Lawrence Berkeley National Laboratory

Recent Work

Title
EFFECTS OF VISIBLE LIGHT EXPOSURE ON MITOCHONDRIAL STRUCTURE AND FUNCTION

Permalink
https://escholarship.org/uc/item/8px4m94z

Author
Aggarwal, Bharat Bhushan.

Publication Date
1977-11-01

eScholarship.org Powered by the California Digital Library
University of California

U U J U 4 9 U U 9 4 9

LBL-6848 c.1
UC-48

RECEIVED
LAWRENCE
BERKELEY LABORATORY

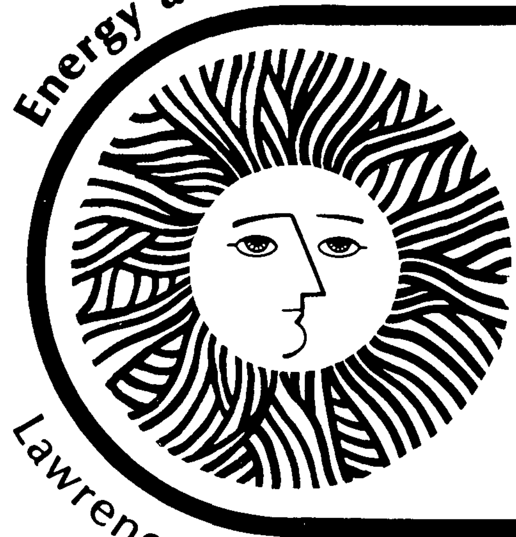
MAR 23 1978

LIBRARY AND
DOCUMENTS SECTION

For Reference

Not to be taken from this room

Energy and Environment Division



Effects Of Visible Light Exposure On Mitochondrial Structure And Function

Bharat Bhushan Aggarwal

(Ph.D. thesis)

November 16, 1977

Lawrence Berkeley Laboratory University of California/Berkeley

Prepared for the U.S. Department of Energy under Contract No. W-7405-ENG-48

LBL-6848 c.1

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

0 0 0 0 4 9 0 0 9 5 0

To the superb guidance of my Father and in the memory of my dear Mother.

0 0 0 0 4 9 0 0 9 5 1

CONTENTS

ABSTRACT v

ACKNOWLEDGMENT vii

ABBREVIATIONS viii

INTRODUCTION 1

BACKGROUND 1

PHOTODYNAMIC ACTION vs. MITOCHONDRIA 10

1. Chromophores 10

2. Oxygen Consuming Processes 24

3. Membrane Damage. 27

4. Structure and Functions. 29

RATIONALE OF PRESENT STUDY 42

EXPERIMENTAL METHODS 43

I. Mitochondrial Preparations 43

II. Illumination Procedure 43

III. Biochemical Assays and Procedures 46

RESULTS AND DISCUSSION 56

I. Coupling and Electron Transport 56

II. Structural Alterations 56

III. Prevention and Amplification 56

GENERAL DISCUSSION 116

Synopsis of Visible Light.Damage to Mitochondria 116

Proposed Explanation of the Mechanism of Visible Light Damage to Mitochondria 119

Visible Light Damage to Mitochondria vs. Cells 128

SUMMARY 131

REFERENCES 133

0 0 0 0 4 9 0 0 9 5 2

EFFECTS OF VISIBLE LIGHT EXPOSURE ON MITOCHONDRIAL STRUCTURE AND FUNCTION

Bharat Bhushan Aggarwal

Membrane Bioenergetics Group
Lawrence Berkeley Laboratory
University of California
Berkeley, California

ABSTRACT

Previous studies have suggested, mitochondria as a target of visible light and oxygen in damage processes to human cells *in culture*. In the present investigation, mitochondria isolated from rat liver cells were used as a model to study such damage. Whole mitochondria and mitochondrial inner membranes were exposed to visible light of wavelength above 400 nm and of intensity 300 mW/cm^2. The temporal sequence of changes in the mitochondrial structure and functions following the onset of illumination were examined.

The sequential changes in the mitochondria were: stimulation of respiration, a decline in ATP synthesis, inactivation of respiration, increased ATPase activity and finally, loss of transmembrane potential. Loss of respiration was mainly due to destruction of dehydrogenases. Among the components of dehydrogenases, destruction of flavins and loss of ubiquinone reduction and sulfhydryl groups were most susceptible to illumination. Succinate dehydrogenase was inactivated more rapidly than NADH and choline dehydrogenases. Redox reactions of cytochromes and cytochrome c oxidase activity were unaffected.

Concomitantly, visible light exposure to mitochondria also resulted in structural damage. This was indicated by release of soluble proteins, decrease in light scattering and increased NADH penetrations into the mitochondria. Microscopically the alterations in the mitochondrial morphology was indicated by dissolution of the inner membrane and distortion of the outer membrane. Electrophoretically the polypeptide profile of the mitochondrial inner membrane was altered after illumination. Besides protein damage, the lipids of the membranes were also peroxidized.

Inactivation of various enzymes was prevented by anaerobiosis and enhanced by increasing O_2 tension. Presence of exogenous flavins greatly enhanced inactivation of all the above mitochondrial activities. Various antioxidants were effective against lipid peroxidation but not against enzyme inactivation. However, it was feasible to protect against both lipid peroxidation and enzyme inactivation by using a combination of substrates and inhibitors of the electron transport chain.

Results obtained above suggests that visible light mediates a flavin photosensitized reaction that initiates damage, involving participation of an activated species of oxygen in the damage propagation. Results also indicate that the damage mechanism to lipids is different from that of the proteins.

0 0 0 0 4 9 0 0 9 5 3

ACKNOWLEDGEMENT

I am graciously thankful to Professor Lester Packer for his excellent guidance and supervision during the course of this investigation. A sincere thanks are also extended to Professor Yoram Avi-Dor (on sabbatical leave from Technion, Haifa, Israel) for teaching me "mitochondriology". A highly valuable criticism offered to improve this dissertation by Professor T. Singer and Professor A. McLaren is acknowledged with deep gratitude and warm regards. I am also thankful to Drs. R. Mehlhorn, A. Quintanilha and J. Walton for their helpful suggestions and collaborations. All the past and present members of Membrane Bioenergetics Group shall never be forgotten for sharing their Academic and Social life. My thanks are also due to Ms. C. Konjevich for hearing my tales of frustration and excitment in academic affairs.

A national scholarship from the Ministry of Education, Government of India and a Patent Fund Award from the Chancellor, University of California, Berkeley is highly acknowledged.

This work was done under the auspices of the U. S. Department of Energy.

0 0 0 0 4 9 0 0 9 5 4

Abbreviations

RLM	Rat liver mitochondria
SMP	Submitochondrial preparations
RCR	Respiratory control ratio
TCA	Trichloroacetic acid
FCCP	p-trifluoromethoxy(carbonyl-cyanide)phenylhydrazone
CAT_{16}	4-(cetyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide
PMS	Phenazine methosulfate
DCPIP	2,6-dichlorophenol indophenol
TMPD	N,N,N ,N -tetramethyl-p-phenylenediamine
ETF	Electron transport flavoprotein
TTFA	Thenoyl trifluoroacetone
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
EDTA	Ethylenediamine tetraacetic acid
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
DCMU	3-(3,4-dichlorophenyl)--1,1-dimethylurea
DMF	Dimethylfuran
DABCO	Diazabicyclooctane
GSH	Glutathione
TBA	Thiobarbituric acid
$K_3Fe(CN)_6$	Potassium ferricyanide

0 0 0 0 4 9 0 0 9 5 5

INTRODUCTION

Although the average life span of a given species is fixed by its genetic clock, a large deviations are observed within a species (1). Precisely what determines such derivations is not well understood, environmental factors have been suggested to play an important role (2). Radiations in the visible region of the spectrum and oxygen are two such environmental agents to which we are exposed everyday and excessive dosage of either of the two agents could be harmful. Studies from Packer's laboratory have indicated that human cells _in vitro_ when exposed to high oxygen concentration (4) or to light in the visible region of the spectrum, (3) show a decline in the average life span. Since mitochondria are the primary site in the cell where oxygen is utilized and since they also contain flavin and heme pigments to absorb visible light, it was decided to investigate the effects of visible light and oxygen on the structure and function of isolated mitochondria.

BACKGROUND

Radiations in the visible region of the spectrum is not ordinarily destructive to the living tissue, however in the presence of certain dyes, it produces marked damaging effects. This phenomenon was first discovered by O. Raab in 1900 (5) when he observed that acridine dye was more toxic to paramoecia in the light than in the dark. Since then, several reports (6) have appeared about the effects of visible light on the biological system both in the presence and absence of exogenously added pigments, of both biological (Hematoporphyrin, chlorophyll, bilirubin, flavins, cytochromes) and non-biological (fluoroscein, xanthene, acridine, phenazine dyes) origin. The evidence for the requirement of oxygen for

light effects stemmed from the photochemical studies (7), in which iodine ion was converted to iodine by Eosine Y only in the presence of oxygen. Later it was also shown in a biological system that oxygen is required for the destruction of rose bengal stained bacteria by light (8). The various effects produced by the combined interaction of light, oxygen and sensitizer are termed photodynamic effects. This term is currently also described as "sensitized photooxidation". The occurances of sensitized photooxidation have been studied extensively at the multi-cellular, cellular, subcellular and molecular level, (6,9) and briefly examined below:

Multicellular Organisms

Small mammals injected with a photosensitizing dye are rapidly killed on exposure to high intensity light. The response of animals to sensitized photooxidation (6) have been divided into three categories: a) sensory stimulation e.g., scratching and hyper-sensitivity; b) skin damage, e.g. erythema and edema resulting from vascular damage, cell degeneration and skin necrosis; c) body changes in areas not reached by light e.g., decreased body temperature, decreased blood pressure, intestinal hemorrhage, generalized circulatory collapse. The latter case could be due to photoproducts produced in the skin and carried to remote parts of the body by the circulatory system.

Photosensitization in man is of common occurrence and it results from ingestion of, or contact with photosensitizing compounds (certain drugs, petroleum and coal tar products, nitrogen dioxide, perfumes, etc.) or from disorders of porphyrin metabolism (10). Similar responses

have also been observed in herbivorous animals following the ingestion of plants such as buckwheat, hypericum, etc. which contain photosensitizing pigments (11). The complexity of multicellular organisms make it difficult to understand the mechanism of such responses, therefore it is important to examine photosensitization at the cellular level.

Whole Cells

The effects of sensitized photooxidation on cells include abnormal cell division (12), depression of glycolysis and respiration (13), inactivation of hill reaction (14) and interference with other metabolic activities such as synthesis of lipids (15), proteins (16), DNA (17) and inactivation of active transport (18). Much of this work has been done with microorganisms and mammalian cells in culture. The work with human cells in culture has indicated a gradual decline in the proliferative capacity of cells on exposure to visible light. An inverse relationship between the age of culture and photosensitivity of cells has been established (17). Since the primary site of oxygen attack or visible light in a cell is its membrane and since latter also contain pigments to absorb visible light, it is important to examine the background information at the cell membrane level.

Cell Membranes

Although membrane damage has been suggested as a primary event resulting in cell death (19), very little is known about sensitized photooxidation at the membrane level. A typical biological membrane primarily consist of lipids and proteins. The unsaturated fatty acids and cholesterol are very labile components of the membrane (20,21)

and could disrupt its structure. The photohemolysis of erythrocyte membranes have been observed by several investigators (22-24) and correlated with the formation of cholesterol hydroperoxides (25). Studies with artificial membrane system as a model for erythrocyte membrane have shown that the lysis of membrane vesicles is due to photoperoxidation of lipids (26). Different kinds of cell membranes differ in the contents of peroxidizable lipids. Thus Tappel laboratory finds that isolated lysosomal membranes undergo lipid peroxidation at one third the rate of mitochondrial membranes and one tenth the rate of microsomal membranes (27). This can be interpretted for higher contents of saturated lipids (non-peroxidizable) in lysosomal membranes than that in the mitochondria or microsomes.

Besides the peroxidation of lipids, membrane damage by photosensitization is also reflected by an increase in permeability (28). destruction of active transport (29) and membrane bound enzymes (30) and release of soluble enzymes (28). The possible mechanism of these effects, whether it is direct or medicated through free radical intermediates of lipid peroxidation, is not clear. Several model studies have been done at the molecular level.

Molecular

The sensitized photooxidation of a wide variety of simple organic molecules has been examined using many different sensitizers. In general, saturated and unsubstituted aliphatic compounds are resistant and hydroxyl or amino groups substistuted aromatic compounds are suceptible to photooxidation (31). Among amino acids, aromatic are very

susceptible and aliphatic are relatively resistant. Histidine, Methionine, tryptophan and tyrosine are rapidly photooxidized in the presence of riboflavin (32). A tripeptide, glutathione is photooxidized by riboflavin, FMN, lumiflavin (but not with FAD) with the production of oxidized glutathione (33) thus forming disulfide bridges.

Photodynamic damage to proteins results from the destruction of amino acid side chains. Histidine and tryptophan residue are destroyed faster in most proteins (34) than tyrosyl, methionyl and cystinyl residues. The rate of photooxidation of these amino acids is several folds slower in proteins than when they are free in solution. This is perhaps due to the fact that in a protein certain fractions of a given amino acid will be exposed at the surface, thus readily accessible to photooxidation, whereas others are "buried" and thus less readily available (35). For example Taka Amylase A is inactivated more rapidly by photodynamic treatment with riboflavin after being unfolded in 8M area (36). Indeed this property of selective modification of certain amino acids in a protein molecule by sensitized photooxidation has become a useful tool to study the structure-function relationship of an amino acid residue to a protein molecule. Thus in the case of Ribonuclease T, photooxidation sensitized with methylene blue results in the destruction of both histidine and tryptophan residues, whereas with riboflavin only histidine residues are lost (37).

Mechanism of Photodynamic Action

Photodynamic systems generally consist of a sensitizer, solvent, oxygen and a substrate (a material being photooxidized). These components interact in a variety of ways as shown in Fig. 1 and the kind of interaction is determined by the sensitizer. In the dark, the sensitizer in a given solvent exists in a ground state. The two electrons in a given molecular orbital are usually spin paired (in opposite directions) and thus do not contriubute to the magnetic moment of the molecule. This condition is termed singlet state, designated ^1S in Fig. 1. When ^1S absorbs a photon, one of the electrons from a filled orbital, is transferred to an unfilled higher energy orbital giving an excited state, S^*. In some cases the spin of the excited electron is reversed giving a paramagnetic state or a triplet state, ^3S of the sensitizer. Triplet states are not produced directly from the ground state but result from intersystem transitions of excited singlet states. The lifetime of triplet states are much longer than those of the excited singlet states.

Photochemical reactions result from deactivation (quenching) of the excited states of the sensitizer by reactants in the system, which could be either oxygen or directly with the substrate. The more commonly proposed mechanisms for photodynamic actions found in the biological literature is also illustrated in Fig. 1. In mechanisms I and II substrate is the primary reactant with excited sensitizer, while in III and IV oxygen is the primary reactant. Mechanism V requires binding of sensitizer to substrate prior to light absorption.

0 0 0 0 4 9 0 0 9 5 8

Fig. 1. Interrelationship of various mechanisms (I-V) of sensitized photooxidation. Notations used are: sensitizer in singlet state (^1S), sensitizer in triplet state (^3S), light quantum (hv), substrate in oxidized state (R), substrate in reduced state (RH_2), sensitizer in excited singlet state (S^*), excited singlet oxygen (O_2^*) and oxyradical (SOO).

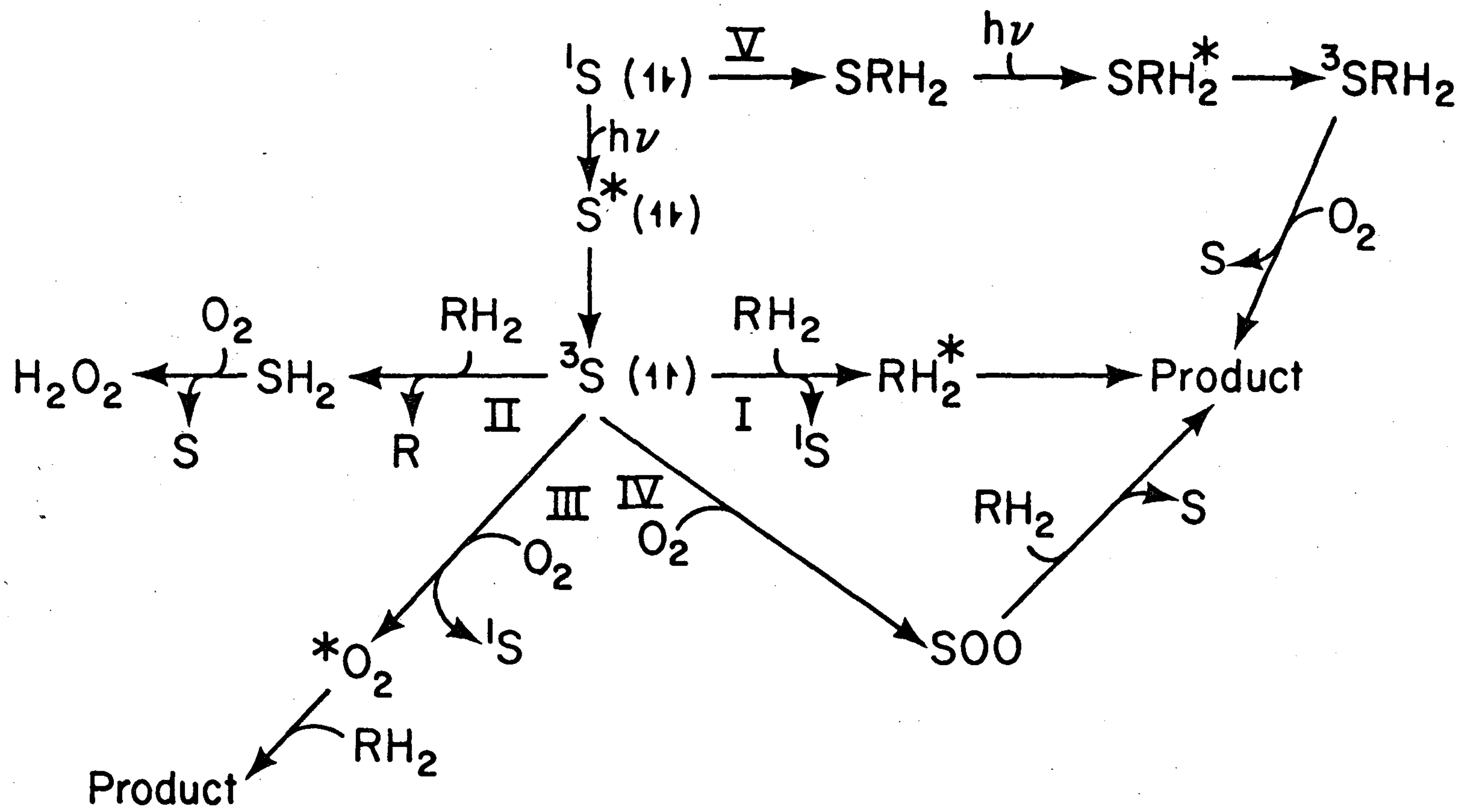


Fig. 1

0 0 0 0 4 9 0 0 9 5 9

There are several possible mechanisms by which excited sensitizer could react with a substrate e.g. by resonance energy transfer, by charge transfer complex formation and by electron transfer reactions. In photodynamic system, electron transfer (hydrogen abstraction), analogous to mechamism III is more frequent. Sensitized photo-autooxidation initiated by energy transfer from a triplet sensitizer to triplet oxygen (type III) with the latter being raised to the singlet state has also been demonstrated (38). This highly reactive oxygen could then react with substrate. In summary, although a number of reaction mechanisms have been proposed to account for photodynamic action (39) there is good evidence that more than one mechanism can occur with a given substrate, depending on the photosensitizer and the reaction conditions.

PHOTODYNAMIC ACTION vs MITOCHONDRIA

Photodynamic systems require a "sensitizer" or a chromophore which can absorb light in the visible region of the spectrum, "oxygen" as a photooxidant and a suitable "substrate" susceptible to photooxidation. In what follows the relevance of these criteria to mitochondria are reviewed.

1. Chromophores

The chromophores present in the mitochondria have been extensively studied and absorb visible light in the range 340-630mm. The absorption maxima, extinction coefficient and concentration of presently recognized components in rat liver mitochondria are given in Table I.

Flavins

Mitochondrial flavins are redox active chromophores of complex I and II of the respiratory chain. Besides complexes, flavin are also associated with several other enzymes listed in Table II. The structure of a flavin coenzyme is given in Fig 2. All flavins show a strong absorption in the blue and ultraviolet region of the spectrum. The optical properties of riboflavin, FMN and FAD in 0.1M phosphate buffer PH 7.0 and lumiflavin and lumichrome in water is as follows:

0 0 0 0 4 9 0 0 9 6 0

Table I. CHROMOPHORES OF RAT LIVER MITOCHONDRIA*

Chromophore	Absorption Maxima nm	Extinction Coefficient $cm^{-1}mM^{-1}$	Concentration nmoles/mg protein
Flavoproteins	465-510	11	0.69
NAD(H)	340-374	6.22	1.90
Ubiquinone	289-272	12.2	2.30
Cytochrome b_{561}	561-575	14.6	0.10
b_{566}	566-575	16.0	
Cytochrome C_1	552.5-540	17.5	0.11
Cytochrome C	550-540	19.9	0.20
Cytochrome a_3	444-445	16.4	0.41
a	605-630	26.4	

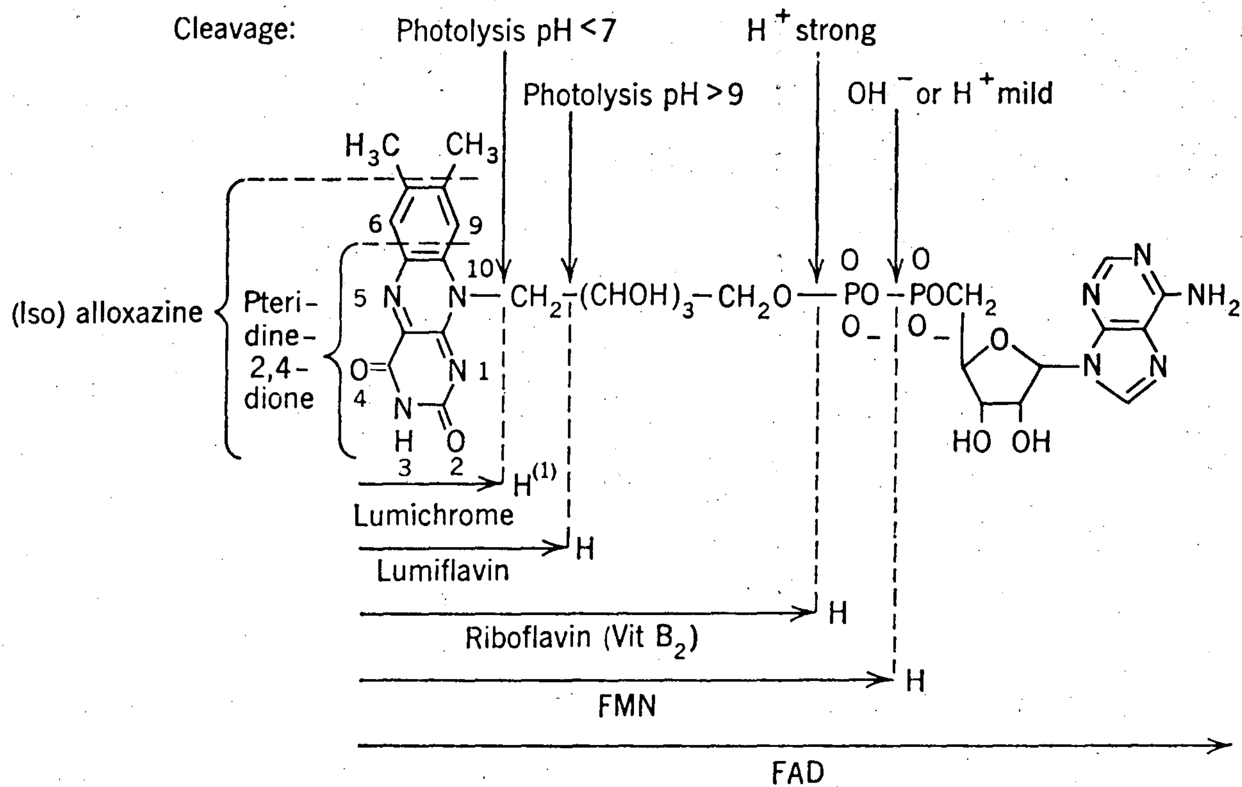
*From handbook of cell biology; compiled and edited by Altman P. L. and Katz D. D. (1976), p 144 Federation of American Societies for Experimental Biology Bethesda, Maryland.

Table II. MITOCHONDRIAL FLAVOPROTEINS.

Flavoprotein	Localization	Nature of Flavin Component	References
Succinate dehydrogenase	Inner membrane	Bound FAD	40
NADH dehydrogenase	Inner membrane	Free FMN	41
Choline dehydrogenase	Inner membrane	Free FAD	47
L-Glycerol 3-phosphate dehydrogenase	Inner membrane	Free FAD	48
Monoamine oxidase	Outer membrane	Bound FAD	42
Sarcosine dehydrogenase	Matrix	Bound FAD	43
Dimethyl glycine dehydrogenase	Matrix	Bound FAD	44
Butyryl CoA dehydrogenase	Inner membrane or Matrix	Free FAD	45
Acyl CoA dehydrogenase	Matrix	Free FAD	46
Electron Transferring flavoprotein dehydrogenase	Inner membrane or Matrix	Free FAD	46

0 0 0 0 4 9 0 0 9 6 1

Fig. 2. Structure and cleavage loci of flavocoenzymes. (From Ehrenberg and Hemmerich 1968, used by permission).



Structure and cleavage loci of flavocoenzyme

XBL 7710-10312

Fig. 2

Flavins	Absorption maxima (nm) (molar extinction coefficient ε x 10^{-3} cm^{-1} in parenthesis)			
Riboflavin		266 (32.5)	373 (10.6)	445 (12.5)
FMN		266 (31.8)	373 (10.4)	445 (12.5)
FAD		263 (38.0)	375 (9.3)	450 (11.3)
Lumiflavin	225.5 (30.3)	265 (32.4)	369 (8.4)	444 (10.9)
Lumichrome	219 (30.1)	261 (30.5)	354 (8.6)	

On reduction of flavins, peak around 445nm is bleached. In mitochondria, flavins are linked with the apoprotein part of the enzyme either covalently or noncovalently. The protein environment around the flavin molecule modifies its optical absorption; for example in case of the complex II 375 nm absorption peak due to FAD is shifted to 345-350 nm (50).

Flavins are well known for their photolability. The cleavage of flavin molecule occurs on illumination (Fig. 2) through a disproportion mechanism which involves dehydrogenation of side chain by the excited state of the heteroaromatic nucleus of the molecule (51). Photochemical methods have been used to generate flavin semiquinone and and dihydroquinone both in free state (52) and in the flavoproteins (53) to study the triplet state of the flavins (54) and their interaction with a variety of aromatic compounds (55). Anaerobically flavins are photoreduced by amines, amino acids, alcohols and reduced pyridine nucleotides (56). The photoreduction can be reversed by

oxidants such as oxygen leading to the formation of superoxide radical (57). When flavoproteins are exposed to light aerobically, a flavin catalyzed denaturation of proteins occurs (58). For example the inactivation of complexes I and II has been shown on exposure to blue light (59).

Flavin chromophore associated with complex I and II is required for their electron transfer activity. Large spectral changes have been noticed on reduction of these complexes with their natural substrates (60). The substrate induced bleaching of the complexes has been attributed in part to the flavin coenzyme. Since the flavin moieties are tightly bound covalently in complex II and noncovalently in complex I, the complete dissociation of the flavins has not been possible without destabilizing the enzymatic activity.

Iron Sulfur Centers

Another important chromophore associated with complexes I, II and III of the mitochondria is the Fe-S centers (Fig. 3). It has been shown that NADH dehydrogenase of complex I absorbs light in the entire visible range with a maximum between 400-420 nm which was assigned mainly to the iron components (60). Quantitatively 16g atoms Fe-S/mole of flavin were determined in purified NADH dehydrogenase (60) and 16-18 Fe-S groups per mole of FMN was shown in complex I (71). Later on this was also confirmed by others (61). If all the iron sulfur centers of complex I are of [4Fe-4S] type, then 4 Fe-S centers and if [2Fe-2S] type then 8 Fe-S centers are expected. By using low temperature EPR spectroscopy, centers N_1-N_4 have been demonstrated unequivocally by various workers (see review 62) which can be characterized on g scale as

0 0 0 0 4 9 0 0 9 6 2

Fig. 3. The mitochondrial respiratory chain showing the site of action of various substrates and inhibitors.

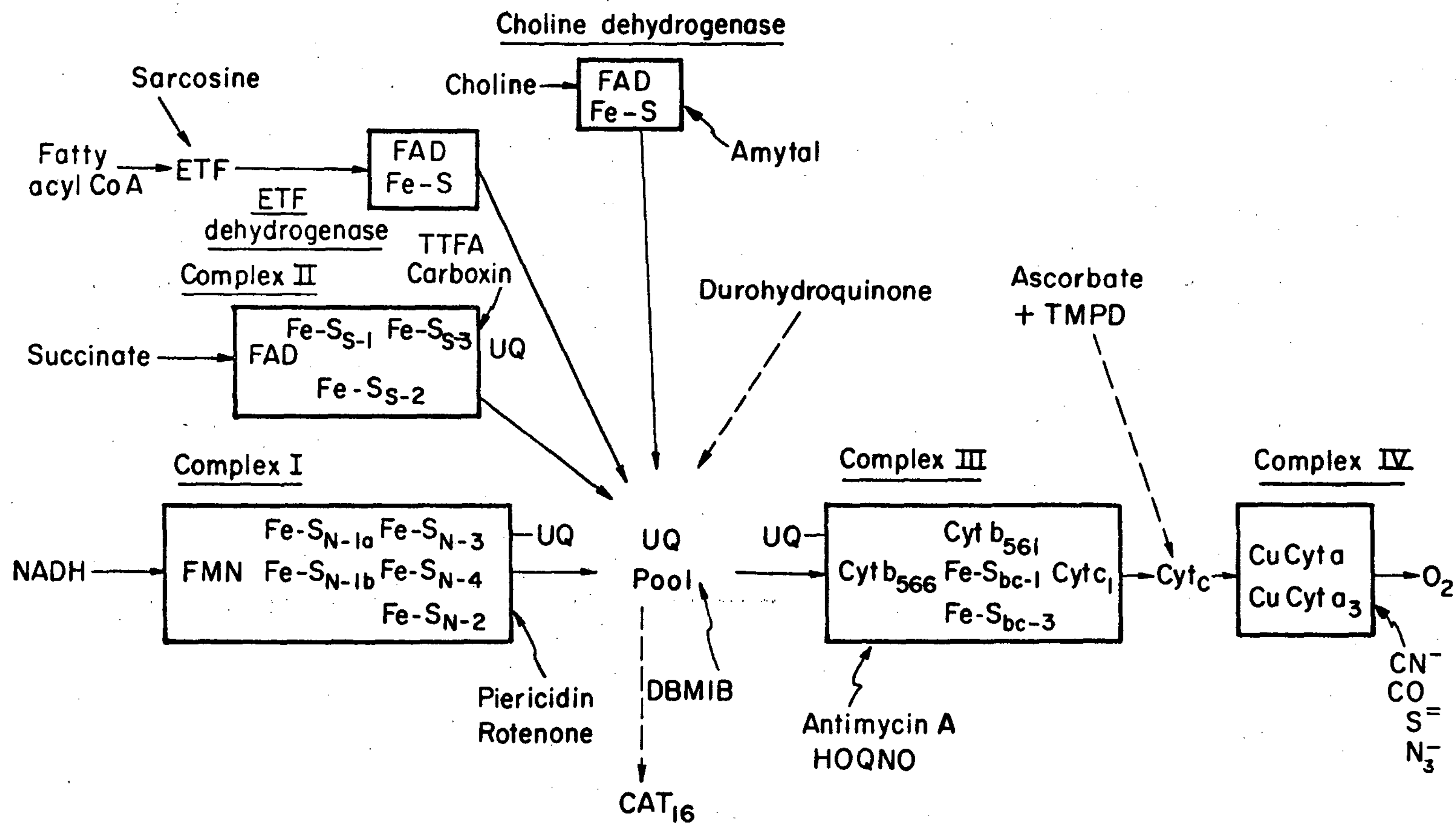


Fig. 3

0 0 0 0 4 9 0 0 9 6 3

N_1 (2.022, 1.938, 1.923), N_2 (2.054, 1.922), N_3 (2.101, 1.886, 1.864) and N_4 (2.103, 1.861). Centers N_5 and N_6 have also been indicated (69) but are in contradiction (70). The function of these various centers is not well understood (see review 73) except N_1 which is shown to be involved in electron transport from NADH to ferricyanide (72).

There are 3 Fe-S centers (S_1, S_2 and S_3) associated with complex II; of which centers S_1 (2.03, 1.93, 1.91 g) and S_2 (2.03, 1.93, 1.91) can be observed in the reduced state while S_3 (HiPIP) can be detected in the oxidized state (62). Centers S_1, S_2 and S_3 can be distinguished by their midpoint potential of 0, -260 and +60 mv respectively. This complex abosrb light in the entire visible range with peaks at 420 and 460 nm and a shoulder at 550 nm (63). Later on it was shown that 420 nm peak and most of the absorbance about 500 nm is due to iron sulfur centers (64).

Iron sulfur centers associated with complex III absorb visible light with maxima at 460 nm and 575 nm and a shoulder at 315 nm (65). There is a great controversy about the number of iron sulfur centers associated with this complex. Center b C_1 (2.025, 1.89, 1.81, 1.78 g) has been isolated and characterized (66), center b′ C_2 (2.086, 1.94, 1.898) which was called "center N_5" (67) of complex I is now considered to be a part of electron transferring flavoprotein dehydrogenase (70); and center b C_3 (2.01 g) which is of HiPIP type (68,69) has been suspected as a contamination from complex II.

Ubiquinone

Ubiquinone (coenzyme Q_{10}) is the major type of quinone present in rat liver mitochondria, associated with complex I, II and III. The amount of ubiquinone is usually several folds (2.30 nmole/mg protein) greater than the other chromophores of the mitochondria (Table I). In the oxidized form pure ubiquinone in ethanol has a prominent absorption peak in the ultraviolet range 270 nm ($\varepsilon_{270nm}^{1\%,cm^{-1}}$ = 165) and a second peak in the visible range at 405 nm ($\varepsilon_{405nm}^{1\%,cm^{-1}}$ = 5.9) (74). The reduced ubiquinone exhibits a single peak at 290 nm ($\varepsilon_{290nm}^{1\%,cm^{-1}}$ = 46.4) During transition from oxidized to reduced state, the absorption peak at 275 nm and 450 nm disappears and the yellow color of the oxidized form fades.

Several reports have appeared about the destruction of ubiquinone by sunlight (76), ultraviolet light (77-81) and near ultraviolet light (82-87). In many investigations of bacterial respiration, near uv has been used to modify quinones in whole cells and in electron transport preparations obtained from them (87). There is one report indicating that pure ubiquinone is 4-8 times more sensitive to light than ubiquinone in the cell (88). These workers also demonstrated that light at 313 nm is 4 times more potent than at 405 nm in the destruction of ubiquinone leading to the formation of several polar photoproducts as separated by thin layer chromatography.

The role of ubiquinone in the transfer of electrons from flavoproteins to cytochromes (89) and in proton translocation across the mitochondrial membrane (92) is well known. The redox potential

0 0 0 0 4 9 0 0 9 6 4

of ubiquinone, E_o = + 98 mv at pH 7.4 and 25°C suggests its position between cytochrome b and c of the respiratory chain (75), but enzymatic studies indicate a function before cytochrome b (89). Recently, Ohnishi has reported a midpoint potential of ubiquinone ($Q \rightarrow QH^{\cdot}$) + 80 mv and ($QH^{\cdot} \rightarrow QH_2$) + 140 mv (94). A large stoichiometric excess of ubiquinone in the mitochondria is ambiguous, since at aesrbic steady state only 50% of the ubiquinone is reduced (90). This raises some doubts about the function of ubiquinone merely as an electron carrier. Both prooxidant and antioxidant role of ubiquinone will be discussed later. There are certain reports suggesting 2 different pools of ubiquinone (86); an active pool which participates in the electron transfer and an inactive pool. There are also some reports suggesting that ubiquinone exists in both free as well as bound form (91,93) in the mitrochondria; whether inactive and active pools are related to the free and bound form of the ubiquinone pools respectively is not known. Recently ubiquinone specific apoprotein has been isolated and characterized (91).

Hemes

This chromophore is associated with the various cytochromes of complex II, III and IV; and cytochrome c of mitochondria. The absorption of a given cytochrome is determined by the heme moiety and its environment. Various cytochromes absorb light in the visible region of the spectrum between 420-620 nm as shown in Table I.

Several reports have appeared about the sensitivity of various cytochromes to visible light exposure. The photodestruction of

cytochrome a_3 of beef heart mitochondria and of complex IV has been shown on exposure to blue light 330-540 nm (101). Furthermore, studies with complex III indicated the destruction of cytochrome b_{560} on blue light exposure (59). The inactivation of cytochrome oxidase has also been reported on exposure to red light (103).

Cytochromes are the active redox components of the respiratory chain. Complex III of this chain contains equimolar amounts of cytochrome C_1 and cytochrome b_{560} and b_{566} (95,96). Cytochrome b_{560} is readily reduced by substrates but cytochrome b_{566} is reduced only in the presence of substrate and antimycin A. These two different kinds of cytochrome b probably correspond to cytochrome b_k and b_t respectively (97). Complex IV is constituted by cytochrome a and a_3 (98) and 2 coppers per heme (99). Substrates are known to reduce only one of the coppers (100) of complex IV and the exact role of second copper is not understood. Cyanide inhibits this complex by blocking the reduction of copper.

Besides various cytochromes associated with complex II, III and IV, there is cytochrome c which is not associated with any of the complexes and serves as a electron carrier from complex III to IV. Moreover this is the only cytochrome which exhibits electrostatic interaction with the mitochondrial membranes and thus can be reversibly removed by salt treatment (102).

<u>Pyridine Nucleotides</u>

Pyridine nucleotides are coenzymes of several mitochondrial dehydrogenases such as NADH and alcohol dehydrogenases. Reduced pyridine nucleotides have a strong absorption peak in ultraviolet

range at 259 nm ($\varepsilon^{mm^{-1}cm^{-1}}_{259nm}$ = 19) and another absorption peak near uv range at 340 nm ($\varepsilon^{mm^{-1}cm^{-1}}_{340nm}$ = 6.22) (104,105). On oxidation 340 nm peak completely disappears and 259 nm peak declines ($\varepsilon^{mm^{-1}cm^{-1}}_{259nm}$ = 14). NADH has a strong fluorescence with emission maxima at 465 nm when excited at 340 nm wavelength (106).

The role of pyridine nucleotides as a carrier of protons from various metabolic reaction is well known (107). Rat liver mitochondria contains both NAD(H) (10 nmoles/mgprotein) and NADP(H) (3.4 nmoles/mg protein) (108).

Respiratory Chain. The various chromophores discussed above are components of a respiratory chain catalyzing the transfer of electrons and protons from NADH and succinate to oxygen. The arrangement of these components along the chain which is most generally accepted at this time is given in Fig. 3. This scheme has been derived from spectroscopic data together with kinetic studies and data on the effects of specific inhibitors (109,110,89). This scheme is also supported by the redox properties of various chromophores and their midpoint potentials.

The approaches involving disruption and reconstitution of the electron transport system (111) has contributed a great deal in understanding the function of an individual component. Methods have been developed for the resolution of the respiratory chain into 4 enzyme complexes viz; NADH-Q reductase (I), succinate-Q reductase (II), QH_2-cytochrome C reductase (III) and cytochrome c oxidase (IV) (112). The composition of each of the complex is given in Table III (61). A reconstitution experiments with complexes

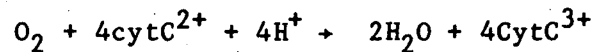
I + II + III + IV indicate that they interact stochiometrically to form an active unit capable of transferring electrons from NADH and succinate to molecular oxygen in the presence of ubiquinone and cytochrome c (113-115).

2. Oxygen Consuming Processes

Besides chromophores, oxygen is also required for photodynamic effects. Both enzymatic and nonenzymatic reactions of the mitochondria consume oxygen.

a) Enzymatic oxygen consumption: Certain oxidases and oxygenases in the mitochrondria utilize oxygen as follows:

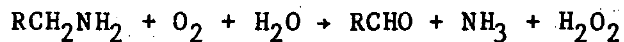
I) Cytochrome oxidase: It has been estimated that 90% of the biological oxygen is consumed through cytochrome oxidase (116) which catalyzes reduction of oxygen to water.



$$O_2 + 4cytC^{2+} + 4H^{+} \rightarrow 2H_2O + 4CytC^{3+}$$

This reaction occurs through two electron step processes (117) and a large amount of free energy released is coupled to oxidative phosphorylation.

II) Monoamine oxidase: It is a mitochondrial outer membrane linked enzyme which catalyzes deamination of primary amines using molecular oxygen.



$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

0 0 0 0 4 9 0 0 9 6 6

Table III. Components of Complexes I, II, III and IV.

Complex		Components	Concentration (per mg protein)
I	NADH-Q reductase	FMN	1.4-1.5 nmole
		Nonheme iron	23-26 ng-atom
		Labile sulfide	23-26 nmoles
		Ubiquinone	4.2-4.5 nmoles
		Lipids	0.22 mg
II	Succiate-Q reductase	FAD	4.6-5.0 nmoles
		Nonheme iron	36-38 ng-atom
		Labile sulfide	32-35 nmoles
		Cytochrome b	4.5-4.8 nmoles
		Lipids	0.20 mg
III	Q H_2-cytochrome c reductase	Cytochrome b	8.0-8.5 nmole
		Cytochrome c_1	4.0-4.2 nmole
		Nonheme iron	10-12 ng-atom
		Labile sulfide	6-8 nmole
		Ubiquinone	2-4 nmole
		Lipids	0.4 mg
IV	Cytochrome c oxidase	Cytochrome a,a_3	8.4-8.7 nmole
		Copper	9.4 ng-atom
		Lipids	0.35 mg

III) Oxygen consumption for H_2O_2 production: Rat liver mitochondria produce H_2O_2 at a rate that accounts for 1-2% of the total uptake of oxygen (109). Since substrate dependent H_2O_2 production in mitochondria is Antimycin A sensitive (118), it suggests dehydrogeneses, ubiquinone or cytochrome b as the site of oxygen reduction. Both NADH and succinate dehydrogenases are iron sulfur flavoproteins whereas cytochrome b is an iron sulfur heme protein. Fridovich's laboratory has shown that iron-sulfur flavoproteins (126), flavoproteins (122), iron sulfur proteins (121) and quinols (122) are capable of generating superoxide radical, a precursor of H_2O_2. The production of superoxide radical in ox heart mitochondria has been demonstrated and iron sulfur centers of NADH dehydrogenase as the source of this radical is suggested(125). Recently in beef heart mitochondria the H_2O_2 production is shown to be due to ubiquinone approximately 0.3-0.6 nmoles/min/mg protein of H_2O_2 production has been assigned to ubiquinone (118,124). Perhaps the semiquinone form of ubiquinone is responsible for the generation of the H_2O_2.

Besides electron transport systems, auxillary mitochondrial dehydrogenases can also generate H_2O_2 by consuming oxygen. Various workers have reported generations of about 0.1 nmole H_2O_2/min/mg protein in rat liver mitochondria as very likely due to dihydroorotate dehydrogenase activity (111,120,123).

IV) Monooxygenases: Besides oxidases, there are certain monooxygenase such as kynurenine 3 monooxygenases in rat liver mitochondrial outer membrane (128) also known to consume molecular oxygen.

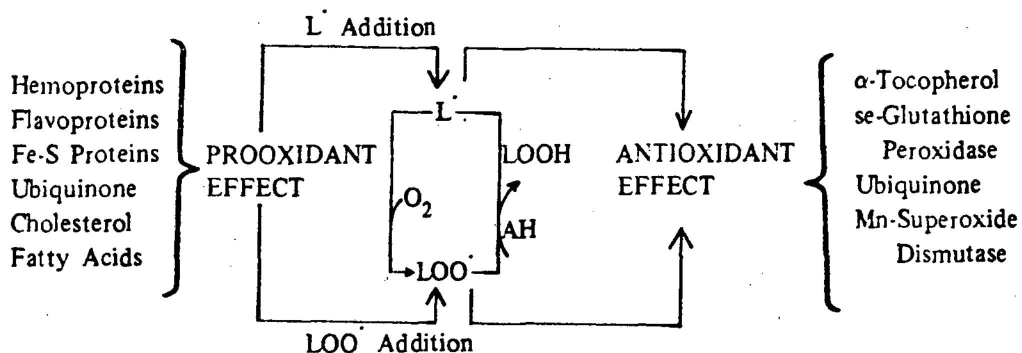
0 0 0 0 4 9 0 0 9 6 7

b) Non Enzymatic Oxygen Consumption: Oxygen is also consumed for the autooxidation process in the mitochondrial membranes due to their high unsaturated lipid contents. The autooxidation of unsaturated lipids has been shown to be induced by mitochondrial iron sulfur flavoproteins (127).

3. Membrane Damage

Besides chromophores and oxygen, mitochondria also provide a substrate for sensitized photooxidation. The mitochondrial membranes consist of lipids and proteins. Data on rat liver mitochondria indicate that approximately 4% of the total mitochondrial protein is in the outer membrane, 21% in the inner membrane and 67% in the matrix (128). Approximately 27% of the mitochondrial dry weight is lipids, of this 90% is in the form of phospholipids, the remaining 10% consists of neutral lipids mainly cholesterol, ubiquinone, α-tocopherol and carotenoids (129,130). The phospholipids belong to three major classes viz; cardiolipins, phosphatidylcholine and phosphatidylethanolamine in the ratio 1:4:4 respectively. The most important feature of the mitochondrial phospholipids is a very high degree of unsaturation of fatty acyl chains. The saturated fatty acids occur only to the extent of 1-2%. The ratio of protein to lipids in the outer and inner membrane of mitochondria is 1:0.28 and 1:0.83 respectively. Qualitatively cardiolipins occur exclusively in the inner membrane while phosphatidlylinositol and cholesterol are much more abundant in the outer membrane.

Mitochondial membranes carrry out a series of free radical mediated oxidation-reduction reactions. Free radicals produced physiologically during such processes are propagated by oxygen, resulting in membrane damage. This occurs through a chain of reactions as shown below:



L· = Lipid radical, LOO· = Lipid peroxide

LOOH = Lipid hydroperoxide AH = Antioxidant

Thus according to above scheme agents which enhances free radical concentration in a system has been regarded as "PROOXIDANTS" and agents that lower free radical concentration are "ANTIOXIDANTS".

In mitochondria, hemoproteins, flavoproteins, iron sulfur proteins, and lipids can be categorized as prooxidants, whereas -Tocopherol, superoxide dismutase, glutathione peroxidase and ubiquinone are as antioxidants. Any interference with the mitochondrial antioxidant mechanisms can result in prooxidant effect. For example vitamin E deficiency in rats is known to enhance the

free radical concentration (131), which is poroxidant effect. The existance of free radicals in the mitochondria have been shown by electron spin resonance studies (132). EPR signals of ubiquinone flavosemiquinone, superoxide and iron sulfur radicals have been identified. The reactivity of a given radical is determined by its local environment, whether aqueous, lipid or surface. Radicals are usually more stable in lipid environment than in aqueous phase. Thus radicals produced in the mitochondrial membranes have higher reactivity due to their longer half life in the lipid phase. The reactivity of the radical is also enhanced by the fluid nature of the mitochondria due to high unsaturated lipid contents.

4. Structure and Functions

Mitochondria in a eukaryotic cell is a tiny unit capable of carrying out several functions autonomously. On the basis of its structure and function, it is frequently compared with bacteria and the latter has been suggested as its ancestors (133). Since most of the studies done earlier on photodynamic action are in bacteria, this should have great relevance to mitochondria. The structure and functions of the mitochondria will be reviewed briefly below:

a) Structure:

Rat liver mitochondrion is a double walled structure consisting of a smooth outer membrane and involuted inner membrane enclosing matrix. The infoldings of the inner membrane gives rise to a structure called cristae, a device known to increase the surface

area of the inner membrane. Most of the energy related functions of the mitochondria are localized in the inner membrane; whereas outer membrane is relatively passive in functions. The outer membrane can be easily removed by hypotonic shock, resulting in a mitoplast which consist of inner membrane and matrix. Sonication of mitoplast releases the matrix and inverses the membrane polarity, leading to a purified submitochondrial (inner membrane) preparations.

Freeze fracture studies have revealed a great deal about the anatomy of the mitochondrial inner and outer membranes (Fig. 4). These studies have shown the size and distribution of various globular proteins (particles) in the membranes (134). In isolated membrane preparations, the particles are attached predominately to one of the two faces of the membrane. The particles appears to be more abundant on the inner membrane surface (136) and still more frequent on the cristae (137). This suggests an asymmetry in the mitochondrial membrane similar to other membranes (135). The asymmetry in the mitochondrial membrane has also been confirmed by chemical labeling (138), by spin labeling (139) and by immunological studies (140).

b) Energy Transduction

The energy released during oxidation of coenzymes through the respiratory chain is coupled to synthesize ATP from ADP. The nature of this coupling has been studied a great deal in isolated mitochondria. If mitochondria in a coupled state are supplied with ADP (the energy acceptor) the rate of oxygen consumption markedly increases (State III), however when the ADP supply is depleted, the

0 0 0 0 4 9 0 0 9 6 9

Fig. 4. Organization of particles in the mitochondrial membranes as revealed by freeze fracture electron microscopy. The model shows the characteristic structural features seen in the half membranes that arise as a result of fracturing the outer and inner membranes. Non-particle areas in the fracture faces are persumed to be pure lipid domains. The B face of the outer membrane and the A face of the inner membrane are revealed as convex fractures, concave fractures show the B face of the inner membrane and the A face of the outer membrane. (From Packer and Worthington, 1974, used by permission).

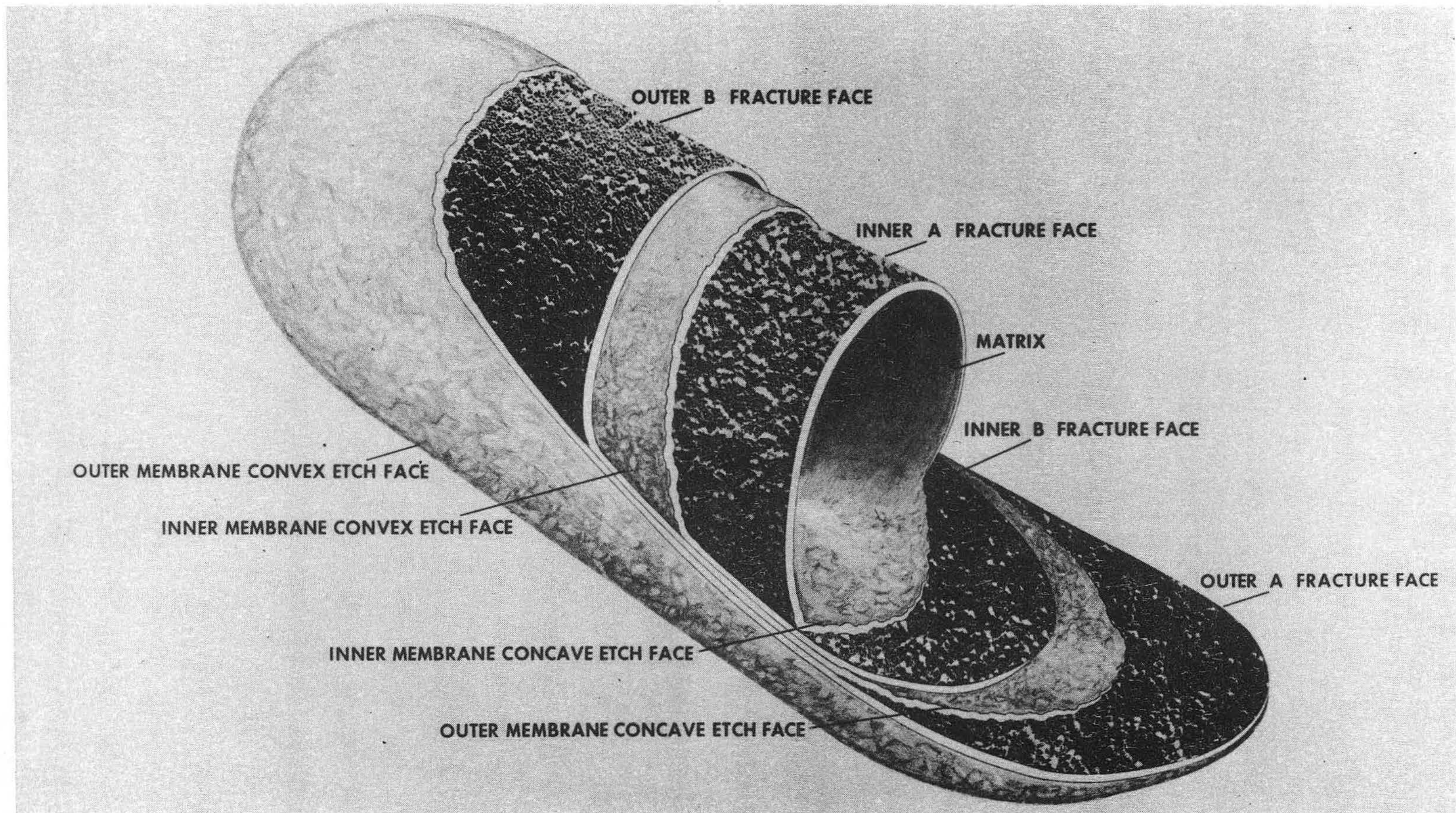


Fig. 4

XBB 770-10583

rate is slowed down (State IV) thus conserving unrequired energy in the form of reduced coenzymes. The conservation of energy released during these oxidative reactions in synthesizing ATP from ADP and inorganic phosphate is known as "Oxidative phosphorylation".

It has been established that three molecules of ATP are synthesized by the oxidation of one molecule of NADH. The synthesis of ATP is coupled to 3 sites of the respiratory chain. They are located on the path of electrons from NADH to ubiquinone (complex I), from ubiquinone to cytochrome c (complex III) and from reduced cytochrome c to oxygen (complex IV). The evidence for location of the energy coupling sites is based on the measurements of the phosphorylating efficiency with various substates and artificial electron donors and acceptors (141), on studies of the steady state kinetics of respiratory chain in the presence and absence of ADP (109), and on direct reconstitation experiments involving individual complexes together with ATP synthesizing system (142).

Much of our knowledge about the coupling of oxidative phosphorylation to electron transport stems from the use of uncouplers and inhibitors of oxidative phosphorylation. Uncoupling may be brought about by treatment of a variety of agents thus affecting respiratory control, oligomycin sensitive ATPase and phosphorylation efficiency. In uncoupled mitochondria, ATP synthesis is replaced by an ATP hydrolysis, respiratory control is abolished and free energy of substrate oxidation is dissipated in the form of heat (143). In general all the procedures which impair the integrity of the inner mitochondrial membranes results in partially or fully

uncoupled preparations (144). The uncoupling has been classified according to the procedures and agents which elicit this kind of response in mitochondria (145). It includes a) Structural uncoupling as induced by mechanical disruption (freezing and thawing, shearing forces, sonication), aging, detergents or phospholipase treatment; b) Uncoupling by cations and ionophores, e.g. cations such as K^+ in the presence of Valinomycin; c) uncoupling involving covalent binding e.g. alkylating agents of mustard gas and electrophiles such as isothiocyanates; d) uncoupling by phenols and other anionic aromatic compounds e.g., classical uncoupler 2,4 dinitrophenols.

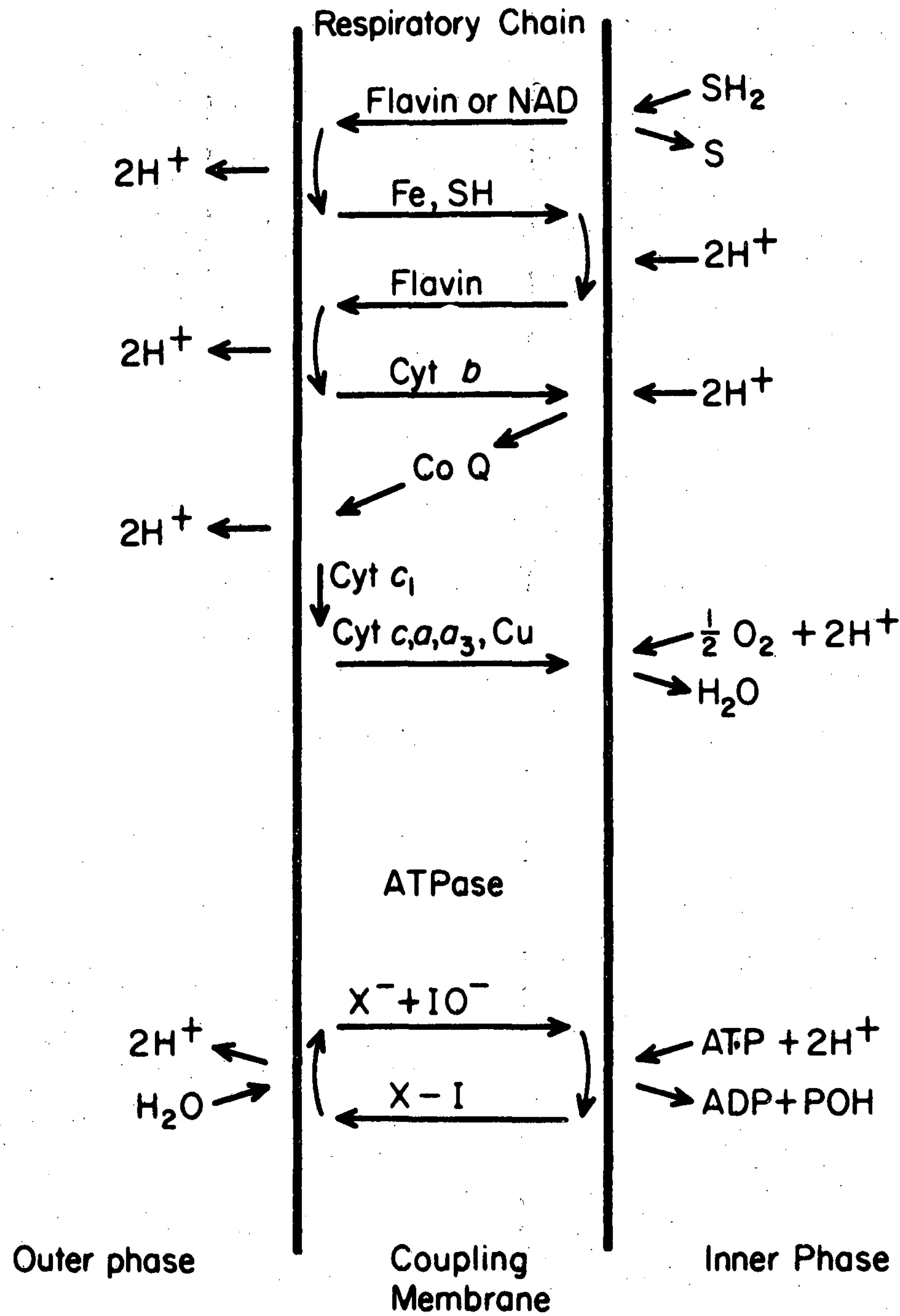
Perhaps the most enigmatic aspect of oxidative phosphorylation is the mechanism of energy coupling from electron transport to ATP synthesis. Most hypothesis of energy conservation differ mainly in what one assumes to be the nature of coupling device. A high energy intermediate has been proposed by Chance (146) and the conformational changes in the ATPase protein molecule has been suggested by Boyer (147). Perhaps the most widely accepted and debated hypothesis of energy coupling is that of Mitchell (148), according to which the energy of electron transport chain is stored in the form of electrochemical gradients. The electrochemical gradient is constituted by a H^+ ion concentration gradient and a potential across the semipermeable membrane, the so called protonmotive force. The electron transport is postulated to be responsible for the gradients in mitochandria by producing an efflux of protons. This is the consequence of an asymmetric proton discharge in the steps

in which reduced H carriers (e.g. reduced CoQ or NADH) are oxidized by the electron carrying cytochromes. Presumably there are three such steps or loops per cytochrome chain (Fig. 5). In this way $6H^+$ are separated between the internal and external phase by the transfer of an electron pair from NADH to oxygen. The experimental evidences supporting the possibility of producing protons in this manner are, that in rat liver mitochondria $6H^+$ are released when -hydroxybutyrate is used as a substrate and $4H^+$ when succinate is used (149). Similar results have been obtained with submitochondrial preparations. (150) However, kinetic and equilibrium behavior studies of the cytochrome system of mitochondria do not support the redox loop concept of proton translocation (151). To explain such anamolies, a concept of the protonmotive ubiquinone cycle and of the cyclic loop 2-3 system has been introduced (152). The stoichiometery of the number of protons released per pair of electrons observed by previous workers (149,150) has also been considered coincidental. Some of the recent studies using oxygen pulse technique, indicate that the H^+/site ratio is 4 (153) instead 2 as it was originally proposed (149). The difference between these results becomes apparent when phosphate movement is taken into consideration. A separation of net 4 charges/site across the mitochondrial membrane with transfer of two electrons has also been suggested by others (154).

The production of a proton gradients concomittant to the electron transport has been found insufficient to support phosphorylation, thus a potential across the mitochondrial membrane has been suggested

Fig. 5. A scheme of proton translocating mitochondrial respiratory chain and ATPase as proposed by Chemiosmotic hypothesis. X-I represents an hypothetical intermediate responsible for proton translocation. (From Mitchell 1969, used by permission).

0 0 0 0 4 9 0 0 9 7 2



XBL 7710-10311

Fig. 5

also in part responsible for the electrochemical gradients (155). The membrane potential is the result of charge imbalance caused by the H^+ efflux accompanying metabolism. Presumably in intact mitochondria the inside phase would be negative. The membrane potential and proton gradient are related by the following equation:

$$\text{Proton motive force } \Delta\rho = \Delta\psi + 2.3\ RT/F\ \Delta\rho H$$

where $\Delta\psi$ is the membrane potential, R the gas constant, T the temperature and F the Faraday. A total protonmotive force of 230 mV has been estimated in state IV mitochondria by valinomycin induced K^+ distribution method (155). However lower values have been reported by others using different methods. The membrane potential measured from the distribution of 2,4 OXOZOlidinedione is 130-150 mV (156); direct determinations using microelectrodes indicated a value 10-20 mV (157); while studies using electrofluorometric dyes reflected membrane potential of 150-180 mV (158). The measurements of the relative contribution of $\Delta\psi$ and $\Delta\rho h$ to the protonmotive force in state IV mitochondria has revealed that $\Delta\psi$ represents more than two thirds of the total force (159). The actual protomotive force which would be needed for phophorylation depends on the concentration of various components. At steady state when ATP, ADP and Pi are in equilibrium, the force required for the synthesis of ATP is approximately 320 mv (160), however lower values have been achieved by others (161). Since the pH component is necessarily limited, a large portion of the protonmotive force would have to correspond to the membrane potential. However, biological membranes generally are not able

to support a potential much in excess of 200 mV, perhaps due to the nature of lipids.

c) Ion Transport: Transport of a majority of ions through the mitochondria occur by means of a special mechanism involving carriers or transporter molecules, which is characterized by a) high specificity of the carrier molecule b) saturation kinetics c) blockage by specific inhibitors and d) a high dependence on temperature. Metabolites such as adenine, glutamate, aspartate, ornithine, etc., are transported actively either against electrochemical gradients or in the direction of the gradients on the expenditure of energy (162). In case of glutamate there is some indication that no energy is required in maintaining its steady state (163).

The study of ion transport in isolated mitochondria has been carried out in a number of ways. An indirect method for following the peneteration of substrates uses the redox state of pyridine nucleotides as an indicator of their entery into the mitochondrial interior (164). The transport of ions can also be followed by ion specific electrodes, in particular cationic electrodes highly sensitive to K^+ or Na^+ (165). The external concentration of Ca^{++} can be monitored accurately and rapidly with Ca^{++} indicator dye, mureoxide (166). Radioactive tracer technique is also useful to estimate cations and anions transport. The permeability and active transport in mitochondria has been studied indirectly by monitoring the light scattering or transmission by the mitochondrial suspension. The light scattering (or the optical density) varies inversely with the mitochondrial volume (167) which changes with the transport

of ions. The alterations in the mitochondrial volume under different metabolic states has also been followed by light transmission (168,169).

The transport of substrate anions across the mitochondrial membrane is carrier mediated; requires catalytic amount of phosphate and is inhibited by mercurial agents (170). Monovalent cations such as K^+ can be exchanged against H^+ gradients in the presence of an antibiotic, valinomycin (171). Phosphate transport is energy dependent and is inhibited by mercurials. The carrier mediated transport of adenine nucleotide involves one to one exchange between ATP and ADP; and is blocked by atractyloside (172). The inhibitors of transport are not always specific. Tetraphenylboron, for example, has been found to block the entery of a variety of ions, presumably by increasing the negative charges on the mitochondrial surface (173). Conversely, cations added to mitochondria suspended in low ionic strength medium increases the uptake of succinate, malonate and phosphate. This effect has been interpreted to be due to the increase in the positive charges on the mitochondrial surface, although alternate explanation such as, co-transport is also possible.

d) Protein Synthesis:

Mitochondria carry out synthesis of about a dozen of hydrophobic polypetides (174) three of which are associated with cytochrome oxidase, four with oligomycin sensitive ATPase and one with cytochrome b. Probably some components of the adenine nucleotide translocator are also synthesized in the mitochondria (175). The nature of the remaining

0 0 0 0 4 9 0 0 9 7 4

polypeptide is not known. Like bacteria, mitochondrial protein synthesis system is inhibited by chloramphanicol, lincomycin and erythromycin (176). Certain mitochrondrial mutants exhibit antibiotic resistance *in vivo* but not *in vitro*. Since the protein synthesizing unit in the mitochandria is membrane bound (177), this may explain the hydrophobic nature of polypeptides produced as they are to be incorporated into the membrane.

RATIONALE OF PRESENT STUDY

In our laboratory, it has been demonstrated that exposure of human cells in culture to light in the visible region of the spectrum or to high oxygen concentration leads to gradual cessation of growth and DNA synthesis (40). Microscopic observations indicate mitochondria as the probable locus of damage in the cell. Therefore, we decided to investigate the effects of visible light and oxygen exposure on the mitochondria with following rationale:

1) Pigments such as flavins, cytochromes and iron sulfurs present in the mitochondria, absorb visible light and thus could act as photosensitizers.
2) Almost 90% of the total cell oxygen uptake is due to the mitochondria, and thus sensitized photooxidation may result.
3) A high unsaturated fatty acid contents of the mitochondrial membranes make it more susceptible to environmental factors.
4) A series of free radical mediated reactions carried out by the mitochondrial biocatalysts make it a self destructure unit.
5) Any damage to the energy synthesis apparatus of the mitochondria may cause inhibition of growth, DNA synthesis and active transport of the cell.

Therefore, mitochondria seem to be a useful test system to study the effects of visible light and oxygen exposure in particular. In the present investigation, attempts have been made to characterize the nature of damage, its probable mechanism and protection.

0 0 0 0 4 9 0 0 9 7 5

EXPERIMENTAL METHODS

I. Mitochondrial preparations: Mitochondria (RLM) were isolated from rat liver according to established procedures (178), in a medium of 0.25 M sucrose, 1mM Tris and 1 mM EDTA (pH 7.4). For the last two centrifugations, RLM were washed in 0.25M sucrose and finally resuspended in this medium at a concentration of 80 mg protein/ml. Submitochondrial preparations (SMP) were made by sonicating mitochondria at 20 kHZ in 30 mM phosphate buffer pH 7.4 with 30 sec periods for a total of 2 min, then removing unbroken mitochondria by centrifugation at 8500Xg for 10 min, centrifuging the supernatant fraction at 100,000Xg for 10 min and resuspending the pellet of SMP in 30 mM phosphate buffer. The method used to prepare cytochrome c depleted mitochondria was essentially that of Jacobs and Senadi (102) which involves incubation of RLM in 15 mM KCl for 10 min at 4°C, centrifugation at 6000 Xg for 10 min, resuspending and incubating the pellet in 150 mM KCl for 10 min at 4°C, centrifugation at 5000 Xg for 10 min, washing twice identically and resuspension in 0.25 M sucrose.

II. Illumination Procedure: Incubation of dark and light treated samples (10 ml at 20 mg protein/ml in 0.25 M sucrose) was carried out in a 50 ml conical flasks at 20°C in a specially designed refrigerated shaking water bath, as shown in Fig. 6. The light source was a battery of 250 W quartz iodide lamps (General Electric) and the net light intensity, as measured by a YSI-Kettering (Model 65) radiometer using Kodak neutral density filters,

Fig. 6. A picture of illumination apparatus used in present investigation.

0 0 0 0 4 9 0 0 9 7 6

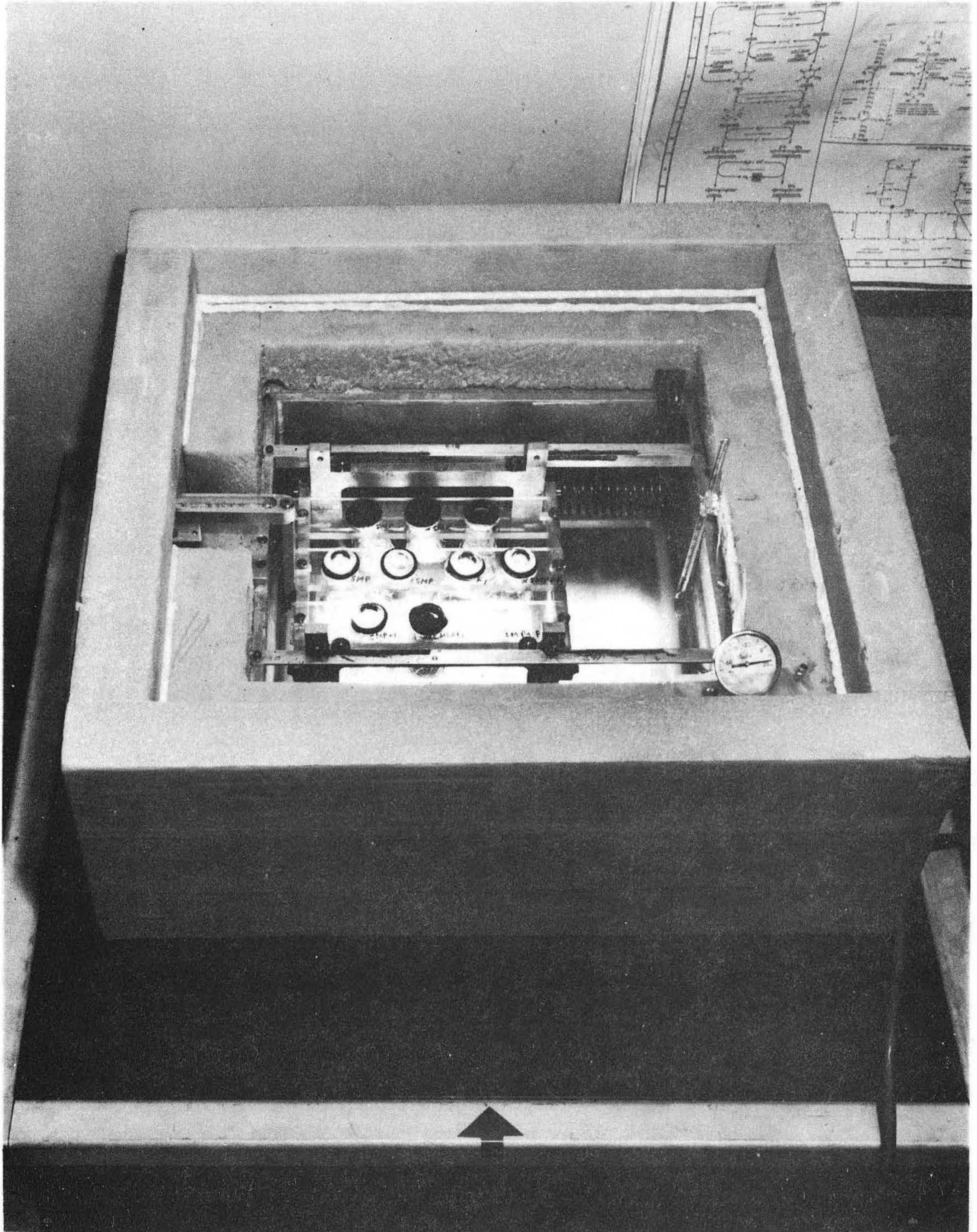


Fig. 6 CBB 761-287

was 300 mW/cm^2 at the sample level. The samples were located at a distance of 8 inches from the light source which was covered by a 400 nm cutoff filter, Corning glass #3389, used to eliminate ultraviolet radiation. The flasks were shaken constantly during illumination. Aliquots were withdrawn at various time intervals and assayed at room temperature.

III. Biochemical Assays and Procedures: Mitochondrial respiration was assayed at protein concentrations of 3 mg/ml in a Rank Oxygen electrode (Rank Brothers, Bottisham, Cambridge, England) attached to a strip chart recorder. The basic reaction medium consisted of 240 mM sucrose, 2 mM KH_2PO_4, 2 mM KCl and 5 mM Tris buffer (pH 7.4). Additions of various substrates (final concentration of 5 mM) were made with a syringe and rates recorded. All substrates were adjusted to pH 7.4 with buffer; durohydroquinone solutions were prepared in dimethylformamide each day before use. The rate of oxygen uptake was expressed as atoms of oxygen consumed/min/mg protein. Respiratory control ratio (RCR) was calculated from the rate of respiration with substrate plus ADP over rate without ADP.

ATP Synthesis and Hydrolysis. The standard assay medium for measurement of ATP synthesis contained 4 mM Tris-HCl buffer (pH 7.4), 10 mM potassium phosphate buffer (pH 7.4) containing ^{32}Pi (2×10^6 c.p.m/ mole), 20 mM glucose, 0.5 mg of hexokinase/ml, 1 mM ADP, 5 mM glutamate and 5 mM malate in a final volume of 3 ml at 25°C. The experiment was initiated by the addition of mitochondria to a final concentration of approximately 6 mg protein/ml. At the exact moment that the sample went anaerobic

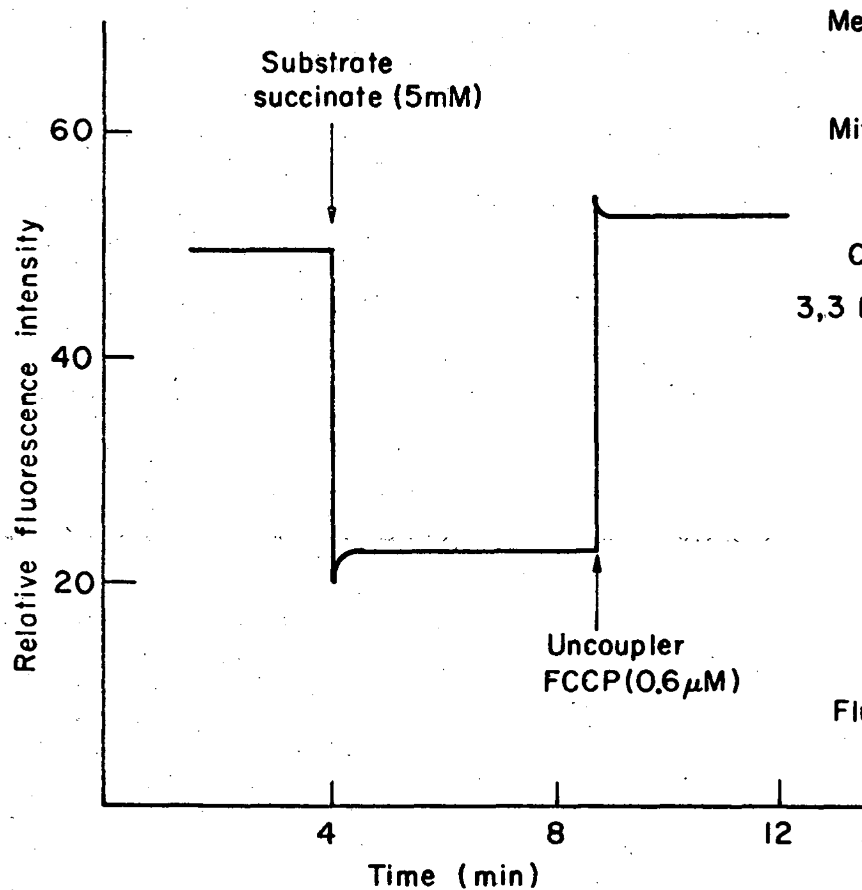
0 0 0 0 4 9 0 0 9 7 7

as determined in a separate experiment on the oxygen electrode, the reaction was stopped by the addition of 0.3 ml of 50% (w/v) trichloroacetic acid. After centrifugation (5000 x g for 10 min), 1 ml of the supernatant was removed and the amount of ^{32}Pi determined by the method of Avron (179). The P/O ratio was calculated as the ratio between the total amount of phosphate esterified to the total amount of oxygen used in the time interval between the addition of mitochondria and the addition of trichloroacetic acid.

ATP hydrolysis was assayed in a reaction medium consisting of 250 mM sucrose, 025 mM $MgCl_2$, 2 mM KCl, 10 mM Tris-HCl buffered to pH 7.4 and 0.1 mg mitochondrial protein in a total volume of 2 ml at 30°C. The reaction was initiated by the addition of ATP (6 mM at pH 7.4) and carried out at 30°C for 20 min after which it was stopped by the addition of 0.1 ml of 40% (w/v) ice cold trichloroacetic acid; the sample was centrifuged (5000 x g for 10 min) and the amount of phosphate in the supernatant determined by the method of Lindberg and Ernster (180). Specific activity was expressed in nmoles of phosphate released/min/mg protein.

Transmembrane Potential. Changes in membrane potential of mitochondrial suspension was estimated by following the fluorescence changes with the cyanine dye, 3,3'-dipropylthiodicarbocyanine iodide at 620 nm excitation/670 nm emission in a Perkin Elmer MPF 44-A spectrofluorometer using a slit width of 5 nm (158). The complete reaction medium contained 240 mM

Fig. 7. Measurement of transmembrane potential by energization and deenergization of the mitochondria using a cyamine dye.

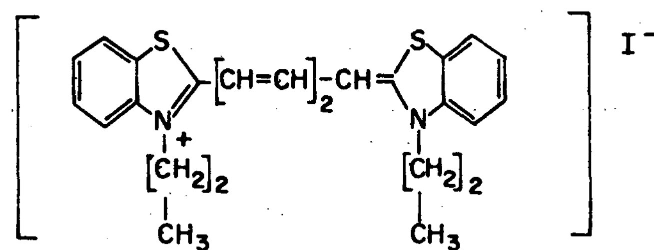


Medium : KCl (150 mM)
Tris: Tris (10mM)
pH 7.4
Mitochondrial protein: 0.25 mg/ml

Cyanine dye: 13 nmoles/mg

3,3 Dipropylthiodicarbocyanine iodide

[diS - C_3 - (5)]



Fluorescence: 620nm excitation/ >670 nm measurement

XBL 768-9198

Fig. 7

sucrose, 10 mM Tris-HCl (pH 7.4), cyanine dye at 13 nmoles/mg protein and 0.75 mg mitochondrial protein in a final volume volume of 3 ml. Changes in the fluorescence intensity were recorded after the successive additions of 5 mM glutamate + malate for the energized condition and 0.6 μM p-trifluoromethoxy (carbonylcyanide) phenyl hydrazone (FCCP) for the deenergized condition (Fig. 7).

Spin Label Assay. The reduction of the spin label CAT_{16} by electron transport from various substrates was followed with a Varian E-109 spectrometer as previously described (181). CAT_{16} is an analogue of cetyl trimethyl ammonium bromide which partitions totally in the membrane; it was synthesized by Dr. R. J. Mehlhorn or our laboratory. The reaction medium used was identical to that used for assaying fluorescence changes described above but at a SMP protein concentration of 6 mg/ml and a spin label concentration of ~ 15 nmoles/ mg protein.

Spectrophotometric Assays: Succinate and choline dehydrogenases activities were measured by phenazine methosulfate (PMS) mediated reduction of 2,6--dichlorophenolindophenol (DCPIP) at 600 nm in a Cary-14 spectrophotometer (182). The reaction (1 ml) contained 50 mM phosphate buffer (PH 7.8), 0.05 mM DCPIP, 0.45 mM PMS, 1.5 mM neutralized KCN and 1 μg Antimycin A. The method of Singer (183) was also used for certain experiments. The latter method requires activation of succinate dehydrogenase and utilizes extrapolated to infinite PMS concentration. However since activities were expressed as percent change of dark control on visible light treatment

0 0 0 0 4 9 0 0 9 7 9

the two methods gave comparable results when examined for succinate dehydrogenase.

NADH dehydrogenase was measured by following the reduction of $K_3Fe(CN)_6$ at 420 nm (184). The assay mixture (1 ml) contained 4 mM Tris-HCl (pH 7.8), 0.75 mM KCN, 1.33 mM $K_3Fe(CN)_6$ and 1μg Antimycin A.

Different spectra of cytochromes were recorded on an Aminco Chance DW--2 dual beam spectrophotometer using a reaction medium consisting of 0.25M sucrose, 0.1 mm KCN and 1mM freshly prepared durohydroquinone.

Lipid Peroxidation. Malondialdehyde a product of lipid peroxidation was measured by modifying a standard method (185) employing thiobarbituric acid, which forms a colored complex. To 1 ml of mitochondrial suspension (containing 0.25-0.5 mg of protein) was added 0.25 ml of 40% (w/v) trichloroacetic acid and 0.25 ml of 2% thiobarbituric acid (pH 7.0 with NaOH). The tubes were boiled for 10 min and then cooled in ice. Flocculant material was removed by centrifugation and 532 nm absorbance measured. The amount of thiobarbituric acid-positive material was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$ (186).

Sulfhydryl Groups. The total sulfhydryl groups of the mitochondria were determined essentially according to the method of Ellman (187). To 1 ml of the sample containing approximately 0.5 mg protein was added 0.01 ml of 10%, sodium dodecyl sulfate to solubilize the mitochondrial membrane. Reaction was started by the addition of 0.02 ml of 3.96 mg ml^{-1} 5, 5'-dithiobis (2-nitrobenzoic acid) and

after 1 hour incubation at room temperature, the absorbance at 412 nm was measured. The concentration of sulfhydryl groups was calculated using an extinction coefficient of 13.6 mM^{-1} cm^{-1}. A calibration curve was made using reduced glutathione in the range 10^{-4}-10^{-5}M.

Amino Groups. The total free amino groups of the proteins were determined by 2, 4, 6 trinitrobenzene sulfonic acid method (188).

Mitochondrial protein was determined by the Biuret method (189) using Bovine serum albumin as a standard.

Flavin Determination. Method employed for the extraction and determination of flavins was essentially that of Singer *et al* (190). Acid extratable flavins were isolated by 5% ice cold TCA precipitation of SMP. One half of the supernatant was neutralized immediately with 2M $KHCO_3$ to pH 7.4, while the other half was incubated at 38°C for overnight in order to hydrolyze the flavin dinucleotide to mononucleotide.

For the isolation of acid non extractable flavins, SMP were precipitated by an ice cold TCA to a final concentration of 5% (w/v), centrifuged and discarded the supernatant. The pellet was resuspended in 2 ml of cold acetone and 0.016 ml of 6N HCl. Centrifuged, discarded the supernatant and resuspended the pellet in 0.2 ml of 1% TCA and 4 ml acetone. The pellet was washed 3 times with 2 ml of 1% TCA, and finally resuspended in 2 ml of 0.1 M Tris-base. Then added 0.1 mg Trypsin and 0.1 mg chymotryprin per mg of SMP protein, and adjusted the pH to 8.0. After incubation at 38°C for 4 hrs with occasional shaking, removed to ice bath and adjusted the pH 0.3-0.4 with 55% TCA. Incubated again at 38°C for overnight and then adjusted the

pH to 3.5 with 2M $KHCO_3$. Centrifuged and measured the flavin contents in the supernatant.

Fluorometry on acid extractable and non-extractable flavins were performed on Perkin Elmer MPF 44A spectrofluorometer with excitation at 375 nm, emission at 526 nm and bandwidth of 5 nm. Riboflavin was used as a standard in 0.1 M phosphate buffer pH 7.4 for acid extractable flavins and in phosphate-citrate buffer at pH 3.4 for acid non-extractable flavins.

Polyacrylamide gel Electrophoresis in SDS. The overall approach was that of Fairbanks (191) with a few modifications. Concentrated stock solutions were mixed in the proportions given in Table IV.

The gel solution was poured into glass tubes (5 mM x 100 mM) to a height of 85-90 mm and overlayed with water. After gelation (60 min at room temperature), the water was replaced by electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.4) and the gels were allowed to sit overnight before being used. Samples (2 mg protein ml^{-1}) were solubilized by mixing 3 parts suspension with one part of solubilization mixture to afford the following final concentrations: sodium dodecylsulfate (1%), mercaptoethanol (1%), glycerol (5%), pyronine Y (12.5 μg/ml) in electrophoresis buffer. The solubilized samples (20-50 μl, 30-75 μg protein) were layered beneath the buffer onto the gel surface. A current of 1 mA/gel was administered until the pyronine Y (tracking dye) entered the gel. The current was then increased to 8 mA/gel until the tracking dye migrated to within 2-3 mm of the end of the gel (approximately 90 min). After electrophoresis, the gels were

removed from the tubes by squirting water from a syringe between gel and glass wall. The position of the tracking dye was marked in each gel by pricking it with a needle dipped in drafting ink.

The gels were stained with coomassie blue in the following stages: 1) 25% isopropyl alcohol, 10% acetic acid, 0.025% coomassie blue - overnight; 2) 10% isopropyl alcohol, 10% acetic acid, 0.0025% coomassie blue - 6-9 h; and 3) 10% acetic acid for several h until the background was clear.

0 0 0 0 4 9 0 0 9 8 1

Table IV. A list of various solutions used for the polyacrylamide gel electrophoresis

SOLUTION A (ELECTROPHORESIS BUFFER pH 7.4)		
	Tris HCl	0.04M
	sodium acetate	0.02 M
	EDTA	0.002M
SOLUTION B		
	Acrylamide	18 g
	Bis-Acrylamide	0.48 g
	Electrophoresis buffer . . . . . . up to	100 ml
SOLUTION C		
	N,N,N',N'-tetramethylethylene diamine	0.15 ml
	Sodium dodecyl sulfate	0.3 g
	Electrophoresis buffer . . . . . . up to	100 ml
SOLUTION D		
	Ammonium persulfate	0.3 g
	Electrophoresis buffer . . . . . . up to	100 ml
SOLUTION E		
	Mix Solutions B:C:D	1:1:1

RESULTS AND DISCUSSION

Section I: COUPLING AND ELECTRON TRANSPORT

Respiration

Oxygen uptake by mitochondria was recorded with substrates which enter the electron transport chain at various points (Fig. 3). The coupled respiration of NAD^+ and flavin dehydrogenase linked substrates (State 4) increased by as much as 3-fold during the first 5-7 hours of illumination almost reaching the value of ADP stimulated rate (Fig. 8A). This indicates that the mitochondria were becoming uncoupled. Subsequently the rate of respiration decreased, becoming almost completely inhibited after 12 hours of illumination. Under the same conditions the ADP stimulated respiration (State 3) decreased steadily from the start of light treatment without showing the initial stimulatory phase. The activity of dark controls remained essentially unchanged during the 12 h of incubation. The state 4 respiration with durohydroquinone as substrate did not decrease significantly, however state 3 respiration decreased (Fig. 8B). Cytochrome c oxidase activity (substrate: ascrobate + TMPD) was not significantly affected by light exposure. The respiratory control ratio (State 3/State 4), an index of electrochemical coupling due to oxidative phosphorylation across the inner membrane was the first parameter to change; it declined rapidly during the first 3-6 h of illumination (Fig. 9). These results suggest that during the first 6 h of illumination the mitochondria become uncoupled. To confirm this it was decided to assay directly ATP synthesis and hydrolysis.

0 0 0 0 4 9 0 0 9 8 2

Fig. 8. Effect of time of illumination on mitochondrial respiration. (-Δ-,-O-) represents illuminated samples and (-▲-, -●-) represent dark controls. A) Natural substrates reacting with dehydrogenases; B) Artificial substrates reacting with ubiquinone and cytochrome c oxidase. Where indicated, 1 mM ADP, 1 mM durohydroquinone or 1 mM ascorbate + 0·1 mM TMPD were present.

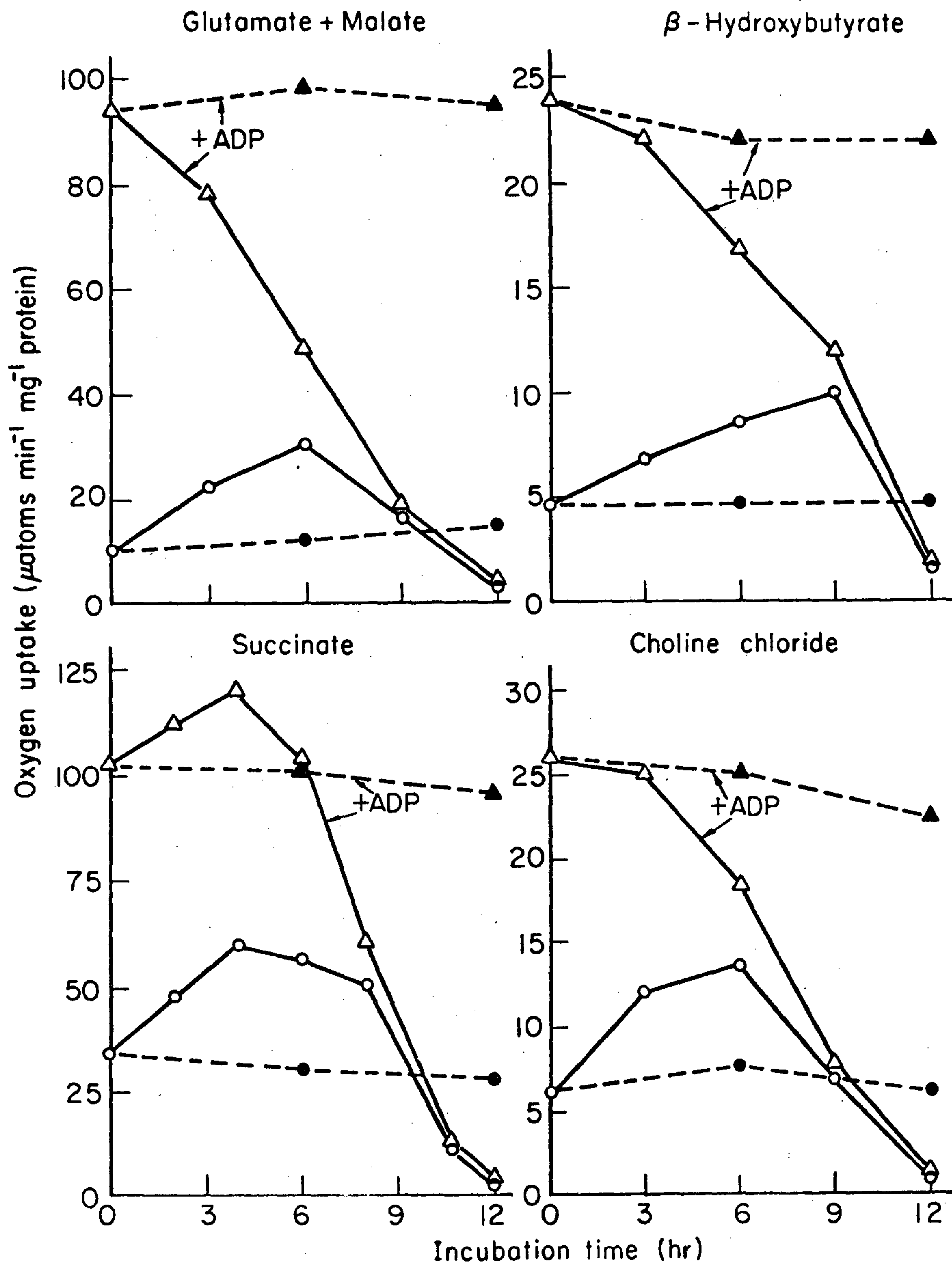


Fig. 8A

0 0 0 0 4 9 0 0 9 8 3

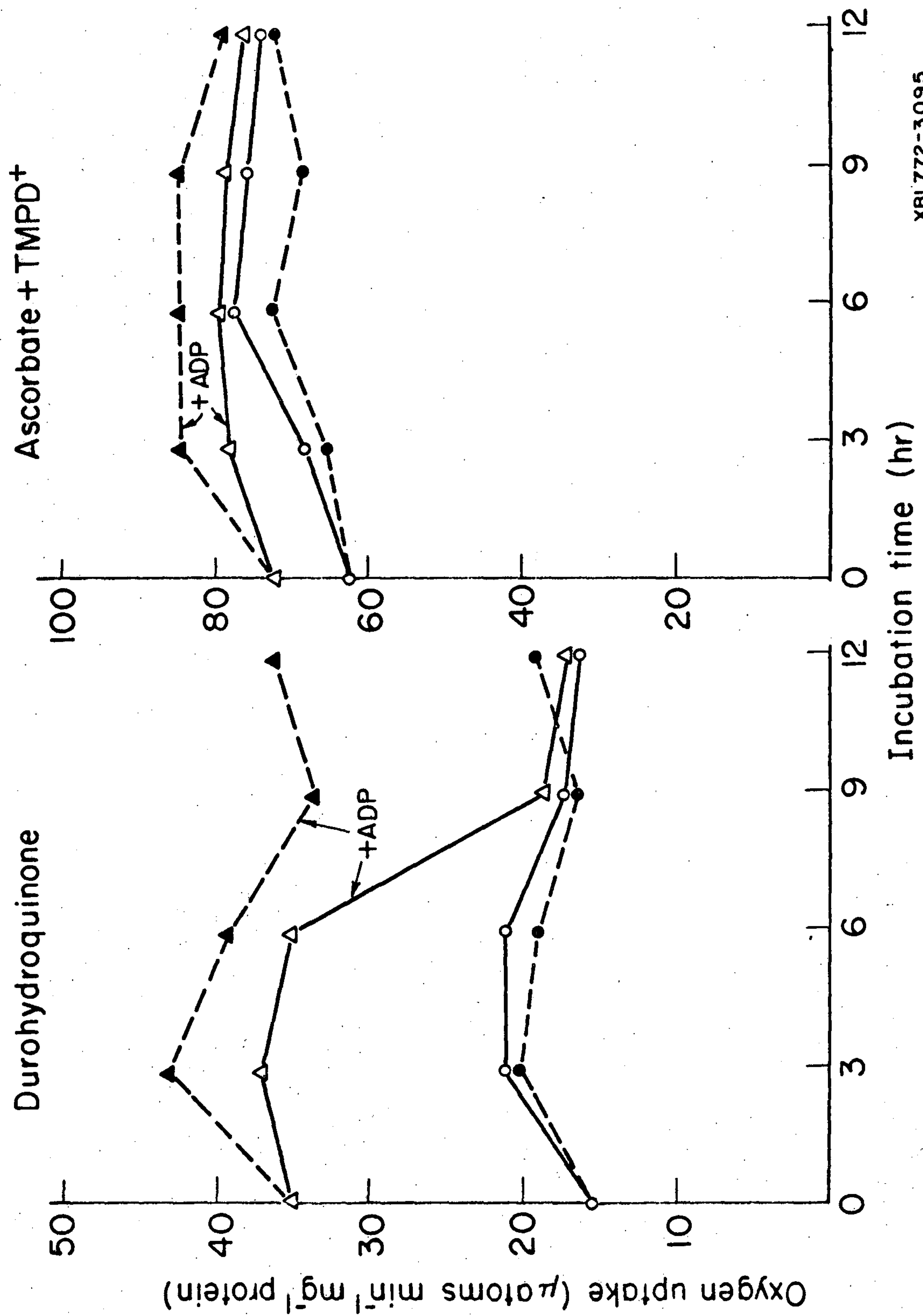
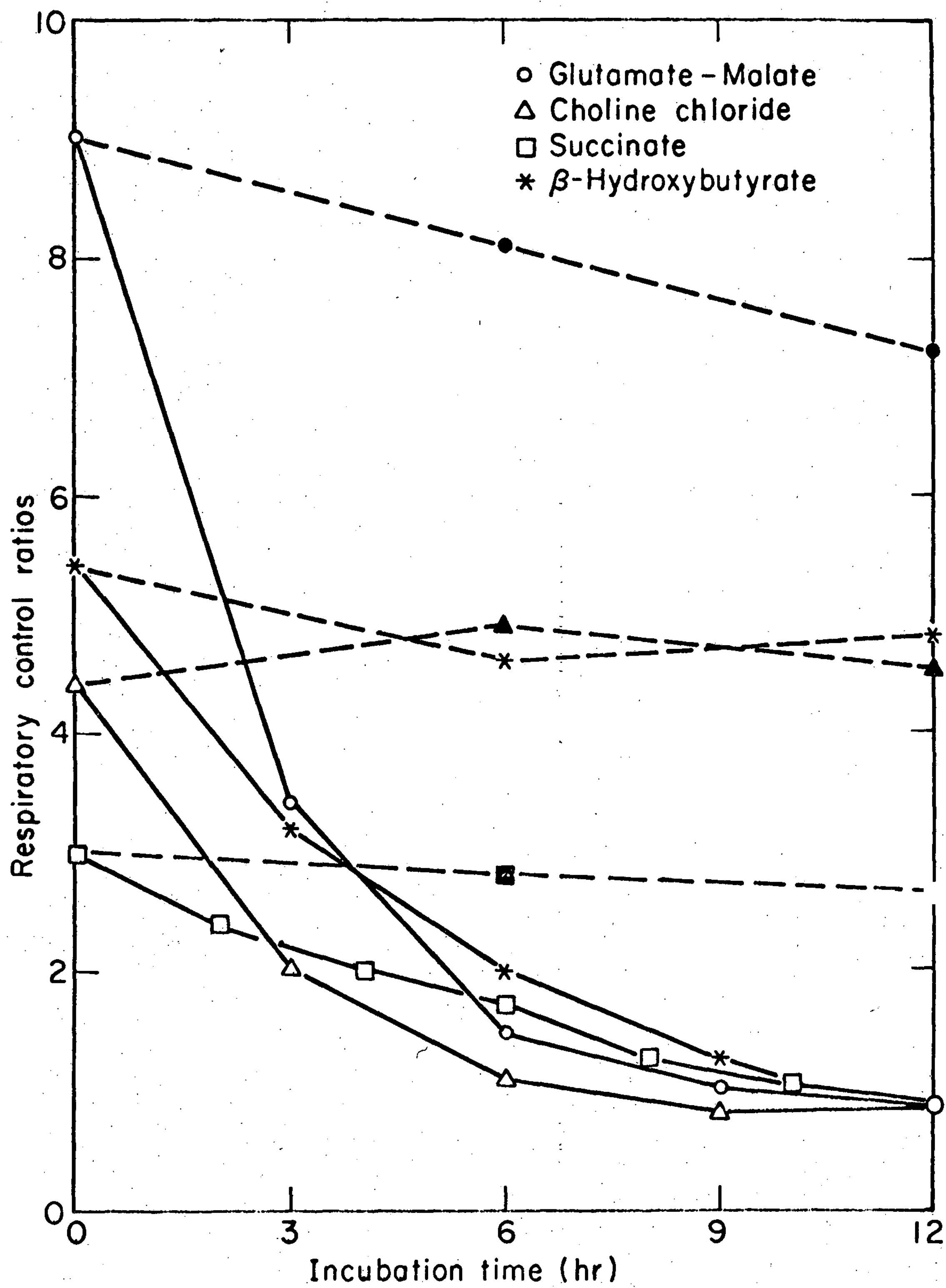


Fig. 8B

Fig. 9. Effect of incubation time on the respiratory control ratios of the mitochondria using various natural substrates. Solid lines represent illuminated and dotted lines non illuminated samples.

0 0 0 0 4 9 0 0 9 8 4



XBL 773-3221

Fig. 9

ATP Synthesis and Hydrolysis

The rate of ATP synthesis showed (Fig. 10) a steady decline over 12 h of illumination. At 12 h of illumination the P:O ratio with glutamate + Malate as substrate was less than 1. It can be seen that the decline in the RCR preceded that of the P:O ratio. A progressive stimulation of ATPase activity was observed during the first 6 h of illumination remaining more or less at this maximal level thereafter. The maximum stimulation of ATPase activity observed was simalar to that measured in the presence of the uncoupler FCCP in the non-illuminated mitochondria.

Transmembrane Potential

A more direct measure of electrochemical coupling due to ion permeability across the mitochondrial membranes is afforded by partitioning of charged dye cations. Mitochondria were energized either by succinate or by glutamate + malate addition, and the fluorescence quenching of the cyanine dye, 3,3'-dipropylthiodicarbocyanine iodide, was followed (Fig. 7). This measure of the transmembrane potential remained unaffected during the first 6 h of illumination but declined rapidly thereafter (Fig. 10).

Destruction of Various Respiratory Components

Dehydrogenases

Illumination caused 90% inactivation of succinate dehydrogenase activity after 12 h of incubation. Under these conditions 65% of choline dehydrogenase and only 40% of NADH dehydrogenase activity was destroyed (Fig. 11). Although the rates of inactivation of various dehydrogenases were different, the rates of inactivation of the

0 0 0 0 4 9 0 0 9 8 5

Fig. 10. Effect of time of illumination on coupled respiration, oxidative phosphorylation, and electrochemical coupling. Solid lines represent illuminated and dotted lines dark control samples. Glutamate + malate was used as substrate.

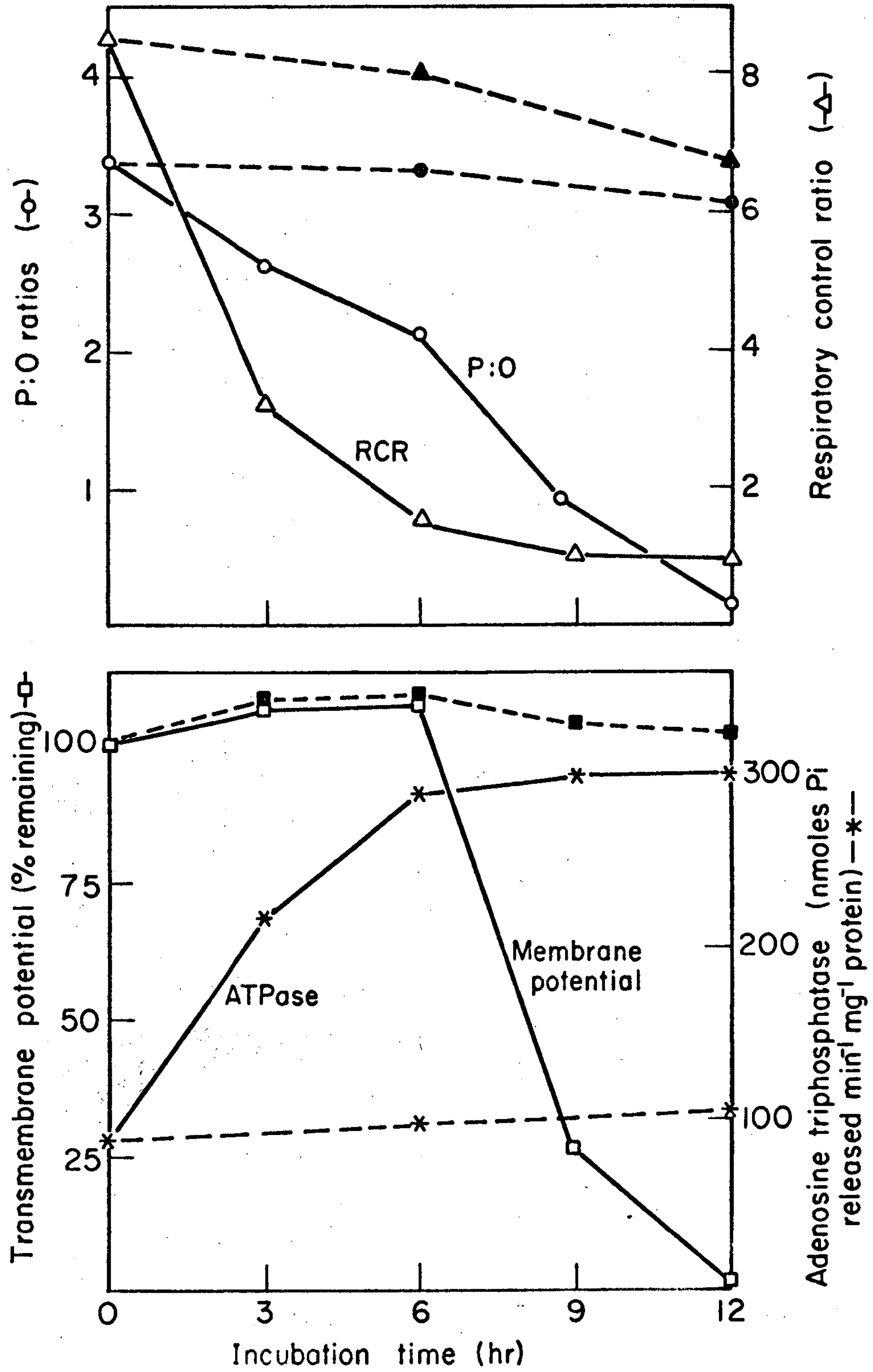


Fig. 10

0 0 0 0 4 9 0 0 9 8 6

Fig. 11. Comparison of the effects of illumination time on dehydrogenase, CAT_{16} reductase and oxidase activities in SMP.

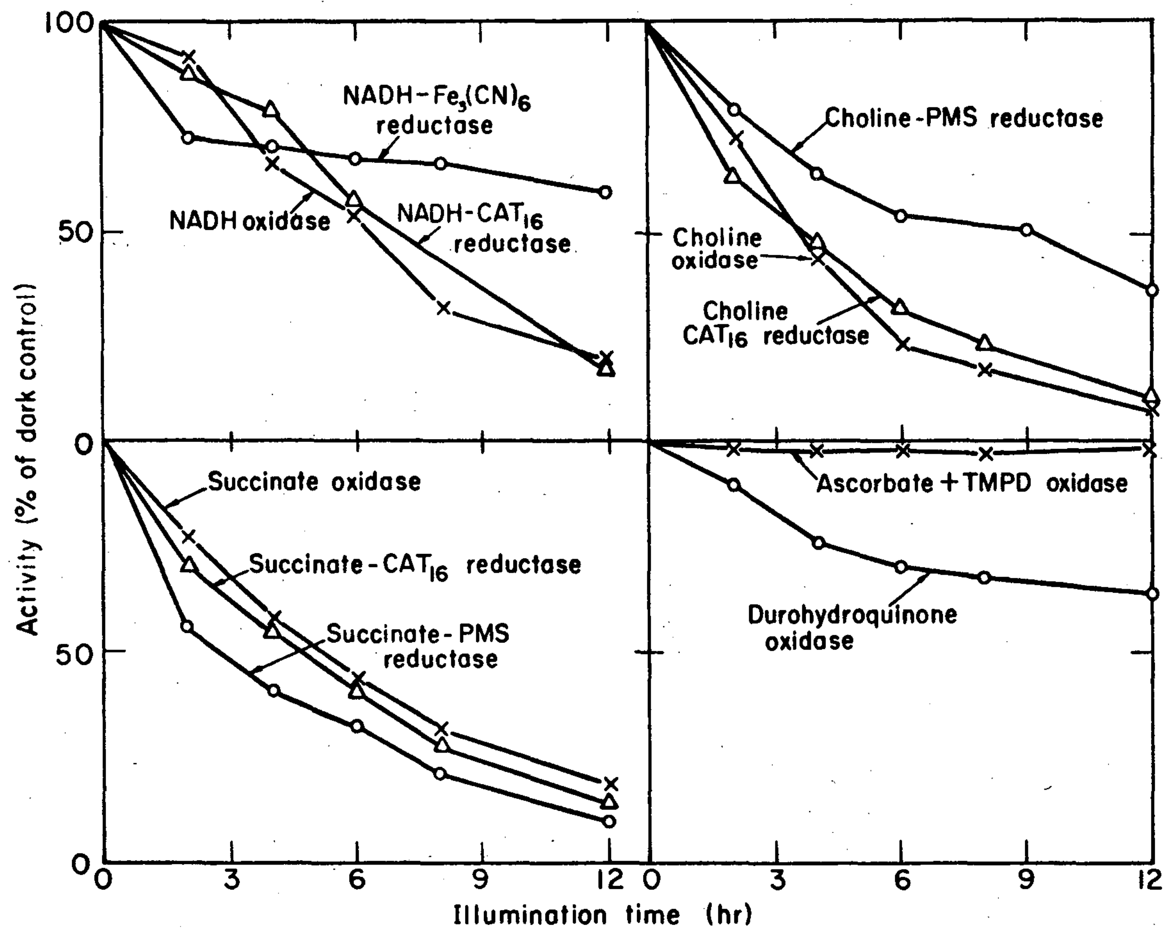


Fig. 11

corresponding oxidase activities were all similar. Approximately 80% of the activity of the succinate, choline and NADH oxidases was lost in 12 h of illumination.

Quinones

The spin label CAT_{16} accepts electrons from the ubiquinone part of the electron transport chain (181). CAT_{16} reductase activity with different substrates was inactivated in parallel with the oxidase activity which implies that illumination leads to inactivation of ubiquinone (Fig. 11). Other evidence indicating that the ubiquinone region of the respiratory chain was damaged by visible light is that oxidation of durohydroquinone, a direct electron donor to the quinone region, was inactivated by 35% over a 12 h period (Fig. 11).

This conclusion is also supported by the rates of inactivation of NADH$_{\rightarrow}$ O_2, NADH CAT_{16} and NADH$_{\rightarrow}$ k_3 Fe $(CN)_6$. Those parts of the chain that involve ubiquinone, namely NADH$_{\rightarrow}$ O_2 and NADH$_{\rightarrow}$ CAT_{16} were inactivated at the same rate, while NADH$_{\rightarrow}$ k_3 Fe $(CN)_6$ which does not require ubiquinone, was inactivated at a relatively slow rate (Table V). This indicates that after exposure to light, damage to ubiquinone is probably the rate limiting step in the flow of electrons from NADH to CAT_{16} or to oxygen. A similar situation holds for choline oxidation. In the case of succinate the rates of inactivation of succinate$_{\rightarrow}$ O_2, succinate$_{\rightarrow}$ CAT_{16} and succinate$_{\rightarrow}$ PMS were dentical indicating that the rate of inactivation of succinate dehydrogenase was $\geq$ to that of the ubiquinone.

Table V. A Summary of 50 Percent Inactivation Time of the Respiratory Chain Using Various Electron Donors and Acceptors

ELECTRON DONOR	ELECTRON ACCEPTOR	50 PERCENT INACTIVATION TIME (HRS.)
NADH	O_2	6·5
NADH	$k_3Fe(CN)_6$	23·0
NADH	CAT_{16}	6·0
Succinate	O_2	4·9
Succinate	PMS-DCPIP	4·5
Succinate	CAT_{16}	5·0
Choline	O_2	5·5
Choline	PMS-DCPIP	10·0
Choline	CAT_{16}	5·0

0 0 3 0 4 9 0 0 9 8 8

Endogenous Cytochrome and Flavins

No significant loss of durohydroquinone reducible cytochromes b_{562}, b_{568}, c_{550} or a_{605} was observed after 12 h illumination. However, exposure of SMP to visible light caused considerable destruction of flavins. The acid extractable FAD and FMN and non-extractable FAD were destroyed at similar rates (Fig. 12). About 50% of total flavins were lost over 12 h of illumination.

Sulfhydryl and Amino groups

Illumination of SMP resulted in approximately 60 percent loss of total free sulfhydryl groups during 12 hrs. of incubation (Fig. 13). Under these conditions no significant loss of total free amino groups was observed.

Discussion

Our results indicate that visible light inactivates the electron transport and energy coupling mechanisms linked to the inner membrane of mitochondria.

Electron Transport

The respiratory chain shows a pattern of inactivation by visible light in the following order of sensitivity: Succinate dehydrogenase $\geq$ ubiquinone > choline dehydrogenase > NADH dehydrogenase. The cytochromes do not seem to be affected. The high sensitivity of succinate dehydrogenase may arise from the covalent binding of the flavin moiety to a histidyl residue of the enzyme (192) which is essential for activity. The flavin sensitised photooxidation of histidine is well known (32,193). In NADH and choline dehydrogenases the flavin is not covalently bound

Fig. 12. Effect of illumination on the flavin content of SMP.

0 0 0 0 4 9 0 0 9 8 9

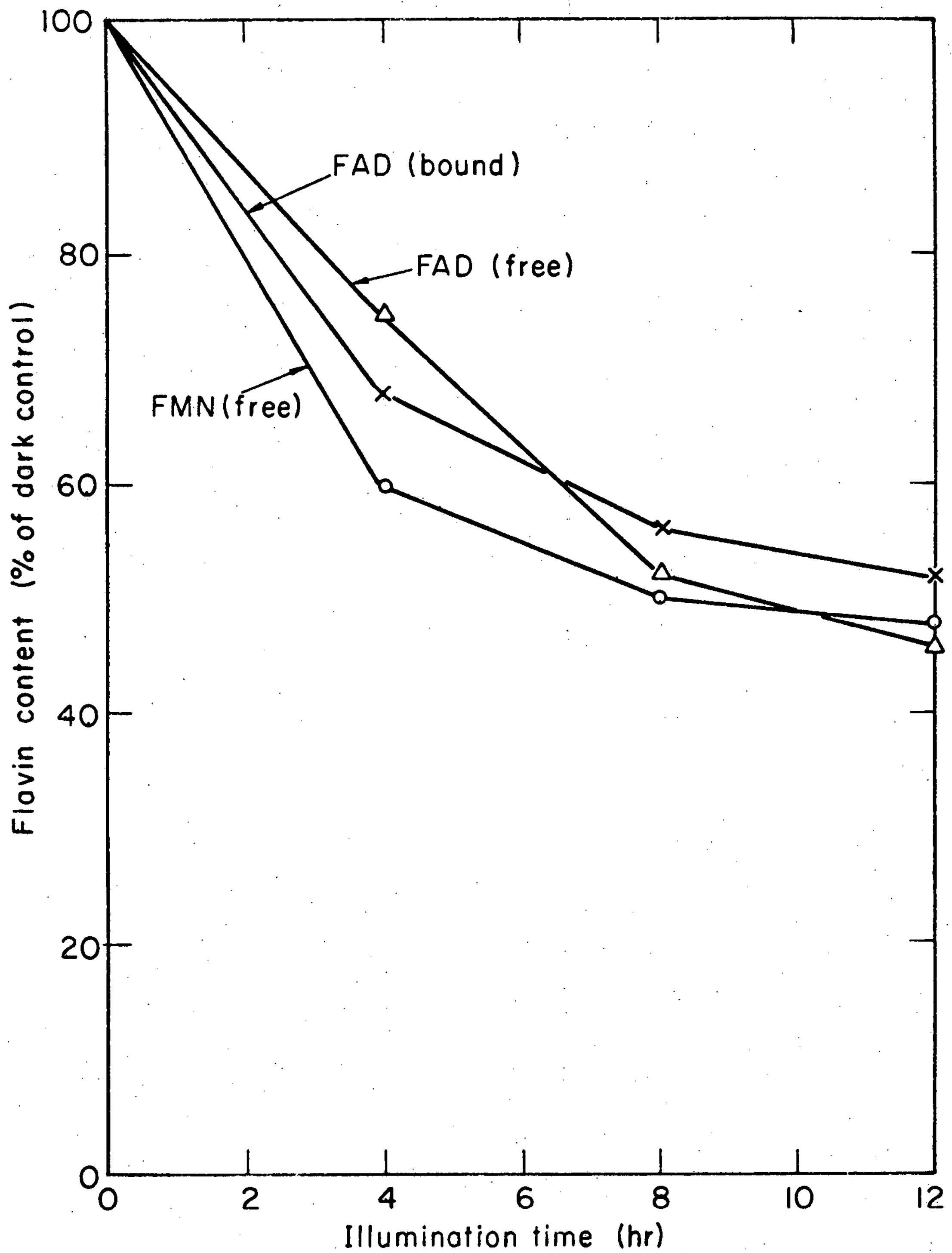


Fig. 12

Fig. 13. Effect of time of illumination on the total free sulfhydryl and amino groups with relation to succinate dehydrogenase activity in SMP.

0 0 0 0 4 9 0 0 9 9 0

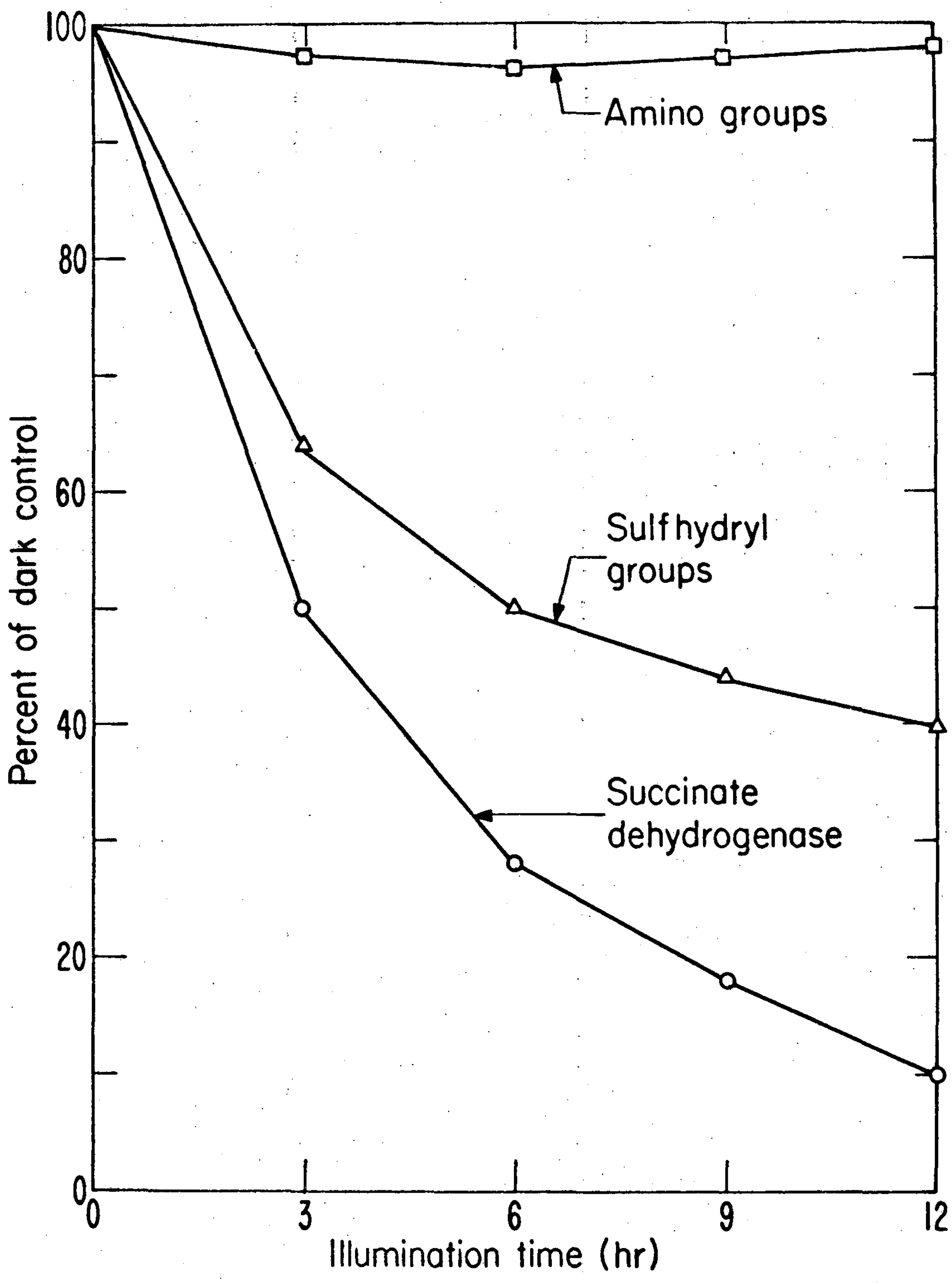


Fig. 13

to histidine which may account for their relative lower sensitivity to visible light. Alternatively, the location of flavin coenzyme within the protein molecule may also be responsible for differential sensitivly. A previous report (59) has also shown that the NADH dehydrogenase (complex I) of beef heart mitochondria is less sensitive to blue light than the succinate dehydrogenase (complex II). The destruction of succinate dehydrogenase on visible light exposure has also been shown in several microorganisms (30,194).

Succinate and NADH-ubiquinone reductases are complex enzymes containing a number of electron transfering groups, flavin, iron-sulfer centers and possibly protein-bound ubiquinone. The activity of this type of enzymes may depend on the integrity of all of their components, making their rate of inactivation more rapid than the rate of loss of any one component; it is not surprising then to find, for example, that the rate of loss of activity of succinate dehydrogenase is more rapid than the rate of loss of bound FAD. The destruction of various dehydrogenases of mitochondria by visible light was also accompanied with the gradual loss of free sulfhydryl groups (Fig. 13). Since the activity of NADH (195), succinate (196) and choline (197) dehydrogenases are dependent on the presence of free sulfhydryl groups, this could explain in part the various visible light effects observed.

The NADH and choline oxidases follow the same inactivation curves as the NADH$\rightarrow$ CAT_{16} and choline$\rightarrow$ CAT_{16} reductases respectively (Fig. 11), while the corresponding NADH$\rightarrow$ $k_3Fe(CN)_6$ and choline-PMS reductases get inactivated much more slowly as a function

of illumination. Since CAT_{16} accepts electrons from ubiquinone (181) these results may indicate a rapid inactivation of the ubiquinone. Succinate oxidase and succinate-PMS or CAT_{16} reductases follow roughly the same rates of rapid inactivation making it difficult to distinguish the effect on the ubiquinone pool. The inactivation of ubiquinone by near ultra violet light has been reported (77) and it has also been shown that pure ubiquinone is 4-8 times more sensitive to visible light than ubiquinone in the cell (88). Several polar photoproducts of ubiquinone have been separated on thin layer chromatography.

Similar to our studies the destruction of succinate oxidase has also been reported in beef heart mitochondria (101) by blue light in the wavelength range 330nm $<\lambda<$ 540 nm (200mW/cm^2) when illuminated at low protein concenteration (~0.2 mg/ml). This destruction was considered to be due to loss of cytochromet oxidase activity, although the kinetics of inactivation did not correlate with that of succinate oxidase; succinate dehydrogenase activity was not reported. By contrast, in our study white light with wavelengths above 400nm (300mW/cm^2) was used and the mitochondria illuminated at protein concenteration 100-fold higher. Under these conditions the inactivation of succinate oxidase proceeded in parallel with loss of succinate dehydrogenase activity, whereas cytochrome c oxidase was unaffected. Thus, the conditions employed in our investigation enable us to detect early stages of visible light damage which involve flavin photodestruction. Cytochrome destruction is probably a later event.

Energy Coupling

The inactivation of energy transducing functions of the mitochondria occurs concomitantly with the inactivation of flavoprotein dehydrogeneses. Loss of energy coupling is shown by a decline in RCR, ATP synthesis, and transmembrane potential and an increase in ATP hydrolysis (Fig. 8) It is interesting to note that the P:O ratio and RCR decreases very significantly and lipid peroxidation increases during the first hours of illumination (appears in Section II) while the ability of the mitochondrial membrane to maintain electrochemical gradients decreases only after approximately 6 hours of illumination. This suggests specific effects on the ATPase-ATP synthetase protein complex or its coupling to the electron transport chain, and that even though ionic gradients can still be formed (as indicated by membrane potential), they can no longer be used for making ATP. The destruction of energy coupling may be due to loss of sulfhydryl groups (Fig. 13) since they are essential for oxidative phosphorylation (198).

SECTION II: STRUCTURAL ALTERATIONS

Early uncoupling effect of visible light exposure suggested structural damage of the mitochondria. The impairment in the structural integrity of the mitochondrial membranes was examined by several different parameters as described below:

Swelling. Illumination of mitochondria resulted in gradual decrease in light scattering and thus increasing swelling (Fig. 14). The swelling of mitochondria was extremely slow during initial phase of stimulation but occurred rapidly thereafter. No swelling was observed in the dark incubated samples.

Protein Release. Concomitant with swelling, the release of proteins from the mitochondria occurred (Fig. 14). The rate of protein release was gradual until the first 3 h of illumination and thereafter increased rapidly.

NADH Accessibility. The impermeability of NADH through mitochondrial inner membrane has been well established (141). How ever if the membrane is damaged, the free permeability of NADH is expected. It was examined by following the oxidation of NADH with malate dehydrogenase which is one of the matrix enzymes. Since oxaloacetate was required for the oxidation of NADH, this suggests its accessibility to malate dehydrogenase. As shown in Fig. 15, NADH became increasingly permeable with illumination time and reached to the same value as mitochondria suspended in hypotonic phosphate buffer. The NADH permeability of mitochondria suspended in isotonic and hypotonic buffer remains unchanged in the dark incubation.

Fig. 14. Effect of illumination time on the mitochondria swelling and protein release.

0 0 0 0 4 9 0 0 9 9 3

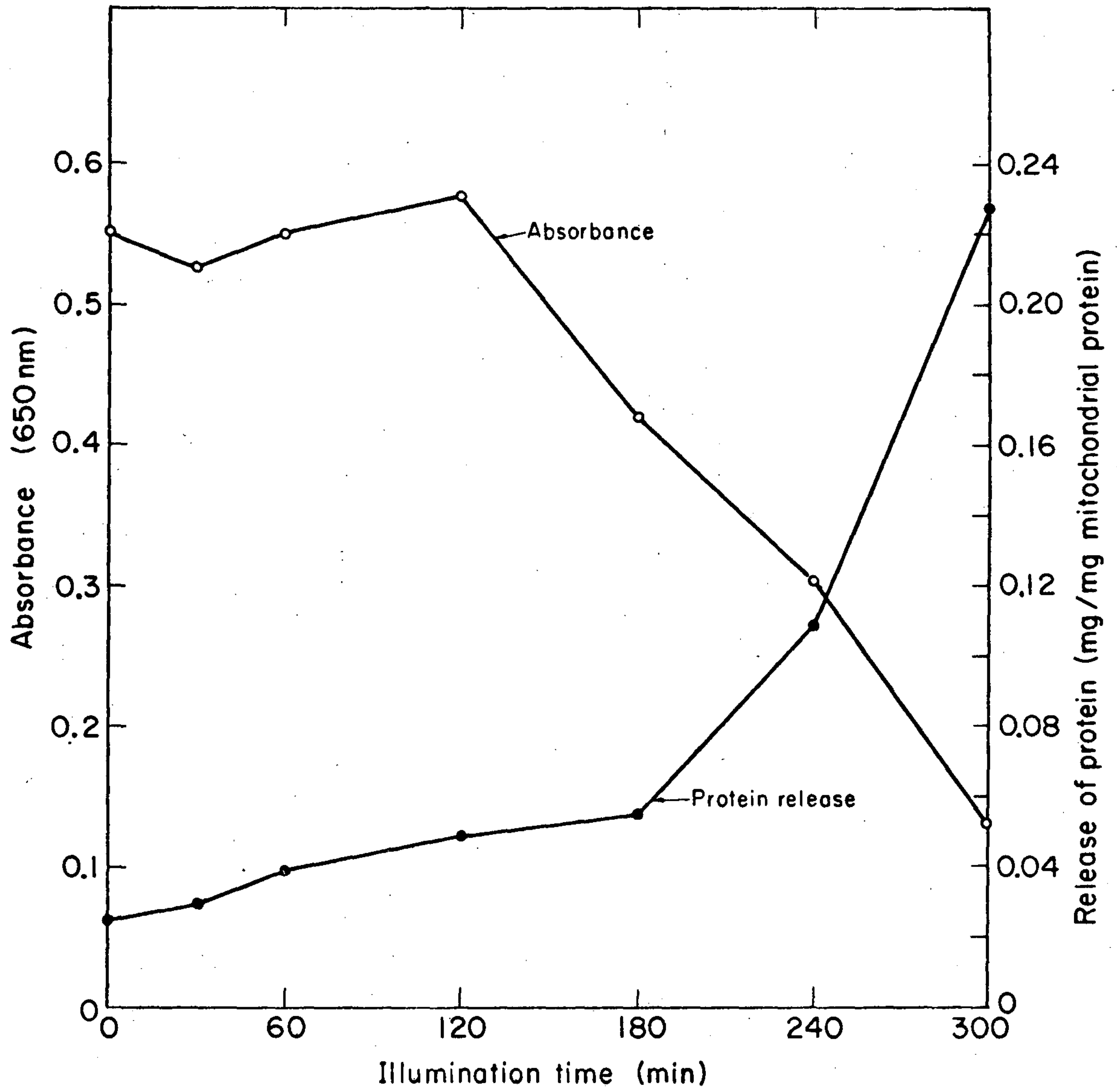


Fig. 14

Fig. 15. Effect of illumination time on the mitochondrial permeability to NADH. The mitochodria suspended in 30 mM phosphate buffer pH 7.4 (-Δ-) are designated as 100% permeable to NADH and the mitochondrial suspended in 0.25 M sucrose (-0-) are 10% permeable to NADH. 1 mM KCN was included in the assay medium to prevent the electron transport mediated oxidation of NADH. The assay medium also contained 0.2 mM NADH, 0.08 mM Na-oxaloacetate, pH 7.4 and 0.1 mg of mitochondrial protein in a volume of 3.0 ml. The oxidation of NADH was followed at 340 nm.

0 0 J 0 4 9 0 0 9 9 4

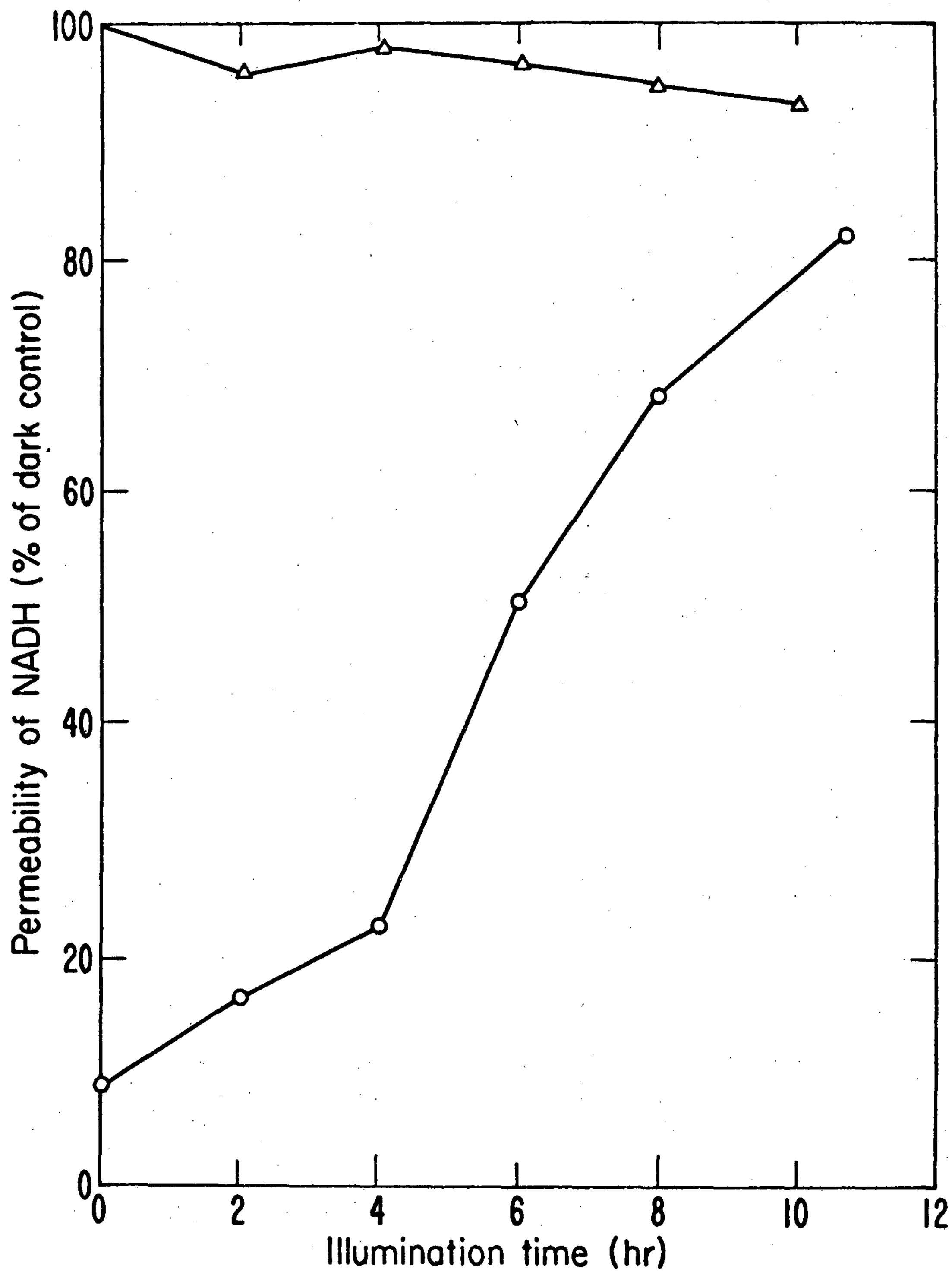


Fig. 15

Morphological Alterations: Changes in the morphology of the mitochondria were followed electron microscopically. The results shown in Fig. 16 indicate a gross structural alteration. An extensive disruption of the mitochondrial membranes occurred on illumination. There appears to be dissolution of inner membranes and loss of matrix contents. The electron micrographs also indicate a complete lack of configuration in the mitochondrial structure on illumination. It is more apparant in isolated mitochondria than mitochondria *in situ.* Under these conditions mitochondria incubated in the dark remain unchanged.

Lipid Peroxidation

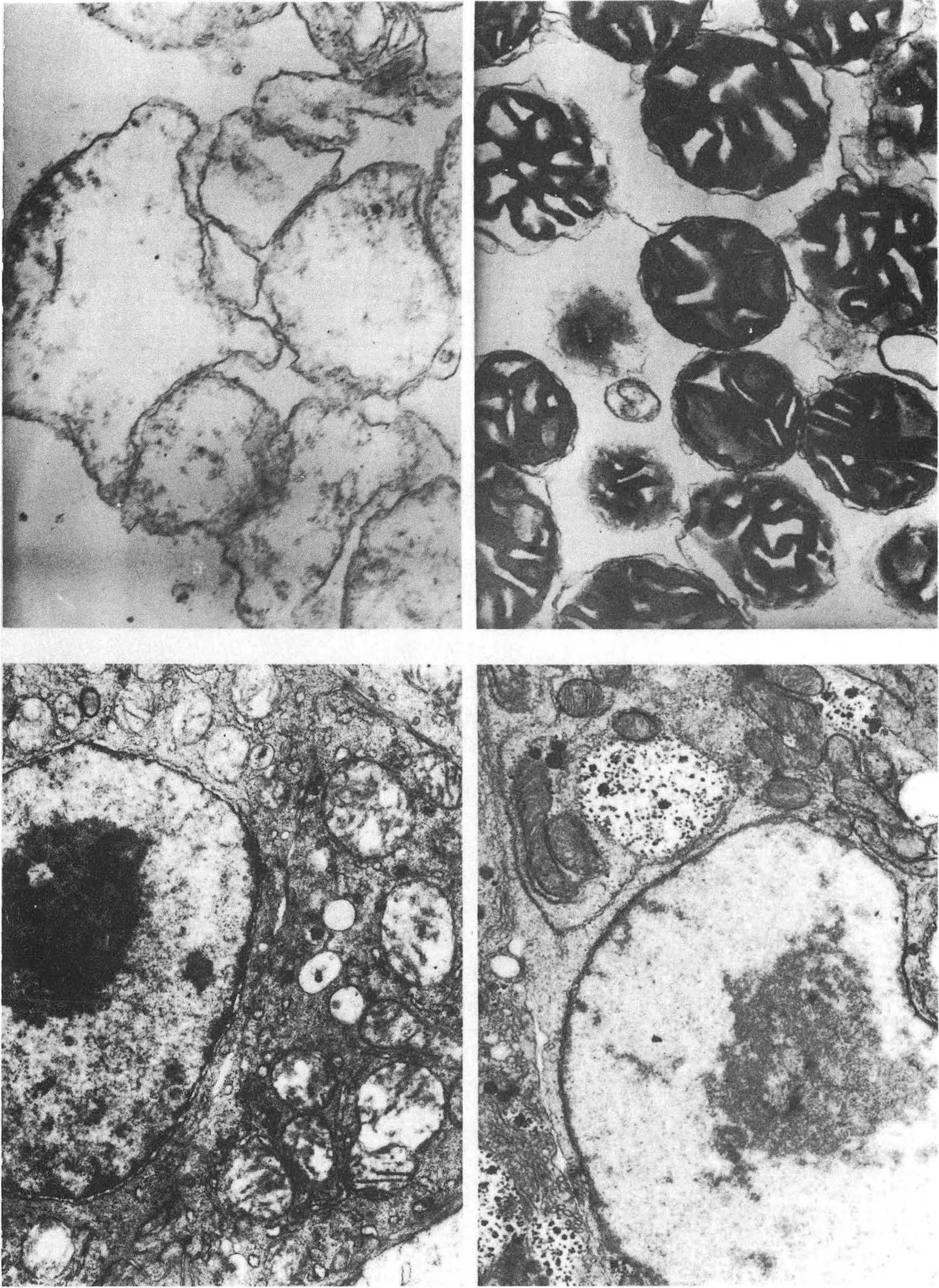
The high contents of unsaturated fatty acids in the mitochondrial inner membranes, provide a greater mobility to the respiratory components but enhance their instability. The peroxidation of such lipids occurred rapidly with increasing time of illumination (Fig. 17). In the dark incubated samples, no lipid peroxidation was observed.

Polypeptide polymerization

Sodium dodecyl sulfate polyacrylamide gel electrophoresis which resolves different proteins as a function of molecular weight was carried out on illuminated and dark control samples of the mitochondrial inner membranes (Fig. 18). The results show a highly polymerized material at the top of the gel on illumination, which is accountable by a decrease in staining intensity of other bands. There seems to be a consistant pattern of polymerization of low molecular weight proteins and aggregation with the high

0 0 0 0 4 9 0 0 9 9 5

Fig. 16. An electron micrograph indicating the effect of illumination on isolated mitochondria (b,d) and the mitochondria *in situ* (a,c). (magnification = 50,000x).



XBB 771-202

Fig. 16

0 0 0 0 4 9 0 0 9 9 6

Fig. 17. Effect of time of illumination on the peroxidation of mitochondrial inner membrane lipids.

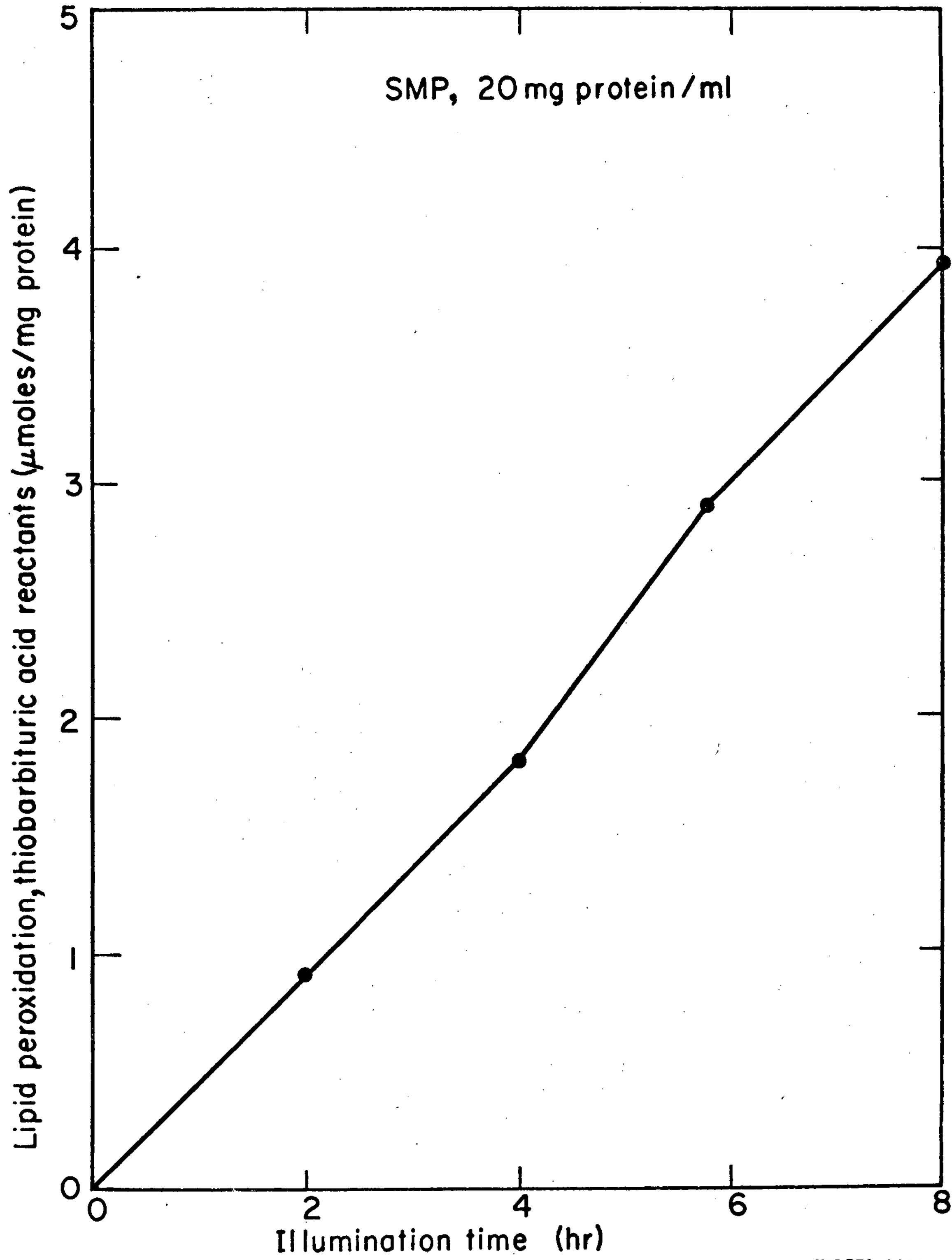


Fig. 17

0 0 0 0 4 9 0 0 9 9 7

Fig. 18. Sodium dodecylsulfate-polyacrylamide gel electrophonesis of illuminated and non-illuminated mitochondrial inner membranes. Inner membranes (2 mg protein/ml) were solubilised in 1% sodium dodecylsulfate and 1% mercaptoethanol and subjected to electrophoresis (in duplicate) in 6% polyacrylamide gels containing 0.1% sodium dodecylsulfate. Each gel contained 50 ug protein. Gels were stained with Coomassie blue and scanned at 550nm.

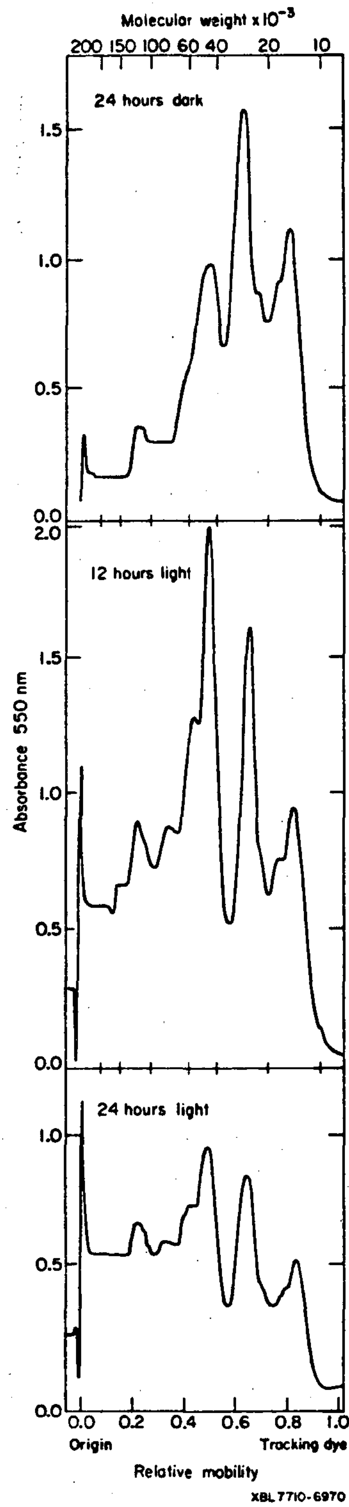


Fig. 18

molecular weight. It was not possible to point out that any particular molecular weight polypeptide is less or more affected than the others.

DISCUSSION

The present study indicates that the exposure of mitochondria to visible light causes several structural alterations which includes swelling, protein release, NADH permeability, lipid peroxidation, polypeptides polymerzation, and morphological deterioration. All these changes reflect a damage to the mitochondrial inner membranes. The swelling of mitochondria has also been reported by other workers on exposure to blue (436nm) and green (556nm) light (199) and to white light in the presence of photosensitizing agents such as eosine y (200) and benzpyrene (201). Various chemicals which are known to induce mitochondrial swelling such as Fe^{++}+ Ascorbate (202), glutathione (203) etc, also cause lipid peroxidation. This was confirmed in our studies as well. The free accessibility of NADH to the matrix enzymes and release of proteins also indicate damage to the mitochondrial inner membranes on illumination.

Electron microscopic studies reported here (Fig. 16) further emphasize that visible light exposure causes dissolution of the inner mitochondrial membranes and loss of structural integrity of its various components. The regular oval shape of the mitochondria disappeared after illumination.

The damage to the mitochondrial membranes was also manifested in the photopolymerization of the proteins (Fig. 18). All the

membrane polypeptides were polymerized although to a variable degree. Flavins are known to initiate the polymerisation of olefins (207) and of acrylamides photochemically. The flavin photosensitised formation of polymers has also reported in a purfied enzyme D-amino acid oxidase (206). The latter studies show that polymers are enzymatically inactive due to probable destruction of active site amino acid residues, therefore it is not very unlikely that the destruction of various flavoprotein dehydrogenases observed in our system is also due to polymerisation. The nature of linkage responsible for polymerisation of protein is not clear. The illuminated samples were treated with sodium dodecylsulfate and mercaproethanol, this suggests the participation of covalent linkages probably other than disulfide bonds. The polymerization resulting in inactivation of enzymes has also been shown by chemical modification of mitochondria with bifunctional alkylating reagent (204). The product of lipid peroxidation is malondialdehyde which could cross link proteins, but the concentration required is several folds higher than actually generated by the membrane (205). Therefore lipid peroxidation does not appear to be a major cause of polymerisation. This is also suggested by our studies that enzyme inactivation occurred eventhough lipid peroxidation was completely suppressed (section III).

0 0 0 0 4 9 0 0 9 9 9

SECTION III ENHANCEMENT AND PROTECTION

In order to understand the mechanism of damage by visible light on the mitochondrial structure and function, the various factors which enhance or suppress the damage were examined.

Wavelength Dependence

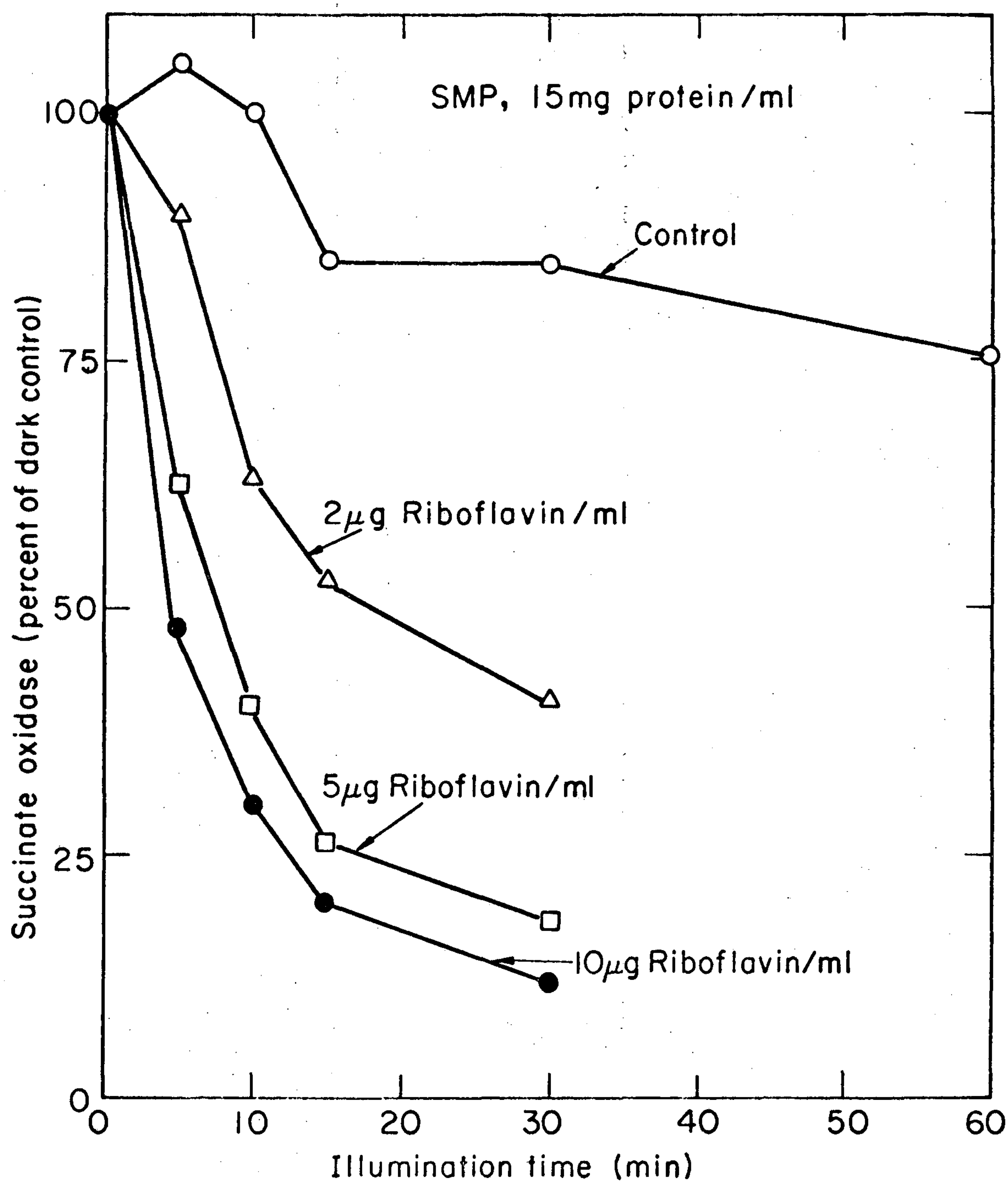
Several filters of broad wavelength were used; this was necessary to obtain measurable rates of inactivation of succinate dehydrogenase at the identical conditions of treatment. Light intensity compensated rates of inactivation were as follows: 350-600nm=1.5; 430-800nm =1.1 and 580-800nm = 0.05, relative to the rate with regular white light which was taken as unity. Thus the major inactivation occurs at wavelengths where flavins absorbs maximum.

Exogenous Sensitizers

The inactivation of succinate oxidase in SMP was enhanced with increasing concentration of exogenous riboflavin (Fig. 19). The rate of mitochondrial uncoupling (Fig. 20) and the inactivation of various inner membrane linked enzymes (Table VI) was also very rapid when exogenous riboflavin was added. Besides riboflavin, the effect of other mitochondrial (FMN, FAD and cytochrome c) and non mitochondrial (Eosine y and Methylene blue) photosensitizers were examined on the light dependent inactivation of succinate oxidase activity. The results shown in Fig. 21 demonstrates that cytochrome c was ineffective; FMN and riboflavin were more effective than FAD; and Methylene blue and Eosine y were the most effective photosensitizers.

Fig. 19 Enhancement of light dependent inactivation of succinate oxidase in submitochondrial preparations by exogenous riboflavin.

0 0 0 0 4 9 0 1 0 0 0



XBL773-3225

Fig. 19

Fig. 20 Effect of exogenous riboflavin and illumination time on the mitochondrial State 3 and State 4 respiration: Riboflavin added was 20 μg/ml. Without riboflavin see Fig 8A.

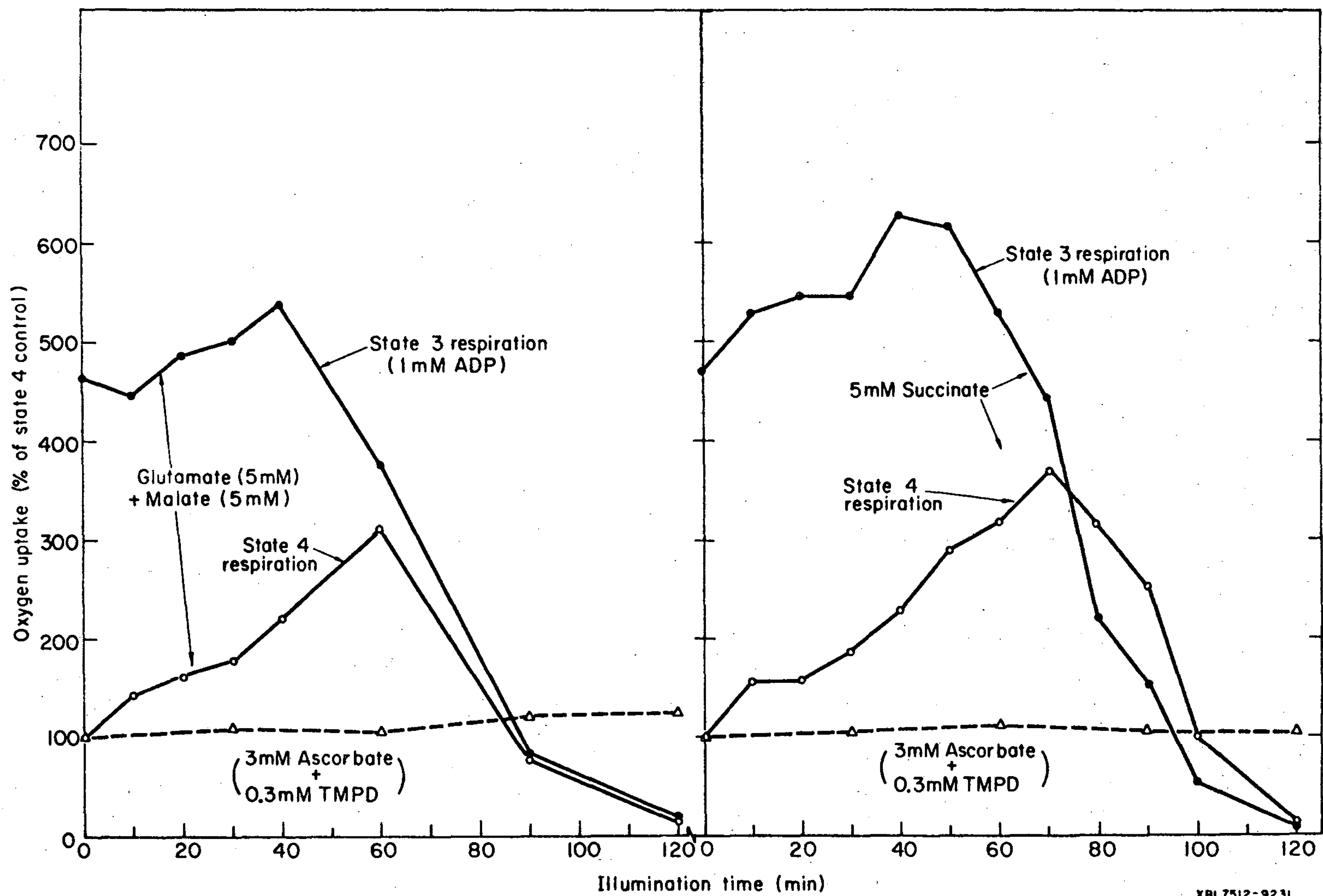


Fig. 20

Oable VI. Effect of illumination time on electron transport and ATPase of submitochondrial preparations in the absence and presence of riboflavin

	RESIDUAL SMP ACTIVITY[a] (% of Non-Illuminated Control)	
	-Riboflavin	+Riboflavin
Succinate Oxidase	48	10
Succinate Dehydrogenase	18	8
NADH Oxidase	70	13
NADH Dehydrogenase	19	15
Cytochrome C Oxidase	136	120
ATPase	61	36

[a]90 min. illumination (3-5° Assay: 25-26°C)
Riboflavin 10 g/ml

XBL7512-9228

0 0 0 0 4 9 0 1 0 0 2

Fig. 21 Effect of exogenous photosensitisers on the rate of inactivation of succinate oxidase in submitochondrial preparations. Cytochrome c was at 1-mM and other substances were at 20 mM concenterations.

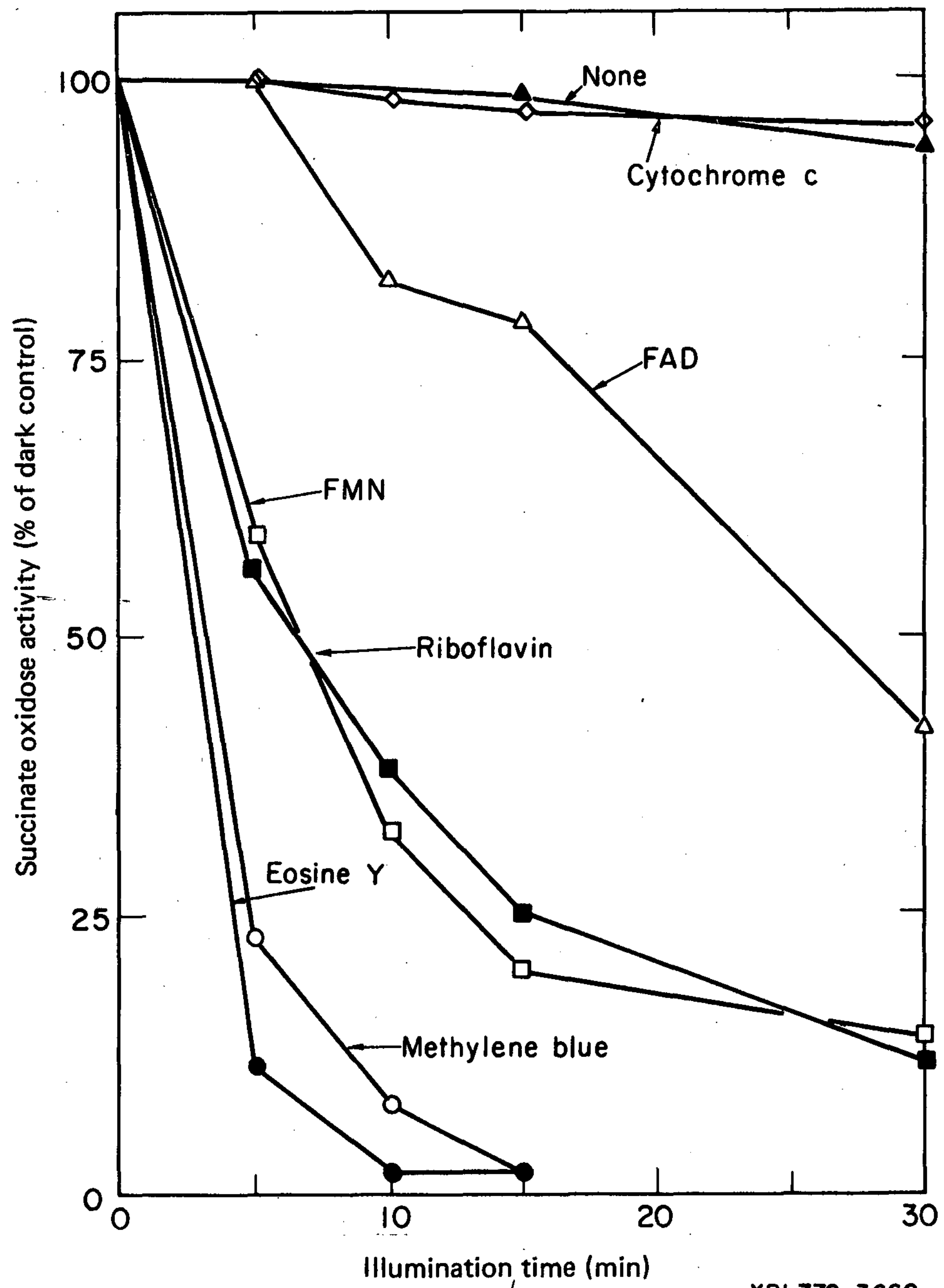


Fig. 21

Oxygen Concentration

Inactivation of succinate dehydrogenase in SMP was also enhanced by incubating the samples under 100% oxygen during the illumination treatment; 81% inactivation was observed after 3 h under 100% oxygen whereas only 55% inactivation occured under 20% oxygen or air (Fig. 22). No significant inactivation of any respiratory component was observed under a 100% nitrogen atmosphere (Table VII).

Respiratory Chain Substrates and Inhibitors

In SMP the sites of interaction of substrates are on the outer exposed surface of the membrane. In this system the protective effect of its can be directly measured without consideration of substrates peneteration or transport. When succinate was present during illumination succinate dehydrogenase was completely protected against inactivation (Fig. 23). The electron transport inhibitors KCN and antimycin A were added to prevent succinate oxidation during the 8 h illumination period. These inhibitors or succinate by itself had no effect on the light dependent inactivation of succinate dehydrogenase (Table VIII). Partial protection of choline dehydrogenase was also observed when illumination was carried out in the presence of choline and Antimycin A or amytal (Table IX). However, little protection of NADH dehydrogenase was afforded by NADH. Protection of ubiquinone was observed when illumination was carried out in the presence of succinate + antimycin A, which keeps ubiquinone in the reduced state. No protection of ubiquinone was observed in the presence of succinate + thenoyl trifluosoacetone (TTFA) when

Fig. 22. Effect of oxygen and illumination time on the succinate dehydrogenase activity of submitochondrial preparations.

0 0 0 0 4 9 0 1 0 0 4

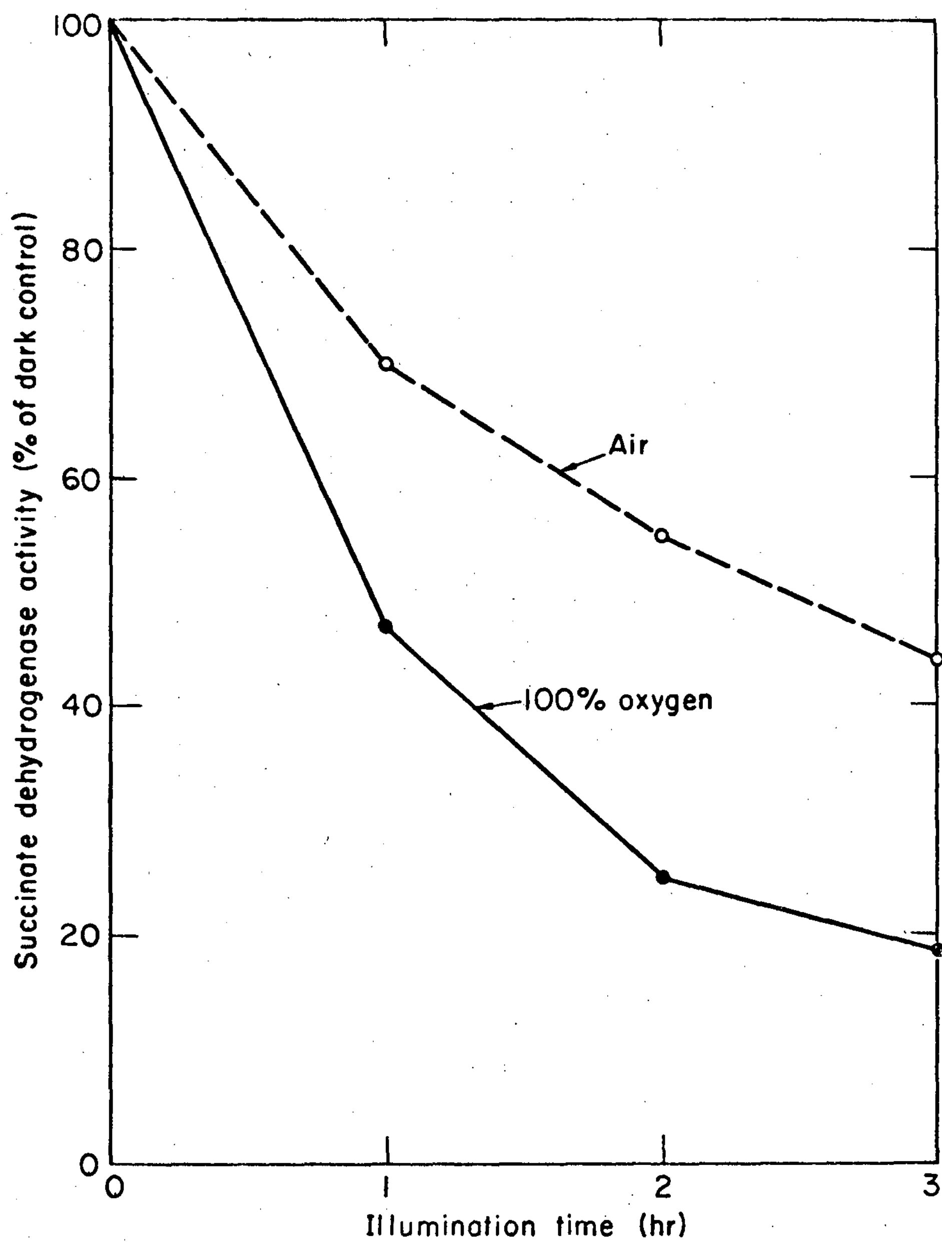


Fig. 22

Table VII

EFFECTS OF ILLUMINATION OF SMP UNDER NITROGEN AND AIR ON ENZYME ACTIVITY AND LIPID PEROXIDATION

SMP, 20 mg protein/ml were incubated 8 h under N_2 or air in the dark or light at 10°C; assays were at 25°C.

Assays	Nitrogen		Air	
	Dark	Light	Dark	Light
	Per cent activity			
Succinate oxidase	120	97	105	32
Succinate dehydrogenase	100	94	115	32
Choline oxidase	92	98	105	20
Choline dehydrogenase	98	100	92	79
NADH oxidase	100	100	110	30
NADH dehydrogenase	97	103	115	80
Adenosine triphosphatase	120	120	110	34
Cytochrome oxidase	105	110	120	110
	µmoles/mg protein			
Lipid peroxidation (Thiobarbituric acid reactions)	0.18	0.175	0.35	3.87

0 0 0 0 4 9 0 1 0 0 5

Fig. 23. Effect of electron transport substrate and inhibitors on the light dependent inactivation of succinate dehydrogenase in submitochondrial preparations. Where indicated 50 mM succinate, 10 mM KCN and 2 μg Antimycin A/mg protein were present.

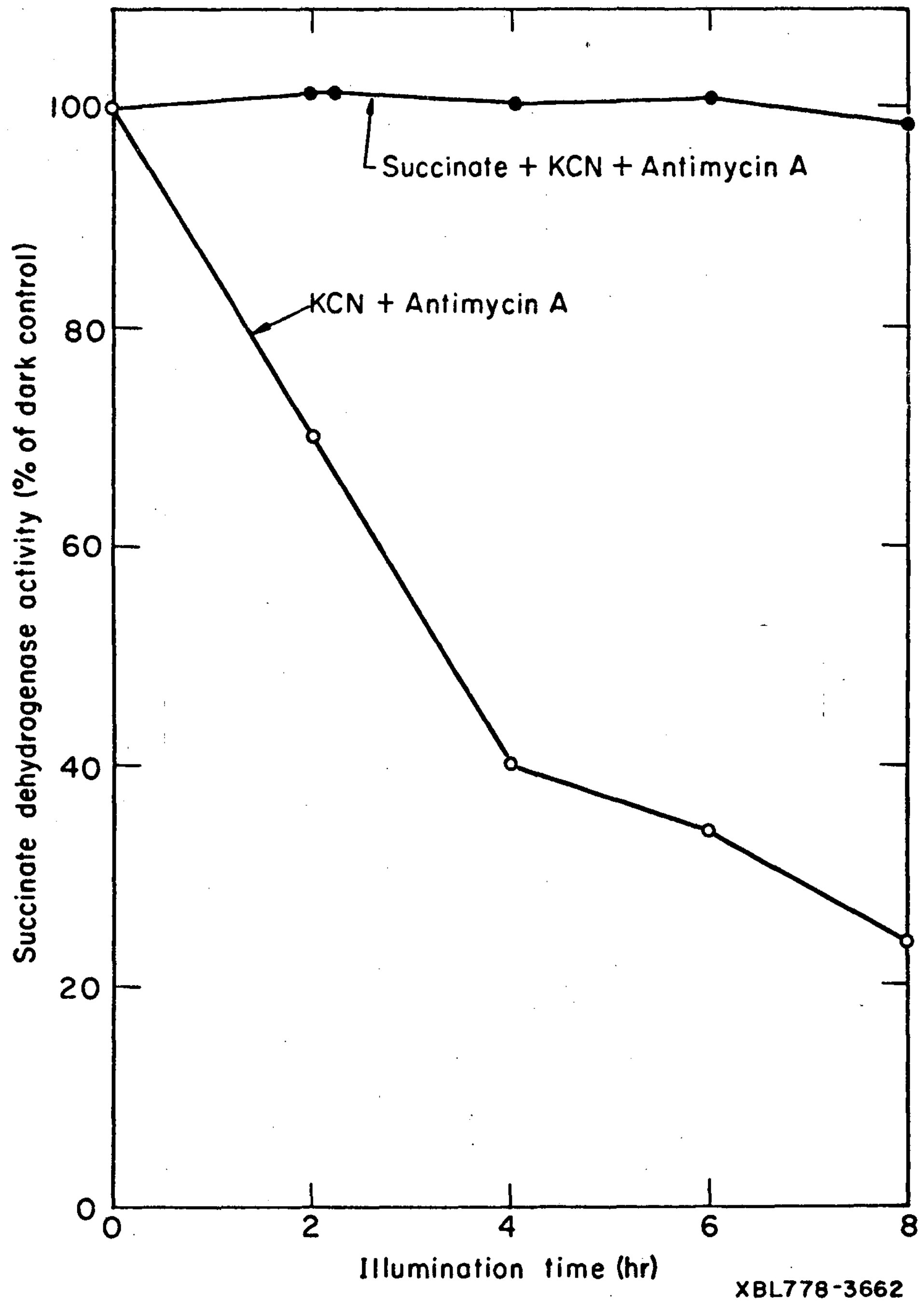


Fig. 23

Table VIII

EFFECT OF RESPIRATORY SUBSTRATE AND INHIBITORS ON LIGHT INACTIVATION OF SUCCINATE DEHYDROGENASE AND ON LIPID PEROXIDATION IN SUBMITOCHONDRIAL PREPARATIONS

		Assays	
Incubation conditions*	Concentration	Succinate dehydrogenase (% residual activity)	Lipid peroxidation (μmoles malondialdehyde/mg protein)
None		26	3.90
Succinate	50 mM	30	3.20
Antimycin A	5 μg/ml	22	1.52
KCN	10 mM	24	1.01
Succinate + KCN + Antimycin A	Same as above	103	0.00
Antimycin A + KCN	"	24	0.83
Succinate + TTFA	40 μg	85	0.12

*SMP, 20 mg protein/ml incubated for 8 hr under air in light or dark at 10°C; assays were at 25°C.

0 0 0 0 4 9 0 1 0 0 6

Table IX

SUBSTRATE PROTECTION OF LIGHT INACTIVATION OF DEHYDROGENASES AND UBIQUINONE IN SUBMITOCHONDRIAL PREPARATIONS

Incubation conditions*	Assays	
	Residual activity (% of dark control)	
	CAT_{16} reductase	Dehydrogenase
	NADH → CAT_{16}	Succinate → PMS
Control	40	30
Succinate + antimycin A	85	88
Succinate + TTFA	30	90
	Choline → CAT_{16}	Choline → PMS
Control	17	50
Choline + antimycin A	72	70
Choline + amytal	15	75
	NADH → CAT_{16}	NADH → $Fe(CN)_6$
Control	36	70
NADH + antimycin A	56	77
NADH + rotenone	21	73

*8 hr, 20 mg protein/ml and as indicated succinate or choline 50 mM, NADH 5 mM, antimycin A 5 μg/mg protein, rotenone 0.1 mM and amytal 1 mM.

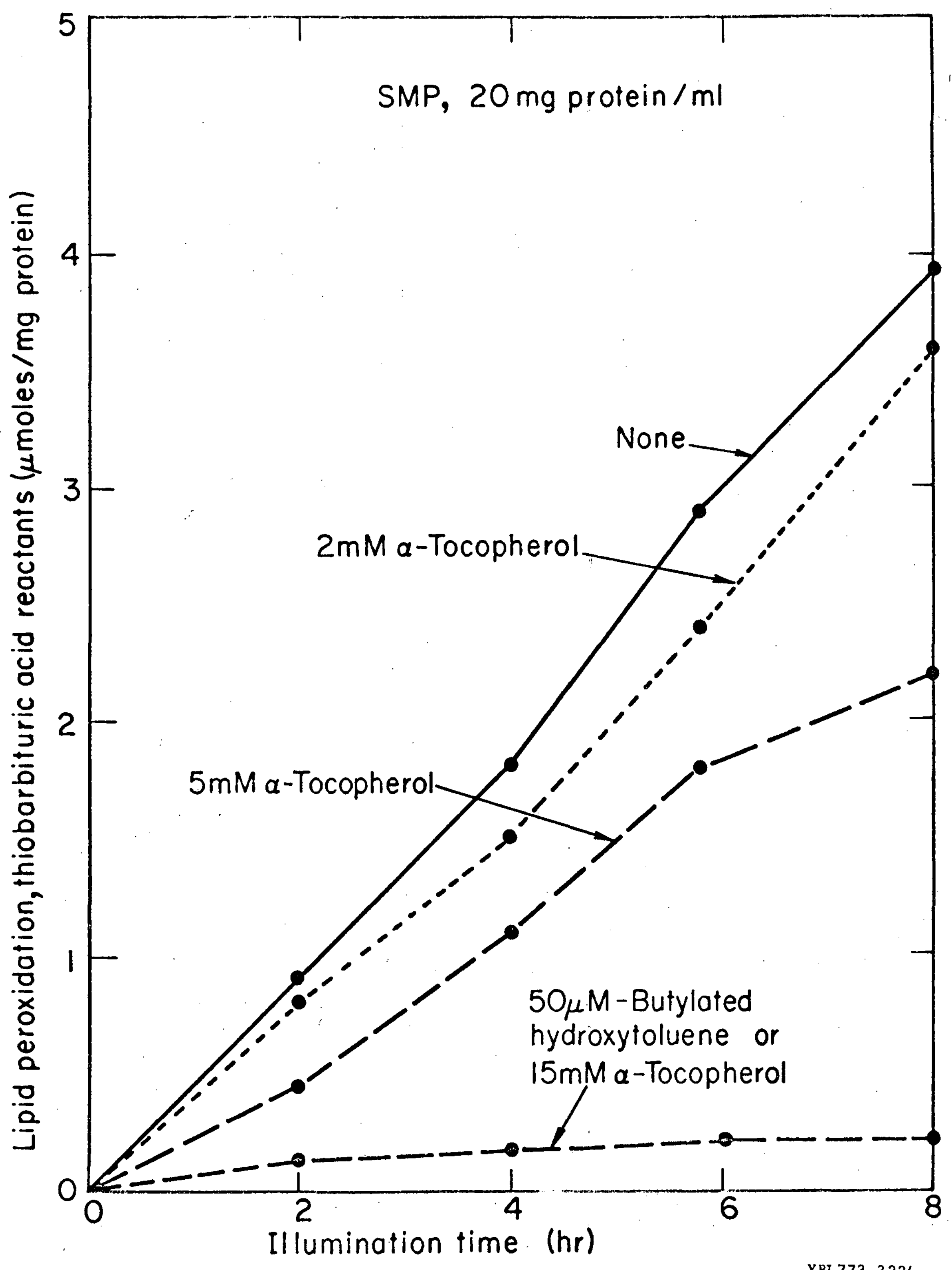
ubiquinone would be in the oxidized state (Table IX). Light dependent peroxidation of membrane lipids was completely inhibited by a combination of succinate + Antimycin A + KCN (Table VIII), but a little protection was observed in the presence of either of these inhibitors or succinate alone.

Free Radical Quenchers and Chelator

Various free radical quenchers and metal chelator were examined for protection against light induced damage to the mitochondrial membranes. As shown in Fig. 24, α-Tocopherol was very effective in suppressing light induced peroxidation of membrane lipids. At 15mM concenteration of α-Tocopherol, lipid peroxidation was completly inhibited. In contrast to α-Tocopherol, Butylated hydroxytoluene, a nonbiological antioxidant was effective at much lower concentration (50 M) to completly suppress lipid peroxidation. Under the conditions when there was no peroxidation of lipids, inactivation of succinate oxidase still occurred (Fig. 25) indicating that the mechanism of damage to lipid is different from that of to the proteins.

Besides vitamin E and Butylated hydroxytoluene, several other protective agents were tried as shown in Table X. EDTA, a divalent cation chelator was effective in suppressing both inactivation of succinate dehydrogenase as well as lipid peroxidation. Among various singlet oxygen quenchers tested, azide (208,209) did inhibit lipid peroxidation to a considerable extent but not enzyme inactivation. DABCO, another well known singlet oxygen quencher (210) was ineffective against both. Bovine serum albumin, which has recently been shown to quench singlet oxygen physically (211) did not

Fig. 24 Effect of α-Tocopherol and Butylated hydroxytoluene on the light induced lipid peroxidation in submitochondrial preparations.



XBL773-3224

Fig. 24

Fig. 25 Effect of vitamin E on light dependent inactivation of succinate oxidase and lipid peroxidation in submitochondrial preparations.

0 0 0 0 4 9 0 1 0 0 9

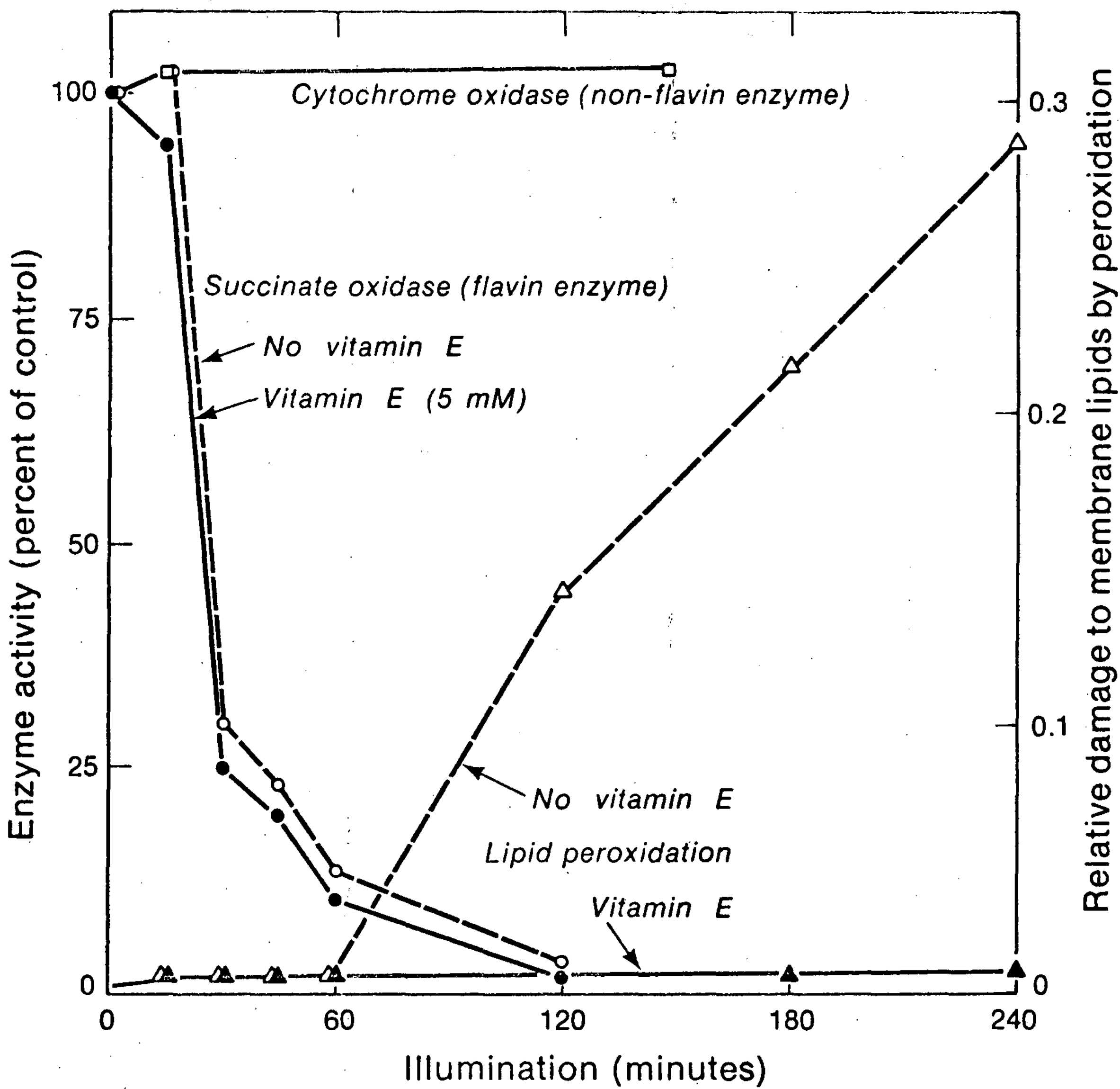


Fig. 25

Table X

TEST FOR QUENCHERS IN INACTIVATION OF SUCCINATE DEHYDROGENASE AND INDUCTION OF LIPID PEROXIDATION IN SUBMITOCHONDRIAL PREPARATIONS

Quenchers	Likely species quenched	Concentration	Succinate dehydrogenase (% residual activity)	Lipid peroxidation (μmoles malondialdehyde/mg protein)
None	–	–	26	3.87
α-Tocopherol	$ROO^{\cdot}$ $O_2^{\bar{}}$ $^1O_2$	15 mM	30	0.02
Butylated hydroxytoluene	$ROO^{\cdot}$	50 μg	27	0.00
1,4-Diazabicyco-[2,2]-octane	$^1O_2$	10 mM	13	4.12
Sodium azide	$^1O_2$	10mM	30	1.02
Bovine serum albumin	$^1O_2$	5 mg/ml	35	2.54
β-Carotene	$^1O_2$	10 mM	29	4.85
Dimethylfuran	$^1O_2$	10 mM	0	5.23
Sodium iodide	$^1O_2$	1 mM	0	4.90
3-(3,4-Dichlorophenyl)-1, 1-dimethylurea	$^1O_2$	0.075 mM	25	3.95
Histidine	$^1O_2$	1 mM	32	2.92
Superoxide dismutase	$O_2^{\bar{}}$	10 μg/ml	30	3.45
Ethanol	$OH^{\cdot}$	2 %	27	3.30
Dimethoxymethane	$OH^{\cdot}$	2 %	30	3.15
Glutathione (reduced)	$ROO^{\cdot}$	1 mM	45	2.20
Ethylenedinitrilotetraacetic acid	Divalent cations	50 mM	63	0.54

SMP, 20 mg protein/ml inducated for 8 hr under air in light or dark at 10°C; assays were at 25°C. Notation used peroxide ($ROO^{\cdot}$), singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bar{}}$), and hydroxyl radical ($OH^{\cdot}$).

0 0 0 0 4 9 0 1 0 1 0

have any significant effect in our system. Various other singlet oxygen quenchers such as Histidine (235), dimethylfuran (234) and β carotene (217), were also ineffective. Ethanol and dimethoxymethane, a hydroxyl radical quenchers (212) had no effect either on lipid peroxidation or on enzyme inactivation. Due to loss of sulfhydryl group on visible light exposure, reducing agent, glutathione was also examined and as shown in Table X, a very little protection of succinate dehydrogenase activity and aganist lipid peroxidation was observed.

DISCUSSION

Present studies indicate that there are various intrinsic and extrinsic factors which can either amplify or suppress the light induced damage. The observation that the rate of destruction of succinate dehydrogenase changes with wavelengths in the order 350-600nm > 430-800nm > 580-800nm suggests the probable involvement of flavins, as endogenous photosensitizer. The role of flavins in light damage is further supported by enhancement of effects by exogenous flavins (Fig. 21) and also that endogenous flavins of the mitochondria are destroyed on illumination (Fig. 12).

Photosensitisation by other components of the respiratory chain appears to be of secondary importance. This is supported by the observation that the rate of inactivation of the respiratory chain did not seem to be affected by the presence of exogenous added cytochrome c (Fig. 21) or depletion of mitochondrial cytochrome c (data not shown). Moreover none of the cytochromes were destroyed under the conditions when 50% of the flavins were lost (Fig. 12).

In the presence of respiratory substrates and inhibitors both the dehydrogenases and ubiquinone were protected if kept in the reduced state (with antimycin A) during illumination. Succinate, NADH and choline dehydrogenases were protected but not the ubiquinone (Table IX) when TTFA, Rotenone or Amytal which inhibit electron flow before the ubiquinone site were present during illumination. This may be due to the fact that the reduced form of flavins absorb very little visible light as compared to their oxidized forms. It is also possible that the binding of substrate to the active site may also help to stabilize the protein. Ray and Koshland (35) have reported that binding of substrate to the enzyme phosphoglucomutase protects the active site amino acid residues against methylene blue sensitised damage.

In addition to visible light and a photosensitiser, the various destructive effects may also require the presence of oxygen, since inactivation did not occur under nitrogen (Table VII) and was significantly enhanced by increasing the oxygen concenteration in the gas phase (Fig. 22). This indicates the likely involvement of some activated species of oxygen, either singlet oxygen ($^1O_2$), superoxide radical (O^-_2) or the hydroxyl radical ($OH^\cdot$), species that are known to cause damage to membrane components (213). Since the rate of destruction of succinate oxidase was higher with eosine y or methylene blue, than that with flavins as exogenous photosensitiser (Fig. 21), it suggests $^1O_2$ as the damaging species. This suggestions is based on the present concensus that flavins generate mainly O^-_2 radical (57) which reacts with its dismutated product to form $^1O_2$ and $OH^\cdot$(214), whereas eosine y or methylene blue produce $^1O_2$ directly (215).

Among various protective agents tested, -Tocopherol and Butylated hydroxytoluene suppressed lipid peroxidation but not enzyme inactivation (Fig. 25). This suggests that either these antioxidants are not accessible to proteins or the mechanism of damage to proteins is different from that of to the lipids. If the damage to protein is due to either $^{-}O_2$ or $^{1}O_2$, then inaccessibility seems more likely since -Tocopherol is known to quench both $^{-}O_2$ (216) and $^{1}O_2$ (217). Perhaps due to similar reasons, other $^{1}O_2$ quenchers also had very little or no effect. Hydroxyl radical quenchers such as ethanol and dimethoxymethane (212) did not protect either lipid or protein damage. The protective effect EDTA on both lipids and proteins (Table X) suggests the involvement of bivalent cations. It is expected since bivalent cation such as Fe^{++} in the mitochondria is known to damage its membrane (218). The respiratory substrates in combination with inhibitors protected both lipids and proteins (Table VIII). This indicates that the damage originates from the proteins, probably the coenzyme part of the molecule, and spread to the lipid components of the membrane by oxygen.

GENERAL DISCUSSION

Synopsis of Visible Light Damage to Mitochondria

Visible light exposure affected both protein and lipid components of the mitochondrial membranes (Fig. 26). Protein damage was indicated by the photopolymerisation and destruction of respiratory chain linked ATP synthetase and dehydrogenases. Formation of lipid peroxides and inactivation of ubiquinone was a measure of lipid damage. Structurally, disruption of mitochondrial membrane was reflected by irreversible swelling, free permeability to NADH, release of matrix contents and dissolution of inner membranes microscopically. The probable chromophore in the mitochondria responsible for absorption of visible light is flavin. The evidence in favor of this suggestion include: destruction of flavoprotein dehydrogenases; loss of bound and free flavins, enhancement of destruction by exogenous flavins and lack of inactivation by a combination of substrate and inhibitors of electron transport chain. Since major inactivation occurred in the wavelength region where mainly flavins and partially cytochromes absorb, our studies do not entirely rule out iron sulfur and heme also as likely photosensitisers, although cytochrome c depletion or exogneous cytochrome c addition did not affect the rate of destruction and neither any cytochromes were inactivated spectrophotometerically.

Besides photosensitiser, oxygen was also needed for visible light induced damage to the mitochondrial membranes. Since under anaerobic conditions no damage occurred, this can be argued against that it is due to the reduced state of flavins. This appears unlikely

0 0 0 0 4 9 0 1 0 1 2

Fig. 26. A synopsis of visible light effects on mitochondria.

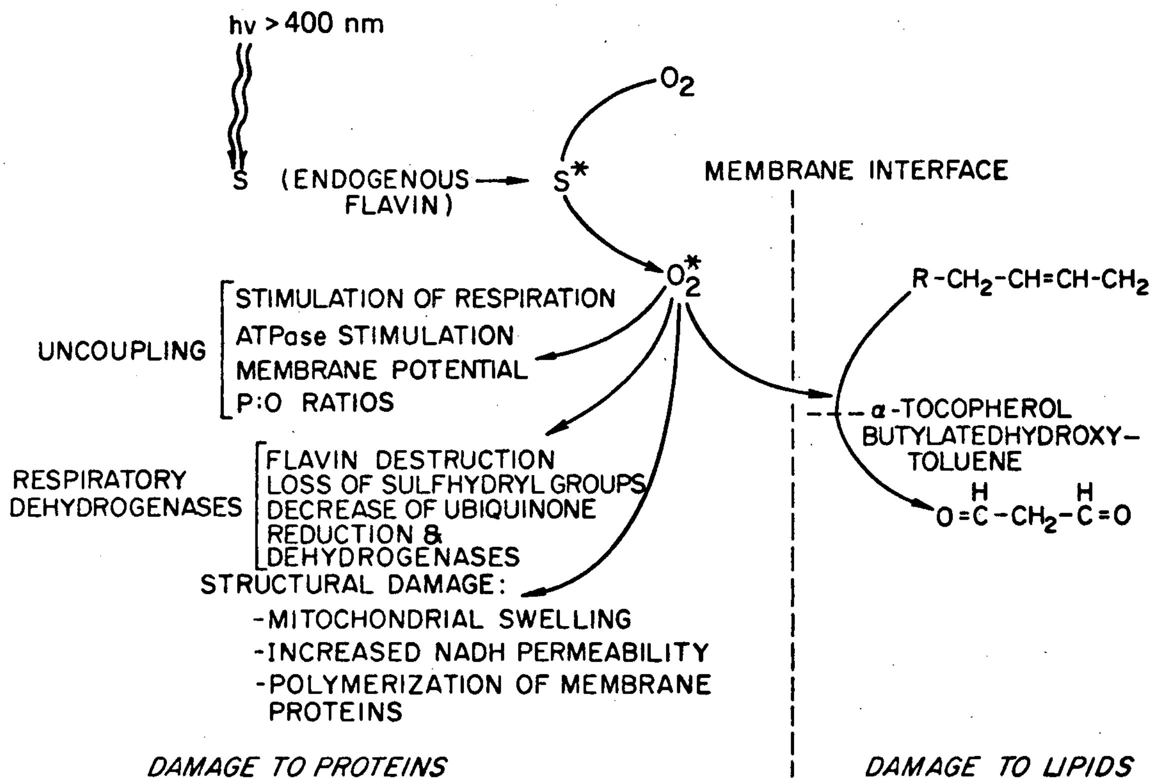


Fig. 26

because rate of damage was enhanced by increasing oxygen concentration.

Proposed Explanation of the Mechanism of Visible light Damage to Mitochondria

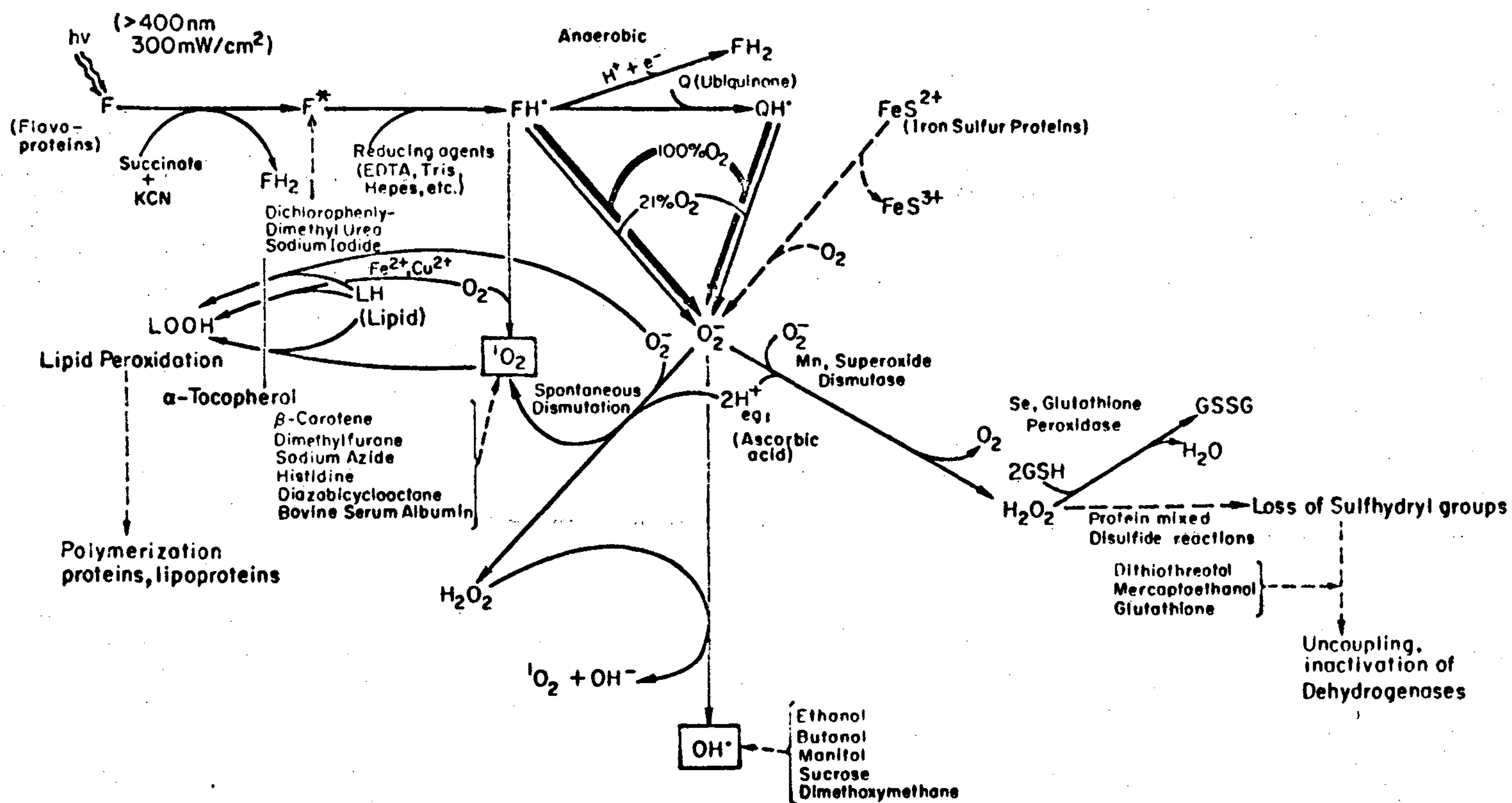
The various steps involved in damage to the mitochondrial membranes on exposure to visible light and oxygen can be conveniently separated into three stages, viz; initiation, propagation and termination. The following discussion is based on the scheme shown in Fig 27 which considers all the three stages of the mechanism of damage.

INITIATION

The chromophore in the mitochondrial membrane which absorbs light in the visible region of the spectrum is the likely site of initiation of damage. Our studies indicate that flavins are the main endogenous photosensitizer. As shown in Fig. 27 flavins(F) on absorption of visible light singlet are excited (F*) from ground to higher singlet and then triplet state (219). Since flavins are the coenzymes of respiratory dehydrogenases, they can be reduced by their substrate. As compared to their oxidized state ($\varepsilon_{450nm}^{mM^{-1}cm^{-1}}=12$), flavins in their reduced state ($\varepsilon_{450nm}^{mM^{-1}cm^{-1}}=0.87$) absorb less than 10% visible light and thus can not initiate damage very effectively. The photoreduction of excited flavin can also be achieved under anaerobic conditions in the presence of amines such as EDTA, Tricine etc (221-223), which act as a proton donors. The formation of dihydroflavin (FH_2) by an abstraction of hydrogen (220) is mediated through a flavosemiquinone free radical which has been

Fig. 27. A proposed mechanism of flavin photosensitized oxidative damage to mitochondria.

FLAVIN PHOTOSENSITIZED OXIDATIVE DAMAGE TO MITOCHONDRIA



XBL7710-3922

Fig. 27

0 0 0 0 4 9 0 1 0 4

demonstrated by electron paramagnetic resonance spectroscopy (229).

The possible role of iron sulfur centers in damage initiation is based on their absorption of light in the visible range ($\varepsilon^{mM^{-1}cm^{-1}}_{450nm}$ =4) and on autooxidation reactions catalysed by iron in the mitochondria (218). The formation of damaging free radicals from purified iron sulfur proteins as they undergo from reduced to oxidized state has been demonstrated (121). Thus it is likely that mitochondrial iron sulfur centers can also contribute to the initiation of damage.

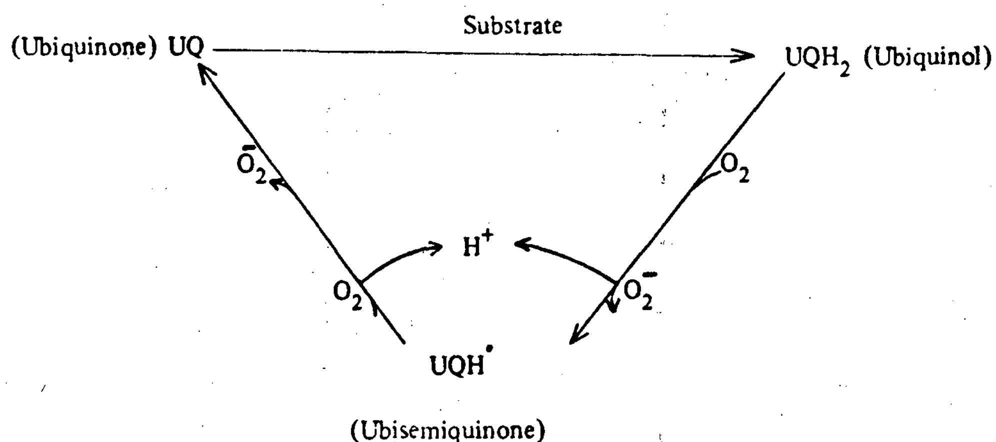
The ubiquinone component of the mitochondria absorb very little light in the visible range ($\varepsilon^{1\%,cm^{-1}}_{405nm}$=5.9). The oxidation reduction cycle of ubuquinone is a one electron transfer process and thus mediated through ubisemiquinone free radical. The latter indicates its potential in initiation of damage to the mitochondrial membranes. The generation of toxic free radicals by ubiquinone has been established (118).

PROPAGATION

The propagation of damage from the site of initiation probably occurs through oxygen. The latter can undergo one electron reduction process giving rise to superoxide (O^-_2) free radical which has been detected by EPR spectroscopy. Under aerobic conditions excited flavins consume oxygen to generate either singlet oxygen ($^1O_2$) (224) or superoxide radical (225). The formation of O^-_2 radical by the reduction of ubiquinone region of the respiratory chain has also been demonstrated by several workers (118,226). Therefore it is

0 0 J 0 4 9 0 1 0 1 5

suggested that flavosemiquinone generated by light, could give rise to ubisemiquinone which in turn may react with oxygen to produce O^-_2 radical by univalent reduction. The ubisemiquinone free radical has been detected in the mitochondrial membranes, amounting to about 0.2-1.5% of the total quinone contents (231). Both quinol and semiquinol forms of ubiquinone can produce O^-_2 radical (122) by the following reaction:



Thus ubiquinone can play an important role in damage propagation.

The iron sulfur centers as the generators of O^-_2 radical has been shown in two systems ; iron-sulfur flavoprotein (227) and iron sulfur protein (121). Although the production of this radical from the mitochondrial iron-sulfur centers has not been demonstrated, but it is possible that univalent reduction of these proteins could also form O^-_2 radical.

The superoxide radical whether generated by flavins, quinones or iron sulfur centers of the mitochondria, is a powerful oxidant and has been shown to cause oxidation of both lipids and proteins (228).

Since in our studies mitochondrial damage was enhanced under high oxygen concentration it may probably be due to increased production of O^-_2 (Thick Arrows in Fig. 27) radical. In order to detoxify this radical generated from various sources, mitochondria contain an enzyme superoxide dismutase which converts O^-_2 to relatively less harmful species, H_2O_2. The latter is further detoxified by the mitochondrial glutathione peroxidase to water. A spontaneous dismutation of O^-_2 could lead to the formation of an highly reactive species, $^1O_2$ (214). Some workers have also suggested that O^-_2 can react with its own dismutated product to form hydroxyl radical ($OH^\cdot$), a powerful oxidant (243).

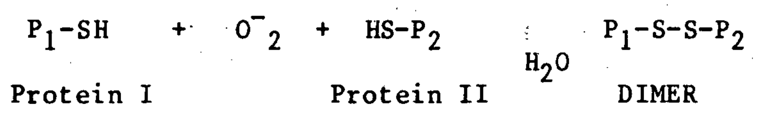
Thus there are several free radical species generated by oxygen which could propagate mitochondrial damage. Oxygen can also spread the damage by carrying out the autooxidation of lipids in the presence of bivalent cations (218), thus resulting in the formation of lipid peroxide (ROO) radicals. The latter can also be produced by direct interaction of lipids with O^-_2 (228), $^1O_2$ (243) or $OH^\cdot$ (214) radicals.

The polymerization of membrane proteins accompanying lipid peroxidation has been shown by several workers (232) but whether one is a consequence of the other, is not well understood. The product of lipid peroxidation is a malondialdehyde. Similar to to other bifunctional alkylating reagents, malondialdehyde has been shown to crosslink proteins resulting in the formation of polymers (232). However, studies with red blood cell membranes have indicated that the concentration of crosslinker required to form polymers is several folds higher than actually produced

0 0 0 0 4 9 0 1 0 1 6

by lipid peroxidation (205). The lack of intermolecular crosslinking of mitochondrial membrane proteins by malondialdehyde has also been demonstrated (238). Our results also confirm that photopolymerization of proteins is independent of lipid peroxidation.

The hypothesis that in a flavin photosensitized damage, the polymerization of proteins does not involve products of lipid peroxidation, is also supported by studies of others, that a purified flavin enzyme on exposure to visible light form dimers, tetramers and polymers which are enzymatically inactive (206). The latter workers have suggested covalent linkages of proteins. Our result show loss of free thiol groups which may indicate formation of mixed disulfide bridges. The The oxidation of thiols by O^-_2 or H_2O_2 has been established (240,241). Thus the polymerization of proteins by thiols could occur as follows:



$$\underset{\text{Protein I}}{P_1\text{-SH}} + O^-_2 + \underset{\text{Protein II}}{HS\text{-}P_2} \xrightarrow[H_2O]{} \underset{\text{DIMER}}{P_1\text{-S-S-}P_2}$$

Therefore protein damage appears to be due to loss of thiols since latter are essential for oxidative phosphorylation (198) and for respiratory chain linked dehydrogenases (195-197).

TERMINATION

The participation of a given radical species in damage propagation is generally identified by a quencher which terminates such damage. Flavin photosensitised lipid peroxidation of the mitochondrial membrane was completely suppressed by α -Tocopherol and butylated hydroxytoluene. This suggests the involvement of lipid peroxide radical (LOO·). The latter can originate either by direct autooxidation of lipids in the presence of bivalent cations or by interaction with an

activated species of oxygen (O^-_2, $^1O_2$ or OH·). Since the formation of peroxide radical was also inhibited by EDTA, this suggests the involvement of bivalent cations catalysing the autooxidation of lipids. The prevention of damage by EDTA in a flavin photosensitised system can also be ineterpretted by its role as flavin reductant.

The suppression of glutathione induced lipid peroxidation of the mitochondrial inner membranes by superoxide dismutase (242) has indicated involvement of superoxide radical. The lack of effect of this enzyme on lipid peroxidation in our system suggests either accessibility barrier or its destruction by visible light or involvement of some other free radical species. A similar rate of peroxidation of lipid observed in mitochondria which contains SOD and in SMP which lacks SOD also indicate that in a flavin photosensitised system O^-_2 may not be involved.

The damage to lipids could also occur by singlet oxygen (217), since β -carotene is known to stop this process (26). Besides β - carotene, there are several other $^1O_2$ quenchers which include DABCO (210), Azide (236), Bovine serum albumin (211), Dimethylfuran (234), Sodium iodide, DCMU (223) and histidine (235). The effectiveness of a given quencher is determined by its physical state and its environment. Sodium azide a freely water soluble compound, suppressed lipid peroxidation significantly in our system, whereas other $^1O_2$ quenchers were relatively unaffective. This could be partly either due to their insolubility in water or their absorption characteristics in the visible range and thus acting as photosensitizers.

The evidence that hydroxyl radical (OH$^{\cdot}$) can induce an oxidative attack on lipids stems from studies on lysosomal membrane, where lysis was enhanced by superoxide dismutase and thus OH$^{\cdot}$ was implicated as causative agent (243). Ethanol and dimethoxymethane, a well known OH$^{\cdot}$ quencher had no effect on the photooxidation of lipids in our system A similar result has also been obtained in a model system study of lipid peroxidation (214).

The damage to mitochondrial proteins in a flavin photosensitized system involves oxidation of free thiol groups, which may result in aggregation of proteins by disulfide linkages. In xanthine oxidase system the thiol oxidation is caused by superoxide radical (241) and also to a lesser extent by H_2O_2 (240). The The oxidation of glutathione by H_2O_2 is a well known reaction reaction catalyzed by a specific mitochondrial matrix enzyme glutathione peroxidase. The disulfide linkages of thiol oxidation can be terminated by reducing agents such as dithiothreotol or mercaptoethanol. None of these aggents were effective when added during light exposure probably due to their role as free radical generators. These reagents also had no effect on the post illuminated samples, it may be due to inaccessibility of the reagent in a photopolymerized material (207).

The damage to both lipids and proteins components of the membrane was terminated by using a combination of respiratory substrates and inhibitors. The probable explanation for this is that under these conditions the flavin coenzyme of the protein molecule is in a reduced state and thus do not absorb enough

0 0 0 0 4 6 0 1 0 1 7

light to cause damage. It also suggests that the damage most likely initiates from the proteins and then propagated on to lipids where it can be terminated by α-tocopherol. Thus our studies seem to indicate that the protein damage is due to loss of sulfhydryl groups, where as peroxide radicals cause lipid damage.

VISIBLE LIGHT DAMAGE OF MITOCHONDRIA vs CELLS

Both near ultraviolet (300-420 nm) and visible (above 370 nm) light is known to effect the survival of cells in culture (Table XI). Microscopic observations have indicated damage to the mitochondria. The light intensity required to diminish cell viability (0.2-0.4 mW/cm^2) is normally much lower than that needed for mitochondrial damage (200-300 mW/cm^2). This may be due to the geometery and the optical density of the sample. Using blue light (330-540 nm) of intensity 200 mw/cm^2, it has been shown that cytochrome oxidase activity of yeast cells, of beef heart mitochondria and of complex IV is destroyed (101,244). Our studies with rat liver mitochondria using white light (400-720 nm) at intensity 300 mW/cm^2 indicate that the destruction of respiratory chain linked flavoprotein dehydrogenases is a primary step in damage process. A similar results have also been obtained in bacterial cell envelops using white light (30) and in complexes I and II of beef heart mitochondria on exposure to blue light (59).

The destruction of ATP synthesizing system of the mitochondria may explain the inhibition of growth of cells by visible light exposure, since ATP is essential for DNA, RNA and protein synthesis

Table XI. VISIBLE LIGHT DAMAGE TO CELLULAR SYSTEMS

	LIGHT INTENSITY mw/cm^2)	WAVELENGTH (nm)	ACTIVITY DESTROYED	REFERENCES
Human Cells (WI-38)	0.18	375-700	Cell viability	17
Human Cells (D98/AH_2)	0.40	300-420	Cell viability	246
Yeast Cells (Saccharomy-ces cerevisiae)	200	330-540	Cytochrome oxidase	244
Bacterial Cells (Micorcoccus roseus)	32	400-720	NADH oxidase, Succinate dehydro-genase, ATPase	30
Mitochondria (Beef heart)	200	330-540	Succinate oxidase Cytochrome oxidase	101
Complexes I-IV (Beef heart)	200	330-540	All complexes	59
Mitochondria (Rat liver)	300	400-720	Flavin dehydro-genases, ATP synthesis	Present investigation

and also for active transport. A temporal sequence of events in damage process might further elucidate the mechanism of photokilling of cells. A kinetic of inactivation of various flavin enzymes located in different subcellular compartments also might help in understanding the visible light damage to cells. Photosensitized damage can be used as a tool to selectively destroy certain kinds of tumor cells which are rich in light absorbing pigments such as hematoporphyrin. Present studies are also relevant to understand the damage caused in those parts of human body regularly exposed to sunlight (e.g., skin epithelium and retinal membranes).

0 0 0 0 4 9 0 1 0 1 9

SUMMARY

1. The effects of visible light of wavelengths above 400 nm on rat liver mitochondria and submitochondrial preparations was investigated.
2. The temporal sequence of changes included: stimulation of respiration, a decline in ATP synthesis, increased ATPase activity, inactivation of respiration and loss of trans-membrane potential.
3. The inactivation of respiration was accompanied with the destruction of dehydrogenases in the order succinate dehydrogenases > choline dehydrogenase > NADH dehydrogenase.
4. Among the components of dehydrogenases, destruction of ubiquinone reduction, and loss of flavins and sulfhydryl groups were most susceptible to illumination.
5. Redox reactions of cytochromes and cytochrome c oxidase activity were unaffected.
6. The structural damage was indicated by release of soluble proteins, decrease in light scattering and increased NADH peneteration into the mitochondria.
7. Microscopically the alteration in the mitochondrial morphology was indicated by dissolution of inner membranes and distortion of the outer membrane.
8. Electrophoretically the polypeptide profile of the mitochondrial inner membrane was drastically altered after illumination.
9. Peroxidation of membrane lipids also occurred.

10. Damage to both lipids and proteins was stopped under anaerobic conditions and enhanced by high oxygen tension.
11. Exogenous flavins enhanced the damage and maximum destruction occurred in the wavelength regions where flavins absorb.
12. Various antioxidants and free radical quenchers were effective against lipid peroxidation but not against enzyme inactivation.
13. Combination of substrates and inhibitors of the electron transport chain suppressed both destruction of enzymes and lipid peroxidation.
14. Our results indicate that visible light initiates a flavin photosensitized reaction that produces damage involving participation of an activated species of oxygen in the damage propagation.

0 0 0 0 4 9 0 1 0 2 0

REFERENCES

1. Kohn, R. R. (1971) in Principles of Mammalian Aging, pp. 105, Prentice-Hall, Inc., Englewood Cliffs, New Jersey.
2. Packer, L., Deamer, D. W. and Heath, R. L. (1967) in Advances in Gerontological Research, vol. 2 (ed. by Strehler, B. L.) pp. 77, Academic Press, New York.
3. Pereira, O. M., Smith, J. R. and Packer, L. (1976) Photochem. Photobiol. 24, 237.
4. Packer, L. and Fuhr, K. (1977) Nature 267, 423.
5. Raab, O. (1900) Z. Biol 39, 524.
6. Blum, H. F. (1964) in Photodynamic Action and Diseases Caused by Light, Reinhold Inc., New York.
7. Straub, W. (1904) Arch. Exptl. Pathol. Pharmacol 51, 383.
8. Jodlbanner, A. and Van Tappeiner, H. (1905) Dent. Arch. Klin. Med 82, 520.
9. Spike, J. D. (1968) in Photophysiology vol. 3 (ed. by Giese, A.) pp. 33, Academic Press, New York.
10. Santamaria, L. (1960) in Recent Contribution to Cancer Research in Italy, Suppl. to Tumori (ed. by Bucalossi, P. and Veronesi, V.) pp. 167, Ambrosiama, Milan.
11. Clare, N. T. (1956) in Radiation Biology vol. 3 (ed. by Hollander, A.) pp. 693, McGraw Hill, New York.
12. Sakawa, Y. and Hase, E. (1967) Plant & Cell Physiol. 8, 509.
13. Epel, B. (1973) in Photophysiology vol. 8 (ed. by Giese, A.) pp. 209, Academic Press, New York.

14. Teresa, G. W., Teresa, N. L. and Milius, P. (1965) Can. J. Microbiol. 11, 1028.

15. Black, H. S. and Rauschkolb, E. W. (1971) J. Invest. Dermatol. 56, 387.

16. Sulkowski, E., Guerin, B. and Defaye, J. (1964) Nature 202, 36.

17. Pereira, O. M. (1975) in Riboflavin Sensitized Visible Light Effects on Human Diploid Fibroblasts in Culture, Masters thesis, University of California, Berkeley..

18. Baran, L. R., Daoust, J. Y., Labelle, J. L., Martin, W. G. and Schneider, H. (1974) Biochem. Biophys. Res. Commun. 56, 522.

19. Alexander, P. and Bacq, Z. M. (1961) in The Initial Effects of Ionizing Radiations on Cells (ed. by R. J. C. Harris) pp. 3

20. Pryor, W. A., Stanley, J. P. and Blair, E. (1977) Lipids 11, 370.

21. Bland, J., Madden, P. and Herbert, E. J. (1975) Physiol. Chem. & Physics 7, 69..

22. Nilsson, R., Swanbeck, A., and Wennersten, G. (1975) Photochem. Photobiol 22, 183.

23. Schothorst, A. S., Steveninck, J. V., Went, L. N. and Suurmond, D. (1972) Clin. Chim. Acta 39, 161.

24. Goldstein, B. D. and Harber, C. B. (1972) J. Clin. Invest. 51, 892.

25. Lamola, A. A., Yamane, T. and Trozzolo, A. M. (1973) Science 174, 1131.

0 0 0 0 4 9 0 1 0 2 1

26. Anderson, S. M. and Krinsky, N. I. (1973) Photochem. Photobiol 18, 403.

27. Tappel, A. L., Sawant, P. L., and Shibko, S. (1963) in Lysosomes (ed. by deReuck, A. V. S. and Cameron, M. P.) pp. 78, Little, Brown & Co., Boston, Massachusetts.

28. Slater, T. F. and Riley, P. A. (1966) Science 209, 151.

29. Koch, A. L., Doyle, R. J. and Kubitschek, H. E. (1976) J. Bact. 126, 140.

30. Ascenzi, J. M. and Cooney, J. J. (1975) Photochem. Photobiol 21, 307.

31. Spike, J. D. and Glad, B. W. (1964) Photochem. Photobiol 3, 471.

32. Tomita, M., Irie, M. and Ukita, T. (1969) Biochem 8, 5149.

33. Sone, K. and Koyanagi, T. (1966) Bitamin 33, 349..

34. Vodrazka, Z. (1959) Chem Listy 53, 829..

35. Ray, W. J., Jr. and Koshland, D. E. Jr. (1962) J. Biol. Chem. 237, 2493.

36. Kim, S. S. (1966) J. Fac. Agr. Kyushu Univ 13, 729.

37. Weil, L. (1965), Arch. Biochem. Biophys. 110, 57.

38. Livingston, R. and Rao, V. S. (1959) J. Phys. Chem. 63, 794.

39. Foote, C. S. (1968) Science 162, 963.

40. Kearney, E. B. (1957) J. Biol. Chem. 229, 363.

41. Cremona, T. and Kearney, E. B. (1963) Nature 200, 542.

42. Kearney, E. B., Salach, J. I., Walker, W. H., Seng, R. L., Kenney, W., Zeszotek, E. and Singer, T. P. (1971) Eur. J. Biochem. 24, 321.

43. Patek, D. R. and Frisell, W. R. (1972) Arch. Biochem. Biophys. 150, 347.

44. Frisell, W. R. and Mackenzie, C. G. (1962) J. Biol. Chem. 237, 94.

45. Mahler, H. R. (1954) J. Biol. Chem. 206, 13.

46. Beinert, H. (1963) in The Enzymes vol 7 (ed. by Boyer, P. D., Lardy, H. and Myrback, K.) 2nd Edition, pp 447, 467, Academic Press, New York.

47. Redina, G. and Singer, T. P. (1959) J. Biol. Chem. 234, 1605.

48. Singer, T. P. (1963) in The Enzymes vol 7 (ed. by Boyer, P. D., Lardy, H., and Myrback, K.) 2nd Edition, pp. 345, Academic Press, New York.

49. Whitby, L. G. (1953) Biochem. J. 54, 437.

50. Kearney, E. B. (1960) J. Biol. Chem. 235, 865.

51. Tegner, L. and Halmstrom, B. (1966) Photochem. Photobiol. 5, 223.

52. Raddha, G. K. and Calvin, M. (1964) Biochem. 3, 384.

53. Massey, V. and Palmer, G. (1960) Biochem. 5, 3181.

54. Lhoste, J. M., Hang, A. and Hemmerich, P. (1966) Biochem 5, 3290.

55. Raddha, G. K. (1966) Biochim. Biophys. Acta 112, 448.

56. Penzer, G. R. and Raddha, G. K. (1967) Quat. Rev. (London) 21, 43.

57. Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Mathew, R. G., Schuman, M. and Sullivan, P. A. (1969) Biochem. Biophys. Res. Commun. 36 891.

0 0 0 0 4 9 0 1 0 2 2

58. Ehrenberg, A. and Hemmerich, P. (1968) in Biological Oxidation (ed. by Singer, T. P.) pp. 239. Interscience, John Wiley, New York.

59. Ninneman, H. (1974) FEBS Lett. 39, 353..

60. Ringler, R. L., Minakami, S. and Singer, T. P. (1963) J. Biol. Chem. 238, 801.

61. Hatefi, Y., Hanstein, W. G., Davis, K. A. and You, K. S. (1974) Ann. N. Y. Acad. Sci. 227, 504.

62. Beinert, H. (1977) in Iron Sulfur Proteins vol. 3 (ed. by Lovenberg, W.) pp. 61, Academic Press, New York.

63. Singer, T. P., Kearney, E. B. and Bernath, P. (1956) J. Biol. Chem. 223, 599.

64. Singer, T. P. and Massey, V. (1957) Rec. Chem. Prog. 18, 201.

65. Rieske, J. S., McLennan, D. H. and Coleman, R. (1964) Biochem. Biophys. Res. Commun. 15, 338.

66. Rieske, J. S. Hansen, R. E. and Zaugg, W. S. (1964) J. Biol. Chem. 239, 3017.

67. Ohnishi, T., Wilson, D. F., Asakura, T. and Chance, B. (1972) Biochem. Biophys. Res. Commun. 46, 1631.

68. Ruzicka, F. J. and Beinert, H. (1974) Biochem. Biophys. Res. Commun. 58, 556.

69. Ohnishi, T., Ingledew, W. J. and Shiraishi, S. (1975) Biochem. Biophys. Res. Commun. 63, 894.

70. Ruzicka, F. J. and Beinert, H. (1975) Biochem. Biophys. Res. Commun. 66, 622.

71. Machinist J. M. and Singer T. P. (1965) Proc. Natl. Acad. Sci. (U.S.) 53, 467

72. Beinert, H. (1965) in Nonheme Iron Proteins (ed. by Sanpietro, A.) pp. 23, Antioch Press, Yellow Spring, Ohio.

73. Singer, T. P. (1977) in Iron Sulfur Proteins vol 1, (ed. by Lovenberg, W.) pp. 225, Academic Press, New York.

74. Crane, F. L., Hatefi, Y., Lester, R. L., Widner, C. (1957) Biochim. Biophys. Acta 25, 220.

75. Moret, V. Pinamouti, S. and Fornasari, E. (1961) Biochim. Biophys. Acta 54, 381.

76. Mee, J. M. L., Brooks, C. C. and Yanagihara, K. H. (1975) Biochem. Biophys. Res. Commun. 65, 228.

77. Kashket, E. R., and Brodie, A. F. (1962) J. Bact. 83, 1094.

78. Baum, R. H. and Dolin, M. I. (1965) J. Biol. Chem. 240, 3425.

79. Knook, L. L. and Planta, R. J. (1971) FEBS Lett. 14, 54.

80. Maraquez, E. D. and Brodie, A. F. (1970) J. Bact. 103, 261.

81. Creed, D., Werbin, H. and Strom, E. T. (1971) J. Am. Chem. Soc 93, 502.

82. Brodie, A. F. (1965) in Biochemistry of Quinoues (ed. by Morton, R. A.) pp. 356, Academic Press, New York.

83. Fujita, M., Ishikawa, S. and Shimazono, N. (1966) in J. Biochem. 59, 104.

84. Knook, L. L. and Planta, R. J. (1971) J. Bact. 105, 483.

85. Bragg, P. D. (1971) Can. J. Biochem. 49, 492.

86. Takamiya, K. I. and Takamiya, A. (1970) Biochim. Biophys. Acta 205, 72.

0 0 0 0 4 9 0 1 0 2 3

87. Fong, F., Peters, J., Pauling, C., and Heath, R. L. (1975) Biochim. Biophys. Acta 387, 451.

88. Werbin, H., Lakchaura, B. D. and Jagger, J. (1974) Photochem. Photobiol. 19, 321.

89. Klingenberg, M. (1968) in Biological Oxidation (ed. by T. P. Singer) pp. 3, Interscience, John Wiley, New York.

90. Palmer, J. M. and Hall, D. O. (1972) in Prog. Biophys. Mole Biol. 24, 127.

91. Yu, C. A., Yu, L. and King, T. E. (1977) Fed. Proc. 36, 2861a.

92. Mitchell, P. (1975) FEBS Lett. 56, 1.

93. Ohnishi, T. (1977) FEBS Lett. in press.

94. Ohnishi, T. (1977) Arch. Biochem. Biophys. in press,

95. Davis, K. A., Hatefi, Y., Poff, K. C., and Butler, W. L. (1972) Biochem. Biophys. Res. Commun. 46, 1984.

96. Davis, K. A., Hatefi, Y., Poff, K. C. and Butler, W. L. (1973) Biochim. Biophys. Acta 325, 341.

97. Chance, B., Wilson, D. F., Dutton, P. L. and Erecinska, M. (1972) Proc. Natl. Acad. Sci. U. S. 66, 1175.

98. Griffiths, D. E. and Wharton, D. C. (1961) J. Biol. Chem. 236, 1850, 1857.

99. Slater, E. C., VanGelder, B. F. and Minnaerl, K. (1965) in Oxidases and Related Redox System vol 2 (ed. by King, T. E., Mason, H. S. and Morrison, M.) pp. 617, John Wiley, New York.

100. Sands, R. H. and Beinert, H. (1959) Biochem. Biophys. Res. Commun. 1, 175.

101. Ninneman, H., Butler, W. L. and Epel, B. L. (1970) Biochim. Biophys. Acta 205, 507.

102. Jacobes, E. E. and Senadi, D. R. (1960) J. Biol. Chem. 235, 531.

103. Rounds, D. E. and Olson, R. S. (1965) NEREM Rec. 7 106.

104. Siegel, J. M., Montgomery, G. A. and Bock, R. M. (1959) Arch. Biochem. Biophys. 82, 288.

105. Kaplan, N. O., (1960) in The Enzymes vol 3 (ed. by Boyer, P. D., Lardy, H. and Myrback, K.) 2nd Edition pp. 105, Academic Press, New York.

106. Weber, G. (1957) Nature 180, 1409.

107. Sund, H. (1968) in Biological Oxidation (ed. by Singer, T. P.) pp. 641, Interscience, John Wiley, New York.

108. Klingenberg, M. (1959) Biochem. Z. 332, 47.

109. Chance, B. and William, G. R. (1956) Adv. Enzymol. 17, 65.

110. Lehninger, A. L. (1965) The Mitochondrion, Molecular Basis of Structure and Function, Benjamin, Inc., New York.

111. Kagawa, Y. (1972) Biochim. Biophys. Acta 265, 297.

112. Hatefi, Y., Hanstein, W. G., Galante, Y. and Stiggall, D. L. (1975) Fed. Proc. 34, 1699.

113. Hatefi, Y., Haavik, A. G. and Griffith, D. E. (1961) Biochem. Biophys. Res. Commun. 4, 441, 447.

114. Hatefi, Y. (1966) Comp. Biochem. 14, 199.

115. Hatefi, Y., Haavik, A. G., Fowler, L. R. and Griffith, D. E. (1962) J. Biol. Chem. 237, 2661.

116. Malmstrom, B. G. (1973) Quart. Rev. Biophys. 6, 389.

0 0 0 0 4 9 0 1 0 2 4

117. Wood, P. M. (1974) FEBS Lett. 44, 22.

118. Boveris, A., Cadenas, E. and Stoppani, A. D. M. (1976) Biochem. J. 156, 435.

119. Forman, H. J. and Kennedy, T. (1974) Biochem. Biophys. Res. Commun. 60, 1044.

120. Forman, H. J. and Kennedy, T. (1975) J. Biol. Chem. 250, 4322.

121. Mishra, H. P. and Fridovich, I. (1971) J. Biol. Chem. 246, 6886.

122. Mishra, H. P. and Fridovich, I. (1972) J. Biol. Chem. 247, 188.

123. Aleman, V. and Handler, P. (1967) J. Biol. Chem. 242, 4087.

124. Boveris, A., Oshino, N., and Chance, B. (1972) Biochem. J. 128, 617.

125. Tyler, D. D. (1975) Biochim. Biophys. Acta 396, 335.

126. Fridovich, I. (1970) J. Biol. Chem. 245, 4053.

127. Kaschnitz, R. M. and Hatefi, Y. (1975) Arch. Biochem. Biophys. 171, 292.

128. Schnaitman, C. and Greenwalt, J. W. (1968) J. Cell Biol. 38, 158.

129. Sottocassa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1968) in Methods in Enzymology vol 10 (ed. by Estrabrook, R. W. and Pullman, M. E.) pp. 443, Academic Press, New York.

130. Chapman, D. and Leslie, R. B. (1970) in Membranes of Mitochondria and Chloroplast (ed. by Racker, E.) pp. 91, Van Strand Reinhold Co., New York.

131. Witting, L. A. (1970) Prog. Chem. Fats & Other Lipids 9, 519.

132. Beinert, H. (1972) in Biological Application of ESR (ed. by Swartz, H. M., Bolton, J. R. and Borg, D. C.) pp. 351, Wiley (Interscience), New York.

133. Fridovich, I. (1975) Ann. Rev. Biochem. 44, 147.

134. Packer, L. (1973) in Mechanism in Bioenergetics (ed. by Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N.) pp. 33, Academic Press, New York.

135. Branton, D. and Deamer, D. W. (1972) in Membrane Structure (ed. by Alfert, M., Bauer, H., Sandwritter, W. and Sitte, P.) Springer--Verlag, Wien-New York.

136. Melnick, R. L. and Packer, L. (1971) Biochim. Biophys. Acta 253, 503.

137. Tewari, J. P., Malhotra, S. K. and Tu, J. C. (1971) Cytobios 4, 97.

138. Tinberg, H. M., Melnick, R. L., Maguire, J. and Packer, L. (1974) Biochim. Biophys. Acta 345, 118..

139. Tinberg, H. M., Packer, L. and Keith, A. D. (1972) Biochim. Biophys Acta 283, 193.

140. Mochan, B. S., Lang, R. W. and Elliott, W. B. (1970) Biochim. Biophys. Acta 216, 106.

0 0 0 0 4 9 0 1 0 2 5

141. Lehninger, A. L. (1955) Harvey Lectures 49, 174.

142. Racker, E. (1974) BBA Library 13, 269.

143. Slater, E. C. (1966) Comp. Biochem. 14, 327.

144. Hall, D. O. and Palmer, L. M. (1969) Nature 221, 717.

145. Hanstein, W. G. (1976) Biochim. Biophys. Acta 456, 129.

146. Chance, B. (1967) in Biochemistry of Mitochondria (ed. by Chance, B., Estabrook, R. W. and Williamson, J. R.) pp. 9, Academic Press, New York.

147. Boyer, P. D. (1974) BBA Library 13, 289.

148. Mitchell, P. (1968) in Mitochondria: Structure and Function (ed. by Ernster, L. and Drahota, Z.) pp. 219, Academic Press, New York.

149. Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147.

150. Hinkle, P. C. and Horstman, L. L. (1971) J. Biol. Chem. 246, 6024.

151. Slater, E. C. (1971) Quart. Rev. Biophys. 4, 98.

152. Mitchell, P. (1976) J. Theoret. Biol. 62, 327.

153. Brand, M. D., Reynafarje, B. and Lehninger, A. L. (1976) J. Biol. Chem. 251, 5670.

154. Wikstrom, M. K. F. (1977) Nature 266, 271.

155. Mitchell, P., and Moyle, J. (1969) Eur. J. Biochem 7, 471.

156. Padan, E. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431.

157. Tupper, J. T. and Tedeschi, H. (1969) Science 166, 1539.

158. Laris, P. C., Bahr, D. P. and Chaffee, R. R. J. (1975) Biochim. Biophys. Acta 376, 415.

159. Rottenberg, H. (1975) Bioenerg. 7, 61.

160. Rosing, J. and Slater, E. C. (1972) Biochim. Biophys. Acta 267, 275.

161. Nicholls, D. G. (1974) Eur. J. Biochem. 50, 305.

162. Azzone, G. F. and Massari, S. (1973) Biochim. Biophys. Acta 301, 195.

163. King, M. J. and Diwan, J. J. (1972) Arch. Biochem. Biophys. 152, 670.

164. Klingenberg, M. (1967) in Mitochondrial Structure and Compartmentation (ed. by Quagliariello, E., Papa, S. Slater, E. C. and Tager, J. M.) pp. 271, Bari:Adriatica Editrice.

165. Pressman, B. C. (1968) Ann. N. Y. Acad. Sci. 148, 285.

166. Scarpa, A. and Graziotti, P. (1973) J. Gen. Physiol. 62, 756.

167. Tedeschi, H. and Harris, D. L. (1955) Arch. Biochem. Biophys. 58, 52.

168. Gooch, V. and Packer, L. (1974) Biochim. Biophys. Acta 346, 245.

169. Packer, L. Utsumi, K. and Mustafa, M. G. (1966) Arch. Biochem. Biophys. 117, 381.

170. Fonyo, A. (1968) Biochem. Biophys. Res. Commun. 32, 624.

171. Harris, E. J., Catlin, G. and Pressman, B. C. (1967) Biochem. 6, 1360.

0 0 0 0 4 9 0 1 0 2 6

172. Klingenberg, M. and Bucholz, M. (1973) Eur. J. Biochem. 38, 346.

173. Meisner, H. (1973) Biochim. Biophys. Acta 318, 383.

174. Schatz, G. and Mason, T. L. (1974) Ann. Rev. Biochem. 43, 51.

175. Haslam, J. M., Perkins, M., and Linnane, A. W. (1973) Biochem. J. 134, 935.

176. Ibrahim, N. G., Burke, J. P. and Beattie, D. S. (1974) J. Biol. Chem. 249, 6806.

177. Linnane, A. and Haslam, J. M. (1970) Cure. Top. Cell Regener. 2, 101.

178. Schneider, W. C. and Hogeboom, G. H. (1950) J. Biol. Chem. 183, 123.

179. Avron, M. (1960) Biochim. Biophys. Acta 40, 257.

180. Lindberg, O. and Ernster, L. (1956) Methods in Biochem. Anal. 3, 1.

181. Quintanilha, A. T. and Packer, L. (1977) Proc. Natl. Acad. Sci. (U.S.) 74, 570.

182. King, T. E. (1967) in Methods in Enzymology vol 10 (ed. Estrabrook, R. W. and Pullman, M. E.) pp. 322 Academic Press, New York.

183. Singer, T. P. (1974) in Methods of Biochem. Analysis Vol. 22 (ed. by Glick, D.) pp. 123, John Wiley & Sons, Inc.

184. Murakami, S., Ringler, R. L. and Singer, T. P. (1962) J. Biol. Chem. 237, 569.

185. Wilbur, K. M., Bernheim, F. and Shipiro, W. O. (1949) Arch. Biochem. Biophys. 24, 305.

186. Sawicki, E., Stanley, T. W. and Johnson, H. (1963) Anal. Chem. 35, 199.

187. Sedlak, J. and Lindsay, R. H. (1968) Anal. Biochem. 25, 192.

188. Fields, R. (1972) in Methods in Enzymology vol 25 (ed. by Hirs, C. H. W. and Timasheff, S. N.) pp. 464, Academic Press, New York.

189. Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751.

190. Singer, T. P., Salach, J., Hemmerich, P. and Ehrenberg, A. (1971) in Methods in Enzymology vol 18B (ed. by McCormick, D. and Wright L. D.) pp. 416 Academic Press, New York.

191. Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochem. 10, 2606.

192. Walker, W. H. and Singer, T. P. (1970) J. Biol. Chem. 245, 4224.

193. Johnson, P. G., Bell, A. P. and McCormick, D. B. (1975) Photochem. Photobiol. 21, 205.

194. Mathew, M. M. and Sistrom, W. R. (1959) J. Bact. 78, 778.

195. Gutman, M., Mersmann, H., Luthy, J. and Singer, T. P. (1970) Biochem. 9, 2678.

196. Hopkin, I. G. and Morgan, E. (1938) Biochem. J. 32, 611.

197. Rothschild, H. A., Cori, O. and Barron, E. S. G. (1954) J. Biol. Chem. 208, 41.

198. Haugaard, N., Lee, N. H., Kostrzewa, R., Horn, R. S. and Haugaard, E. S. (1969) Biochim. Biophys. Acta 172, 198.

0 0 0 0 4 9 0 1 0 2 7

199. Fredrick, J. (1958) Arch. Biol. 69, 169..

200. Haga, J. Y. and Spikes, J. D. (1967) Fed. Proc. 26, 668.

201. Santamaria, L. and Fanelli, O. (1961) in Progress in Photobiology pp. 348. Elsevier, Amsteradam.

202. Hoffstein, P. E. Hunter, E. E., Gebicki, J. M. and Weinstein, J. (1962) Biochem. Biophys. Res. Commun. 7, 276.

203. Hunter, E. E. and Weinstein, J. (1963) Biochem. Biophys. Res. Commun. 11, 456.

204. Tinberg, H. M. and Packer, L. (1976) in The Enzymes of Biological Membranes, vol I (ed. by Montonossi, A.) pp. 171, Plenum Press, New York.

205. Girotti, A. W. (1975) Biochem. 14, 3377.

206. Tu, S. C. and McCormick, D. B. (1973) J. Biol. Chem. 248, 6339.

207. Oster, G. (1954) Nature 173, 300.

208. Hasty, N., Merkel, P. B., Radlick, P. and Kearns, D. R. (1972) Tetrahedron Lett. pp. 49.

209. Gollnick, K., Haisch, D. and Schade, G. (1972) J. Am. Chem. Soc. 94, 1747..

210. Ouannes, C. and Wilson, T. (1968) J. Am. Chem. Soc. 90, 6527.

211. Matheson, I., Etheridge, R., Kratowich, N., and Lee, J. (1975) Photochem. Photobiol. 21, 165.

212. Dorfman, L. M. and Adams, G. E. (1973) Reactivity of the Hydroxyl Radicls in Aqueous Solutions, NSRDS-NBS No. 46, U. S. Department of Commerce, National Bureau of Standards.

213. Mead, J. F. (1976) in Free Radicals in Biology, vol I (ed. by Pryor, W. A.) pp. 51, Academic Press, New York.

214. Kellogg, E. W. and Fridovich, I. (1975) J. Biol. Chem. 250, 8812.

215. Foote, C. S. (1976) in Free Radicals in Biology, vol II (ed. by Pryor, W. A.) pp. 85, Academic Press, New York.

216. Nishikimi, M. and Machlin, L. J. (1975) Arch. Biochem. Biophys. 170, 684.

217. Fahrenholtz, S. R., Doleiden, F. H., Trozzolo, A. M. and Lamola, A. A. (1974) Photochem. Photobiol. 20, 505.

218. McKnight, R. C. and Hunter, E. E. (1966) J. Biol. Chem. 241, 2757.

219. Tether, L. R. and Turnbull, J. H. (1962) Biochem. J. 85, 517.

220. Halmstrom, B. (1964) Ark. Kemi 22, 329.

221. Nelson, N., Nelson, H. and Racker, E. (1972) Photochem. Photobiol. 16, 481.

222. Frisell, W. R., Chung, C. W. and McKenzie, C. G. (1959) J. Biol. Chem. 234, 1297.

223. Palmer, G. R. and Raddha, G. K. (1968) Biochem. J. 109, 259.

224. Penzer, G. R. (1970) Biochem. J. 116, 733.

0 0 0 0 4 9 0 1 0 2 8

225. Massey, V., Palmer, G. and Ballou, D. (1971) in Flavins and Flavoproteins, vol III (ed. by Kamin, H.) pp. 349. University Park Press, Butterworths.

226. Loschen, G., Azzi, A., Richter, C. and Flohe, L. (1974) FEBS Lett. 42, 68.

227. McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6056.

228. Fridovich, I. (1976) in Free Radicals in Biology, Vol I. (ed. by Pryor, W. A.) pp. 239, Academic Press, New York.

229. Commoner, B. and Lippincott, B. B. (1958), Proc. Natl. Acad. Sci. (U.S.) 44, 1110.

230. Merkel, J. R. and Nickerson, W. J. (1954) Biochim. Biophys. Acta 14, 303.

231. Backstrom, D., Norling, B., Ehrenberg, A. and Ernster, L. (1970) Biochim. Biophys. Acta 197, 108.

232. Tappel, A. L. (1965) Fed. Proc. 24, 73.

233. Foote, C. S. and Denny, R. W. (1968) J. Am. Chem. Soc. 90, 6233.

234. Foote, C. S. (1971) Pure Appl. Chem. 27, 635.

235. Nilsson, R., Merkel, P. B. and Kearns, D. R. (1972) Photochem. Photobiol. 16, 117.

236. Foote, C. S., Fujimoto, T. T. and Chang, Y. C. (1972) Tetrahed. Lett. p. 45.

237. Doleiden, F. H., Fahrenholtz, S. R., Lamola, A. A. and Trozzolo, A. M. (1974) Photochem. Photobiol. 20, 519.

238. Horton, A. A. and Packer, L. (1970) J. Gerontol. 25, 199.

239. Steck, T. L. (1972) Biochim. Biophys. Acta 225, 553.

240. Weisiger, R. A. (1973) Ph.D. Thesis Duke University, Durham, North Carolina.

241. Mishra, H. P. (1974) J. Biol. Chem. 249, 2151.

242. Zimmerman, R., Flohe, L., Wesser, U. and Hartman, H. J. (1973) FEBS Lett. 29, 117.

243. Fong, K. L., McCay, P. B., Poyer, J. L., Keele, B. B. and Mishra, H. (1973) J. Biol. Chem. 248, 7792.

244. Ninneman, H., Butler, W. L. and Epel, B. (1970) Biochim. Biophys. Acta 205, 499.

245. Packer, L. and Worthington, L. (1974) in The Biogenesis of Mitochondria pp. 537, Academic Press, Inc. New York.

246. Stoien J. D. and Wang R. J. (1974) Proc. Natl. Acad. Sci. (U.S.) 71, 3961

0 0 3 0 4 9 0 1 0 2 9

This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

TECHNICAL INFORMATION DEPARTMENT
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720