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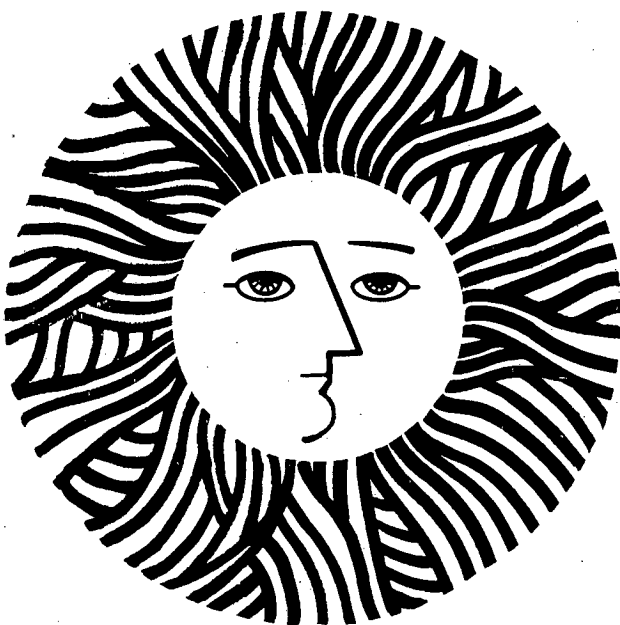
UNIVERSITY OF CALIFORNIA

ENERGY & ENVIRONMENT DIVISION

THE MEMBRANE ORGANIZATION OF CYTOCHROME c OXIDASE

Maurice Scott Swanson
(Ph.D. thesis)

October 1979



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THE MEMBRANE ORGANIZATION OF CYTOCHROME c OXIDASE

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ABSTRACT

The membrane organization of mitochondrial cytochrome c oxidase, and the role of intracomplex, intercomplex, rotational and lipid versus protein motion to that organization, has been investigated by amino acid chemical modification and electron paramagnetic resonance.

Crosslinking of purified and reconstituted cytochrome c oxidase and mitochondria with biimidates, in the presence and absence of cytochrome c, has been used to investigate oxidase subunit interactions, and whether cytochrome c mobility is required for electron transport activity. Biimide treatment of oxidase induces crosslinkage between all of the oxidase protomers except subunit I when >20% of the free amines are modified. Steady-state activity is much more inhibited by biimide than monoimide treatment. Transient kinetics of ferro-cytochrome a reduction indicate inhibition results from an effect on heme a to a₃ electron transfer. Crosslinking between oxidase molecules to form large aggregates displaying rotational correlation times >1 ms does not affect oxidase activity inhibition.

Crosslinking of mitochondria results in the covalent coupling of complexes bc₁ and aa₃ with cytochrome c, and inhibits steady-state oxidase activity more than in the case of purified oxidase. Addition

of cytochrome c to the purified oxidase or to c-depleted mitoplasts increases the extent of activity inhibition by biimidates slightly. Cytochrome c oligomers formed by biimidate treatment act as competitive inhibitors of native c, however crosslinking of cytochrome c to c-depleted mitoplasts or purified oxidase results in a catalytically inactive complex.

The rotational mobility of spin-labeled oxidase in purified form, and incorporated into liposomes, was also studied to gain information on the membrane organization of oxidase. A rigidly attached maleimide spin label permitted the measurement of the protein's overall rotational mobility by saturation transfer electron paramagnetic resonance. A long-chain maleimide spin label was used to detect the fluidity of the lipid hydrocarbon region adjacent to the protein by conventional EPR. Oxidase activity and cytochrome c binding were not affected by labeling. Reconstituted membrane vesicles, containing functionally incorporated enzyme (as indicated by high electron transport activity and respiratory control), were prepared by either sonication or cholate dialysis. One method of preparing the purified enzyme resulted in a high degree of protein rotational mobility at 4°C both in the purified detergent-solubilized enzyme (effective rotational correlation time of 100 ns) and in reconstituted membranes (correlation time of 40 μ s). By contrast, another purification procedure resulted in little or no sub-millisecond protein rotational mobility both in purified form and in reconstituted membranes, suggesting the presence of large protein aggregates. Thus, the state of aggregation of cytochrome oxidase in membranes appears to depend on the state of aggregation prior to reconstitution. The mobile

and immobile enzymes had the same high activity.

In both reconstituted preparations the bulk of the lipid was quite fluid at 4°C, as probed by a free fatty acid spin label. The lipid hydrocarbon region adjacent to the protein, as probed by the long-chain maleimide spin label, was also quite fluid in the membranes containing mobile enzyme, but was strongly immobilized in the membranes containing immobile enzyme.

These findings have led to a model for the structure and membrane organization of cytochrome oxidase, and the role of cytochrome c rotational and/or translational motion on the mitochondrial inner membrane surface.

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ABBREVIATIONS

ETC	Electron transport chain
ESR	Electron spin resonance
DPG	Diphosphatidyl glycerol
<u>aa</u> ₃	Cytochrome <u>c</u> oxidase
EPR	Electron paramagnetic resonance
NMR	Nuclear paramagnetic resonance
CO	Carbon monoxide
T _c	Transition temperature
DMS	Dimethyl suberimide dihydrochloride
EA	Ethyl acetimidate
FNPA	4-fluoro-3-nitrophenyl azide
TMPD	<u>N,N,N',N'</u> -tetramethylphenylenediamine
DNPA	2,4-dinitro-5-fluorophenyl azide
DMA	Dimethyl adipimidate dihydrochloride
DTBP	Dithiobispropionimidate dihydrochloride
MA	Methyl acetimidate
MBI	Methyl butyrimidate
MMBI	Methyl mercaptobutyrimidate
MSL	4-maleimido-2,2,6,6-tetramethylpiperidinoxy
FASL(1,14)	2-(14-carboxyl-tetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy methyl ester
MSL(1,14)	2-(14-carboxyl-tetradecyl-N-ethyl maleic ester)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy
MP	Melting point

DOC	Deoxycholate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
SDS	Sodium dodecyl sulfate
MW	Molecular weight
FCCP	Carbonyl cyanide, p-trifluoromethoxyphenylhydrazone
RCR	Respiratory control
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate

INTRODUCTION

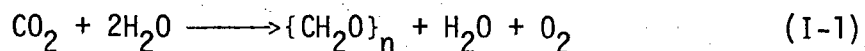
Aerobic cells derive the energy essential for the maintenance of life from biological oxidation reactions. The most common of these processes are dehydrogenations utilizing molecular oxygen as an electron acceptor. In eukaryotes, these substrate oxidations are catalyzed by aerobic dehydrogenases, or oxidoreductases, which are positioned within the mitochondrial inner membrane as a series of reduction-oxidation complexes of increasing midpoint potential. Since these enzymes which comprise the electron transport chain are integral membrane proteins their diffusion is limited within the plane of the bilayer, and yet electron transport, which involves the dynamic interaction of redox centers positioned within these catalytic complexes, is an extremely efficient and regulated process involving not only electron transport and transhydrogenations, but also phosphorylation of ADP. Knowledge about macromolecular dynamics of mitochondrial inner membrane components is thus essential to understanding how cells derive their energy by substrate oxidations.

This dissertation is a study of the terminal oxidoreductase of the electron transport chain (ETC), cytochrome c oxidase. The rationale of this project is that since cytochrome oxidase has been postulated to be a central control point of electron transport (1-3), and since it is the only enzyme complex which reacts with both oxygen and the only water soluble (peripheral) protein of the ETC, cytochrome c, information about how motion within the cytochrome oxidase complex, and between it and other ETC components, might provide an insight into how spatial relationships within and between the complexes could modulate electron flow down the chain.

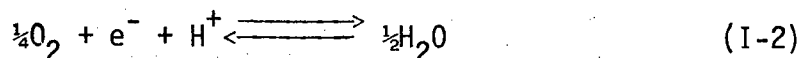
In the present investigation, we have attempted to describe the types of motion which occur within cytochrome c oxidase by chemical modification and electron spin resonance(ESR) studies. Moreover, it has been possible to not only define rotational mobility parameters of the oxidase complex diffusing in various environments by saturation transfer spin resonance techniques, but also the relative motion between protein and lipid within the complex by conventional EPR.

BACKGROUND

Why Cytochrome c Oxidase ?- Photosynthesis, the biochemical process which converts light energy into potential chemical energy, results in the evolution of oxygen by the following process:



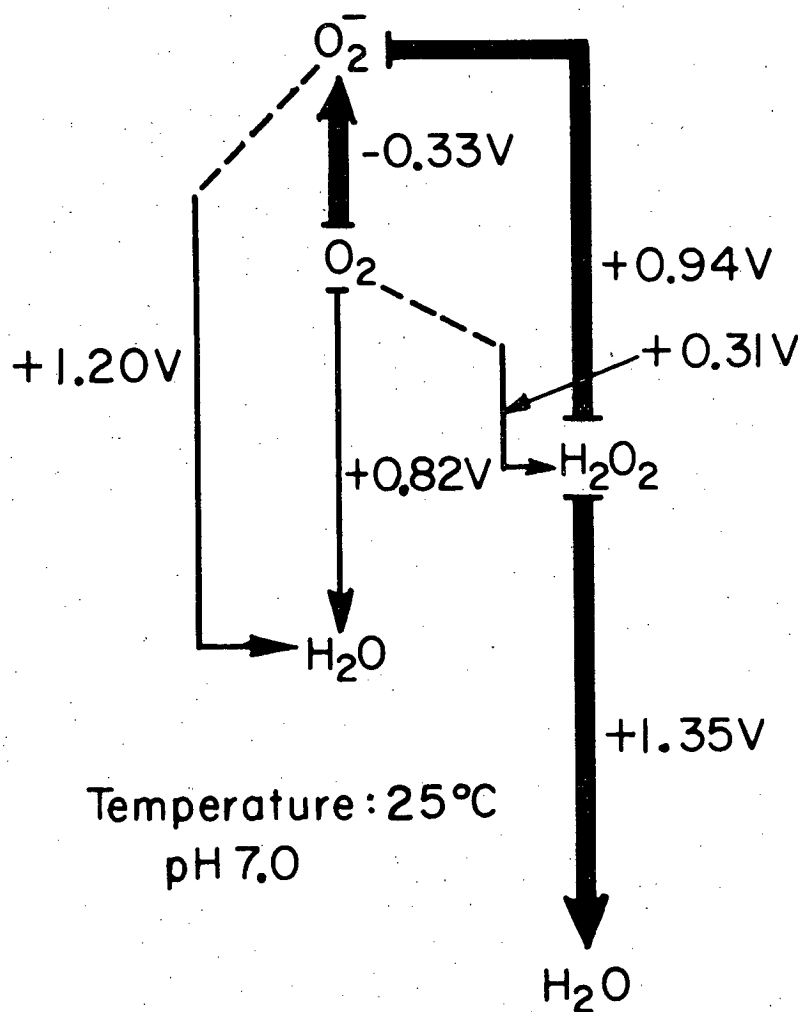
The oxidizing power of this evolved oxygen may be expressed in terms of the standard potential of the reaction:



At pH 7.0 this potential value is +0.820 V(1). The availability of molecular oxygen, which is a relatively stable and strong oxidant, is then utilized for the process of electron acceptance in eukaryotic mitochondrial electron transport.

As Fig. 1 illustrates, the reduction of molecular oxygen to water must proceed via a one electron step to superoxide, a reaction which is not thermodynamically favorable since the standard reduction potential is -0.330 V(2). This disfavored one-electron step is the reason that molecular oxygen is relatively chemically inert, and does not spontaneously transform into water. Therefore, in order for oxygen reduction to occur either a relatively large amount of energy must be utilized as a driving force or a two-electron step to peroxide must occur. Another reason for the chemical inertness of dioxygen concerns its atomic structure in that, although dioxygen has an even number of electrons, it is paramagnetic since its two highest occupied molecular orbitals each contain an unpaired electron. Reactions involving molecular oxygen and peroxide or oxide, which are not paramagnetic, are forbidden since spin reversal must occur. However, if molecular oxygen is allowed to interact with the paramagnetic center of a transition metal, which then

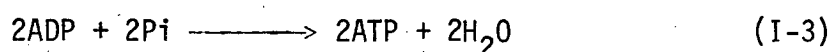
Fig. 1. Conversion of molecular oxygen to water: standard oxidation-reduction potentials. The large arrows refer to pathways which involve one-electron steps.



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acts as a reaction catalyst, then reduction to water can occur.

Cytochrome c oxidase therefore performs three essential functions in its mediation of molecular oxygen reduction. First, it is responsible for circumventing the restriction imposed by the oxygen molecule in reducing dioxygen to water. Secondly, it allows delivery of oxidizing equivalents from the high oxygen potential to an acceptor of suitable potential in order not to release energy all at once. Thirdly, it couples the energy transformation of a reduction-oxidation potential to a phosphate potential for the synthesis of ATP:



where the phosphate potential is defined as:

$$\Delta G_p = \Delta G_p^0 + RT \ln \frac{\{\text{ATP}\}}{\{\text{ADP}\}\{\text{P}_i\}} \quad (\text{I-4})$$

Cytochrome c Oxidase and the Electron Transport Chain: Structure-Protein and Lipid Components

The electron transport chain of eukaryotes is composed of four oxidation-reduction complexes, designated as complexes I-IV, which contain protein, lipid, transition metals, nucleotides, quinones, and labile sulfur(3-6). Table I defines the complexes in terms of the type and quantity of their constituents, however it is not possible presently to fully characterize the membrane displacement of the complexes in relation to one another. The simple concept that the electron transport complexes are arranged in a linear sequence of increasing midpoint potential(7) has evolved into a more complicated, but not necessarily more accurate, view of the complexes as being relatively randomly distributed and even self-aggregating assemblies(8). For instance, Hackenbrock and Hammon(9), have demonstrated by using ferritin-labelled

Table I
Oxidation-Reduction Components of
The Electron Transport Chain

<u>Complex</u>	<u>Enzyme</u>	<u>Components</u>	<u>Concentration</u> per mg protein
I	NADH:ubiquinone 10 oxidoreductase (1.6.99.3)	Flavin mononucleotide non-heme iron labile sulfide ubiquinone lipid	1.4-1.5 nmol 23-26 ng atom 23-26 nmol 4.2-4.5 nmol 0.22 mg
II	succinate: ubiquinone 10 oxidoreductase (1.3.99.1)	Flavin adenine dinucleotide non-heme iron labile sulfide cytochrome b lipid	4.6-5.0 nmol 36-38 ng atom 32-38 nmol 4.5-4.8 nmol 0.2 mg
III	ubiquinol: cytochrome <u>c</u> oxidoreductase (1.10.2.2)	non-heme iron labile sulfide ubiquinone cytochrome b cytochrome <u>c</u> ₁ lipid	10-12 ng atom 6-8 nmol >2 nmol 8.0-8.5 nmol 4.0-4.2 nmol 0.4 mg
IV	cytochrome <u>c</u> : oxygen oxidoreductase (1.9.3.1)	cytochromes <u>aa</u> ₃ copper lipid	10.5-14.0 nmol 11-12 ng atom 0.02-0.4 mg

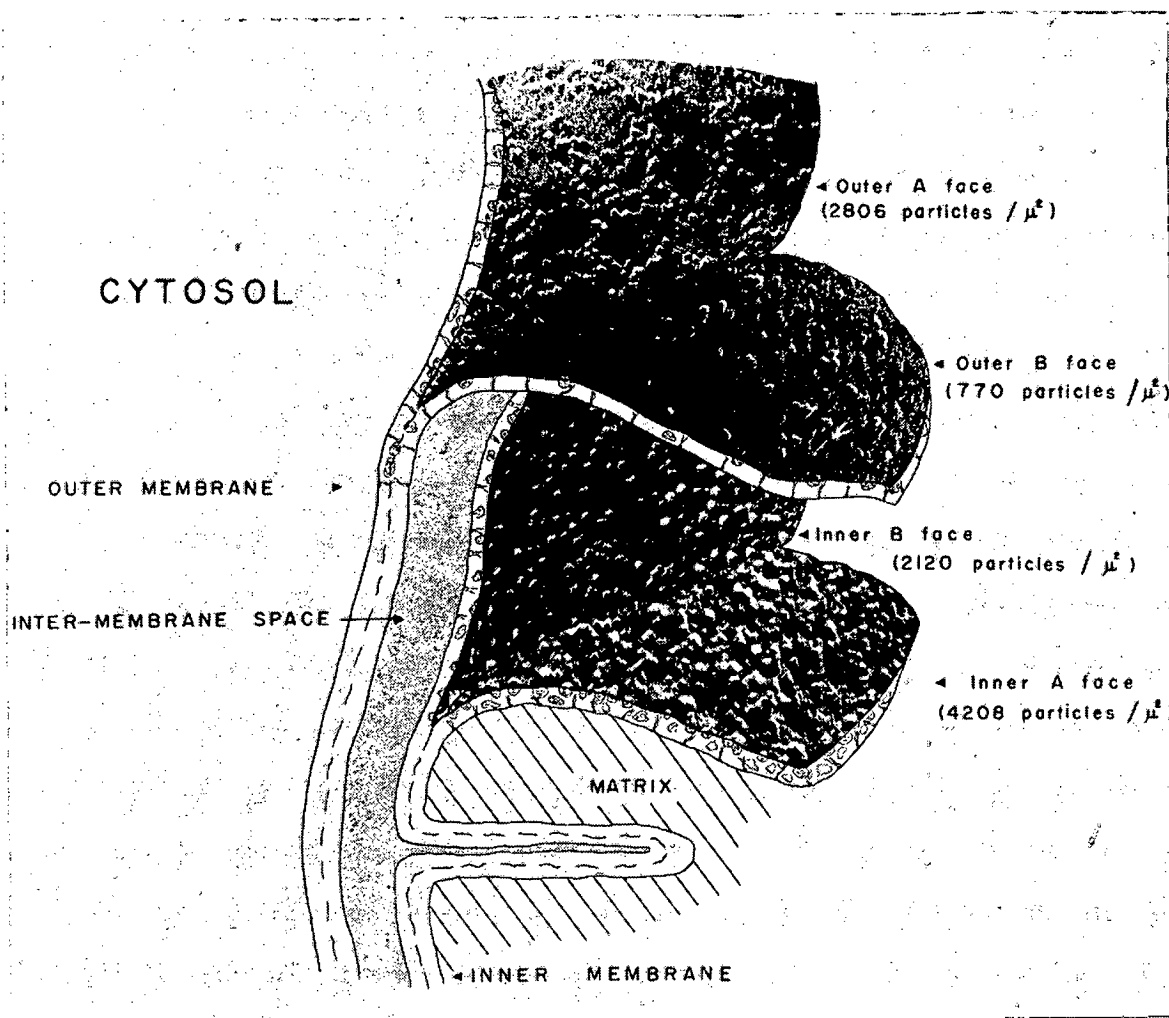
Compiled from information in Reference (4).

and affinity-purified IgG immunoglobulins monospecific for cytochrome oxidase that approximately 2000 cytochrome c oxidase binding sites exist on the surface of the inner membrane which contains an average of 17,000 oxidase monomers. This implies that since the active unit of oxidase is a dimer, at least when it is isolated, that tetramers or higher oligomers of these dimeric units commonly occur in vivo.

Previously, a structural concept of the energy transducing membrane of mitochondria based upon the distribution of particles detectable by freeze-fracture electron microscopy suggested that particle distribution was not only asymmetric but that particles, assumed to be protein, were immersed in a lipid 'sea'(10)(Fig. 2). This model is an adaptation of the fluid mosaic model of membranes as popularized by Singer and Nicolson(11). By contrast, Sjöstrand and coworkers(12,13) have produced evidence that cristae membranes contain densely packed protein, suggesting that the inner membrane of mitochondria may be highly viscous. In this latter model apposed cristae membranes contain a small amount of bilayer and an enormous concentration of protein somewhat similar to a 'brick wall' where the 'bricks', or protein, are interspersed with lipid 'mortar'(12). This question of the displacement of proteins in the inner mitochondrial membrane has not yet been resolved.

Within the inner membrane, cytochrome oxidase as the terminal enzymatic component is positioned so that it can accept electrons from reduced cytochrome c and donate them to molecular oxygen(Table I). Cytochrome c oxidase is thus a transmembrane and oligomeric enzymatic complex consisting of 6-10 polypeptides, two copper atoms, two a-type hemes and lipid(2-40%, by weight)(14). The cytochrome oxidase complex

Fig. 2. Mitochondrial particle distributions as detected by freeze-fracture electron micrographs of inner and outer membranes, after Packer (10). The diagram was constructed from representative replicas of half membranes created by the freeze fracture technique(used by permission).



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is large with dimensions of $52 \times 60 \times 85 \text{ \AA}$, and can only be removed from the mitochondrial membrane by detergents(15).

Table II lists the molecular weights of the oxidase polypeptide subunits determined by Swanson et al(16,17), and the range found in the literature. Since the polypeptide weight of complex IV equals 122,500 and the average lipid content is 0.2 mg/mg protein then the average monomer of the enzyme, assuming no subunit duplications occur, should have a molecular weight equal to 147,000. The only reported case in which the enzyme has been found to exist in an active form in this molecular weight range is (31), although in our hands this preparation only retains 10% of its original activity. Therefore it appears that the isolated cytochrome c oxidase complex tends to be a self-aggregating system, and that at least dimerization is required for efficient electron shuttling.

Table II also summarizes what is known about the structure of each of the oxidase subunits and their disposition in relation to one another and to the membrane. Table III lists the amino acid composition of purified oxidase and whole mitochondria. Table IV illustrates the variation in the lipid content of purified oxidase and whole beef heart mitochondria. It is important to note that the negative phospholipid, diphosphatidyl glycerol(DPG), is a major component of isolated oxidase.

Cytochrome Oxidase:Hemes and Copper Atoms- Another aspect of cytochrome oxidase structure concerns the prosthetic groups, hemes a and a₃, and the relationship of the iron within these structures to the copper atoms in the complex. Fig. 3 shows the chemical structure of heme a; heme a₃ possibly differs only in the fact that it is positioned

BEEF HEART CYTOCHROME c OXIDASE

Subunit	Molecular Weight ^{18,19}	Membrane Disposition ^{3,6}	Polar Amino Acids (%) ^{20,21}	Nearest Neighbors ¹⁴	Characteristics
native enzyme I - VII	122,500 (~101,000 - 145,000)	transmembrane complex	—	—	functional unit: dimer (4 Cu ⁺⁺ , 4 Fe ⁺⁺) ²² conformations: reduced: open ²³⁻²⁵ oxygenated: intermediate oxidized: compact
I	35,500 (33,000 - 43,000)	intramembrane [40,000 (I)] ^a	35.5	not cross-linked	hydrophobic
II	22,000 (19,000 - 27,000)	cytoplasmic [22,500 (II)] II & III unresolved	44.7	V	hydrophobic-hydrophilic cytochrome c binding ²⁶
III	20,500 (19,000 - 25,000)		39.9	V	hydrophobic N-ethyl maleimide reactivity (pH 7.0) ¹⁹
IV	16,500 (13,800 - 17,200)	matrix [15,000 (III)]	48.6	VI	hydrophilic
V	12,400 (6,000 - 13,000)	intramembrane [11,200 (IV)]	47.8	II, III, VII	hydrophilic possible heme a binding subunit (11,600) ²⁷
VI	8,800 (6,700 - 11,200)	cytoplasmic [9,800 (V)]	49.7	IV	hydrophilic N-ethyl maleimide reactivity (pH 7.0) ¹⁹
VII	6,800 (3,400 - 9,000)	cytoplasmic [7,300 (VI)]	53.7	V	hydrophilic
cytochrome c	12,400	—	—	II ²⁶	binding to aa ₃ : primarily electrostatic ^{28,29} binds to III in yeast ³⁰ binds to II in beef heart ²⁶

a. Original nomenclature of (3).

Numbers refer to reference from which information was derived.

Table III
Amino Acid Compositions of Beef Heart Mitochondria and
Purified Cytochrome c Oxidase

Amino Acid	Purified Cytochrome Oxidase		Chymotrypsin-	Mitochondria ^a
	{heme a}=11.5 ^a	{heme a}=10.5 ^b	oxidase ^c	
Lysine	28	39	25	435
Histidine	20	30	20	150
Arginine	21	31	17	310
Aspartic Acid	52	60	41	562
Threonine	51	53	40	364
Serine	53	54	31	396
Glutamic Acid	52	60	40	686
Proline	48	46	35	339
Glycine	53	59	46	580
Alanine	55	62	46	628
Cysteine	7	7	7	70
Valine	45	51	39	482
Methionine	13	35	35	181
Isoleucine	40	43	37	396
Leucine	79	87	64	654
Tyrosine	29	33	23	188
Phenylalanine	43	47	38	303
NH ₃	63	59	-	507
Tryptophan	27	30	27	77
Ethanolamine	-	-	-	148
Total Residues ^d	716	827	601	6801

^aRef. (32) ^bRef (33) ^cRef (34) ^dBased on per mole heme a

Table IV

Phospholipid Content of Beef Heart Mitochondria andPurified Cytochrome c Oxidase

<u>Preparation</u>	<u>Extraction Procedure</u>	<u>Percent Total Phospholipid</u>				
		<u>DPG</u>	<u>PE</u>	<u>PC</u>	<u>PI</u>	<u>others</u>
Mitochondria	Chloroform-Methanol ^a	16	31	37	10	5
	Alkaline Chloroform-Methanol ^b	18	30	38	5	9
Cytochrome c Oxidase	<u>Enzyme Type</u> <u>(Isolation Detergent)</u>					
	Membranous ^c (Triton)	50.4	13.4	30.1	-	6.0
	Lipid-free ^c (Triton)	73.0	-	-	-	27.0
	Membranous ^d (DOC & cholate)	30.0	30.6	32.0	-	7.0

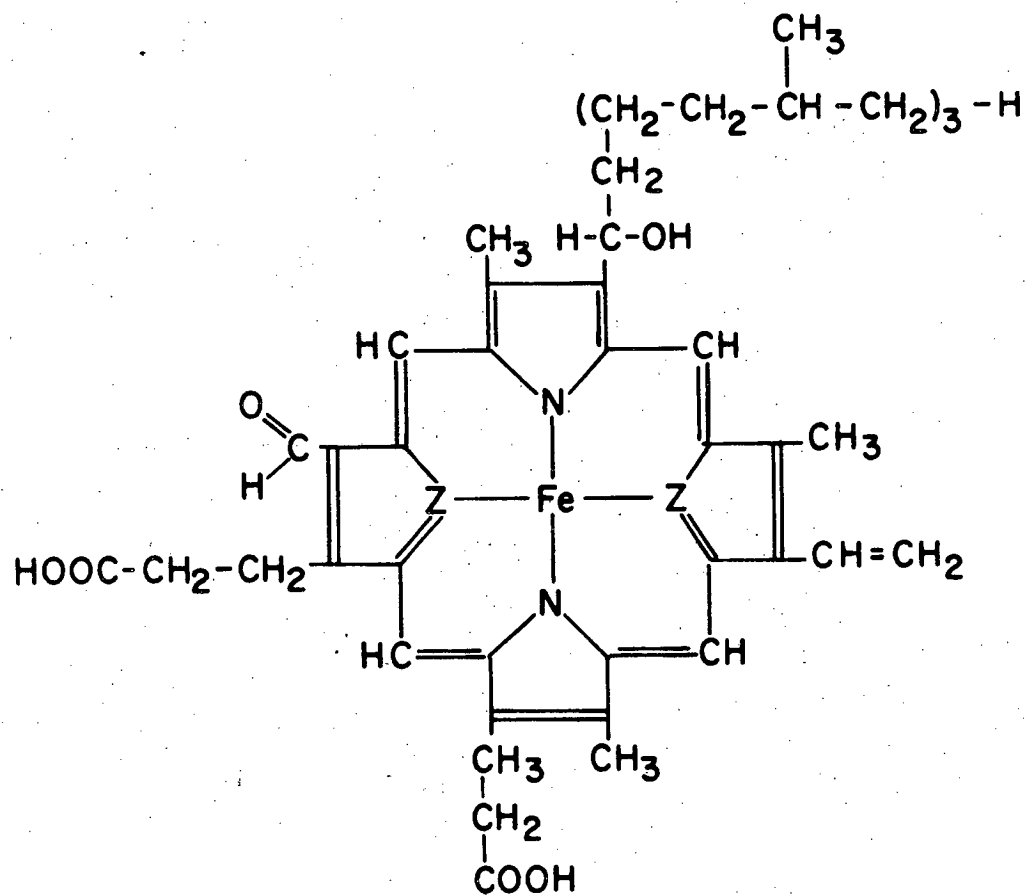
^aRef (35) ^cRef (37,38)^bRef (36) ^dRef (39)

Fig. 3. The hemes of cytochrome oxidase.

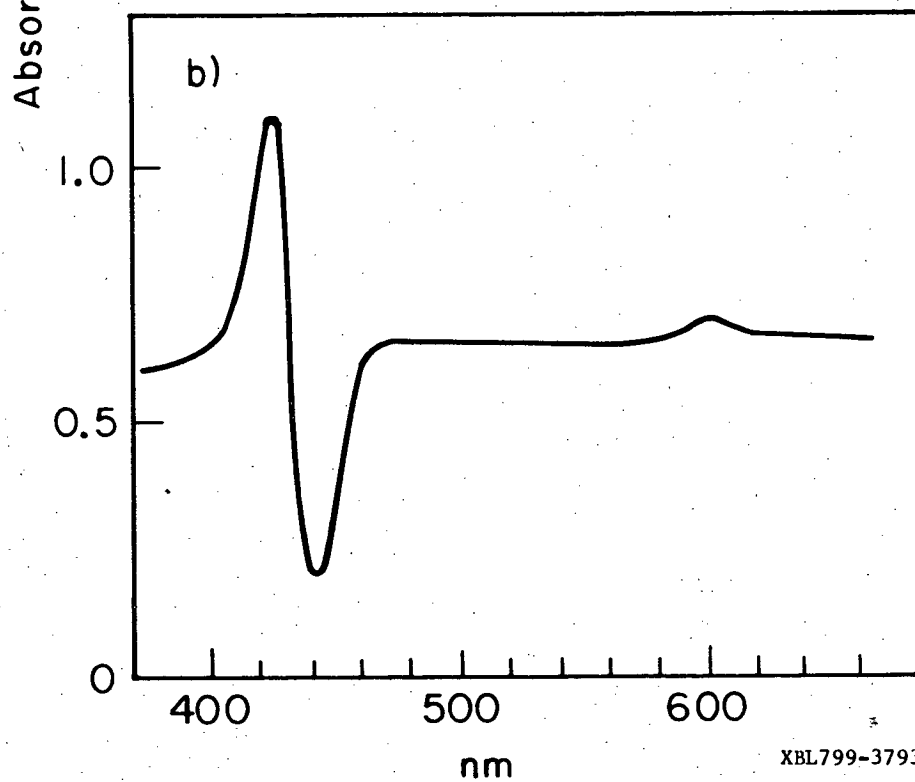
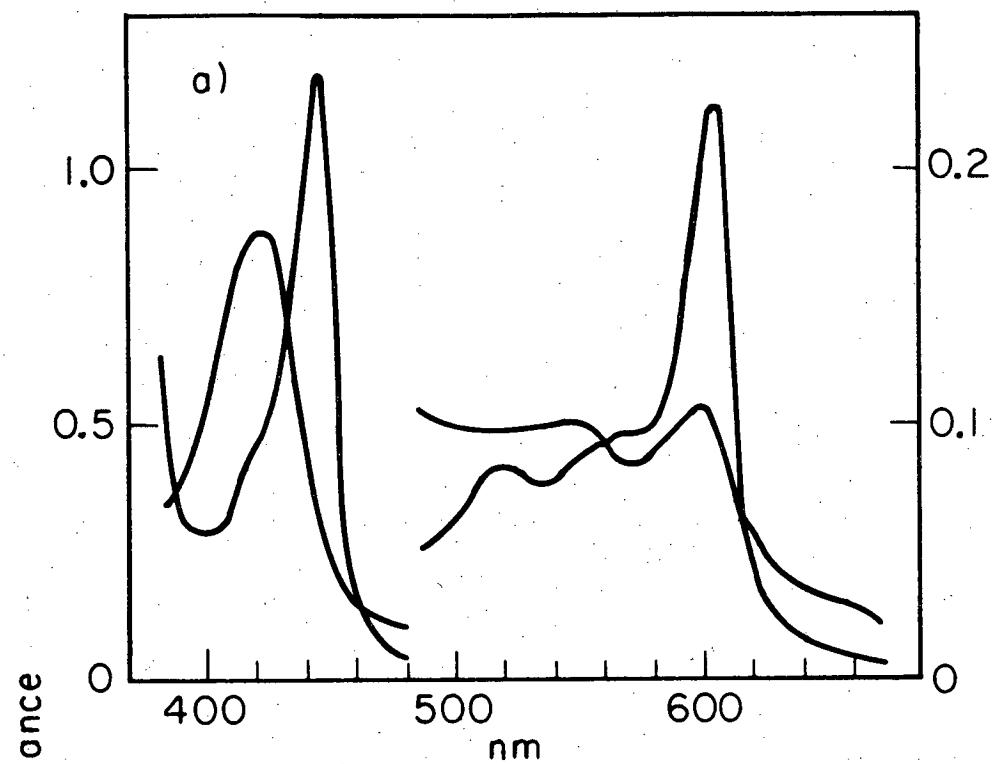
a) The chemical structure of the prosthetic group of cytochromes of class A(heme a).

b) Absorption spectra of oxidized, reduced, and CO-liganded cytochrome c oxidase.

The aa₃ concentration in the cuvette was 10 μ M.



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in another region of the enzyme and ligands CO and oxygen, among others, and thus can be spectrophotometrically distinguished.

Keilin and Hartree(40,41) first postulated the existence of two types of hemes in cytochrome oxidase on the basis of visible spectrum changes induced by cyanide and CO. Since then it has been possible to demonstrate that both components ligand in a 1:1 molar ratio to cytochrome aa₃ (42-44), and therefore only one of the hemes must bind these ligands. Neither the hemes nor the copper atoms of cytochrome oxidase are covalently linked to the protein and are released when the enzyme is disrupted by strong detergents or organic solvents(14). Fig. 3 shows the absorption spectra which are obtained for the oxidized and reduced enzyme, and also when CO is liganded to heme a₃ where the absorption maximum wavelength in the Soret region shifts from 445 to 428.5 nm. Studies by Wilson and coworkers(45,46) on hydrated and oriented multi-layers of 'membranous' cytochrome oxidase and whole mitochondrial membranes have indicated that the hemes are oriented in both systems with the normal to the heme plane lying approximately in the membrane plane.

Cytochrome Oxidase:Midpoint Potentials- Another aspect of both hemes and copper atoms of cytochrome oxidase is that they have characteristic half-reduction or midpoint potentials. Potentiometric titrations, based upon the absorption of the hemes, yield reduction-oxidation potentials for these various components. Wilson et al(47,48) have concluded from these studies that two separate one electron acceptors exist in the oxidase complex which differ in midpoint potential($E_{m7.2}$) by 150 mV. Cytochrome a is defined as the low potential heme with an $E_{m7.2}=0.210$ V and cytochrome a₃ as the high potential heme with an $E_{m7.2}=0.385$ V.

The low potential copper, which is detectable by various physical methods, has an $E_{m7.2} = 0.245$ V, and the so-called invisible copper has an $E_{m7.2} = 0.340$ V. These workers suggest, on the basis that the extinction coefficient of cytochrome a is increased when heme a₃ binds CO, that heme-heme interactions only occur when heme a₃ is liganded(49,50). Malmström(51), however, has observed that the assignment of the high and low potentials to heme a and a₃, respectively, is somewhat arbitrary, and that the two hemes may not reside in two different environments. Nevertheless, it does appear that reduction, or oxidation, does proceed through two iron-copper pairs with one at high potential and one at low potential(2) in the cytochrome c oxidase complex. It should also be noted that conclusions about the relationships between the iron and copper in these pairs/^{are} at present, unjustified since the oxidase complex is a highly cooperative system and the intrusion of one electron into this system may strongly perturb the electron affinities of the other electron active centers.

Cytochrome Oxidase:EPR- Electron spin resonance signals from cytochrome c oxidase results from the oxidized iron and copper moieties, Fe^{+3} and Cu^{+2} . Cu^{+2} yields an intense and sharp EPR signal at $g=2$ in both model complexes and in oxidase. The copper signal of purified oxidase can be due to adventitiously-bound Cu^{+2} , which is possibly a Type I or tetrahedrally-distorted coordination, and integrally-bound Cu^{+2} , which is possibly Type II or rhombohedrally-distorted. This inherent copper signal, Type II, is not removed by chelating agents (52), and not saturable at relatively high microwave power(53). It was noted quite early that only 40% of the total Cu^{+2} which is observed chemically(54) was EPR detectable(55), and it now appears that 80%

of the signal from one Cu^{+2} and none from the other copper is distinguished by EPR(56). This EPR invisibility probably arises from interactions between copper and other oxidase metal centers which would be expected to quench the total copper signal.

Oxidized iron, Fe^{+3} , in cytochrome oxidase is characterized by a major EPR signal at $g=3$ with minor components at $g=2.2$ and $g=1.45$. Because of the line width of the iron signal, and its partial placement under the copper signal, it is difficult to quantitate this signal. Once again, the total intensity of the Fe^{+3} signal is only 40% of the chemically observable iron(56), and thus the low signal intensity of low spin Fe^{+3} must be due to interaction with another paramagnetic center. This type of interaction is poorly understood although heme-heme and heme-copper interactions have been suggested(46).

Several investigators have attempted assignments of the EPR detectable iron and copper to cytochromes a and a₃. During reductive titrations the low spin signal at $g=3$ disappears, with a corresponding midpoint potential of $+0.340 \text{ V} \pm 0.02 \text{ V}$ (57), and a high spin signal at $g=6$ appears. The high spin signal, composed of at least one axial and one rhombic species, is maximized in the half-reduced enzyme and it disappears upon further reduction with a midpoint potential of $0.210 \pm 0.20 \text{ V}$.

Some controversy exists as to how the signals should be assigned. Wilson and Leigh(50) have interpreted this signal modification induced by partial reduction as being indicative of the $g=3$ signal belonging to heme a₃ since the low spin signal disappears together with the reduction of the high potential component(47). Hartzell and Beinert(57) suggest that the spin state of cytochrome a changes with the redox state of cytochrome a₃. That implies that cytochrome a is low spin

when a_3 is oxidized and high spin when a_3 is reduced, and that the a_3 is EPR 'invisible'. Recently, Beinert and Shaw(58) have shown that if the enzyme is fully reduced in the presence of an equimolar quantity of cytochrome c then anaerobic reoxidation by ferrocyanide or porphyrin-ide results in simultaneously intense high and low spin heme signals at 0.1-2 s after mixing. These investigators have shown that about 80% of the total heme can be detected under these conditions. In addition, 70-100% of one heme, represented by the low spin, and 60-70% of the other heme, represented by the rhombic high spin signal, can be accounted for as well as 8-20% of one heme being present in the axial high spin signal. This work implies that, although the low spin signal can still be assigned to cytochrome a , the rhombic high spin signal must be attributed to cytochrome a_3 .

Cytochrome c Oxidase and the Electron Transport Chain:Function-

The electron transport chain is situated within the inner mitochondrial membrane such that it can accept reducing equivalents, in the form of NADH, succinate or any of the other reducing substrates, which creates electron flow down a potential gradient with molecular oxygen being the final electron acceptor. Table V lists the various natural and artificial enzymatic reactions which the oxidation-reduction complexes, I-IV, catalyze. We are interested in reviewing the reaction which cytochrome c oxidase catalyzes principally because we will be analyzing alterations of this activity by various protein chemical modification reagents. Cytochrome c oxidase is the terminal oxidase of respiratory metabolism in all eukaryotic and some prokaryotic organisms and catalyzes the reaction:

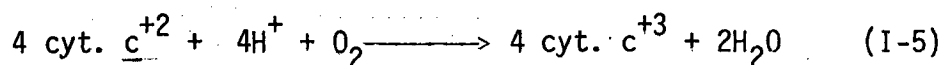
Table V

Redox Reactions Catalyzed By The Isolated Respiratory Complexes

Complex	Reaction Catalyzed	Activity ^a	Conditions
I	NADH→ubiquinone-1 NADH→ferricyanide	25 685	V _{max} with respect to acceptor concentration
II	Succinate→ubiquinone-2 Succinate→PMS(DCIP) ^b	50-55 50-55	- V _{max} with respect to {PMS}
III	Reduced ubiquinone-2→ cytochrome <u>c</u>	300-500	-
IV	Ferrocytochrome <u>c</u> →O ₂	140-180	infinite {cytochrome <u>c</u> } by extrapolation of rates measured in range of 10- 30 μM ferrocytochrome <u>c</u>
	Ascorbate + TMPD + ferri-cytochrome <u>c</u> → ferrocytochrome <u>c</u> →O ₂	400 electrons/s	polarographic measurement in 67 mM KH ₂ PO ₄ (25°C)
		50 nmoles O ₂ -min	polarographic measurement [†] in 25 mM Tris-acetate (25°C)

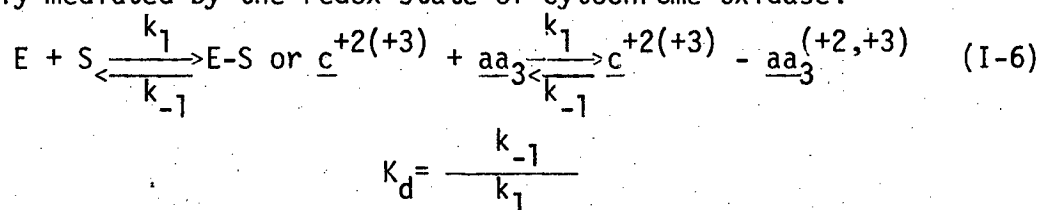
^aActivity is expressed as μmoles of substrate oxidized, exchanged or hydrolyzed per min per mg protein at 38°C except c) & d).

^bPMS, phenazine methosulfate; DCIP, 2,6-dichloroindophenol



The only known electron donor for ferricytochrome c oxidase under physiological conditions is ferrocytochrome c, although the enzyme is also reducible by dithionite(59), NADH(60), ascorbate(61), and dichloro-hydroquinone(62). We will review how the reaction of both dioxygen and cytochrome c with cytochrome oxidase results in net electron transfer.

The Cytochrome c-Cytochrome Oxidase Reaction: Cytochrome c Binding Properties- In order for ferrocytochrome c to donate an electron to the oxidase complex it must first bind to the active site on the enzyme. However, since the interaction between c and aa₃ is primarily electrostatic(63) this presents a conceptual difficulty since the inner membrane surface possesses net negatively-charged lipids and protein moieties. However, this apparent paradox is resolvable because of the heterogeneity of these negatively-charged lipid and protein binding sites. In other words, the enzyme possesses a certain complement of extremely high affinity binding site(s) and a multitude of lower affinity sites. The association-dissociation reaction is then possibly mediated by the redox state of cytochrome oxidase:



Unfortunately, both ferro- and ferricytochrome c also bind small anions and cations(64-66). Studies on the solution electrophoretic properties of ferricytochrome c(64) indicate that anions bind to both regions of high lysine and arginine density on the c molecule in the order(at pH

6.9):

phosphate>chloride>iodide>sulfate>cacodylate

Some of these anions, such as phosphate, are bound so tightly they are only removed by electrodialysis(64). For instance, the K_d (apparent) of $H_2PO_4^-$ is 25-100 μM , while chloride is 50-100 μM (67,68).

Ions such as chloride actually distinguish the cytochrome c redox state in that they preferentially bind to either the ferrous or ferric forms while others, such as phosphate, bind with equal affinity(65). Obviously then buffer ionic strength becomes very important in determining the binding affinity of cytochrome c for ions, lipids, and proteins.

Margoliash and coworkers(69) have utilized this facet of cytochrome c binding to study the binding kinetics of various eukaryotic cytochromes c to purified and Keilin-Hartree particle cytochrome c oxidase. They have shown that at low ionic strength in cacodylate buffer, cytochrome c binds to purified oxidase in an approximately 1:1 molar ratio, and this complex is not dissociated by gel filtration. Association and dissociation of c from its high affinity oxidase binding site does occur(70) however with time, and this can be expected to increase the number of c molecules which rebind randomly to low affinity sites. Definition of these high and low affinity sites in terms of whether they are composed of lipid and/or protein is not presently possible, however Van Gelder et al(71) have suggested that the high affinity site is surrounded by negative charges and buried within the complex since the enzyme is not reduced, in the absence of cytochrome c which is reduced readily, by hydrated electrons. Koppenol et al(72) have calculated the electric potential fields around ferri- and ferro-

cytochrome c and have suggested that the c dipole is attracted and then forced to align in the direction of the electric field vector which points into cytochrome oxidase.

Binding Domains of Cytochrome c on Cytochrome Oxidase- Recently, the cytochrome c domain which interacts with cytochrome oxidase with extremely high affinity has been defined by Margoliash and coworkers (69,70), and has been mapped to the left of the upper half of the exposed heme edge with the center of contact at the β -carbon of phenylalanine 82. This delineation of the molecular arrangement of cytochrome c with respect to its high affinity binding site on the oxidase was possible not only because of the availability of monocarboxydinitro-phenyl derivatives of lysines 60, 39, 22/99, 27, 8, 87, 72, and 13 of the cytochrome c molecule(73), but also the development of a kinetic assay which allows the calculation of a dissociation constant(K_d) for different populations of chemically modified molecules based upon the finding that TMPD and ascorbate, but not ascorbate alone, are able to reduce c bound to its high affinity site($K_d=0.03 \mu\text{M}$) at low ionic strength in the absence of polyvalent anions which bind to the c molecule, such as phosphate.

Cytochrome c Binding to Lipids- Cytochrome c also binds avidly to negatively charged lipids. For instance, c will bind to lipid vesicles composed of 80% phosphatidyl choline and 20% cardiolipin in 15 mM KCl (pH 8.0) with a $K_d \approx 200 \mu\text{M}$. Since the low affinity sites, as determined by the previously described method(70), have a $K_d \approx 1 \mu\text{M}$ these will bind quantitatively more c, however this lipid affinity does affect the Eadie-Hofstee-Scatchard analysis(this thesis) of both the high and low affinity sites.

Brown and Wüthrich(74) have investigated the binding of ferricytochrome c to 1:4 cardiolipin:phosphatidyl choline single bilayer vesicles by ^1H and ^{13}C NMR combined with EPR detection of a spin probe covalently attached to the c molecule at methionine-65. The vesicles were approximately 300 Å in diameter both in the absence and presence of the protein. They discovered that the binding of cytochrome c affects phospholipid distribution within the bilayer in that c apparently binds to cardiolipin head groups and causes clustering of this phospholipid; the interior of the bilayer is unaffected by the presence of the protein. Phospholipid clustering leads to segregation of immobilized cardiolipin, which displays lower rotational movement because of its interaction with cytochrome c, and mobile phosphatidyl choline populations. Evidence was also obtained that cytochrome c binds to these vesicles with its heme group pointing away from the phospholipid-protein interface. This work implies that cytochrome c binds to pure lipid systems by its 'backside'; we have already presented evidence that this c binds to purified oxidase, reductase, and Keilin-Hartree particles near its heme crevice or 'front-side'.

Enzyme Kinetics:Ferrocytochrome c to Ferricytochrome a- Keilin(75) was the first to study the relationship between cytochrome c concentration and the rate of oxygen uptake by Keilin-Hartree particles. He interpreted his results in terms of Schütz's law(76):

$$v = k_p \sqrt{[E]_t [S]_t} \quad (I-7)$$

where the velocity of an enzyme catalyzed reaction is directly proportional to the square root of the product of the enzyme and substrate concentrations and time. Interestingly, this equation is a better approximation for some cytochrome c catalyzed reactions than the

Michaelis-Menten derivation of the Henri equation(77):

$$v = \frac{k_p \{E\} + \{S\}}{K_m + \{S\}} \quad (I-8)$$

A point which should be introduced here is the types of assays which are used to determine steady-state and transient kinetics of the cytochrome c reaction. The most simple experimental assay involves following the oxidation of ferrocytochrome c(Table VI) at 550 nm by cytochrome oxidase in the absence of a regeneration system, such as ascorbate, for ferrocytochrome c once it is oxidized. This system is ideal for studying pre-steady state or transient kinetics of the reaction of heme c with heme a, since the oxidation of the former can be followed at a wavelength of 500 nm and the reduction of the latter at 605 nm or 445 nm(62). An interesting aspect of this work is that the addition of ferrocytochrome c to an anerobic suspension(10^{-7} M O_2) of cytochrome oxidase initiates biphasic absorbance changes at the above wavelengths. The first, or burst, phase is believed to correspond to the formation of ferrocytochrome a and the oxidation of ferrocytochrome c and is complete in 10 ms; the same type of reaction occurs in the presence of cyanide which ligands to cytochrome a₃(62)(see Table VII). The slow phase, which lasts several seconds, appears to relate to the intra-cytochrome oxidase complex electron transfer rate from ferrocytochrome a to ferricytochrome a₃. Examination of the near infrared region of the visible spectrum during these transient kinetic experiments also indicated that cytochrome a is in rapid equilibrium with a second site, identified as the visible copper because of its 830 nm absorbance(81). As shown on Table VI, the reaction of ferrocytochrome c with oxidase

Table VI

Steady-State and Transient Kinetic Activity Determinations ofIsolated Cytochrome c Oxidase

Determination	Reaction ^a	Rate ^b	Temperature °C
<u>Transient</u>			
spectrophotometric Δ550 nm absorbance	$\underline{c}^{+2} + \underline{a}^{+3} \xrightarrow{\underline{c}^{+3}} \underline{a}^{+2}$	10^6-10^7 (rate constant)	20°C
spectrophotometric Δ605 nm absorbance	$\underline{a}^{+2} + \underline{a}^{+3} \xrightarrow{\underline{a}^{+3}} \underline{a}^{+2}$	750 s ⁻¹	20°C
spectrophotometric Δ445 nm absorbance	$\underline{a}_3^{+2} + O_2 \xrightarrow{\underline{a}_3^{+3}} H_2O$	150 s ⁻¹	20°C
<u>Steady-State</u>			
polargraphic	{TMPD + ascorbate} + $\underline{c}^{+3} \xrightarrow{\underline{c}^{+2}} \underline{c}^{+3} + \underline{a}^{+3} \xrightarrow{\underline{c}^{+2}} \underline{a}^{+3}$ $\xrightarrow{\underline{c}^{+3}} \underline{a}^{+2} + \underline{a}^{+3} \xrightarrow{\underline{a}^{+3}} \underline{a}^{+2}$ $+ O_2 \xrightarrow{\underline{a}_3^{+3}} H_2O$	250-450 s ⁻¹	25

^aThe reactions are only representations of the pathways and are not balanced. In the polarographic example cytochrome c dissociation can, but does not have to, occur after heme a reduction.

^bRates are dependent on the type of preparation, and are expressed as TN. °C Ref (62)

Table VII

Inhibitors of Isolated Cytochrome c Oxidase

Ligand	Inhibition Type	K_i (M)	Group ^d
CO	competitive	0.32×10^{-6}	I
azide	uncompetitive	33×10^{-6}	I
fluoride ^c	complex	3500×10^{-6}	II
formate	uncompetitive	1500×10^{-6}	-
hydroxylamine ^b (NO)	uncompetitive ^a	500×10^{-6}	II
cyanide	noncompetitive	0.2×10^{-6}	I
sulfide	noncompetitive	0.2×10^{-6}	I
semicarbazide ^b	-	0.17	III
hydrazine ^b	-	0.11	III
bisulfite ^b	competitive ^e	1.0×10^{-2}	I
salicyaldoxime ^b	-	4.4×10^{-3}	III
ethylxanthate ^b	-	4.8×10^{-3}	III

Determined from reciprocals, $1/v$ versus $1/[O_2]$ by Ref (78).

^a metabolism of hydroxylamine generates NO, which is inhibitory.

^b value is K_d determined by (79), values are for active transport at pH 7.4.

^c determined by (80), value is determined at saturating $\{aa_3\}$.

^d Group I: an inhibitor forms a completely inactive 1:1 enzyme inhibitor complex

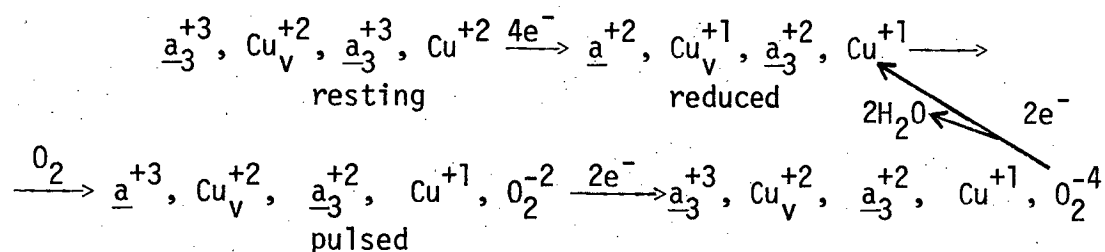
Group II: a 1:1 enzyme inhibitor complex still retains some residual activity and only a 1:2 complex is completely inactive

Group III: Both 1:1 and 1:2 complexes are formed, and any aa_3 -inhibitor complex is inactive

^e competitive with respect to azide and cyanide

consists of several phases the first of which is a second order reaction with a rate constant of 10^6 - 10^7 $\text{M}^{-1}\text{sec}^{-1}$. The transfer of electrons within the complex is slow under anerobic conditions in the isolated oxidase, 0.6 electrons/s/ aa_3 unit, as monitored by the appearance of photosensitivity of the CO-aa_3 complex(CO only ligands a_3^{+2})(82). Chance et al (83) however have shown that the rate limiting intracomplex electron transfer in intact mitochondria when oxygen is pulsed into the medium is on the order of 400 electrons/s/ aa_3 unit for the burst phase. This paradox has not yet been resolved.

Antonini et al(82) have proposed a scheme for the transfer of electrons from reduced cytochrome c to cytochrome oxidase as follows:



where $\text{Cu}_v^{+2,+3}$ is the visible and $\text{Cu}^{+2,+3}$ the invisible copper. The interesting aspect of this model is that it predicts the rate of internal electron transfer from cytochrome a in the pulsed enzyme will be considerably faster than in the resting oxidase, an observation which can be demonstrated experimentally(82).

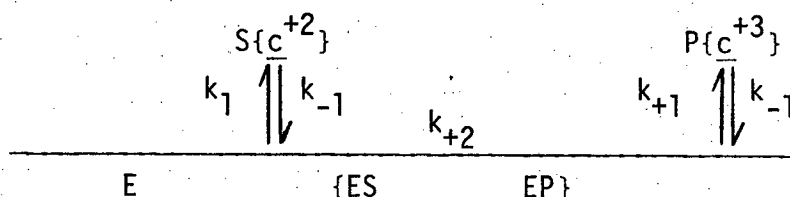
Steady-state kinetics can be examined by the addition of substrates and electron donors which regenerate ferrocycytochrome c after it is oxidized by ferricytochrome a (Table VI). In this type of system the reaction velocity is proportional to the ferrocycytochrome c concentration at saturating ascorbate concentrations:

$$v_R = k_o \{ \text{AH} \} \{ \text{c}^{+3} \} \quad (\text{I-9})$$

The steady-state kinetics measured by the various assay techniques should apply to the same empirical rate equation(84):

$$v_R = \frac{V_{\max} \{c^{+2}\}}{K + \{c^{+2}\} + \{c^{+3}\}} = \frac{V_{\max} \{c^{+2}\}}{K + \{c_{\text{total}}\}} \quad (\text{I-10})$$

This rate equation implies that even at saturating substrate concentration the reaction is first order. Explanations of this rate equation were first provided by Minnaert(85) in the form of six possible mechanisms, however his mechanism IV has prevailed:



The rate equation of this mechanism would be:

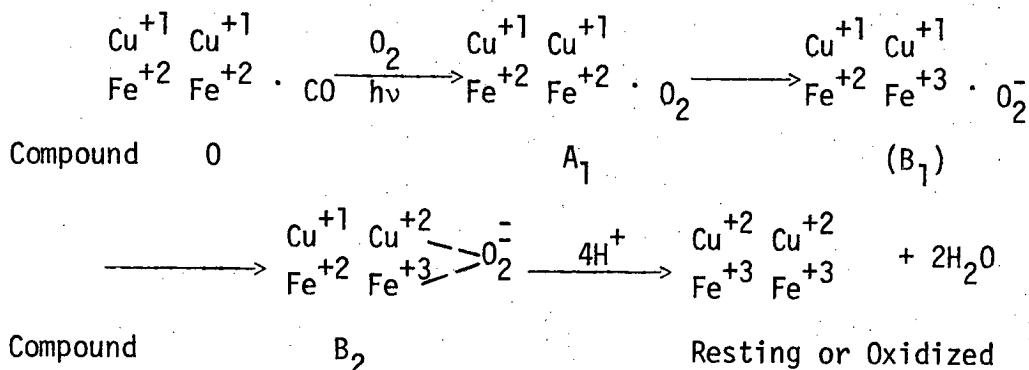
$$v_R = \frac{\frac{k_{+2}k_{-1}}{k_{+2} + k_{-1}} e\{c^{+2}\}}{\frac{k_{-1}}{k_{+1}} + \{c_{\text{total}}\}} \quad (\text{I-11})$$

It is interesting to note that this mechanism suggests that ferri-cytochrome c is a competitive inhibitor of ferrocytochrome c.

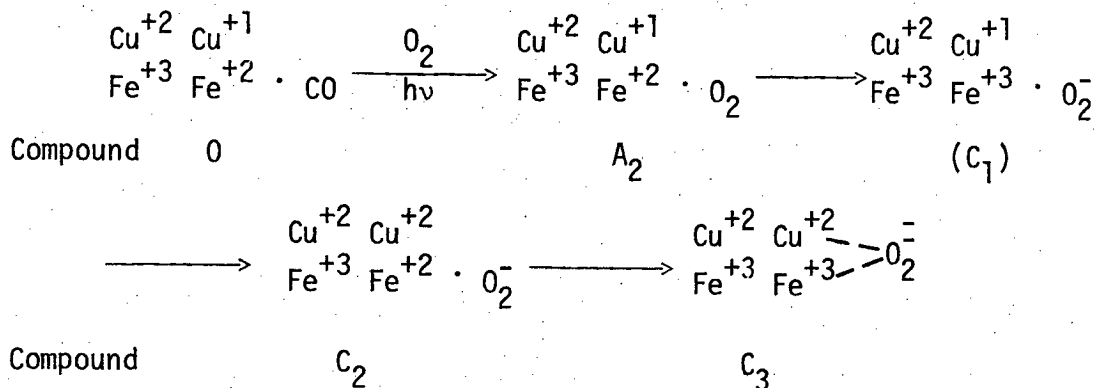
Enzyme Kinetics: Cytochrome c Oxidase to Oxygen- Cytochrome c oxidase is a bisubstrate enzymatic complex which produces ferricytochrome c from ferrocytochrome c, and water from molecular oxygen. The reaction of oxidase with oxygen at low temperatures has been most extensively studied by Chance and coworkers(86,87). Three cytochrome oxidase compounds, designated as A, B, and C, are formed at significant conc-

entrations at low temperatures, as detected by near infrared absorption spectra. Compound A appears to be oxycytochrome oxidase, Compound B has been determined to be peroxycytochrome oxidase(86), and Compound C is thought to be ferrous-cupric copper-superoxide anion intermediate. An idea of how these compounds arise is depicted below:

UNIFORM VALENCE:



MIXED VALENCE:



After the photolysis of the reduced CO-oxidase compound by light, oxygen binds and the various intermediates are formed, however it should be stressed that this phenomenon is only detectable at temperatures around -40 to -100°C . Compound A, the first intermediate formed, has a second order rate constant of formation of $685 \text{ M}^{-1}\text{s}^{-1}$ at -94°C , a K_d at -100°C equal to $300 \mu\text{M}$ while the apparent activation energy(E_a) is 9.9 kcal/mole (87-89). However, the K_m for oxygen at room temperature is equal

to 0.05-0.1 μM ; the reason for this discrepancy is unknown.

As the sample is warmed to above -90°C compound A is converted to compound B which appears following first order kinetics ($k=0.45 \text{ sec}^{-1}$ at -78°C) and an $E_a=12.5 \text{ kcal/mole}$. As the above figure shows this is thought to correspond to the oxidation of the invisible copper.

The figure also demonstrates that compound A can be formed from a mixed valence state oxidase and when electron transfer is allowed to proceed above -90°C then compound C is formed from A.

Another spectrally distinct form of cytochrome c oxidase at room temperature was first discovered by Okunuki et al(90), who observed that during aeration of the reduced oxidase a spectral intermediate, with an absorption maximum of 428 nm, appeared which was converted to the fully oxidized form (absorption maximum 418-423 nm) in the reaction catalyzed by cytochrome c. They termed this form 'oxygenated' cytochrome aa₃, and suggested that this distinct spectral form represented a functional intermediate in the reduction of oxygen. The functional significance of this intermediate, which has also been analyzed with spin label probes(25) is not clear.

Wilson and coworkers(91,92), based upon an analogy with CO binding, have suggested that oxygen forms a 'bridged' compound between the cytochrome a₃ iron and the 'invisible' copper. The bound peroxide, which is formed by a two-electron reduction of the oxygen, is then further reduced to water in either one two-electron step or two one-electron steps.

Steady-State and Transient Kinetics- Schindler(93) has introduced evidence that the relationship between respiration rate, v_R , and oxygen partial pressure, P_{O_2} , cannot be described by a Michaelis-Menten

type rate equation, but instead can be fitted to the empirical rate equation proposed by Bander and Kiese(94):

$$v_R = \frac{V_{\max} (P_{O_2})^{1.4}}{K + (P_{O_2})^{1.4}} \quad (I-12)$$

By measuring the k_{+1} as $10^7 \text{ M}^{-1} \text{ s}^{-1}$ and relating this to values for the apparent K_m for oxygen and turnover numbers(TN) of the oxidase, he obtained the following relationship:

$$K_{m_{\text{app}}} = \frac{n \text{TN}}{4k_{+1}} \quad (I-13)$$

where $n=0.65$. This equation implies that the $K_{m_{\text{app}}}$ is linearly related to the reaction rate at saturating oxygen concentrations.

Degn and Wohlrab(95) have measured the steady-state respiration rate of rat liver mitochondria while simultaneously monitoring the redox state of the cytochromes. At the $K_{m_{\text{app}}}$ for oxygen, a strong dependency existed on the mitochondrial energy state and variations in the steady-state cytochrome oxidation levels as a function of the rate of oxygen consumption between coupled and uncoupled respiration. This observation appears to be in conflict with the last equation since K_m decreases with mitochondrial de-energization or uncoupling.

Transient kinetics of the reaction between cytochrome c oxidase and oxygen can be accomplished by the stopped-flow or flash-flow method where CO-reduced oxidase is mixed with oxygen and the CO compound is photolyzed(96,97). The apparent second order rate constant using isolated enzyme was $8.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (96). Using the method of Degn and Wohlrab(95), Petersen(1) has also investigated the reaction of oxygen

and reduced cytochrome a_3 using oxygen steady-state kinetics and has calculated a second-order rate constant in agreement with that obtained by transient kinetics, approximately $10^8 \text{ M}^{-1}\text{s}^{-1}$.

Motion in Enzyme Complexes- The idea that the myriad of static chemical structures, which conceptualize such entities as sugars, fatty and amino acids to us, combine to form macromolecular assemblies, such as membranes, is fundamental to the understanding of life processes. However, organized motion of these chemicals at the expense of negative entropy is what appears to constitute life. All molecules and their constituents undergo random thermal motion at temperatures exceeding absolute zero. By motion we mean from atomic transitions and oscillations to macromolecular rotational and translational movement. This observation introduces a dilemma elucidated by Erwin Schrodinger in his book, "What is Life", which is: how do life processes which appear to critically depend on events on the atomic and molecular level function in an orderly fashion under the continuous bombardment of random thermal energy? The answer appears to be that cells expend energy to minimize entropy although the manner in which this is accomplished is only vaguely understood. It would appear that the process of electron movement in the electron transport chain, which we have described as an organized series of reduction-oxidation catalysts, is not able to tolerate substantial randomness in the organization of the mitochondrial inner membrane. Each of the complexes must precisely associate with their redox partner in order for electron transport to be an efficient process. A central question in modern biophysics and biochemistry is not only how random motion is minimized between redox catalysts, but how within a complicated membrane enzyme complex

can efficient electron shuttling occur at the expense of negative entropy? Our concern is to define the types of macromolecular motion displayed by cytochrome c oxidase, and to relate these motile properties to structural and functional aspects of this enzyme.

Prior to analyzing data on the role of motion within cytochrome c oxidase to the organization and functioning of that protein-lipid complex, we must first define the types of motion with which we are concerned and the physical basis for analyzing that motion. The components which comprise the inner mitochondrial membrane with which we are concerned are the lipid, heme, copper, and protein moieties integral to the enzyme.

Motion of Membrane Components- Biological membranes are held together by non-covalent interactions between lipid and protein molecules. This non-covalent bonding allows motional freedom of these membrane components.

Lipid Motion- In membrane bilayers lipids undergo transverse, rotational, and lateral, as well as segmental, motion. Jain and White(98) have described five types of motion in biological membranes: 1) gauche-trans transitions; 2) segmental motion; 3) transverse motion; 4) rotational motion; and 5) lateral diffusional motion.

A gauche-trans jump involves rotation about a C-C bond in the saturated acyl chain moiety of phospholipids. The frequency of this transition is $\sim 10^9 - 10^{10} \text{ s}^{-1}$ (98) while C-C bond oscillation frequency is $3 - 6 \times 10^{12} \text{ s}^{-1}$, and the transition energy required to jump is $\sim 3.6 \text{ kcal/mole}$ (99). Segmental motion refers to the flexing of the fatty acid chains and their oscillatory motion about an axis normal to the membrane plane. McFarland and McConnell(100) have proposed a general membrane

model which accounts for the 'flexibility gradient' or the observed increase in the hydrocarbon chain motion as one approaches the terminal methyl groups of the fatty acid chain. Their model suggests that a cooperative tilt of the acyl chains occurs near the polar head groups. The head groups are tilted relative to the normal to the membrane surface whereas the region of the chain near the terminal methyl groups is perpendicular to the surface. This process results in reduced motional freedom in the head group area. Furthermore, when the fatty acid chains are tilted at a 30° angle, the carbon atom density near the head groups is ~13% higher than in the region near the terminal methyls. However, $\text{trans} \rightarrow \text{gauche}^+ \rightarrow \text{gauche}^-$ isomerizations are less probable in this region than near the terminal methyl groups. This process increases the carbon atom density gradient in the direction of the normal to the bilayer, and thus the carbon atom density throughout the hydrocarbon region is nearly constant across the bilayer(101).

The time constant for these types of motion are less than 10^{-9} s, and since more disorder exists in the center of the bilayer this introduces packing problems. This problem can be overcome by bending the chains as explained above, or by decreasing the packing density in the glycerol backbone region where the extra space is filled by water molecules(102).

Transverse motion or the flip-flop of lipid molecules from one half of the bilayer occurs quite slowly in artificial systems, or a half-time of 4-11 days for phosphatidyl choline flip-flop in PC vesicles (103,104). McNamee and McConnell(105) measured the flip-flop rate of a head group phospholipid spin label in vesicles prepared from the

electroplax of Electrophorus electricus and found it to be much greater or $\sim 4.7 \text{ min}^{-1}$ at 15°C . They have speculated that denatured protein could possibly catalyze the higher flip-flop rates seen in biological membranes while Bretscher(106) has postulated an enzyme, 'flippase', which is able to catalyze phospholipid flip-flop in biological membrane systems.

The rotational motion of the phospholipids of sonicated liposomal membranes about an axis normal to the bilayer plane occurs in the 10^{-6} - 10^{-9} sec range above T_c (107,108). The idea of phospholipid rotational motion is intricately tied to lateral diffusional motion which is the movement of phospholipid molecules past each other in the membrane plane. This concept may be visualized as a compact ensemble of spinning 'tops', placed on a plane, where each 'top' or lipid molecule possesses random, or nearly random, diffusional properties. Table VIII shows that for both artificial and native lipid systems the lateral diffusion constant has been calculated to be on the order of $10^{-8} \text{ cm}^2 \text{ s}^{-1}$. Another aspect as to how quickly phospholipids will diffuse laterally in a bilayer involves the fact that different fatty acid chains display different transition temperature values for the fluid to solid, or rigid, state. Transition temperature is dependent on chain length, degree of unsaturation and branching(98). For instance, if the fatty acid chain length is varied for phosphatidyl choline the transition temperature can range from 0°C for L- α -dilauroyl to 41°C for L- α -dipalmitoyl. Defined transition temperatures or cooperative endothermic transitions exist in homogeneous lipid suspensions because of the organization of the polymethylene chains. Above the transition temperature (T_c) the chains exist in a liquid crystalline phase or a mixed gauche-

Table VIII
Lateral Diffusion of Phospholipids in Natural
and Artificial Membranes

<u>Membrane</u>	<u>Diffusing Species</u>	<u>Detection</u>	<u>Temp(°C)</u>	<u>D^a</u>
Lipid vesicle (109)	dipalmitoyl phos- phatidyl choline + cholesterol	ESR	20-50	0.1-10
Lipid vesicle (109)	egg phosphatidyl choline	NMR	20	0.5
Lipid vesicle (110)	dipalmitoyl phosphatidyl choline	ESR	25	1.8
Lipid vesicle (111)	dimyristoyl ethanolamine	ESR	59	2.8
Lipid vesicle (111)	dipalmitoyl phosphatidic acid	fluorescence	60	17.0
Sarcoplasmic reticulum(112)	heterogeneous phospholipid	ESR	40	10.0
Sarcoplasmic reticulum(109)	heterogeneous phospholipid	NMR	31	0.4
Electroplax(109)	heterogeneous phospholipid	NMR	33	>0.1
Sciatic nerve (109)	heterogeneous phospholipid	NMR	31	0.5
<u>Escherichia</u> <u>coli(113)</u>	heterogeneous phospholipid	NMR	31	1.8

Numbers in parentheses refer to Reference numbers.

^a values are expressed as $D \times 10^{-8} (\text{cm}^2 \text{s}^{-1})$.

trans conformation, which confers a degree of disorder to the region near the terminal methyl group. Below the T_c the lipid exists in the gel phase where the chains are in the all-trans orientation. This variability in chain length and head group structure in biological membranes has been hypothesized to result in the lateral phase separations in the membrane plane(114). The extent of these zones of fluid and solid lipid depends on the temperature and the molecular structure of the phospholipids involved.

This phenomenon has been studied in binary mixtures of purified phospholipids by McConnell's group(115) using phase diagrams to predict bilayer structure. For instance, in mixtures of dielaidoylphosphatidyl choline and dipalmitoylphosphatidyl choline one obtains a three region phase diagram where the phases are separated by the 'fluidus' or fluid and 'solidus' or solid temperature versus mole percent curves. At temperatures above the fluidus curve the mixture of phospholipids is homogeneously fluid with all of the molecules undergoing rapid lateral bilayer diffusion and below the solidus curve the mixture is also homogeneous but the rate of lateral motion is less. However, between these curves a coexistence of two domains occurs where the lipids are either relatively fluid or solid. These phase separation domains have been visualized in artificial mixed lipid systems by freeze-fracture electron microscopy(115), differential scanning calorimetry(116), reflectivity measurements(117), X-ray diffraction studies(118), and selected dark field electron microscopy(119). These studies have all indicated that the cooperative gel phase domains exist and are up to several micrometers in diameter containing several hundred molecules.

Returning then to our spinning top model we encounter several

interesting facets of our 'membrane' structure which arise out of consideration of rotational and lateral motion. Since the population is heterogeneous, and since the composition of the components affects their rotational and lateral mobility, then the membrane, instead of existing as a system of phospholipids(or tops) displaying random walk, will contain patches of lipid displaying low rotational and translational motion and others displaying high rotational and translational motion; these domains will be in constant flux. These events will also possibly depend upon and, in turn, affect the degree of segmental and transverse motion of the component phospholipids. It is beginning to be difficult to visualize such a dynamic system, and yet we are still far displaced from natural membranes where local ion concentrations, and peripheral and intrinsic membrane proteins, might also be expected to affect the types of motion which phospholipids in the bilayer undergo.

Of the methods used to detect the various types of lipid motion, the spectroscopic techniques of EPR, NMR, and fluorescence have been the most predominantly used. This is due, in part, to the availability of spin-labeled analogues of fatty acids and phospholipids which are assumed to mimic the behavior of the native lipids. Since these probe molecules only 'see' an approximation of the natural membrane, the question which arises is how much do these structure reporters perturb the environment in which they are located. There is evidence that certain types of spin-labeled molecules tend to fluidize the local membrane area at concentrations in excess of 1 mole%(120), and that the nitroxide in spin-labeled fatty acids has a tendency to orient

itself at the air-water interface. However, studies using these probes are useful for comparative purposes.

Protein Motion- The inherent complexity of membrane-bound enzyme complexes dictates that the types of motion which we have to consider for the protein moiety of cytochrome c oxidase will be manifold and difficult to analyze. Nevertheless it is possible to extrapolate what is known about protein structure and conformational changes although the accuracy of this approach is not assumed.

Protein structure is divided into four levels of organization based on the level of interaction required for the maintenance of that structure. We will be particularly concerned with the alterations in tertiary and quaternary structure of cytochrome c oxidase. Tertiary structure is defined by the manner in which the secondary structure is folded, due to amino acid interactions as hydrogen-bond formation, Van der Waals interactions, charge-transfer forces and salt-linkages. Interactions between these polypeptides, which assume various tertiary structures based on their amino acid sequence and hydrogen-bonding interactions between carbonyl oxygen and amide nitrogen atoms, yield aggregates of individual molecules.

The types of motion which we will consider may be divided into two groups or motion within a polypeptide chain and motion between protomers of an oligomeric protein. Unfortunately the description of the types of motion which membrane proteins undergo cannot be simply described as in the case of lipid since each protein has a different structure, and this will affect its molecular motion characteristics. However, we will be interested in motion within a protein which can

be detected by reporter molecules such as spin labels or fluorescent probes, and rotational and translational, or lateral, motion of an entire lipid-protein complex within the membrane. Since the dynamic nature of an enzyme is directly related to its function, I will attempt to describe the types of motion which we have found to be relevant to cytochrome c oxidase without detailing general examples taken from other protein systems, such as hemoglobin.

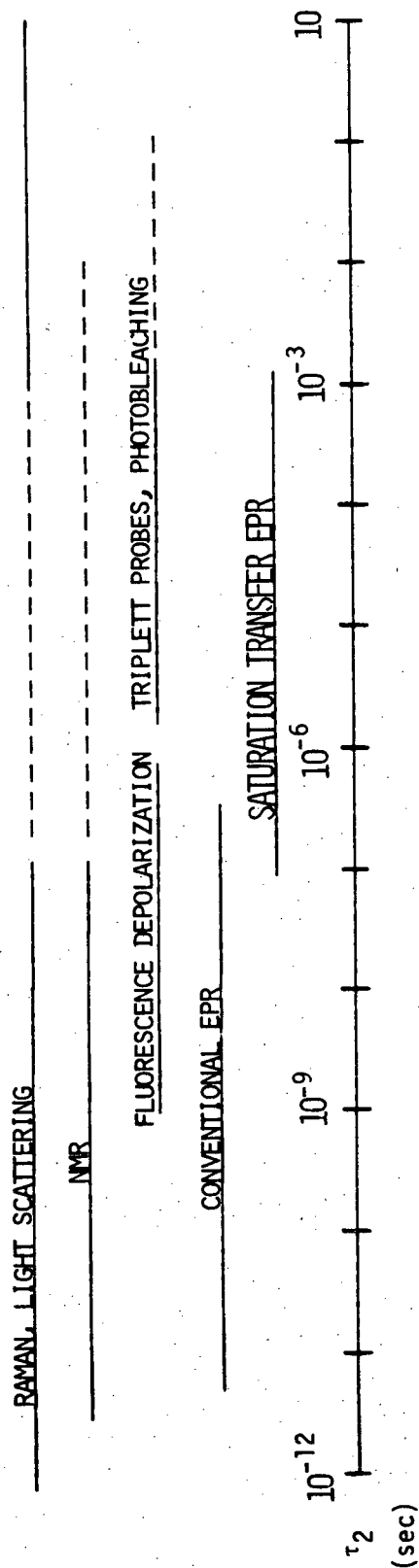
The detection methods used to study the types of motion which occur within membrane enzymes involve studying changes in the catalytic activity which are induced by such things as inhibitors(1), temperature (96), cofactors(63), ions(69), lipids(121), and chemical modification reagents(122). Both subtle and large conformational changes in the secondary, tertiary, and quaternary structures of proteins can also be detected by the use of EPR(25), NMR(123), and fluorescent(124) probes attached specifically or non-specifically to amino acids.

Fig. 4 shows the methods which have been used to study the rotational motion of soluble and membrane-bound proteins. It is important to note that the time scale which is applicable to some techniques is irrelevant to others. Electron microscopy has been used in conjunction with fluorescent(126), and ferritin-labeled(127,128) antibodies specifically elicited to certain membrane proteins in order to study the translational diffusion of membrane proteins.

Types of Motion in the Cytochrome c Oxidase Complex- The types of motion which we will be investigating in the role of motion in the membrane organization of cytochrome oxidase may be divided into four sections , or intracomplex, intercomplex, rotational, and lipid versus

Fig. 4. Methods used to estimate rotational correlation times of proteins, after Thomas(125).

MEASUREMENT OF ROTATIONAL CORRELATION TIMES (τ_2)



protein motion. The rationale for this division of motion parameters important for oxidase activity involves a pragmatic aspect of studying protein-lipid macromolecules. Oxidase intra- and intercomplex motion involves a myriad of chemical events including peptide bond rotations, minor and major oscillations of secondary, tertiary, and quaternary protein structure, and a variety of lipid motion described in the previous section. Of course, it is not possible to study all of these motion characteristics in detail, and therefore we are left with observing the effects of restricting motion by chemical modification. The idea of investigating the rotational motion of the entire complex IV is less complicated, and methods have recently been developed for this type of determination. The motion of protein in relation to lipid within the enzyme is also an aspect which can be empirically investigated utilizing existing spectroscopic technology. However, cytochrome oxidase is a dynamic enzyme system, and it should be recalled that the types of motion we will be discussing concern defined protein and lipid movement which is indirectly observed; we must always be concerned with the 'uncertainty' principle when examining molecular motion.

Prior to describing the objectives of this dissertation, it will be useful to review the literature which is concerned with protein and lipid motion of complex IV in the order described above.

Intracomplex Motion- One of the basic postulates of the conformational coupling hypothesis of oxidative phosphorylation(129) is that each of the energy transducing complexes of the respiratory chain is oscillating between different conformational states during electron transport. Since the chemiosmotic and chemical coupling hypotheses do

not depend on the idea of various energetic protein conformations as much, many researchers have attempted to demonstrate that dramatic intracomplex motion occurs during the redox cycle of cytochrome oxidase, since it is not only believed to be a control point of electron transport, but it is also an ATP coupling site.

A considerable amount of experimental evidence indicates that the conformation of the oxidized oxidase varies from that of the reduced form. Nicholls and coworkers(130) have suggested that since iron atom spin state changes have been linked to protein conformational changes in hemoglobin(131-133), and are part of haem-haem interactions responsible for sigmoidal oxygen binding curves, then cytochrome oxidase, which also displays haem-haem spin state changes(134,135) which can be related to oxygen binding, might also undergo conformational changes. The rationale of this approach has been that since heme ligands as sulfide and cyanide form such tight complexes with heme a_3 (Table VII), and since heme a and a_3 interact so strongly(136), then these events suggest that the oxidase has a tendency to assume specific conformations under a variety of environmental stimuli. It is important to define the term 'conformational change' in this context, where a conformational state is defined as a spectroscopic state. Therefore, any changes observable spectroscopically either due to changes in the environment of a chromophore or paramagnetic absorption of an intrinsic or extrinsic protein moiety is the result of a 'conformational change'. For instance, cyanide and sulfide complexes are always in the red-shifted or low-spin conformation, while the formate complex is blue-shifted and high spin(130). Oxygen also induces a catalytic cycle of reduced to oxygenated to oxidized spectroscopic

forms, which we discussed previously. Wikström(137,138) has also studied conformational changes induced in the oxidase during energization of the mitochondrial inner membrane, and has proposed a model that implies that the induction of a membrane potential generates an electrical field which causes a conformational change within the oxidase complex. This protein change is suggested to be more than just a minor molecular rearrangement in that it alters the pK_a of certain amino acids exposed to both the matrix and the inner membrane space, and is responsible for an hypothesized proton translocation function for the oxidase. Proton translocation by cytochrome oxidase is a matter of considerable debate(139-141).

Another way in which intracomplex motion has been studied is through the use of spin label probes attached to oxidase protein. Dasgupta and Wharton(25) have studied the motion and environment of an N-ethylmaleimido spin label in the oxidized, oxygenated, and reduced conformations. They discovered that the spin label was most immobilized in ferrocycytochrome c oxidase and least in the ferricytochrome form. Evidence that the oxygenated form is a conformational variant has also been provided by Myer(142), who analyzed the circular dichroic spectra of the oxidized and oxygenated enzyme.

In recent years, several investigators have attempted to elucidate the role polypeptide mobility plays in the functioning of several electron transport enzymes by employing chemical probes which form covalent bonds with amino acids(122). Imidoesters, a class of reagents which react with primary amines to form cationic amidines, have been utilized by this laboratory to demonstrate inhibitions of ATPase, NADH oxidase, succinate oxidase, and ascorbate-TMPD oxidase to varying

degrees in rat liver mitochondria(143,144). It was shown that crosslinking by the bifunctional reagent DMS(11 Å between reactive groups) is much more inhibitory than simple amidination induced by a monofunctional reagent, EA. The conclusion from these studies was that bifunctional, and not monofunctional, alkylating reagents inhibit enzyme activity by limiting molecular motion, but the type of mobility limitation has not been delineated(145). Recently, Bonaventura et al(146), described some functional and structural alterations in cytochrome oxidase induced by DMS and concluded that DMS treatment of isolated oxidase did not alter the reduction of heme a by unmodified heme c, but affected the electron transport rates between hemes a and a₃. These results indicate that during the redox cycle molecular motion on the scale of 10 Å or greater occur. This indicates that major conformational variants arise during electron transport through complex IV.

Intercomplex Motion- The close association of cytochrome c oxidase with cytochrome c reductase is implied in all models of electron transport, even if cytochrome c acts as an electron shuttle. The fact that electron transport down the chain from complexes I→IV is such an efficient process implies that random motion of the complexes is minimized. By intercomplex motion we mean the motion experienced by cytochrome oxidase in relation to cytochrome c and the other complexes.

The cytochrome content data(147,148) of beef heart mitochondria indicates that the inner membrane contains the ATPase complex, complex III, cytochrome c, and complex IV in the approximate ratios 1:1:2:2. Ernster(149) has hypothesized, based upon this and other data, that the cytochromes and ATPase combine to form redox assemblies, however no evidence exists for this idea.

The role of motion between cytochrome c and cytochrome c oxidase however has been extensively studied using a variety of approaches. Margoliash and coworkers, using chemically modified cytochromes c(70) and site-specific anti-cytochrome c antibodies(150), have demonstrated that the binding site on the cytochrome c molecule for the reductase and oxidase complexes are essentially identical(151). This suggests that cytochrome c mobility is required for electron shuttling between these two complexes. It has also been reported that alterations in the affinity of cytochrome c to the oxidase inhibit the rate of electron transfer within the oxidase complex even after the modified c-oxidase complex is formed(152). Chance and coworkers(153), have also suggested a 'branching' mechanism role for cytochrome c mobility based upon studies which indicate that cytochrome c not only mediates electron flow between reductase and oxidase molecules that belong to a single 'assembly', but also it can mediate between those which belong to different 'assemblies'. Chance has defined lateral diffusion of cytochrome c on the membrane plane as motion: 1) across the bilayer surface between complexes; 2) across the surface of one complex to another; and 3) microscopic vibrations and translations which bring the hemes of the donor and acceptor into close proximity(154).

In agreement with the results obtained by Margoliash's laboratory, as described previously, Azzi and coworkers(155) have reported that covalent binding of cytochrome c to oxidase, by the photoaffinity label FNPA, causes a severe inhibition of ascorbate-TMPD oxidase activity in the case where linkage was made between oxidase and lysine-13 of cytochrome c; linkage through lysine-22 caused a smaller(10%) inhi-

bition. However, it should be noted that in the absence of added cytochrome c, the complexes of cytochrome c-aa₃ were inactive.

In contrast to these crosslinking studies, Erecinska et al(156) have reported that horse heart cytochrome c can be covalently bound to c-depleted rat liver mitochondria via a 2,4-dinitro-5-fluorophenylazide (DNPA) linkage. When the mitochondria are disrupted by detergent a 1:1 complex of c:aa₃ can be purified which retains activity. The addition of native cytochrome c to this complex of c-aa₃ does not result in an increase in activity.

Rotational Motion- Rotational motion of the cytochrome c oxidase complex within the mitochondrial inner membrane may be expected to be affected by a variety of interactions between oxidase molecules and other redox components. Hbchli and Hackenbrock(127,128), have demonstrated using mitoplasts, which had been converted from the complicated native configuration to simple spheres by treatment in hypotonic medium, and freeze-fracture electron microscopy that, if the inner membranes are equilibrated at -10°C in 30% glycerol(w/v) and then rapidly frozen, the fracture faces of both membrane halves reveal lateral separation between clusters of intramembrane particles, which are assumed to be protein. However, when the membranes at 25°C are rapidly frozen no lateral phase separation of protein and lipid occurs (127). These investigators have also employed ferritin-labeled immunoglobulins mono-specific for cytochrome c oxidase to demonstrate that clustering of oxidase, through the crosslinking action of the immunoglobulins, occurs at both 25°C and -10°C, but that warming of the -10°C sample back to 25°C does not result in separation of the clusters treated with the monospecific immunoglobulin(128). These

observations suggest that, at least in these perturbed inner membranes, cytochrome oxidase diffuses freely at physiological temperatures, and therefore must possess a high degree of rotational freedom.

The rotational mobility of the heme a_3 component of cytochrome oxidase in situ in native rat liver mitochondria has been studied by Junge and coworkers(157) by observation of the time-dependence of linear dichroism following flash photolysis of a CO- aa_3 complex. They have interpreted their data to suggest that the rotational correlation time of the heme a_3 is at least 100 ms(158). In agreement with the idea that cytochrome oxidase is rotationally immobilized, several laboratories have demonstrated that it is possible to obtain a 'membranous' preparation of oxidase which is active(150,160), and in which the enzyme appears to be packed in an ordered two-dimensional array(161). Henderson et al(162) have obtained electron microscopic evidence that this crude preparation of oxidase is vesiculated and that the vesicles are collapsed. They suggest that oxidase molecules on apposed membrane layers interlock, but that adjacent enzymes do not touch being separated by lipid bilayer areas. Blasie et al(163) have studied the other 'membranous' oxidase preparations by X-ray diffraction, optical polarization, and EPR spectroscopy, and have demonstrated that the oxidase molecules in these bilayers are asymmetrically distributed, span the membrane, and that their distribution is non-crystalline. Whether or not oxidase exists as a crystalline, and therefore aggregated, structure in the inner mitochondrial membrane has not been investigated. The rotational mobility of the enzyme certainly might depend on its aggregation state, but this possibility has not been approached experimentally.

Lipid Versus Protein Motion- As opposed to the rotational mobility of the entire cytochrome c oxidase complex, many investigators have been concerned with the relative motion of lipid intrinsic and extrinsic to the enzyme. It has been known for quite some time that not all of the phospholipids within the oxidase complex can be removed without protein denaturation(36). This implies that phospholipids are trapped within the tertiary or quaternary structure of the protein. Crane and coworkers(37,38) have demonstrated that highly lipid-depleted oxidase preparations are reactivated by phospholipid or non-ionic detergents such as Tween, Emasol, or Triton, and have implied that phospholipids not only enhance the interaction between cytochrome c and cytochrome oxidase but that they also aid in maintaining molecular arrangements favorable to efficient intracomplex electron transfer. The requirement of a fluid intramolecular lipid environment has also been demonstrated using phospholipid-detergent exchange chromatography (22). These workers found no phospholipid head group specificity for reconstitution of enzyme activity after lipid depletion, but they did discover that the longer, and therefore the more fluid, the fatty acid chain the higher the activity(22). These studies suggest that phospholipid fluidity within the oxidase complex is important for activity and suggests a highly randomized configuration for the acyl chain.

In contrast, Jost et al(164-166) have produced evidence, obtained with spin-labeled fatty acids(165) and phospholipids(166), that a tightly bound lipid 'annulus' exists around each cytochrome oxidase molecule in 'membranous' and lipid-deficient enzyme preparations. Using cytochrome oxidase-dimyristoyl phosphatidyl choline complexes, where a small proportion(2 mole% relative to the unlabeled phospholipid) of the

total phospholipid population was spin-labeled, Marsh et al(167) have demonstrated several distinct components of the EPR spectrum corresponding to lipid-boundary layer immobilization and bilayer perturbation by protein packing. The latter component is only seen in bilayer containing high concentrations of proteins.

As opposed to the results obtained with EPR, analysis of cytochrome oxidase-deuterated phosphatidyl choline complexes by deuterium Fourier-transform NMR indicates that the fatty acid chains are highly disordered. No evidence was found in these studies for immobilized or boundary lipid. The discrepancy in the EPR and NMR studies may result from different phospholipid-enzyme preparative techniques, and the time scale resolvable by the two resonance methods(168,169).

Objectives and Significance of this Study

Hopefully, it has become apparent that the role of intracomplex, intercomplex, rotational, and lipid versus protein motion in efficient electron transfer from cytochrome c to oxygen is poorly understood at present. This field of study is beset by poorly defined systems and controversy as to the artificiality of studies in vitro with the isolated cytochrome c oxidase complex. The ultimate objective of this study is to provide a clearer understanding of how motion, within the framework mentioned above, affects oxidase activity in defined systems. More specific objectives are:

- 1) to understand the requirement of motion within the inner mitochondrial membrane by inducing structural mobility restraints through the use of bifunctional imidates.
- 2) clarification of how both mono- and bifunctional alkylation of primary amines affects cytochrome c oxidase activity of the purified and reconstituted enzyme, and when it is in situ in the inner membrane.
- 3) to understand how cytochrome c-cytochrome oxidase binding interactions are affected by variations in the intra-molecular and rotational mobility of the enzyme by using a water-soluble spin probe which has been developed in the course of this research, an imidoester spin label.
- 4) to determine if cytochrome c covalently bound to its high affinity binding site on the cytochrome oxidase complex is capable of accepting electrons from ascorbate or ascorbate-TMPD.

- 5) to determine if purified cytochrome oxidase is rotationally mobile or immobile when it is reconstituted into the bilayer of a lipid vesicle.
- 6) to determine whether rotational mobility of complex IV is required or not required for activity.
- 7) to understand how boundary lipid fluidity within the enzymatic complex contributes to the function of cytochrome oxidase.
- 8) to determine whether the rotational mobility of cytochrome oxidase is affected by the type of lipid used to reconstitute the enzyme, and whether this effect alters activity.
- 9) to summarize the types of motion which are detectable by the techniques previously mentioned, and to assess ideas as to the membrane organization of cytochrome oxidase which can be accommodated by the motile properties of the complex.

EXPERIMENTAL METHODS

MATERIALS:

Lipids and Detergents- Phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, and diphosphatidyl glycerol were purchased from Avanti Biochemicals, Inc., and were greater than 99% pure. Asolec-tin containing 95% soy phosphatides was obtained from Associated Concentrates, Woodside, Long Island, N.Y.. Cholic and deoxycholic acids were from Aldrich and were recrystallized twice from ethanol. Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 80 (polyoxyethylene sorbi-tan monooleate), Triton X-100 and X-114 were purchased from Sigma Chemical Co.

Imidoesters and Heterobifunctional Reagents- Dimethylsuberimide dihydrochloride(DMS), dimethyladipimide dihydrochloride(DMA), dithio-bispropionimide dihydrochloride(DTBP), methyl acetimide(MBI), methyl mercaptobutyrimide(MMBI), p-azidophenylacetyl bromide and 4-fluoro-3-nitrophenyl azide(FNPA) were obtained from Pierce Chemicals.

Micellaneous- All of the gel filtration resins employed in this study as well as Ficoll 400 and Percoll were obtained from Pharmacia and electrophoresis purity reagents were from Bio-Rad. Cytochrome c (Type VI) was from Sigma Chemicals.

Spin Labels- The commercially obtained spin labels used in this study were from Syva, and included 4-maleimido-2,2,6,6-tetramethyl-piperidinoxyl(MSL), and 2-(14-carboxyltetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy methyl ester(FASL(1,14)). Dr. P. Devaux kindly provided 2-(14-carboxyltetradecyl-N-ethyl maleic ester)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy(MSL(1,14)). These various spin labels are

diagrammed in Fig. 5.

Imidoester Spin Label- The imidoester spin label was synthesized according to the following protocol.

3-Cyano-2,2,5,5-Tetramethylpyrroline-1-Oxyl:

The method of Krinitskaya(170) was followed with slight modification. To a 250 ml flask equipped with a magnetic stirring bar, 7.0 gm (0.084 M) 2,2,5,5-tetramethyl-2-pyrrolinyl-1-oxyl-3-carboxamide(Eastman Kodak) and 125 ml anhydrous pyridine were added. The orange solution contained a tan insoluble impurity which was removed by filtration. The clear solution was cooled under nitrogen to -30°C and tosyl chloride(27.2 gm., 0.143 M) was added in increments over a 5 min. period whereupon the temperature rose to -15°C . The resulting clear solution was allowed to warm to room temperature and remained sealed under nitrogen for six days. The solution was adjusted to pH 10.0 by addition of 96 ml of 4 M KOH and heated for 1 hour at 80°C to destroy excess tosyl chloride. After cooling, the alkaline solution was extracted four times with 150 ml portions of ether. The extract was subjected to 2 x 350 ml of 10% HCl washes, 2 x 350 ml saturated Na_2CO_3 , 1 x 350 ml H_2O . After Na_2SO_4 drying, evaporation of the ether yielded 5.5 gm of a yellow-orange solid which was dissolved in 250 ml hexane at 22°C , filtered, evaporated to 55 ml, and chilled to -18°C to yield 3.4 gm light yellow crystals. M.P. $62.5-63.5^{\circ}\text{C}$ (Literature(170) 62.5-63), and infrared(KBr disk) revealed 2250 cm^{-1} characteristic of CN absorbance. The 1680 cm^{-1} band due to amide was absent.

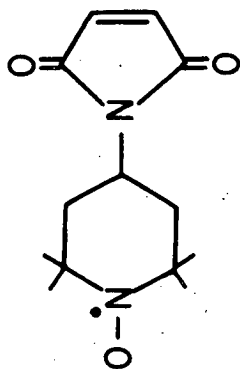
Methyl-3-imidate-2,2,5,5-Tetramethylpyrroline-1-Oxyl-Hydrochloride:

The method of base catalyzed imidoester formation as described by

Fig. 5. Spin labels used in this study for determination of protein rotational correlation times, and lipid versus protein motion.

Spin label I: MSL

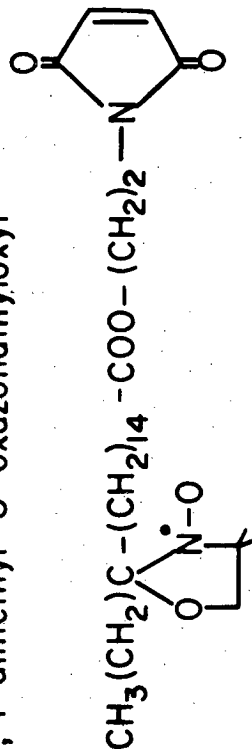
4 - Maleimido-2,2,6,6-tetramethylpiperidinoxy



Spin label II: MSL (1,14)

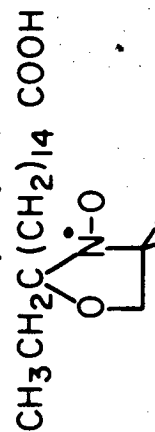
2 - [14 - carboxytetradecyl - N - ethyl maleic ester] - 2 - ethyl

4,4 - dimethyl - 3 - oxazolidinyloxy



Spin label III: FASL (1,14)

2 - (14 - carboxytetradecyl) - 2 - ethyl - 4,4 - dimethyl - 3 - oxazolidinyloxy

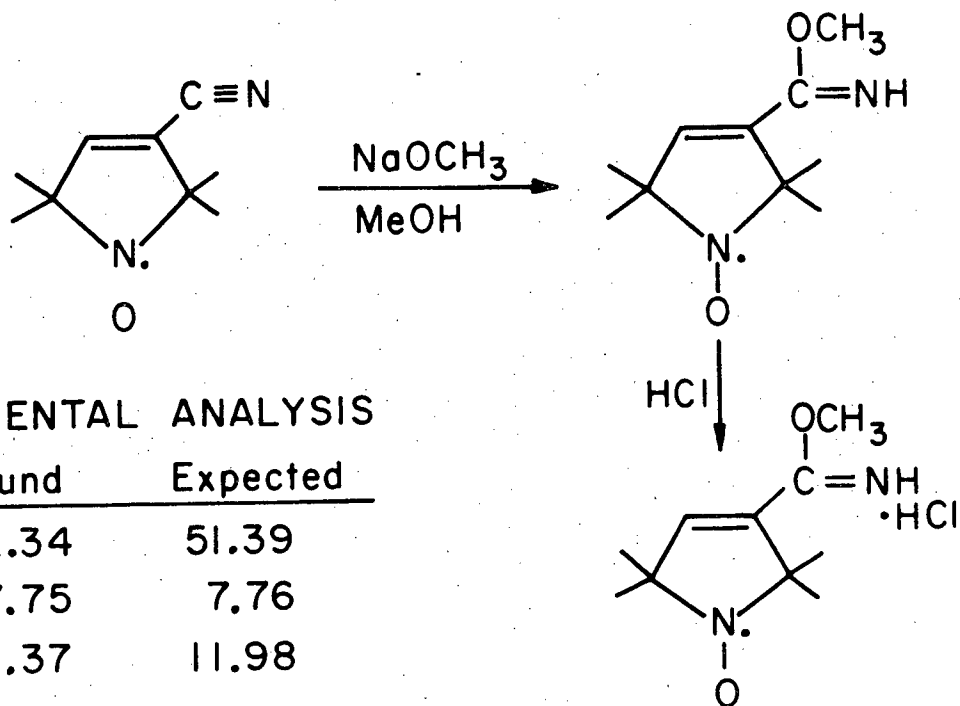


Schaefer was used(171). In a 50 ml flask, 3.0 gm of 3-cyano-2,2,5,5-tetramethylpyrroline-1-oxyl and 26 ml of 0.687 M NaOCH₃ in methanol were placed. The clear solution was allowed to stand at 22°C for 15 min. At the end of this period, sodium methylate was destroyed by gassing the solution with dry CO₂ to saturation. Following filtration of the sodium methylate-CO₂ adduct, the clear solution was evaporated to dryness under vacuum yielding a light orange oil. Gas chromatographic analysis of the reaction product revealed several impurities including a large amount of unconverted nitrile starting compound. A small sample of the reaction product(as an ether solution) when washed with 0.1 N HCl/H₂O and resubjected to gas chromatographic analysis showed a complete and selective removal of one of the reaction products which was assumed to be the desired free imidoester. On this basis, the remaining reaction product was dissolved in 25 ml anhydrous ether to which ether saturated with anhydrous HCl was added dropwise(causing an orange precipitate to form) until gas chromatographic analysis of the supernatant revealed only a trace of the desired compound to remain in solution. The precipitate was filtered under nitrogen, washed with fresh ether and dried under vacuo. Yield was 700 mg. Infrared(KBr) reveals strong absorbance at 2860 cm⁻¹ and 1625 cm⁻¹ characteristic of imidoester hydrochloride. The spectrum showed a complete absence of nitrile. This synthesis is shown diagrammatically in Fig. 6.

METHODS:

Biological Preparations- Beef heart mitochondria for the preparation of cytochrome c oxidase, kindly provided by Drs. T.P. Singer and D. Green & H. Tisdale, were prepared by the method of Blair(172). For the

Fig. 6. The synthesis of the imidoester spin label used to probe cytochrome c-cytochrome oxidase interactions.

PREPARATION OF IMIDOESTER SPIN LABEL

ELEMENTAL ANALYSIS

	Found	Expected
C	52.34	51.39
H	7.75	7.76
N	12.37	11.98
Cl	12.76	15.17

Precipitate From Ether
Overall yield = 5.7%

study of imidoester effects on whole beef heart mitochondria, small scale preparations were performed by the method of Smith(173). Rat liver mitochondria were isolated according to previously published methods(174). Mitoplasts were prepared by rupturing the outer membrane in hypotonic salt(15 mM KCl) and c-depleted mitoplasts were then prepared according to Jacobs and Sanadi(175).

For the isolation of phospholipids from both beef heart and rat liver mitochondria, a chloroform-methanol extraction was performed (176,177). Purified phospholipids were stored at -20°C for no longer than 24 hr prior to use. Asolectin was purified by acetone extraction (178) and stored at -20°C under nitrogen.

Cytochrome c oxidase was isolated by variety of techniques(Table IX). For studies on intramolecular modification, the enzyme was prepared from beef heart mitochondria as described by Capaldi and Hayashi(183). Test runs were made to determine the optimal concentrations of K^{+} cholate and ammonium sulfate(22°C saturated) to use at each fractionation step. For comparison studies, cytochrome oxidase purified from Keilin-Hartree particles by the method of Van Buuren(181) and enzymatically characterized by Nicholls et al(130) was employed.

For experiments on the rotational motion of lipid and protein, the oxidase complex was isolated by two techniques. In order to obtain a lipid-rich preparation(40% lipid, by weight), the mitochondria were first fractionated with deoxycholate and then with cholate according to the procedure of Errede et al(184), and this enzyme will be referred to as DOC-oxidase. The lipid-poor(2-6% lipid, by weight) preparation was isolated by fractionating the mitochondria first with Triton X-114

Table IX

Beef Heart Cytochrome c Oxidase

Isolation Procedure	Detergents	Salts	Lipid Content mg/mg protein	Heme a Concentration nmol/mg protein
Yonetani(179) ^a 1 preparation	cholate	(NH ₄) ₂ SO ₄	0.15 {0.10}	8.6 {8.1-8.5}
Kuboyama et al(180) ^a 1 preparation	cholate Tween 20	(NH ₄) ₂ SO ₄	0.26 {0.20}	9.5 {11.0}
Van Buuren(181) ^a 3 preparations	DOC cholate	KCl (NH ₄) ₂ SO ₄	0.26-0.31 {0.20-0.30}	8.6-11.7 {10.0-12.0}
Fowler et al(39) ^b 2 preparations	DOC	KCl (NH ₄) ₂ SO ₄	0.31-0.42 {0.30-0.40}	8.2-9.1 {8.4-8.7}
Wharton & Tzagoloff ^b (182) 1 preparation	DOC cholate	KCl (NH ₄) ₂ SO ₄	0.35 {0.30}	8.0 {7.7-8.5}
Capaldi & Hayashi ^b (183) 5 preparations	DOC cholate	KCl (NH ₄) ₂ SO ₄	0.15-0.32 {0.17-0.25}	8.0-10.1 {9.4-10.6}
Errede et al(184) ^b 3 preparations	DOC cholate	KCl (NH ₄) ₂ SO ₄	0.35-0.42 {0.30-0.40}	9.0-10.5 {10.5-10.9}
Hartzell & Beinert ^b (185) 3 preparations	Triton cholate	KCl (NH ₄) ₂ SO ₄	0.02-0.06 {0.02-0.05}	10.0-11.8 {13.0-14.0}

Numbers in brackets refer to literature values
Starting preparations were: ^aKeilin-Hartree particles or ^bbeef heart mitochondria

0 0 0 0 5 5 0 4 2 8 4

and then with cholate(185), and this preparation will be called Triton-oxidase. The Triton-oxidase preparation is generally insoluble at low enzyme concentrations especially if extensive fractionation by ammonium sulfate is required to obtain a highly purified oxidase (heme a > 10 nmoles/mg protein). Therefore refractionation of the enzyme in Tween 20 with ammonium sulfate results in a highly purified and soluble preparation(16), required for the EPR experiments. Further purification of all oxidase preparations was performed, in some cases, by gel filtration essentially according to Briggs et al(186) using 25 mg of purified oxidase in a 1.5 x 65 cm Sepharose 4B column equilibrated with 0.09M NaCl. 0.02 M Tris-Cl pH 8.0 and 0.5% Tween 80.

All of the enzyme preparations were stored by adding aliquots dropwise into a Nalgene beaker filled with liquid nitrogen in order to form small beads which were then stored in liquid nitrogen. This method is convenient because it allows the removal of small quantities of enzyme at a time, and the activity remains constant for at least six months.

Chemical Modifications- Stock imidate solutions were prepared by the method of Tinberg et al(143) using 0.133 M triethanolamine-0.33 M sucrose pH 9.0 for mitochondrial preparations. DMA, DTBP, and DMS were used as 5-40 mM incubation solutions, and MA, EA, and MBI as 10-80 mM. Amidination was accomplished by incubating mitochondria(6 mg/ml), isolated oxidase(1.23 mg/ml), cytochrome c(6.5 mg/ml), and vesicles (0.25 mg/ml) for 30 min at room temperature. Substitution of 0.133 M KH_2PO_4 -0.33 M sucrose, for the mitochondrial preparations, or 0.133 M KH_2PO_4 , for the isolated oxidase, as the incubation buffer resulted in identical imidate effects. Immediately after the incubation time had

expired, all of the preparations were placed on ice, the ascorbate-TMPD oxidase activities were determined polarographically, and 60 μ l 3.0 M sodium acetate pH 5.0 was added to hydrolyze excess reagent. Both five-fold dilution and centrifugation of the mitochondrial samples or immediate dialysis of purified and reconstituted oxidase yielded identical results. All samples were then dialyzed immediately against 1000 volumes of deionized water overnight at 3°C.

Amidinated cytochrome c was prepared as above except that the sodium acetate addition was omitted. Immediately after overnight dialysis against deionized water, the DMS-treated cytochrome c (1.5 ml) was subjected to gel filtration on a 1.0 x 50 cm column equilibrated in 0.05 M KH_2PO_4 pH 7.4, and elution was carried out with the same buffer. After chromatography pooled fractions corresponding to oligomers and monomers of cytochrome c were dialyzed against 1000-fold volumes of deionized H_2O since phosphate buffers inhibit the flow rates of Amicon UM filters. The samples were then concentrated in a Model 12 Amicon Cell equipped with a UM 2 membrane and concentrated at 3°C to at least 0.2 mM. In order to obtain complete separation of monomeric cytochrome c from the oligomeric population, DMS-treated cytochrome c was chromatographed on a 0.7 x 30 cm of Sephadex G-75 Superfine equilibrated in 50 mM Tris-acetate pH 7.8.

Spin Labels- Stock solutions of the imidoester spin label, 20 mM, were prepared as described(187) using 0.133 M KH_2PO_4 as the stock buffer. Reduced and oxidized cytochrome c (6.5 mg/ml) preparations were incubated for varying periods of time up to 24 hr at 22°C at an imidoester concentration of 10 mM. The degree of amidination resulting from imi-

doester treatment was determined by following the loss of free amino groups after Sephadex G-25 chromatography in 50 mM KH_2PO_4 pH 7.4. The spin content of each sample was calculated from the second integral of the spectra using a PDP 11/34 computer interfaced with the EPR spectrometer.

In order to study protein rotational mobility, cytochrome oxidase was spin-labeled with MSL using 2 moles of label per mole of Triton-oxidase, and 5 moles per mole of DOC-oxidase. Preparations were incubated 10 min at 22°C and then non-covalently attached label was eliminated by Sephadex G-25 gel filtration in 50 mM KH_2PO_4 pH 7.5, 0.25% Tween 20, 0.05% cholate. In the case of both the Triton-oxidase and DOC-oxidase, it was necessary to treat the spin-labeled oxidase with 1 mM cysteine and 10 mM potassium ferricyanide for 60 min in order to minimize the weakly immobilized signal. At the end of the incubation period, the sample was diluted ten-fold in the above buffer and precipitated with ammonium sulfate(0.35 saturation). This pellet was brought up in either 0.25 M sucrose for incorporation into liposomes or detergent-containing buffer(50 mM KH_2PO_4 pH 7.5, 0.25% Tween 20, 0.05% cholate) for the determination of the rotational mobility of the soluble oxidase. Labelling of cytochrome c oxidase with the fatty acid maleimide spin probe, MSL(1,14), was performed according to Favre et al(188) at a spin label to aa₃ molar ratio of 2:1. The amount of MSL(1,14) bound to lipid in the cytochrome oxidase complex was determined after alkaline chloroform-methanol extraction of the enzyme (36).

The fluidity of lipid associated with both soluble and reconstituted cytochrome oxidase was studied using a fatty acid spin label, FASL(1,14).

For studies on the intracomplex motion of cytochrome oxidase, 3.0 mg of the MSL probe were added to 15 mg of enzyme (in a total volume of 0.5 ml) and the mixture was incubated for 15 min at 22°C. This solution was then chromatographed to remove non-covalently bound spin on G-25 (Sephadex) in 50 mM KH_2PO_4 pH 7.5, 0.25% Tween 20, 0.05% cholate.

Cytochrome Oxidase Subunit Separation- The subunits of the cytochrome c oxidase complex were dissociated in detergent and then chromatographed in order to determine the binding sites of the various spin labels. Spin-labeled cytochrome oxidase (20 mg) was dissolved in 1.0 ml of buffer containing 5% SDS, 8 M urea, and 0.05 M NaH_2PO_4 pH 7.2 by heating at 37°C for 30 min (20). Chromatography was carried out at 20°C in a column (1.5 x 85 cm) of Sephacryl S-200 Superfine equilibrated with 5 mM SDS, 8 M urea, 0.05 M NaH_2PO_4 pH 7.2 at a flow rate of 3 cm/hr. Fractions were collected in three pools corresponding to elution peaks and lyophilized after desalting by dialysis against deionized H_2O . After lyophilization, the samples were brought up in 0.4 ml of the elution buffer and EPR conventional spectra (V_1) were recorded.

High-affinity Binding as Measured by Gel Filtration- The technique used to bind ferricytochrome c to cytochrome oxidase in a 1:1 molar ratio was essentially that of Ferguson-Miller et al (69), except that 300 μl samples were applied to a 1.5 x 50 cm column of Sephadex G-75 at 25°C and a 1.5 x 30 cm column at 4°C. Cytochrome c was added in a five-fold molar excess to cytochrome aa₃, which had been dialyzed against 25 mM Tris-cacodylate pH 7.8, 0.25% Tween 20, 0.05% cholate, and the resulting mixture was applied to the G-75 column. Prior to binding of the spin-labeled c to cytochrome oxidase, monomeric ferricytochrome c was obtained by gel filtration through G-75 Superfine in a

column(0.7 x 30 cm) equilibrated in 50 mM Tris-acetate pH 7.5(189).

Electrophoretic Analyses- SDS gel electrophoresis was carried out in a Bio-Rad Model 220 Dual Vertical Slab Cell using either 1.5 mm spacers and 20 well combs(mitochondria and isolated oxidase) or 3.0 mm spacers and 10 well combs(vesicles) at 50 mA per gel for 90-120 min. Gels were made according to the discontinuous Tris-glycine system of Laemmli(190) using 3% stacking and 12.5% separation gels(acrylamide/bisacrylamide, 30/0.8%) for the separation of mitochondrial samples. For isolated oxidase and vesicles 5% stacking and 13.5-16.0% gradient gels(acrylamide/bisacrylamide, 30/1.6%) were utilized in order to achieve acceptable resolution of small molecular weight polypeptides. Gradient gels were prepared at 3°C with pre-cooled solutions by means of a Buchler gradient former and peristaltic pump. Immediately prior to electrophoresis, samples were either not heated, heated at 37°C for 30-120 min or at 100°C for 1.5 min in a solubilization cocktail comprised of glycerol, SDS(1%), stacking buffer, mercaptoethanol, and a trace of bromophenol blue tracking dye. The samples were not frozen at any point in the procedure since this led to irreversible aggregation. Gels were fixed and stained with coomassie blue as described by Fairbanks et al(191) in a stirred Corning ACI electrophoresis chamber. Densitometric tracings of stained gels were made at 545 nm using a linear transport accessory on a Gilford Model 240 spectrophotometer. Gels were calibrated for molecular weight using phosphorylase a, bovine serum albumin, ovalbumin, chymotrypsin A, myoglobin, lysozyme, cytochrome c, and bovine trypsin inhibitor as standard proteins. Gels of all crosslinked samples were calibrated against appropriate controls to determine the amount of protein not entering the separation gel by weighing reproductions of

densitometer tracings. The calculation of the 'oligomer spread', which is due to heterogeneous monofunctional attack and hydrolysis of the bifunctional imidoesters was calculated assuming certain molecular weights for the reacted imidates (DMA=231; DMS=259). The calculation of the increase of polypeptide molecular weight due to monofunctional imide reaction was also performed in this manner.

SDS-urea gel electrophoresis was performed according to Downer et al (20) using a Bio-Rad Model 150A Tube Gel Electrophoresis Cell. For comparative purposes continuous buffer phosphate gels were also made and run as described by Weber and Osborn (192).

Enzyme Assays- Two types of assays were employed for the determination of cytochrome c oxidase activity. Polarographic assays, in 4.0 ml of buffer at 25°C, were carried out either according to Nicholls et al (130) in the presence of 1% asolectin in 67 mM KH_2PO_4 and 0.5% Tween 80 pH 7.4 using 12.5 mM potassium ascorbate, 0.5 mM TMPD, and 2.5 to 25 μM cytochrome c or according to Ferguson-Miller et al (70) for the determination of the dissociation constant for high affinity binding using 25 mM Tris-acetate pH 7.8 and 7 mM potassium ascorbate, 0.7 mM TMPD, 0.004 to 4.0 μM cytochrome c and 50 nM cytochrome aa₃ (final concentration). In the latter case, monomeric ferrocytochrome c was obtained by gel filtration through Sephadex G-75 Superfine as described (189).

Assays for the activity and orientation of oxidase incorporated into liposomes was performed at 20°C in 50 mM KH_2PO_4 pH 7.5 using 25 mM potassium ascorbate and 1 mg/ml cytochrome c (193). Uncoupling was achieved by a mixture of 0.5 $\mu\text{g/ml}$ valinomycin and 2.5 μM FCCP while

orientation was determined by comparing rates of O_2 consumption in the absence and presence of 3% Tween 80(w/v). The baseline rate in the absence of cytochrome c was determined in all cases and subtracted from the rates obtained at the various cytochrome c concentrations.

For sample comparison, cytochrome oxidase activity was also determined spectrophotometrically at 25°C in a buffer containing 0.05 M KH_2PO_4 pH 7.0 and 0.5% Tween 80 by following the oxidation of ferro-cytochrome c. Activities were expressed as MA_0 in moles of cytochrome c oxidized/mole heme a₃, and MA_{omax} and K_{mapp} were determined according to Vanneste et al(194).

Transient kinetics of ferrocytochrome c oxidation by cytochrome oxidase were also performed spectrophotometrically as described above using an Aminco-Morrow Stopped Flow attachment. Data were collected on a storage oscilloscope, and analyzed according to Gibson et al(62).

Micellaneous Assays- Heme a concentration was determined using an Aminco DW-2 spectrophotometer from the reduced minus oxidized difference spectrum of cytochrome oxidase in 0.1 M KH_2PO_4 and 0.05% deoxycholate pH 7.0 using a $\Delta\epsilon_{605-630}=13.2 \text{ mM}^{-1}\text{cm}^{-1}$ (194). Heme a₃ concentration was determined from reduced versus reduced plus CO difference spectrum using a $\Delta\epsilon_{428.5-445}=148 \text{ mM}^{-1}\text{cm}^{-1}$ (194). Cytochrome c concentration was also determined spectrophotometrically in a 0.05 M KH_2PO_4 pH 7.4 buffer using a $\Delta\epsilon_{540-550}=20 \text{ mM}^{-1}\text{cm}^{-1}$ (70).

Free amino groups were detected fluorometrically on a Perkin-Elmer MPF-44A fluorescence spectrophotometer using fluorescamine by a modification of Bohlen et al(195) using 1% SDS(144). Protein concentrations were determined by the method of Lowry et al(196) using defatted bovine serum albumin as a standard; a trichloroacetic acid precipitation

modification of the procedure was used when solvent or salt interferences were a problem. For rapid analysis of mitochondrial and crude oxidase protein concentrations, the rapid biuret procedure of Van Buuren(181) was employed.

Phospholipid content analyses were performed by a total phosphorous determination of an alkaline chloroform:methanol(2:1) extract and concentration was expressed as mg/mg protein. Sulfhydryl content was determined using 5,5'-dithiobis-(2-nitrobenzoic acid), or DTNB, using the method of Ellman(197) in 0.1% SDS. Midpoint potential measurements of cytochrome c were performed according to previously published procedures(198,199) at 22°C.

Osmotic sensitivity measurements were performed essentially according to Tinberg et al(144) by incubating mitochondria(0.5-1.0 mg protein/ml) in either 0.25 M sucrose or deionized H₂O. The samples were allowed to equilibrate for 15 min at 25°C in a controlled temperature water bath, and the absorbance was recorded at 546 nm. The empirical index of osmotic sensitivity was taken as the ratio($Ab_{546}(H_2O)/Ab_{546}(sucrose)$).

Liposome Preparation- Liposomes composed of partially pure asolectin, mitochondrial, and purified phospholipids were prepared by cholate dialysis(200) or by a modification of the sonication procedure of Racker(201). For the sonication procedure, phospholipids(25 mg) were dried under a stream of nitrogen, redissolved in a small volume of ether and dried again under nitrogen for 5-10 min. To this dried preparation 1 ml of 50 mM KH₂PO₄ was added and sonication of this suspension was performed in a bath-type sonifier under argon for 15 min at 22°C. Immediately after sonication, 1.25 mg of cytochrome oxidase was added and the mixture incubated at 22°C for 30 min. At the end of this incubation

period, the mixture was sonicated for 5 min under argon. Concentration of both types of vesicle suspension was achieved by centrifugation at 45,000 RPM in a 50 Ti rotor for 90 min. The resulting pellet was resuspended to a final aa_3 concentration of 30-50 μ M. In order to obtain an immobilized vesicle suspension all preparations were centrifuged in an EPR capillary which was inserted into a specially designed Lucite tube. This preparation was centrifuged in a Type 42.1 rotor at 25,000 RPM for 24 hr.

Liposomes were also reconstituted with pure neutral phospholipid according to the procedure of Karlsson et al(202) in a 50 mM borate buffer pH 7.7 containing 150 mM NaCl. Phosphatidyl choline from egg yolk was suspended in the above borate buffer to a concentration of 25 mg/ml and sonicated under a water-saturated argon stream for 30 min using a conical centrifuge tube positioned in an ice bath. After centrifugation of this suspension at 14,000 g for 60 min 1 ml of the supernatant was removed and mixed with 0.2 ml of aa_3 to obtain a final concentration of 30 μ M aa_3 . In the case of the Triton-oxidase, the enzyme was incubated with the liposomes for 60 min at 0°C prior to sonication since immediate sonication led to inactivation of the oxidase. The mixture was then sonicated 1 min on/1 min off/1 min on at 0°C.

For the studies on intracomplex motion of the enzyme, vesicles were also obtained by sonication of a mixture of 10 mg purified oxidase, 400 mg crude soybean asolectin, and 16.0 ml of 67 mM KH_2PO_4 pH 7.4 using a Branson Model 350 tip sonifier. Sonications were performed at 0°C (70 W, half-inch tapped tip) for a total of 60 min using a 2 min on/2 min off cycle to prevent temperature increases under an argon atmosphere, which was water saturated. Immediately after sonication

multilamellar liposomes and titanium particles were removed by centrifugation at $180,000 \times g$ using a 50 Ti rotor. The light green supernatant was removed carefully with a Pasteur pipette leaving the residue.

Ficoll Gradient Analysis- Discontinuous Ficoll gradients were prepared according to Carroll and Racker(193) in 50 mM KH_2PO_4 pH 7.5 using steps of 5, 6, 8, 10, 15, and 30% Ficoll. After temperature equilibration of the gradients at 4°C for 4 hr, 1 ml aliquots of the cytochrome oxidase liposomal suspension were carefully applied to the top and centrifugation was performed in an SW 41L swinging bucket rotor at $150,000 \times g$ for 17 hr at 4°C . Each step of the gradient was analyzed for phospholipid content, enzyme activity, protein concentration, and respiratory control.

EPR Experiments: Practical Aspects(see Appendix II for Theoretical Aspects)- A Varian E109E X-band spectrometer was employed in the absorption mode for the first harmonic in phase(V_1) and the second harmonic out of phase(V_2'). A 0.5 mm inside diameter glass capillary cell was used together with a quartz dewar. The temperature was controlled with a Varian E4540 Variable Temperature Controller. The microwave field strength received by the sample was determined by calibration with a solution of Fremy's salt(203). The microwave setting was 10 mW corresponding to a field strength of 0.13 gauss. Conventional EPR spectra(V_1) were recorded with a 100 kHz field modulation(1 gauss peak to peak amplitude). Saturation transfer spectra(V_2') were recorded with a field modulation of 50 kHz(6.3 gauss amplitude) and phase sensitive detection at 100 kHz(second harmonic), out of phase. The phase was set by minimizing the signal at low microwave(0.5 mW) power. The microwave power setting was then increased to 40 mW corresponding

to a field strength of 0.25 gauss and the V_2' spectrum was recorded.

RESULTS AND DISCUSSION

Section I: INTRACOMPLEX VERSUS INTERCOMPLEX MOTION

To distinguish how the requirement for motion within the cytochrome oxidase complex affected activity, the purified and reconstituted oxidase was treated with mono- and biimidates (Fig. 7). Results on the inhibition of the purified enzyme were then compared to those obtained when whole mitochondria or mitoplasts were treated. The role of cytochrome c as a mobile electron carrier between cytochrome c reductase and oxidase was studied by following the effect of mono- and biimidates on the steady-state kinetics of the cytochrome oxidase reaction using cytochrome c supplemented and depleted mitochondria and purified enzyme.

RESULTS

Purified Cytochrome Oxidase: Crosslinking- To detect crosslinking during biimide treatment of purified cytochrome oxidase, samples were subjected to SDS gel electrophoresis (Fig. 8). Interpolypeptide crosslinking results in the disappearance of bands and the appearance of new bands of lower relative mobilities. Polypeptide aggregates exceeding 110,000 daltons are excluded from the separation gel. To eliminate the possibility that aggregation created the altered electrophoretic patterns seen in the imide-treated samples several solubilization methods were used (Methods), but the gel patterns showed that extensive crosslinking only occurred with bifunctional imidates. Polypeptide I appears resistant to crosslinking, whereas disappearance of all of the other subunits coincided with the formation of a new band of MW~87,000. This might correspond to a crosslinked aggregate

Fig. 7. An illustration of possible mono- and biimidate reactions in the mitochondrial inner membrane.

MONOFUNCTIONAL AMIDINATION AND CROSSLINKING BY IMIDOESTERS

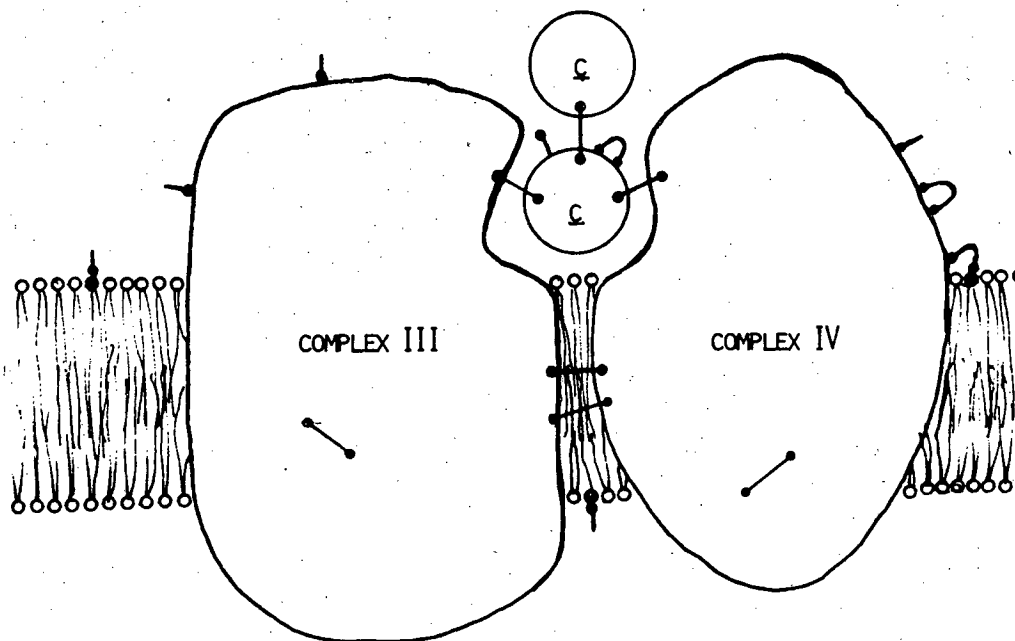
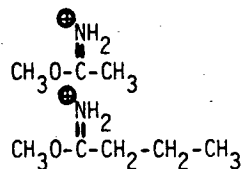
Biimidates: (● — ●)

Chemical Name	Formula	Maximum Link (Å)
Dimethyl adipimide (DMA)	$\text{CH}_3\text{O}-\overset{\text{NH}_2}{\underset{\parallel}{\text{C}}}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{NH}_2}{\underset{\parallel}{\text{C}}}-\text{OCH}_3$	9
Dimethyl suberimide (DMS)	$\text{CH}_3\text{O}-\overset{\text{NH}_2}{\underset{\parallel}{\text{C}}}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{NH}_2}{\underset{\parallel}{\text{C}}}-\text{OCH}_3$	11

Monoimidates: (● —)

Methyl acetimidate
(MA)

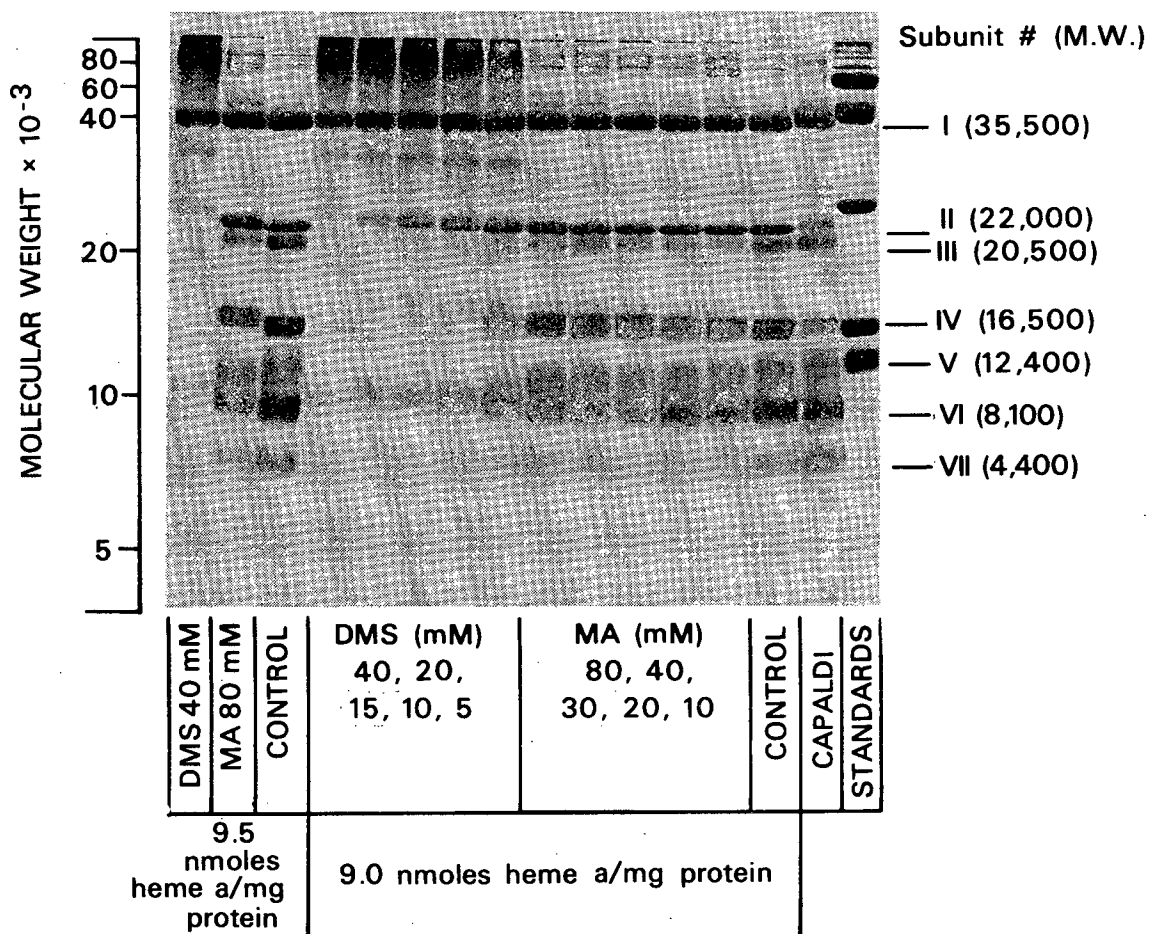
Methyl butyrimide
(MBI)



XBL 799-12053

Fig. 8. SDS gel electrophoresis of control and imidoester-treated cytochrome c oxidase. Purified oxidase (10 μ M) was incubated at various imidate concentrations, dialyzed, and then 30 μ g protein were loaded per channel.

CYTOCHROME OXIDASE



XBB 770-11453

of polypeptides II-VII with an apparent $MW=85,000 \pm 2,000$. Treatment of cytochrome oxidase with 5-40 mM DTBP, a cleavable biimidate, results in electrophoretic patterns similar to those shown for 5-40 mM DMS in the absence of a disulfide reducing agent. Addition of mercaptoethanol results in uncrosslinked patterns similar to those shown for MA-treated samples. Unfortunately, DTBP strongly inhibits cytochrome oxidase at very low concentration (~ 1 mM), because of H_2S , which is apparently generated during biimidate treatment. Sulfide has been shown to be a noncompetitive inhibitor towards oxygen with a $K_i=0.2 \mu M$ (78).

The gradient gels used to separate the oxidase protomers resolves not only the seven components and two contaminants reported by Downer et al (20), but also higher molecular weight contaminants. These larger polypeptides, which can be eliminated by Sepharose 4B chromatography, did not affect the crosslinking patterns significantly (data not shown). The number of primary amino groups modified is compared in Table X with the percent of total protein not entering the separation gel. The assay for crosslinking underestimates the amount of interpolypeptide coupling since if two polypeptide VII's are coupled ($MW=4400$) these crosslinked products will still enter the separation gel and be scored as uncrosslinked protein.

The electrophoretic profile of cytochrome oxidase is not markedly changed when a 1:1 mixture of cytochrome c and oxidase is treated with MA, but small differences are detected in the case of DMS. Subunits II and III stain less intensely at 5 mM DMS when the enzyme is incubated with cytochrome c. The variations in staining intensity are small however, and conclusions as to the cytochrome c binding site(s) on

Table X

Imide Treatment of Oxidized Cytochrome c + Purified Oxidase

<u>Imide Concentration</u>	<u>Primary Amino Group Decrease</u>	<u>Separation Gel Excluded Protein</u>	<u>Oxidase Activity Remaining</u>
(mM)	(%)	(%)	(%)
MA 10	12	<1	99
80	66	<1	55
MBI 10	30	<1	100
80	54	<1	47
DMS 5	20	5	62
40	64	23	11

Values are the mean of at least five determinations

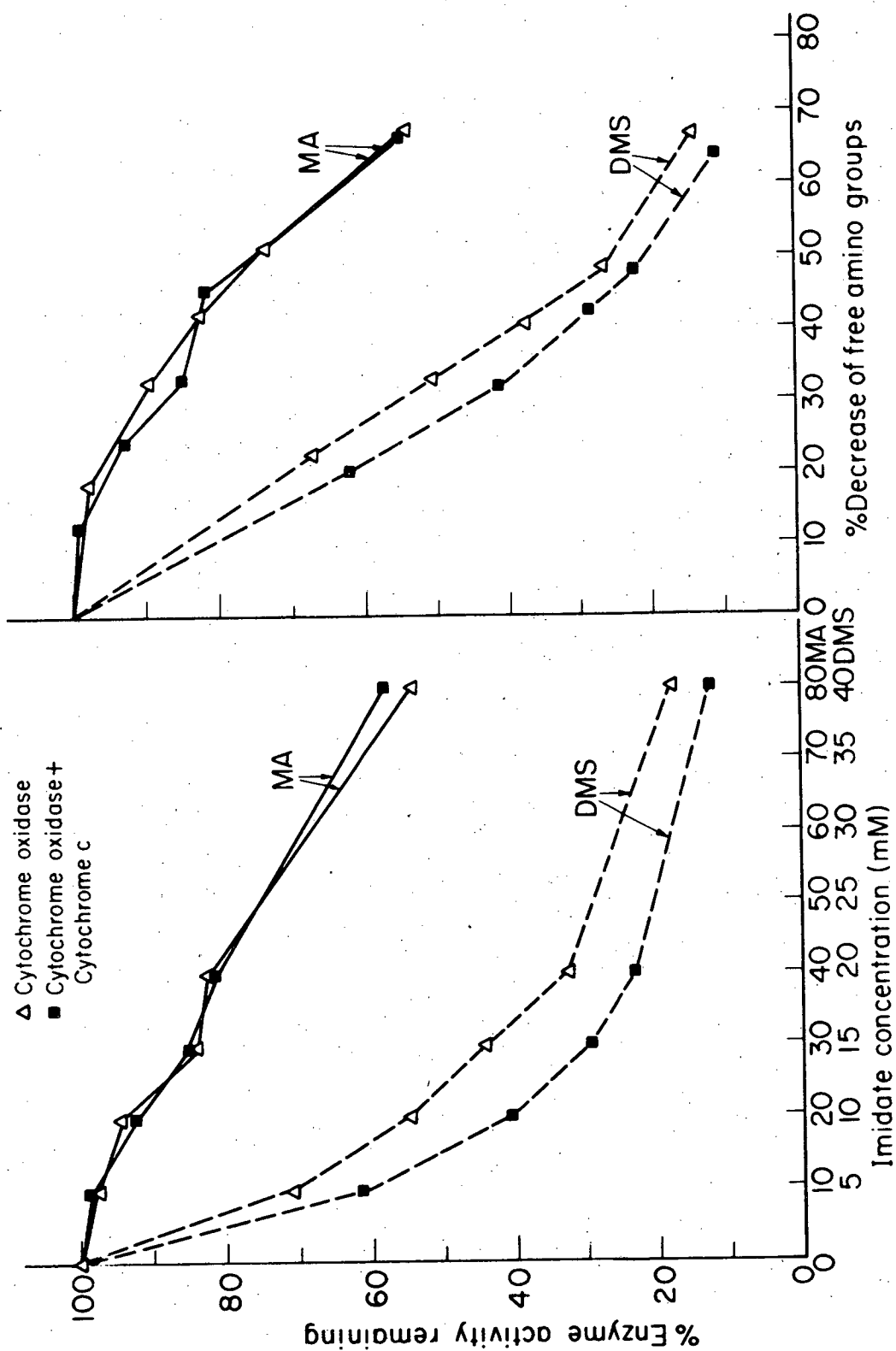
0 0 0 0 5 5 0 4 2 9 3

the oxidase complex cannot be drawn because of the high ionic strength at which imidate treatments are performed(70). Sephadex G-75 gel filtration of cytochrome oxidase, which had been incubated with cytochrome c and treated with 5 mM DMS, resulted in the elution of two peaks.

The first peak contained all of the cytochrome oxidase and 0.2-0.4 cytochrome c molecules/aa₃ unit, but this fraction did not possess ascorbate-TMPD oxidase activity in the absence of added cytochrome c.

Enzyme Activity- MA and DMS inhibit oxidase activity(Fig. 9) although the bifunctional is much more effective at concentrations <10 mM. Table X compares the enzyme inactivation versus the percent separation gel excluded protein. At DMS concentrations >20 mM the opacity of the isolated oxidase solutions increased. Sepharose 4B chromatography showed that intermolecular crosslinking occurred. However, increasing the concentration of enzyme in the incubation mixture enhances activity both in control and 20 mM DMS-treated oxidase(Fig. 9). Although DMS inhibition of ferro- and ferricytochrome oxidase activity is identical, MA inhibition of the activity of the reduced form is ~15% greater than the oxidized form. The smaller MA inhibition appears to be due to the reaction of 5-10% more free amines in the reduced as compared with the oxidized enzyme. MA and DMS appear as noncompetitive(irreversible) inhibitors as depicted on a Lineweaver-Burk plot for both ferro- and ferricytochrome oxidase. Fig. 9 also demonstrates that the addition of cytochrome c to the enzyme during imidate treatment slightly affects the extent of activity inhibition. However, DMS inhibition in the presence of cytochrome c is only 5% more than in its absence, and the disparity between the enzyme activity at a particular degree of amine

Fig. 9. Inhibition of ascorbate-TMPD oxidase activity by imidate treatment of cytochrome oxidase. Cytochrome oxidase (10 μ M) was incubated either in the absence or presence of cytochrome c (10 μ M). When the effect of dilution was studied the incubation concentration of cytochrome oxidase varied between 0.15 to 15 μ M.



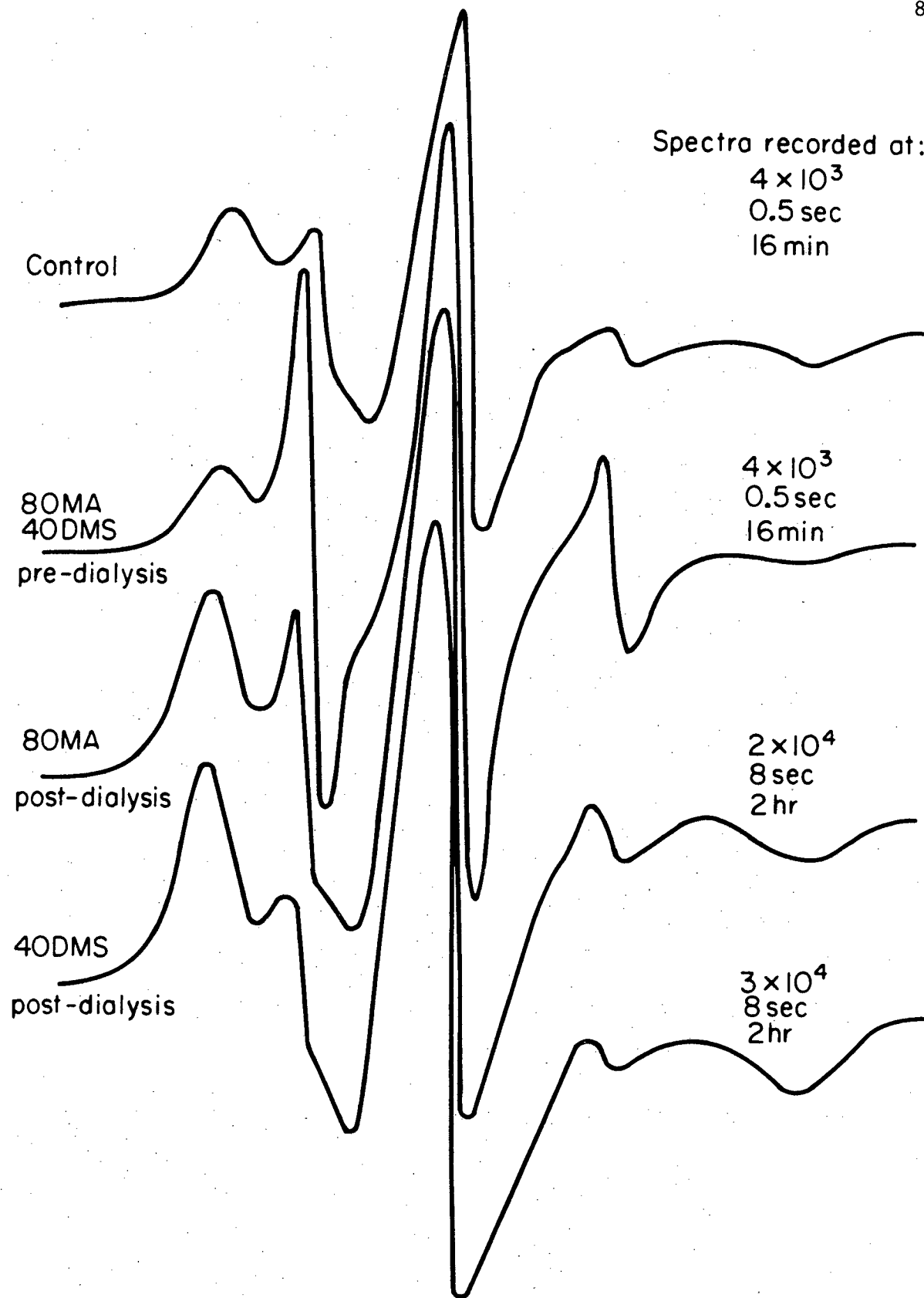
XBL7711-3946

modification is constant in the two cases. Under pre-steady state assay conditions, transient oxidation rates of ferrocytochrome c by control and crosslinked preparations(20% free amines modified) are identical with a second order rate constant of $\sim 3 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$.

Imidate treatment of cytochrome oxidase reconstituted into lipid vesicles composed of phosphatidyl choline and diphosphatidyl glycerol resulted in activity inhibition similar to the detergent-solubilized enzyme. If partially pure asolectin or mitochondrial lipids were used to reconstitute higher imidate concentrations were required to obtain a particular degree of activity inhibition and crosslinking due to the presence of primary amine-containing lipids. It is interesting that crosslinking of reconstituted oxidase did not affect the respiratory control ratio displayed by any of these vesicle types(RCR=3-5, depending on the preparation).

Spin Label Studies- To detect micro-environment alterations in the oxidase complex, a maleimide spin label which binds covalently to a group on the protein independent of imidoester reaction sites was chosen. This label reacts approximately 1000 times more readily with sulfhydryls at pH 7.0 than with lysines(204). It was found that 4-6 spin labels were attached per aa₃ unit at pH 7.0, and the imidoester reaction was performed at pH 8.5-9.0. While at low imidate concentrations(10 mM for DMS or 20 mM for MA) the spectrum of spin-labeled oxidase is unaffected, at higher concentrations an increase in the weakly immobilized signal is detectable(Fig. 10). This weakly immobilized signal increase appears to occur because of a detergent-like effect of imidoester hydrolysis products(205) since spectra

Fig. 10. Electron paramagnetic resonance spectra of control, MA, and DMS-treated cytochrome oxidase labeled with a short-chain maleimide spin label. Spectra were examined before and after dialysis.



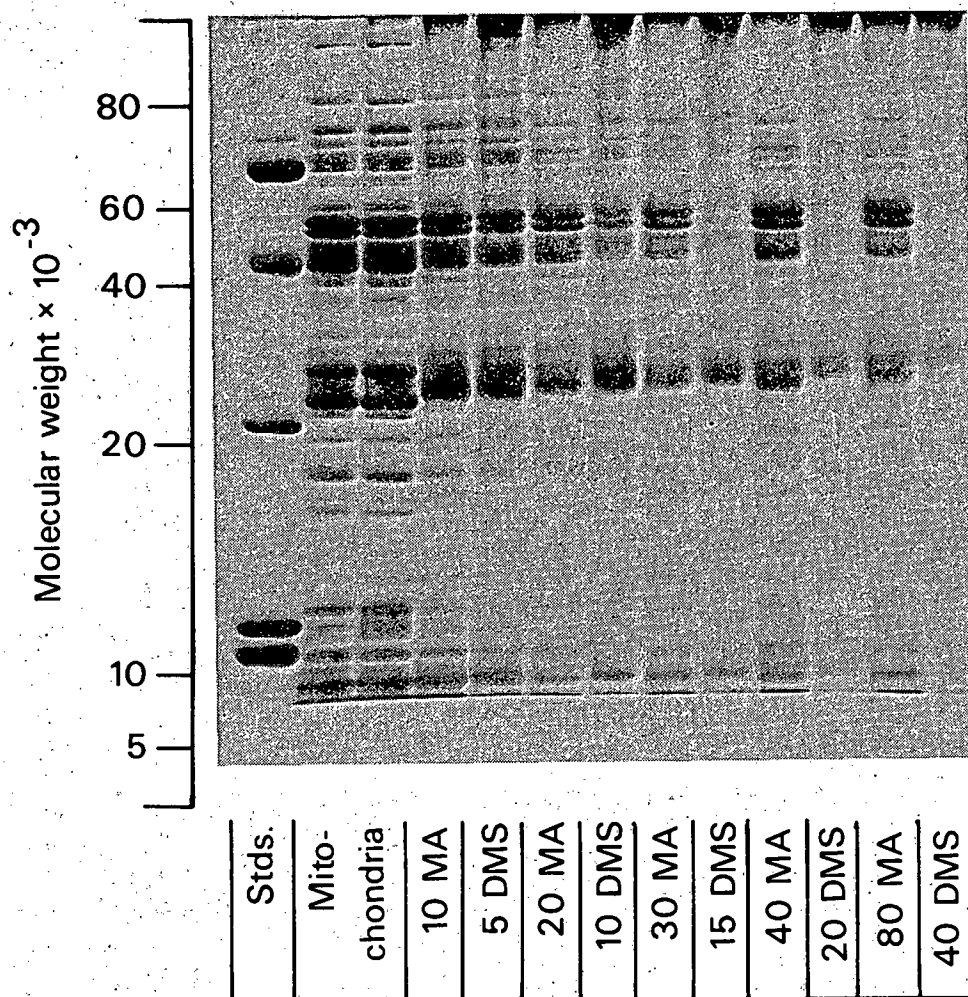
XBL799-3796

obtained after dialysis show that the weakly immobilized signal has returned to the line height of the control for the MA-treated preparation. In agreement with the results of Tinberg et al(143), the hydrolysis product of DMS, dimethylsuberate, was not inhibitory to ascorbate-TMPD oxidase activity at lower concentrations(<10 mM), but was at higher concentrations. This indicates that the inhibition of activity at DMS concentrations >10 mM is not only a product of cross-linking, but also results from other factors such as a possible detergent effect of dimethylsuberate. The strongly immobilized signal in the DMS-treated sample though becomes much larger than the weakly-immobilized signal after dialysis. Oxidase activity of spin-labeled and imidoester-treated preparations were identical to samples not labeled.

Mitochondria:Crosslinking- At DMS concentrations >10 mM(Fig. 11), some crosslinked material is unable to enter the stacking gel which implies that the apparent molecular weight of these crosslinked aggregates exceeds 500,000 daltons. The electrophoretic resolution of polypeptides which are not major components of mitochondria is not achieved due to their low concentration. MA appeared to disrupt the electrophoretic profile of mitochondria more than in the case of the purified enzyme. Nevertheless, mitochondria treated with 200 mM MA show hypotonic lysis behavior essentially identical to control preparations as detected by light scattering(Methods) indicating that treatment with the monofunctional reagent does not cause sufficient crosslinking to effect the osmotic properties of the inner membrane. Mitochondria treated with the cleavable biimidate, DTBP, only rupture in a similar manner to untreated preparations after the disulfide bond is cleaved

Fig. 11. SDS gel electrophoresis of control and imidate-treated beef heart mitochondria. Mitochondria(10 mg/ml) were incubated with various imidate concentrations, dialyzed, and then 30 μ g were loaded per channel.

BEEF HEART MITOCHONDRIA



XBB 799-12718

by dithiothreitol, while mitochondria treated with DMA or DMS concentrations >2 mM were osmotically insensitive. Crosslinking of oxidase polypeptides to other complexes is believed to have occurred since less than 1% of the electrophoretically resolvable material remains in the separation gel at high DMS concentrations. By crosslinking mitochondria at low biimidate concentration (5 mM) and subsequent detergent fractionation and Octyl-Sepharose 4B hydrophobic chromatography (206) it was not possible to purify cytochromes aa_3 independent of cytochromes bc_1 or to entirely separate cytochrome c from cytochromes aa_3 .

Mitochondrial Enzyme Activity- MA and DMS inhibit ascorbate-TMPD oxidase activity in mitochondria (Fig. 12, Table XI). DMS-induced inhibition is linear up to 60% free amine modification while MA is much less inhibitory. The increased inhibition by MA at 50% free amine modification might result from introduction of the bulky N-alkyl acetamidine group onto protein and/or lipid sites since the larger monoimidate, methyl butyrimidate (MBI), increases the inhibition only 5-8% more than MA, while the inhibition pattern remains quite similar. Addition of cytochrome c during incubation of mitochondria with MA does not affect inhibition of the activity, but in the case of DMS inhibition was significantly increased. Hypotonic lysis of the outer membrane and salt-induced removal of cytochrome c results in mitoplasts which are less inhibited by biimidate crosslinking whereas the decline of native mitochondrial oxidase activity is intermediate between mitochondria which are c -supplemented and those which are c -depleted.

Cytochrome c Crosslinking- Cytochrome c slightly increases DMS

Fig. 12. Inhibition of ascorbate-TMPD oxidase activity in intact, cytochrome c-depleted, and cytochrome c-supplemented beef heart mitochondria. Mitochondria were at 10 mg/ml protein in the incubation mixture.

XBL799-3795

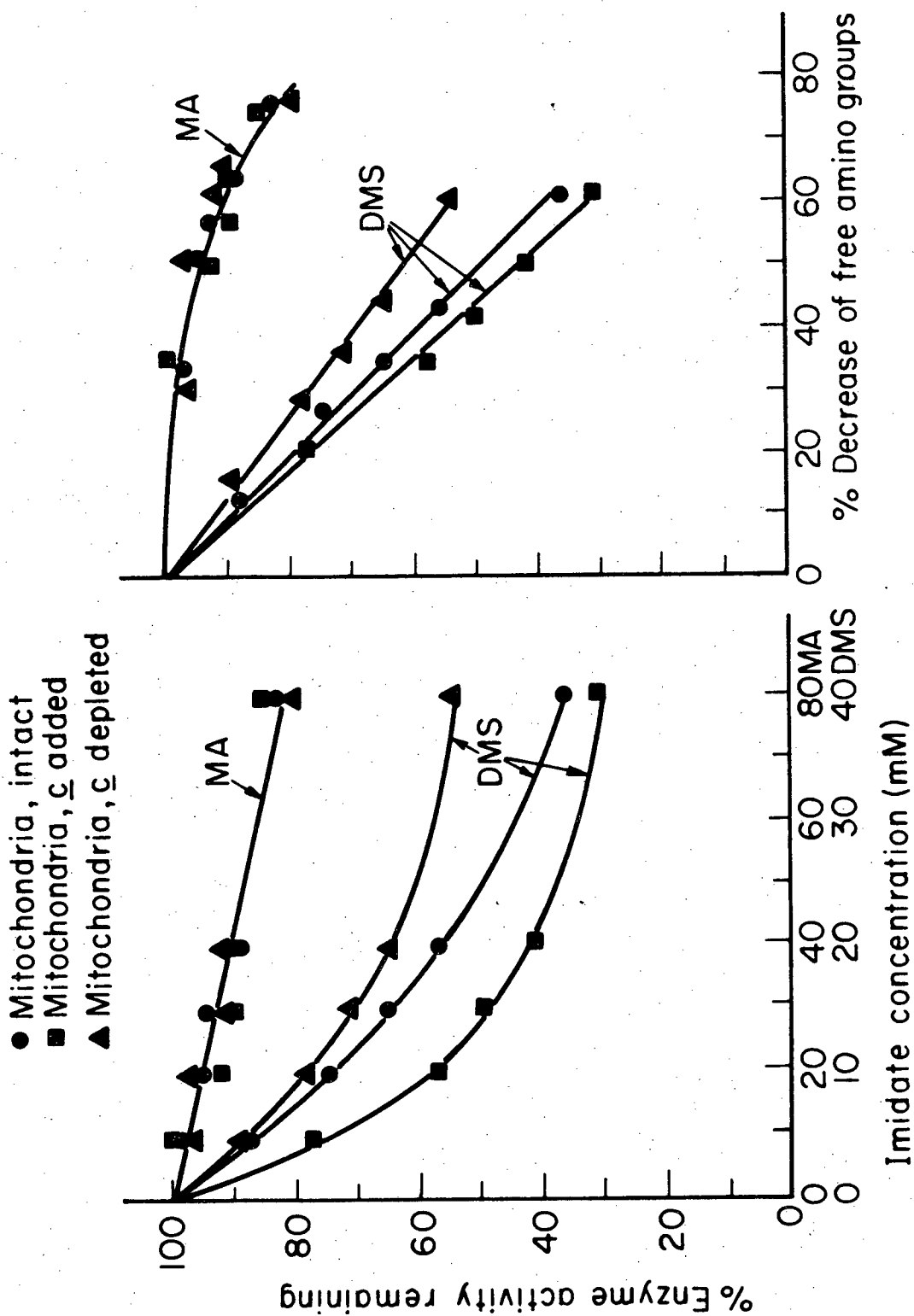


Table XI

Imidate Treatment of Purified Cytochrome Oxidase and Cytochrome c-depleted

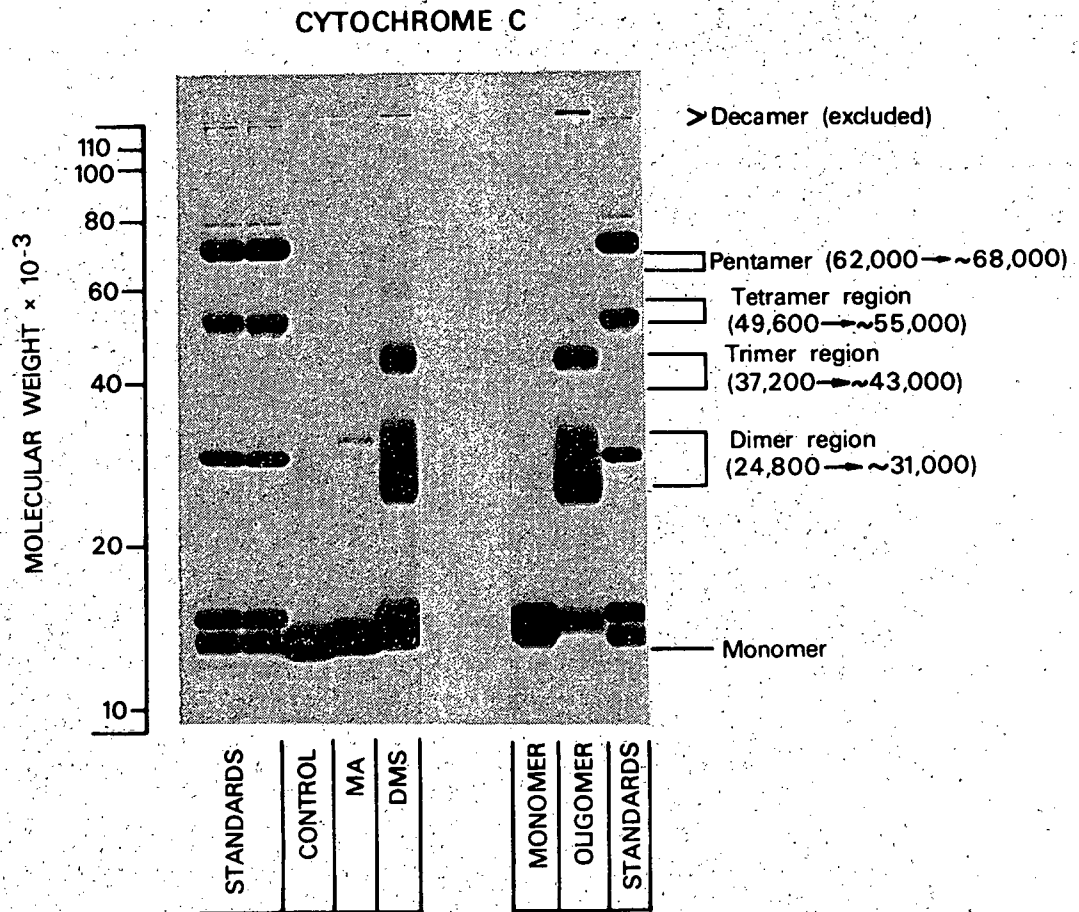
<u>Sample</u>	<u>Imidate Concentration</u>	<u>Beef Heart Mitochondria</u>		
		<u>Primary Amino Group Decrease</u>	<u>Separation Gel Excluded Protein</u>	<u>Oxidase Activity Remaining</u>
	(mM)	(%)	(%)	(%)
Purified Oxidase	MA 10	6	1	98
	80	67	1	54
DMS	5	8	5	71
	40	67	23	18
Beef Heart Mitochondria	MA 10	31	15	96
	80	75	40	78
DMS	5	17	24	89
	40	60	72	53

Values are the mean of at least five determinations

inactivation of cytochrome c oxidase. Since cytochrome c contains 19 lysine residues which constitutes 20% of the residues in its primary structure, c could be randomly crosslinked to various portions of the oxidase (or to the inner membrane surface) and inactivate it indirectly, or it could be immobilized at its active site. Cytochrome c was therefore treated with MA or DMS, and the relative abilities of these modified cytochrome c molecules to reduce unmodified purified cytochrome oxidase were assayed. DMS-treated cytochrome c was separated on Sephadex G-75 into monomer and oligomer fractions. SDS gel electrophoresis shows that separation of monomers from oligomers was complete, but some monomers are present in the oligomer fraction (Fig. 13). It is noted that in MA- and DMS-treated samples band diffusiveness increases in these gels particularly around the dimer region. This may result from random alkylation of free amines; this has been used to calculate effective 'oligomeric regions' as is shown in Fig. 13. Crosslinking at this cytochrome c concentration (0.5 mM) also results in some polymer formation (>decamer). The midpoint potentials ($E_{m7.0} = 285 \pm 5$ mV) of native, MA, and DMS-treated cytochrome c were unchanged.

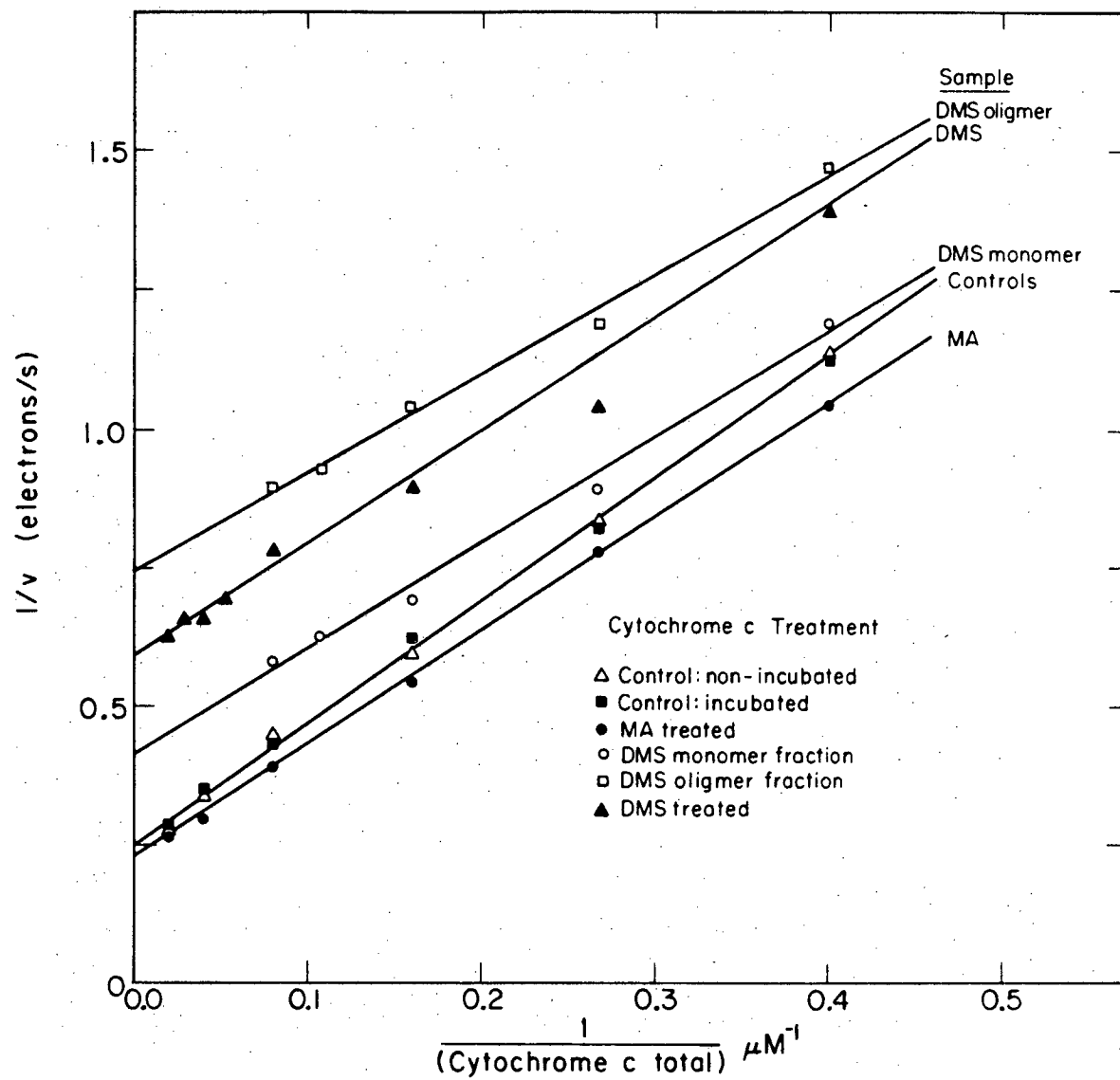
A Lineweaver-Burk plot of ascorbate-TMPD oxidase activity (Fig. 14) using control and imidoester-modified cytochrome c molecules as substrates, indicates that MA and control samples have nearly identical V_{max} and identical K_m values. The unseparated DMS-treated sample possessed a much lower V_{max} and higher K_m , but when the DMS-treated sample was separated by column chromatography, the monomer fraction which was recovered was only slightly inhibited while the oligomer fraction was very inhibited. One possibility is that oligomer formation was decreasing the effective concentration of ferrocytochrome c. This

Fig. 13. SDS gel electrophoresis of imidate-treated cytochrome c. Each channel was loaded with 40 μ g protein.



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Fig. 14. Double reciprocal plot of ascorbate-TMPD oxidase activity using MA- or DMS-treated cytochrome c as substrate.

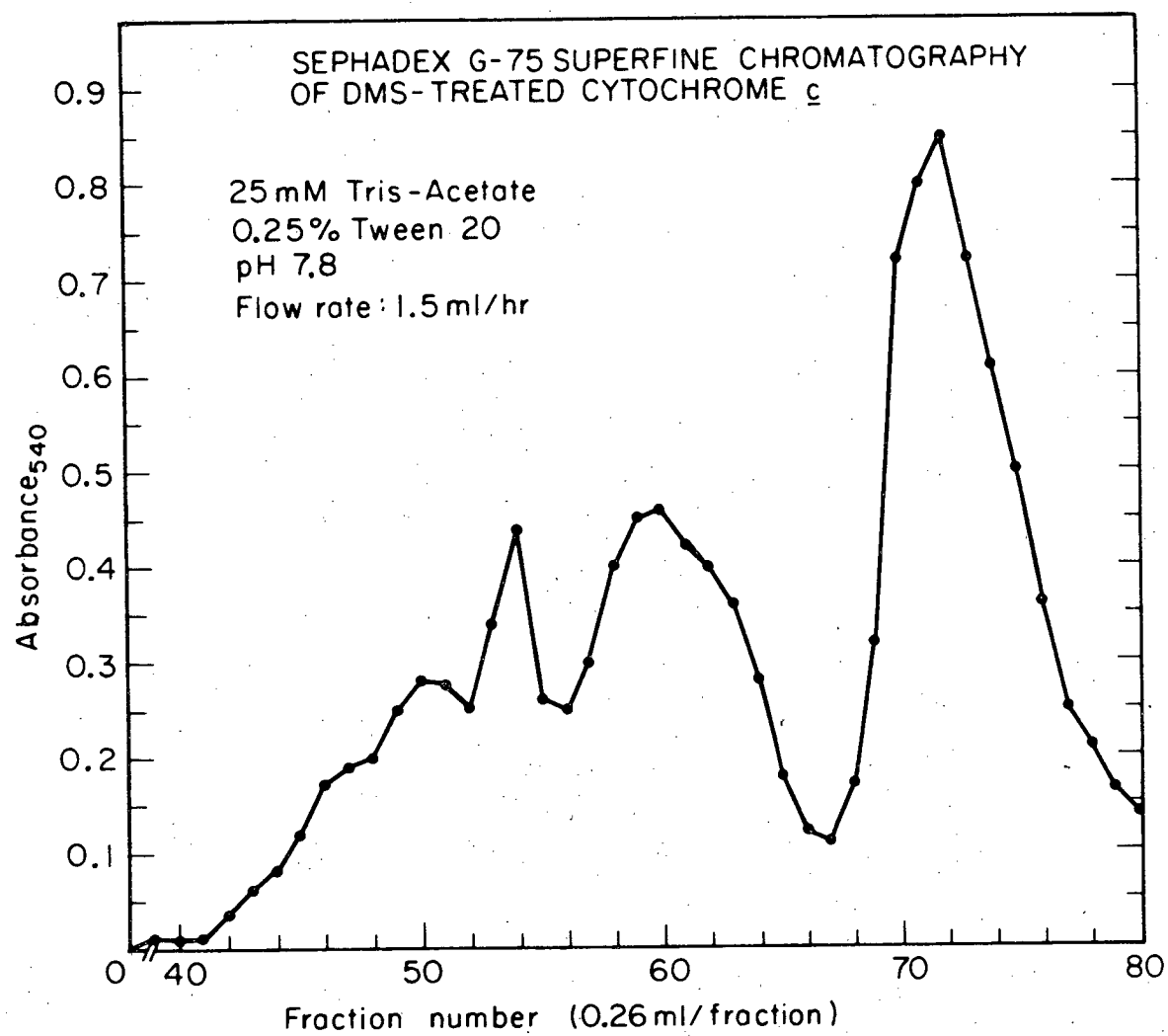


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possibility appears to be eliminated by the finding that oligomeric c is a partial competitive inhibitor of native c with a $K_i = 5.4 \mu\text{M}$. Complete separation of oligomeric from monomeric cytochrome c was achieved at low ionic strength (Fig. 15). Eadie-Hofstee-Scatchard plots demonstrate that the K_d for the high affinity site is similar for native, MA, and $\text{DMS}_{\text{monomeric}}\text{-}\underline{\text{c}}$ ($\sim 3 \times 10^{-8} \text{ M}$) but much greater for $\text{DMS}_{\text{polymeric}}\text{-}\underline{\text{c}}$ ($\sim 1.1 \times 10^{-6} \text{ M}$).

Heterobifunctional Crosslinking Studies- Since cytochrome c covalently bound to its high affinity interaction site on cytochrome oxidase might inhibit electron transport between heme c and heme a, attempts were made to crosslink c to oxidase using the combination of methyl mercaptobutyrimidate (MMBI) and p-azidophenylacetyl bromide (APAB) (207). The advantage of this two-step crosslinking reaction is that disulfide exchange between MMBI-labeled cytochrome c SH groups and cysteine residues on the oxidase does not occur, and the crosslink is stable to disulfide reducing conditions. When modified cytochrome c and cytochrome oxidase are combined and chromatographed on a Sephadex G-75 column at low ionic strength, they elute together in the range 0.8-1.1:1 (c:aa₃). This complex is not cytochrome c stably bound to its high affinity interaction site (70), however it is expected that a significant proportion of the total c population does remain bound at this site. After activation of the nitrene (207) this mixture was again chromatographed on Sephadex G-75 in 50 mM KH_2PO_4 pH 7.4. All of the cytochrome c which is not covalently bound should dissociate and elute later, however a significant amount of the modified cytochrome c remained bound to the oxidase (c:aa₃ ratio in the first elution peak equals 0.5-0.6:1). Precipitation of the enzyme with

Fig. 15. Elution profile of DMS-treated
cytochrome c from a Sephadex G-75 Superfine
column.



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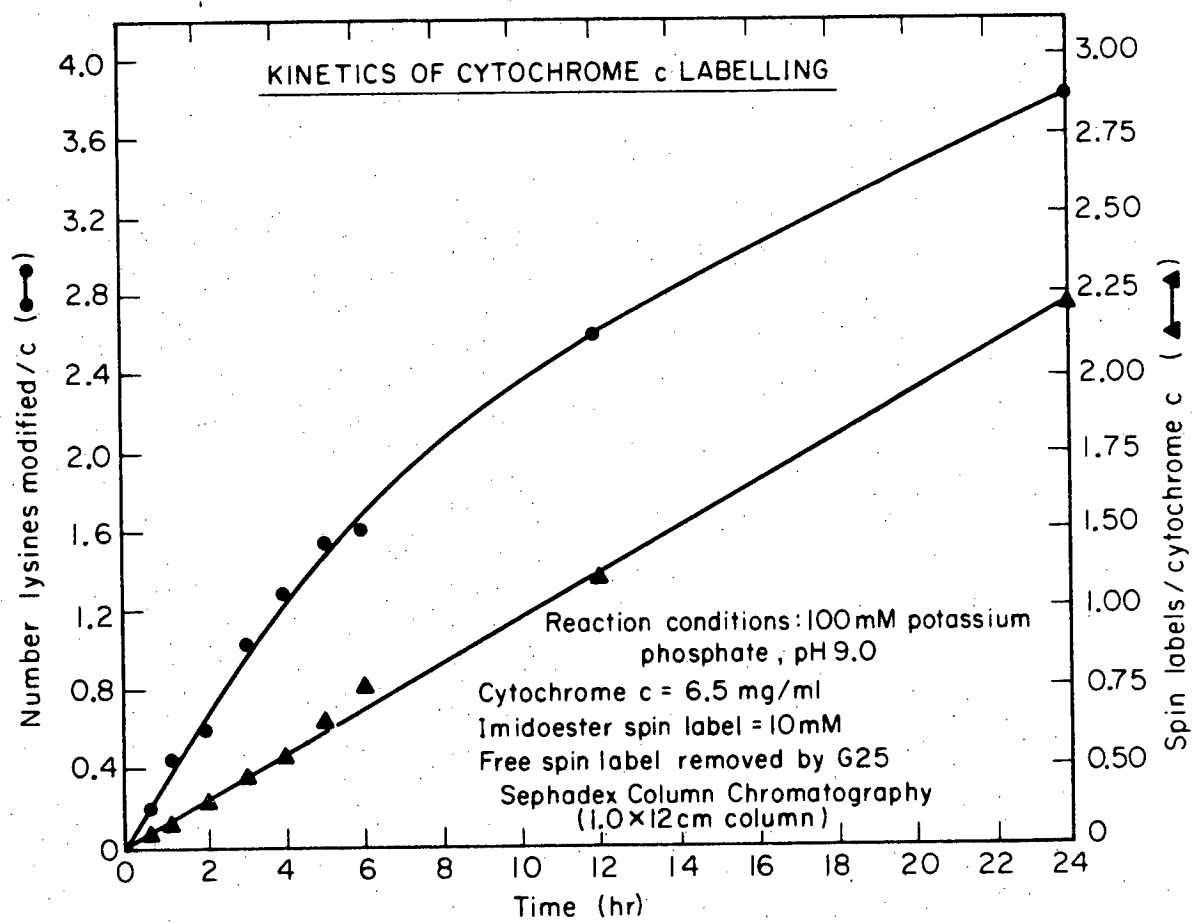
ammonium sulfate(0.35 saturation) did not alter the concentration of cytochrome c with respect to the enzyme.

Margoliash and coworkers(70) have shown that cytochrome c bound to its high affinity site on cytochrome oxidase can be reduced by ascorbate plus TMPD but not by ascorbate alone at low ionic strength in a buffer containing no competitive ions, such as phosphate. Polarographic tracings of experiments conducted in 25 mM Tris-cacodylate pH 7.8 with ascorbate plus TMPD as reductants show that oxygen utilization is quite low, however addition of native cytochrome c appreciably increases the rates in both buffers. This suggests that c is not bound to its high affinity interaction site. Similar results were obtained with another heterobifunctional reagent, 4-fluoro-3-nitrophenyl azide(FNPA), although modification of cytochrome c by this reagent significantly increases the K_d for the high affinity site ($K_d \sim 0.13 \times 10^{-6}$ M). These samples were also subjected to SDS gel electrophoresis. The cytochrome c-oxidase complex showed decreased staining intensity of both subunits II and III.

An Imidoester Spin Probe of Cytochrome c-Cytochrome Oxidase Interactions- As specified in the introduction the tertiary structure of cytochrome c is arranged such that lysines 87, 72, 27, and 8 are at the periphery of the high affinity binding site, and lysine 13 is near the center(70). A water-soluble spin-labeled imidoester was prepared as specified in Methods and used to probe this interaction of a water-soluble and membrane-bound protein.

Reaction of the spin label with cytochrome c occurs slowly with a substantial amount still reacting at 24 hr(Fig. 16). Double integration

Fig. 16. Time course of imidoester spin-labeling of cytochrome c.

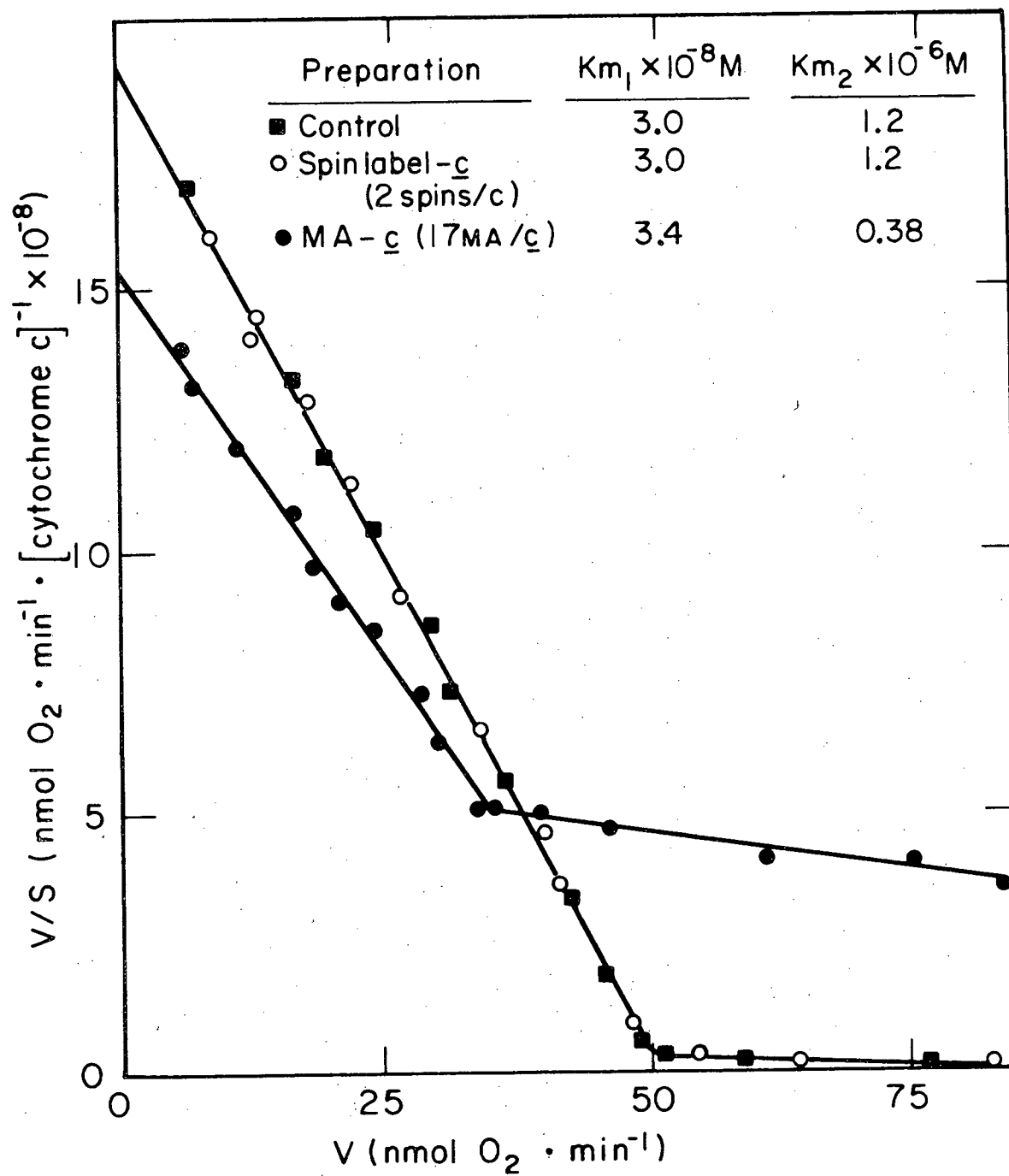


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of the first derivative EPR spectra shows that after 24 hr of treatment only 2.2 molecules are bound per molecule of cytochrome c, while analysis of the free amino groups indicated 3.8 lysines had reacted. However, since 30% of the total spin of the reaction mixture is lost in 24 hr this suggests that only 2.7 spins should be detected on the labeled c population, in good agreement with the EPR spectra. By comparison, labeling of cytochrome c with methyl acetimidate ($t_{1/2}$ hydrolysis=27 min) and methyl benzimidate ($t_{1/2}$ hydrolysis=55 hr) at pH 8.5 shows that 17 and 15 of the total lysines are modified in 30 min by 40 mM reagent, respectively. Perhaps the double bond within the ring structure of the label stabilizes the imidoester and slows its reaction. Under these labeling conditions the oxidized and reduced spectra of spin-labeled cytochrome c is unchanged. SDS gel electrophoresis of the spin-labeled cytochrome c did not reveal the presence of oligomers; crosslinking by other monofunctional reagents has been hypothesized to occur(208).

Since the imidoester labeling of proteins is specific and preserves charge, it was of interest to see if labeled cytochrome c molecules retained similar electron transport and binding activities as the native protein. Eadie-Hofstee-Scatchard analysis(70) demonstrated that the spin-labeled cytochrome c molecules(2 spins/c) were identical to the native protein, and similar to cytochrome c extensively modified with methyl acetimidate(17 lysines modified)(Fig. 17). Chromatography at low ionic strength in the presence of cytochrome oxidase resulted in a 0.8-1.1:1(c:aa₃ molar ratio) complex for both native and modified cytochrome c.

Fig. 17. Eadie-Hofstee-Scatchard plot
of native, imidoester spin-labeled, and
MA-treated cytochrome c.



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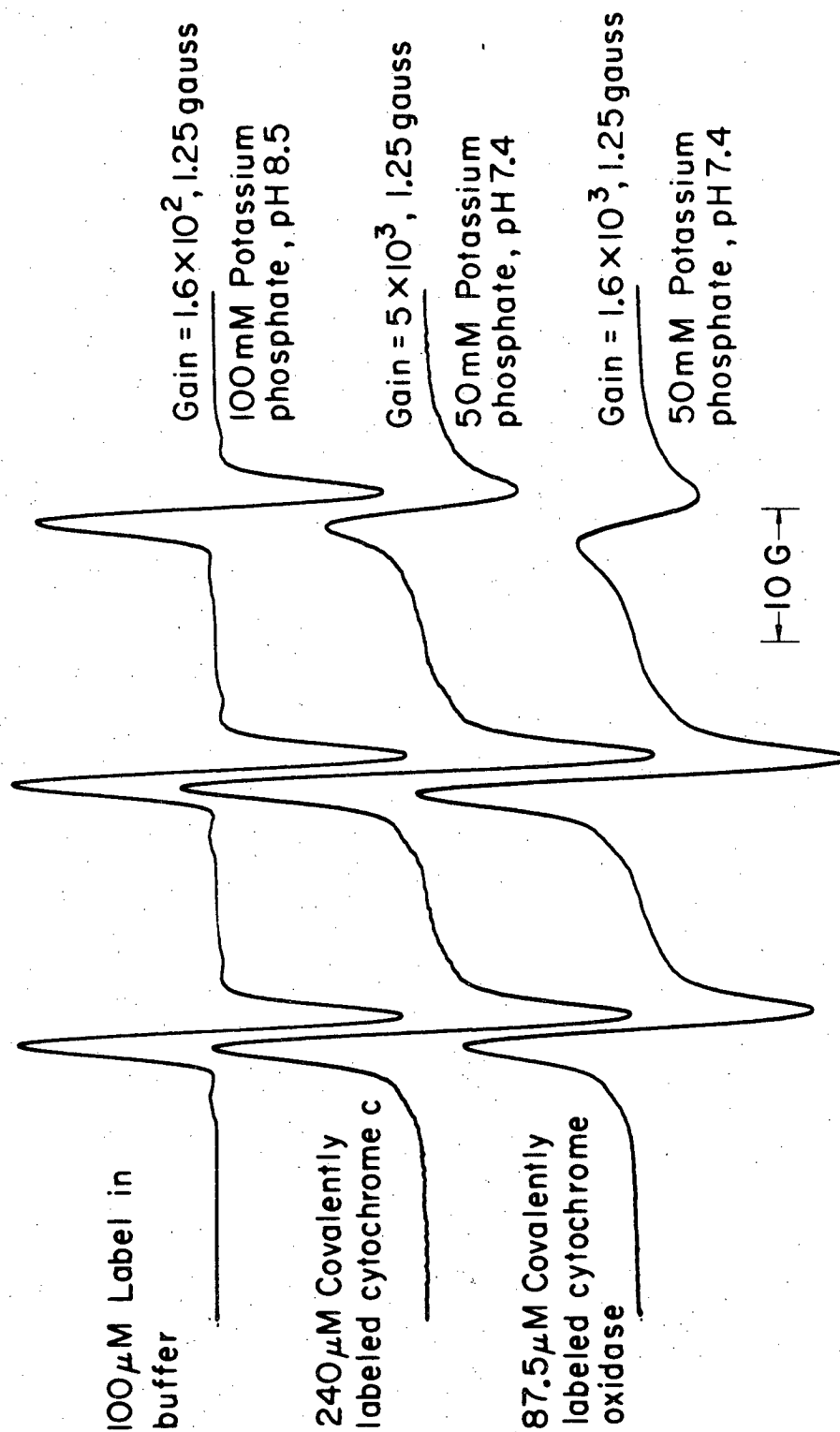
The spectra in Fig. 18 demonstrate that the imidoester spin label is weakly immobilized on both cytochrome c ($\tau_c = 9.3 \times 10^{-10}$) and cytochrome oxidase molecules. These results are similar to those reported by Drott et al.(209) using N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidiny1-bromoacetamide to label the free sulfhydryl of yeast cytochrome c ($\tau_c = 1.0 \times 10^{-9}$) and identical to the results of Azzi et al(210) using N(2,2,5,5-tetramethyl-3-pyrrolidyl-1-oxyl) iodoacetamide to label methionine-65 on horse heart cytochrome c ($\tau_c = 9.3 \times 10^{-10}$). However, one of the principal advantages of using an imidoester spin label for protein spin labeling is its water solubility. The weak immobilization and hyperfine coupling(15.5 ± 0.2 gauss) indicate the nitroxide is exposed to a polar environment.

Reaction of the imidoester occurs with unprotonated lysines and is thus favored at high pH. The high positive charge density of cytochrome c tends to repel the positively charged imidoester and also raise the local pH as a function of proton repulsion. As these two effects tend to cancel each other it is difficult to predict which lysine residues on the protein will react using criteria of local charge density. The binding of cytochrome c to cytochrome oxidase in 25 mM Tris-cacodylate and in 50 mM phosphate was studied. In order to obtain a cytochrome c-cytochrome oxidase complex where c was only bound to its high affinity site, chromatography at low ionic strength was performed (2). Since this has been kinetically described as cytochrome c bound only to its high affinity site further immobilization of the nitroxide might be expected to occur if there had been substantial labeling of the lysines near the heme crevice. The spectra of the imidoester-

Fig. 18. ESR spectra

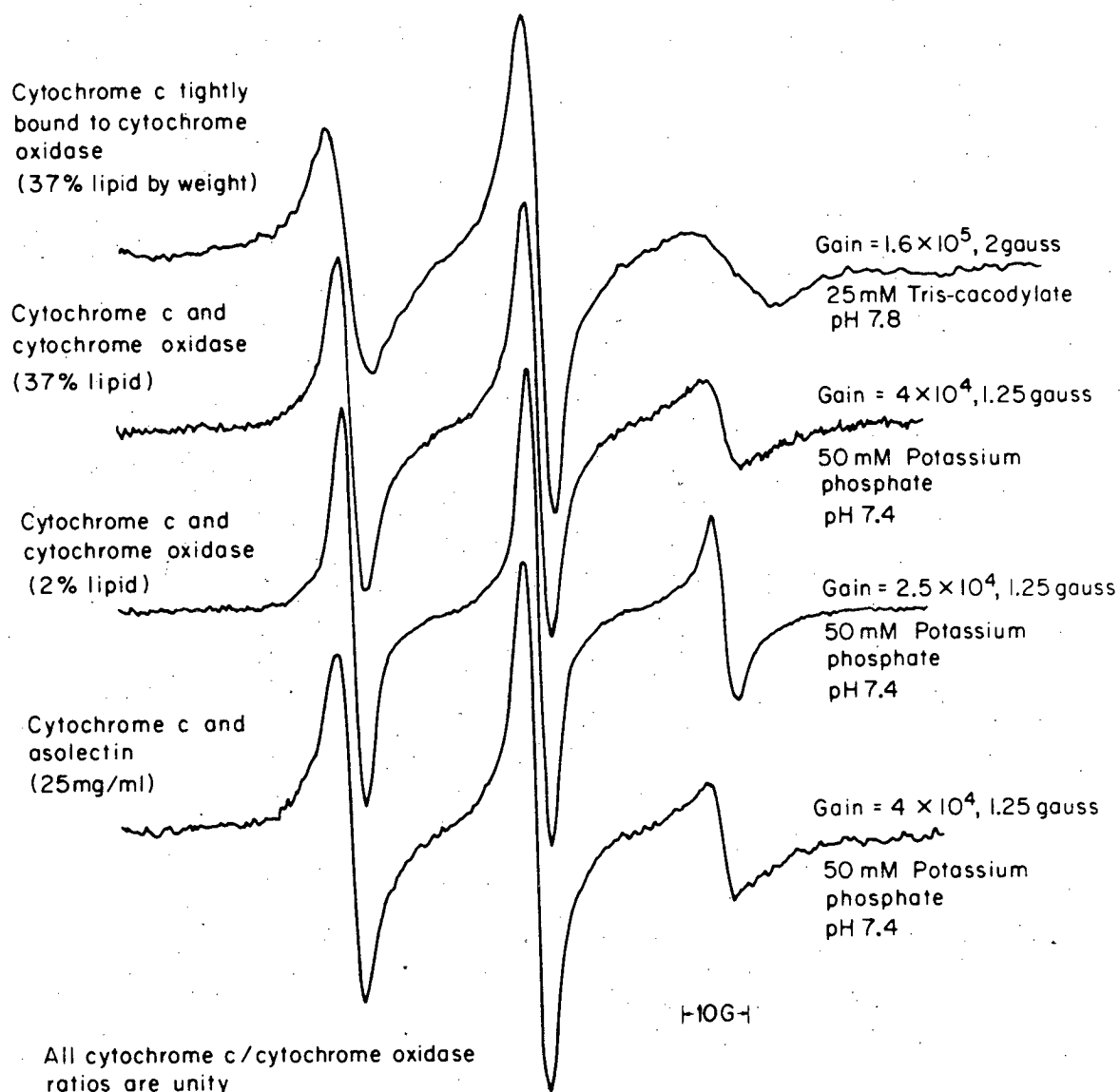
- a) free imidoester spin label, and covalently attached to cytochrome c and cytochrome oxidase.
- b) labeled cytochrome c bound to lipid-rich and lipid-poor cytochrome oxidase and asolectin vesicles.

BOUND AND FREE IMIDOESTER SPIN LABEL



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BINDING OF SPIN LABELED CYTOCHROME c



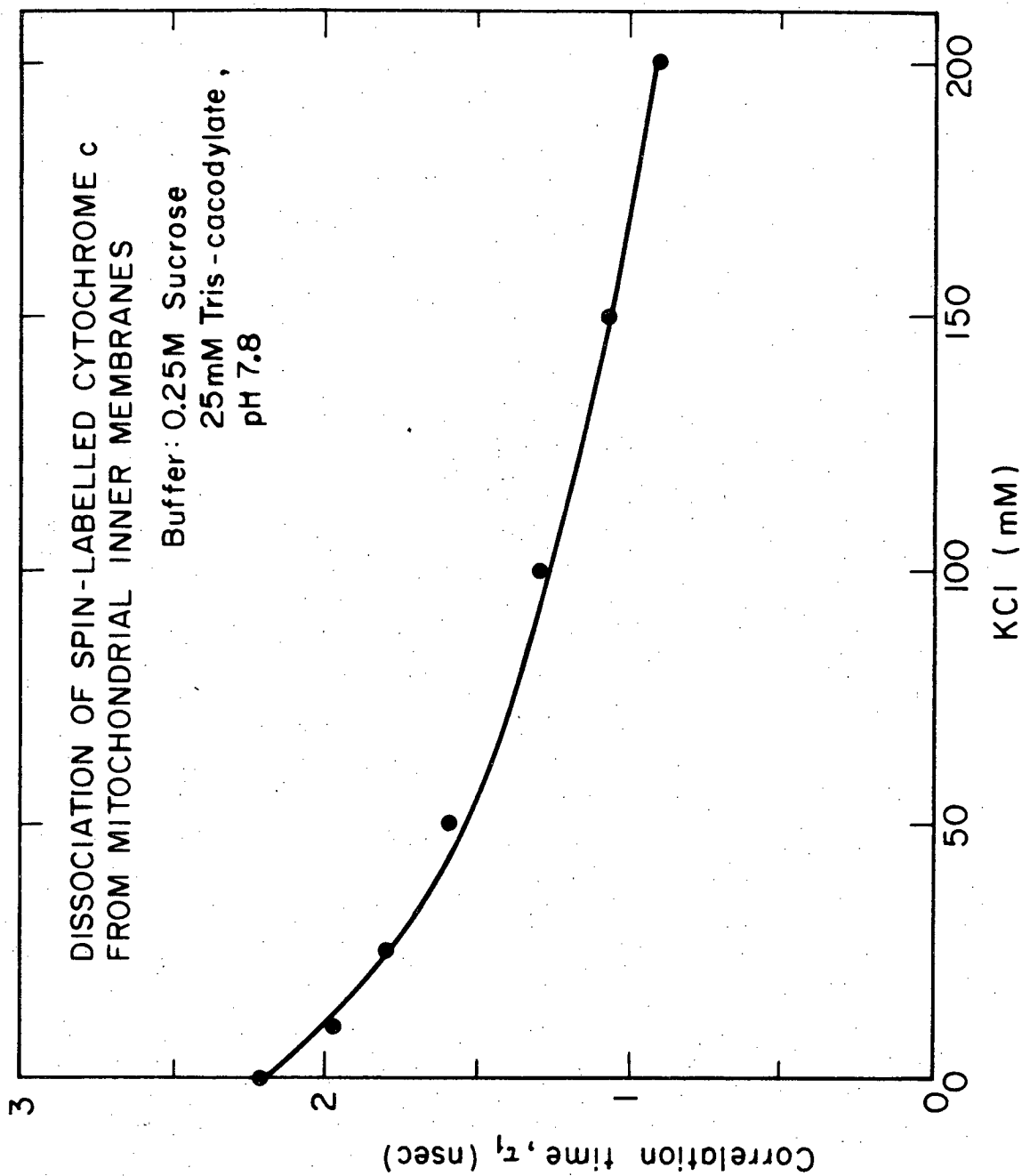
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labeled cytochrome c, shown in Fig. 18, indicated that most of the spins become more immobilized upon binding of the protein to its binding site($\tau_c = 2.4 \times 10^{-9}$). This implies that most of the labeling has occurred at or near the binding site, or (less likely) that upon binding there is an alteration of the cytochrome c structure leading to changes in the environment of spins that are distant from the binding site. The splitting of the three nitroxide lines (hyperfine coupling) remains about 15.2 gauss upon binding and therefore the environment of the spins remains polar as the spins become immobilized.

Binding of spin-labeled cytochrome c to a lipid-poor (185) cytochrome c oxidase results in significantly less immobilization of the nitroxide than the lipid-rich preparation (184). This indicates that the cytochrome c binding sites on these two oxidase preparations are dissimilar even though they are kinetically indistinguishable at low ionic strength. Binding of spin-labeled cytochrome c to negatively-charged phospholipid vesicles also immobilizes the nitroxide but to a lesser extent than cytochrome oxidase ($\tau_c = 1.8 \times 10^{-9}$).

The binding of spin-labeled cytochrome c (2 spins/c) to cytochrome c-depleted mitochondrial inner membranes was salt dependent (Fig. 19). The correlation time of the bound c ($\tau_c = 2.2 \times 10^{-9}$) was similar to that of the bound cytochrome c specifically labeled at methionine-65 (210) ($\tau_c = 2.3 \times 10^{-9}$). This indicated that most of the spin became immobilized upon binding to the salt-depleted membranes.

Fig. 19. Dissociation of imidoester spin-labeled cytochrome c from cytochrome c-depleted inner mitochondrial membranes.



DISCUSSION

The results of the present investigation, and other using chemical crosslinking(143-145) and different methods(16), show that long-range motion of cytochrome oxidase with respect to other components in the respiratory chain is not required for activity. Some degree of motion is, however, clearly essential for a fully functional enzyme, and for it to interact normally in the electron transport chain. The problem is to identify the degree to which such motion is required and the specific groups involved on the interacting proteins. Evidence pertaining to these two points is discussed further below.

Interaction and Motion of Oxidase Polypeptide Subunits and the Role of Lipid- Crosslinking of purified oxidase inhibits heme a to heme a₃ electron transfer. Thus under pre-steady state conditions transient ferrocytochrome c oxidation and ferricytochrome a reduction is similar for control and crosslinked preparations. Bonaventura et al (146), who also investigated transient kinetics of ferrocytochrome c oxidation by control and crosslinked oxidase, arrived at similar conclusions. Furthermore, they have demonstrated that the rate of CO ligation to heme a₃ is identical for both the control and crosslinked enzyme.

Intermolecular crosslinking of oxidase with DMS did not appear to affect enzyme activity. Oxidase can be isolated as a 'membranous' patch(159,160) in which it is regularly ordered in a two-dimensional crystalline array(161,162). Highly purified oxidase preparations when reconstituted into lipid vesicles also display a tendency to aggregate (16), and there have been speculations(14) that the active enzymatic

unit is a dimer of four hemes and four copper atoms. Hackenbrock and Hammon(9) have provided evidence by freeze-fracture electron microscopy that cytochrome oxidase may exist in an aggregated form within the mitochondrial inner membrane. The experiments reported here indicate that intermolecular crosslinking of oxidase does not affect cytochrome c accessibility to the heme c-heme a electron transfer site. This implies that intermolecular crosslinking creates, or possibly stabilizes, regularly ordered cytochrome oxidase aggregates. Thus using saturation transfer EPR techniques, our laboratory has recently demonstrated(16) that the rotational correlation time of oxidase reconstituted into lipid vesicles is insensitive to biimidate-induced intersubunit crosslinking. Oxidase could be reconstituted in a relatively immobile aggregated patch($\tau_2 > 1$ ms) or in a highly mobile form($\tau_2 = 40$ μ s), but activity of the mobile and immobile oxidase complexes were identical($V_{\text{max}} = 400$ electrons/s), and reconstituted vesicles displayed high respiratory control. Kunze and Junge(158) have shown by linear dichroic measurements that the cytochrome a₃ component of the oxidase complex displays no rotational motion <100 ms. Taken together these results imply that cytochrome oxidase exists in an aggregated form in the inner mitochondrial membrane.

Our studies(16) also show that the fluidity of lipids adjacent to the oxidase depends on the rotational mobility of the protein. If oxidase is not aggregated when it is reconstituted into vesicles, lipid spin probes covalently bound to the protein do not display a strongly immobilized EPR spectral component, whereas if the protein displays little rotational motion a strongly immobilized lipid component is also

observed. However, the activity of cytochrome oxidase with highly fluid and strongly immobilized lipid is identical. Hence, lipid fluidity is not required for electron transport through the oxidase complex.

Intercomplex Motion and Cytochrome c-Oxidase Interactions- Cross-linking of oxidase to cytochrome c reductase occurs concurrently with inhibition of ascorbate-TMPD oxidase activity in mitochondria. The question which arises is whether crosslinking of the oxidase and reductase complexes to each other is, in part, responsible for the inhibition of enzyme activity or whether the results may be entirely explained by crosslinking of the oxidase subunits which we have shown to be inhibitory. Chemical modification results with mitochondria and isolated oxidase cannot be directly compared because of unavoidable differences in reagent accessibility and amine content. Assuming that the inner membrane contains ~25-29%(211) of the total mitochondrial protein, and 20% of this is oxidase protein(14), then oxidase comprises ~5% of the total mitochondrial protein. Since the lysine content of purified oxidase(4-5%) and mitochondria(6%), and the amine phospholipid content, are similar, then at a specific percentage of free amine modification the inhibition of mitochondrial oxidase activity should be at least an order of magnitude less than purified oxidase if the two preparations are incubated with imidates at the same heme a concentration. The assumptions are that oxidase polypeptides are not more accessible than the other complexes' polypeptides, and that cytochrome accessibility remains similar in the two cases. Experiments reported here demonstrate that the inhibition of mitochondrial oxidase activity is considerably more than expected based on comparative

studies with the purified oxidase. This suggests that intercomplex crosslinking also affects cytochrome oxidase activity.

Rendon et al(212) have reported that rat liver mitochondria treated with DMS at 0°C retain respiratory control. Proton permeability, state IV succinate oxidase and adenine nucleotide translocase activities were not affected by crosslinking when assayed at 30°C, but ATPase and ADP-stimulated succinate oxidase activities were inhibited. We have demonstrated that when oxidase vesicles having high respiratory control are treated with DMS, ascorbate-TMPD oxidase activity is inhibited but respiratory control is not affected. Hence, crosslinking minimally perturbs membrane integrity but inhibits enzyme activities differentially.

Addition of cytochrome c during biimidate treatment increases the extent of inactivation, but our results using heterobifunctionally-derivatized cytochrome c indicate that it is crosslinked at a site which does not interfere with the binding of native c. Formation of a covalent complex of cytochrome c-oxidase results in very little endogenous electron transport.

Bisson et al(155) have used FNPA to derivatize cytochrome c. This reagent suffers from the disadvantage that it changes positively-charged lysine residues to uncharged secondary amines, and elimination of positive charge near the oxidase high affinity binding domain on the c molecule is known to alter c-aa₃ binding interactions(70). These investigators also noted that FNPA-c-aa₃ complexes were incapable of electron transport in the absence of added native cytochrome c.

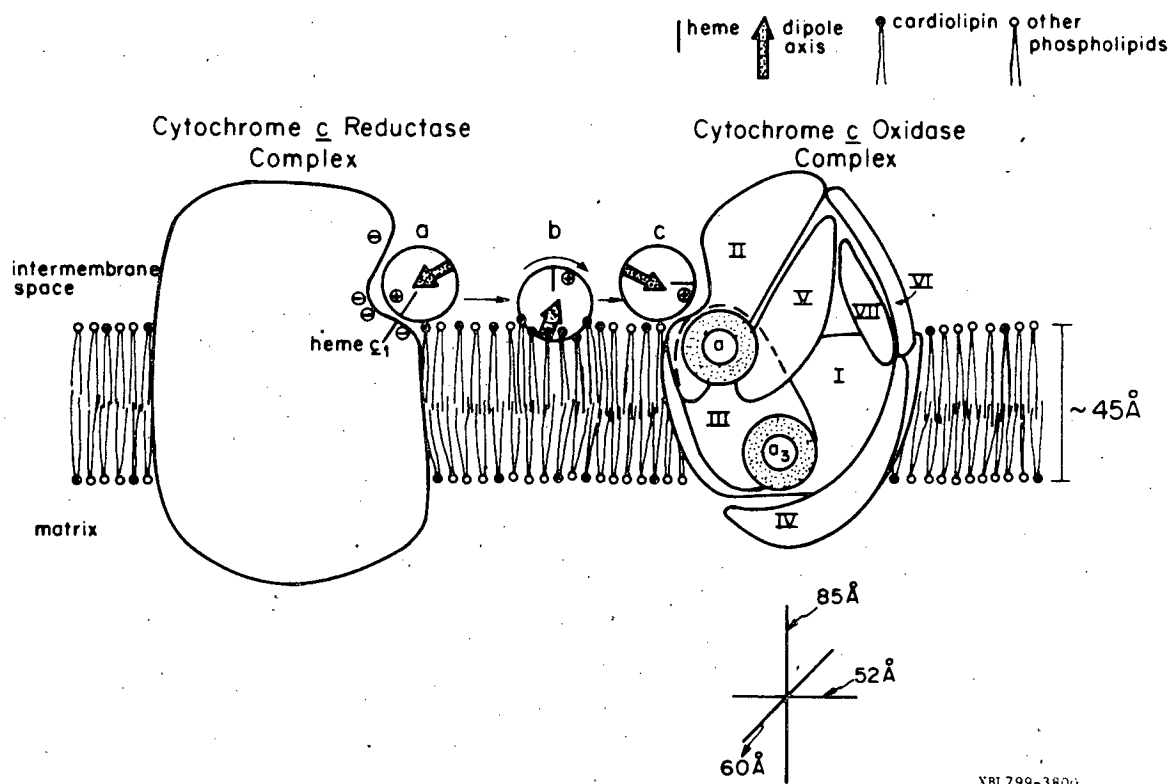
Erecinska et al(156) have used a 2,4-dinitro-5-fluorophenyl azide(DNPA)

-cytochrome c derivative which displayed an apparent K_m for the reaction with cytochrome oxidase 5-6 fold greater than native c. The addition of these DNPA-c molecules to cytochrome c-depleted rat liver mitochondria, and light activation of the nitrene, resulted in a 1:1(c:aa₃) complex. In contrast to our results and those of Bisson et al(155), DNPA-c-aa₃ complexes were active. Moreover, electron transport rates were only slightly stimulated by added native c.

A Model of Cytochrome c-Cytochrome Oxidase Interactions in the Inner Mitochondrial Membrane- Margoliash and coworkers(70,150,151) have demonstrated by selective chemical modification and antibody studies that interaction domains on the cytochrome c molecule for the reductase and oxidase are essentially identical. Brown and Wüthrich(74) have shown that cytochrome c binds to phosphatidyl choline-diphosphatidyl glycerol vesicles by its 'backside'(near residue 60) and causes clustering of diphosphatidyl glycerol. Our studies indicate that covalent bonding of cytochrome c to oxidase can be obtained, but that this complex is incapable of electron transfer activity. These results indicate that cytochrome c rotational and/or lateral movement on the inner membrane surface is required for electron shuttling. The results suggest a model(Fig. 20) for the role of motion in electron transport between the reductase and oxidase complexes. It is suggested that the ferricytochrome c dipole axis(72) preferentially orients towards its high affinity interaction site near heme c₁ on the reductase. When electron transfer results in the reduction of heme c, charge distribution of the high affinity interaction site changes and this results in dissociation of c from the

Fig. 20. A model for the structure of cytochrome c oxidase, and hypothetical rotational motion of cytochrome c. The dimensions illustrated are for a 'mem-branous' enzyme preparation(159,160), while the subunit disposition(3) and heme displacement are from previous studies(27, 213).

STRUCTURE OF CYTOCHROME OXIDASE AND HYPOTHETICAL
ROTATIONAL MOTION OF CYTOCHROME c



reductase and rebinding to phospholipid sites. The c dipole axis reorients towards the high negative charge density on the oxidase interaction site and binding occurs; electron transfer then takes place between heme c and heme a.

Section II: ROTATIONAL AND LIPID VERSUS PROTEIN MOTION

Cytochrome c oxidase appears to be a closely associated complex of polypeptides and lipid which requires a degree of intracomplex fluidity in order to undergo its catalytic cycle. Electron transport in mitochondria however might also involve the rotational motion of the individual enzyme complexes, and this property could affect the organization of membrane-bound cytochrome oxidase. Therefore spin labels were used to study the motion of lipids and the rotational mobility of the spin-labeled protein(214) in various environments, including when it is functionally incorporated into the bilayer of a lipid vesicle. This approach, which has proven useful in the study of other membrane-bound proteins(188) was used to assess the importance of lipid and protein mobility for enzyme function.

RESULTS

Characteristics of Enzyme Preparations- The preparations which were utilized for the determination of the rotational mobility of the soluble and membrane-bound cytochrome oxidase are described in Table

Data for chymotrypsin-treated preparations is also presented for comparison with previous results(193). Triton-oxidase had the smallest amount of polypeptide contamination, while DOC-oxidase was much more contaminated as shown in Fig. 21. It should be noted that this discontinuous buffer gradient gel allows the resolution of many high molecular weight contaminants not detected on continuous buffer gels employing urea and SDS(20). As was noted in the previous section, these high molecular weight contaminants could be eliminated by further purification using cholate and ammonium sulfate(193) and gel filtra-

Table XII
Characteristics of the Cytochrome Oxidase Preparations

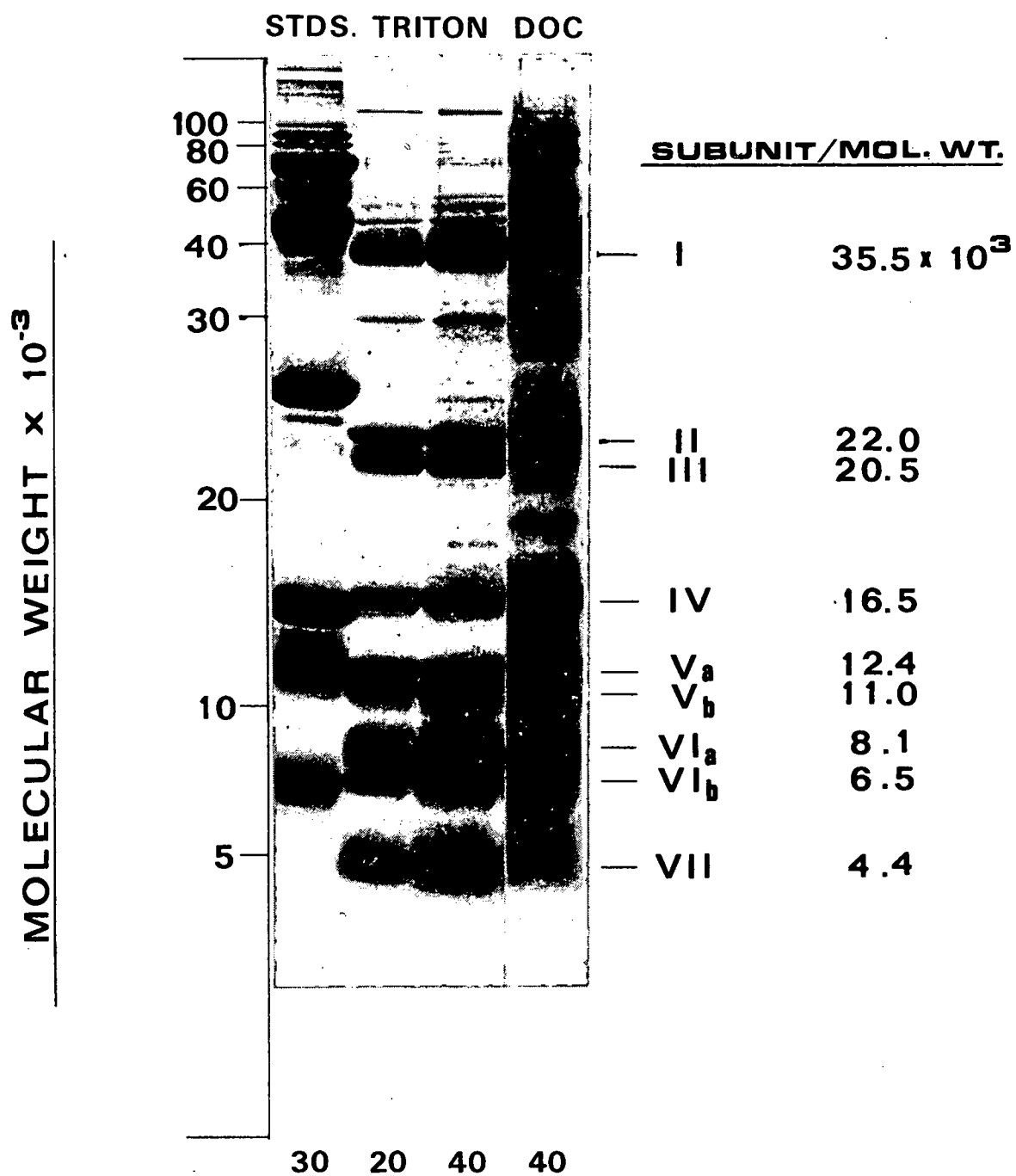
<u>Preparation</u>	<u>Purity</u>	<u>Activity^a</u>	<u>Lipid Content</u>	<u>SH Groups^b</u>
	nmoles heme a/mg protein	electrons/ s per aa ₃	mg lipid/ mg protein	
DOC-oxidase	9.0-10.5	400	0.35-0.42	9
Triton-oxidase	10.0-11.8	400	0.02-0.06	7
Chymotrypsin DOC-oxidase	10.0-11.7	140	ND ^c	7
Chymotrypsin Triton-oxidase	10.5-12.0	400	ND	7

^aActivity is expressed as $V_{\max}$ value in 67 mM KH_2PO_4 pH 7.4, 0.5% in the presence of 1% asolectin at 25°C

^bDetermined according to (197) in 0.1% SDS. Values, the mean of at least three determinations, were rounded to the nearest whole number.

^cNot determined

Fig. 21. SDS gel electrophoresis of the cytochrome oxidase preparations used in the rotational mobility study. The numbers at the bottom of the gel profile refer to the number of μg loaded per channel. The stacking gel was removed for convenience, and did not contain any stained material.

CYTOCHROME OXIDASE

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tion on Sepharose 4B(20). The nine polypeptides seen in Fig. 21 are the seven polypeptides of bovine heart cytochrome c oxidase plus the two contaminants Va and VIb described by Downer et al(20); these could not be entirely eliminated by incubation of the enzyme with chymotrypsin(10 μ g/mg oxidase protein) at 25°C in 0.1 M NaH_2PO_4 pH 7.8, 0.5% cholate for two hours(34,193). In the case of the Triton-oxidase no reduction of the specific activity occurred as a result of the proteolytic digestion, whereas in the case of the DOC-oxidase the activity declined from 400 to 140 electrons/s per aa_3 unit.

Assays of the activity of spin-labeled Triton-oxidase and DOC-oxidase are shown in Fig. 22. No differences could be detected between the native and spin-labeled enzymes. As shown in Fig. 22, only the lipid-poor Triton-oxidase preparation could be activated by the addition of lipid, as previously reported(202). The binding affinity constant (K_d) of cytochrome c at the high affinity site is 0.03 μ M in good agreement with other results(70).

As can be seen from Table XIII approximately one MSL spin label per oxidase remained after spin-labeling and the cysteine and ferricyanide treatment. In order to determine whether the spin population was bound to one distinct subunit of the enzyme, the preparation was dissociated with 2% SDS and 8 M urea and chromatographed as detailed in the Methods section(Fig. 23). EPR was used to determine the amount of spin associated with each fraction. SDS-urea gel electrophoresis was then performed as in (20), and it was found that spin was associated only with fractions containing subunits II and III(MW 22,000 and 20,500); these were not resolved further because of spin loss which occurred

Table XIII

Reduction of the Covalently-Bound MSL Spin Probe

<u>Preparation</u>	<u># Spin Labels Added/aa₃</u>	<u># Spin Labels^a Reacted/aa₃</u>	<u># Spin Labels^b Not Reduced</u>
Triton-oxidase	2	1.5	1.1
DOC-oxidase	5	3.2	1.4

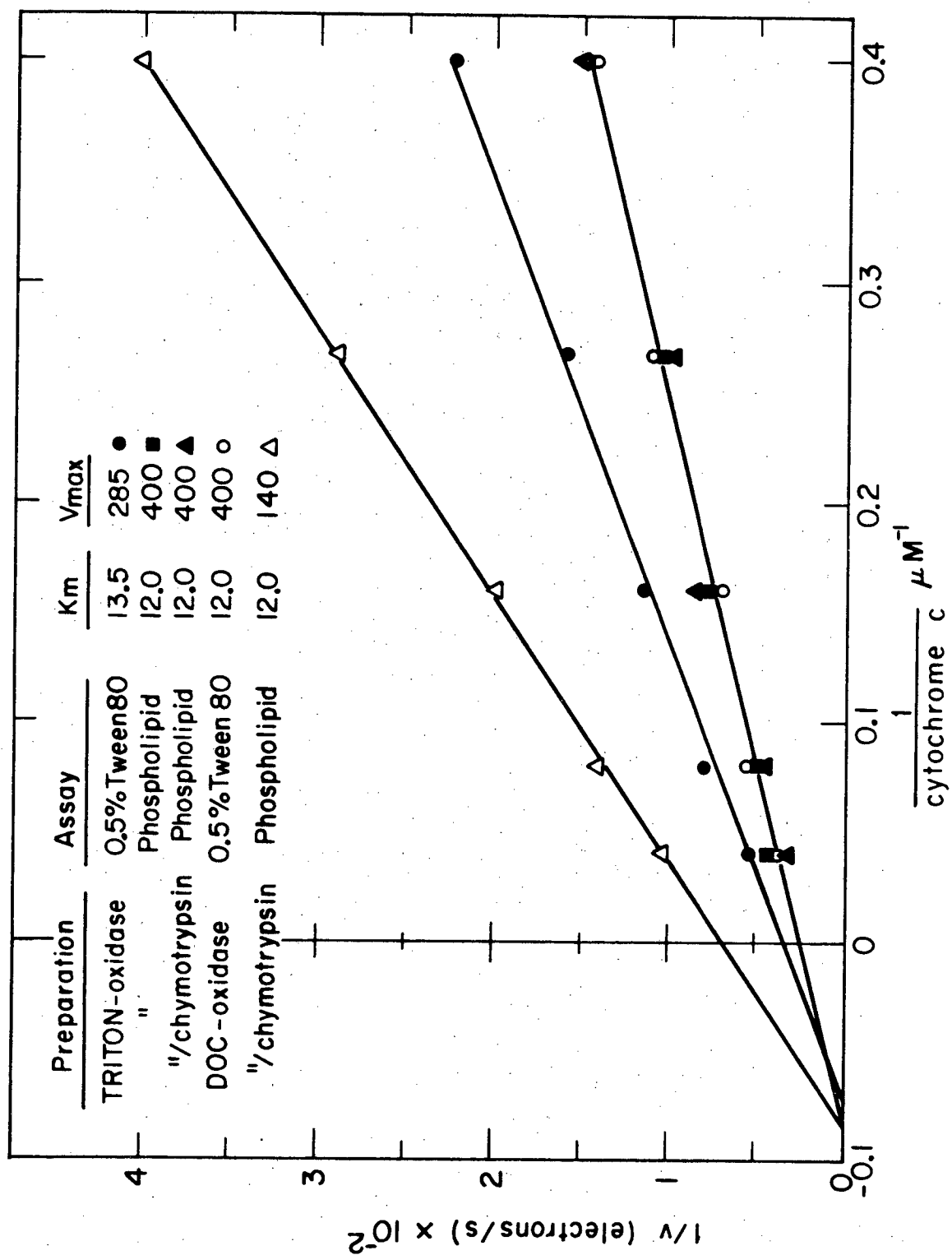
^aBased on the number of SH groups for DTNB reaction (197) before and after spin-labeling(mean of 5 determinations).

^bCalculated by double integration of spectra taken before and after cysteine and ferricyanide treatment(mean of 5 determinations).

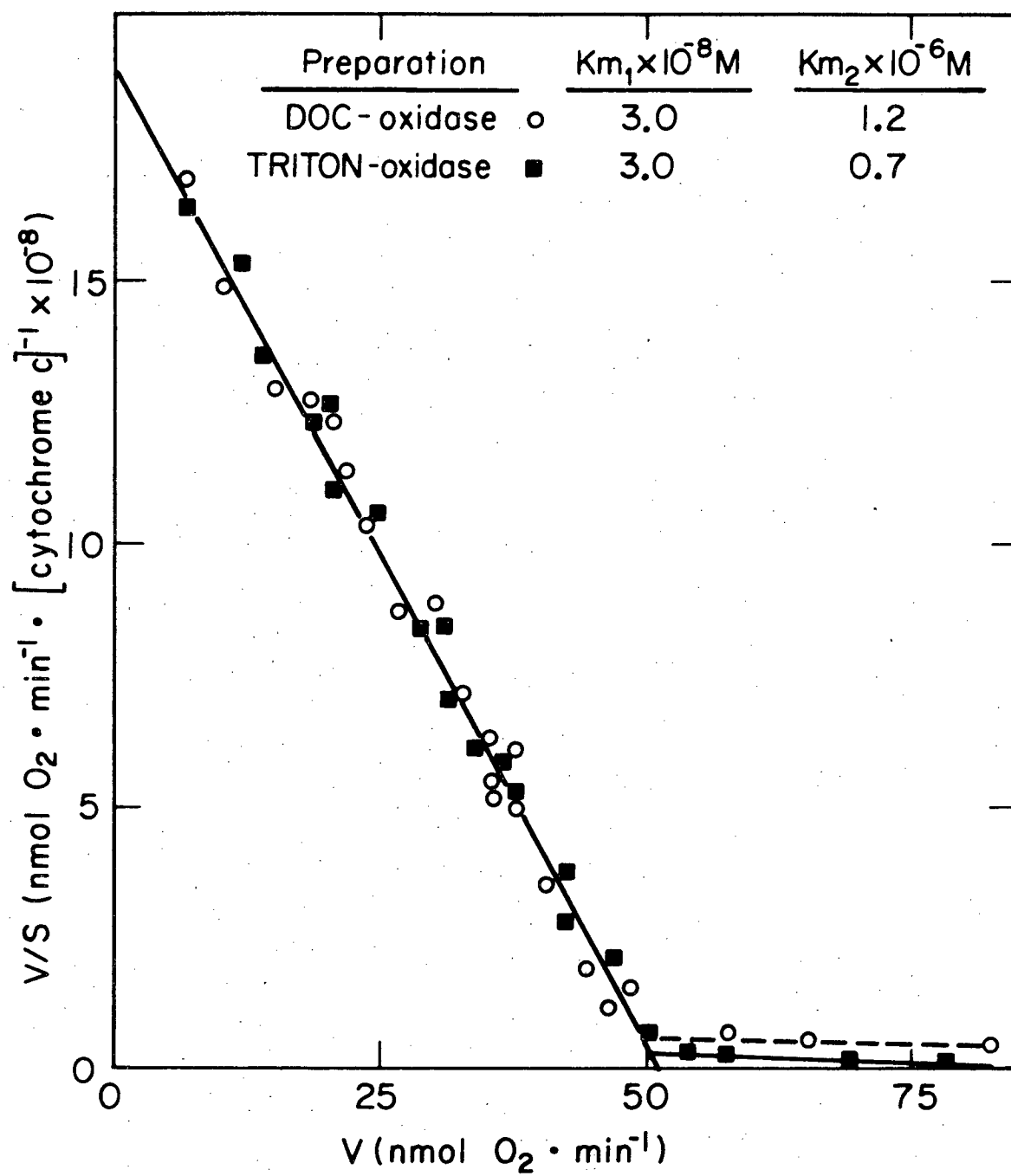
Fig. 22. Polarographic activity assays of spin-labeled cytochrome c oxidase.

- a). Lineweaver-Burk plot of steady-state O_2 consumption.
- b). Eadie-Hofstee-Scatchard plot showing high affinity binding kinetics of cytochrome c.

All points represent mean averages of at least five determinations. Activity assays of native and spin-labeled cytochrome oxidase yielded identical results.

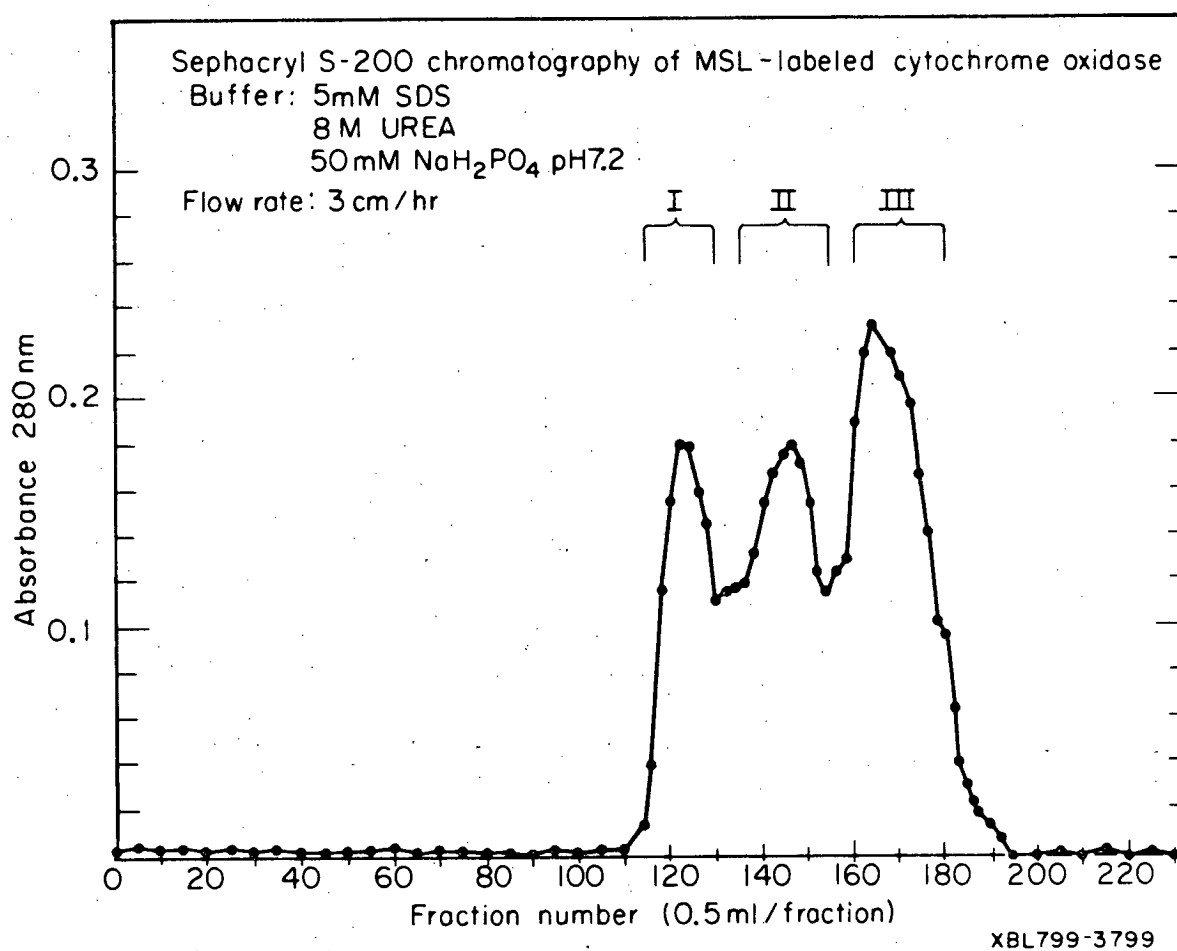


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Fig. 23. An elution profile from a
Sephacryl S-200 column of detergent(SDS)-
solubilized cytochrome c oxidase.

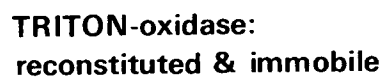
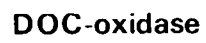


during the chromatographic procedure. Kornblatt et al(215) have previously demonstrated that (^{14}C)-NEM and MSL both react with a subunit of MW 25,000 at pH 8.5, but subunits II and III were not resolved in their electrophoretic study. Recently, these investigators have produced evidence that (^{14}C)-NEM reacts with subunits III and VIa at pH 7.0 with the labeling of subunit III being predominant(19).

Reconstitution Using Phosphatidyl Choline- Following spin labeling and chromatography the DOC-oxidase eluted always from the column as a turbid suspension, whereas the Triton-oxidase remained optically clear. The turbid suspension had the opalescent appearance characteristic of a vesicle suspension. An electron micrograph of a negatively-stained preparation of DOC-oxidase after chromatography(Fig. 24), demonstrates that it consists of a very heterogeneous population of vesicles with diameters ranging from 500 to 2500 Å; some multilamellar vesicles were also detected, but we found no respiratory control in these preparations.

Since saturation transfer EPR experiments require spin label concentrations on the order of 10 μM or higher, a reconstitution procedure developed by Karlsson et al(202) was used at a low lipid to protein ratio(1:1, by weight), permitting convenient concentration of the enzyme to 50 μM . Negatively-stained preparations of DOC-oxidase reconstituted in this manner, showed vesicles of mean diameter 1020 ± 100 Å(Fig. 24), but with no detectable respiratory control; an analysis of these samples on a discontinuous Ficoll gradient(Fig. 25), demonstrated little or no enzyme incorporation in the phosphatidyl choline vesicles, in agreement with previous results(216). Using

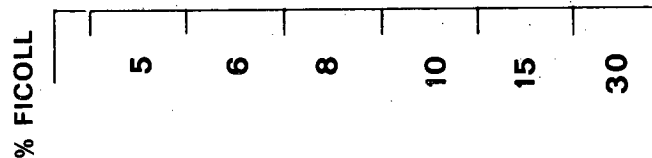
Fig. 24. Electron micrographs of preparations negatively-stained with 1% phosphotungstate(pH 7.0) followed by 1% uranyl acetate.



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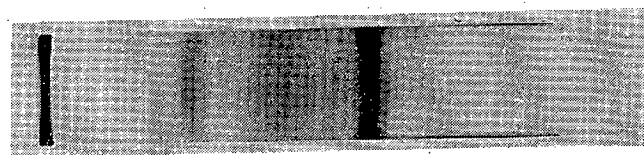
Fig. 25. A Ficoll discontinuous gradient analysis of protein incorporation into lipid vesicles. The sonication procedure of (202) was employed using preformed phosphatidyl choline vesicles and DOC-oxidase, and the cholate dialysis procedure of (201) was used employing either mitochondrial lipids or partially-purified asolectin and the Triton-oxidase, either chromatographed or not. The use of mitochondrial lipids in the cholate dialysis procedure did not change our results in any detectable fashion.

SONICATION: PHOSPHATIDYLCHOLINE
DOC- oxidase



PERCENT OF TOTAL		<u>RCR</u>
<u>PROTEIN</u>	<u>PHOSPHOLIPID</u>	
0	58	-
0	14	-
0	3	-
6.0	1	1.0
25.2	4	1.0
68.8	21	1.0

CHOLATE DIALYSIS: ASOLECTIN
TRITON- oxidase



PERCENT OF TOTAL		<u>RCR</u>
<u>PROTEIN</u>	<u>PHOSPHOLIPID</u>	
0	19	-
37.3	53	4.5
50.0	20	3.6
12.7	5	3.0
0	3	-
0	0	-

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partially purified asolectin we were able to obtain 20% incorporation of cytochrome oxidase by this technique(as detected by Ficoll gradient analysis). However, with 80% of the enzyme unincorporated, the respiratory control ratio was very low($RCR=1.3\pm0.2$).

Preparations with much greater respiratory control ratios were obtained by different reconstitution procedures using much higher lipid to protein ratios.

Reconstitution with High Respiratory Control- These cytochrome oxidase vesicles were prepared by a modification of Racker's sonication technique(201), and cholate dialysis technique(200), using partially purified asolectin or mitochondrial lipids. Only Triton-oxidase was used, which after spin-labeling and chromatography can be shown to be in a disaggregated form(see later Results).

The complete incorporation of Triton-oxidase into partially purified asolectin vesicles is shown in Fig. 25. Similar results were obtained with mitochondrial lipids. Electron micrographs of these negatively-stained preparations are shown at the bottom of Fig. 24 (mean vesicle diameter 420 ± 30 Å).

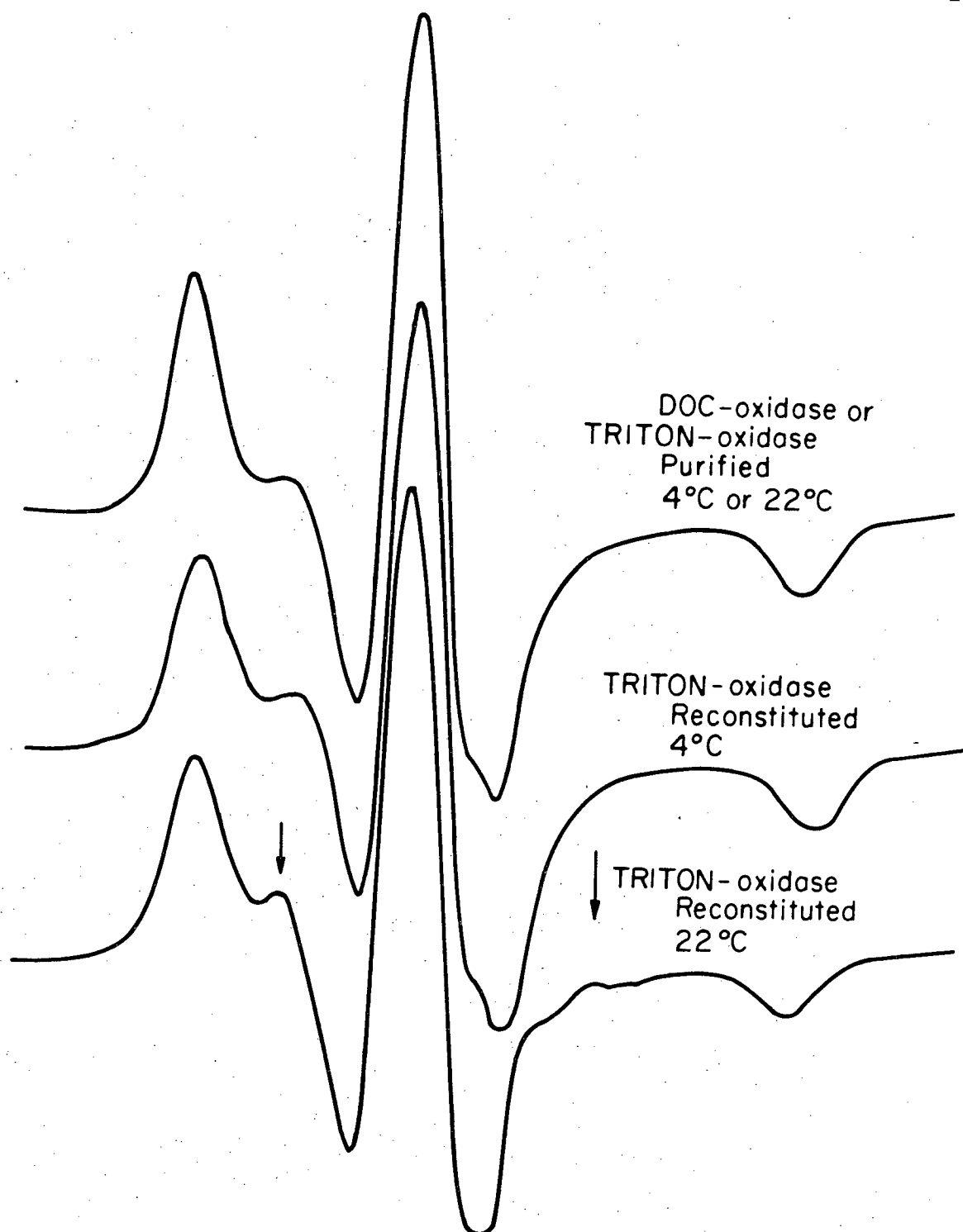
EPR Spectra of the Various Preparations- Determination of rotational motion of proteins by the technique of saturation transfer EPR requires that a spin label be bound to a macromolecule such that the population of weakly immobilized spins is minimized as detected on the V_1 spectrum. The molar ratio of MSL to cytochrome c oxidase, the incubation time and temperature were adjusted to minimize this weakly immobilized population, but in the case of both the DOC- and Triton-oxidase additional treatment with cysteine and ferricyanide was

required. This treatment affected neither the activity of the enzyme nor the K_d for high affinity binding of cytochrome c measured at low ionic strength. Both DOC- and Triton-oxidase preparations possess nearly identical V_1 spectra at 4°C (Fig. 26), but when the Triton-oxidase is reconstituted by the cholate dialysis or sonication (our modification of (201)) procedures, a small weakly immobilized population of spins (at arrows) can be seen at 22°C but not at 4°C. To avoid ambiguities all the spectra were recorded at 4°C.

Lipid Versus Protein Mobility- As described by Jost et al (164,165), when lipid (including some spin-labeled hydrocarbon chains) is added to oxidase which has been partially phospholipid depleted, the spin labels become strongly immobilized. The DOC-oxidase was clearly in a membranous form (Fig. 24). As shown in Fig. 27, the fatty acid spin label FASL(1,14), becomes immobilized (V_1 spectrum) when it is added to this preparation, in agreement with results obtained with similar oxidase preparations (166). However, Fig. 27 also shows that the rotational correlation time of MSL spin-labeled DOC-oxidase is >1 ms (V_2' spectrum).

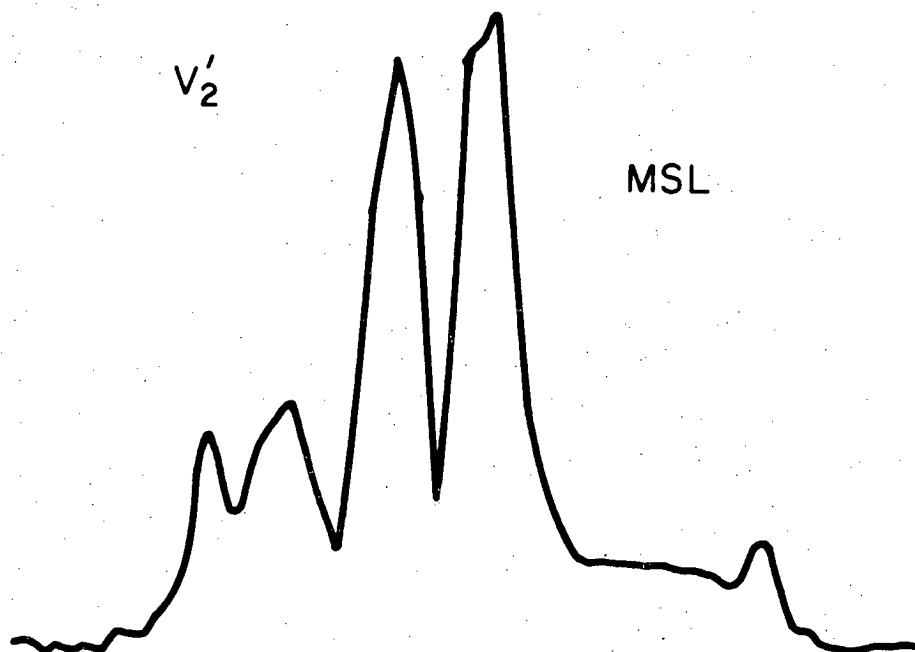
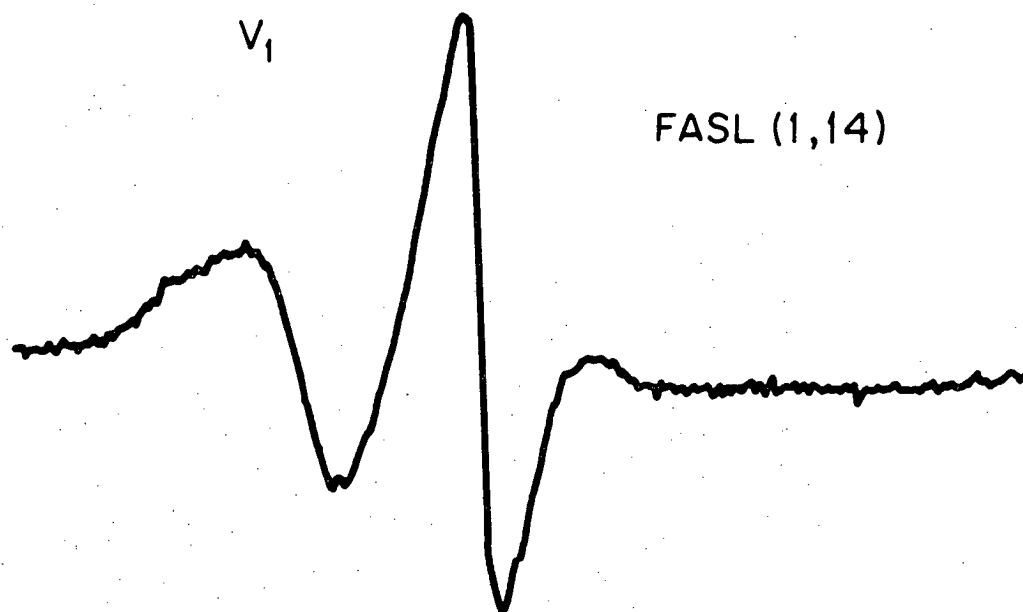
Rotational Correlation Times of Purified and Reconstituted Triton-oxidase- A preparation of Triton-oxidase was spin-labeled and either (a) chromatographed and then treated with cysteine and ferricyanide or (b) directly treated without chromatography. Both preparations were then precipitated at 0.35 saturation of ammonium sulfate and redissolved in detergent-containing buffer. These two types of spin-labeled Triton-oxidase were then reconstituted using partially purified asolectin or mitochondrial lipids by the cholate dialysis procedure.

Fig. 26. Conventional(V_1) spectra of the purified(in detergent) and reconstituted cytochrome oxidase(50 μ M) preparations spin-labeled with MSL. The arrows designate the weakly immobilized signal present at 22°C in the Triton-oxidase preparations reconstituted by the cholate dialysis method.



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Fig. 27. A conventional(V_1) spectrum of added FASL(1,14) and second harmonic(V_2') spectrum of covalently bound MSL to purified DOC-oxidase (50 μ M) in 50 mM KH_2PO_4 pH 7.5, 0.25% Tween 20, prior to lipid addition.



Similar results were obtained by our modification of the sonication procedure(201).

Saturation transfer spectra of the four types of preparations are shown in Fig. 28; indicated are the parameters L , L'' , C , and C'' used to estimate the rotational correlation times. The Triton-oxidase, which was chromatographed following spin-labeling, possessed an effective rotational correlation time(τ_2) of about 100 ns, as determined from the above parameters of its spectrum(see Fig. 29). This is the correlation time predicted for a sphere with a molecular weight of ~200,000 at 4°C(16). This preparation contains only 4% lipid (by weight), and the protein's estimated MW is 140,000(17); it appears therefore that the chromatographed Triton-oxidase exists in a disaggregated form, in agreement with analytical ultracentrifugation studies in Triton X-100(22) and Emasol 1130(32).

The Triton-oxidase that was not chromatographed prior to cysteine and ferricyanide treatment displayed a rotational correlation time of >1 ms, indicating protein aggregation. Since a large part of this preparation aggregated on the Sephadex G-25 gel filtration column when chromatographed, and could only be solubilized by continued elution, it appears that the enzyme exists in a heterogeneous mixture of particle sizes and that gel filtration in detergent slowly eliminates the higher molecular weight aggregates from the population.

As can be seen in Fig. 28, the Triton-oxidase which was mobile in the chromatographed form remained mobile when reconstituted($\tau_2 \sim 40 \mu s$), while the immobilized unchromatographed preparation remained immobilized when reconstituted. The vesicles made from the immobile and the mobile

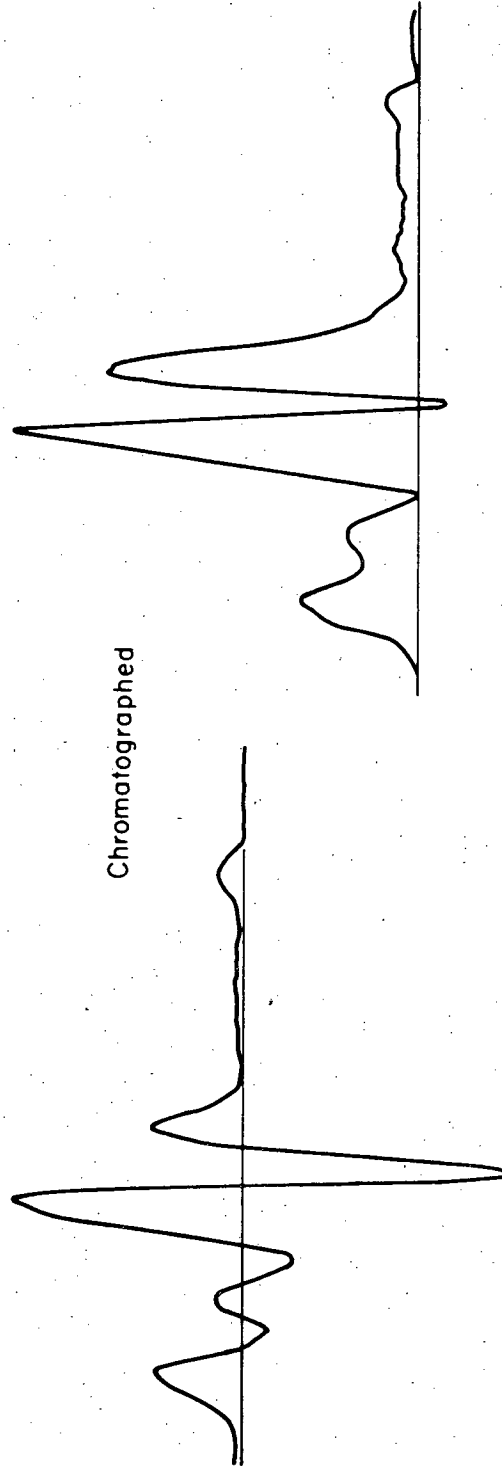
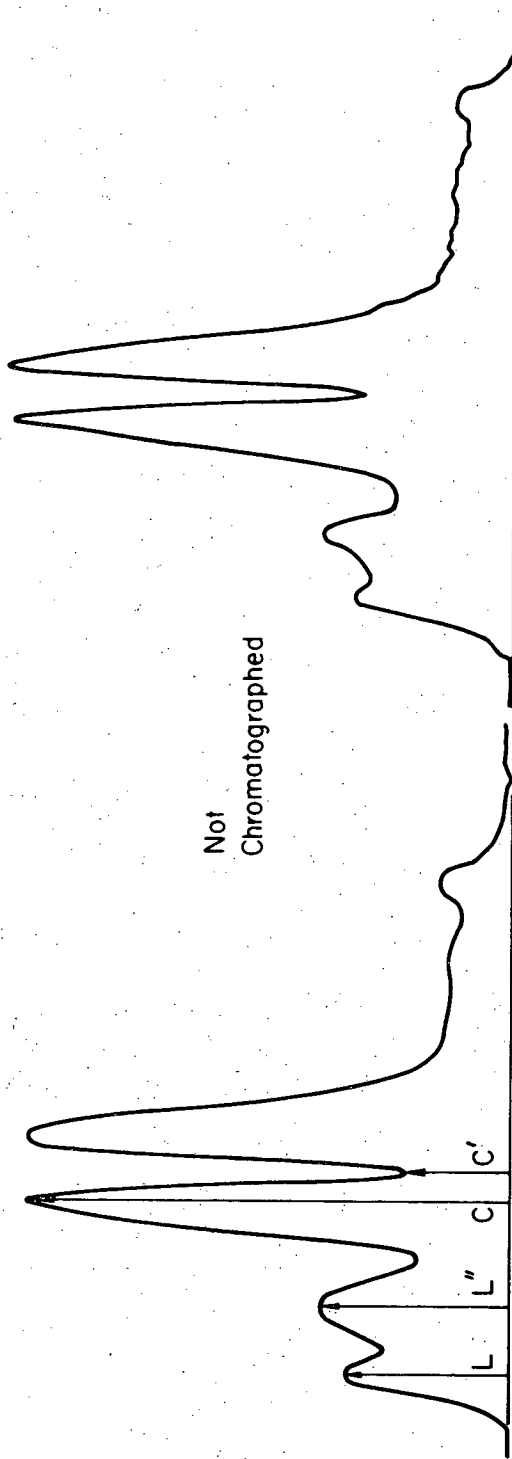
Fig. 28. Second harmonic(V_2') spectra of purified and reconstituted Triton-oxidase preparations which were either not chromatographed or chromatographed after spin labeling. The spectral parameters L , L' , C , C' are used to calculate effective rotational correlation times as described in Methods.

PURIFIED

RECONSTITUTED

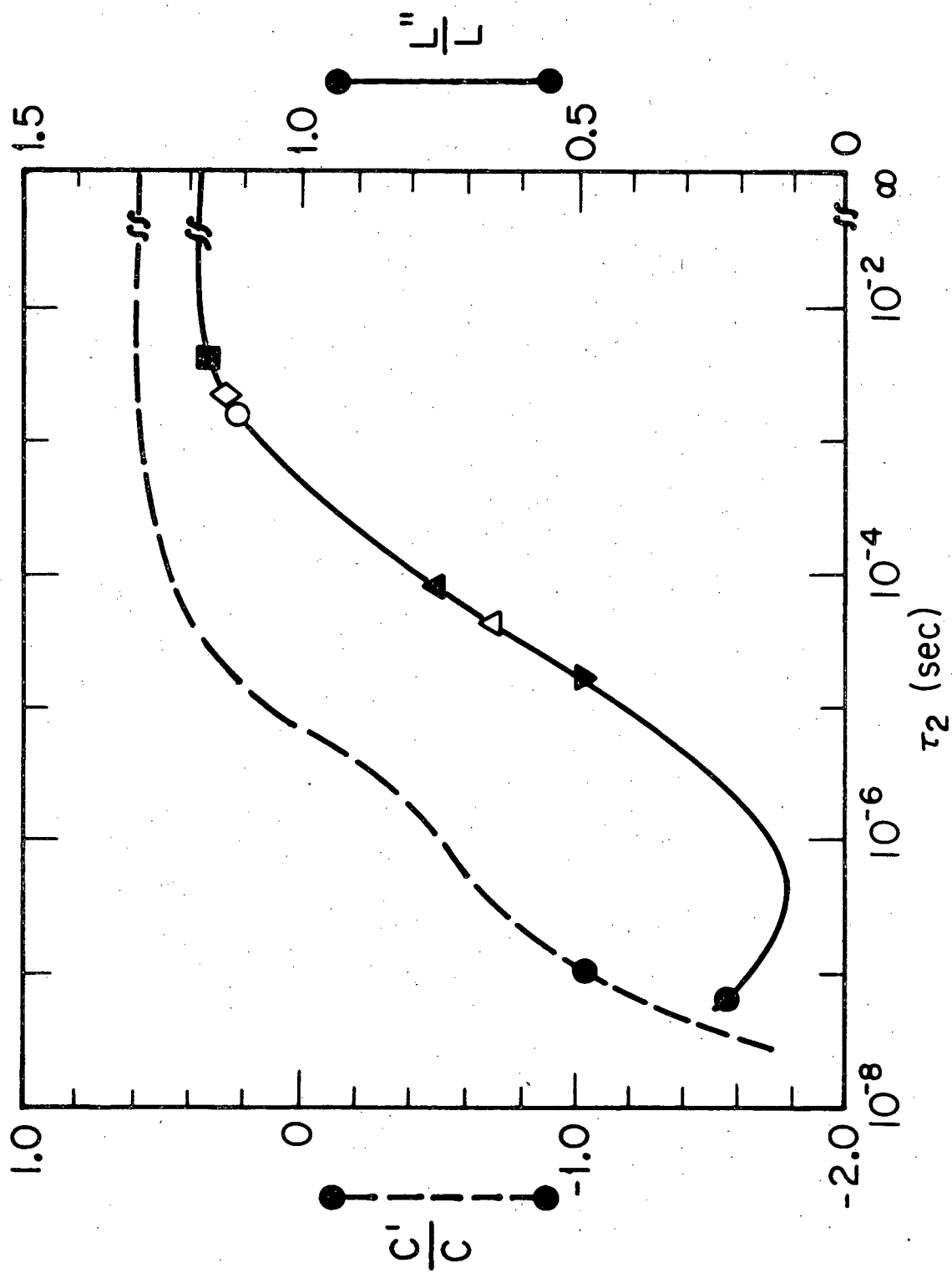
Not
Chromatographed

Chromatographed



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Fig. 29. Plots of the spectral parameters C'/C and L''/L and their relationship to the rotational correlation time, τ_2 , illustrating the procedure used to determine effective correlation times for nitroxide spin label saturation transfer EPR spectra(V_2'). The curves were obtained from theoretical studies and model system experiments on isotropic rotational diffusion(203). Purified Triton-oxidase(in detergent) after chromatography($\bullet\text{---}\bullet$); Purified DDC-oxidase(in detergent) after chromatography($\circ\text{---}\circ$); Triton-oxidase, chromatographed and then reconstituted($\Delta\text{---}\Delta$); Triton-oxidase, not chromatographed and then reconstituted($\blacksquare\text{---}\blacksquare$); rhodopsin, as determined by(188)($\blacktriangledown\text{---}\blacktriangledown$); Ca^{++} -ATPase, native lipids($\blacktriangle\text{---}\blacktriangle$); Ca^{++} -ATPase, lipids replaced with dipalmitoyl lecithin($\blacklozenge\text{---}\blacklozenge$). Both of the Ca^{++} -ATPase values were determined by(214).



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preparations were quite similar in size with diameters of $410 \pm 30 \text{ \AA}$ and $420 \pm 30 \text{ \AA}$ respectively (Fig. 24). Both preparations were fully active ($V_{\text{max}} = 400 \text{ electrons/s}$) and possessed high respiratory control ratios (in some cases close to 5), indicating that most of the enzyme was functionally incorporated (193).

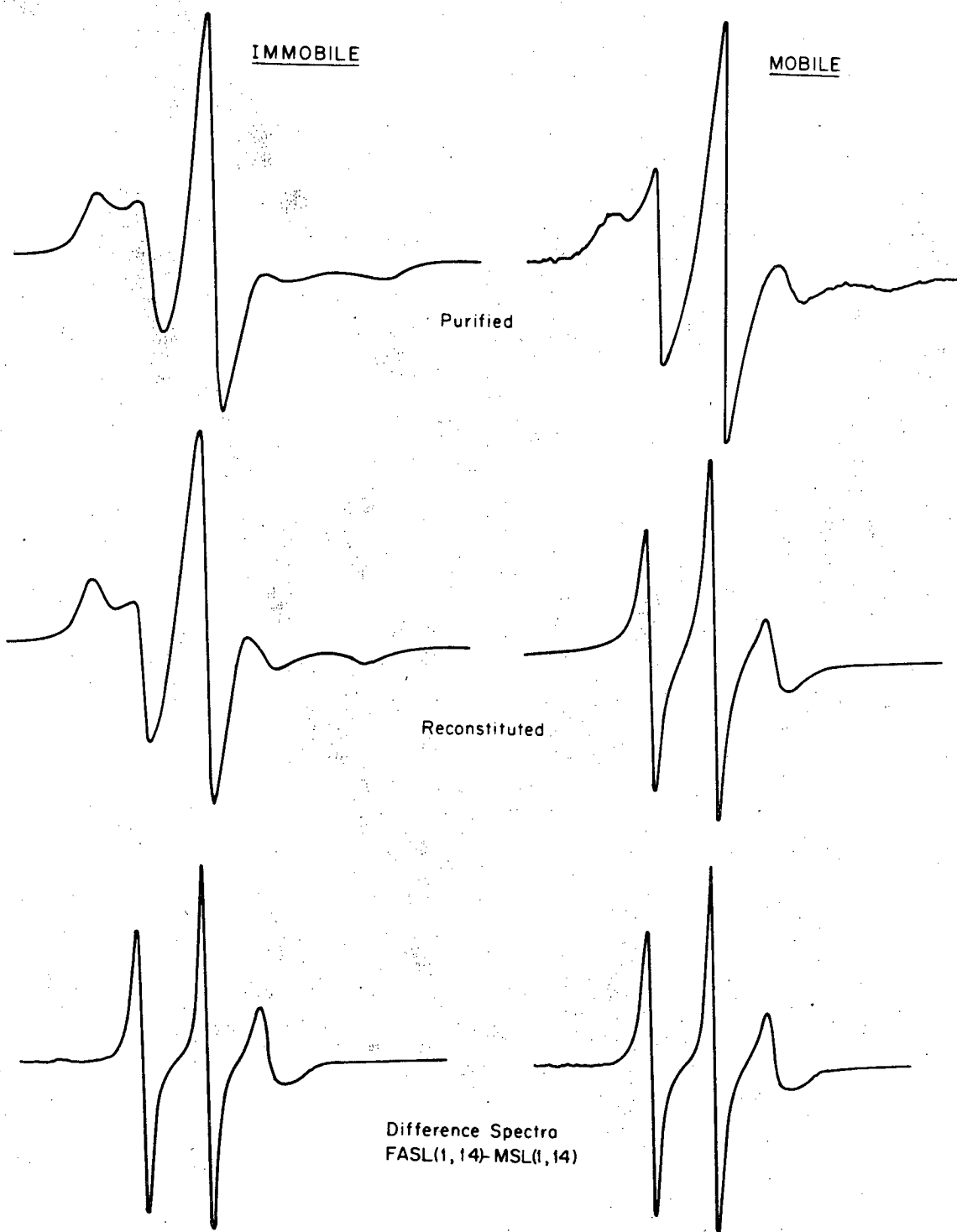
The rotational time of reconstituted cytochrome oxidase appeared to be independent of vesicle mobility, since centrifugation of the vesicle suspension to form a compact gelatinous pellet did not alter the V_2' spectra. Treatment of the mobile Triton-oxidase in reconstituted vesicles, at low oxidase concentration (0.38 mg/ml) with the bifunctional imidoester, dimethyl suberimidate, at a final concentration of 10 mM does not result in a further immobilized spectrum. Electron micrographs of these vesicle preparations indicate that no inter-vesicular crosslinking occurs under these conditions, whereas SDS gel electrophoresis demonstrates extensive polypeptide coupling of all the oxidase subunits, except for subunit I (MW 35,500) (217). Since the MSL spin probe is bound to either subunit II or III, our crosslinking results indicate that we are not observing the independent rotational motion of these subunits. Under these conditions no inter-cytochrome oxidase crosslinking is believed to occur (217). Glutaraldehyde treatment (40 mM final concentration) of concentrated vesicle suspensions (5 mg/ml) does result in an immobilized V_2' spectrum of the mobile preparation (τ_2 increases from $40 \text{ \mu s}$ to $>1 \text{ ms}$); glutaraldehyde treatment of the immobile reconstituted oxidase did not appreciably alter the V_2' spectrum (L''/L increased from 1.20 to 1.25).

A summary of the saturation transfer data obtained for the various

preparations of cytochrome oxidase used in this study (Fig. 29), shows how effective correlation times were estimated (214). The spectral parameter C'/C is especially sensitive in the 10^{-6} to 10^{-8} s range, while L''/L is sensitive in the range 10^{-3} to 10^{-6} s.

Lipid Mobility Correlates with Protein Mobility- Since the reconstituted Triton-oxidase vesicle preparations contained a lipid to protein molar ratio of >3000:1, it is virtually impossible to selectively study the lipid adjacent to the protein using free spin probes of the lipid phase. Therefore a lipid probe was used that was covalently attached to the protein, MSL(1,14). The nitroxide group of this probe is attached near one end of the acyl chain and the other end reacts with the sulfhydryl group at pH 7.0 through the N-ethyl maleimide linkage. This probe has been used to study the lipid environment of rhodopsin in retinal disk membranes (188). MSL(1,14) was added at a molar ratio of 2:1 to aa_3 . Determinations of the total spin bound to the enzyme, following elimination of the non-covalently bound MSL(1,14), were performed as in (188) and lipid was then extracted by the alkaline chloroform-methanol procedure described in Methods. These results indicate that $90 \pm 5\%$ of the total spin was bound to the protein portion of the enzyme. As can be seen in Fig. 30, MSL(1,14) is immobilized on both the chromatographed (mobile protein) and the non-chromatographed (immobile protein) purified Triton-oxidase, but a greater proportion is immobilized on the non-chromatographed preparations. When these two types of Triton-oxidase were reconstituted, the preparation containing mobile protein displayed no evidence of a strongly immobilized MSL(1,14) (Fig. 30, center right), whereas the

Fig. 30. Conventional(V_1) spectra of MSL(1,14) covalently bound to Triton-oxidase. The difference spectra of FASL(1,14)-MSL(1,14) at the bottom of the figure were obtained by computer subtraction using a PDP 11/34 computer attached to the EPR recorder. Purified $\underline{aa}_3$ concentration was 100 μM in 50 mM KH_2PO_4 pH 7.5, 0.25% Tween 20, 0.05% cholate. Reconstituted $\underline{aa}_3$ concentration was 50 μM in 50 mM KH_2PO_4 pH 7.5.



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preparation containing immobile protein showed a large amount of immobilized MSL(1,14)(Fig. 30, center left). The spectra of added FASL(1,14)(free fatty acid) to these preparations(molar ratio of FASL(1,14):oxidase was 1:1) are depicted at the bottom of Fig. 30. As expected, neither Triton-oxidase vesicle type immobilized a significant fraction of this free fatty acid label at these high lipid to protein ratios. It is interesting to note that the spectrum of the MSL(1,14) in the mobile Triton-oxidase(Fig. 30, center right) is slightly broader than that of the FASL(1,14) - MSL(1,14) difference spectrum in the same preparation(Fig. 30, bottom right). Thus, although the adjacent hydrocarbon chains are not strongly immobilized by the protein, they are slightly less mobile than the lipid chains far from the protein. The hyperfine splitting of the covalently labeled MSL(1,14) differs at most by 0.2 gauss from that of FASL(1,14) in the reconstituted systems indicating that the two probes are probably in similar hydrophobic environments.

DISCUSSION

The rotational mobility of isolated and reconstituted cytochrome c oxidase from beef heart mitochondria has been studied and correlated with the fluidity of the hydrophobic environment adjacent to the protein. Prior to chromatography, the spin-labeled complex never displays rotational motion in the sub-millisecond range of correlation times (Fig. 28). Chromatographed DOC-oxidase, which contains considerably more lipid and contaminant polypeptides (Fig. 21) than the Triton-oxidase preparation, has a tendency to vesiculate and/or form enzyme sheets even in detergent, and shows no sub-millisecond rotational motion. However, chromatographed preparations of spin-labeled Triton-oxidase, in the presence of detergent, display rotational correlation times in the 100 ns range (Fig. 28), indicating that the enzyme is probably disaggregated. These results suggest that, as isolated, cytochrome oxidase is probably in the form of aggregated patches (as previously observed, Reference 165), and that chromatography in the presence of detergent can, under certain conditions, disaggregate these patches.

Functional incorporation of cytochrome oxidase into lipid vesicles was achieved at high lipid to protein ratios (12.5:1, by weight), by the cholate dialysis or sonication procedures, but this did not seem to modify the aggregation state of the oxidase. Following the incorporation of non-chromatographed samples of cytochrome oxidase into liposomes, the rotational correlation time of the protein remains >1 ms; incorporation of chromatographed oxidase into liposomes results in a rotational correlation time in the 40 μ s range, indicating that

the oxidase probably remains in a disaggregated form in the membrane.

The activity of the complex in the reconstituted preparations does not seem to depend on its rotational correlation time; the respiratory control ratios are also very similar in the two kinds of reconstituted preparations and so are the lipid to protein ratios of the various populations of vesicles obtained in the Ficoll gradients. These results are independent of whether mitochondrial or partially purified asolectin is used for reconstitution. Therefore, sub-millisecond rotational mobility of cytochrome c oxidase is not required for electron transfer from cytochrome c (its natural donor) to oxygen). Aggregated patches of the complex will efficiently transfer electrons from cytochrome c to O_2 and may in fact be present in the inner membrane of mitochondria, as previously suggested(9).

In contrast to non-chromatographed oxidase, both Ca^{++} -ATPase (MW ~100,000) from sarcoplasmic reticulum(214,218) and rhodopsin (MW ~40,000)(219) display a high degree of sub-millisecond rotational mobility both in native and reconstituted membranes(Fig. 29). In addition, when the mobility of the Ca^{++} -ATPase is inhibited, the enzyme activity is also inhibited. Like the immobile cytochrome oxidase preparations, spin-labeled recombinant acetyl choline receptors have no sub-millisecond rotational mobility, indicating protein aggregation(220).

The results with reconstituted cytochrome oxidase covalently labeled with MSL(1,14) at a lipid to protein ratio of 12.5:1(by weight) indicate that the hydrophobic environment of the oxidase is very viscous in the aggregated form, and very fluid in the disaggregated form(Figs. 28 and 30). It is difficult to identify precisely the region around

the oxidase sampled with this probe; the maximum distance from the maleimide moiety to the nitroxide radical is about 30 Å and therefore no more than the first three layers of lipid around the enzyme are sampled by the probe. It is not surprising that, in the aggregated form, the immediate environment of the oxidase should be so viscous; in this case only a few lipid molecules are expected to be trapped between the oxidase complexes with their fatty acid chains in a constrained environment.

Earlier EPR studies with spin-labeled fatty acid chains and lipids in cytochrome oxidase preparations have shown a high degree of immobilization of lipids at low lipid to protein ratios(165-167). The results obtained with DOC-oxidase and the fatty acid spin probe FASL(1,14)(Fig. 27) have confirmed this. In the previous studies (165-167), the amount of strongly immobilized lipid was shown to be proportional to the amount of protein, and the results were interpreted to mean that each protein independently immobilized a constant number of lipid molecules. This was visualized as a boundary layer of strongly immobilized lipid. An equally probable explanation for these results could have been the immobilization of lipid trapped within protein aggregates which do not disaggregate at high lipid concentrations. The latter explanation is supported by the results of the present investigation. Furthermore, in the disaggregated form of the reconstituted enzyme, the fluidity of the hydrophobic environment around the oxidase measured with the MSL(1,14) probe is only slightly less than the fluidity of the bulk lipid hydrophobic environment measured with the FASL(1,14) probe(Fig. 30).

Theoretical considerations(221) and NMR data(168,222-224) suggest that, if a boundary layer of lipids exists around proteins then, compared to the bulk lipids, its fatty acid environment may be more fluid below the bulk lipid phase transition and less fluid above the phase transition. The protein may act like an impurity or defect in the lipid lattice, altering somewhat the ordering of the fatty acid chains near its hydrophobic environment. Dahlquist and coworkers (222,226) have performed deuterium NMR experiments on a DOC-purified oxidase preparation. They found evidence for two distinct populations of lipid hydrocarbon chains, one strongly immobilized and one approximately as mobile as pure lipid in the absence of protein. Under similar conditions of preparation, the DOC-oxidase used in the present investigation is aggregated. Oldfield et al(168) performed analogous NMR experiments on a cholate-purified cytochrome oxidase preparation and have not detected an immobilized phospholipid population. If this latter preparation contained disaggregated enzyme, then the discrepancy between these two NMR studies may be explained by our analysis; that is, a distinct strongly immobilized population of hydrocarbon chains exists only when the enzyme is aggregated.

A similar situation arises in rhodopsin-containing membranes(188), where the immobilization of the protein and lipid occur only at low lipid to protein ratios.

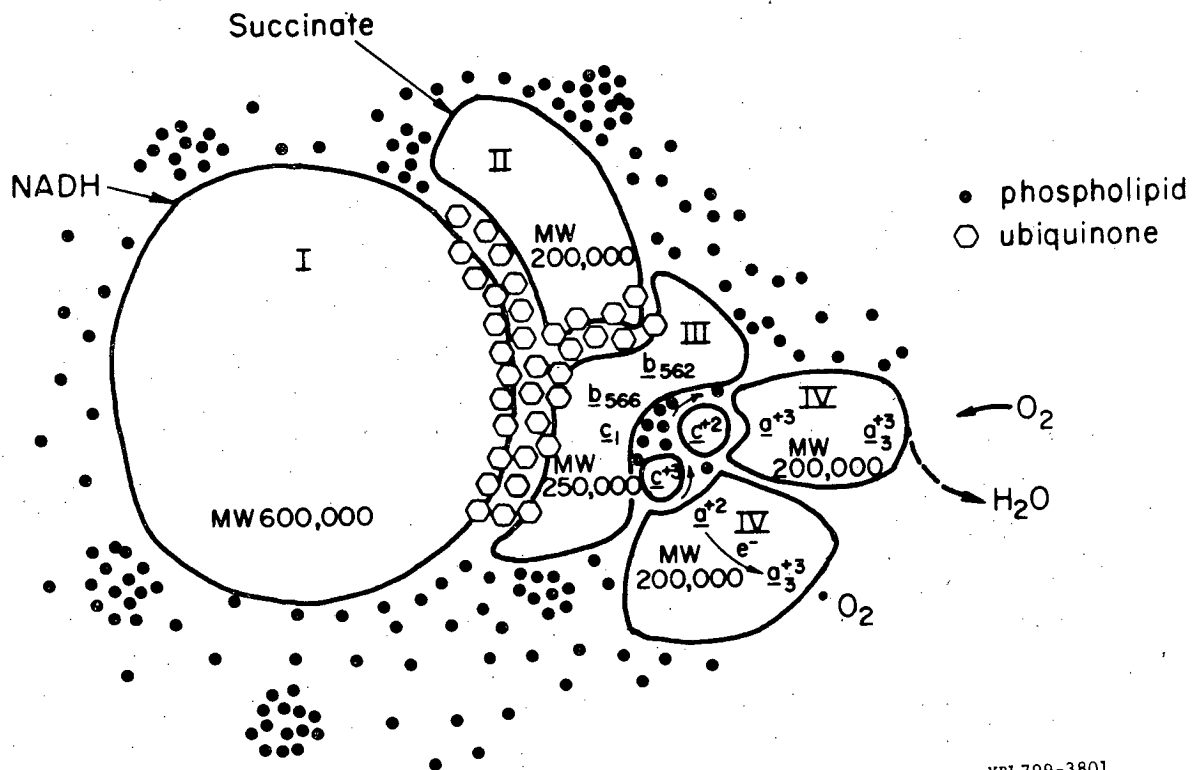
GENERAL DISCUSSION

The results of this investigation on the membrane organization of cytochrome c oxidase have indicated that the oxidase complex is a closely-knit functional unit which requires a certain degree of polypeptide flexibility for electron transport activity. Crosslinking between oxidase molecules to form large aggregates does not affect activity inhibition by biimidates whereas covalent coupling of oxidase to complex bc₁ does. Furthermore, it appears that oxidase-mediated electron transport is not affected by immobilization of the protein in a lipid bilayer, and lipid fluidity within the oxidase complex is not required for activity.

These results suggest a model for the organization of cytochrome c oxidase within the mitochondrial inner membrane as shown in Fig. 31. The electron transport chain is illustrated as being composed of closely associated protein-lipid complexes with ubiquinone mediating electron transport between complexes I, II, and III. The ratio of complex I:II:III:c:IV is shown as 1:1:1:2:2 based upon(147,148). Although this diagram shows only one electron transport chain it is likely that many chains are clustered together, as shown by electron microscopy using ferritin-labeled immunoglobulins monospecific for cytochrome oxidase(9). Cytochrome oxidase is illustrated as being a functional dimer based upon the inner membrane cytochrome content(147,148), analytical ultracentrifugation(14), saturation transfer EPR(16) and crosslinking studies. The oxidase complex is also shown in two different conformational states. The partially reduced oxidase(a⁺², a⁺³) is illustrated as being more compact based upon analytical ultracentrifugation(23) and chemical modification(217) studies. Cytochrome c is depicted to be

Fig. 31. An illustration of the possible organization of the mitochondrial electron transport chain. The conformations of the complexes are arbitrary.

THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN



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mobile as explained in Fig. 30. Lipid organization of the inner membrane is shown as being non-uniform. The various transition temperatures(114) and micro-environmental effects of mono- and divalent cations(98), and protein concentration(169) might dictate whether certain lipids, such as diphosphatidylglycerol(74), will be dispersed or clustered.

Implications of the Model- The most obvious implication of the model is that the inner mitochondrial membrane is not organized as a fluid mosaic of protein and lipid. Since cytochrome oxidase, and possibly the other respiratory complexes, do not undergo rotational motion on the sub-millisecond time scale, then electron transport activity requires that the reduction-oxidation centers of the complexes be within a few angstroms of each other. If the complexes are immobilized, one way to modulate electron flow down the chain is for the proteins to assume different conformational states so that, for instance, the hemes are apart under conditions of high energy charge so that electronic communication between them is inhibited. Another way in which electron movement down this pseudo 'solid-state' electron chain is for some components of the chain, such as ubiquinone and cytochrome c, to be mobile. The function of these mobile carriers is to regulate the amount of energy generated by electron flow down the potential gradient. For instance, when energy charge is high, ATP binds to cytochrome c and restricts its binding to both the reductase and oxidase complexes(227).

SUMMARY

- 1) The dynamics of electron movement within the mitochondrial inner membrane appears to require that the electron transport chain complexes be closely associated.
- 2) In the present study, the relationship of intracomplex, intercomplex, rotational, and lipid versus protein motion to the membrane organization of cytochrome c oxidase has been studied.
- 3) Crosslinking of purified or reconstituted cytochrome oxidase, which results in the covalent coupling of subunits II-VII, is much more inhibitory to steady-state oxidase activity than monoimide treatment.
- 4) Since the chain length of the biimides employed in this study is 11 Å or less, these oxidase subunits are closer to one another than this distance.
- 5) At lower extents of amine modification(<20%) biimide crosslinking of oxidase does not affect heme c-heme a electron transfer rates.
- 6) These results indicate that even though the subunits of the cytochrome oxidase complex are closely associated as a functional unit, a certain degree of polypeptide flexibility is required for activity.
- 7) The role of intracomplex motion has been studied in mitochondria and purified oxidase in the presence and absence of cytochrome c.
- 8) Crosslinking of mitochondria by biimides appears to be more inhibitory to steady-state oxidase activity in the case of mitochondria than for purified oxidase.
- 9) Biimide treatment of mitochondria results in the crosslinking of complexes bc₁ and aa₃ with cytochrome c.
- 10) Crosslinking of cytochrome c to c-depleted mitoplasts or purified oxidase resulted in a catalytically inactive complex.

- 11) These results suggest that the electron transport chain complexes are closely associated in the inner membrane, and that cytochrome c mobility is required for electron transfer between complexes III and IV.
- 12) The rotational mobility of cytochrome oxidase was measured by saturation transfer EPR.
- 13) The purified oxidase could be prepared in a form which displayed a high($\tau_2=100$ ns) or a low($\tau_2>1$ ms) degree of rotational mobility.
- 14) When reconstituted, the rotational mobility of membrane-bound oxidase depended upon its rotational mobility prior to reconstitution. However, the mobile and immobile enzymes had the same high activity.
- 15) Lipid motion within the oxidase complex was also studied by conventional EPR, and was found to be related to protein rotational mobility. If the protein was highly mobile, then the lipid was also quite fluid. Conversely, if the protein was immobilized on the sub-millisecond time scale, the lipid displayed a strongly immobilized spectral component.
- 16) These results have been used to construct models for the intramolecular arrangement of cytochrome oxidase polypeptide subunits, and the disposition of the oxidase complex within the electron transport chain.

APPENDIX I

Theoretical ESR Aspects

Conventional 1st Harmonics(53, 228)- All of the electron paramagnetic resonance work reported in this paper was performed using stable nitroxide radicals. As previously mentioned, we are interested in examining both rapid (10^{10} to $5 \times 10^8 \text{ s}^{-1}$), intermediate(5×10^8 to 10^7 s^{-1}), and slow(10^7 to 10^3 s^{-1}) motions of these radicals when they are covalently bound to protein and when they are free. In order to describe these types of motions it is necessary to provide a theoretical foundation for data analysis for both rapid(1st harmonic) and slow(2nd harmonic) motions(203).

Conventional EPR is capable of detecting motion in the time range 10^{10} to 10^7 s^{-1} , and it is useful in determining the motion of free and bound(whether covalently or not) populations of nitroxides. Basically, analyses of motion in both conventional and second harmonic EPR depend on spectral changes in line widths and shapes which can be related to specific rates of motion by theoretical and empirical analysis.

EPR is a spectroscopic technique which allows detection of electronic transitions between the Zeeman levels of a paramagnetic system positioned in a static magnetic field. EPR detection of nitroxide radicals therefore depends on the intrinsic angular momentum of the unpaired electron spin. Contributions to non-zero angular momentum, J , results from spin angular momentum, S , and orbital angular momentum, L . The magnetic moment of an atom in an electronic state defined by the factors L , S , and J is dictated by quantum theory as:

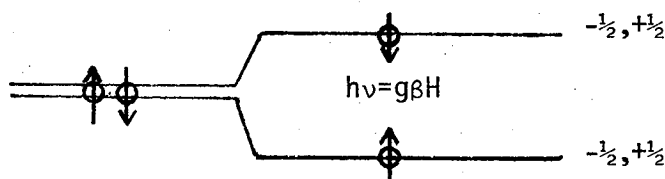
$$\mu = -g\beta_e J$$

$$\text{where } g = \frac{1 + J(J + 1) + S(S + 1) - L(L + 1)}{2J(J + 1)}$$

β_e = Bohr magneton, $|e| \hbar / 2mc$

g = spectroscopic splitting factor

When radicals are situated in a constant magnetic field of strength, H_0 , the unpaired spins orient themselves in relation to the field. This is because electron spins are quantized and are allowed only certain orientations in the field, each corresponding to a discrete energy. There are only two allowed orientations given for the unpaired electron, the spin-up or parallel and spin-down or anti-parallel projection. This is depicted below in an energy diagram:



As shown, in the absence of a magnetic field both orientations possess equal energy and thus are equally favorable. The imposition of a magnetic field, H_0 , results in the spin-down possessing considerably more energy; it thus becomes the less favorable orientation. The factor which makes it possible to use this phenomenon to study atoms in various states results from the fact that electromagnetic radiation can induce transitions in this system from the spin-up to spin-down projection. The basis for the spectroscopic transition is given by:

$$h\nu = g\beta H$$

where h = Planck's constant

β = Bohr's magneton

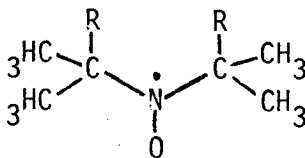
g = 2.0023 for a free electron

The g -value defines the spectral absorption position for a particular magnetic species, but usually deviates (from 2.0023) because of orbital magnetism which arises from electron orbital motion, which in turn is dependent on the molecular environment of the electron. That is perpendicular superposition of an electromagnetic field of frequency ν will result in spin reorientation and equalization of the population of the two Zeeman levels if the energy of the field ($h\nu$) equals the energy required for reorientation. EPR as a spectroscopic technique relies on restoring a spin system perturbed away from thermal equilibrium (relaxation) by the induction of a magnetic field. As electromagnetic radiation is absorbed by the spin system this loss of energy ($h\nu$) is detected by the spectrometer and recorded much as a light spectrometer detects light absorption. Loss of absorbed electromagnetic radiation by the spin system depends on the environment of the electron or, in other words, how quickly it is able to lose that absorbed radiation and reabsorb more.

The ability of a spin system to lose this absorbed energy is characterized by its spin-spin (τ_1) and spin-lattice (τ_2) relaxation. Spin-spin relaxation time (τ_1) results from variation in local magnetic fields induced by surrounding electronic and magnetic moments. While this results in a frequency broadening of the resonance transition between the Zeeman levels, it does not change the average energy of the spins in these levels; this effects the width (broadness) of the absorption lines. Spin-lattice (τ_2) relaxation time is the result of spin

interactions with surrounding(lattice) electronic and magnetic fields of varying magnitudes and direction. This effects population distributions in the Zeeman levels, or the average energy of the spin system, and therefore alterations are induced in the absorption line height.

ESR Spectral Characteristics:Rapid Motion and EPR- Nitroxide free radicals containing a paramagnetic moiety are relatively stable and inert because of the protective effect of the four methyl groups as depicted below:



ESR spectra of derivatives of this structure are composed of three sharp and well-resolved hyperfine lines which result from the coupling of the unpaired electronic spin with the nuclear spin of the nitrogen. The unpaired electron is localized on a $2p\pi$ nitrogen orbital. These spectra are not only sensitive to molecular motion, but also the solvent in which they are dissolved. This arises from the fact the magnetic parameters of radical species are sensitive functions of molecular electronic distribution. The unpaired electron in the nitroxide radical is in the spherically symmetrical S state where it is delocalized over the oxygen and nitrogen. Isotropic hyperfine structure therefore results from spin interaction with the nitrogen nucleus which causes splitting of the EPR absorption lines into three components corresponding to a nuclear spin orientation along ($M=+1$),

perpendicular to ($M=0$), and against ($M=-1$) the constant magnetic field, where M is defined as the nuclear magnetic quantum number.

Anisotropic hyperfine structure results from the fact the electron can also be in an unsymmetrical P orbital where the type of electron-nuclear interaction is independent of the rotational angle between the radical and the magnetic field. The type of interaction thus becomes dependent on the angle between the direction of the p orbital axis and the constant magnetic field. The resonance condition for an electron interacting with a nucleus with a magnetic moment $M=+1,0,-1$ is changed by the additional local field induced by the nucleus. Anisotropic hyperfine structure is averaged to zero in a low viscosity medium by rapid thermal motion of the radical. In a system of randomly oriented nitroxide molecules each case possesses its own set of local fields induced by nuclei surrounding unpaired electrons due to varying orientation of the radical molecular axes relative to the magnetic field direction. In the case of rapidly tumbling molecules, the EPR linewidth narrows due to the fact that this anisotropic component is averaged out. Another type of spectral anisotropy, g factor anisotropy, is produced because of the influence of an asymmetrical electronic component and orbital magnetism results in g -factor deviation. This deviation is dependent on radical orientation and the intensity of its motion. When the radical is unable to undergo rotational and translational motion the simplified absorption spectrum is the result of the superposition of microspectra of a large number of radicals, all with different orientations of hyperfine and g -factor tensors relative to the applied magnetic field.

Slow Motion and EPR(229,230)- For the determination of rotational correlation times in the range 10^{-7} - 10^{-3} s⁻¹, spectra are obtained empirically and compared to theoretical analyses. In this time range the principle difficulty is contributions from anisotropic motion.

Slow motion may be studied by saturation of the spin system in three ways: 1) electron-electron double magnetic resonance(ELDOR); 2) steady-state saturation; and 3) passage or modulation spectroscopy. In ELDOR experiments, the saturation transferred from an intensely irradiated ('pumped') resonance frequency to a weakly irradiated ('observed') frequency is observed. This technique unfortunately suffers from very low sensitivity. Steady-state saturation involves observing changes in signal amplitude and lineshape which accompany microwave power increases, but these spectral changes are small compared to the signal amplitude. In passage, or saturation transfer, EPR the spin system is displaced from thermal equilibrium by partial saturation, and the transfer of saturation, as the spins diffuse between a saturated and unsaturated spectral position, is observed. Therefore this type of spectroscopy depends on the rate of spin passage through resonance as a result of an imposed modulation of the resonance condition. The modulation employed is a sinusoidal modulation of the Zeeman magnetic field or:

$$\frac{1}{2}H_m \cos(w_m t)$$

where H_m =peak to peak amplitude

w_m =frequency of modulation.

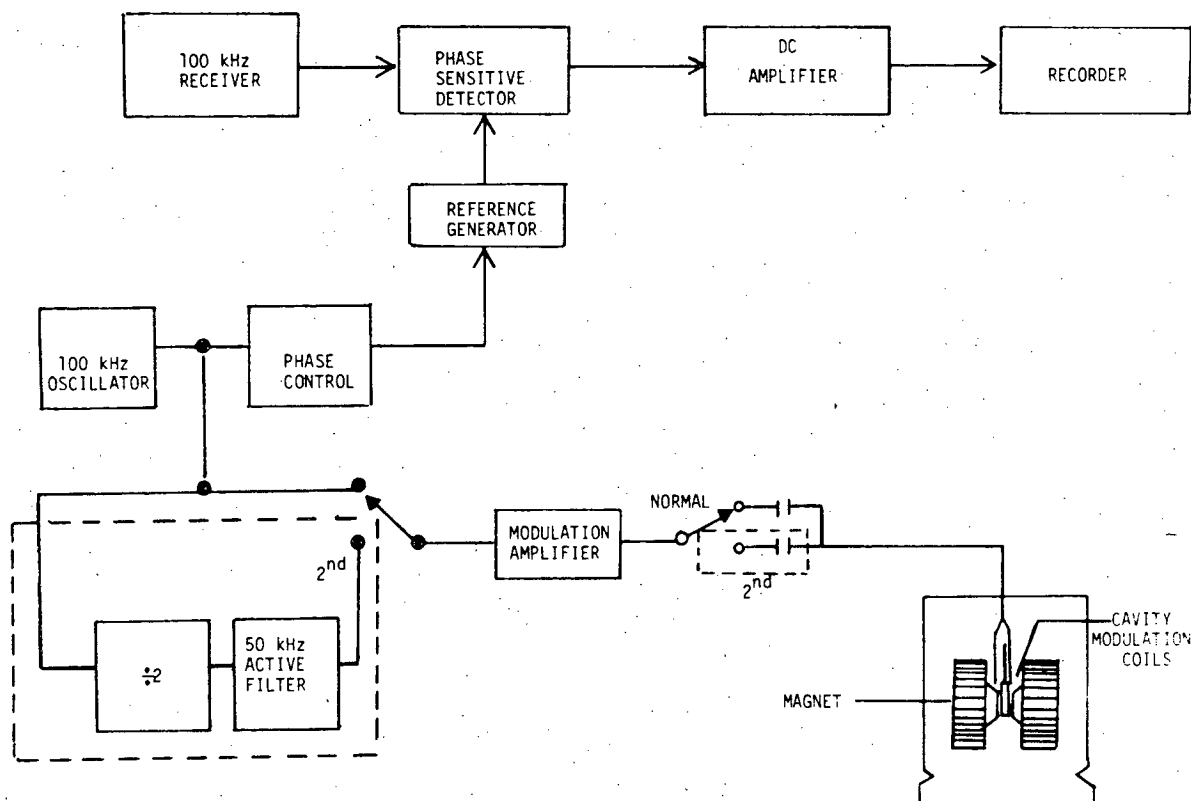
Therefore saturation transfer is based on the competition between field modulation and the rotational diffusion in regulating spin passage

through resonance. When $\omega_m^{-1} = \tau_2 = \tau_1$, the response is most sensitive to changes in τ_2 , and for nitroxides this equals $\approx 10^{-5}$ s.

Instrumentation: Saturation Transfer- Saturation transfer spectra may be recorded on a conventional spectrometer which is outfitted for second harmonic detection (as shown in Fig. 1.). Spectra are recorded in phase (V_1) and out of phase (V_2'), but the modification permits modulation of the magnetic field at $\frac{1}{2}$ the frequency of phase sensitive detection (second harmonic detection). In this case, the field is modulated at a frequency ($\omega_m/2\pi$) of 50 kHz and phase sensitive detection is used to observe the signal component at 100 kHz.

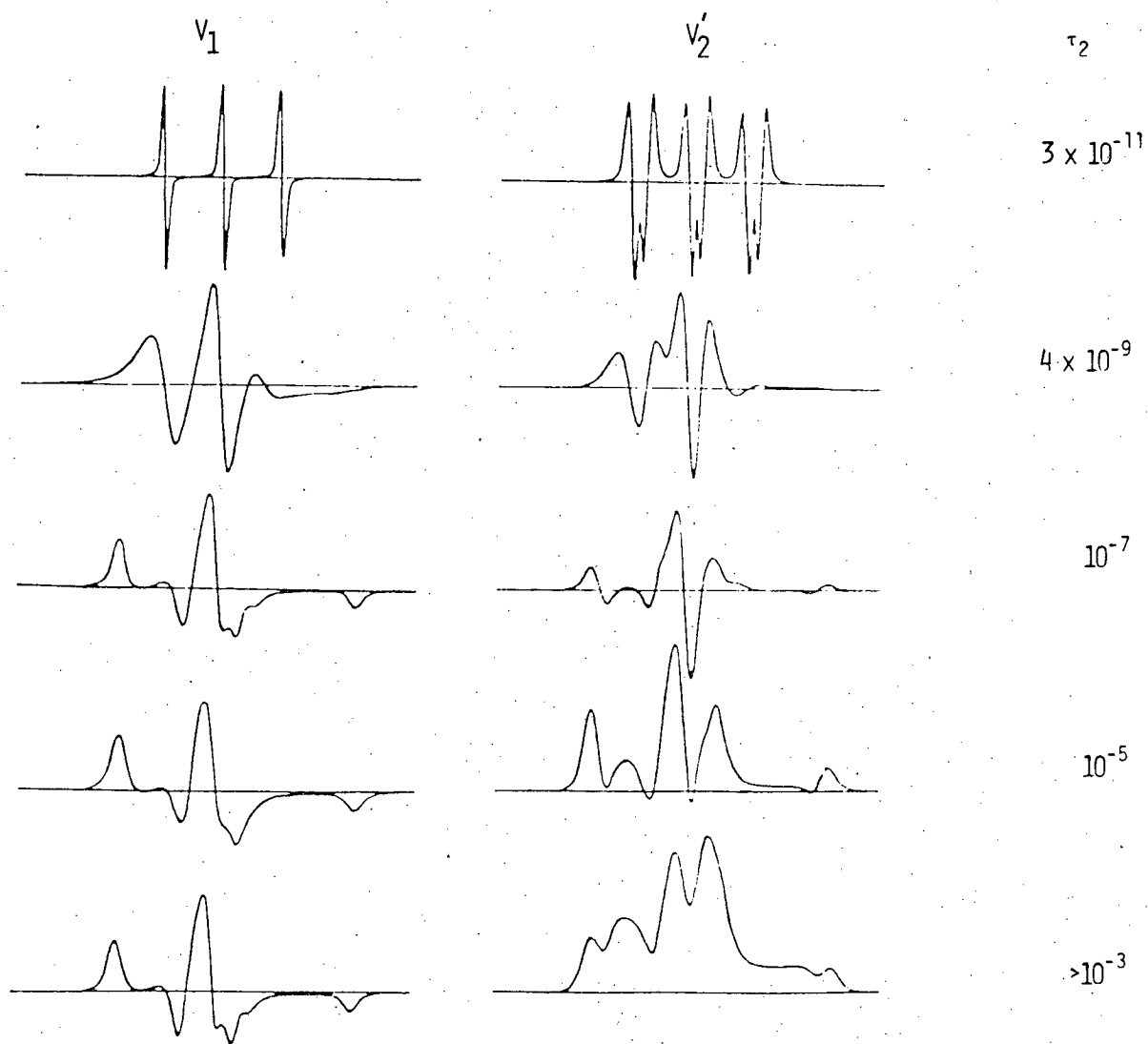
Spectral Analysis: Saturation Transfer- Thomas et al (203), using computer simulation and experiments with various free spin labels and spin-labeled proteins, have succeeded in analyzing second harmonic spectra in terms of rotational correlation time. Fig. 2 shows spectra obtained from a free spin label, Tempol, in water and increasing concentrations of glycerol, as well as a short-chain maleimide, which is strongly immobilized on hemoglobin. As can be seen the V_2' spectra undergo distinctive changes between 10^{-7} to 10^{-3} s, whereas the conventional V_1 spectra do not. Fig. 3 demonstrates that these workers have been able to correlate the spectral parameters C'/C , L''/L , and H''/H to the second harmonic correlation times (τ_2) of these various molecules.

Fig. 1.(Appendix I) Schematic diagram illustrating modulation and phase-sensitive detection circuits used for conventional and second harmonic analysis. The 100 kHz system is shown, and dashed lines denote components which are required for saturation transfer analysis, after Thomas et al(203).



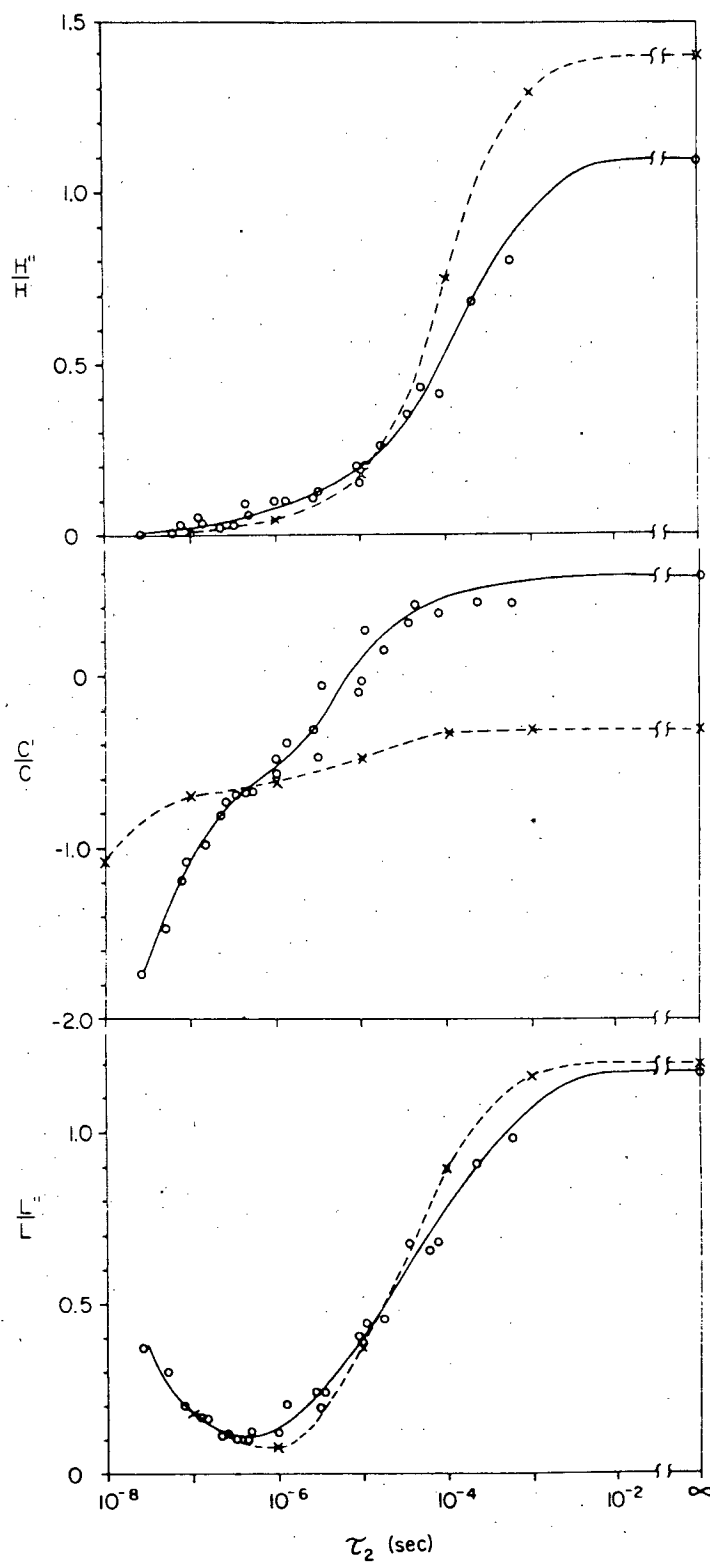
XBL 799-12041

Fig. 2.(Appendix I) Conventional(V_1) and second harmonic(V_2') spectra illustrating spectral changes associated with various rotational correlation times of isotropically tumbling nitroxide spin labels. The top two pairs represent a small spin label, tempol, in water & glycerol. The middle two pairs are from MSL-labeled hemoglobin in glycerol and water solutions while the bottom pair of spectra are from MSL-hemoglobin precipitated with $(\text{NH}_4)_2\text{SO}_4$, after Thomas(230)(used by permission).



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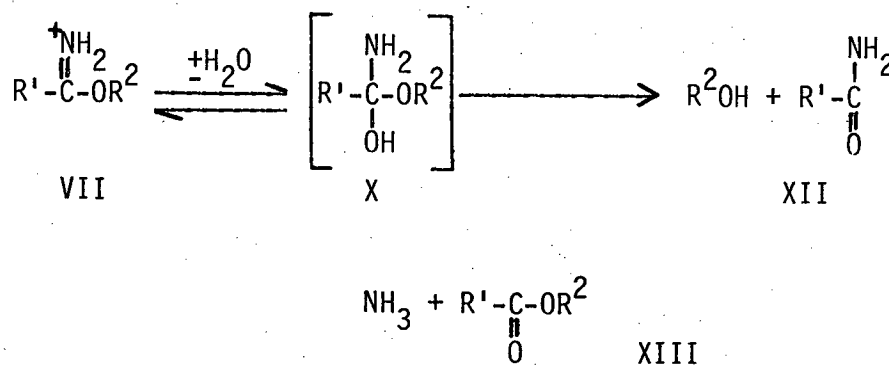
Fig. 3.(Appendix I) The spectral parameters C'/C , L''/L and H''/H plotted against the rotational correlation time (τ_2) determined from saturation transfer spectra(solid lines), and from theory and computer simulation(dotted lines), after Thomas(230) (used by permission).



APPENDIX II.

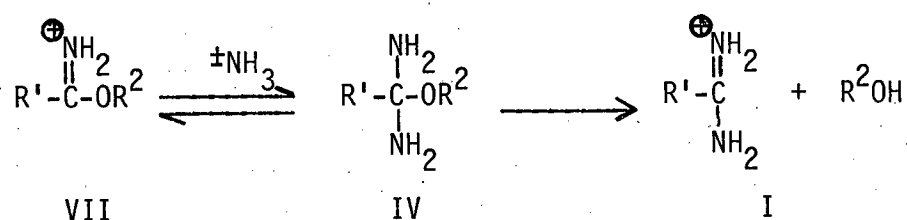
Problems Associated with Imidoesters as Crosslinking Reagents

Chemical modification reagents are useful tools for studying structure-function relationships in proteins, however they suffer from problems associated with reaction heterogeneity, charge alteration, and breakdown product interference. Imidoesters are particularly valuable for studying how the induction of motion restraints on polypeptide subunits of oligomeric enzymes affects enzyme activity. The addition of these amino acid modification reagents to a solution containing free amines results in a variety of products being formed depending on the structure and concentration of the imide, the nature of the attacking amine(s), temperature, pH, and buffer concentration. The starting imidoester will undergo various chemical reactions when added to an aqueous buffer such as hydrolysis (Roman Numerals are from (231)):

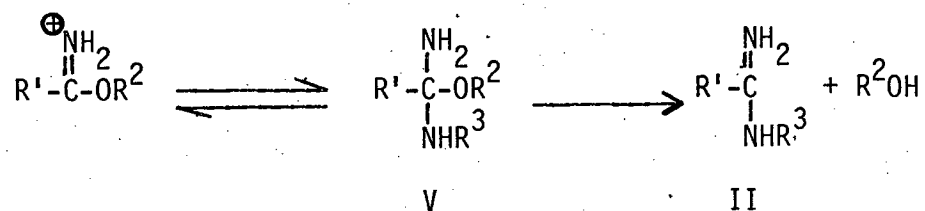


where the principle products are the amide and alcohol or the ester and ammonia.

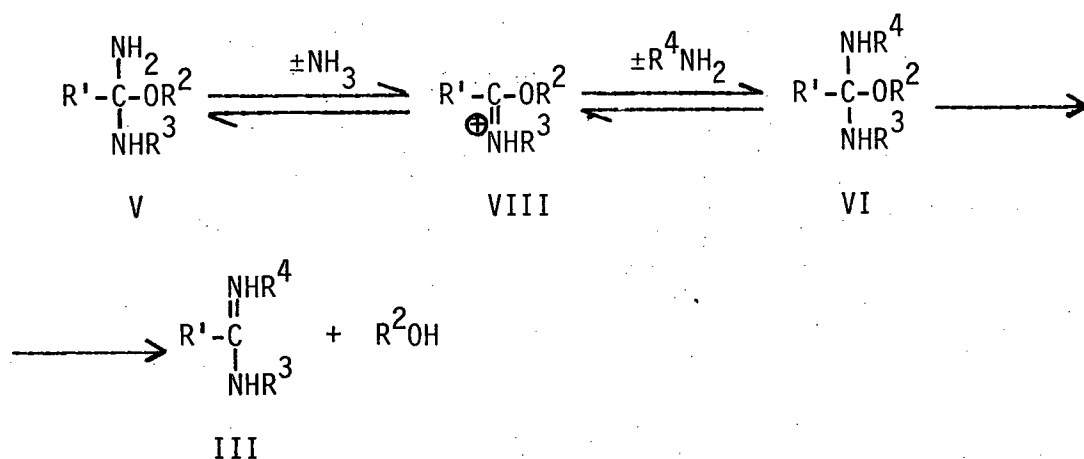
The ammonia which is generated may then react with the imidoester to form an amidine(I):



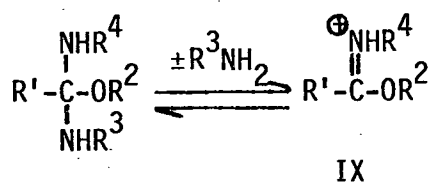
Reaction of the imidoester with a primary amine on a protein will generate another amidine(II):



This amidination reaction results in the covalent bonding of the imidoester to the protein. Unfortunately, other reactions may occur in that the intermediate amine may react with ammonia to generate the following series of reactions:



The tetrahedral intermediate can also react with another R^3NH_2 as follows:

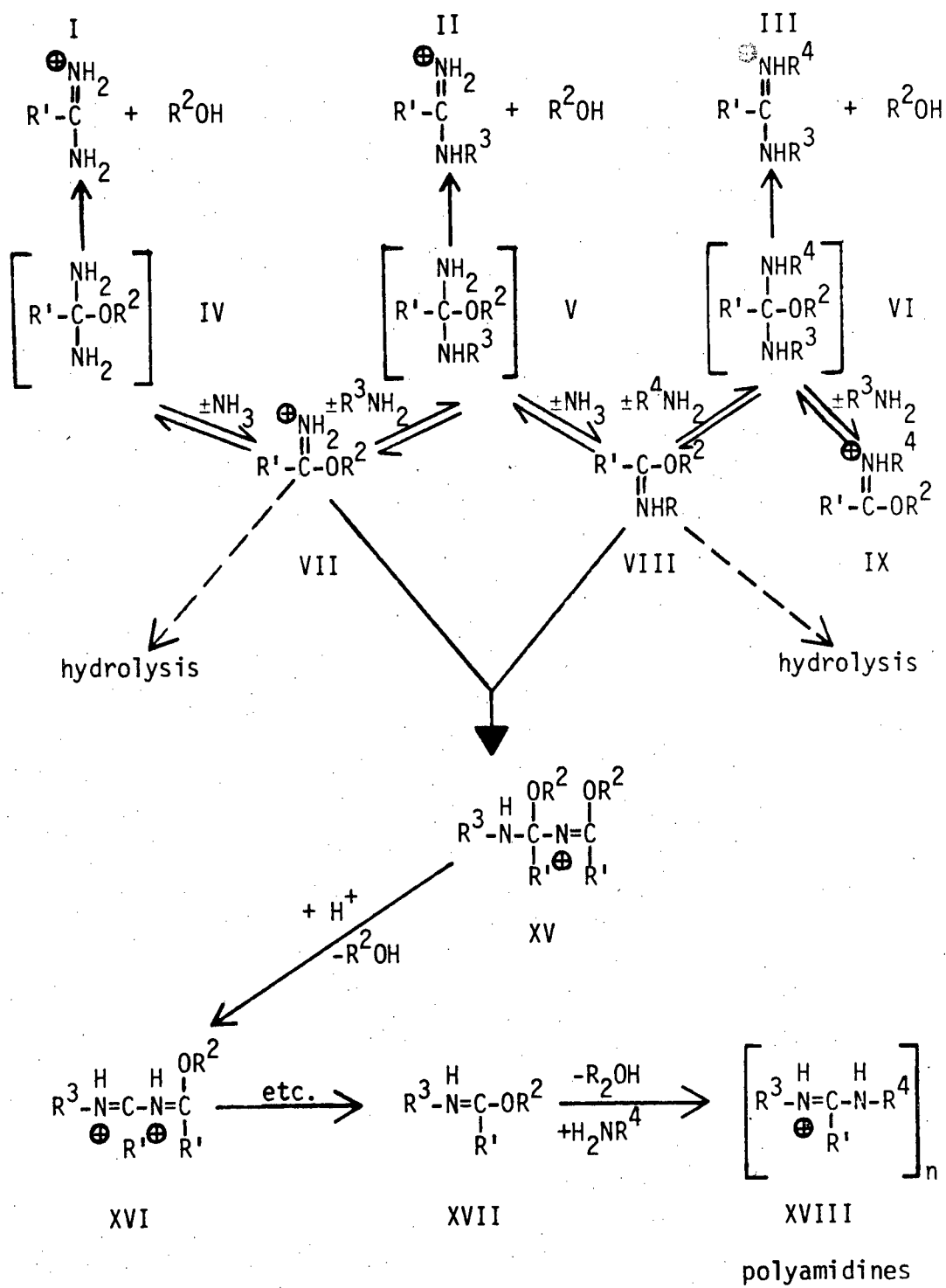


The VIII product may also undergo hydrolysis to form the alcohol and regenerate free R^3NH_2 .

Hand and Jencks(232) have demonstrated that the observed rate constant follows a bell-shaped curve for the disappearance of imidoesters in the presence of amines as a function of pH. The intermediates and side reactions described above are therefore strongly pH dependent. Low pH tends to favor hydrolysis, resulting in the formation of the ester XIII from the conjugate acid form of X or XI, and aminolysis favoring the formation of the imidates VII or VIII from the conjugate acid of V. High pH favors hydrolysis of the conjugate base of X or XI to form the amide XII and aminolysis of the conjugate base of V to form amidines I or II.

Browne and Kent(208) have employed proton NMR spectroscopy to determine the half-time of imidoester disappearance in aqueous solution at 22°C and 35°C. They found that at 0.2 M ethyl acetimidate (EA) the $T_{1/2}$ for imidoester disappearance was <5 min at pH 8.5 for both temperatures and at pH 10.0 at 22°C the $T_{1/2}$ was >25 min. Hunter and Ludwig(233) however found that the $T_{1/2}$ for methyl acetimidate(MA) disappearance in aqueous buffer at pH 8.5 was approximately 33 min. The kinetics of reaction product formation which resulted when either MA or EA were combined with primary amines, n-propylamine and ϵ -aminocaproate, at pH 8.0 and 10.0 were also studied. At lower pH, both imidoesters reacted very rapidly(the reaction was 85% complete <1 min) and essentially quantitatively to form N-alkyl imidates(VIII) from the primary amines(see Fig. 1). These products were then converted slowly($T_{1/2}$ = 4-7 min depending on the imidoester used) to yield

Fig. 1.(Appendix II) An illustration of the types of reaction products which can result when imidates are added to a solution containing primary amines, after (231,234).



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two parts ester(XIII) and one part N-alkyl acetamidine(II), however there was an initial lag of 1-2 min before II was produced in significant amounts. If the reaction of EA with ϵ -aminocaproic acid was carried out at pH 10.0 in 0.5 M borate buffer the yield of N-alkyl acetamidine was two to three fold higher(60% versus 20-30%) than at the lower temperature, and the formation of II was 90% complete in <3 min after mixing. However, these experiments were conducted using an initial amine concentration of 0.38 M and 0.40-0.46 initial imidate concentrations for EA and MA, respectively. At the lower imidate concentrations used in this study less ammonia is produced and therefore there is less competition with the hydrolysis reaction.

These results imply that at pH 8.5-9.0, where all of the imidate treatments reported in this dissertation were performed, hydrolysis to regenerate the protein amino groups is faster compared with amidine formation since conversion of VIII to V to II will be a relatively slow process. Fig. 1 shows that the N-alkyl imidate VIII could also react with another protein amino group which would result in an N,N'-disubstituted amidine(III). A monofunctional reagent could, by this pathway, produce a one carbon atom crosslink. This figure also demonstrates that reaction of the N-alkyl intermediate(VIII) with the imidoester(VII) could occur repetitively causing the addition of one positive charge and 2.7 Å chain length per step. Hydrolysis could then occur which would end the multiple amidination or XVII could react with ammonia or an amino group to form a polyamidine(see Fig. 1).

Siezen(234) has presented evidence for polyamine formation at pH 8.0 during the crosslinking of the A + B subunits of α -crystallin by

dimethyladipimide(DMA) and dimethylsuberimide(DMS). This resulted in the individual chain being charge modified. For these experiments 0.2-25 mM reagent and 0.35-0.50 mM ϵ -amino groups of A₂ and B₂ chains were used at 20°C for 2 hr.

In our experiments with cytochrome c oxidase, care was taken to avoid reagent excesses and long incubation times which might be expected to lead to monofunctional crosslinking and polyamide formation. We have already demonstrated that monofunctional crosslinking, if it did occur, could not be detected by SDS gel electrophoresis to a significant extent in isolated oxidase. In the case of mitochondria, alteration of the gel pattern did take place however. It has been demonstrated that minimal crosslinking by monofunctionals under these conditions occurs(143). The concentrations of free enzyme-specific amines and imidoesters were also controlled so that even the highest imidoester concentration employed, 80 mM monofunctional and 40 mM bifunctional, the molar ratio of imidoester to primary amine did not exceed 100:1. Sieze did not see significant polyamide formation with the imidoester to amine molar ratio of 10:1. In the case of cytochrome c a small amount of intermolecular(<1%) monofunctional crosslinking did occur when the incubation ratio of monofunctional imidoester was 4:1.

At the lower concentration of imide used(5-10 mM) however, the ratio of imidoester to free amine was on the order of 10-20:1. It is likely that under the conditions used, the effect of imides on enzyme activity at high reagent concentration is due to both N-alkyl acetamide formation and to other side reactions. It is for this reason / ^{that} analysis of the effect of crosslinking on enzyme activity

is most valid at incubation imidoester concentrations <10 mM.

Problems Associated with Nitrenes as Crosslinking Reagents

Knowles(235), who was responsible for the introduction of nitro-phenylazide groups as nitrene precursors, has reviewed how the nitrene is generated from the azide and the possible reaction products. Table I shows that once the nitrene is generated it can undergo a variety of reactions. The advantage of using aryl azides is that they can be photolyzed at 350 nm unlike the alkyl azides which have their absorption maximum at ~290 nm, a region where significant protein absorption occurs. Although the nitrenes can react in non-polar environments and at very low temperatures, the numbers of products generated presents a problem. This difficulty is insignificant when one is only attempting to distinguish if a protein is labelled(crosslinked), but is great if reaction site specificity is important.

It is also important to note that crosslinking by putatively mono-functional azide reagents can occur by the mechanism in the last part of Fig. 1. Nevertheless, these reagents are very useful as general heterobifunctional crosslinking reagents.

Table I



II. Nitrene Fates-

REACTANTS		PRODUCTS		REACTION TYPE
a) $\text{R-N} \cdot$	$+ \text{H-C} \equiv$	$\longrightarrow$	$\text{RNH} \cdot + \cdot \text{C} \equiv$	hydrogen abstraction
b) $\text{R-N} \cdot$	$+ \text{H-C} \equiv$	$\longrightarrow$	$\text{R-NH-C} \longrightarrow$	insertion at C-H
c) $\text{R-N} \cdot$	$+ \text{C} \equiv \text{C} \equiv$	$\longrightarrow$	$\text{R-N} \begin{array}{c} \diagup \quad \diagdown \\ \text{C} \quad \text{C} \end{array}$	addition
d) $\text{R-N} \cdot$	$+ \text{R-N} \cdot$	$\longrightarrow$	R-N=N-R	condensation
e) $\text{R-N} \cdot$	$+ (\text{R}')_2\text{NH}$	$\longrightarrow$	$\text{R-NH-N} \begin{array}{c} \text{R}' \\ \diagup \quad \diagdown \\ \text{R}' \end{array}$	insertion at N-H
f) $\text{R-N} \cdot$		$\longrightarrow$		rearrangement products

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