity of this comparison is discussed in detail by Shulman et al. (14). We see that as the ligand geometry proceeds from square planar to tetrahedral the 4s-4p separation

TABLE 1. X-Ray Absorption Edge Data for Cu(I) Compounds*

Compound	Transition Energies (eV)		
	1s → "4s"	1s → 4p	1s → 5p
$Cu(Me_2PIMI)CO$	8982.2	8988.4	8996.0
CuCN	8982.9	8988.3	8997.0
CuCl	8984.0	8987.8	
CuI	8984.0	8986.6	
$[Cu(HB(pz)_3)]_2$	8984.8		8996.7
$CuCO(HB(pz)_3)$	8985.2		8997.0

*The absorption edge spectra of these compounds are presented in Appendix I.

TABLE 2. A Comparison of the (4s - 4p) Energy Level Separations for the Zn(II) Ion and the Cu(I) Compounds Studied

Compound	Description	Energy Level Separation (eV) (4s - 4p)
Zn(II)	free ion	6.1
$Cu(Me_2PIMI)CO$	square planar	6.2
CuCN		5.4
CuCl	tetrahedral-ionic	3.9
CuI	tetrahedral-ionic	2.7
$[Cu(HB(pz)_3)]_2$	tetrahedral-covalent	~ 0
$CuCO(HB(pz)_3)$	tetrahedral-covalent	~ 0

decreases, which is what one would expect for increased s-p mixing. In the tetrahedrally coordinated covalent pyrazolyl borate complexes, the 4s-4p splitting seems to have disappeared altogether, indicating complete s-p mixing resulting in a single hybrid state. Note that the energy of the transition in this case is between that of the $1s \rightarrow 4s$ and $1s \rightarrow 4p$ transitions of the other Cu(I) complexes. Thus, for these two compounds, it is not valid to speak of the $1s \rightarrow 4s$ or the $1s \rightarrow 4p$ transition separately. However, for the sake of tabulation, the energy of this $1s \rightarrow (4s\text{-}4p)$ transition is listed together with the $1s \rightarrow 4s$ transitions of the other Cu(I) compounds. For this reason, we denote this set of transitions as $1s \rightarrow$ "4s". The spectral features above the 4p transition are presumably due to transitions from the 1s to 5p and higher p orbitals.

Table 3 shows the edge data of a number of Cu(II) compounds. The lowest energy absorption is very weak and has been assigned to the symmetry-forbidden $1s \rightarrow 3d$ transition. The second peak or shoulder at higher energies corresponds to the $1s \rightarrow 4s$ transition, and the maximum corresponds to the $1s \rightarrow 4p$ transition. The energy differences among these transitions are compared with the energy level separation among the 3d, 4s, and 4p levels of the Zn(III) ion in Table 4. We see that the most ionic of the Cu(II) compounds, $CuCl_2 \cdot 2H_2O$, bears the closest fit to the ionic Zn(III) levels, as expected, since atomic level comparisons are only relevant to very ionic compounds.

The Cu(III) spectral data are shown in Table 5. In the biuret, oxide, and periodate complexes, the $1s \rightarrow 3d$ transitions are extremely

TABLE 3. X-Ray Absorption Edge Data for Cu(II) Compounds*

Compound	Transition Energies (eV)		
	1s → 3d	1s → 4s	1s → 4p
$K_2Cu(bi)_2$	8979.8	8986.2	?
CuO	8979.4	8986.2	9000.0
$Cu_2(PAA)_2en$	8979.9	8986.5	9000.0
$Cu(II)(H_{-3}G_4)^{2-}$	8978.1	8986.8	8997.9
$Cu(tren)Im(PF_6)_2$	8979.2	8987.0	8997.8
Cs_2CuCl_4	8978.5	8987.1	8993.7,8997.0
$Cu(dien)(NO_3)_2$	8979.4	8987.6	8995.0
$CuCl(HB(pz)_3)$	8979.1	8988.1	8996.5
$CuCl_2 \cdot 2H_2O$	8978.8	8988.2	8995.9
Cu(II)-periodate	weak	weak	8998.0
binuclear Cu(II) complex	8979.3	weak	9000.0

*The absorption edge spectra of these compounds are presented in Appendix II.

TABLE 4. A Comparison of the (3d - 4s), (4s - 4p), and (3d - 4p) Energy Level Separations for the Zn(III) Ion and the Cu(II) Compounds Studied

Compound	Energy level separation (eV)		
	(3d-4s)	(4s-4p)	(3d-4p)
Zn(III)	10.0	7.9	17.8
$K_2Cu(bi)_2$	6.4		
CuO	6.8	13.8	20.6
$Cu_2(PAA)_2en$	6.6	13.5	20.1
$Cu(II)(H_{-3}G_4)^{2-}$	8.7	11.1	19.8
$Cu(tren)Im(PF_6)_2$	7.8	10.8	18.6
Cs_2CuCl_4	8.6	6.6(9.9)	15.2(18.5)
$Cu(dien)(NO_3)_2$	8.2	7.4	15.6
$CuCl(HB(pz)_3)$	9.0	8.4	17.4
$CuCl_2 \cdot 2H_2O$	9.4	7.7	17.1
binuclear Cu(II) complex			20.7

TABLE 5. X-Ray Absorption Edge Data for Cu(III) Compounds*

Compound	Transition Energies (eV)		
	1s → 3d	1s → 4s	1s → 4p
Cu_2O_3	8978.6	8985.7	8998.5
$KCu(bi)_2$	8978.4	8986.4	8998.6
Cu(III)-periodate	8978.0	8987.0	9000.0
$Cu(III)(H_{-3}G_4)^-$	8980.6	8987.8	9001.0

*The absorption edge spectra of these compounds are presented in Appendix III.

weak and occur roughly 7-9 volts below the $1s \rightarrow 4s$ transition. In the tetraglycine the $1s \rightarrow 3d$ peak is stronger but shifted about 2 volts to higher energy with a similar shift in the $1s \rightarrow 4s$ peak. The $1s \rightarrow 4p$ peaks occur 20-22 volts above the 3d peak and the spectra are fairly featureless above this peak. Zn(IV) spectroscopic data are not available for comparison.

Cytochrome c Oxidase. The Cu K-edges of both the oxidized and the reduced cytochrome c oxidase are shown in Figure 5. Upon comparison of the spectra of the oxidized and the reduced protein, one immediately notices a net shift in intensity towards lower energies upon reduction, as well as a definite change in the shape of the edge. Five peaks or shoulders are observed in the oxidized spectrum, and at least four are observed in the reduced spectrum. The assignment of the peaks will be discussed below.

Figure 6 compares the results obtained with the model compounds and cytochrome oxidase. The data from the model compounds establish an energy range for each of the transitions in the various oxidation states of copper. The peaks occurring in the cytochrome oxidase spectra are represented as discrete lines. A fair amount of overlap is seen among the transitions of the different oxidation states which gives rise to some ambiguity in the assignment of peaks in the oxidase spectra. In particular, all the transitions for Cu(II) are indistinguishable from those of Cu(III). However, the $1s \rightarrow$ "4s" transitions of the Cu(I) compounds, at about 8983 eV, do not overlap any transitions of the Cu(II) or Cu(III) compounds. Thus, the existence of a transition in this range

Figure 5. The copper K-edge spectra of oxidized—and reduced---cytochrome c oxidase.

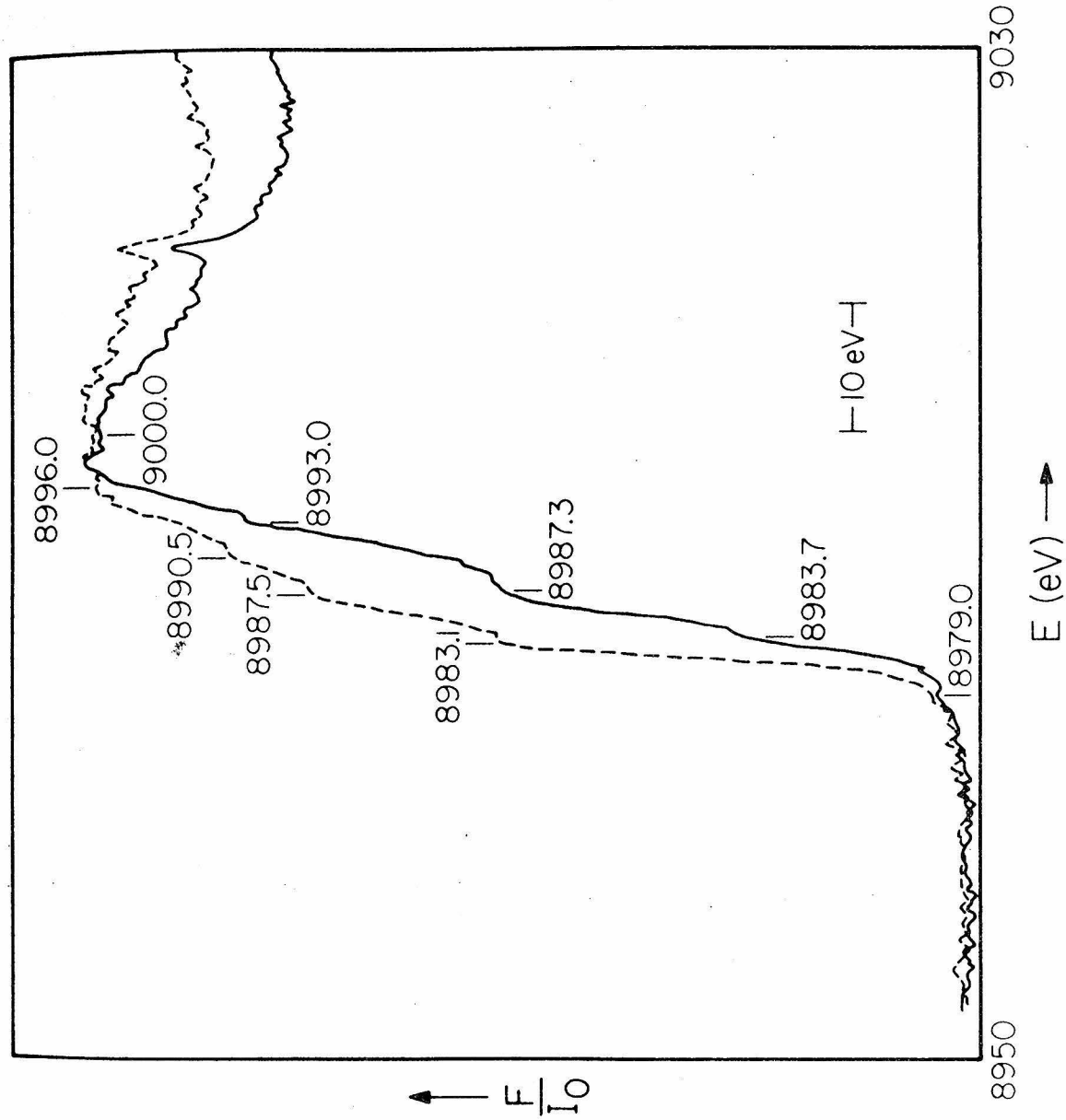
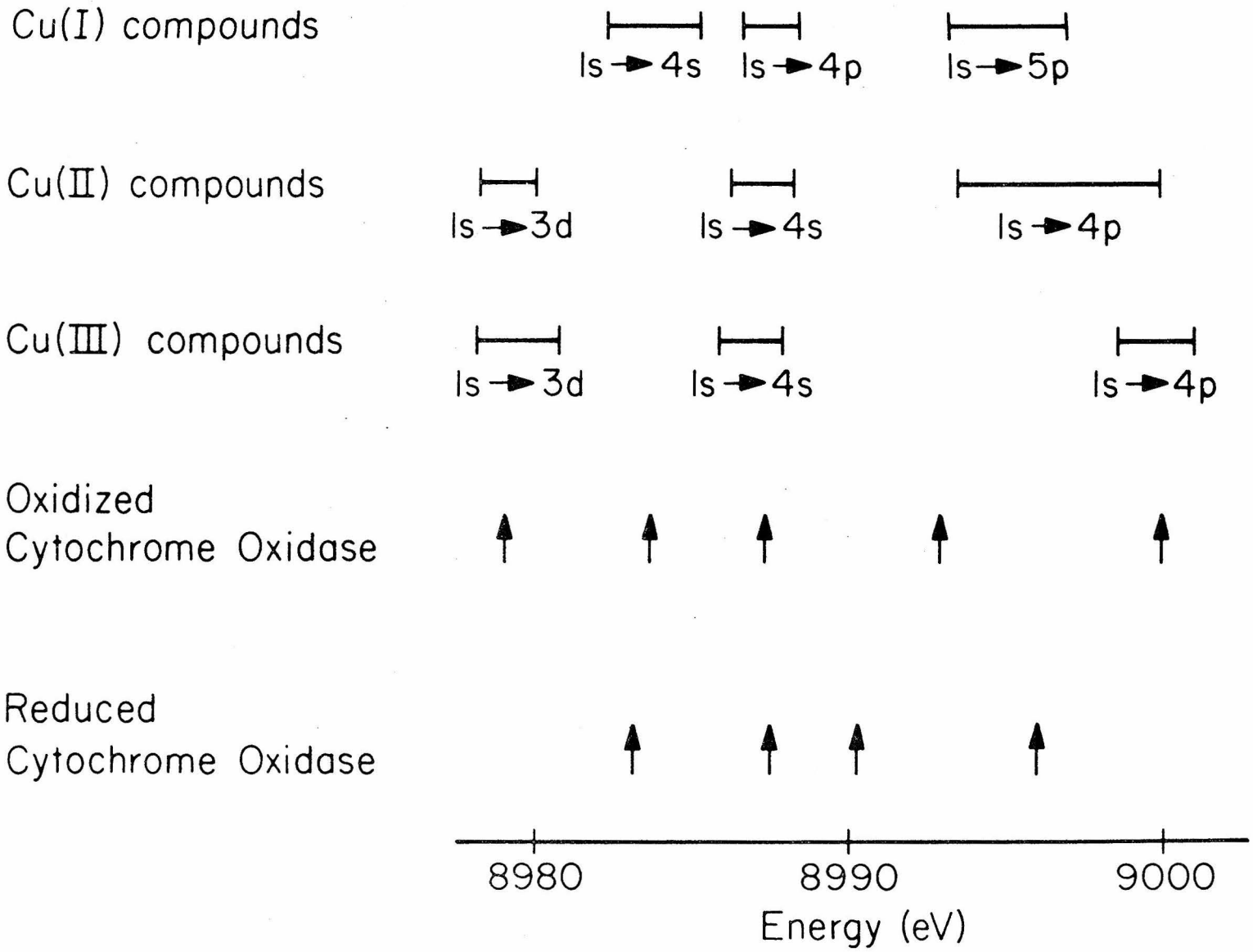

8996.0
9000.0
8990.5
8993.0
8987.5
8987.3
8983.1
8983.7
8979.0
10 eV
9030
8950
E (eV)
F/I0

Figure 6. A graphical comparison of the transition energy data for the Cu(I), Cu(II), and Cu(III) model compounds with the transitions observed in oxidized and reduced cytochrome c oxidase.


Cu(I) compounds
1s → 4s
1s → 4p
1s → 5p
Cu(II) compounds
1s → 3d
1s → 4s
1s → 4p
Cu(III) compounds
1s → 3d
1s → 4s
1s → 4p
Oxidized
Cytochrome Oxidase
Reduced
Cytochrome Oxidase
8980
8990
9000
Energy (eV)

is strong evidence for the presence of Cu(I).

In the spectrum of reduced cytochrome oxidase, we see that the lowest energy peak falls at 8983.1 eV which is well within the range for the 1s → "4s" transition of Cu(I). The next few peaks fall in the range for 1s → 4p and 1s → higher p transitions of Cu(I) compounds. No attempt is made to make separate peak assignments for the two individual coppers in cytochrome oxidase.

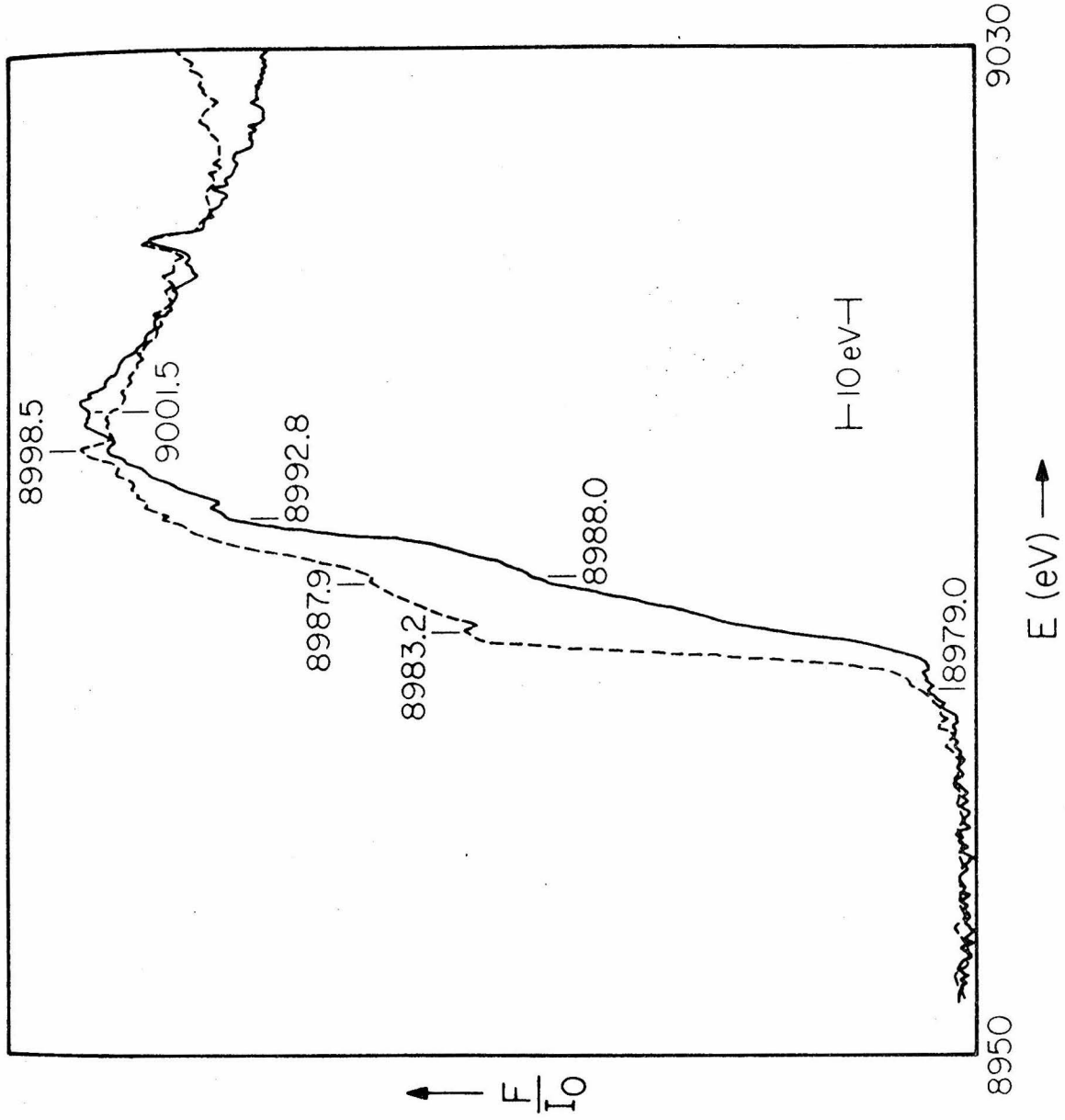
The oxidized oxidase spectrum exhibits a very weak, barely visible 1s → 3d transition at 8979 eV. According to the model compound data, this may be attributed to either a Cu(II) or a Cu(III). The next transition at 8983.7 eV, however, clearly corresponds to a 1s → "4s" transition of a Cu(I). Intensity measurements on properly normalized spectra show that it is half the intensity of the corresponding 1s → "4s" peak in the reduced oxidase spectrum (see Table 6). The remaining peaks fall in the region of overlap between the 1s → 4p transition of Cu(I) and the 1s → 4s and 1s → 4p transitions of Cu(II) and Cu(III). Thus, it is not possible to make unambiguous assignments for these peaks.

Plastocyanin. The K-edge absorption spectra of oxidized and reduced bean plastocyanin are shown in Figure 7. In both oxidized and reduced states, the plastocyanin spectra are quite similar to the Cu K-edges of the oxidase in the oxidized and in the reduced state respectively, especially in the high energy regions. The prominent exception is that the low energy peak assigned to the 1s → "4s" transition of Cu(I) in oxidized cytochrome oxidase is not present in the spectrum of oxidized plasto-

TABLE 6. Relative Intensity Data for the Cu(I) 1s → 4s Transition of the Cu(I) Model Compounds, Reduced Plastocyanin, and Cytochrome c Oxidase

Compound	Relative Intensity
$Cu(Me_2PIMI)CO$	0.128
CuCN	0.136
CuCl	0.075
CuI	0.112
$[Cu(HB(pz)_3)]_2$	0.069
$CuCO(HB(pz)_3)$	0.074
Reduced plastocyanin	0.122
Cytochrome c oxidase	
· reduced	0.102
· oxidized	0.057

Figure 7. The copper K-edge spectra of oxidized—and reduced---bean plastocyanin


8998.5
9001.5
8992.8
8988.0
8987.9
8983.2
8979.0
10 eV
9030
8950
E (eV)
F/I0

cyanin.* This peak, however, is present in the spectrum of the reduced protein and is assigned to the $1s \rightarrow$ "4s" transition of Cu(I). Note that the lowest energy transition in the oxidized spectrum which is assigned to the $1s \rightarrow 3d$ transition of Cu(II) apparently disappears upon reduction. This is consistent with Cu going from Cu(II) to Cu(I).

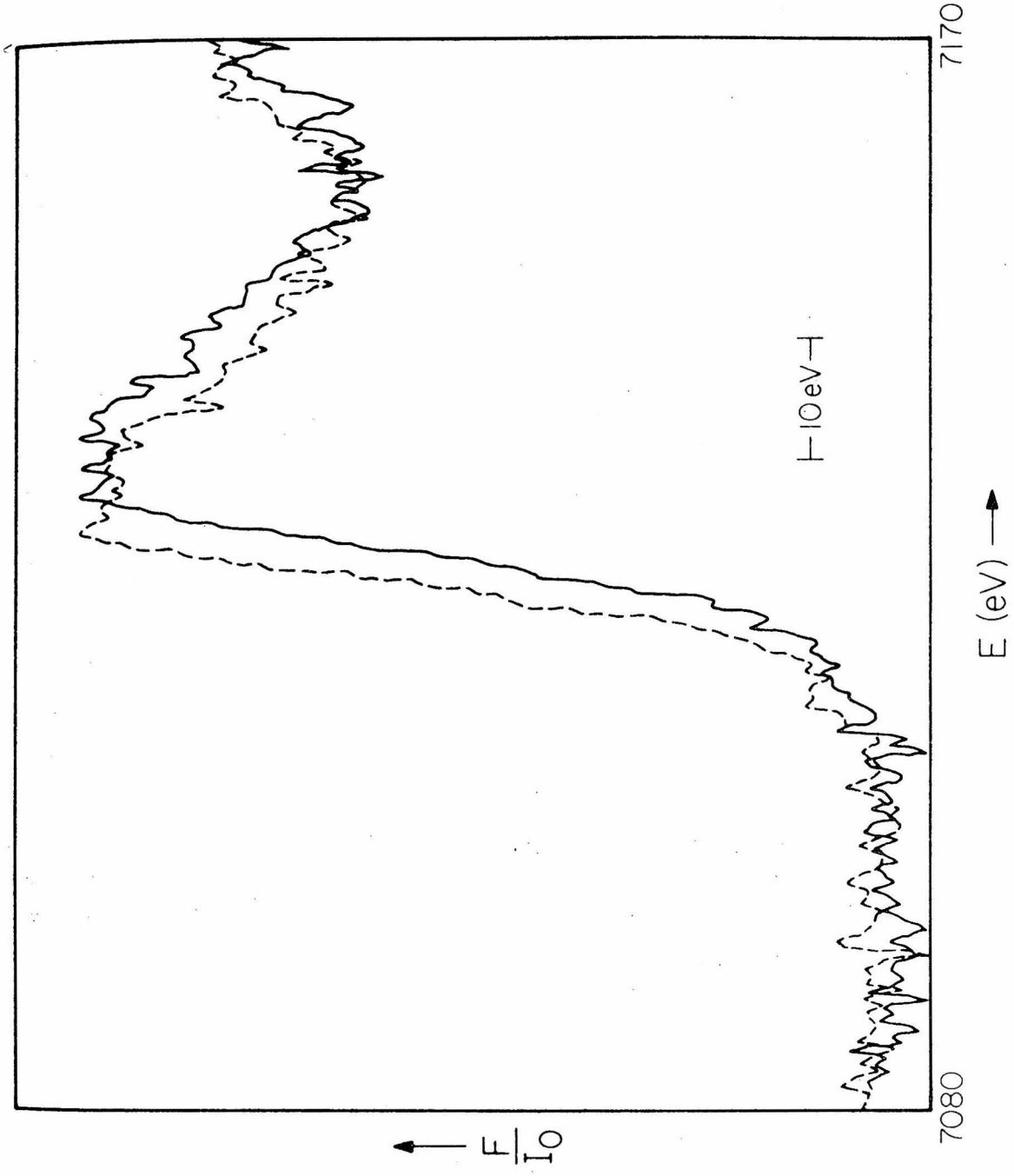
Fe K-Edge of Cytochrome Oxidase. The iron absorption edge spectra of cytochrome oxidase were also obtained for the oxidized and the reduced protein. As shown in Figure 8, there is a small shift of about 2 eV to lower energy in going from the oxidized to the reduced state. This is considerably less than the shift observed by others for ionic Fe complexes, but of comparable magnitude for shifts obtained with covalent Fe complexes and the heme Fe of cytochrome c (11). However, the edge spectra due to the two individual heme irons are not resolved here. In fact, the edge is fairly narrow.

4. Discussion

The data on the model compounds show that the energies of the $1s \rightarrow 3d$ and/or $1s \rightarrow 4s$ transitions for each oxidation state of copper fall within a very narrow range. Considering the diversity of the ligands involved, this is remarkable. In comparing the data from the Cu(I)-d^{10} and Cu(II)-d^9 compounds, we immediately note the appearance of the

*Intensity measurements of the very slight inflection in the edge spectrum of oxidized plastocyanin in the region of the $1s \rightarrow$ "4s" transitions show that the strength of this transition is less than 10% of the $1s \rightarrow$ "4s" transition in the corresponding region of the spectrum of reduced plastocyanin. This small component of Cu(I) intensity in the oxidized spectrum is attributed to the small fraction of auto-reducible protein which has been detected by other means (H. B. Gray, personal communication).

Figure 8. The iron K-edge spectra of oxidized—and reduced---cytochrome c oxidase.


7170
7080
10eV
E (eV)
F/I0

$1s \rightarrow 3d$ transition in the d^9 system. In addition, we see an overall shift of the $1s \rightarrow 4s$ and $1s \rightarrow 4p$ transitions towards higher energies. This is expected, since it requires more energy to move an electron away from a nucleus with a greater positive charge. However, in going from Cu(II) to the analogous Cu(III) compounds, there is no general upward shift in the energies for the three transitions. This suggests that although the Cu in these compounds carries a formal charge of +3, the electron density about the nucleus is essentially the same as for Cu in the +2 oxidation state. This may be due to enhanced $L \rightarrow M$ π bonding. Thus, on the basis of the X-ray absorption data alone, it is not possible to distinguish between formal +2 and +3 oxidation states. It is possible, however, to identify a Cu(I) compound by its very characteristic $1s \rightarrow$ "4s" transition. Since there is no overlap between this transition and any of the transitions of Cu(II) and Cu(III), a Cu(I) assignment for a peak occurring in this region is fairly straightforward.

Comparison of the absorption edge fine structure of cytochrome c oxidase with that of the model compounds indicates that Cu(I) is present not only in the reduced but also in the oxidized protein. The normalized intensity of the $1s \rightarrow 4s$ signal in the spectrum of the reduced oxidase is 0.10, twice that of the Cu(I) $1s \rightarrow 4s$ signal in the spectrum of the oxidized protein. For the model Cu(I) compounds, the normalized intensity of the $1s \rightarrow 4s$ transitions is within the range of 0.07-0.14; the strength of the corresponding transition in reduced plastocyanin is 0.12 (See Table 6). These results strongly suggest that one of the two Cu ions in oxidized cytochrome c oxidase is Cu(I).

Considering the importance of this conclusion, we have considered and rejected several alternative explanations of the transition at 8983.7 eV. One might argue that radiation damage in the sample might partially reduce the oxidized sample. However, no change in the X-ray absorption spectra was observed with time. Also, the optical spectra of the irradiated samples agreed well with the published data. Finally, the data from three separate experiments on two different batches of cytochrome oxidase are identical, the ratio of Cu(I) in the reduced to oxidized protein being 2:1 in each case. Thus, we feel that we can reasonably exclude the possibility of Cu(I) as being merely an experimental artifact.

We also considered the possibility that our model compounds were not sufficient representatives of copper in a protein environment. For this reason, we have examined the Cu edge spectra of another copper protein, bean plastocyanin. The X-ray absorption edge spectra of oxidized and reduced plastocyanin indeed bear a close resemblance to the respective spectra of oxidized and reduced cytochrome oxidase, as a comparison of Figures 1 and 3 will show. Note, however, that oxidized plastocyanin does not contain a transition in the energy range that encompasses the $1s \rightarrow$ "4s" transitions of Cu(I). A further comparison of the plastocyanin data with that of the model compounds shows that the transition energies in oxidized and reduced plastocyanin are well within the energy ranges established by Cu(II) and Cu(I) model systems respectively. Thus, we see that the electronic environment of copper in a protein is not significantly different from that of copper in the inorganic models studied.

This argues in favor of our assignment of the 8983.7 eV transition in the spectrum of oxidized cytochrome oxidase to Cu(I), since a transition in this region is characteristic of all of the Cu(I) models and is present in none of the Cu(II) models.

Thus, of the two coppers in cytochrome c oxidase, only one of the coppers formally undergoes a change in oxidation state between Cu(I) and Cu(II) or Cu(III), while the other is always effectively Cu(I). Reductive titration experiments show that four electron equivalents can be reversibly taken up by the protein, and it is generally assumed that the four metal atoms are involved in oxidation and reduction (5). The notion that the redox-active sites are restricted to the metal ions would be compatible with our findings if the reducible Cu in the oxidized protein were Cu(III). However, our X-ray absorption data are ambiguous with respect to the oxidation state of this second copper, and there is no other experimental evidence that even suggests the possible occurrence of Cu(III) in oxidized cytochrome oxidase. On the other hand, there is no direct evidence that both coppers partake in the oxidation-reduction reactions. The EPR signal at $\sim g = 2$, which has been attributed to Cu(II) in the oxidized protein, at most accounts for only half of the total copper in cytochrome oxidase (1,4). In addition, there is a question as to whether this signal is actually due to copper or to a sulfur radical (15). Thus, there is a serious possibility that both coppers are EPR-silent and that one of the four electron acceptors is really a protein ligand. In fact, it has been previously postulated that a Cu(I)-disulfide system might exist in the protein such that the

complex as a whole participates in electron transfer, with the ligand being the prime reversible electron acceptor (16). Such model systems are discussed by Hemmerich (17). The results obtained in our X-ray absorption experiments are not inconsistent with such a model. However, since none of the ligands to copper are known, a clarification of this point awaits future investigation.

In summary, on the basis of X-ray absorption edge studies of cytochrome oxidase, plastocyanin, and a host of model compounds, evidence is presented that one of the coppers in oxidized cytochrome c oxidase is in the +1 oxidation state. The implications of this finding are that it calls for a reinterpretation of some of the existing data on the protein, perhaps a redefinition of "invisible copper", and a re-examination of the mechanisms proposed for electron transfer mediated by cytochrome oxidase.

This research would not have been possible without the overwhelming generosity of a number of people. Drs. Tsoo E. King, Chang-An Yu, and Linda Yu of the State University of New York at Albany generously supplied us with purified and concentrated cytochrome c oxidase. Drs. Robert Gagné, Harry Gray, and Mr. Dave Dooley provided us with many of the model compounds referred to in this chapter. Dr. William Blumberg made available to us his data on a number of model compounds prior to publication. To all these individuals we are extremely grateful. We would also like to thank Drs. W. E. Blumberg, R. Gamble, H. B. Gray, and R. G. Shulman for many stimulating discussions and their con-

tinued interest and encouragement throughout the course of this work.

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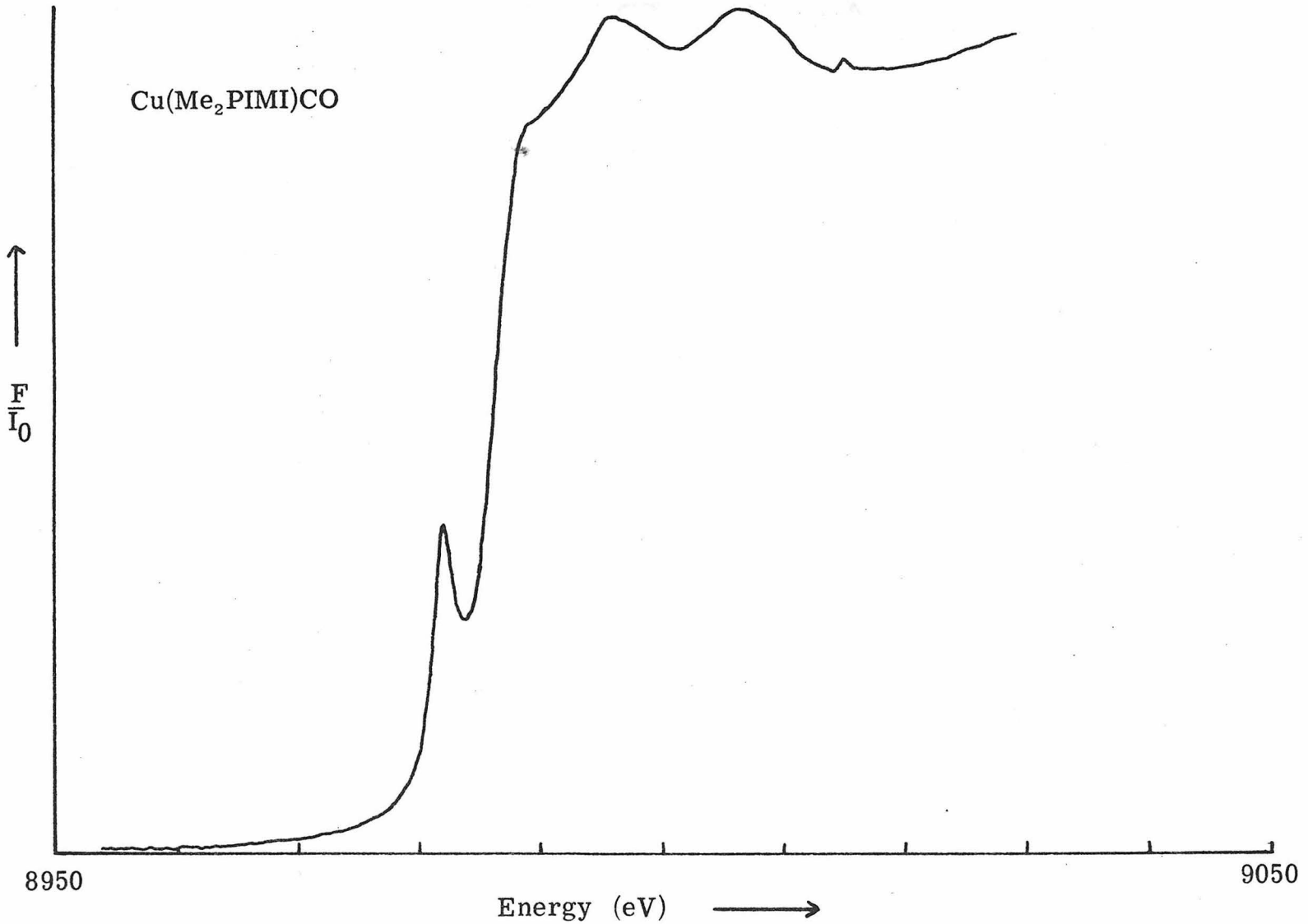
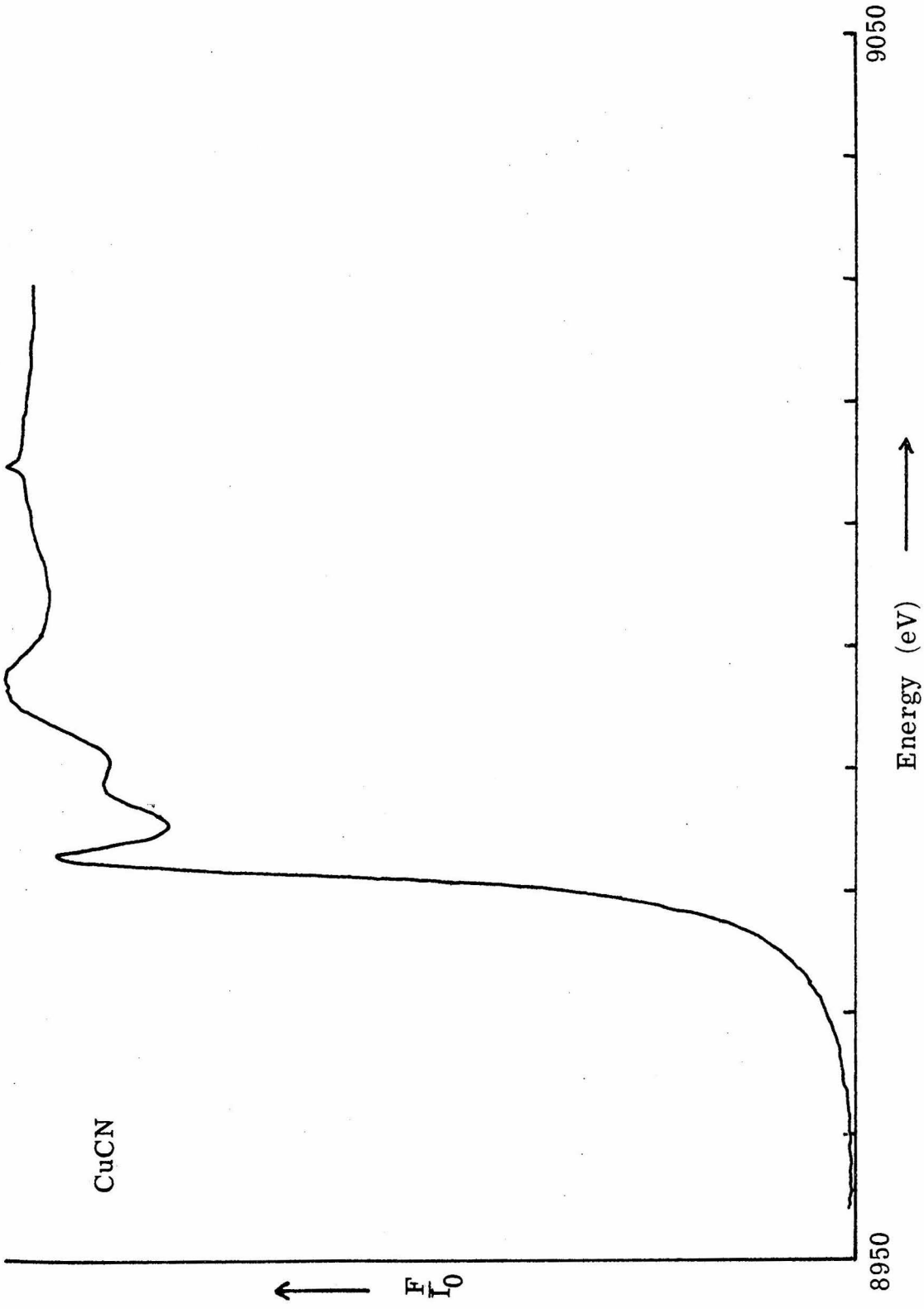
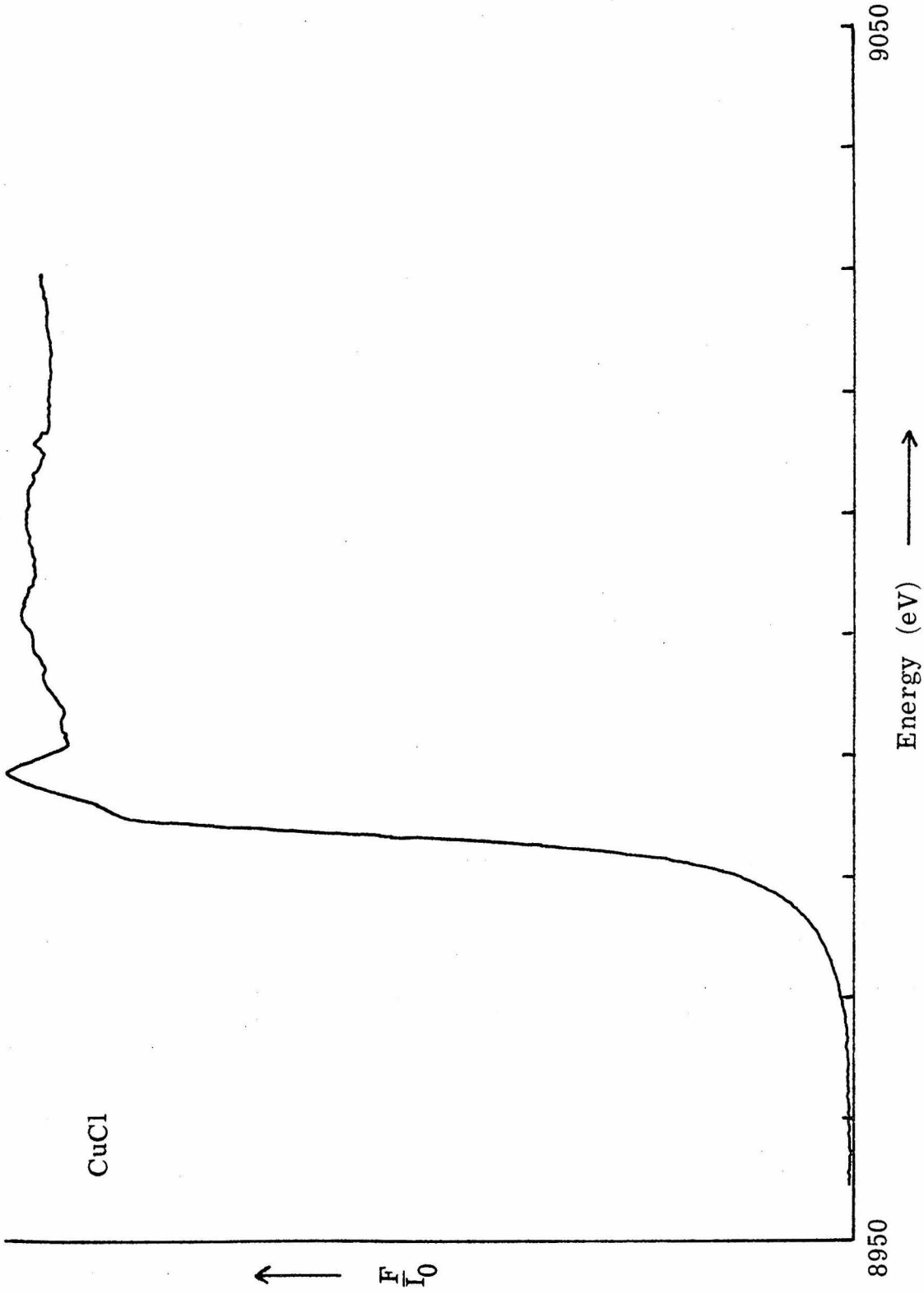
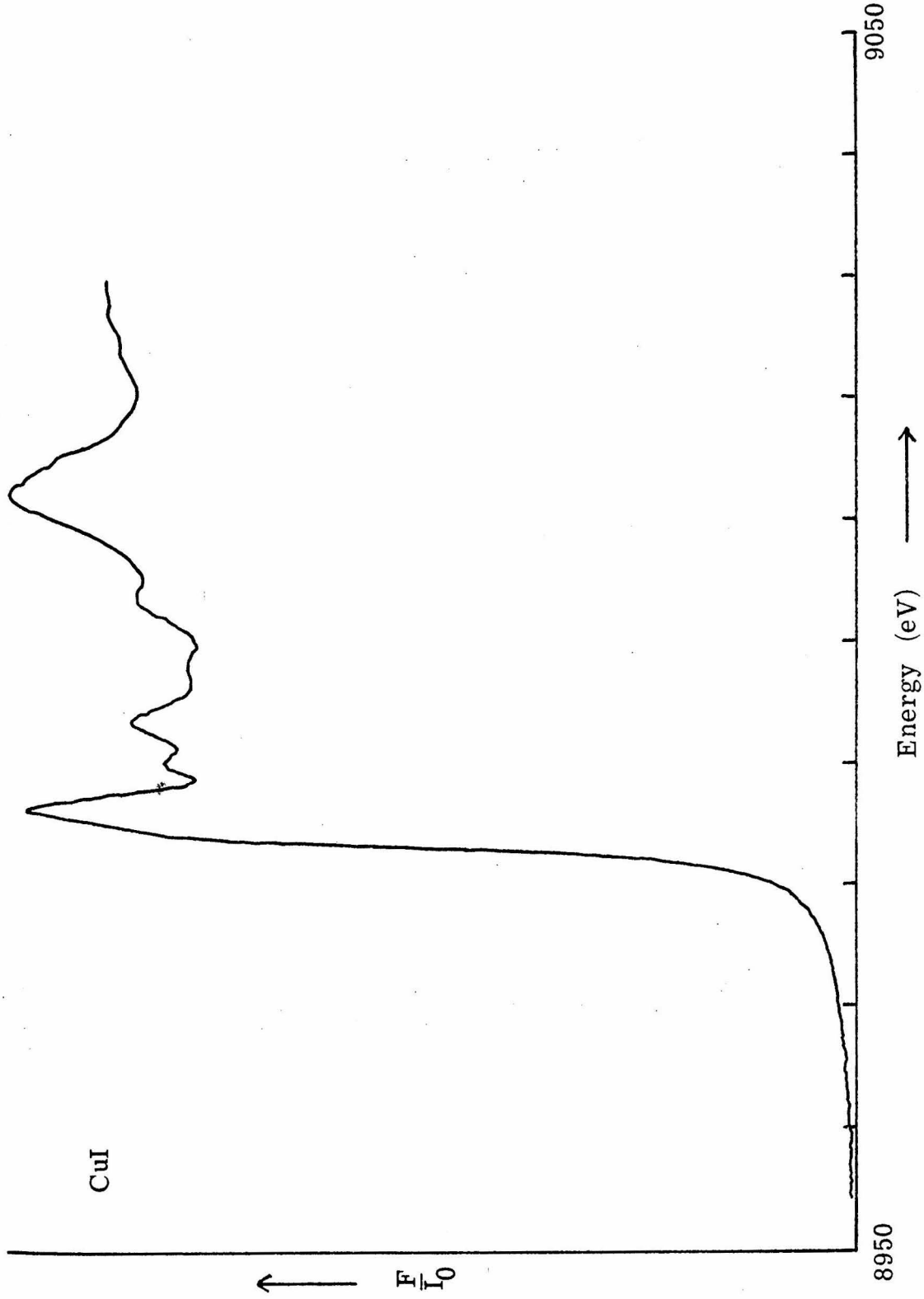
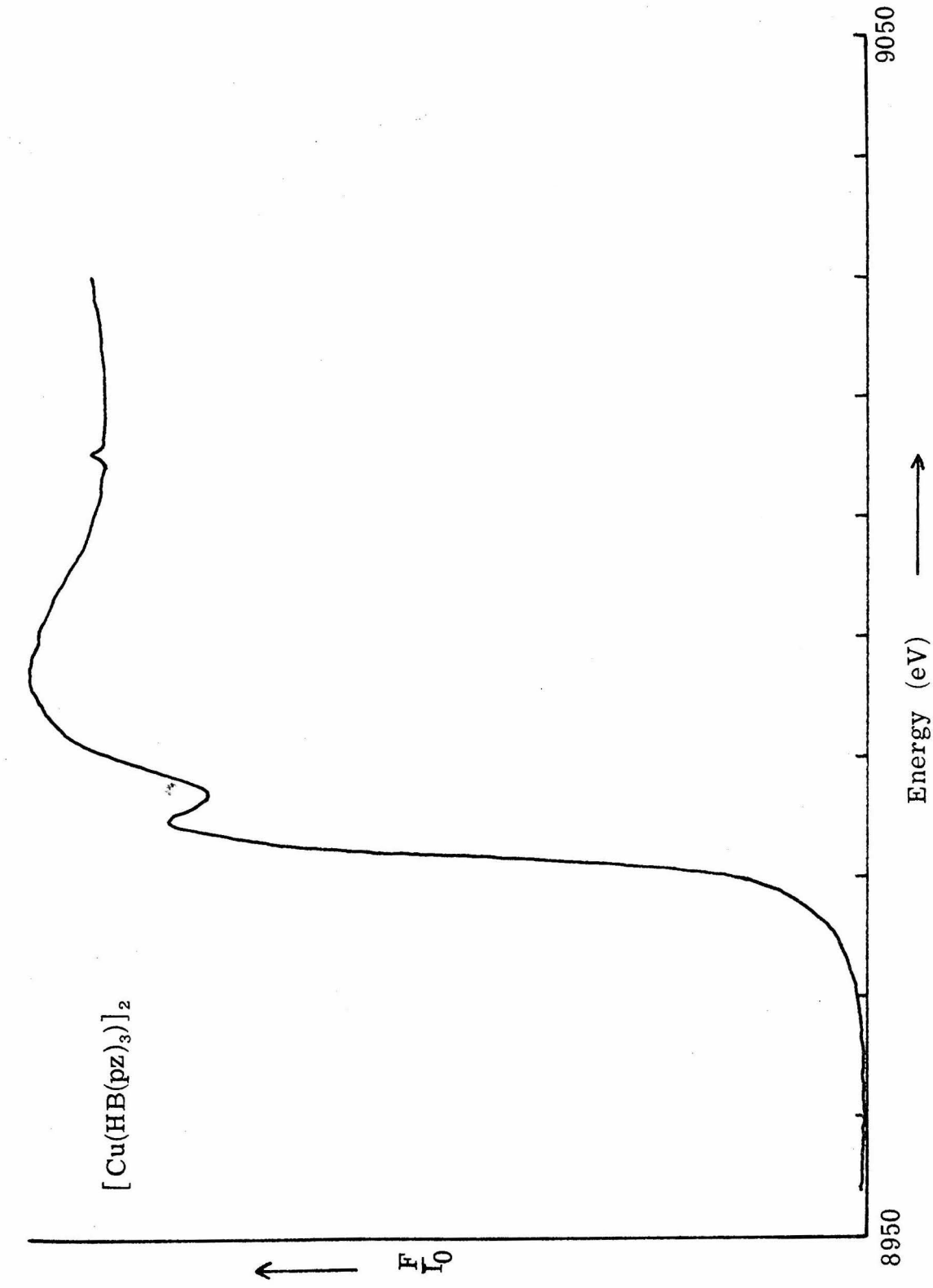
APPENDIX I. X-RAY ABSORPTION EDGE SPECTRA OF THE Cu(I) MODEL COMPOUNDS

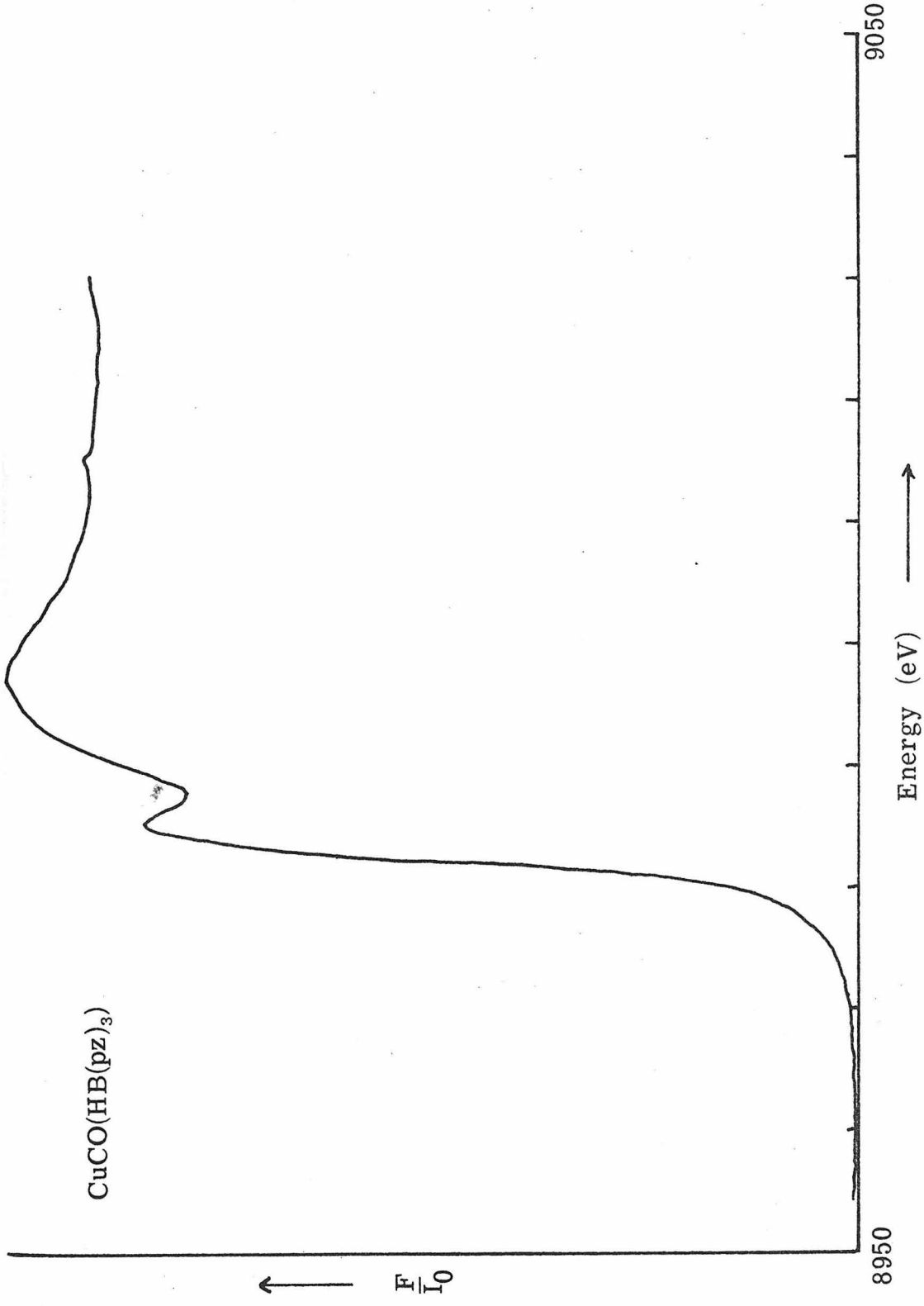

Cu(Me2PIMI)CO
F/I0
8950
9050
Energy (eV)


CuCN
E_{L_0}
8950
9050
Energy (eV)

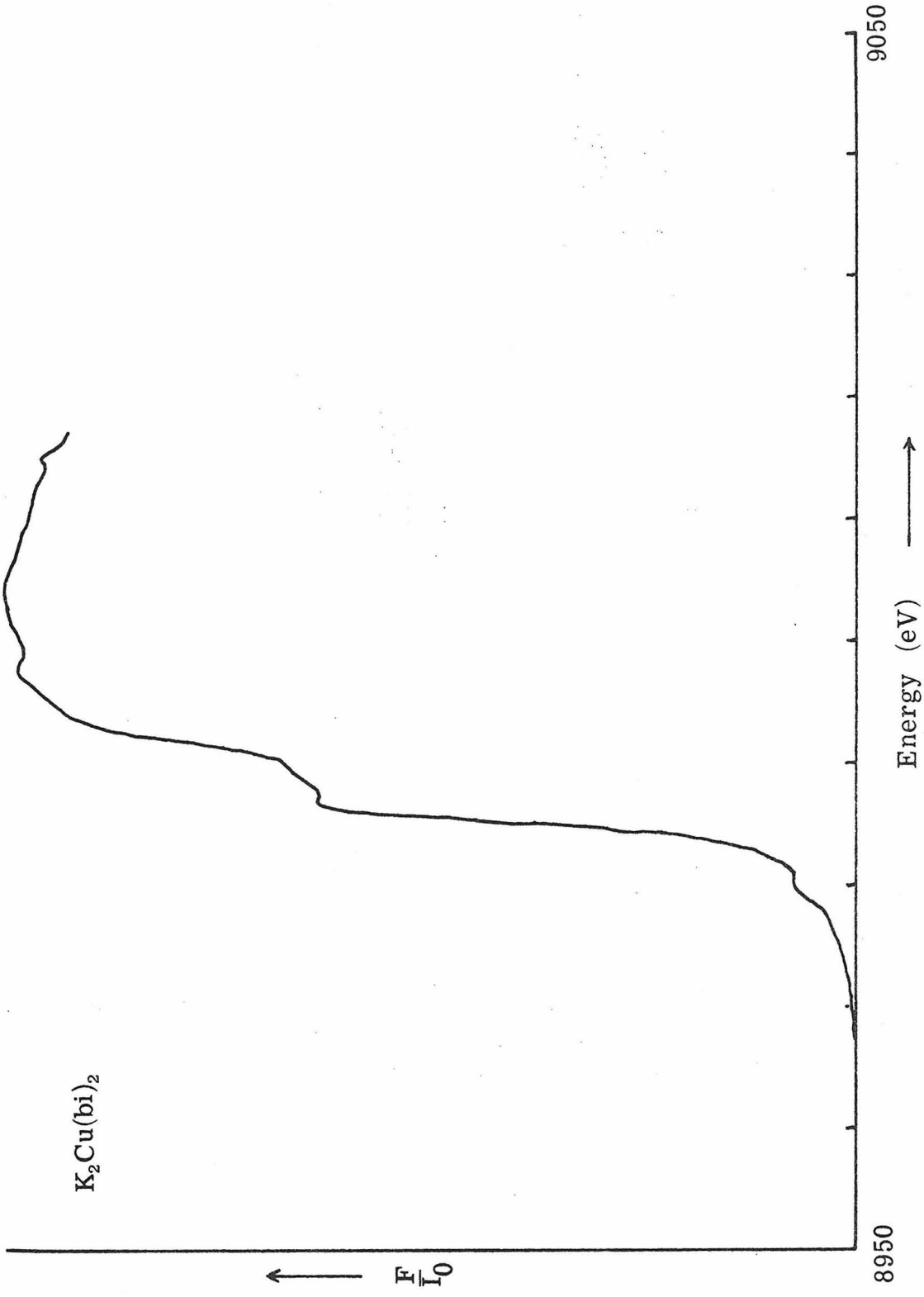

CuCl
$\frac{F}{I_0}$
8950
9050
Energy (eV)

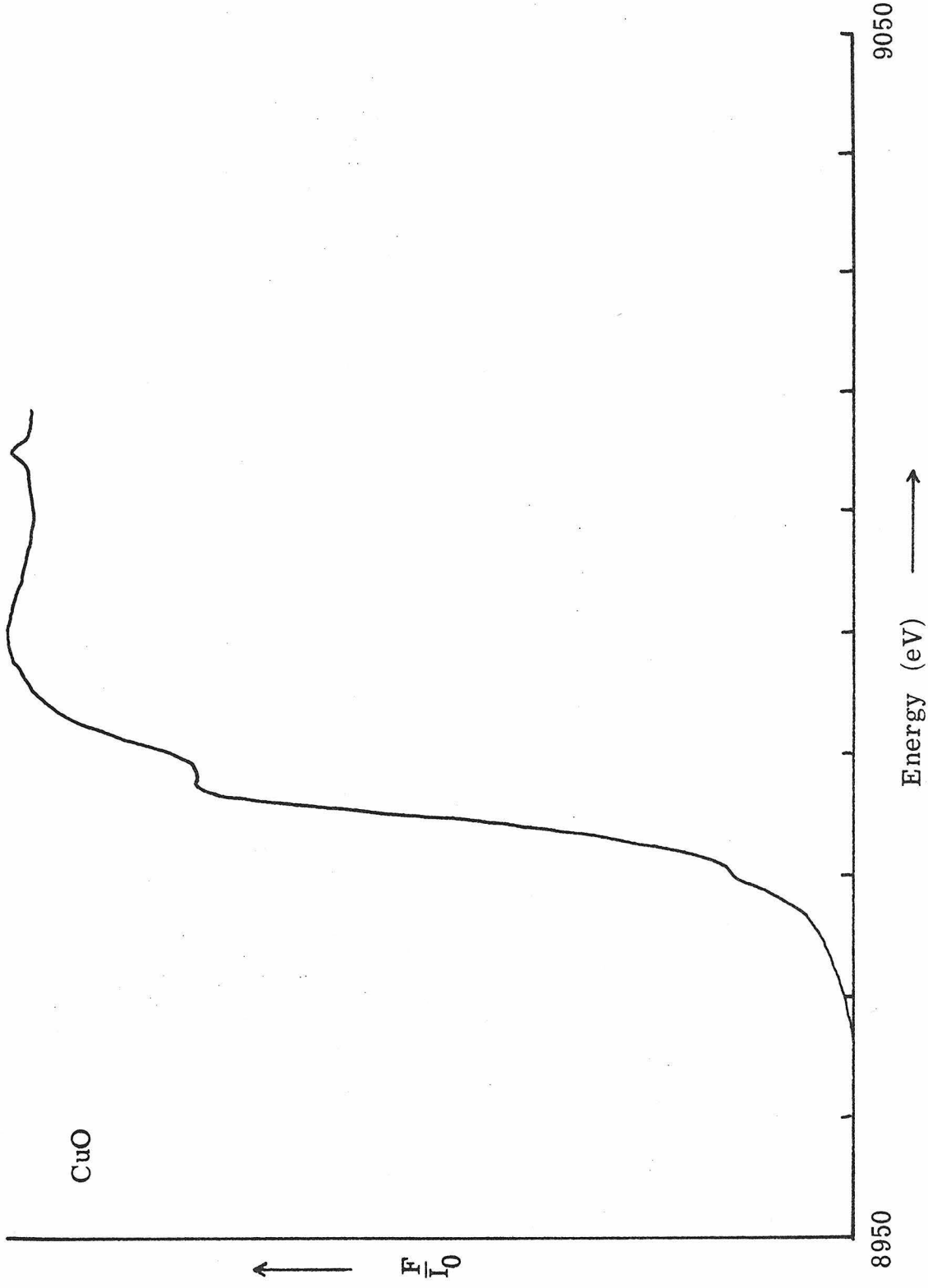

CuI
$\frac{F}{I_0}$
8950
9050
Energy (eV)

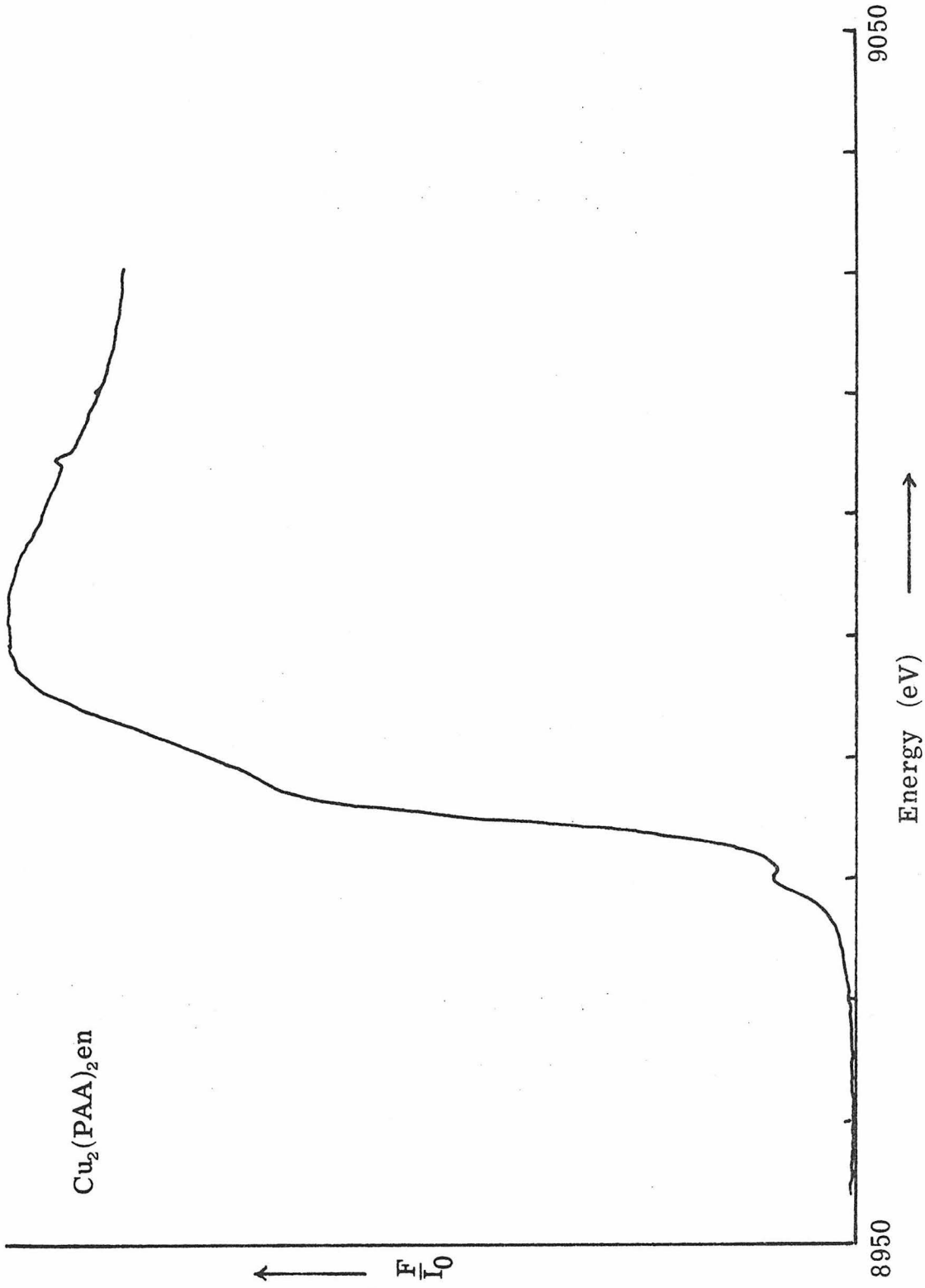

[Cu(HB(pz)3)]2
F/I0
8950
9050
Energy (eV)

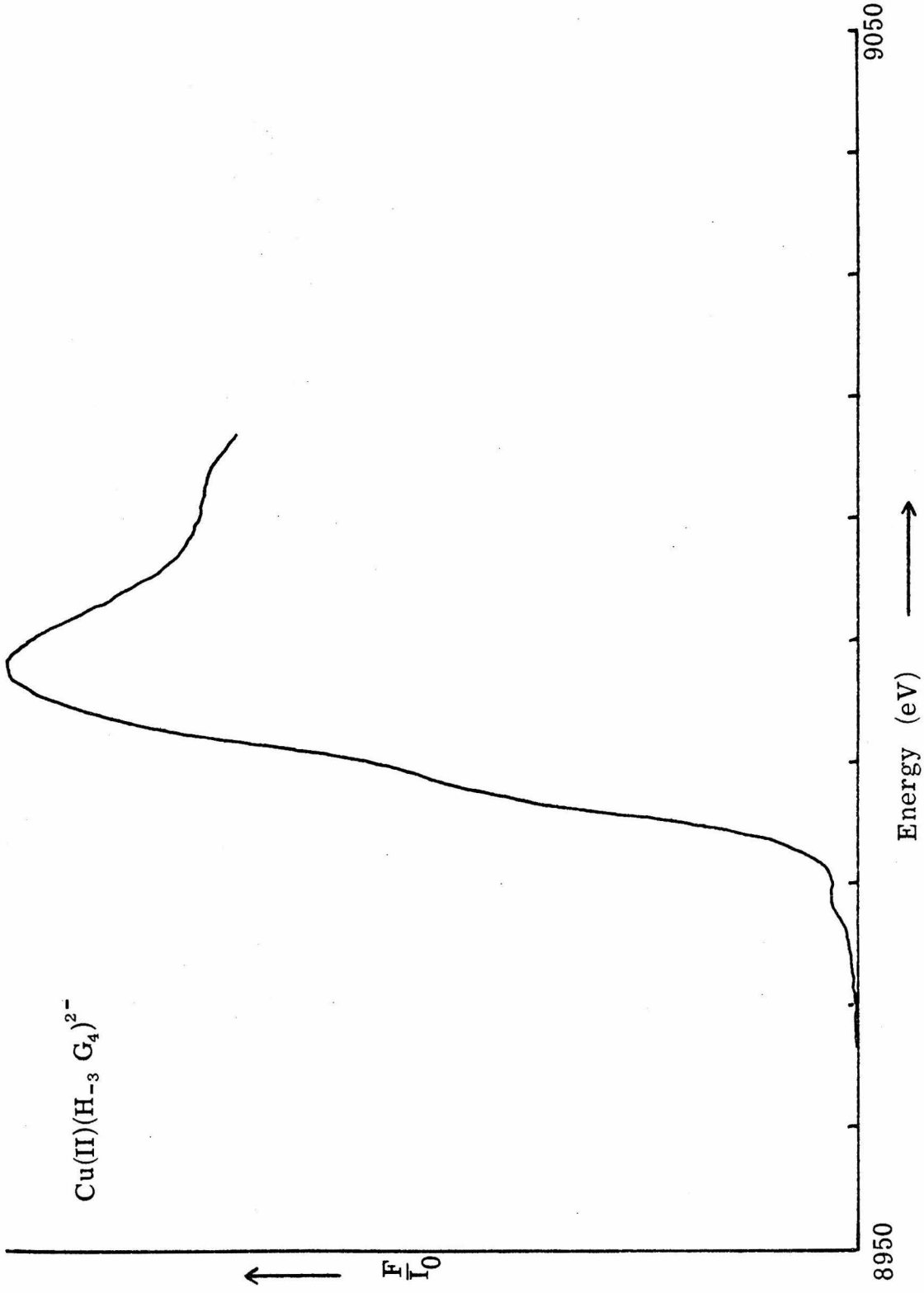

CuCO(HB(pz)3)
F/I0
8950
9050
Energy (eV)

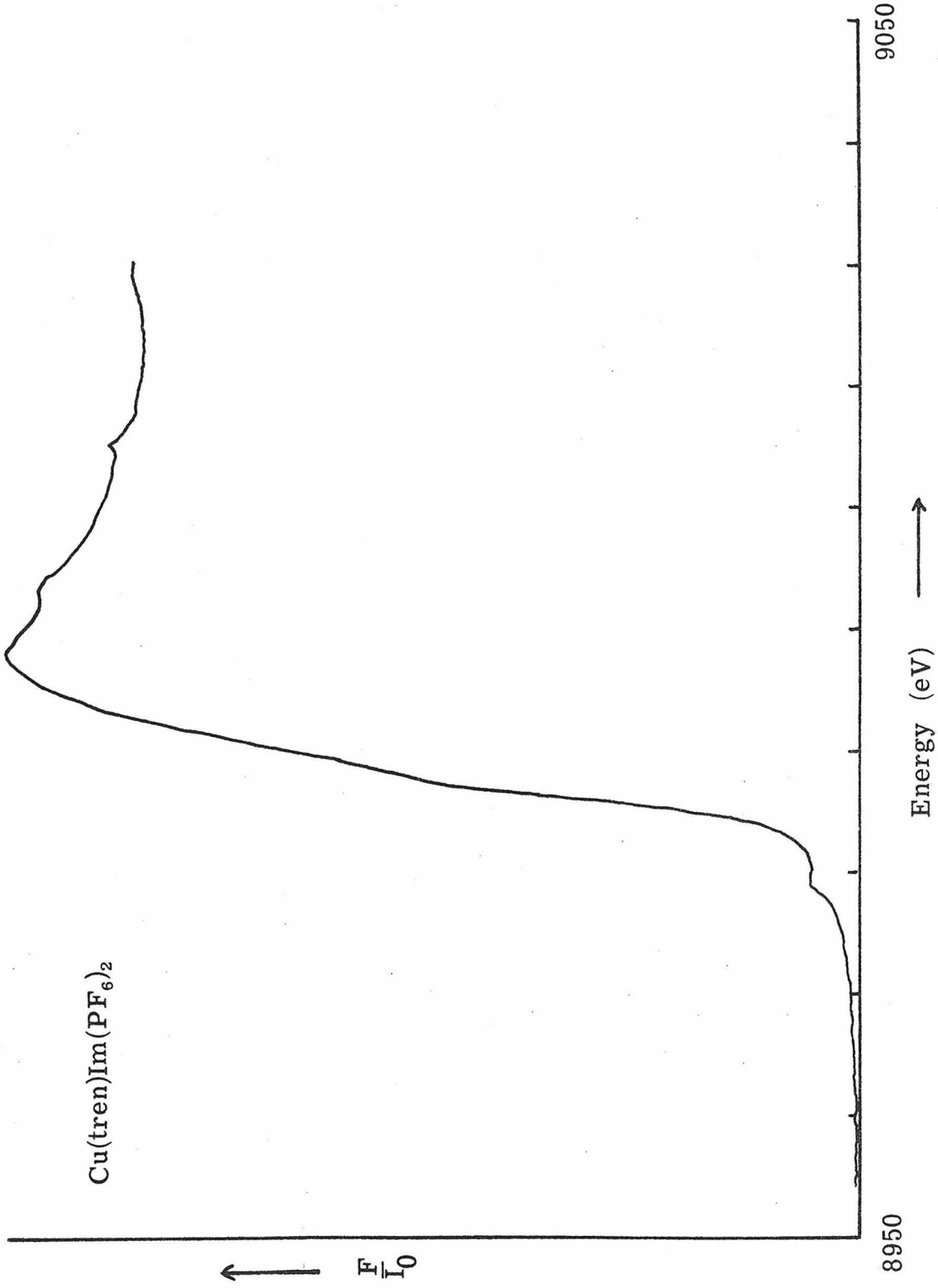
APPENDIX II. X-RAY ABSORPTION EDGE SPECTRA OF THE Cu(II) MODEL COMPOUNDS

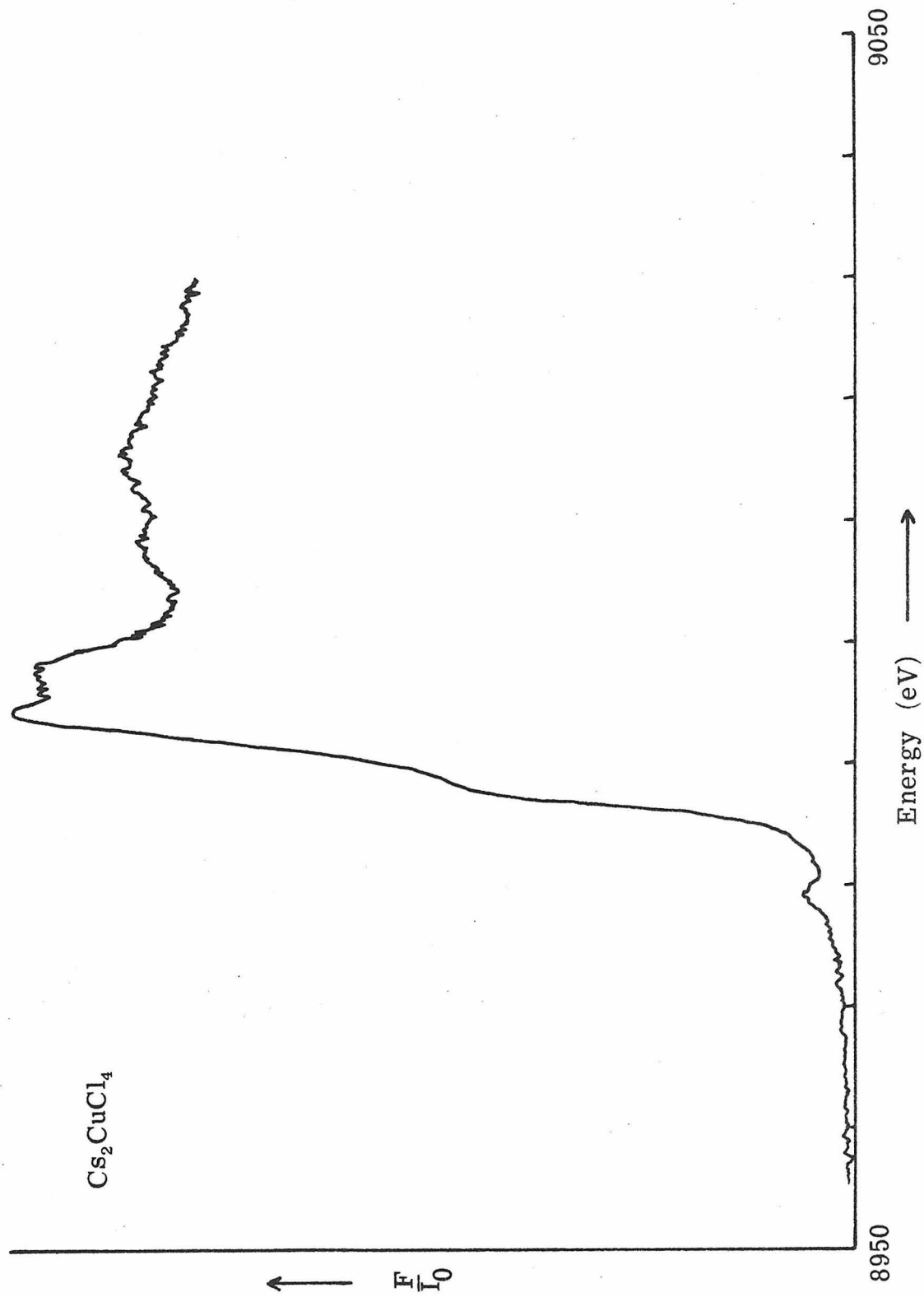

$K_2Cu(bi)_2$
$\frac{F}{I_0}$
8950
9050
Energy (eV)

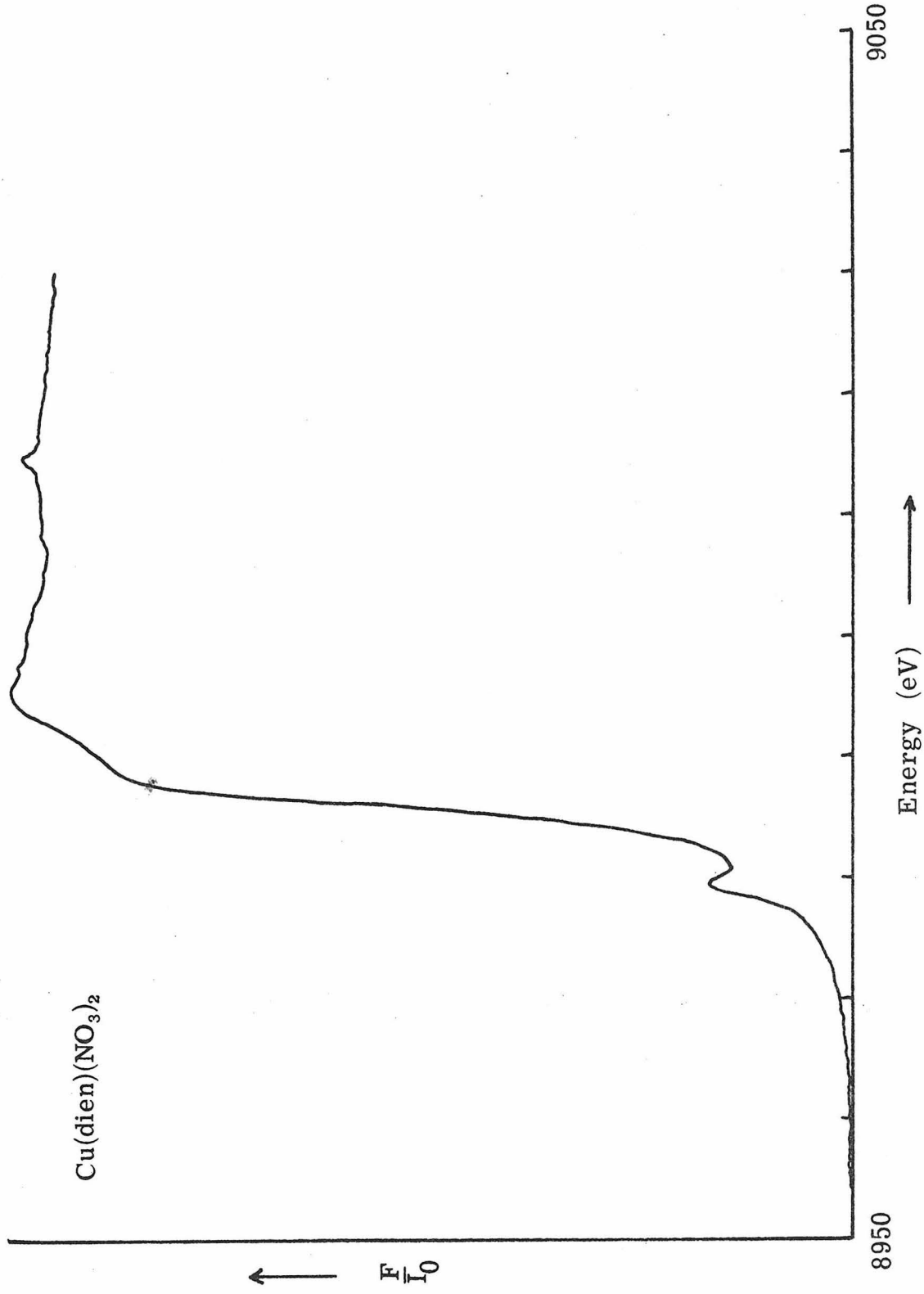

CuO
$\frac{F}{I_0}$
8950
9050
Energy (eV)

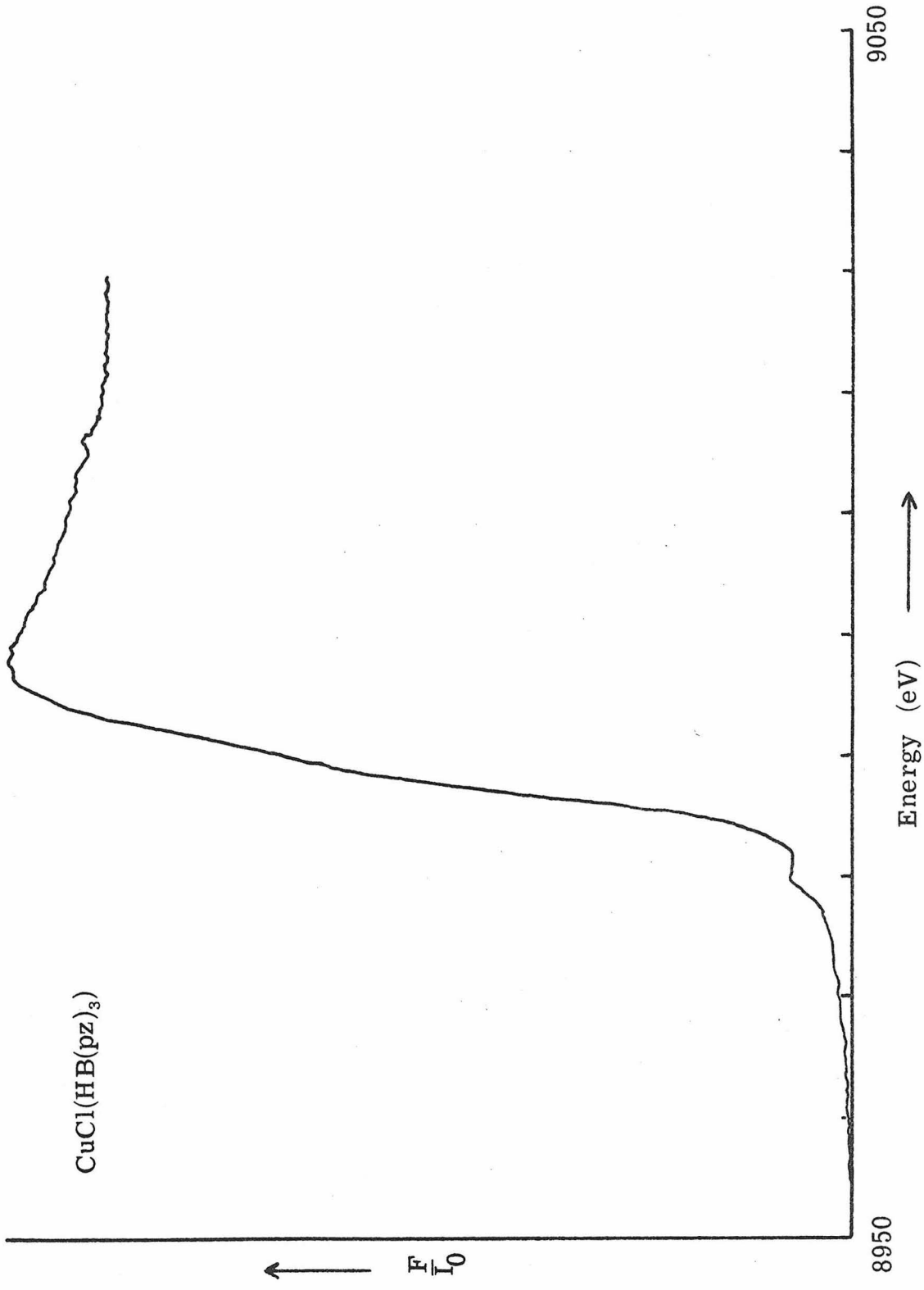

$Cu_2(PAA)_2en$
$\frac{F}{I_0}$
8950
9050
Energy (eV)

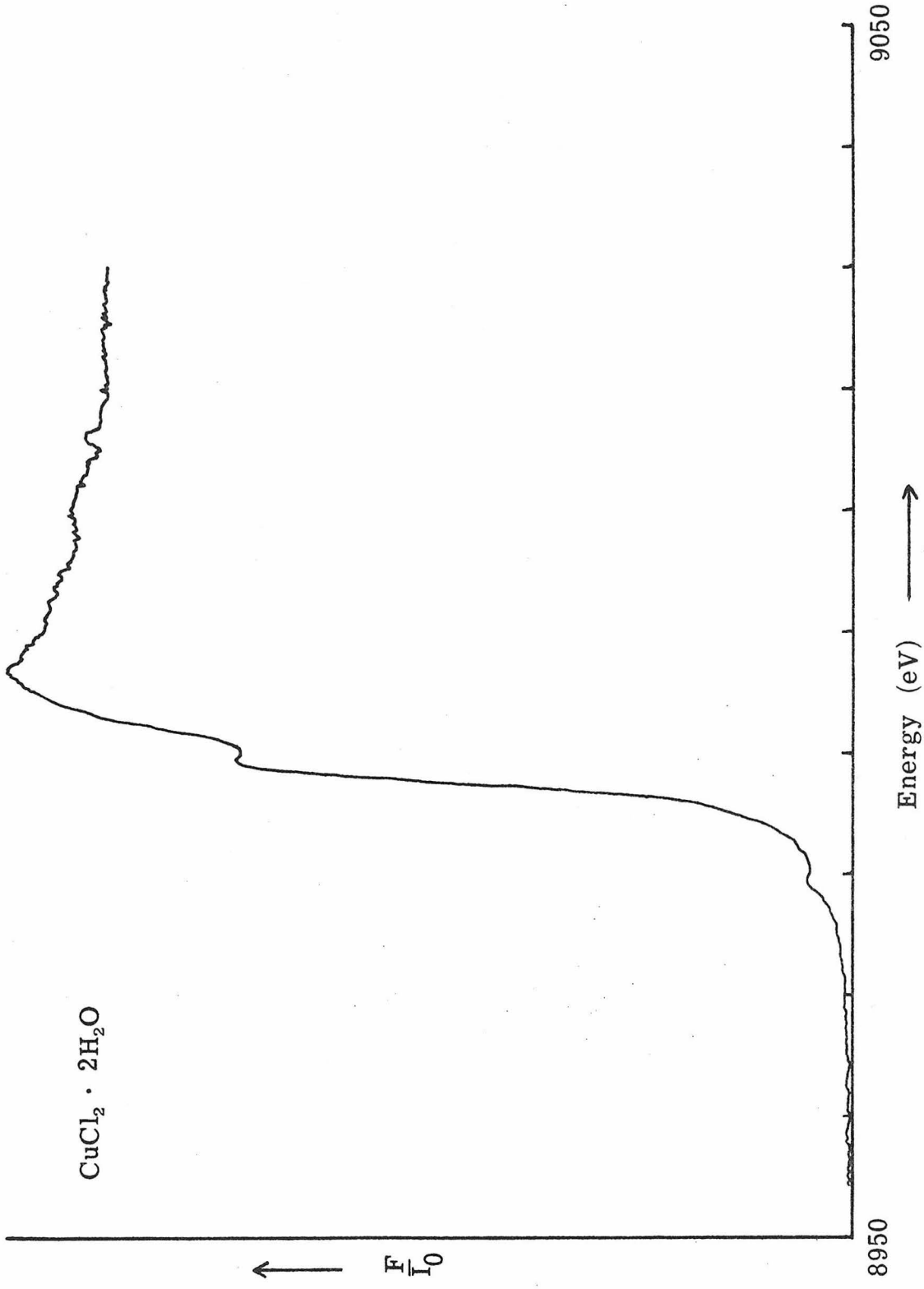

Cu(II)(H-3 G4)2-
F/I0
8950
9050
Energy (eV)

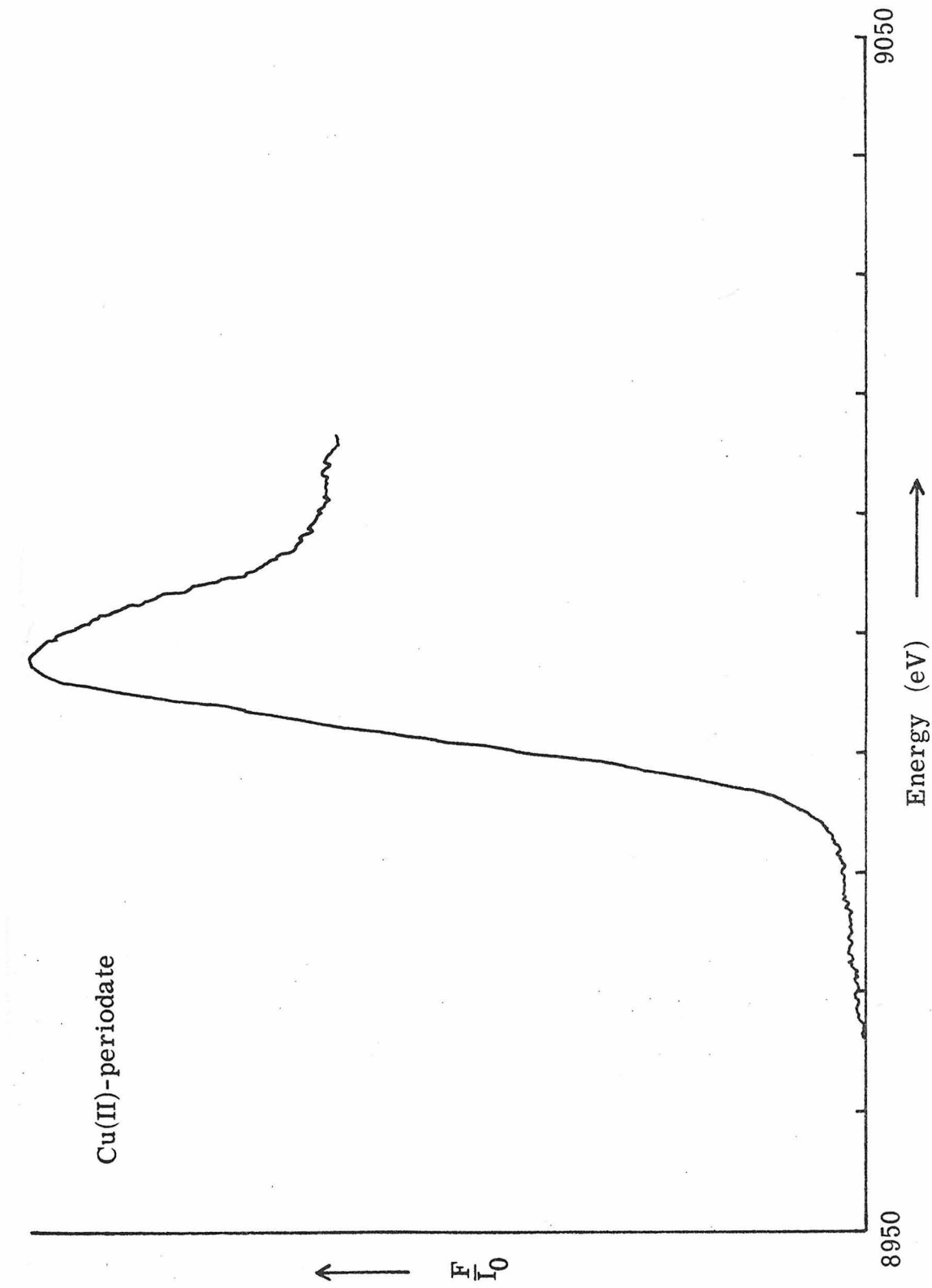

Cu(tren)Im(PF6)2
F/I0
8950
9050
Energy (eV)

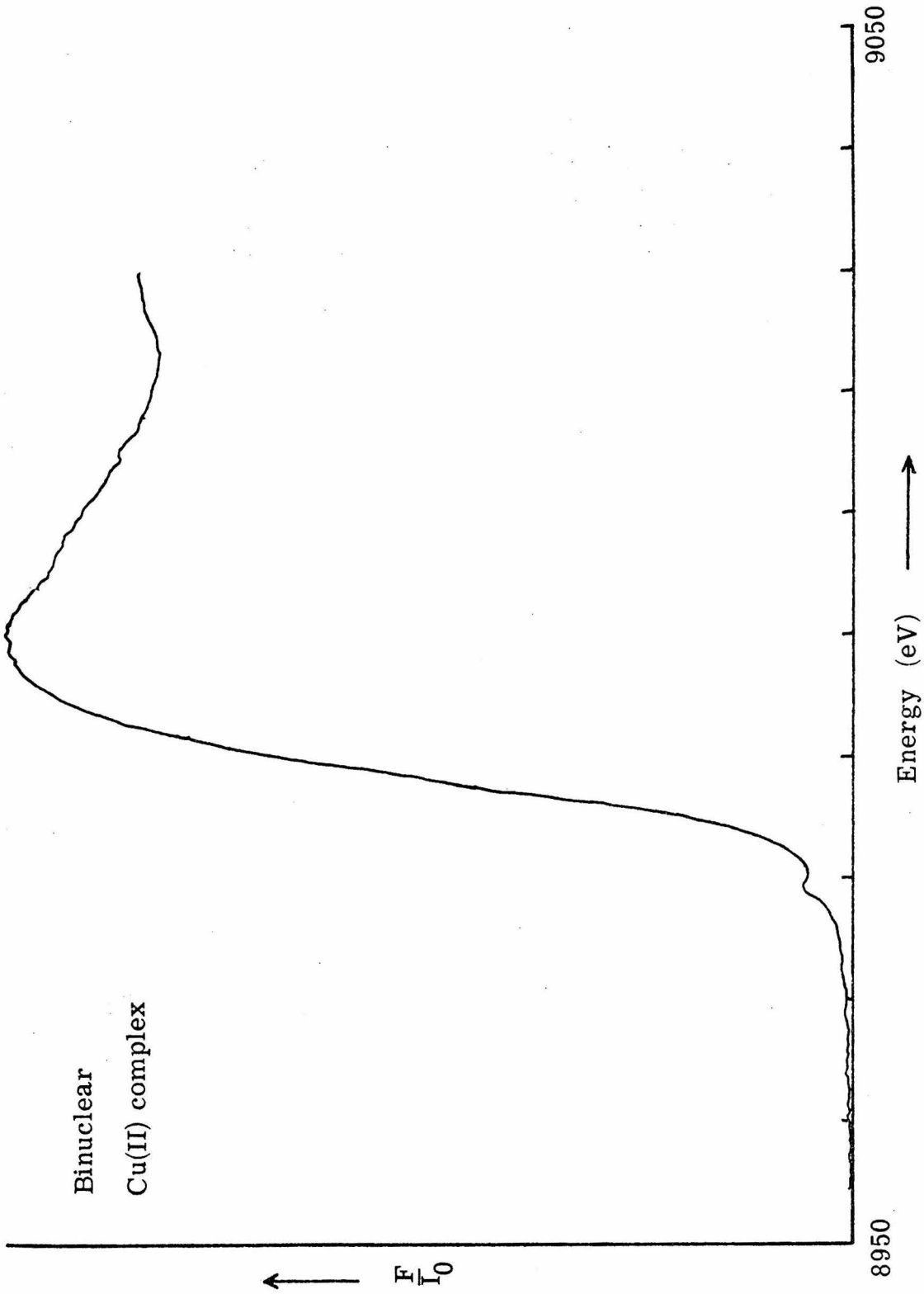

Cs_2CuCl_4
$\frac{F}{I_0}$
8950
9050
Energy (eV)


Cu(dien)(NO3)2
F/I0
8950
9050
Energy (eV)


CuCl(HB(pz)3)
F/I0
8950
9050
Energy (eV)

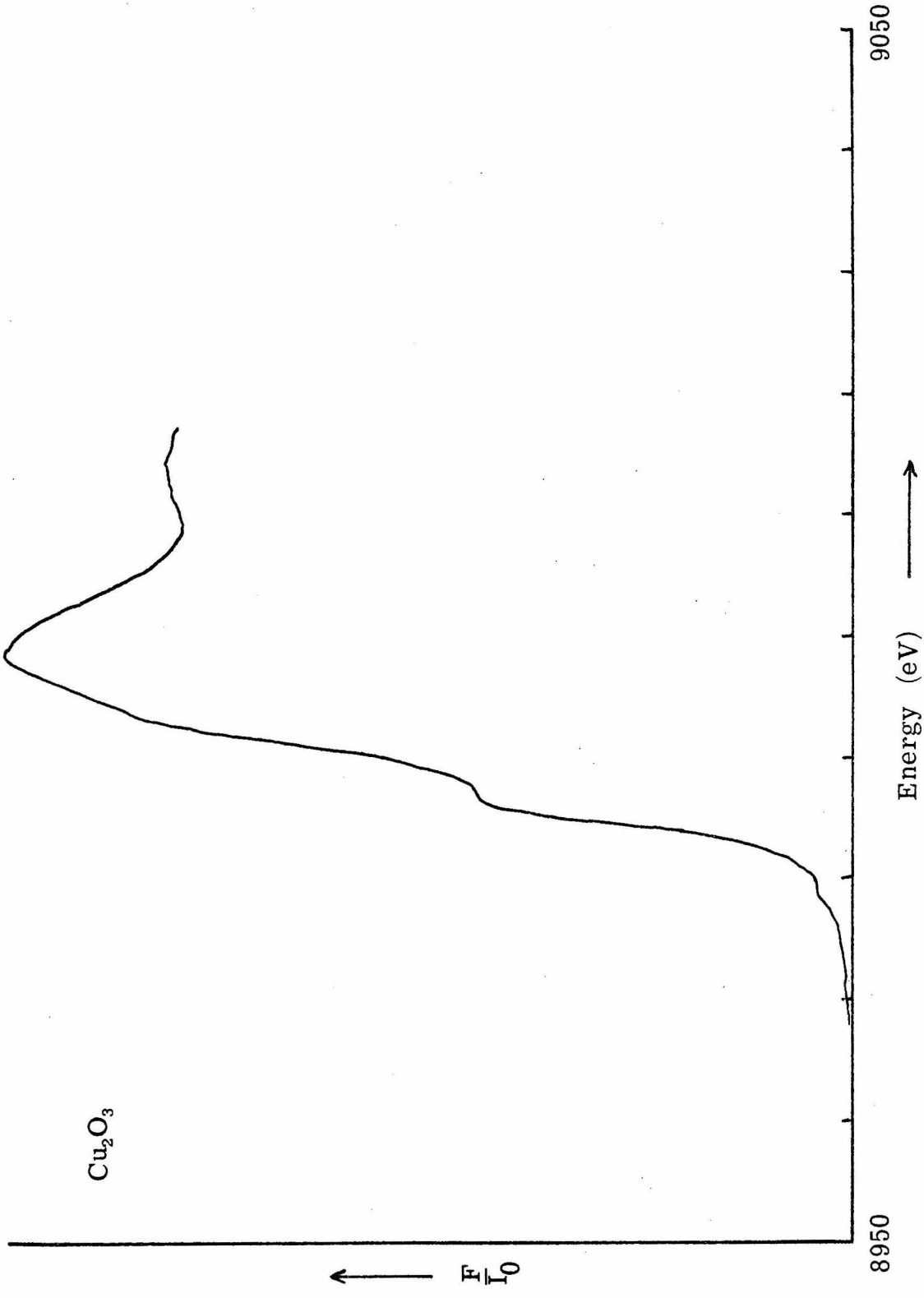

$CuCl_2 \cdot 2H_2O$
$\frac{F}{I_0}$
8950
9050
Energy (eV)

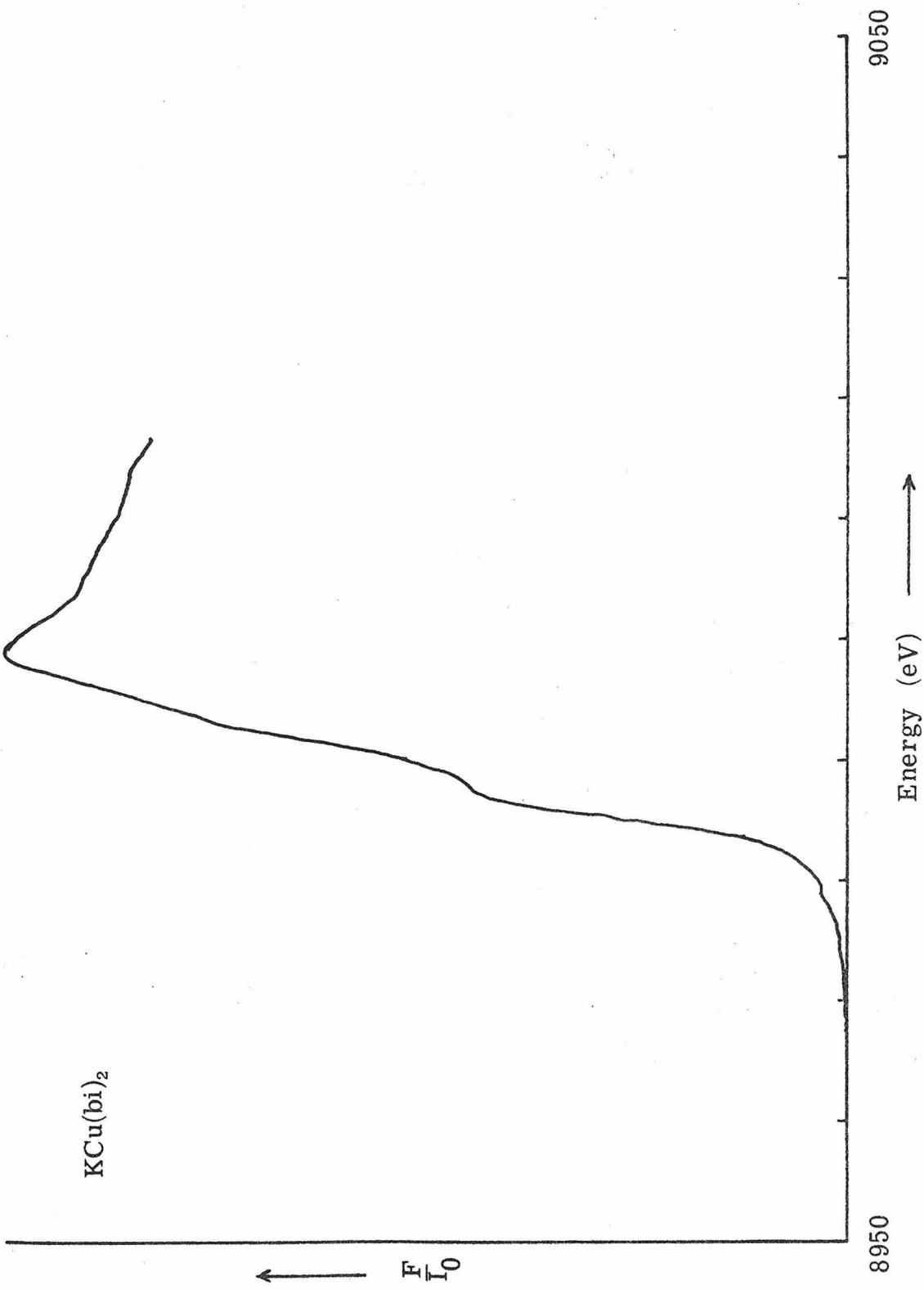

Cu(II)-periodate
F/I0
8950
9050
Energy (eV)

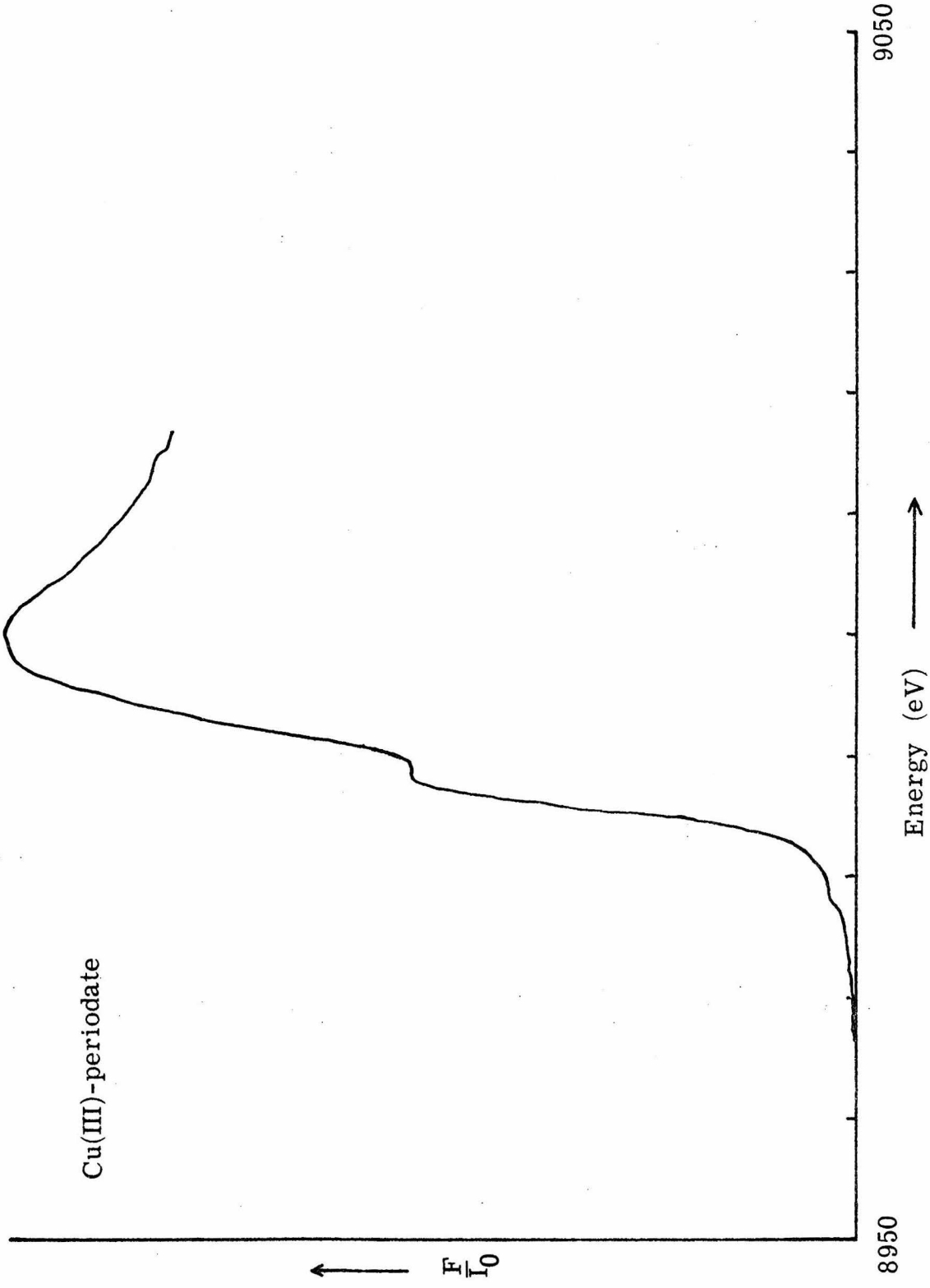

Binuclear
Cu(II) complex
$\frac{F}{I_0}$
8950
9050
Energy (eV)

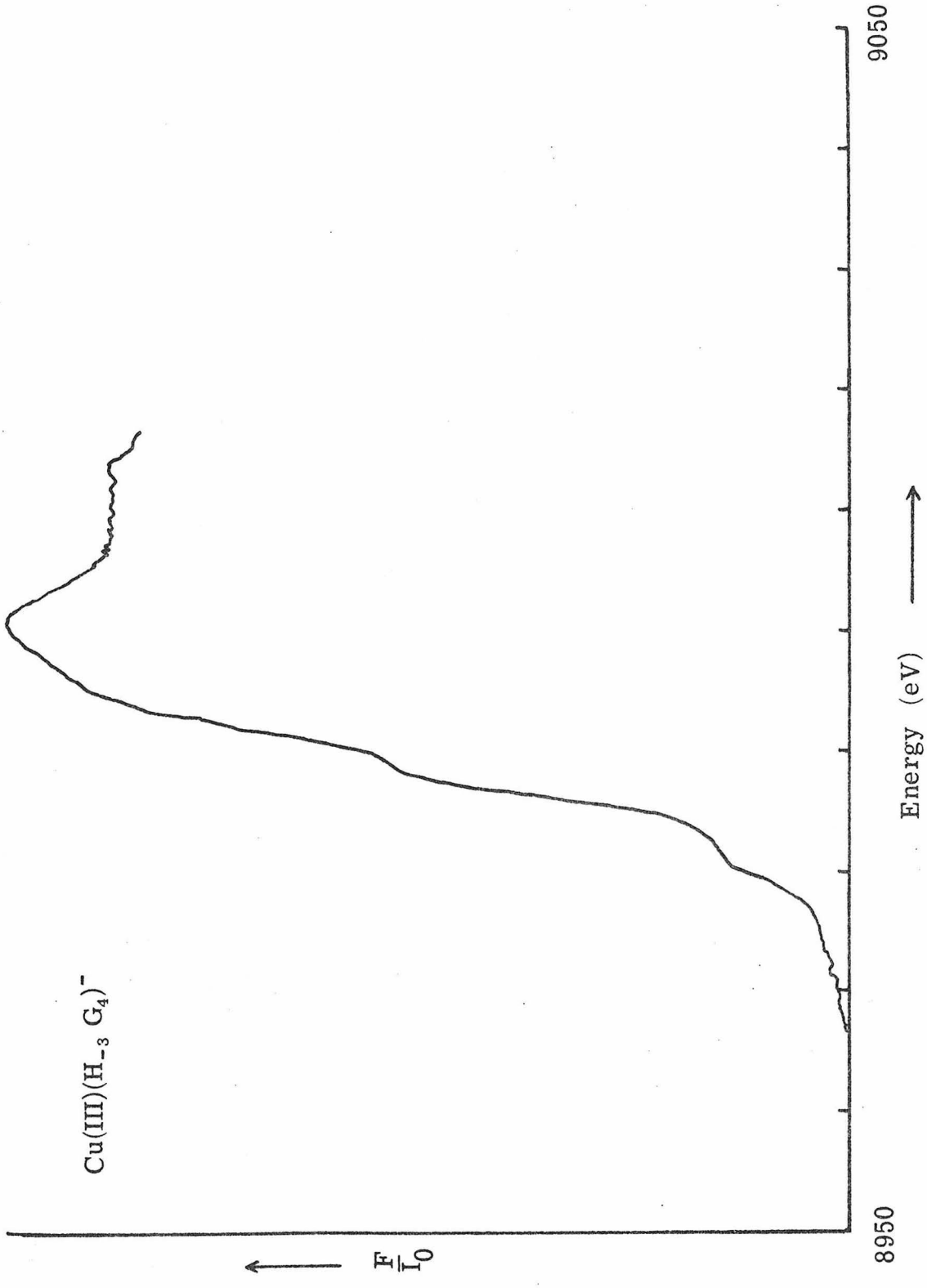
APPENDIX III. X-RAY ABSORPTION EDGE SPECTRA OF THE Cu(III) MODEL COMPOUNDS

(courtesy of W. E. Blumberg)


Cu_2O_3
$\frac{F}{I_0}$
8950
9050
Energy (eV)


KCu(bi)$_2$
$\frac{F}{I_0}$
8950
9050
Energy (eV)


Cu(III)-periodate
$\frac{F}{I_0}$
8950
9050
Energy (eV)


Cu(III)(H_-3 G_4)^-
F/I_0
8950
9050
Energy (eV)

III. X-RAY ABSORPTION EDGE STUDIES ON CYANIDE-BOUND CYTOCHROME c OXIDASE

1. Introduction

Cytochrome c oxidase (ferrocytochrome c: oxygen oxidoreductase; EC 1.9.3.1) is the terminal enzyme in the mitochondrial respiratory chain and, as such, reacts directly with molecular oxygen. The mechanism of this four-electron reduction is currently a topic of intense investigation and controversy [1,2]. Recently, there has been a great deal of interest in the chemical and physical properties of various inhibitor complexes of cytochrome c oxidase [3-7]. In particular, considerable effort has been spent on studying the cyanide- and carbon monoxide-bound protein with the hope of trapping one or more of the metal components in the oxidized or reduced form [8-11]. The rationale for such studies is the possibility that "mixed valence" forms of the CN- or CO-inhibited protein might provide some clues about the mechanistic pathway of electron transfer to oxygen as well as the interaction of the four metal components in the functional protein. In fact, on the basis of spectroscopic and electrochemical properties of the CO and azide complexes of cytochrome c oxidase, Wilson et al. have proposed that there exists a direct heme-heme interaction in the protein [9]. On the other hand, Yong and King have postulated a heme-copper-heme interaction based on their studies of a "half-reduced" and a fully-reduced CN-complex of the oxidase [11]. To our knowledge, this discrepancy has not yet been resolved.

One of the major difficulties in the above studies has been the lack of knowledge of the redox states of all four metal atoms (2 heme Fe and 2 Cu), especially in the partially reduced complexes of the protein. Recently, Anderson et al. reported that the CO-complex of cytochrome oxidase is titratable with three electrons [3], a result in contradiction to an earlier report that the formation of the CO-complex involves a two-electron reduction [6]. Part of the confusion here may be attributed to the fact that at least one of the coppers ("invisible" Cu) is not directly detectable by EPR or spectrophotometric techniques. Furthermore, the contribution of the remaining three components to the optical spectra is still unsettled [1,2,12]. Similarly, the partially-reduced CN-complex of cytochrome oxidase obtained in the presence of reducing agents has not been definitively characterized with respect to the oxidation states of the metal atoms. Yong and King have prepared a form of the CN-complex which they called "half-reduced" in which heme a and one of the two copper centers are reduced, while heme a_3 remains oxidized [11]. However, there is really no direct evidence for the oxidation state of the second copper atom.

Recently, we have demonstrated that X-ray absorption spectroscopy can be of great value in defining the oxidation states of the metal atoms in metalloproteins. Its usefulness arises from the fact that the position of the absorption edge and the absorption edge fine structure are sensitive to the charge density and point symmetry about the atom of interest. Our studies on the oxidized and reduced forms of cytochrome oxidase provide evidence which strongly suggests that one of the coppers

in the oxidized protein exists in the reduced state [13]. Thus, in view of this result, it was of interest to apply this technique to the determination of the oxidation state of the second copper in the dithionite-reduced CN-complex of oxidase as well as to investigate any changes which occurred in the X-ray absorption spectra of cytochrome oxidase upon complexation with cyanide.

Preliminary results on the oxidized and partially-reduced CN-complexes of cytochrome oxidase indicate that: 1) CN has no effect on the Cu edge spectrum of the oxidized protein; 2) the intensity of the $1s \rightarrow 3d$ transition in the Fe edge spectrum of the oxidized protein is slightly enhanced in the presence of cyanide; 3) both Cu atoms of the CN-complex are reduced (i.e., Cu^{+1}) in the presence of excess dithionite, a result which in conjunction with EPR data [4,14] suggests that three out of the four electron acceptors in the complex are reduced.

2. Materials and Methods

Purified cytochrome c oxidase was generously provided by Drs. Tsoo E. King, Chang-An Yu, and Linda Yu of SUNY at Albany, New York. It was suspended in 0.5% sodium cholate/50 mM phosphate buffer pH 7.4 at a concentration of 100 mg/ml. To form the oxidized CN-complex, 17 µl of 0.25 M potassium cyanide solution pH 7.2 was added to 0.4 ml of the concentrated oxidase solution and the resultant solution was left standing under argon for 20-30 minutes. The so-called "half-reduced" complex was prepared by adding the oxidized cyanide complex (0.4 ml) to an excess amount (10 mg) of solid dithionite under argon. The

samples were kept on ice until the X-ray absorption measurements were made.

The Cu and Fe K-edge absorption spectra were obtained using synchrotron radiation from SPEAR at the Stanford Linear Accelerator Center. The broadband radiation was passed through a channel-cut silicon crystal monochromator and the Cu or Fe fluorescence Kα radiation from the target was monitored using a nine-element array of NaI scintillation counters. Details of the experimental set-up and data analyses are described elsewhere [13,15,16]. Since the samples were dilute, multiple scans were recorded and later summed.

3. Results and Discussion

The Cu K-edge spectrum of the oxidized CN-complex showed virtually no change from that of the fully oxidized cytochrome c oxidase (Fig. 1). On the other hand, the Fe K-edge of the oxidized CN-complex shows a slight increase in the intensity of the $1s \rightarrow 3d$ transition, although the position of the edge as a whole remains unchanged (Fig. 2). These results may be taken to mean that while cyanide does not grossly affect the charge density about either the Cu or the Fe atoms in the protein, it does enhance the probability of the $1s \rightarrow 3d$ transition of the iron chromophore. This may be interpreted as evidence for binding of cyanide to iron (presumably heme a_3). The enhanced $1s \rightarrow 3d$ transition is to be expected, since π-bonding of cyanide to iron introduces some p character to the iron d orbitals.

Reduction of the CN-complex in the presence of excess dithionite results in the Cu K-edge absorption spectrum shown in Figure 3. For

Figure 1. The copper K-edge spectra of a) the oxidized CN-complex of cytochrome c oxidase, and b) oxidized cytochrome c oxidase

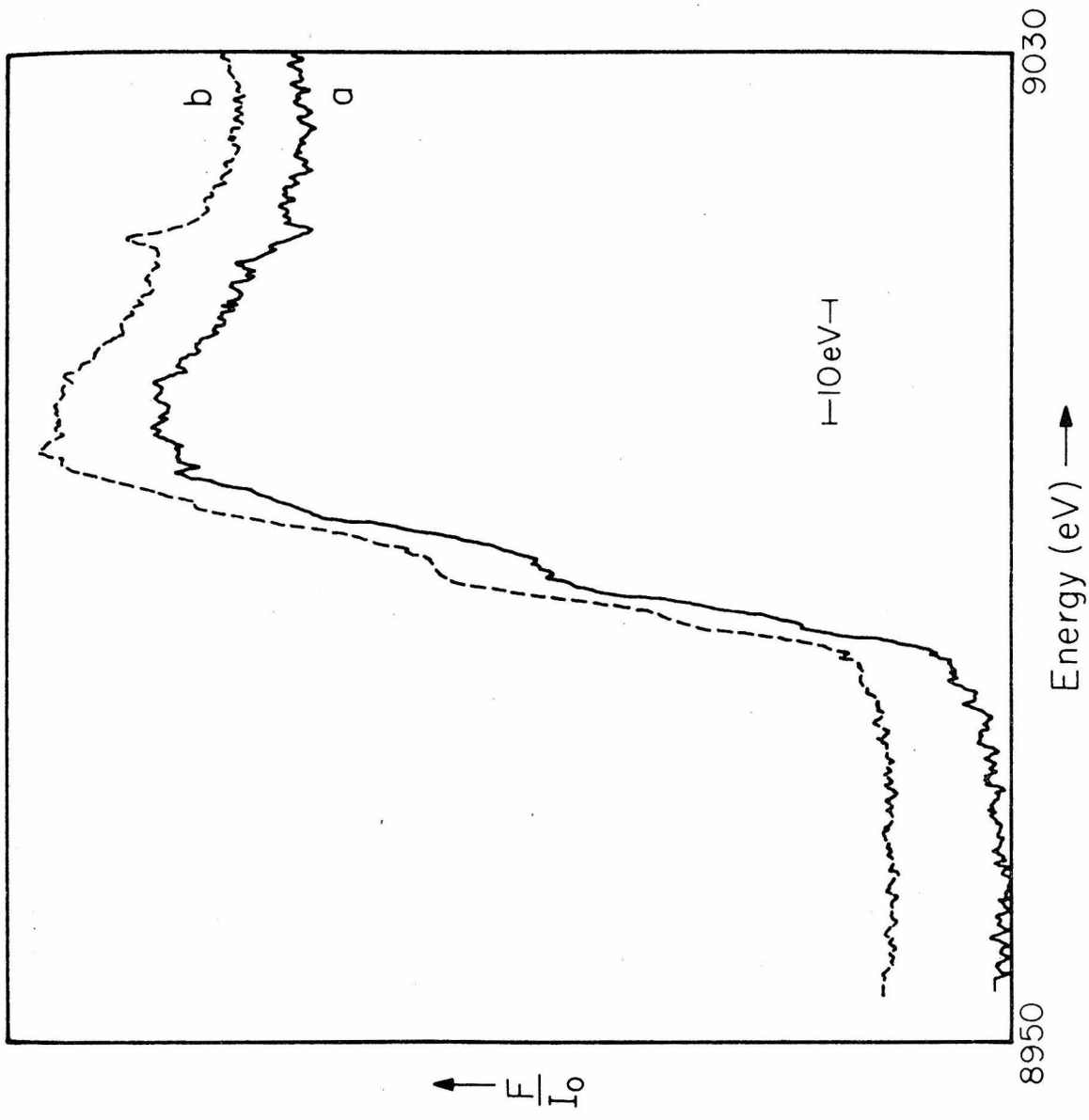

b
a
10eV
9030
8950
Energy (eV)
F/I₀

Figure 2. The iron K-edge spectra of a) the oxidized CN-complex of cytochrome c oxidase, and b) oxidized cytochrome c oxidase

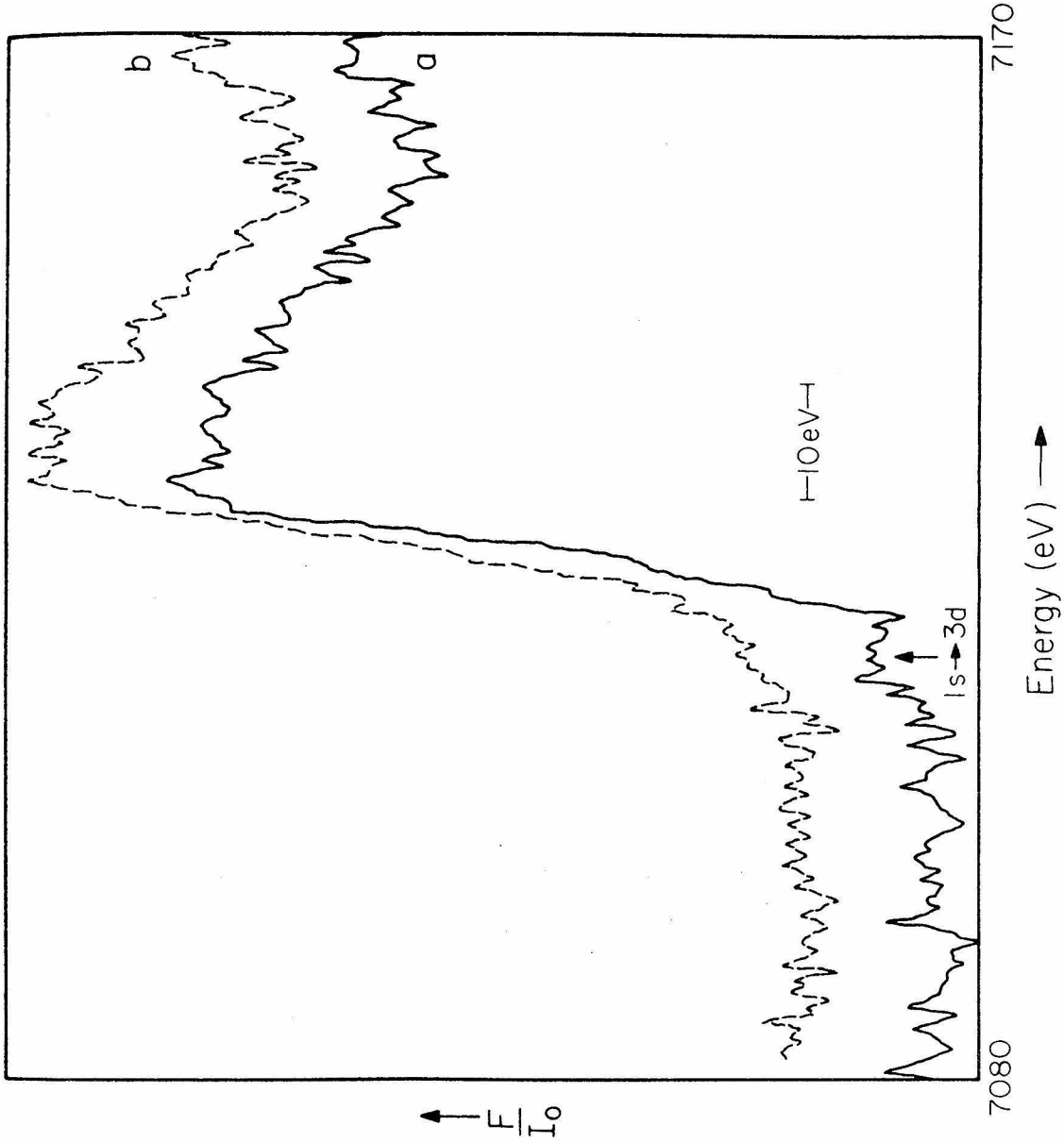
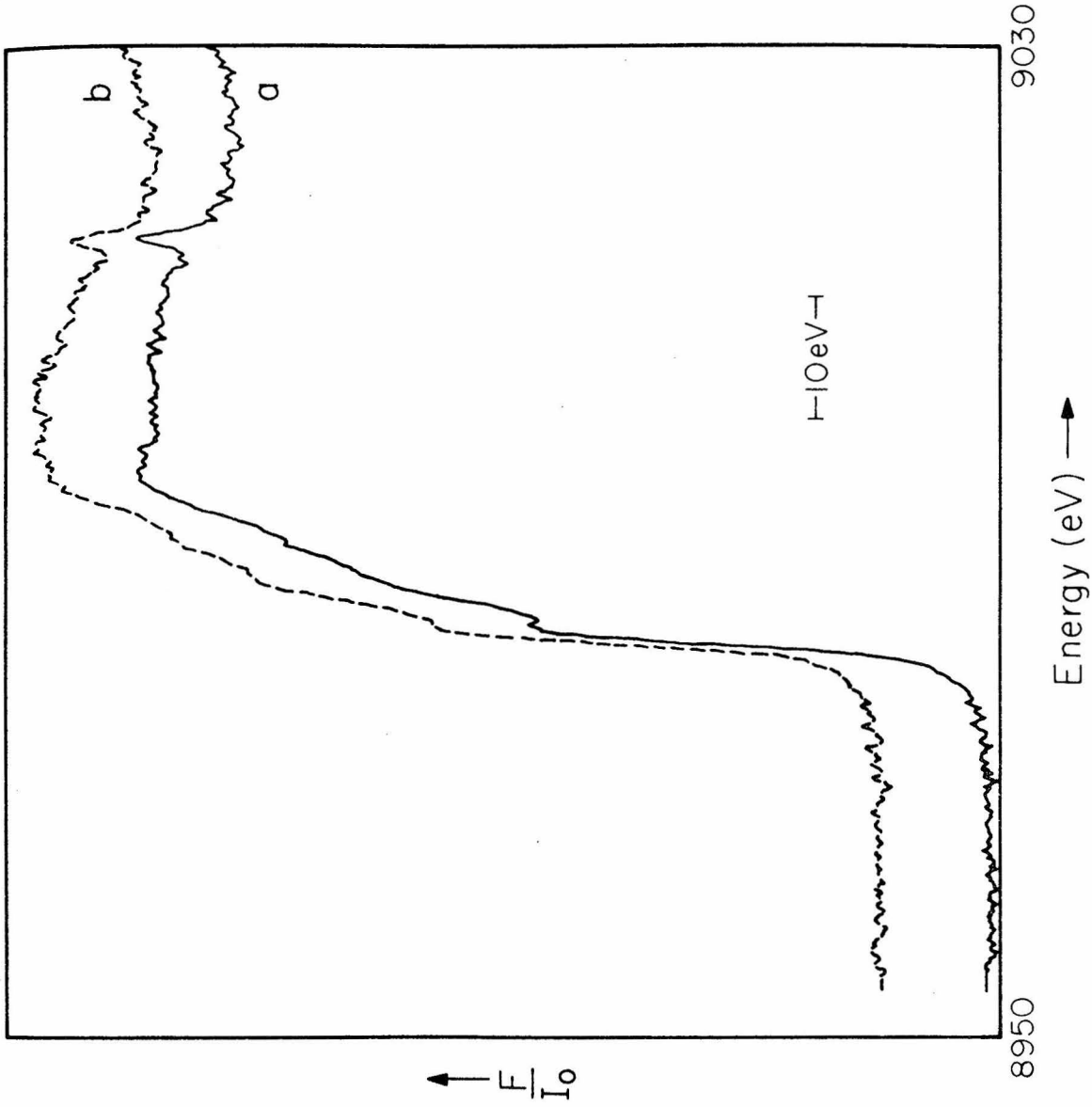

b
a
10eV
1s→3d
7080
7170
Energy (eV) ⟶
F/Io

Figure 3. The copper K-edge spectra of a) the dithionite-reduced CN-complex of cytochrome c oxidase, and b) reduced cytochrome c oxidase


b
a
10eV
8950
9030
Energy (eV)
F/Io

comparison we also show the spectrum of the fully reduced oxidase. There is no apparent spectral difference between the "half-reduced" CN-complex and the fully reduced protein as far as the copper edge is concerned. This implies that both coppers are in the +1 oxidation state in the so-called "half-reduced" complex described by Yong and King [11]. This result is entirely consistent with the available EPR data [4,11,14]. The addition of excess dithionite to cyanide-treated cytochrome c oxidase results in the disappearance of the low-spin ferric heme signal at g = 3.03, as well as a decrease in the very weak high-spin heme signal at g = 5.86. Concomitantly, a new signal appears at g = 3.58. The latter has been attributed to a low-spin ferric heme of the cyano-complex of cytochrome a_3 [4]. In addition, Yong and King have shown that the g = 2 signal disappears in the "half-reduced" complex. We have confirmed this observation. Yong and King have attributed the disappearance of the g = 2 signal to the reduction of one of the coppers [11]. However, they did not explicitly assign an oxidation state to the second copper, which is presumably EPR-silent.

Taken together, the EPR spectra of the dithionite-treated CN-complex show the loss of two paramagnetic entities (centered at g = 3.03 and g = 2) and the gain of one new paramagnetic entity, heme a_3-CN. The latter is supposedly antiferromagnetically coupled to the EPR-invisible Cu in the fully oxidized protein [17]. If the coupling between heme a_3 and invisible Cu is broken and heme a_3 becomes EPR-visible, the EPR-invisible Cu should also become detectable by EPR if it were still oxidized. However, no Cu EPR signals are observed, implying that both coppers are reduced.

Recent X-ray absorption studies on oxidized and reduced cytochrome c oxidase provide evidence that one of the coppers in the oxidized protein is already in the +1 oxidation state [13]. This would imply that either the EPR signal at g = 2 is due to the remaining copper (which would be in contradiction to other studies which suggest antiferromagnetic coupling between a heme and a copper in the oxidized protein) or that it is due to some other paramagnetic species that accepts electrons reversibly. Hu et al. have postulated that one of the four redox sites is a protein ligand perhaps coupled to Cu^{+1} in the oxidized protein [13]. A Cu(I)-disulfide system has been previously discussed as a possible electron acceptor in cytochrome c oxidase [18]. In addition, the possibility that the EPR signal at g = 2 is due to a sulfur radical in a particular environment has also been raised [19]. However, regardless of the assignment of the g = 2 EPR signal, the X-ray absorption and EPR studies together show that the CN-complex of cytochrome c oxidase in the presence of excess reducing agent can accept three reducing equivalents.

We are deeply indebted to Drs. Tsoo E. King, Chang-An Yu, and Linda Yu of the State University of New York at Albany for generously supplying us with the cytochrome c oxidase used in this experiment.

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IV. SUMMARY

The major objective of the studies described in the preceding chapters has been the characterization of the metal centers in cytochrome c oxidase using x-ray absorption spectroscopy. In particular, we were interested in determining the oxidation state of the spectroscopically invisible copper, since this information is not directly obtainable by other methods. In Chapter II, we presented evidence that indicates that one of the two copper ions in cytochrome oxidase is in the +1 oxidation state in the oxidized protein. This result, while apparently resolving the issue of EPR-silent Cu, raises doubt regarding the identity of one of the four redox sites in cytochrome oxidase. To accommodate four electrons in oxidized cytochrome oxidase, we have therefore suggested that one of the four electron acceptors is an amino acid residue of the protein, perhaps coupled to Cu(I). Since none of the ligands to Cu are known, current studies in our laboratory are directed toward this problem.

With respect to the oxidation states of the metal ions in cytochrome oxidase, we were also interested in determining the state of reduction of the protein in a "mixed valence" compound of cytochrome oxidase. In examining the x-ray absorption spectra of the "half-reduced" cyanide complex of cytochrome oxidase (Chapter III), we found that both coppers were reduced. In conjunction with the available EPR data, this result indicates that not two but three of the four electron acceptors in cytochrome oxidase are reduced in the dithionite-treated cyanide complex of cytochrome c oxidase.

Our studies on the Fe K-edge of oxidized and reduced cytochrome oxidase, though limited, indicate that, in terms of electronic environment, the heme irons of cytochrome oxidase more closely resemble the covalent iron hexacyanides and the heme of cytochrome c than the ionic iron fluorides. In addition, the effect of ligand binding to the heme iron can also be observed. Thus, these studies demonstrate the potential usefulness of x-ray absorption edge spectroscopy in obtaining information about the charge density of the metal components in metalloproteins.

PART II. AN INVESTIGATION INTO THE LIPID FACTORS AFFECTING PROTEIN ACTIVITY AND RESPIRATORY CONTROL IN RECONSTITUTED CYTOCHROME c OXIDASE MEMBRANES

I. GENERAL INTRODUCTION

The mitochondrion (Fig. 1) is a subcellular compartment whose primary function is the production of energy in the form of ATP through the coupled processes of electron transport and oxidative phosphorylation. The enzymes that are responsible for these processes are situated in the inner mitochondrial membrane where the electron transport components make up what is collectively known as the respiratory chain. Though highly complex in structure and composition, the respiratory chain can be broken down into four functionally discrete complexes, each catalyzing a specific set of redox reactions (Fig. 2). The most studied of these complexes is Complex IV which is at the terminal end of the respiratory chain. Complex IV catalyzes the oxidation of ferrocytochrome c by molecular oxygen and, in addition, couples the energy released in the oxidation to the synthesis of ATP. The enzyme that makes up this complex is known as cytochrome c oxidase and contains as its prosthetic groups two heme A's and two copper ions.[1] Phospholipids also have been found to be an important part of this complex, since their removal from a cytochrome oxidase preparation results in a loss of protein activity.[2,3] This activity is restored upon reconstitution of the inactive protein with certain phospholipids. Thus, the essentiality of phospholipids for cytochrome oxidase activity has been demonstrated. However, it has not yet been established what role the lipids play in the activation of the protein.

In this respect, the inner mitochondrial membranes from a number of tissues and species display a remarkable similarity in lipid

Fig. 1. Schematic representation of mitochondrial structure

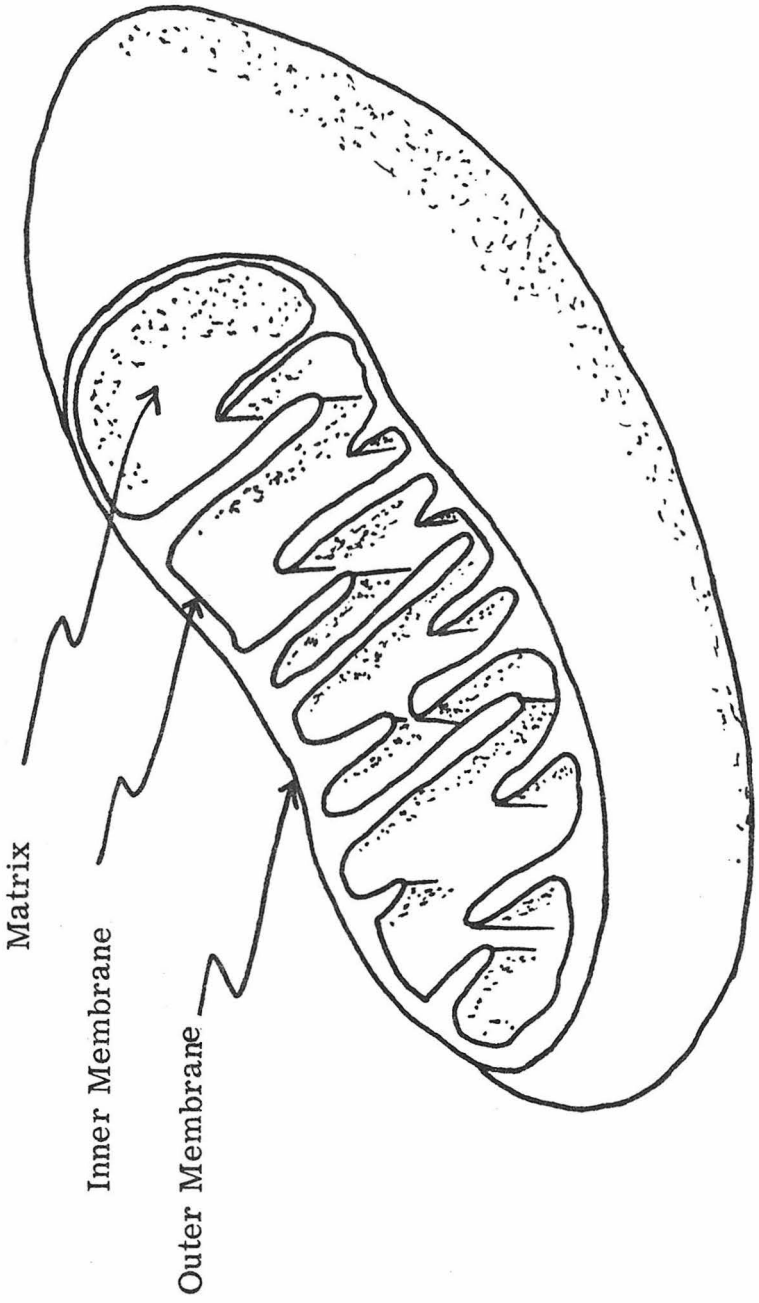
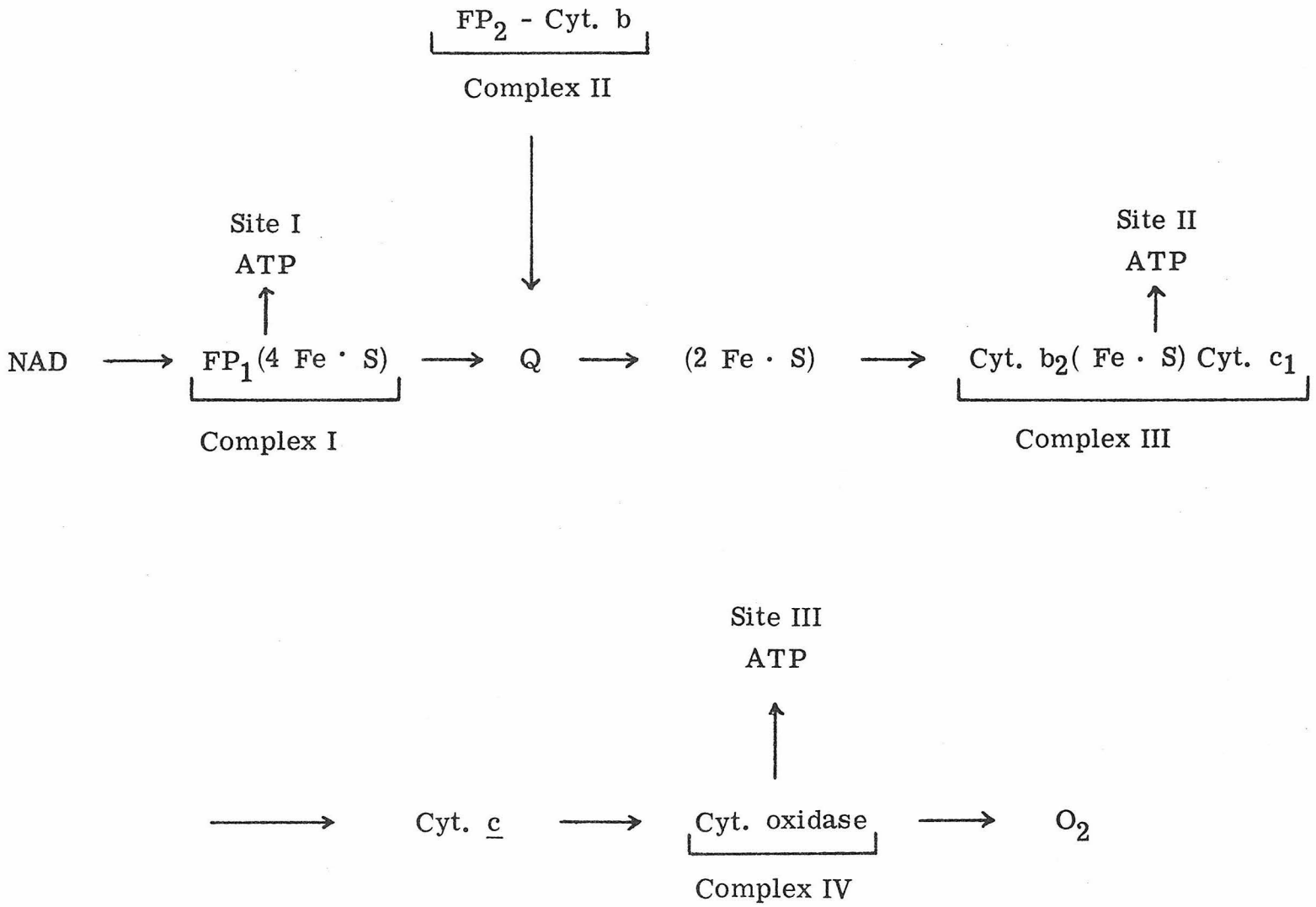

Matrix
Inner Membrane
Outer Membrane

Fig. 2. The respiratory chain


FP2 - Cyt. b
Complex II
Site I
ATP
Site II
ATP
NAD
FP1 (4 Fe · S)
Q
(2 Fe · S)
Cyt. b2 (Fe · S) Cyt. c1
Complex I
Complex III
Site III
ATP
Cyt. c
Cyt. oxidase
O2
Complex IV

composition. In general, they contain roughly 28% lipid by weight, of which more than 90% is phospholipid. Of the phospholipids present, 37% is phosphatidylcholine, 31% phosphatidylethanolamine, and 16% cardiolipin(diphosphatidylglycerol).[3] This constancy in the ratio of lipid to protein, as well as in the relative amounts of different lipids, implies a structure-function relationship which is common to almost all biological systems. Yet, the details of the relationship are unclear.

When cytochrome oxidase is fractionated from the inner mitochondrial membrane, the lipid component that is tightly associated with the protein is highly enriched in cardiolipin (an acidic phospholipid).[4] Upon further stripping of the lipids from the enzyme, it was found that a minimum of 1-2 molecules of cardiolipin per molecule of oxidase is absolutely essential for maintaining the protein in a form which can be activated by the addition of more phospholipids.[4] It has been suggested that these few residual molecules of cardiolipin (sometimes called Awasthi lipids) are necessary for maintaining the structural integrity of the protein. A rather detailed study of the tightly-bound lipid reveals a slightly higher level of saturated fatty acids in this component than in the bulk lipid, and a resistance of this lipid fraction to phospholipase A.[4] Also, it was found that of the several natural lipid components, cardiolipin was most effective in restoring protein activity. Moreover, the requirement for cardiolipin is implicated in the coupling process between electron transport and oxidative phosphorylation. Chemical agents, called uncouplers, which are highly effective in destroying the coupling of ATP synthesis to the redox reactions of the

respiratory chain *in vivo* and *in vitro* have been shown to greatly enhance the activity of reconstituted cytochrome oxidase, particularly in membranes containing cardiolipin.[5] This link between cardiolipin and chemical uncoupling makes it a prime target in the study of the role of phospholipids in energy coupling, or respiratory control, processes.

In addition to the specific lipid composition of mitochondrial membranes, it is now evident that the physical properties of the membrane as a whole influence the various membrane-associated processes. With respect to energy coupling, the structural and spatial integrity of the membrane is important. Disruptive procedures (such as detergent solubilization) have been shown to destroy the coupling between oxygen reduction and ATP synthesis without affecting the ability of the protein to carry out the electron transfer reactions.[6,7] Furthermore, it has been demonstrated that an asymmetric arrangement of proteins with respect to the membrane is necessary for the membranes to exhibit coupling phenomena. For example, reconstituted membranes containing both the cytochrome *c* oxidase and the ATP synthetase complexes are incapable of ATP synthesis unless the reductant, ferrocytochrome *c*, is confined to one side of the membrane.[8] This demonstration of the vectorial nature of the redox and coupling processes has been taken to be strongly supportive of Mitchell's chemiosmotic hypothesis of energy coupling.[9-11] This and other coupling hypotheses will be discussed in Chapter III of this section.

In terms of protein activation, there is yet another bulk membrane property that is important. It has been demonstrated that a

number of physiological functions (such as amino acid and carrier-mediated ion transport across membranes, and the activity of most membrane-bound enzymes) are highly sensitive to the physical state of the membrane lipids.[12-16] Usually, the rate of transport or the protein activity is greater when the lipids are in the liquid crystalline, or fluid, state than when they are in the gel state. Although it has been postulated that the fluidized membrane enhances protein activity by easing configurational restraints on the protein,[13,17] other explanations are possible. For example, in the case of cytochrome oxidase membranes, phase-dependent aggregation or diffusion processes may also control the overall reaction rates.

In the work that follows, we have attempted to systematically study the lipid factors affecting protein activity and respiratory control in reconstituted cytochrome oxidase membranes. In earlier studies of this nature, the heterogeneity of the lipids used in the reconstitution of the protein as well as the high level of natural phospholipid ($\sim$ 20% by weight) associated with the protein itself, have led to confusing and sometimes contradictory results.[1,2,4,18] Thus, we have carried out our studies with cytochrome oxidase membranes which were reconstituted with well-defined lipids and delipidated cytochrome c oxidase ($<$ 3% lipids by weight).

In Chapter II of this section, we investigate the activity dependence of cytochrome oxidase on the physical state of the membrane lipids. We do this by varying not the temperature of the samples, but the ratio of the two phospholipids (dimyristoyl- and dipalmitoylphosphatidylcholine)

used in the reconstitution. In this manner, we obtain samples whose respective phase transition temperatures vary linearly with the ratio of the two components over a relatively narrow temperature range. Thus, we are able to monitor the effect of lipid fluidity on the activity of cytochrome oxidase at a set temperature. The advantages of this approach are discussed in the following chapter.

In Chapter III, we present the results of a number of studies directed toward the elucidation of lipid factors governing uncoupler sensitivity in reconstituted cytochrome oxidase membranes. In particular, the effects of the lipid to protein ratio and the presence of cardiolipin on the development of respiratory control in these model systems have been investigated. On the basis of our results, several ideas are presented regarding the influence of acidic phospholipids on the responsiveness of the membranes towards chemical uncouplers.

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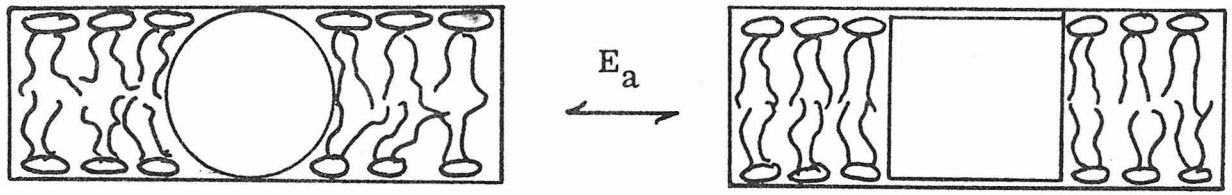
II. THE DEPENDENCE OF CYTOCHROME c OXIDASE ACTIVITY ON THE GEL TO LIQUID CRYSTALLINE PHASE TRANSITION OF THE PHOSPHOLIPIDS IN MODEL MEMBRANES

1. Introduction

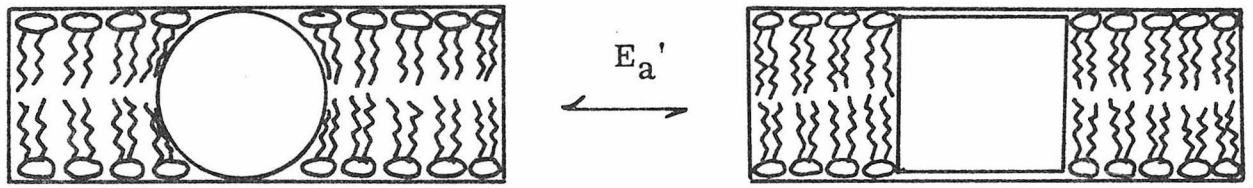
In recent years, it has become increasingly clear that the lipid-related phase transitions occurring in biological membranes have a marked effect on a variety of membrane-associated functions [1-6]. The influence of the gel to liquid crystalline phase transition on the kinetics of some respiratory enzymes is of particular interest, since most of these proteins are tightly associated with membrane lipids. Previous results with intact mitochondria have indicated that there exists a temperature-induced change in the activation of certain membrane-bound enzymes which is related, at least in part, to the physical state of membrane lipids [7,8]. This has been inferred from the discontinuities in the Arrhenius plots of spin-label and oxidative activity studies for a variety of mitochondrial enzymes from homeothermic animal and chilling-sensitive plant tissues [8].

It has been suggested that lipids in the gel state impose a physical constraint on protein conformational changes that are associated with the proper functioning of the enzyme. This constraint is partially relieved when the lipids are above their respective phase transitions. This idea is best summarized in Figure 1. However, the results reported for cytochrome c oxidase from two different laboratories are incongruent [9,7]. Furthermore, the breaks in the Arrhenius curves do not always coincide with the phase transition temperatures of membrane lipids [9-11].

Figure 1. Model for the effect of the physical state of the lipids on protein conformational changes



Liquid Crystalline State ($>T_c$)



Gel State ($< T_c$)

$E_a' > E_a$

This may be due to the heterogeneity of lipids in natural membranes complicated by the possible presence of separate lipid phases with different transition temperatures.

Thus, it is desirable to study the effects of the physical state of the lipids on enzyme activity in model membranes in which the protein and lipid composition can be rigorously controlled. In the work reported here, we have attempted to evaluate the role of phospholipids on the activation of cytochrome c oxidase, the terminal enzyme in the respiratory process, using a simplified membrane system. Our experimental approach involved varying the lipid composition rather than the temperature in assaying for the effect of the gel to liquid crystalline phase transition temperature on the activity of the protein. In synthetic systems, the phase transition temperature of the model membrane can be easily varied by choosing appropriate mixtures of phospholipids.

We have reconstituted delipidated cytochrome c oxidase into membranes containing varying ratios of dimyristoyl and dipalmitoyl phosphatidylcholine, and have characterized these reconstituted membranes by activity assays, electron microscopy and turbidity measurements. Our results demonstrate that, at a given temperature, the oxidase activity depends on the composition of the lipid bilayer vesicles, being higher in those membranes which are in the liquid crystalline phase. These results corroborate the findings on intact mitochondria that the oxidase activity is a function of the physical state of the membrane lipids; however, they bypass some of the problems inherent in the interpretation of the earlier experiments involving natural membranes. On the basis of

our findings, a number of possibilities are discussed for the effect of the phospholipid phase transition on the turn-over rate for the reduction of oxygen by membranous cytochrome c oxidase.

2. Materials and Methods

Materials. Cytochrome c oxidase from beef heart mitochondria was generously provided by Drs. Tsoo E. King, Chang-An Yu, and Linda Yu at SUNY, Albany, New York. The preparation used in these studies was a stock solution of delipidated cytochrome oxidase (3 μmol P/μmol heme A) suspended in 1% sodium cholate in a 50 mM phosphate buffer pH 7.4 at a protein concentration of 81 mg/ml. The activity of this solution was less than 0.06 nmole O_2 uptake per nmole heme a per sec, which is 0.34% of the activity of phospholipid-rich cytochrome oxidase [12].

Horse heart cytochrome c Type VI and dioleoyl lecithin (DOL) were purchased from Sigma. Of the other phospholipids, β, γ-dimyristoyl lecithin (DML) and β, γ-dipalmitoyl lecithin (DPL) were purchased from Calbiochem, and beef heart cardiolipin (DPG) was obtained from Serdary Research Laboratories, ascorbate from Matheson, Coleman and Bell, sodium cholate from Schwarz-Mann, and Sigma 7-9 Biochemical Buffer (Tris) from Sigma. Egg yolk lecithin (EYL) was purified from egg yolks following published procedures [13]. All lipids used showed single spots on thin layer chromatographic plates.

Mixed lipid samples were prepared by dissolving the appropriate amounts of pure lipid in chloroform and lyophilizing the solution first in a hot sand bath and then on a high vacuum line for about one day.

Preparation of Reconstituted Cytochrome c Oxidase Membranes. A 2% solution of the appropriate phospholipid in 10 mM Tris buffer pH 7.4 was sonicated until clear (∿ 15-20 min) with a Bronson microtip sonicator in a glycerol bath. After a brief (∿ 10 min) centrifugation to remove any unsonicated material and probe particles, an equal volume of 2% Na-cholate in 10 mM Tris buffer was added to the vesicle solution. After a light heating with a heat gun, the solution became completely clear. To this clear solution was added stock cytochrome oxidase in 1% Na-cholate. The amount of protein added was approximately 20% of the weight of the phospholipid, unless otherwise stated. After a 2-3 hour incubation on ice, the samples were dialyzed extensively for 1.5 - 2 days against at least 600 volumes of the buffer without cholate. Dialysis was carried out in a refrigerator either at 13°C, or at 38°C, which is above the phase transition temperature for all samples except that of pure DPL.

Cytochrome c Oxidase Activity Assay. Temperature-dependent polarographic assays of cytochrome c oxidase activity were obtained by measuring the uptake of oxygen by a solution containing ascorbate, cytochrome c, and reconstituted cytochrome c oxidase membranes according to a procedure described by Smith and Camerino [14] and elaborated on by Dr. Chang-An Yu [15]. A Yellow Springs Instrument oxygen monitor (Model 53) and oxygen probe were used to follow this process.

Electron Microscopy (EM) Studies. EM grids were prepared using the standard drop method with 0.5% potassium phosphotungstate pH 7.5 as the negative stain. Both sample and stain were allowed to remain on the

grid for 30 seconds before their respective removal by washing or by draining with a piece of filter paper. The grids were examined on a Phillips 201 electron microscope. The size of the lipid structures was determined by using actual film magnifications calibrated to the magnification settings on the microscope. Electrostatically charged carbon-coated parlodion or formvar was used for specimen support on all of the grids.

Turbidity Measurements. The optical densities (at λ = 600 nm) of the lipid solutions were obtained using a Beckman Acta CIII spectrophotometer equipped with a T_m programming unit, which allowed continuous variation of the cell temperature.

3. Results

Protein Activity versus Lipid Composition. We have reconstituted cytochrome c oxidase with a variety of lipids (Table I) and have found that the activity in these systems compared favorably with that obtained by other workers using mainly mitochondrial and mixed soybean lipids (asolectin) [16,17]. The activity values obtained range from 10 to 60% of the maximal activity reported by Yu et al. for phospholipid-rich oxidase containing about 20% mitochondrial lipids assayed in the presence of cholate [12]. Although the oxidase activity in DML is lower than the activity in any of the unsaturated lipids, it is nevertheless a 30-fold increase in activity over that of the delipidated protein. In addition, if one allows for the two possible vectorial orientations of the protein across the bilayer and the presence of a certain amount of multilamellar

TABLE I: Activity of Cytochrome c Oxidase Reconstituted with Various Lipids (Lipid/Protein = 20/1 (w/w))

Phospholipid	Activity at 30°C[a,b]
Asolectin	6.0
Cardiolipin (DPG)	6.3
Egg Yolk Lecithin (EYL)	5.6
EYL + DPG (10 mole %)	6.5
Dioleoyl Lecithin (DOL)	8.0
DOL + DPG (10 mole %)	6.2
Dimyristoyl Lecithin (DML)	1.8
DML + DPG (10 mole %)	3.1

For comparison:		Ref.
Purified mitochondrial lipids	2.3-5.5[c,d]	16
Mixed mitochondrial lipids	1.2[c]	17
Asolectin[e]	17.5	12

[a]The activity is given in units of nmol O_2/nmol heme A/sec

[b]All samples were assayed in 50 mM phosphate buffer, pH 7.4

[c]The activity values reported in Ref. 15 and 16 have been converted to the units stated in a.

[d]Assayed at room temperature

[e]In the presence of cholate

structures, the highest activity measured is probably only a lower bound value for this reconstituted system. Thus, we feel that we have successfully attained a sufficiently active model membrane for our studies.

The rate of oxygen uptake was measured for cytochrome c oxidase which was reconstituted in DML, DPL, and various mixtures of the two lipids. Each set of measurements was performed at a constant temperature. This had the advantage of permitting us to monitor the effect of the lipid phase transition on enzymatic activity by compositional changes in the membrane rather than by temperature changes which affect the activation energy of the protein and consequently may mask true lipid effects. Furthermore, since the phase transition temperatures of the samples investigated cover only a narrow temperature range with a difference of just a few degrees between consecutive samples, this experimental design is capable of accentuating activity differences that are dependent only on the physical state of the lipids at a given temperature. This is in contrast to activity measurements performed at a constant temperature on samples with vastly different transition temperatures (Table I).

In Figure 2 the oxidase activity of various samples corresponding to different DML/DPL compositions is plotted versus the difference between the temperature of measurement (T_m) and the phase transition temperature (T_c) of the respective lipid medium for T_m's of 28 and 32°C. For each isotherm, T_m is kept constant, while T_c, and hence the lipid composition, varies. This manner of plotting the data is better understood if one considers the relation of T_m to the phase diagram of the two-component DML-DPL system (water not included in the diagram). From

Figure 2. Activity versus $(T_m - T_c)$ for samples of different lipid compositions dialyzed at 38°C and assayed at 28.4°C (●) and at 32.0°C (▲).

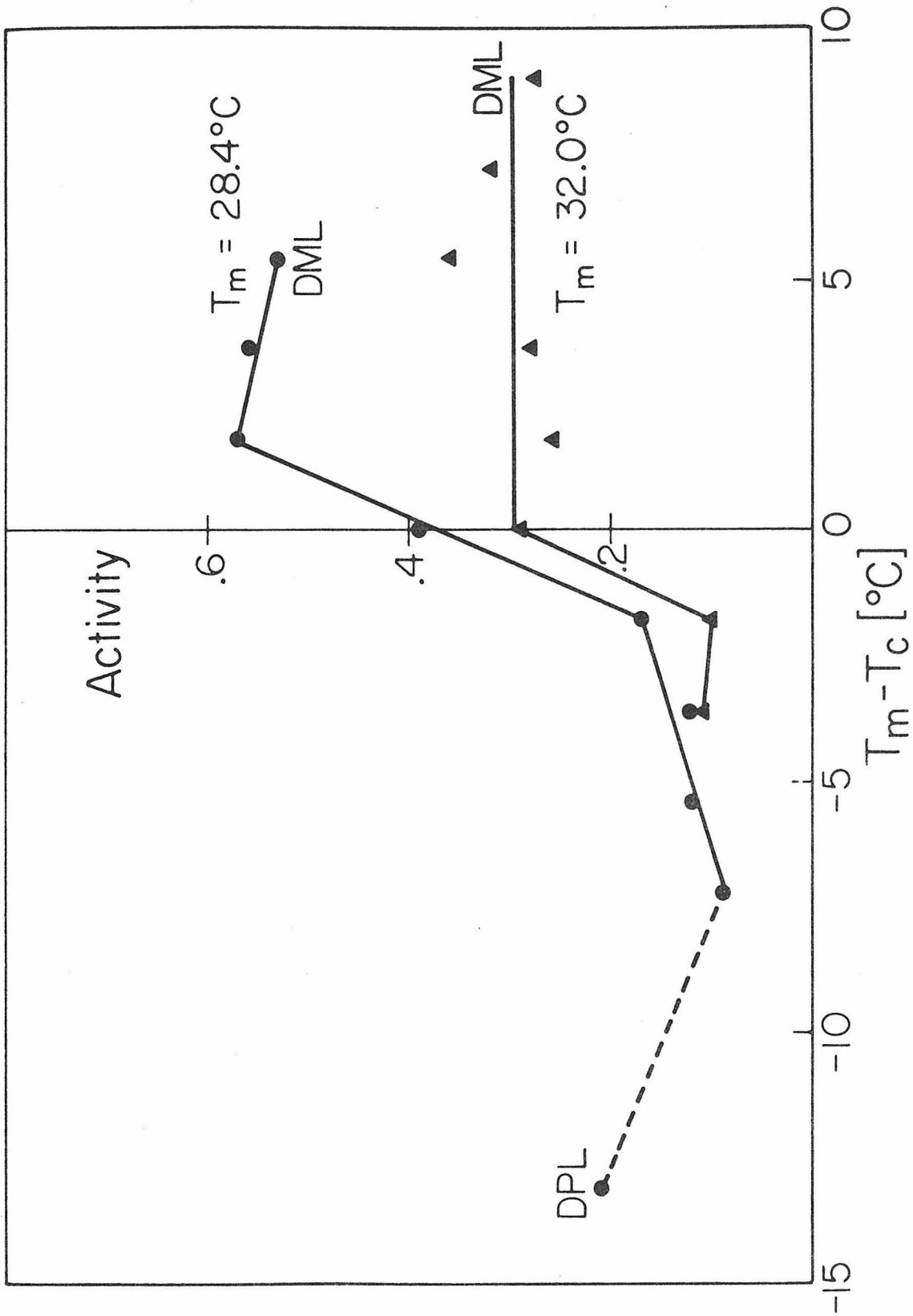

Activity
.6
.4
.2
0
-5
-10
-15
10
5
$T_m - T_c$ [°C]
T_m = 28.4°C
DML
T_m = 32.0°C
DML
DPL

Figure 3. Relation of T_m to a phase diagram of the DML-DPL system. o represents the midpoints of the phase transition for the respective lipid mixtures as determined by turbidity versus temperature measurements

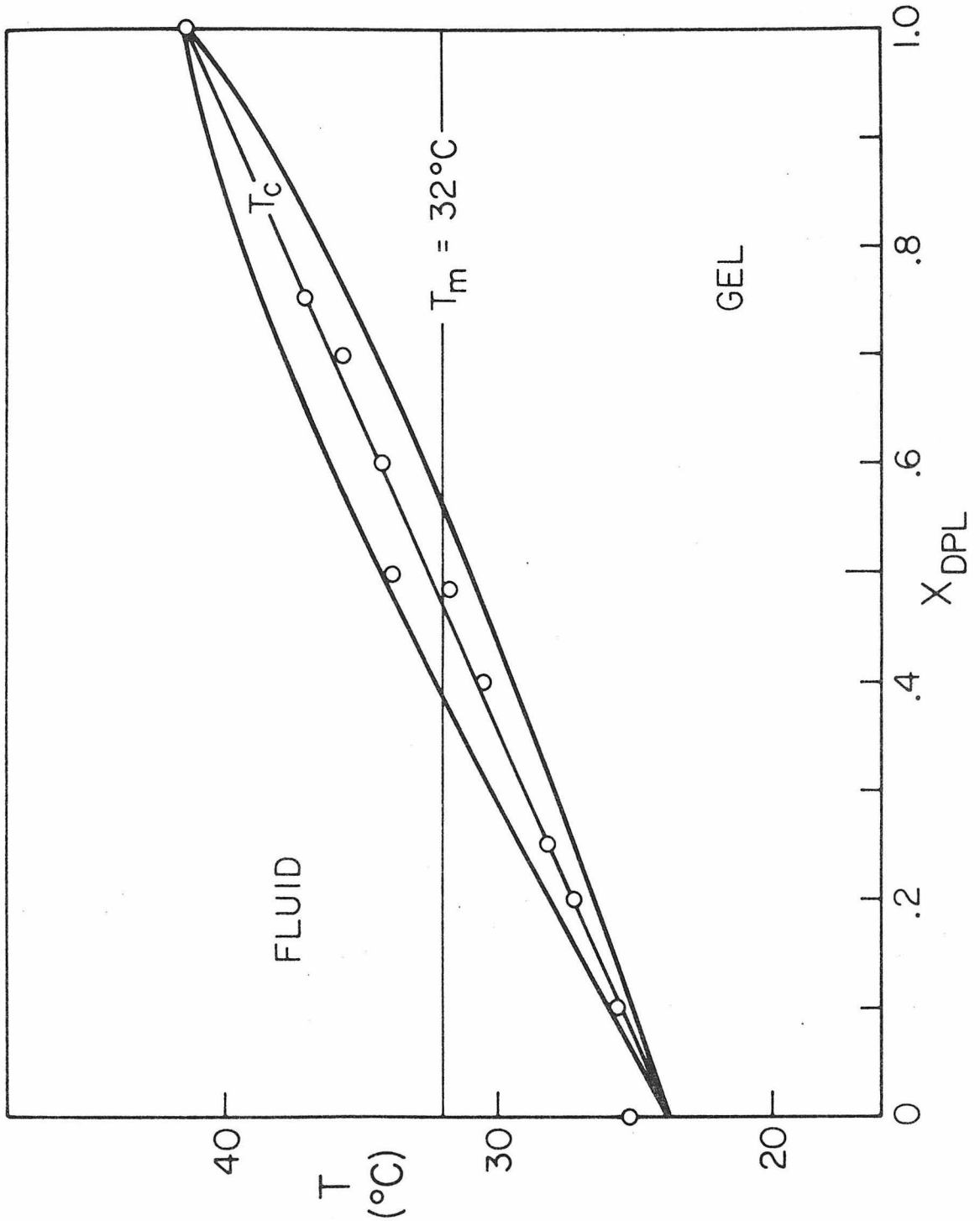

FLUID
GEL
T_c
T_m = 32°C
T (°C)
40
30
20
0
.2
.4
.6
.8
1.0
X_{DPL}

TABLE II: Absorbance vs. Temperature Heating Curves for Samples Dialyzed at 38°C

DML:DPL (w:w)	T_c(calc.) (°C)	T_c(exp) (°C) onset	midpoint	end	transition breadth	$\lvert T_c(\text{exp}) - T_c(\text{calc}) \rvert$
10:0	23.7	24.4	25.1	25.75	1.35	1.4
9:1	25.45	24.8	25.5	26.3	1.50	.05
8:2	27.05	26.0	27.1	28.0	2.0	.05
6:4	30.5	29.2	30.3	31.3	2.1	.2
5:5	32.6	33.3	33.9	34.5	1.2	1.3
4:6	33.85	33.3	34.2	35.0	1.7	.35
3:7	35.8	35.2	35.7	36.5	1.3	.1

TABLE III: Absorbance vs Temperature Heating Curves for Samples Dialyzed at 13°C

DML:DPL (w:w)	T_c(calc.) (°C)	T_c(exp) (°C) onset	midpoint	end	transition breadth	$\lvert T_c(\text{exp}) - T_c(\text{calc.})\rvert$
10:0	23.7	24.4	24.8	25.2	.8	1.1
7.5:2.5	28.1	27.2	28.2	29.2	2.0	.1
5:5	32.6	30.2	31.9	33.5	3.3	.7
2.5:7.5	37.0	35.0	37.0	38.8	3.8	0
0:10	41.5	40.8	41.3	41.7	.9	.2

TABLE IV: Absorbance vs. Temperature Heating Curves for Samples Dialyzed at 13°C and Annealed at 37°C

CML:DPL (w:w)	T_c(calc.) (°C)	T_c (exp) (°C) onset	midpoint	end	transition breadth	$\lvert T_c(\text{exp}) - T_c(\text{calc.}) \rvert$
10:0	23.7	25.2	25.9	26.6	1.4	2.2
7.5:2.5	28.1	27.7	28.9	30.0	2.3	.7
5:5	32.6	31.1	32.3	33.4	2.3	.3
2.5:7.5	37.0	34.8	36.4	38.0	3.2	.6
0:10	41.5	40.2	40.7	41.2	1.0	.8

Figure 3 it is easily seen that all compositions which have phase transition temperatures below T_m, and therefore positive values for T_m-T_c, will be in the liquid crystalline state at the assay temperature. Conversely, all samples with phase transition temperatures above T_m (i.e., with negative T_m-T_c values) will be in the gel state during the assay. The difference between T_m and T_c reflects the extent to which the lipids are above or below their respective phase transition temperatures during the assay. For the sake of simplicity, T_c of each of the mixed lipid samples has been taken to be the temperature corresponding to the appropriate mole fraction of DML and DPL along the straight line connecting the phase transition temperatures of pure DML and pure DPL suspensions in the phase diagram. These temperatures represent the midpoint of the phase transition of the respective lipid suspensions if one assumes ideal mixing of the lipids. In fact, the experimental values for the transition midpoints determined by turbidity versus temperature measurements on these samples (represented by circles on the graph in Figure 3) are in close agreement with the values used (see also Tables II, III, and IV).

As can be seen in Figure 2, samples which are above their phase transitions show a noticeably higher oxidase activity over those which are still below their phase transitions. As the assay temperature is changed by just a few degrees, the proportion of samples which exhibit higher activity changes accordingly (Figure 2). These results with a well-defined lipid system confirm the earlier finding that cytochrome c oxidase activity is a function of the physical state of the membrane

lipids [7,8]. It should also be pointed out that there is very little variability in activity among DML/DPL samples which are above their respective transitions and similarly among samples which are below their respective transitions. In addition, in the mixed lipid samples, the change in activity is correlated with the phase transition of the mixture, rather than that of either pure component. These results may be taken as evidence that the protein has very little preference for either of the lipids used, and that the lipids are in a homogeneous phase.

In the course of this study, it was noted that samples dialyzed below the lipid phase transitions do not show a pronounced increase in activity upon passing through the gel to liquid crystalline transition. This behavior is depicted in Figures 4a and 5a. However, when these samples are subjected to an "annealing" process which involves heating the lipid solutions above their respective phase transition for a short time, the stepped activity versus $T_m - T_c$ plot is again observed (Figs. 4b and 5b). While there is a decrease in activity for samples above T_c at T_m upon annealing, the drop in activity for samples below T_c at T_m is even greater. It has also been noted that samples which are heated to within a few degrees of their phase transitions but never beyond it exhibit higher than expected activities in the gel state after heating. These results demonstrate differences in protein-lipid interactions which are dependent on the manner in which the membranes are prepared. Since these differences seem to be related to the physical state of the lipids during the reconstitution process, one might expect to find a

Figure 4. Activity versus ($T_m - T_c$) curves for (a) samples dialyzed at 13°C (●), and (b) samples dialyzed at 13°C and annealed at 33°C (■). Samples to the left of the arrow have phase transition temperatures above 33°C, the chosen "annealing" temperature

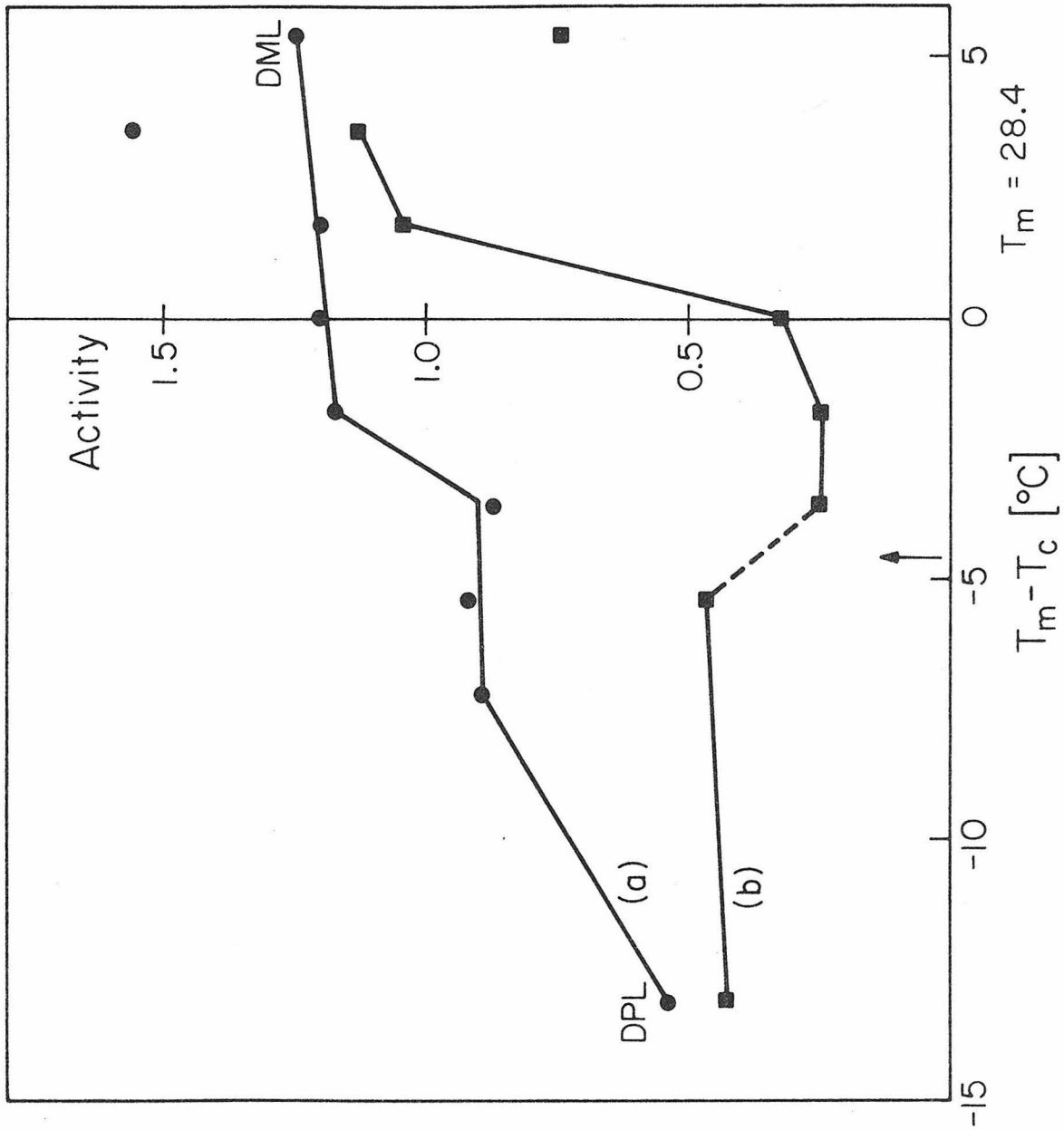
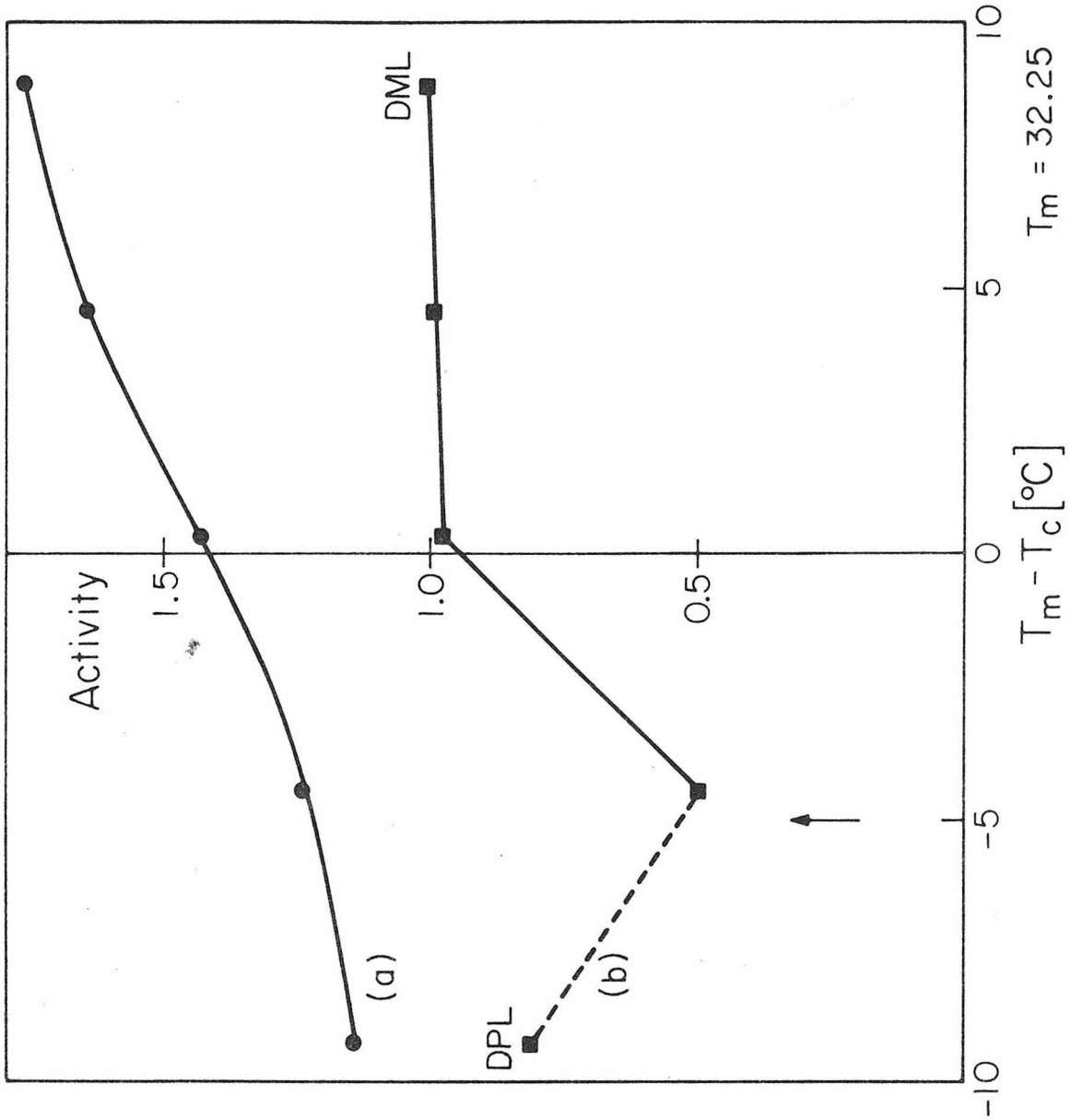

Activity
1.5
1.0
0.5
DML
DPL
(a)
(b)
0
-5
-10
-15
$T_m - T_c$ [°C]
T_m = 28.4

Figure 5. Activity versus ($T_m - T_c$) curves for (a) samples dialyzed at 13°C (●), and (b) samples dialyzed at 13°C and annealed at 37°C (■). DPL has a phase transition temperature of 41.5°C and thus samples incorporated into this lipid are not annealed at 37°C


Activity
1.5
1.0
0.5
DML
DPL
(a)
(b)
-10
-5
0
5
10
$T_m - T_c$ [°C]
T_m = 32.25

lipid-associated structural difference between the membranes prepared above and below their phase transitions.

Electron Microscopy Studies. A structural difference between samples dialyzed above and below the phase transition is indeed borne out in the electron micrographs of the samples. The low temperature samples are generally huge, one micron or more, conglomerates of lipid material, while the high temperature samples are more discrete, apparently single-walled vesicles which are a few thousand angstroms in diameter (cf. Figs. 6a and 6b). In view of this difference in the two systems, it would be of interest to investigate the nature of protein-lipid associations during dialysis above and below the phase transition of the model membranes used. This may well lend insight into the mechanism of protein incorporation into lipid bilayers.

Turbidity Measurements. A number of earlier studies on the temperature dependence of the optical properties of phospholipid dispersions demonstrate changes in the refractive index of the solutions at the phase transition temperature of the lipids studied [18-20]. These changes in the optical properties of the colloidal lipid suspensions have been attributed to changes in the specific volume of the bilayers which in turn affects the refractive index upon melting of the bilayer membrane [19-21]. The special advantage of this method is that it allows one to monitor the phase transitions in vesicles (similar to monitoring T_c in multilayers by thermal methods) with virtually no perturbation of the experimental system (e.g., by spin probes in ESR partition studies). Thus we have elected to measure the turbidity of our

Figure 6. Electron micrograph of (a) a cytochrome oxidase-DML sample dialyzed at 7°C ($< T_c$), and (b) a cytochrome oxidase-DML sample dialyzed at 27°C ($> T_c$).

reconstituted systems as a function of temperature in order to ascertain the phase transition temperatures of the various reconstituted cytochrome c oxidase membrane samples.

The data obtained from these thermal-turbidimetric studies are summarized in Tables II, III, and IV for samples prepared under different conditions. Results show that the midpoint of the gel to liquid crystalline transition in the reconstituted cytochrome c oxidase membranes generally occurs within 2° and often within 1° of the temperature predicted, on the basis of ideal mixing of the lipids. We take this as supportive evidence against gross phase separation of the two component lipids in the model membranes studied. It should be noted, however, that the experimental values for the onset, midpoint, and end of the phase transitions are derived only from the heating curves, since there is an apparent hysteresis in the cooling curve (Fig. 7). Although this hysteresis is minimized when the rate of cooling is decreased, it is nonetheless absent in pure lipid systems [18,20,21]. This result suggests that the transition hysteresis is induced by the proteins in the bilayer.

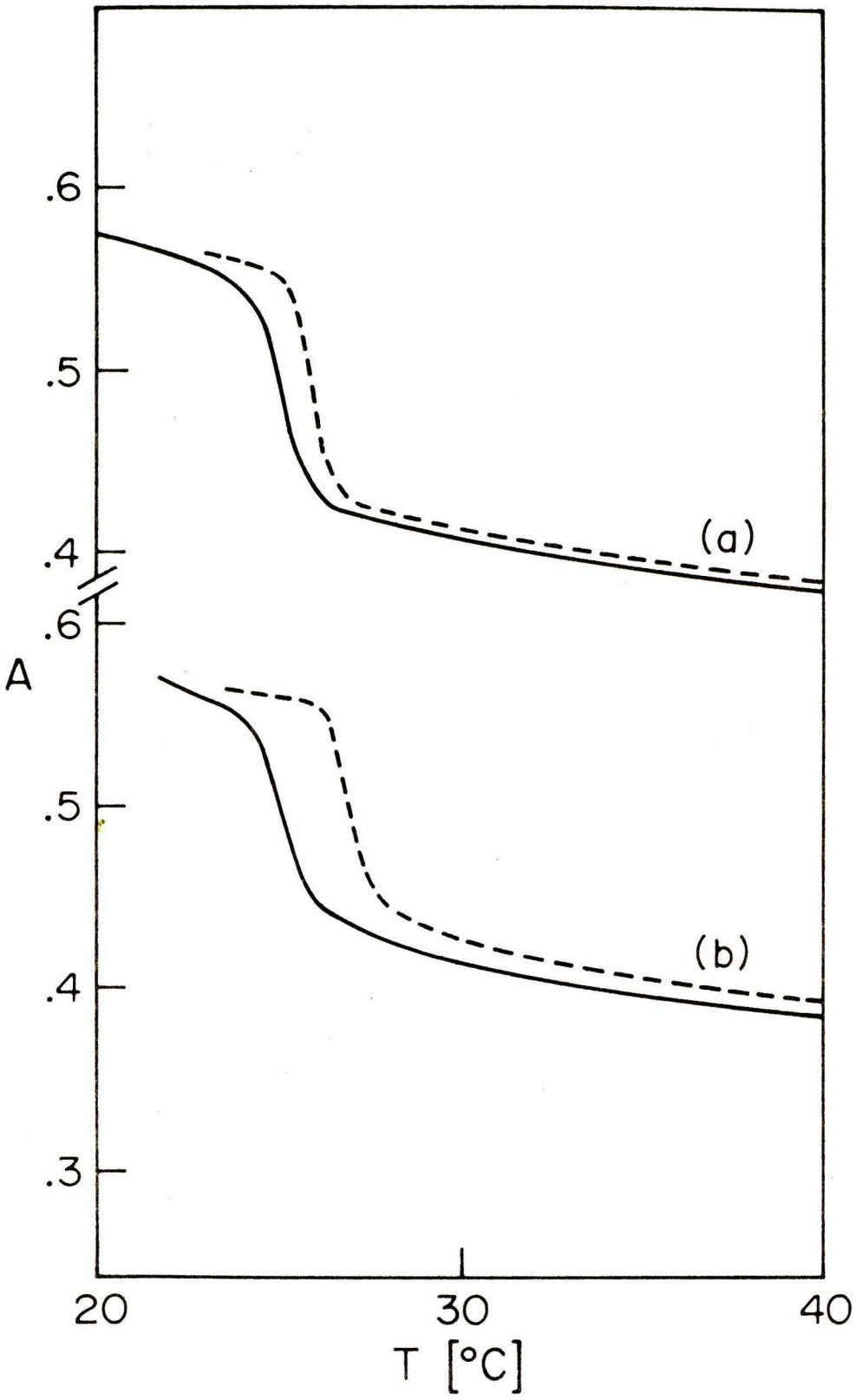
A number of interesting details may also be pointed out. First, the phase diagrams which can be constructed from the data in Tables II, III, and IV indicate that the transition breadth is slightly narrower (1.2 - 3.3°C at DML/DPL = 1) than that obtained by Shimschick and McConnell [22] for mixtures of DML and DPL without protein (5° at DML/DPL = 1). Second, the first heating curve behaves anomalously for all samples dialyzed at 13°C. In addition, "annealing" seems to affect

Figure 7. Absorbance versus temperature curves for a cytochrome oxidase-DML sample dialyzed at 38°C

_____ heating curve; -------- cooling curve

(a) heating rate = 1°C/min; cooling rate = 0.4°C/min

(b) heating and cooling rate = 2°C/min


.6
.5
.4
(a)
.6
A
.5
(b)
.4
.3
20
30
40
T [°C]

the shape of the anomalous first heating curve. Third , broad pretransitions are observed in the region of 30-33.5°C for several of the samples containing higher amounts of DPL, as well as for the neat DPL sample. The nature of these pretransitions is currently not well understood.

4. Discussion

Using well-defined lipid systems, we have demonstrated that cytochrome c oxidase activity is higher in the liquid crystalline phase of the reconstituted membranes than in the gel phase. It is significant that these results were obtained by controlled variation of the membrane composition rather than by the usual temperature-dependent activity assays on natural membranes of heterogeneous composition. It is also important that our turbidity studies show no phase separation of the lipids in the DML-DPL samples. Thus, we can assume that our reconstituted oxidase membranes each have a homogeneous lipid distribution. Previous studies on the relationship of lipid phase transitions and enzyme activity in E. coli membranes have shown disparities between the phase transitions of the lipids and the breaks in the Arrhenius plots of proline transport and succinic dehydrogenase activity [11]. These results have been attributed to the compositional heterogeneity of lipids in the natural membrane. For such studies, the importance of a uniform lipid distribution and hence the usefulness of model membrane systems are not over-emphasized.

We also noted that the oxidase-activity dependence on the phase transition arises only if the reconstitution was carried out above the

phase transition of the lipids used, or if the sample is annealed, following dialysis below the phase transition of the lipid. One possible explanation for this annealing effect is that cytochrome c oxidase is just surface-bound to the phospholipid bilayers formed during dialysis below the phase transition. If so, heating the samples above T_c might cause a reorientation of the protein such that it now intercalates the bilayer and thus becomes sensitive to changes in the fluidity of the hydrocarbon region of the bilayer (for example, by modification in the tertiary folding or quarternary structure). Differences in protein disposition in the bilayer as a function of reconstitution temperature have been demonstrated for M13 virus coat protein in DML vesicles [23]. When reconstituted at the T_c for DML (23°C), the protein orients unidirectionally across the bilayer with the N-terminus located outside. Below 23°C, the linear protein orients itself in a U-shaped manner, with both amino and carboxy-termini exposed to the external medium. However, no changes in orientation are observed upon heating these latter samples above 23°C [24].

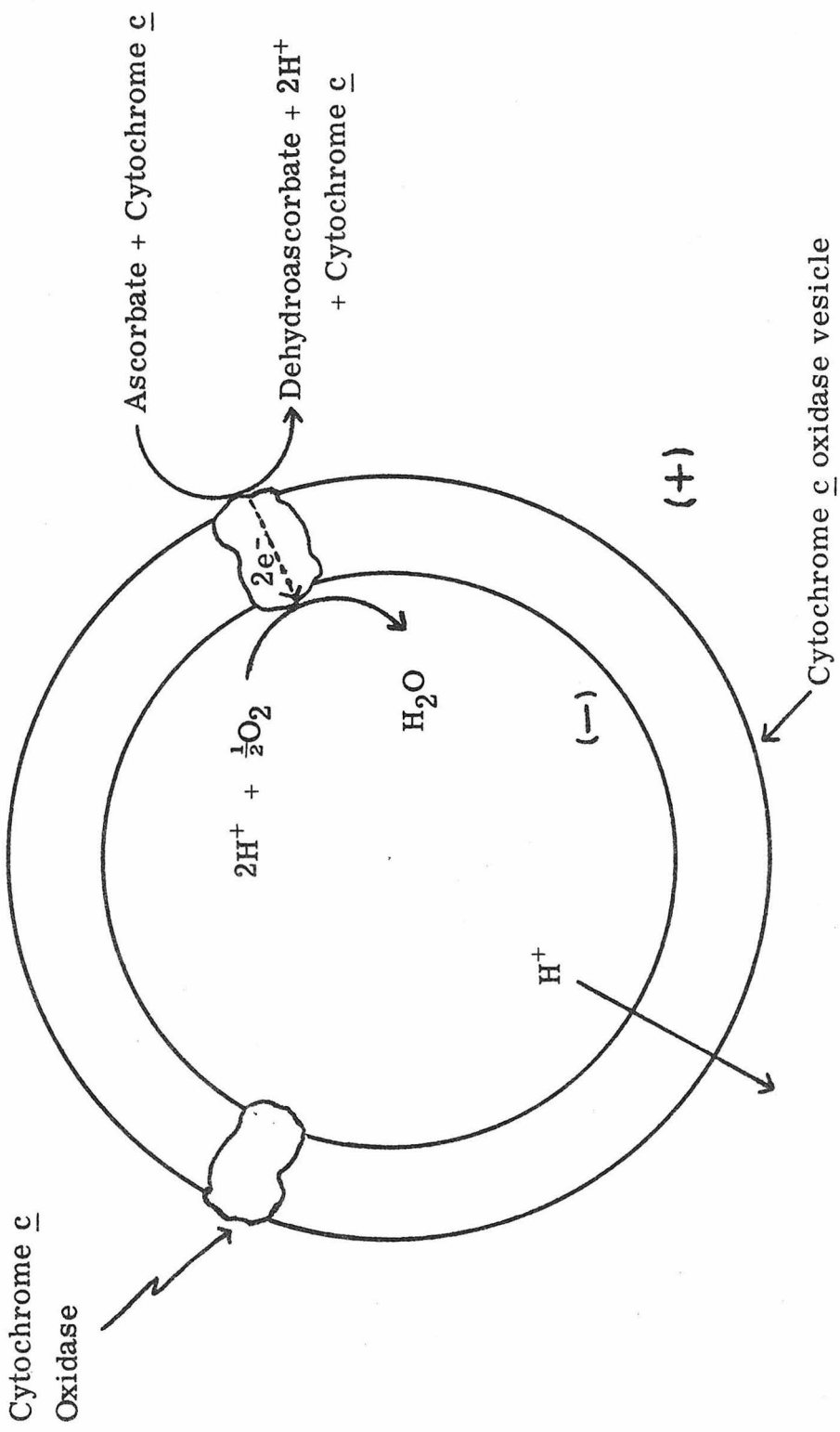
Another, perhaps more plausible, explanation presents itself in the light of recent studies from this laboratory concerning the effects of structural defects in phospholipid vesicles on ion permeability. It has been discovered that preparation of lipid vesicles either by the cholate dialysis procedure or by sonication at temperatures below their respective phase transitions leads to the production of lipid structures that are highly permeable to europium ions [25]. Furthermore, there is a pronounced tendency for these vesicles to fuse. However, when leaky

vesicles are heated above their phase transition temperatures, they become impermeable to ions and are more stable towards fusion.

These results, we believe, are pertinent to the studies on cytochrome c oxidase model membranes. It is generally accepted that electron transport between cytochrome c oxidase and oxygen is accompanied by the formation of a proton gradient across the mitochondrial membrane [26,27]. This proton gradient is attributable, at least in part, to the occurrence of proton-releasing and proton-consuming redox reactions on opposite sides of the membrane. Thus, the gradient formed is a physical consequence of membrane asymmetry. Although reconstituted cytochrome oxidase membranes are generally symmetric (i.e., bidirectional) with respect to protein orientation across the bilayer, membrane asymmetry can be imposed on these model systems by placing the membrane-impermeable reductant, cytochrome c, on only one side of the membrane. In the case of our model membranes which contain cytochrome c in the external vesicle solution, the vesicle interior is expected to become more negative with respect to the exterior (see Fig. 8). As the charge gradient increases, the turnover rate of the oxidase should decrease. In the steady state condition, the gradient established during electron transport is offset by diffusion of protons or some other charged species back into the vesicle. When such a situation exists, the rate of electron transfer and consequently the rate of oxygen consumption can well be expected to be limited by the rate of passive back-diffusion of charge across the membrane.

Results obtained with annealed and unannealed cytochrome c oxidase membranes support the idea of a diffusion-limited step in the

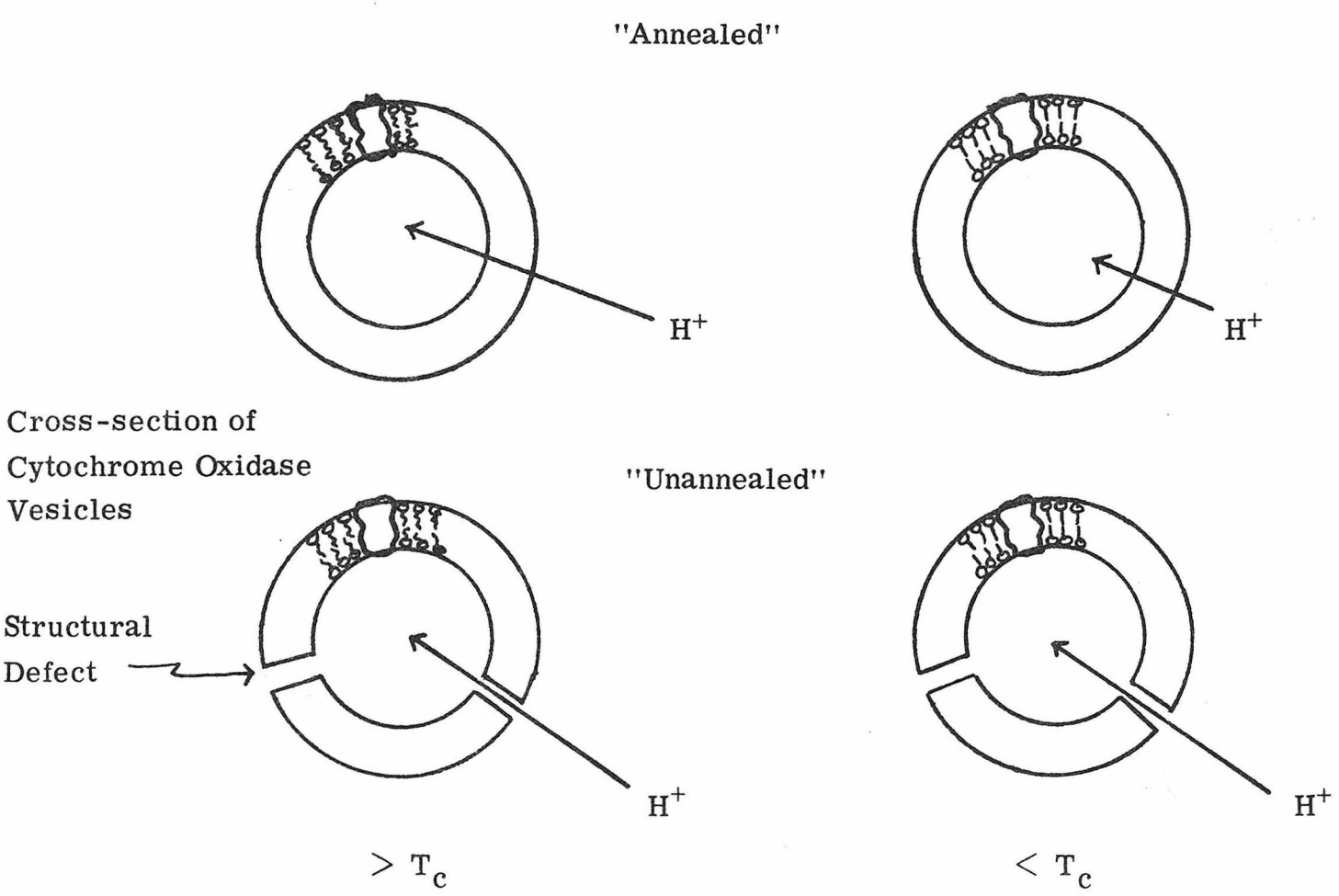
Figure 8. The formation of a proton gradient across reconstituted cytochrome oxidase membranes as a result of imposed membrane asymmetry


Ascorbate + Cytochrome c
Dehydroascorbate + 2H+
+ Cytochrome c
2e-
2H+ + ½O2
H2O
(-)
(+)
H+
Cytochrome c oxidase vesicle
Cytochrome c
Oxidase

reduction of oxygen. Without annealing, i.e., when the vesicles are leaky, the oxidase activity is not strongly dependent on the physical state of the lipids. Under these conditions, there is no barrier to passive back-diffusion of ions across the membrane; consequently, no charge gradient builds up and the membranes can be said to be effectively uncoupled. In addition, the structures observed by electron microscopy are extraordinarily large, which is probably indicative of extensive fusion during the reconstitution and assembly process, a characteristic property of vesicles with structural defects. When the samples are annealed, however, these structural defects are annihilated or are reduced in number, and the effect of the phase transition on protein activity is clearly demonstrated. If the rate of charge diffusion across the bilayer membrane is different above and below the lipid phase transition in sealed vesicles, it would lead, in effect, to a difference in the rate of discharge of the membrane potential established during respiration which, in turn, could explain the observed dependence of oxidase activity on the phase transition of the lipids in the reconstituted membrane. These ideas are summarized in Figure 9.

Finally, our turbidity data suggest an additional interpretation for the effect of the lipid phase transition on oxidase activity. It was noted that a heating/cooling hysteresis is present in the optical density versus temperature curves of membranes containing cytochrome oxidase, but is absent in the curves of protein-free lipid bilayers. At the same time, we noted that for the mixed lipid samples, the transition temperature determined from the heating curve is very close to the

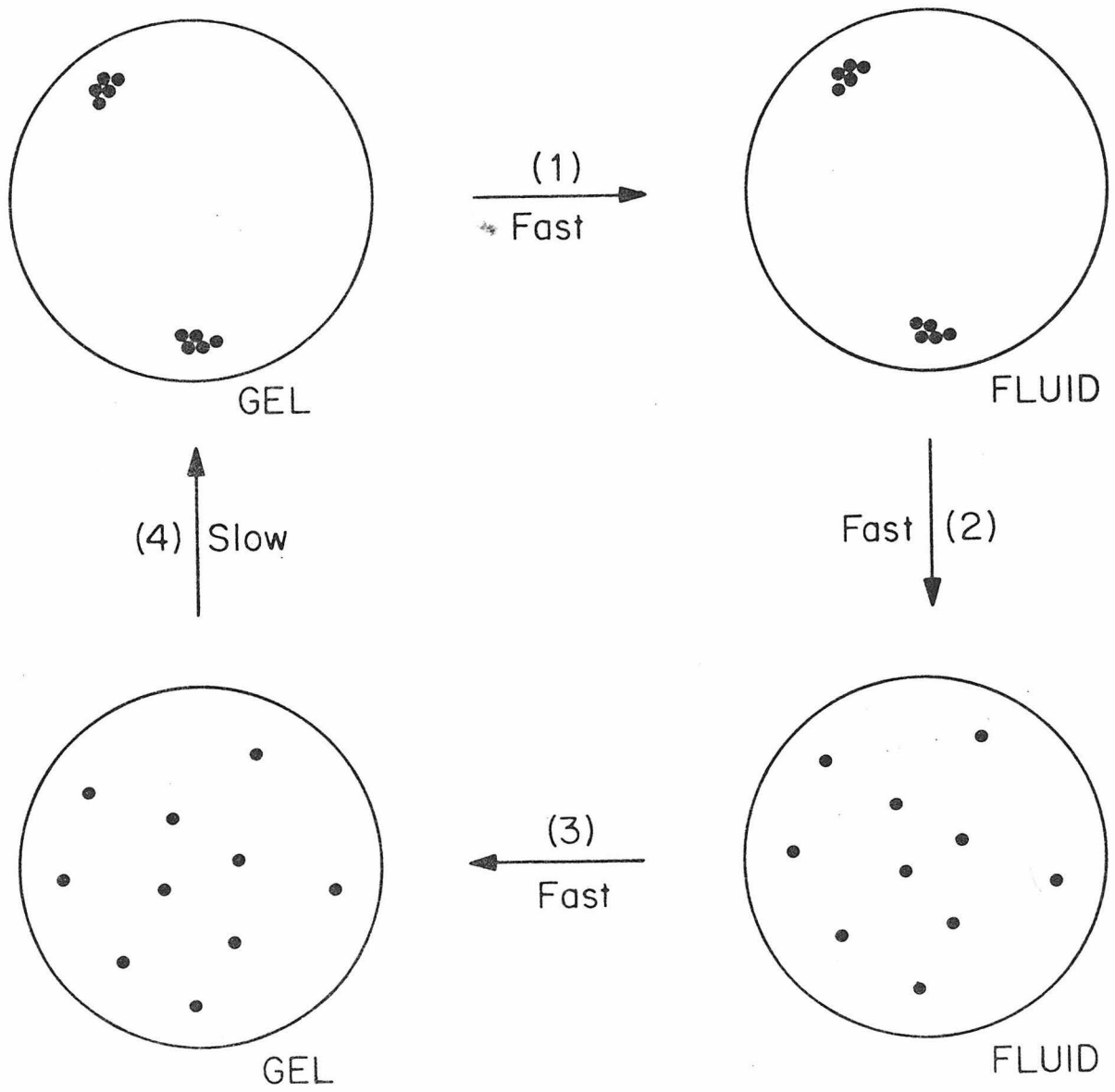
Figure 9. A passive charge diffusion model for the activity dependence of cytochrome oxidase on the physical state of the membrane lipids. (The length of the arrows represents the degree of passive back diffusion of protons into the vesicle in response to the proton gradient generated by electron transport processes).


"Annealed"
H+
H+
Cross-section of
Cytochrome Oxidase
Vesicles
"Unannealed"
Structural
Defect
H+
H+
> Tc
< Tc

temperature calculated for ideally mixed two-component lipid systems. The latter observation indicates that, at a protein-lipid ratio of 0.2, the effect of the protein on the melting of lipids is not large. On the other hand, the transition midpoints obtained from the cooling curves are generally one to several degrees higher than those obtained from the heating curves, depending on the rate of cooling. A number of recent experiments provide evidence for a layer of restricted boundary lipid surrounding integral membrane proteins [28-30]. It has also been suggested that these integral proteins have, in addition, a long range ordering effect on the lipids in their immediate environment [31,32]. A recent theoretical analysis predicts that this long-range interaction between the proteins and the surrounding lipids leads to a lipid-mediated protein-protein interaction which is dependent on the phase of the lipids [33]. As the temperature of the membrane is lowered towards the phase transition, the range of protein interactions increase, leading eventually to protein aggregation. Experimentally, freeze fracture studies support the idea of differential states of aggregation of membrane proteins above and below the phase transition of the membranes [34-38].

Our results are compatible with the idea of a reversible protein aggregation and dispersion in the bilayer which is dependent on the phase of the lipids. In Figure 10, we outline a possible sequence of events during the lipid melting/freezing process which may account for our observations. The heating/cooling hysteresis may be understood if steps (1), (2), and (3) are reasonably fast, but step (4) is slow, which

Figure 10. A model for protein aggregation and dispersal in the bilayer as a function of the physical state of the phospholipids


(1)
Fast
GEL
FLUID
Fast
(2)
(4)
Slow
(3)
Fast
GEL
FLUID

is to be expected. If the proteins are clustered in the gel phase, their interaction with the lipids will be minimized and the transition expected upon heating will be essentially that of the pure lipids unperturbed by the presence of protein. However, if the proteins are dispersed throughout the bilayer in the fluid phase, their sphere of influence on the lipids is maximized and the transition temperature observed upon cooling will reflect this increased protein-lipid interaction. Furthermore, if the proteins are given more time to reaggregate during the cooling process (such as by a slower cooling rate), the hysteresis should disappear. In fact, decreasing the rate of cooling does decrease the amount of hysteresis between the heating and cooling curves (Fig. 7). By contrast, the heating curve is not affected by changing the rate of heating. Taken together, these results lend support to the model presented. If cytochrome oxidase activity is indeed dependent on the state of aggregation of the proteins in the membrane, a difference in the distribution of the proteins in the plane of the bilayer in the gel and liquid crystalline states may well account for the observed activity dependence on the phase transition of the lipids.

Earlier studies which monitored the temperature-dependence of mitochondrial protein activity attribute abrupt changes in the Arrhenius plots in the vicinity of the membrane lipid phase transition to changes in the activation energy of the membrane-associated reaction. It had been further suggested that these changes in the activation energy were, in turn, related to the ease with which necessary configurational changes in the protein occur in the two phases of the lipids. Although our results, at present, do not preclude the idea of a conformational

change taking place in the oxidase during electron transfer, they suggest that there may be additional, or perhaps alternative, explanations for the activity dependence on the physical state of the membrane lipids. In particular, we propose that differential diffusion effects and/or differences in the state of protein aggregation above and below the lipid phase transition are also compatible with the results.

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III. LIPID FACTORS INFLUENCING RESPIRATORY CONTROL IN RECONSTITUTED CYTOCHROME c OXIDASE MEMBRANES

1. Introduction

The efficiency of mitochondrial respiration is highly dependent on tight coupling between the energy-yielding reactions of the electron transport chain and the energy-conserving reaction of ATP synthesis. When this coupling is weakened or destroyed, the breakdown and oxidation of food molecules proceeds rapidly without the concomitant formation of ATP. In such cases, "respiratory control" has been lost and the mitochondria are said to be "uncoupled".

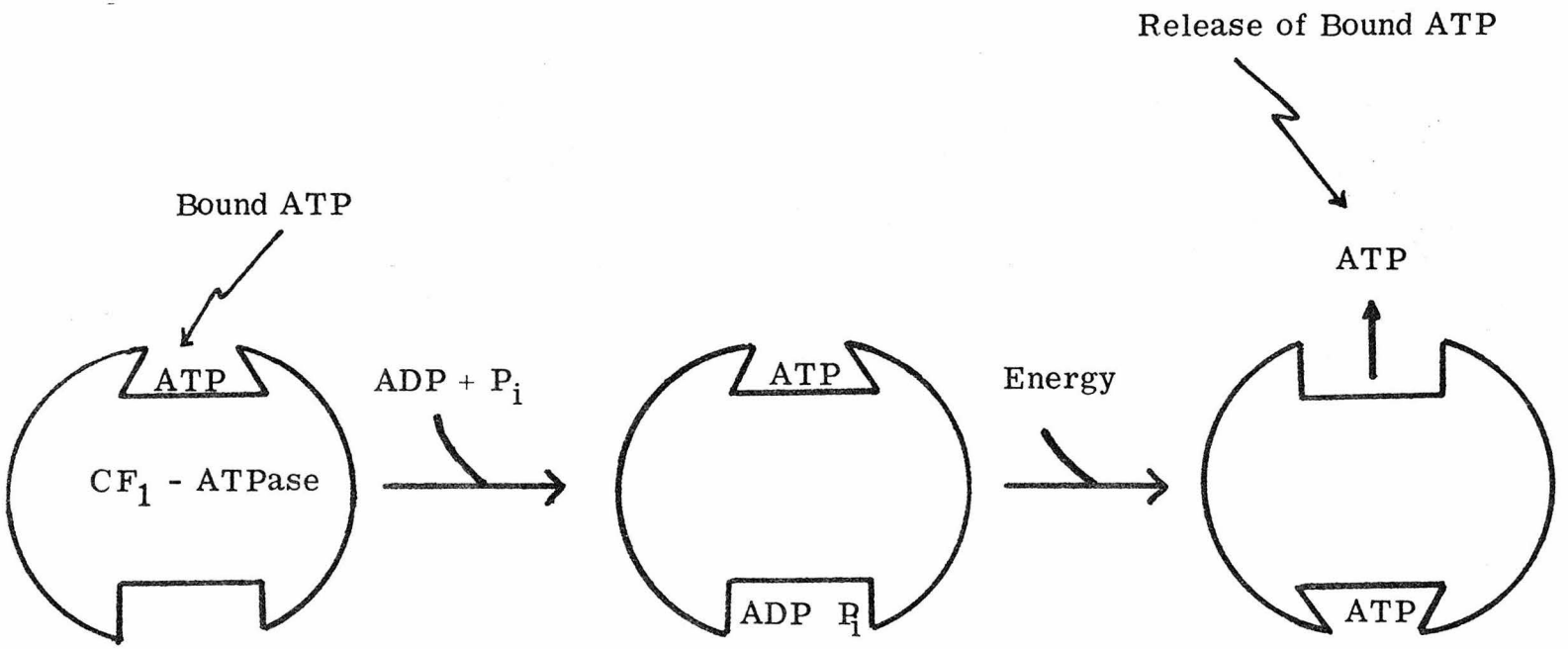
A number of chemical agents of diverse structure are potent uncouplers of respiration, though their mechanism of action is still poorly understood [1-5]. It is assumed that uncouplers destroy the coupling between electron transport processes and oxidative phosphorylation by interacting directly with the high energy coupling intermediate. However, the nature of this high energy intermediate is still being hotly debated [6].

Over the years, three major hypotheses have emerged to explain the mechanism of coupling between electron transport and ATP synthesis. The chemical hypothesis proposed very early by Slater [7] is based on the existence of high energy chemical intermediates, similar to those found in the reactions of glycolysis. Unlike glycolytic phosphorylations, however, membrane structure is a prerequisite for respiratory chain phosphorylations, and the chemical intermediates which were postulated to be the precursors of the high energy phosphate bond of ATP have never been found.

Somewhat akin to the chemical hypothesis is the conformational hypothesis originally proposed by Boyer [8] which found some support in the structural changes observed in the inner mitochondrial membrane during active and resting periods of respiration. The high energy intermediate, in this case, was supposed to be a conformational change in an electron carrier protein which catalyzes the formation of ATP. More recently, the chemical and the conformational hypotheses have merged into what is sometimes called the Boyer-Slater hypothesis [6,9]. Basically, the energy-linked step in this proposal is a conformational change in CF_1-ATPase which results in the release of enzyme-bound ATP. The formation of ATP, on the other hand, is said to be an energy-independent step involving enzyme-bound ADP and P_i. However, the nature of the coupling between the proposed conformational change and the electron transport reactions is still not clear. A schematic diagram of this most recently formulated conformational coupling mechanism is presented in Figure 1. With regard to the uncoupling mechanism, this hypothesis would imply that uncouplers act either by interfering with the proper binding of the substrates to the protein or by inhibiting the conformational changes necessary for phosphate bond formation.

The final hypothesis of energy coupling was formulated by Mitchell on the theory that the osmotic properties of the inner mitochondrial membranes were crucial to the synthesis of ATP [10,11]. Inherent in this chemiosmotic hypothesis were the ideas of supramolecular assembly of the enzyme systems concerned and vectorial processes. According to this hypothesis, the high energy intermediate involved in energy coupling is

Figure 1. The conformational coupling mechanism of ATP synthesis



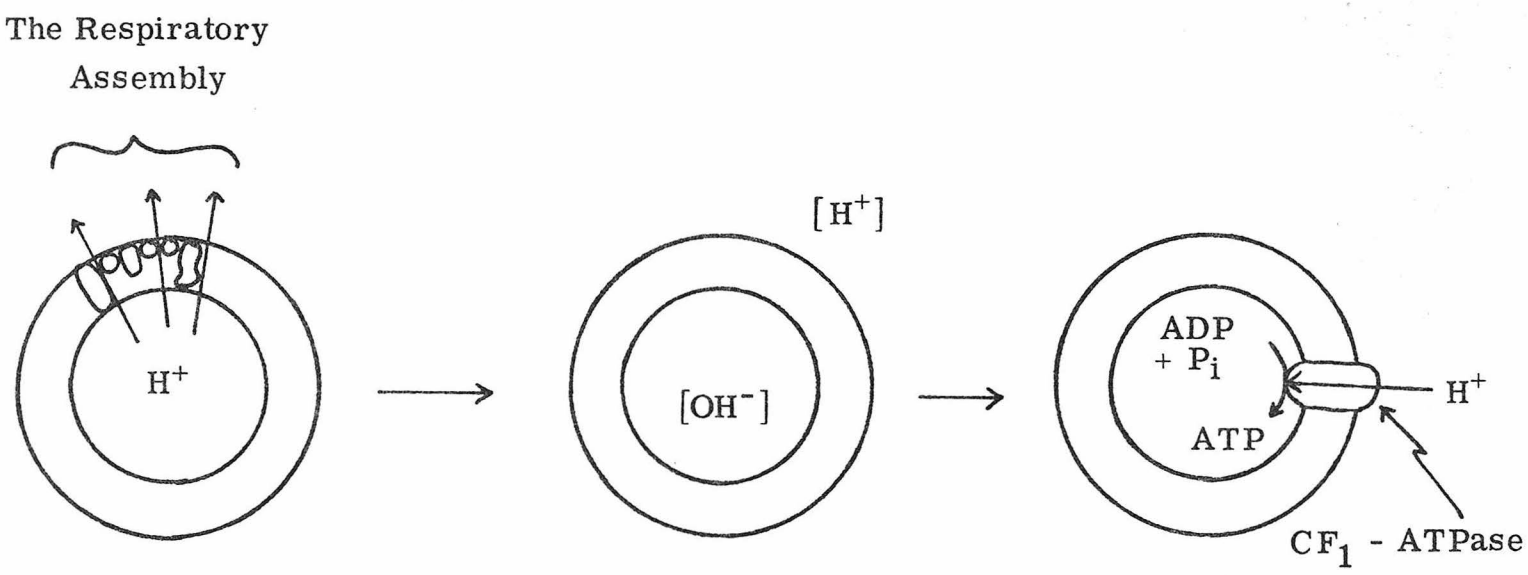
Binding of ADP and P_i

Synthesis of ATP from Bound ADP and P_i

an electrochemical gradient of protons across the membrane generated by the vectorial reactions of the electron transport chain which serve primarily as a proton pump. In turn, the proton pump drives the ATP synthesis reaction which is associated with a reverse proton pump. The scheme of this coupling mechanism is summarized in Figure 2. Since the physical separation of charges is central to the argument of chemiosmotic coupling, a charge-impermeable membrane is an essential and integral part of the coupling mechanism. It is clear, then, that the chemiosmotic hypothesis would predict that any procedure or chemical agent that disrupts membrane structure or causes ion leakage across the membrane would result in uncoupling.

Given that a direct relationship exists between the mechanism of uncoupling and that of coupling, a sizeable effort has been made towards understanding various aspects of the uncoupling process. In particular, the physico-chemical properties of uncouplers and their interactions with model and mitochondrial membranes have been of great interest. Several recent studies have demonstrated that a number of chemical uncouplers are capable of increasing ion conductance across lipid bilayers [12-14]. In addition, a correlation between uncoupler-induced mitochondrial swelling and uncoupling has been established [1]. Both these findings have been taken to be supportive of the chemiosmotic hypothesis. On the other hand, Ting et al. found no simple correlation between the ion conducting and uncoupling abilities of a variety of uncouplers [15]. Instead, it was proposed that uncouplers act as acid or base catalysts of a hydrolytic reaction occurring in the nonpolar region of the

Figure 2. The chemiosmotic coupling mechanism of ATP formation


The Respiratory
Assembly
H+
[H+]
[OH-]
ADP
+ Pi
ATP
H+
CF1 - ATPase
Electron Transport
Processes
Proton Gradient
Established
ATP
Synthesis

mitochondrial membrane [4]. Further support for a chemical uncoupling mechanism comes from the following findings: 1) a stoichiometric relationship between uncoupling agents and "active phosphorylation sites" [16,17], 2) a specific binding site in mitochondria for a class of uncouplers [18], and 3) an effective uncoupler (picrate) that is neither membrane permeable nor ionophoric [2]. Thus, the mechanism of action of uncouplers is still very much unsettled.

Another active area of research involving uncouplers has been the study of uncoupler-membrane interactions. It has been found that uncoupler binding to liposomes and mitochondrial membranes both modulates and is modulated by membrane surface potential [19,20]. As a result, it has been suggested that the binding of uncouplers to membranes may be an important aspect in determining its action as an uncoupler. This, in turn, may be related to a possible role for membrane phospholipids in uncoupling activities.

In fact, an early study by Racker shows that the exhibition of respiratory control (defined in model membrane systems by their sensitivity toward uncouplers) by reconstituted cytochrome oxidase membranes is indeed a complex function of lipid polar head groups, pH, the concentrations of monovalent and divalent cations, and the amount of cholate present in the assay mixture [21]. However, since natural phospholipids and lipid-rich protein (20% lipid; w:w) were used, it is difficult to evaluate what specific lipid factors influenced the establishment of respiratory control in these model systems. It therefore seemed necessary to investigate the lipid requirements for respiratory control in a more well-defined system.

For this reason, we have reconstituted delipidated cytochrome c oxidase (containing less than 3% lipids; w:w) with a number of pure lecithins as well as with lecithin-cardiolipin mixtures, and tested the sensitivity of the reconstituted membranes to several uncouplers. Asolectin (a mixture of natural soy bean lipids) was used as a control, since it has previously been shown that asolectin is capable of reconstituting cytochrome oxidase vesicles with respiratory control [14]. Our results show that 1) respiratory control requires the presence of some acidic phospholipids, and 2) the respiratory control ratio depends on the lipid/protein ratio in these model systems.

2. Materials and Methods

Delipidated cytochrome c oxidase was the gift of Drs. Tsoo E. King, Chang-An Yu, and Linda Yu of the State University of New York at Albany. It was suspended at a concentration of 100 mg/ml in 0.5% cholate/50 mM phosphate buffer, pH 7.4. The model membranes were prepared by a cholate dialysis procedure as described previously [21,22].

The lipids used were obtained from the following distributors: Asolectin from Associated Concentrates; cardiolipin (DPG) from Serdary Research Laboratories; dioleoyl lecithin (DOL) from Sigma; dimyristoyl lecithin (DML) from Calbiochem. Egg yolk lecithin (EYL) was purified from egg yolks following published procedures [23]. All lipids show a single spot on thin layer chromatographic plates developed in chloroform:methanol:water (65:25:4).

Mixed lipid samples were prepared by dissolving the appropriate amounts of pure lipid in chloroform and lyophilizing the solution on a

high vacuum line for about one day.

Horse heart cytochrome c Type VI was purchased from Sigma, ascorbate from Matheson, Coleman and Bell, and sodium cholate from Schwarz-Mann.

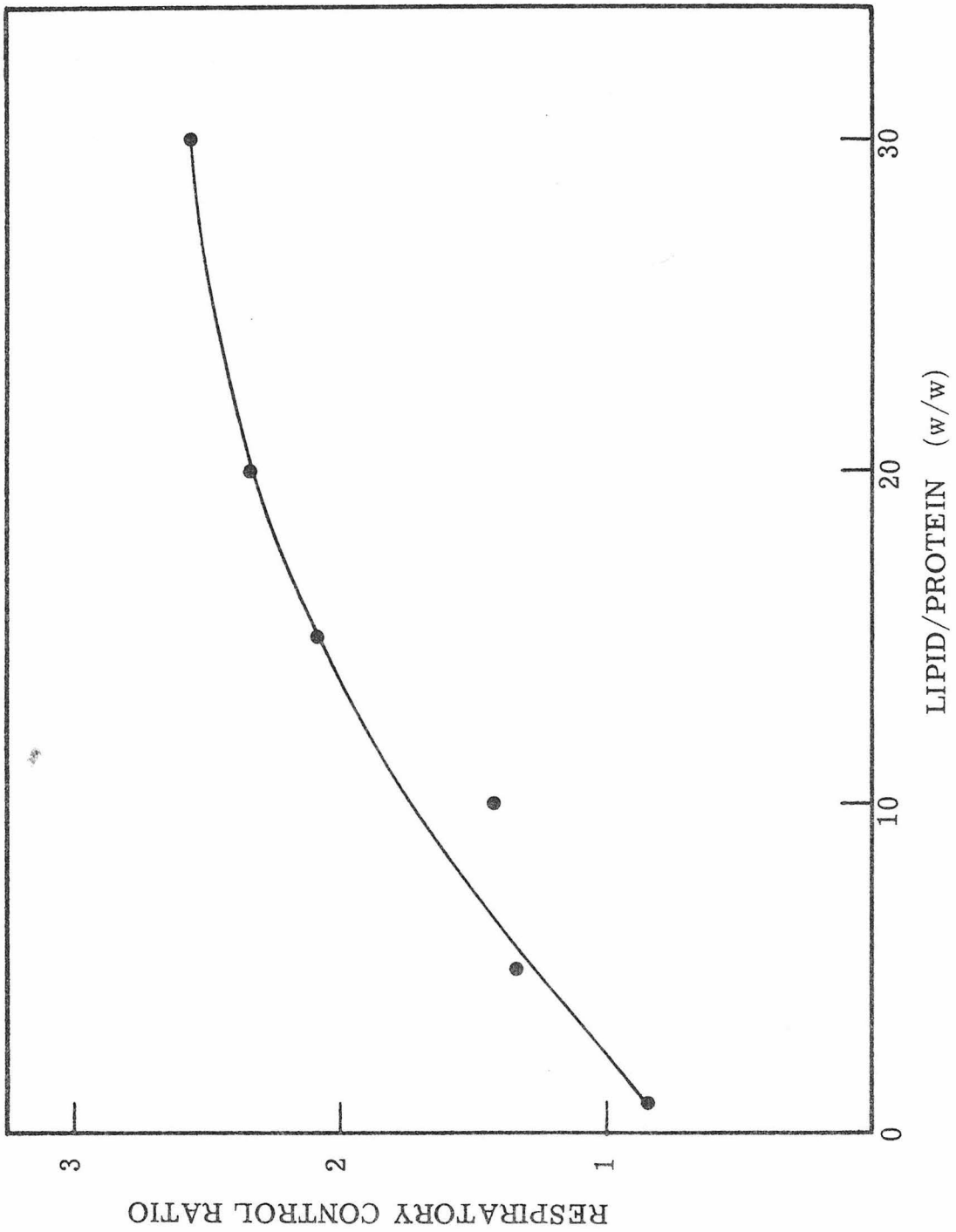
The activity assays were performed polarographically using a YSI oxygen monitor and electrode. The assay solutions contained 0.1 nmol heme A, 24 nmol cytochrome c, and 42 µmole ascorbate in a total volume of 1.5 ml 50 mM potassium phosphate buffer, pH 7.4. During the measurement, uncouplers in ethanolic solution were added to the reaction chamber via a syringe. The final concentrations of uncouplers in the assay medium were 166 µM DNP and 3.3 µM mCCCP. Valinomycin was also present at a final concentration of .007-.03 mg/ml.

3. Results

A. The Effect of Lipid/Protein Ratio on Respiratory Control

Figure 3 shows that the sensitivity of the reconstituted asolectin-cytochrome oxidase membranes to dinitrophenol (DNP) as evidenced by their respective respiratory control ratios increases with increasing lipid concentrations. These results are in accord with studies by Racker [21] and by Hunter and Capaldi [16] in which respiratory control is achieved in cytochrome oxidase vesicles containing relatively high lipid/protein weight ratios of approximately 15/1. In contrast, the lipid/protein ratio in the inner mitochondrial membrane is much lower (0.39/1) [24]. Thus, it is not clear why high protein concentrations in the model membranes inhibit the manifestation of respiratory control.

Figure 3. Respiratory control as a function of the lipid/protein ratio in reconstituted asolectin/cytochrome oxidase vesicles


RESPIRATORY CONTROL RATIO
3
2
1
0
10
20
30
LIPID/PROTEIN (w/w)

On the other hand, cytochrome oxidase activity seems to show an inverse relationship to lipid concentration (Figure 4) except at very low concentrations. Addition of cholate to the reconstituted samples stimulates activity by nearly a factor of 2 (Figure 5) but destroys the membranes' ability to be further stimulated by uncouplers. Since the detergent presumably disrupts membrane structure and makes both right-side-out as well as inside-out cytochrome oxidase molecules available for reaction with membrane-impermeable reducing agents, the results show that, in vesicles reconstituted with asolectin, approximately half of the oxidase molecules are oriented right-side-out, and further imply that the action of uncouplers does require an intact membrane.

B. Effect of Acidic Headgroups on Respiratory Control

Table I lists the activities and respiratory control ratios for membranes reconstituted with various lipids. The data show that cardiolipin (or other acidic phospholipids such as sulfolipids and sulfatides in the case of asolectin) is essential for the conferral of uncoupler sensitivity on cytochrome oxidase membranes. However, it may not be a sufficient prerequisite for the establishment of respiratory control, since the DML-cardiolipin sample does not respond to uncouplers even though the activity of the protein in this sample is comparable to that of the DOL and EYL samples containing the same mole% cardiolipin. It is also interesting to note that all of the mixed lipid samples containing cardiolipin (except for sample 8) have roughly the same activity as the sample containing only cardiolipin. Furthermore, the activity of the DML-cardiolipin sample dialyzed at 3°C is high even though the saturated

Figure 4. Cytochrome oxidase activity as a function of the lipid/protein ratio in reconstituted asolectin/cytochrome oxidase vesicles

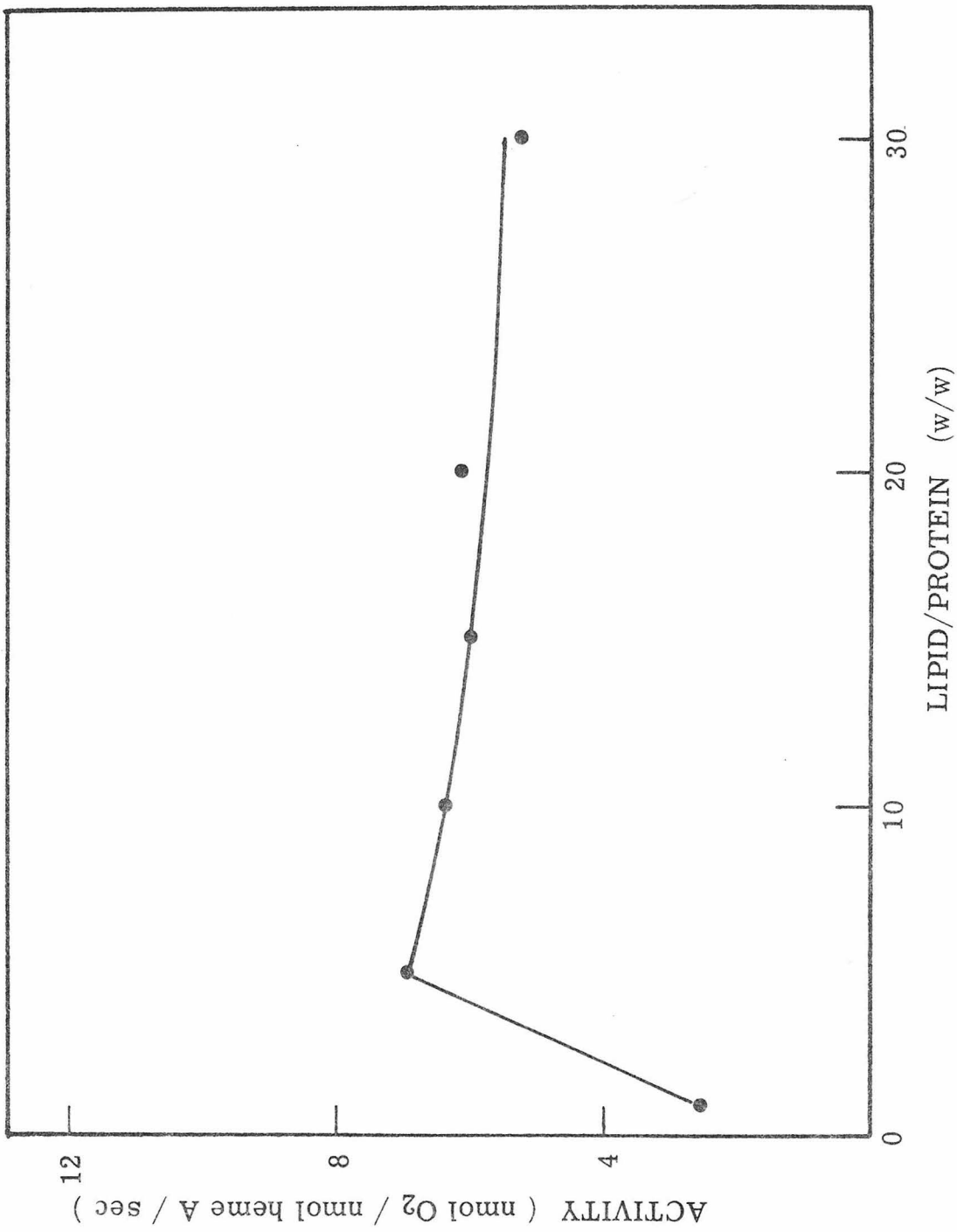

ACTIVITY (nmol O_2 / nmol heme A / sec)
12
8
4
0
10
20
30
LIPID/PROTEIN (w/w)

Figure 5. The effect of cholate on the activity of reconstituted cytochrome oxidase/asolectin vesicles. [2% cholate (w/v) was used to solubilize the reconstituted membranes (1-2% lipid; w/v) prior to the assay].

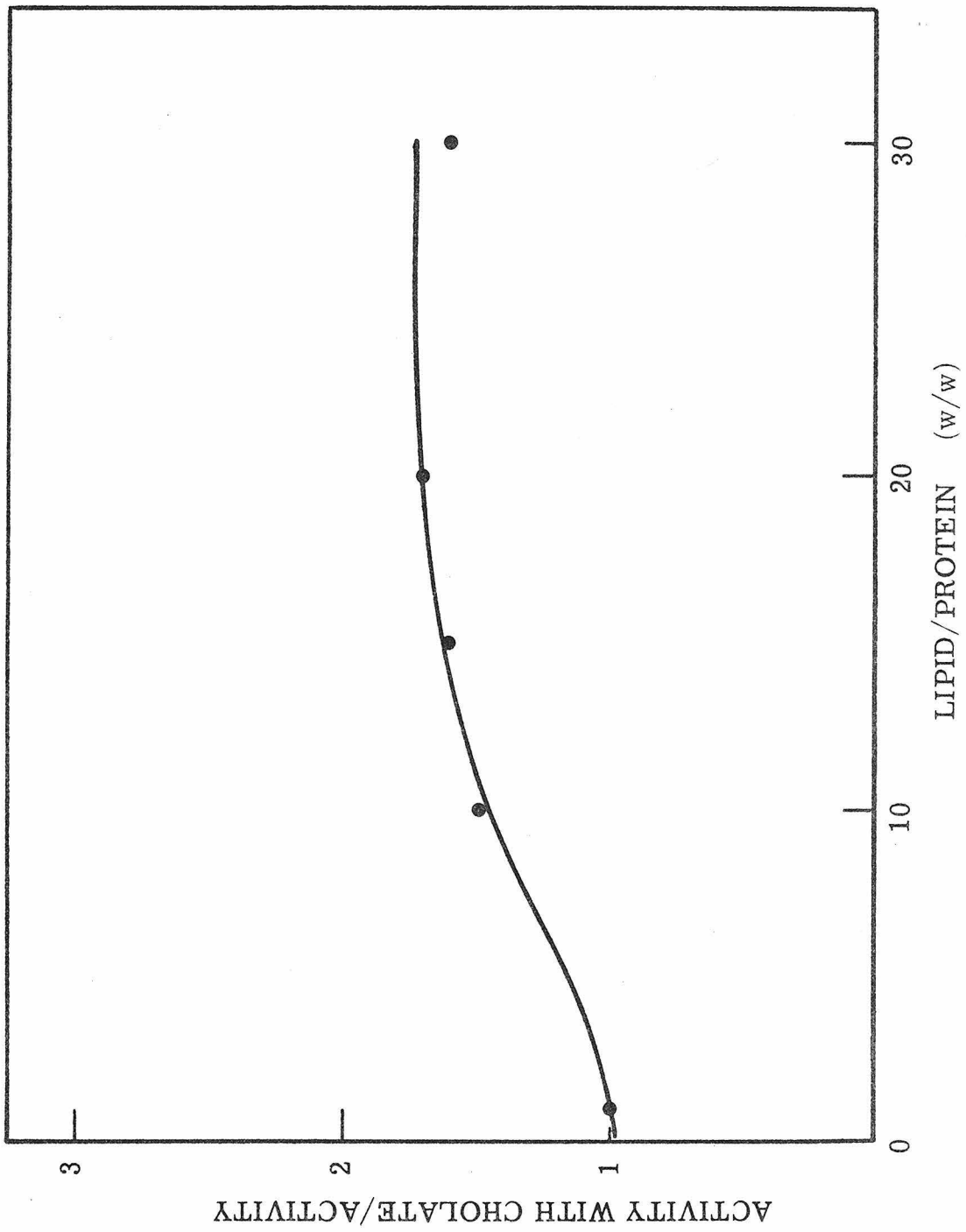

ACTIVITY WITH CHOLATE/ACTIVITY
3
2
1
0
10
20
30
LIPID/PROTEIN (w/w)

TABLE I. Activity and Respiratory Control Ratios for Cytochrome c Oxidase Reconstituted with Various Lipids [Lipid/Protein = 20/1 (w/w)]

Sample #	Phospholipid	Activity at 30°C[a,b]	RCR[c]
1	Asolectin	6.0	2.3 (3.2)
2	Cardiolipin (DPG)	6.3	1.33
3	Egg Yolk Lecithin (EYL)	5.6	-
4	EYL + DPG (10 mole %)	6.5	1.7 (1.6)
5	Dioleoyl Lecithin (DOL)	8.0	-
6	DOL + DPG (10 mole %)	6.2	1.6 (2.1)
7	Dimyristoyl Lecithin (DML)[d]	1.8	-
8	DML + DPG (10 mole %)[d]	3.1	-
9	DML + DPG (10 mole %)[e]	6.3	-

[a]The activity is given in units of nmol O_2/nmol heme A/sec

[b]All samples were assayed in 50 mM phosphate buffer, pH 7.4

[c]Activity in the presence of mCCCP/Activity without mCCCP. (The values in parentheses were obtained with DNP in place of mCCCP).

[d]Dialyzed at 26°C

[e]Dialyzed at 3°C

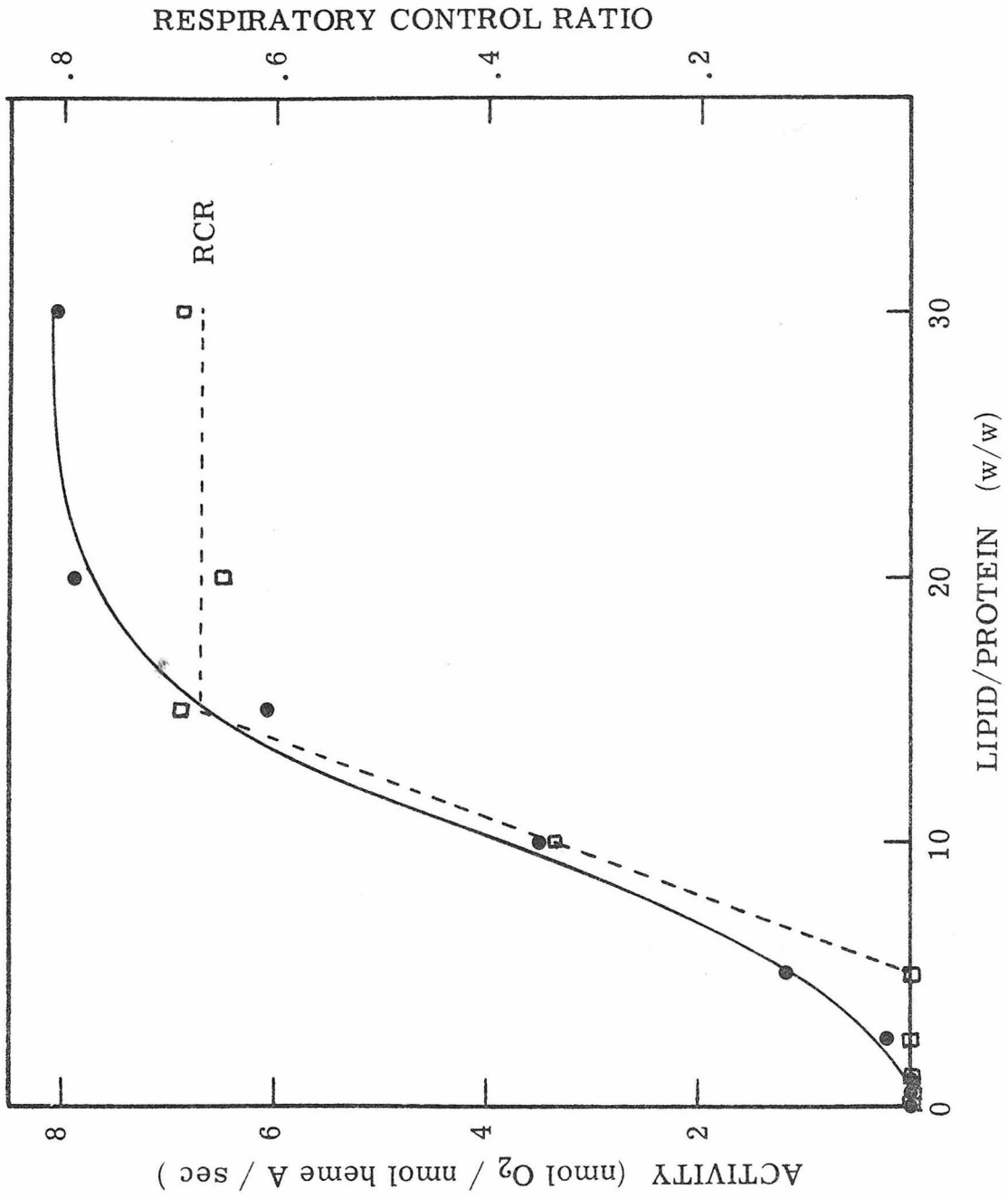
lipid DML by itself seems to be inhibitory towards oxidase activity. These results suggest that cytochrome oxidase may preferentially seek out and surround itself with cardiolipin, which is not surprising, since cardiolipin binds most tightly to cytochrome oxidase during protein extraction from the mitochondrial membrane [25].

C. The Effect of Reconstitution Procedure on Respiratory Control

We have shown earlier that asolectin can be used to reconstitute cytochrome oxidase vesicles with respiratory control using a cholate-dialysis procedure described by Racker [21]. Recently, Eytan et al. described a new method for the reconstitution of cytochrome oxidase vesicles [26]. This procedure involves incubating cytochrome oxidase at room temperature with liposomes containing an acidic phospholipid (e.g., PS, PI, and DPG) without added detergent. This reconstitution procedure was reported to yield vesicles with a unidirectional orientation of the protein and high respiratory control ratios.

We have attempted to apply this method of reconstitution to the preparation of vesicles from sonicated asolectin suspensions and delipidated cytochrome oxidase. Figure 6 shows the activity and respiratory control profile of the samples as a function of lipid concentration. We find that the activity increases as a function of lipid concentration and that the samples do not exhibit respiratory control, the respiratory control ratio being less than 1 in all cases. Thus, both the activity and respiratory control dependence on lipid concentration is not the same as for the asolectin-oxidase samples prepared by the cholate-dialysis method. Perhaps a crucial difference between our experiment and Eytan's is that

Figure 6. Activity and respiratory control profile of cytochrome oxidase/asolectin vesicles as a function of the lipid/protein ratio. These samples were prepared by the method described by Eytan et al. [26]


RESPIRATORY CONTROL RATIO
.8
.6
.4
.2
RCR
ACTIVITY (nmol O2 / nmol heme A / sec)
8
6
4
2
0
10
20
30
LIPID/PROTEIN (w/w)

our protein is "delipidated" (less than 3% phospholipids by weight) and their protein is lipid-rich (containing 20% mitochondrial phospholipids by weight). It is possible that reconstitution of the protein by the Eytan method requires protein-associated lipid in order to facilitate fusion between the protein-complex and the preformed liposomes. In the case of cholate-dialysis, both proteins and lipids are solubilized in cholate prior to the formation of the membranes during dialysis.

4. Discussion

Our results clearly show a requirement for acidic phospholipids in reconstituted cytochrome c oxidase membranes exhibiting respiratory control. Indeed, Racker had previously reported that cardiolipin enhances the uncoupler sensitivity of cytochrome oxidase membranes prepared from purified mitochondrial lipids and cytochrome c oxidase [21]. However, his vesicles which were reconstituted with pure phosphatidylcholine or pure phosphatidylethanolamine also exhibited some measure of respiratory control. In the latter cases, it is possible that residual amounts of cardiolipin bound to the lipid-rich cytochrome oxidase used in these studies may account for the low ratios of respiratory control obtained upon reconstitution with the neutral lipids. In fact, a more recent study by Eytan et al. [26] demonstrates that delipidated cytochrome c oxidase which is incubated with small amounts of neutral (zwitterionic) phospholipids does not form uncoupler sensitive membranes even after subsequent reconstitution with a mixture of PC:PE:PS(1:3:2). This mixture, by itself, is capable of forming oxidase vesicles with

respiratory control. On the other hand, the respiratory control ability of the control enzyme (enriched in mitochondrial phospholipids) was not affected by incubation with neutral phospholipids. These results imply that it is the lipids in the immediate vicinity of the protein which determine whether or not the reconstituted membrane will be responsive to uncouplers. Furthermore, the acidic phospholipids must be intimately associated with the protein in order to fulfill their role in the development of respiratory control.

However, the role that acidic phospholipids play in mediating respiratory control is still unclear. The effect of the lipid/protein ratio on respiratory control seems to indicate that the state of aggregation of the protein is important since respiratory control is achieved only when the protein is diluted out with a large excess of lipid. Presumably, the average distance between protein molecules increases with increasing lipid concentration. Therefore, it could be the function of acidic phospholipids to act as membrane-associated dispersing agents in maintaining a minimum distance between the individual oxidase molecules by virtue of charge repulsion. It is conceivable that an aggregate of proteins in the membrane could inhibit respiratory control by either masking the site of uncoupler binding on the protein, or by rendering the membrane leaky to protons or other ions within or around the protein cluster through disruption of the local bilayer structure. However, given that the intact mitochondrial inner membrane is roughly 60 times richer in protein content than the coupled model membrane systems, the above explanation may not be too plausible unless the inhibitory effect of aggregation is specific for oxidase clusters.

Acidic phospholipids may also exert a more direct effect on uncoupling by modifying the interaction or binding of uncouplers with the oxidase membranes or with the protein itself. In particular, Bakker et al., in studying the binding of several different uncouplers to rat-liver mitochondria and synthetic liposomes, conclude that the uncouplers associate predominantly with the negative (acidic) phospholipids [20]. Thus, cardiolipin could serve as an uncoupler binding site. On the other hand, Hanstein suggests that at least some uncouplers, such as picrate, interact with mitochondrial proteins at specific binding sites accessible only from the matrix side of the inner mitochondrial membrane [2]. It is not inconceivable that such specific binding is also modulated by the presence or absence of acidic phospholipids which are tightly bound to the oxidase.

With respect to Mitchell's chemiosmotic hypothesis, tight binding of acidic phospholipids to cytochrome oxidase may in itself be sufficient for generating the uncoupler sensitivity of reconstituted membranes. If it is assumed that uncouplers act by collapsing the proton gradient established by electron transport, any structural fault in the membrane that facilitates ion translocation across the membrane would short-circuit the uncoupling process. It is possible that, in the absence of acidic phospholipids, membranes may be permeable to ions through the binding sites normally and exclusively occupied by acidic lipids. In other words, acidic phospholipids may act passively as plugs to fill in the holes in, or peripheral to, cytochrome oxidase, thus allowing the membranes to be more responsive to uncouplers. It is therefore essential to fully characterize the ion permeability properties of these membranes.

Assuming that uncouplers interfere directly with the high energy intermediate that couples respiration to phosphorylation, it is evident that each of the possible roles considered above for acidic phospholipids implies a different mechanism of uncoupling. At this time, it is not possible to narrow the options. In any case, it is clear that a comprehensive mechanism for uncoupling must provide an explanation for the effects of cardiolipin and other acidic phospholipids on respiratory control, and, in addition, resolve the discrepancy between the lipid/protein ratio that is optimal for respiratory control in natural and reconstituted membranes.

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IV. SUMMARY

The studies described in Part II of this thesis have been primarily concerned with the manner in which the physical and chemical properties of phospholipids influence the natural functions of a membrane-bound protein. Consequently, we have investigated the dependence of cytochrome oxidase activity on the gel to liquid crystalline phase transition in a number of model systems. On the basis of our results, we have tried to correlate the observed dependence to two likely phase-dependent phenomena: the passive diffusion of ions across the lipid bilayer in response to electron transport processes and the lateral mobility of proteins in the plane of the membrane.

We have also investigated, to some extent, the lipid requirements for the manifestation of respiratory control in reconstituted cytochrome oxidase membranes. We have found that both lipid head group composition as well as lipid/protein ratio have a modulating effect on this activity. In particular, the presence of cardiolipin or other acidic phospholipids seems to be essential for the establishment of respiratory control. Although it is known that cardiolipin binds most tightly to cytochrome oxidase during purification of the protein, it is not clear how they are associated. Therefore, in order to fully assess the role of cardiolipin in the respiratory control process, it first seems necessary to characterize the specific interactions between cytochrome oxidase and cardiolipin. The following proposition describes how this might be accomplished.