THE PHOTOSENSITIVITY OF THE RESPIRATORY CHAIN IN *NEUROSPORA CRASSA*

A Thesis Presented

by

ZEINAB MOHAMED RAMADAN TALIB

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Department of Biochemistry,
Bedford College,
University of London,
London.

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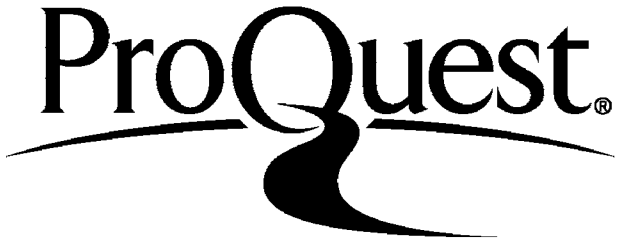

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ABSTRACT

The photosensitivity of respiration in a carotenoid synthesising strain (FGSC 987) and a carotenoidless mutant (FGSC 16) of Neurospora crassa has been studied. Although I have failed to find any effect of light on growth, violet light (385-495 nm, 150 W/m^2) inhibits respiration in both strains. However, mycelia of the yellow strain grown in the light have a high carotenoid content and show a smaller inhibition of respiration than those of the white or the dark-grown yellow strain.

Respiration of isolated mitochondria from yellow and white strains are also inhibited by violet light. Three photosensitive sites have been identified in the succinoxidase of both strains. These are:

1. A photosensitive site identified as ubiquinone

2. A sensitive site repaired by treatment with thiol reagents such as cysteine, glutathione and dithiothreitol

3. A weakly sensitive site associated with succinate dehydrogenase assayed with phenazine methosulphate as electron acceptor. The cytochrome oxidase is not light sensitive unless given prior treatment with a lipid-oxidising enzyme (lipoxygenase).

The NADH-oxidising pathway was less photosensitive than succin-oxidase. The photosensitivity of a sulphydryl group and ubiquinone have been demonstrated in the NADH pathway.

Growth of the pigmented strain in light and good aeration produce hyphae with a high carotenoid content. Ubiquinone in the mitochondria from these mycelia is much less photosensitive when irradiated with yellow light (440 + nm, 532 W/m^2) than is the ubiquinone of mitochondria from the white strain or the dark-grown yellow strain. Mitochondria from the yellow strain are found to contain carotenoid. In dark-grown

cultures this pigment is located in the outer membrane, while in light-grown cultures the pigment is found in both mitochondrial membranes. It is concluded that in cultures grown in the light, carotenoids protect ubiquinone from photodestruction when they are located in the inner membrane.

Carotenoids, even at low levels, seemed to protect kynurenine hydroxylase, the outer membrane enzyme, against the effect of violet light. Protection of this enzyme against H_2O_2 has also been demonstrated but this requires high levels of carotenoid.

CONTENTS

ABBREVIATIONS USED IN THIS THESIS

CoQ_{10}	coenzyme Q, ubiquinone
DCPIP	2,6-dichlorophenol indophenol
DTT	Dithiothreitol
GHS	Glutathione
MB	Methylene blue
Md	Menadione
PCMB	Parachloromercuribenzoic acid
PMS	Phenazine methosulphate
TMPD	Tetramethyl-para-phenylene diamine
W/m^2	$Watts/metre^2$
F.C.	Foot candle
nm	nanometres
U.V.	ultra-violet

I GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. PHOTOSENSITIVITY OF MICRO-ORGANISMS

Neurospora crassa, a member of the non-photo-synthetic eukaryotic ascomycetes, known as red bread mold, produces only traces of carotenoids when grown in the dark. However, a brief exposure to light results in substantial carotenoid synthesis (Batra,1971). An illuminated environment is not entirely beneficial. Blue and near ultra-violet radiation is inhibitory or lethal to a wide variety of prokaryotic and eukaryotic micro-organisms and to a number of tissues of higher plants and animals. Among the prokaryotes, visible plus near ultra-violet radiation, primarily in the range 330-490 nm, has been reported to kill or inhibit the growth of a considerable range of bacteria including, for example, Pseudomonas aeruginosa (Kashket and Brodie,1962).

Burchard and Dworkin (1966) found that dark-grown carotenoidless cells of the soil bacterium Myxococcus xanthus lsyed when exposed to low intensity visible light but only during the stationary phase. Mathews and Sistrom (1959b) reported that the carotenoidless mutant of the non-photosynthetic bacterium Sarcina lutea was killed in an oxygen-dependent reaction by sunlight.

Among eukaryotic micro-organisms, light has been reported to be inhibitory to the colourless alga Prototheca zopfii and to the yeast Saccharomyces cerevisiae (Epel and Krauss,1966). Goldstrohm and Lilly (1965) reported that unpigmented cells of the dark-grown fungus Dacryopinax spathularia and Sporidiobolus johnsonii are killed in an O_2-dependent reaction by sunlight.

Ninnemann and Epel (1968) have shown that respiration and growth of higher plant tissues are also inhibited by blue/violet light. Ninnemann et al (1970b) found that O_2 uptake by isolated beef-heart

mitochondria with succinate as substrate was inhibited by irradiation with blue light. Also cytochrome oxidase (a_3) was destroyed as assayed by the absorption band for the reduced cyanide-cytochrome a_3 complex at 587 nm in the low temperature absorption spectrum.

Ninnemann et al (1970a) suggested that the action of near ultraviolet in yeast involved the inactivation of quinone in the electron transport chain as well as cytochrome oxidase. However, Brodie and Kurup (1966) did not find any effect of 360 nm light on cytochrome in Mycobacterium phlei.

The physiological and action spectra data of Epel and Butler (1970) and of Epel and Krauss (1966) strongly suggested that the target site for the photo-inhibition of growth lay in the respiratory electron transport chain.

Maxwell et al (1971), using Rhodotorula glutinis, obtained evidence suggesting that damage to the membrane-bound enzymes of the mitochondria was the likely site of the lethal action of light.

Many light absorbing pigments such as flavins, quinones and porphyrins (e.g. cytochromes) which could act as endogenous photosensitisers, are normally present in biological systems; such compounds may make the biological material sensitive to the destructive effect of light energy. These sensitisers absorb light which in turn may cause photochemical oxidations in the cell. The nature and the localisation of the endogenous photosensitiser is not very clear.

The photosensitiser may also be an exogenous compound such as a dye or a pigment (Harrison,1967; Mathews-Roth,1967). Exogenous photosensitisers produce comparable effects to those of endogenous ones. It is then intuitively obvious that most organisms must possess a protective or active repair mechanism since sunlight does not appear to be directly detrimental in many cases.

It has been shown that carotenoid pigments protect both photosynthetic and non-photosynthetic organisms against damage by visible radiation (Krinsky,1968). Bacteria, such as Sarcina lutea with carotenoids remain alive in the light for a longer time than carotenoidless mutants of the same strain.

Mathews and Sistrom (1959a) and Salton et al (1965) found that the intracellular location of carotenoids was in the cell membrane (presumably along with the photosensitiser) which also carries the respiratory electron transport chain. Here, the membrane may be the site of photodynamic action.

Several authors have suggested the presence of carotenoids in mitochondria (Crane and Sun,1972). Neupert et al (1972) have shown the presence of substantial amounts of carotenoids in the outer membrane of Neurospora mitochondria.

Working with whole cells and isolated cell membranes of S.lutea Prebble and Huda (1973) and Huda (1970) found photodynamic damage to the respiratory chain using exogenous or endogenous photosensitisers. The respiratory activity of both whole cells and isolated membranes of a non-pigmented mutant showed similar photodynamic damage whereas in carotenoid-containing cells and membranes, these enzyme activities were much less damaged. Prebble and Anwar (1975) concluded that high light-intensity destroys S.lutea quinone (menaquinone,vitamin K) in the absence of carotenoids but not in the presence of these pigments. Thus quinone may be the site of the lethal action of light. Earlier, Santamaria et al (1957) had observed that vitamin K can photosensitise Escherichia coli.

In fungi, a protective role of carotenoids has been shown in some species (Goldstrohm and Lilly,1965). Maxwell et al (1966), using Rhodotorula glutinis, found that younger cultures of the white mutant

were more susceptible to lethal photosensitisation than were the older wild strains, therefore sensitivity is related to age as well as to carotenoid content. In earlier studies by Ryan et al (1943) light did not seem to influence the rate of growth in either the white mutant or the wild-type mycelium of N.crassa. Recently, Blanc et al (1976) have pointed out that carotenoid-containing wild-type conidia of N.crassa were less sensitive to black light radiation (300 to 425 nm) than albino conidia. The kinetics of black light inactivation are similar to those of photodynamic inactivation by visible light in the presence of a photosensitising dye (methylene blue). Only limited inactivation by visible light in the absence of exogenous photosensitisers was observed.

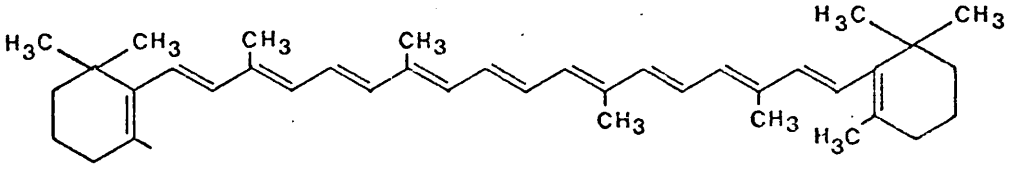
Rilling (1962) and Harding et al (1969) have pointed out that carotenogenesis is frequently stimulated by light in bacteria or fungi. In an ecological sense, this becomes intelligible as an adaptive process leading to protection from photosensitised photo-oxidation. The evidence presented in this thesis is that mitochondrial respiration is light sensitive and that a protective effect of carotenoids in respiration may also be operative in the mold Neurospora crassa.

2. CAROTENOIDS

(a) Definition

Carotenoids are fundamentally tetraterpenes, having 8 isoprene units, which arise through the "tail-to-tail" condensation of two identical 20-C units. It is the series of conjugated olefinic bonds that constitutes the chromophoric group of a carotenoid, which in naturally occurring pigments is usually red, orange or yellow.

Although cyclisation is not as extensive as in the triterpenes (e.g.sterols), carotenoids may be cyclised. Typical of "cyclic carotenoids" is β-carotene (1) which contains two β-ionone residues in its molecules.



Carotenoids can be sub-divided into

a) C_{40} hydrocarbons

b) C_{40} xanthophylls, sub-divided into mono-, di- and poly-hydroxy compounds, ethers, aldehydes, ketones and acids

c) Carotenoids with more than 40 C atoms in the carbon framework

d) Apo-carotenoids with less than 40 C atoms in the carbon framework, including the 30 C carotenoids of Staphylococcus (Taylor and Davies,1976).

Carotenoids are a widely distributed group of pigments present in all higher plants and in many protista, both photosynthetic and non-photosynthetic as well as many animals.

(b) Carotenoids in fungi

Not all fungi synthesise carotenoids, but certain statements can

be made regarding the distribution of these pigments in fungi.

(i) β-carotene is not universally present although it is well distributed in the Mucorales.

(ii) The presence of the major xanthophylls characteristic of higher plants has never been unequivocally established and such minor components as zeaxanthin and cryptoxanthin occur only very occasionally.

(iii) Characteristic fungal carotenoids are frequently acidic, for example, torularhodin and neurosporaxanthin.

(c) Carotenoids in N.crassa

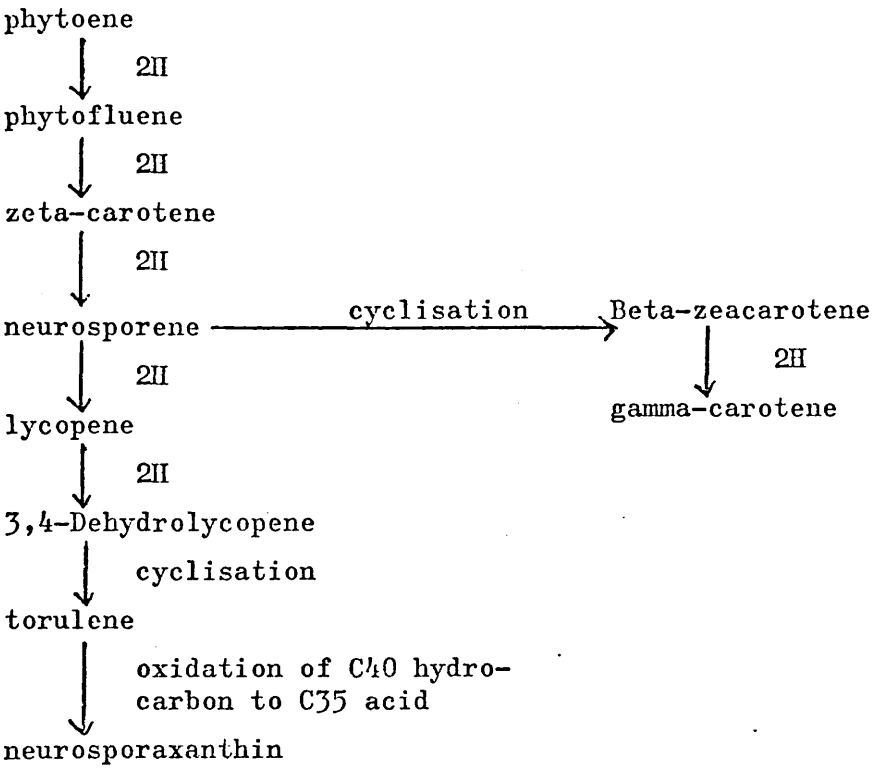
Interest in the Neurospora pigments stems from the early observations of Went (1901,1904) and van Deventer (1930). Both workers noted that cultures of the mold grown in the dark were colourless, but these cultures turned a bright orange when exposed to light.

In the course of extending these investigations, the carotenoids of N.crassa have been studied by many workers, first by Haxo (1949) who identified the polyene compounds in Neurospora in a qualitative manner. Davies et al (1963) showed that in N.crassa, as in other carotenogenic organisms, phytoene and not lycopersene is the first C40 compound formed in carotenoid biosynthesis. Zalokar (1954) reported that zeta-carotene is synthesised by the N.crassa mycelium, and the carotenoid previously identified as spirilloxanthin was shown to be 3,4-dehydrolycopene by Aasen and Liaaen Jensen (1965). In addition, Aasen and Liaaen Jensen (1965) reported for the first time the presence of torulene.

The acidic carotenoid first designated neurosporaxanthin by Zalokar (1957) is responsible for the pink colour of the mycelium, that develops when the dark-grown mycelium is exposed to light and air. In another investigation by Jensen (1965) the chemical structure of the acidic pigment, neurosporaxanthin, was elucidated, and it was

suggested that gamma-carotene or torulene is the precursor of this pigment. Hallermayer and Neupert (1974) in a study of mitochondrial lipids showed that the acidic pigment neurosporaxanthin is the only carotenoid occurring in Neurospora mitochondria.

Harding et al (1969) proposed that, since phytoene accumulates in dark-grown cultures, the biosynthetic step which is blocked in the dark is the conversion of phytoene to phytofluene. Consequently, the effect of light is probably to induce the production of phytoene dehydrogenase. Neurospora crassa, after light-induction, produces phytoene, phytofluene, ζ-carotene, neurosporene, β-zeacarotene, lycopene, 3,4-dehydrolycopene, γ-carotene, torulene, and neurosporaxanthin (Jensen,1965; Harding et al,1969), β-carotene is not synthesised.



The proposed pathway of carotenoid biosynthesis in N.crassa after photo-induction (Harding et al,1969)

Effect of light on carotene levels in N.crassa by Davies (1972)

Carotene	Concentration (µg/100g wet wt.)*	
	Dark	Light
Phytoene	2370	4650
Phytofluene	74	189
θ-carotene	47	364
Neurosporene	79	143
Lycopene	10	102
3,4-Dehydrolycopene	** tr.	18
β-Zeacarotene	tr.	91
ɣ-Carotene	12	533
Torulene	tr.	31.7 or tr.
β-Carotene	11	225
Total acyclic carotenes	2580	5466
Total cyclic carotenes	23	849
Total carotenes	2603	6315

* Yield of mycelium from 500 ml culture medium (static) was 110 g in dark and 116 g in light

**Trace (< 5 µg/100 g)

(d) Photo-induction of carotenoid synthesis

Some organisms are photochromogenic, that is they will synthesise pigments only when exposed to light.

The action spectrum of photo-induction for pigment formation of Mycobacterium marinum and also another unidentified species of Mycobacterium suggested that a flavin was the likely photoreceptor (Rilling,1964) and a porphyrin for M.marinum (Batra and Rilling,1964).

Zalokar (1955) determined an approximate action spectrum of carotenogenesis in N.crassa. This action spectrum showed a plateau between 449 and 488 nm, wavelengths longer than 520 nm were ineffective. The action spectrum corresponded best to a spectrum of a riboflavin derivative and no other pigments with a similar spectrum could be detected in Neurospora. Therefore it was assumed that flavin (probably as flavoprotein) was the photoreceptor. Recently Munoz and Butler(1975), using the N.crassa mycelium, concluded that flavin is the photoreceptor molecule for all of these "blue light responses" and that a light-induced redox change is the primary step in the photocontrol mechanism. De Fabo et al (1976) have determined the action spectrum for light-induced carotenoid biosynthesis in N.crassa. The spectrum has maxima at 450 and 481 nm in the visible range and at 280 and 370 nm in the ultra-violet. They proposed β-carotene, a pigment synthesised by Neurospora whose absorption spectrum resembles the action spectrum, as the receptor molecule.

Rilling (1962), using Mycobacterium sp., Rau et al (1968) and Harding et al (1968), using N.crassa, have shown that photo-induced carotenoid synthesis consists of at least three phases. The photo-chemical reaction is followed by a series of dark reactions (possibly synthesis of carotenogenic enzymes) and finally carotenoid production.

The photo-chemical reaction is temperature independent and in

addition to light requires oxygen (Zalokar,1955; Howes et al,1969). One can speculate on the role of oxygen in the photo-induction. Oxygen could function (a) as an electron acceptor to keep certain redox compounds such as the flavin or the cytochromes in the proper oxidation state or (b) as a direct molecular participant in the light-photoreceptor reaction. In this connection Foote and Denny (1968) have reported that light is capable of producing singlet oxygen by exciting certain redox substances (flavin and porphyrins). Singlet oxygen could be produced during this photochemical reaction and thus participates in the primary photo-oxidation process. At present this idea is speculative.

Rau (1969) has reported that when a suspension of dark-grown Fusarium aquaeductuum or N.crassa was gassed with nitrogen (N_2) and then illuminated, the organism produced 10-75% of the carotenoids as compared to those that were illuminated in an 0_2 atmosphere. This has led to the suggestion that 0_2 does not directly participate in the primary photochemical reaction but functions as an electron acceptor to keep the photoreceptor in the proper oxidation state. A requirement for 0_2 in the dark reactions has also been established (Rilling,1962; Batra et al,1969).

In studies on carotenoid production as a function of time in a dark incubation, there is always a definite lag period following the photochemical reaction before any new carotenoids are formed. The lag period in Mycobacterium sp. and N.crassa is somewhere between 40 minutes and $1\frac{1}{2}$ hours (Rilling,1962; Rau et al,1968).

Zalokar (1954), using N.crassa, reported that illumination as short as one minute stimulated production of full carotenoid colour, but only in the presence of sufficient oxygen and an adequate light intensity (a dose of 10^5 erg/cm^2 at 465 nm was required, Zalokar,1955).

The dark-grown non-photoinduced cultures contained 24 µg/100 g mycelium mostly phytoene. When these cultures were placed in the light for one hour and then incubated for 24 hours in the dark, the total carotenoid increased to about 70 µg/100 g mycelium of which 38 µg were phytoene.

The question can be asked: are the newly synthesised carotenoids after photo-induction deposited on the preformed cell membranes or are they added to a new membrane that is formed concurrently with the carotenoids? Mathews (1966) showed that in prokaryotes (Sarcina lutea) the new carotenoids are added to the preformed membrane.

(e) Relationship to photodynamic action

A comparison of the photochemical reactions in photo-induced carotenoid synthesis and photodynamic action (Spikes,1968; Krinsky, 1968; Wright and Rilling,1963) reveals striking parallels between them. Both are temperature independent, require O_2, and utilise visible light. Thus, both appear to involve a photo-sensitised oxidation of certain cell constituents. Photodynamic action,however, leads to detrimental effects resulting in the death of the organism (Krinsky,1968), while in the second case carotenoid production is stimulated. The quantity of light needed is much larger (about ten-fold) than that needed for the stimulation of carotenogenesis (Wright and Rilling,1963). The same substances might act as the photoreceptor for both photo-responses (Burchard and Dworkin,1966; Burchard and Hendricks,1969).

(f) Photofunction of carotenoids

The protective action of carotenoid pigments against lethal photo-oxidation has been studied in both photosynthetic and non-photosynthetic organisms and this may well be the universal function of carotenoids.

The most striking demonstration of the protective action of carotenoids against chlorophyll (CHL) photosensitisation has emerged from studies with photosynthetic bacteria by Stanier and his colleagues (Griffiths et al,1955). Rhodopseudomonas spheroides normally grows under anaerobic conditions but it will also thrive under aerobic conditions. However, a green mutant which synthesises no coloured carotenoids will photosynthesise normally under anaerobic conditions and will grow heterotrophically in oxygen in the dark, but is killed after 12-14 hours exposure to light and oxygen. Thus carotenoids protect the cells against photosensitisation, which from action spectrum studies is caused by bacteriochlorophyll (BCHL) itself. Griffiths et al (1955) pointed out that carotenoids are universally associated with photosynthetic tissue probably because they can protect cells from photodynamic destruction catalysed by either CHL or BCHL.

In addition to mutation, Cohen-Bazire and Stanier (1958) produced the same effect by growing photosynthetic bacteria (Rhodospirillum rubrum) in the presence of diphenylamine (DPA) which inhibits the production of coloured carotenoids, but which has no significant effect on bacteriochlorophyll synthesis. If DPA-cultured cells are washed free from the inhibitor and illuminated anaerobically, coloured carotenoids are gradually synthesised. There was a simultaneous reduction of photosensitivity of the cells as the carotenoids are synthesised. It is clear that any mutation which prevents carotenoid synthesis without affecting chlorophyll synthesis is lethal in the normal environment, that is in the presence of oxygen and light. This suggests a photodynamic action as defined by Blum (1941).

The general function of carotenoids as protective agents led Cohen-Bazire, Sistrom and Stanier (1957) to suggest that carotenoids characteristically synthesised by many non-photosynthetic bacteria and

fungi might perform a similar function in these organisms against detrimental effects of light. Here, the possibility of photo-oxidative damage by chlorophyll does not exist, but chemotrophic organisms do contain pigments, e.g., porphyrin derivatives and flavins, with intrinsic ability to catalyse photo-oxidation (Burchard and Dworkin,1966; Burchard et al,1966) which might sensitise the cell to visible light. Such a function could explain why so many bacteria commonly found in habitats exposed to sunlight are pigmented by carotenoids, the high concentration of carotenoids in reproductive cells liable to intense light exposure (e.g. the conidia of Neurospora and other fungi, Carlile,1965), and the stimulation of carotenoid production by light both in bacteria (Mycobacterium sp.) and in fungi (Neurospora, Phycomyces).

Investigations of this phenomenon have been made in many non-photosynthetic organisms such as Corynebacterium poinsettiae (Kunisawa and Stanier,1958), Sarcina lutea (Mathews and Sistrom,1959b; Mathews,1964) and Mycobacterium spp (Mathews,1963; Wright and Rilling, 1963). In all these cases it has been shown that the absence of carotenoids is associated with a lack of protection against lethal photodynamic action in the presence of a photosensitiser. In Corynebacterium lacking carotenoids, whether DPA-treated normal cells or pigmentless mutants, cells were only killed on exposure to light in the presence of the exogenous, photosensitising dye, toluidine blue. Evidently the cells do not contain sufficient amounts of natural photosensitising pigments to manifest endogenous photo-sensitisation even under the most severe conditions of light exposure that were tested (approximately 400 F-C for 4 hours).

The ecological importance of this phenomenon was first demonstrated by Mathews and Sistrom (1959b) working on the non-photosynthetic

bacterium S.lutea. In this organism 99% of carotenoidless mutant cells were killed in 2 hr on exposing them to direct sunlight in the presence of oxygen without the addition of an exogenous photosensitising pigment. Normal cells were unaffected by light whether in atmospheres of oxygen or nitrogen and even mutant cells were unaffected by light in an atmosphere of nitrogen. Mathews and Sistrom therefore concluded that the effect they observed wasphotodynamic and that carotenoid protects even non-photosynthetic bacteria from lethal effects of light and oxygen in the absence of an exogenous sensitiser.

Wright and Rilling (1963) working on Mycobacterium sp. found photodynamic killing in mutant cells by using an endogenous photosensitiser and bright light (7400-10,000 foot candles) between 360-590 nm. The killing did not occur in nitrogen and they also found that the rate of killing was slower in pigmented than in carotenoidless cells.

Mathews and Sistrom (1960) performed experiments to determine the location of the sensitive site which is protected by the carotenoids. The first step was to see if photodynamic killing of S.lutea was caused directly by a photochemical reaction. This was done by determining the effect of temperature on the rate of killing, on the premise that a purely photochemical reaction would not have a temperature coefficient of much more than unity. They found from the viable count curve of S.lutea using toluidine blue as photosensitiser that cell death was the same at 6.5°C as at 34°C; this suggested that the death of the cell was caused directly by a photochemical reaction which agreed with the experiments of Dworkin (1958) on the temperature independence of the rate of photodynamic killing of a carotenoidless mutant of R.spheroides. He found a temperature

coefficient (Q_{10}) of 1 for photodynamic killing between 4°C and 40°C and no measurable destruction of BCHL at low temperatures. He concluded that an energy transfer must occur from BCHL (which was the photosensitiser in the bacterium used) to an adjacent acceptor molecule. Since BCHL is located in the membrane associated chromatophores, Dworkin proposed that the membrane must be the site of lethal photo-oxidation.

Thus, if the photosensitiser molecules are very close to the sensitive site, so also must be the molecules which protect this site from the effect of the sensitiser. Mathews and Sistrom (1959a) had already demonstrated that the carotenoids of S.lutea are localised in or on the cell membrane. Therefore, Mathews and Sistrom (1960) were led to suggest that the site of the lethal photo-oxidation is on the cell membrane in non-photosynthetic bacteria. They also found that two enzymes, succinate dehydrogenase and NADH oxidase, were inactivated almost totally when the mutant strain was illuminated in air and toluidine blue. The rate of enzyme inactivation was temperature independent suggesting that the primary event in photodynamic killing may be the inactivation of different enzymes.

Mathews-Roth (1967) in extending her work on the cellular site of lethal photosensitisation, studied the effect of acridine orange, a photo-sensitising dye which is known to affect cellular DNA. Comparing the pigmented wild type and carotenoidless mutant strains of S.lutea in the presence of acridine orange she found that carotenoid pigments failed to prevent the lethal action of light in the presence of acridine orange. It was also found that the pigmented strain treated with the dye in the light gave rise to colourless mutants in numbers much greater than the natural mutation rate. This indicated that exposure to acridine orange and light resulted

in changes in the DNA of the exposed cells. The effect of acridine orange was also studied on the membrane enzymes and it was found that NADH oxidase which is a membrane-bound enzyme (Mathews and Sistrom,1959a) was destroyed in colourless cells but that in pigmented cells it was not affected, whereas adenosine deaminase, a cytoplasmic enzyme (Mathews and Sistrom,1959a) was inactivated to the same extent in both pigmented and non-pigmented cells.

From this she concluded that carotenoids which are located in the cell membranes were capable of preventing the destruction of membrane bound enzymes by acridine orange in the presence of light, but failed to prevent the lethal action of this dye.

It may further be noted that the membrane associated adenosine triphosphatase activity of the colourless Mycoplasma laidlawii was also appreciably destroyed when a cell suspension was exposed to visible light in the presence of toluidine blue (Rottem et al,1968). The pigmented cells exposed to light under the same conditions lost only a small amount of enzyme activity.

Along similar lines Prebble and Huda (1972,1973) and Huda (1970), working on the respiratory electron transport chain of S.lutea, reported that NADH, malate and succinate oxidase activities were affected by illumination with visible light (with and without an exogenous photosensitiser). They reported the following sites of the electron transport chain were affected by light without an exogenous photosensitiser:

a) Malate dehydrogenase flavoprotein

b) A site on the reducing side of the malate dehydrogenase complex

c) A site beyond the dehydrogenase complex which was observed in non-pigmented membranes only.

They suggested that this last site which is beyond the dehydrogenase complex is protected by carotenoids.

Furthermore, Prebble and Anwar (1975) working on S.lutea found that high intensity visible light destroys quinone in the absence of carotenoids but not in the presence of these pigments. Thus they concluded that quinone may be the site of the lethal action of light. To investigate whether carotenoid pigments play a role in stabilising the cell membranes Mathews and Krinsky (1970c) used protoplasts of a colourless mutant strain of S.lutea and a colourless culture produced by adding DPA to the medium of the wild type strain. There were no significant differences in the osmotic fragility of the protoplasts produced from the three cultures. On this basis they concluded that the carotenoids do not play a role in stabilising cell membranes.

Mathews (1964) studied the photosensitivity of a pigmented strain of S.lutea at $4^{o}C$ and $34^{o}C$ and found that at $4^{o}C$ carotenoid protection is not as great as at $34^{o}C$, regardless of whether an endogenous or an exogenous photosensitiser was used. She also observed a shorter lag period before the onset of cell death at $4^{o}C$ than at $34^{o}C$. She suggested that this could be due to the inhibitory effect of enzymes necessary for the protective mechanism at low temperature.

Wright and Rilling (1963) working on Mycobacterium sp. observed photo-killing of the pigmented cells at $0^{o}C$ and a lag period before the onset of the killing of the cell. They also found that the carotenoids of their Mycobacterium sp. were rapidly bleached under the conditions of high light intensity which they used. The greater protection by carotenoid at higher temperature observed by Mathews (1964) and the greater carotenoid pigment formation at $30^{o}C$ (Mathews,1963) led Wright and Rilling (1963) to provide an explanation

for how the carotenoids protect. They suggested that either carotenoid synthesis occurs at the same rate as the carotenoid bleaching which would allow constant protection or the carotenoid is involved in an enzymic process which would serve to protect the cells from the lethal photodynamic effects or by serving in a purely physical manner as shading pigments preventing light from reaching the photosensitising pigment such as flavin.

(g) Photoprotection and chromophore length

It has been shown by Stanier (1959), Crounse et al (1963) and Mathews and Krinsky (1970b) that the ability of carotenoids to provide protection against aerobic photosensitivity in vivo is related to the length of the conjugated chain of double bonds of the carotenoid molecule.

Foote et al (1970a) reported that the rate of quenching of singlet oxygen is dependent on the length of the conjugated polyene chain and parallels the protective action of carotenoids. When singlet oxygen (produced with methylene blue as sensitiser) quenching rates were plotted against the number of conjugated double bonds in the polyene chain, it was found that the rate fell off sharply with decreasing chain length and that the sharpest drop occurred between 7 and 9 conjugated double bonds.

Photobleaching of chlorophyll has also been reported to be protected by carotenoids but only if the chromophoric group of the polyene contains a threshold value lying between 7 and 9 conjugated double bonds. The protection does not depend on the total number of oxidisable double bonds (Claes,1960).

Quenching values for lycopene (11 conjugated double bonds) were also found to be similar to those of β-carotene with 9 conjugated double bonds (Foote et al,1970a). Mathews-Roth et al (1974) who

reached a similar conclusion to Foote et al (1970a) in S.lutea that pigment P.438 with 9 conjugated double bonds was two to three times more effective as a quencher than pigment P.422 with 8 conjugated double bonds. Phytofluene (5 conjugated double bonds) and phytoene (3 conjugated double bonds) were respectively 100 and 1,000 times less efficient than β-carotene for quenching singlet oxygen.

In addition to the length of the conjugated chain of double bonds many workers emphasise the importance of other factors, such as concentration and location of pigment in relation to photosensitiser and sensitive cellular site.

Mathews and Krinsky (1970a) worked on wild-type and mutant strains 2a and 4b of S.lutea. Both mutants contain considerably less total carotenoid pigment than does the wild-type. When they used toluidine blue as an exogenous photosensitiser, although mutants 2a and 4b are partially protected against lethal photodynamic effects by their carotenoid pigments, the protection was quite small in comparison to the wild-type cells. The carotenoids as well as the endogenous photosensitiser have been shown to be localised in the membrane of S.lutea (Mathews and Sistrom,1959a). Thus, with a lower concentration of carotenoid per cell in the mutant there is less chance that the pigment will be close to the photosensitiser. Hence there will be less protection if protection involves direct interaction between carotenoids and photosensitiser. They suggested that a possible mechanism by which carotenoids could protect cells would be an inhibition of photo-oxidation by quenching the excited singlet state of oxygen (Foote and Denny,1968). In this case the inhibition is dependent on the concentration of carotenoid used.

(h) Mechanism of photoprotection

There have now been many reports of carotenoid involvement as

protective agents against aerobic photosensitisation. Since the original observations of Stanier and his colleagues (Griffiths et al, 1955; Sistrom et al,1956) there can be little doubt that these compounds carry out this important function.

What remains to be done is to determine the molecular mechanisms involved in this process, since the precise way in which this protective function is performed is still unknown. It must be emphasised that carotenoids are only effective in those systems which have a true photodynamic action as defined by Blum (1941). This requires the simultaneous interaction of three components: visible light, a photosensitising dye to absorb it, and O_2.

Mathews (1963), while working with different photosensitisers, showed that some of them do not require oxygen. She was comparing lethal photosensitisation of S.lutea using toluidine blue or 8 methoxypsoralen. She noticed that the latter compound could exert its lethal photosensitisation effect in both aerobic and anaerobic conditions. Further, photokilling of bacterial cells sensitised by 8-methoxypsoralen was not protected by carotenoids, whereas photokilling sensitised by toluidine blue was oxygen dependent and carotenoids showed protection.

In many cases of photosensitisation it has not been shown whether either visible light as opposed to near-UV or O_2 is required for the effect.

Both Kunisawa and Stanier (1958), and Mathews-Roth and Krinsky (1965) have demonstrated that the presence or absence of coloured carotenoids have no effect on the viability of bacterial strains exposed to ultra-violet light. Working with pigmented and colourless strains of the yeast Rhodotorula glutinus, Maxwell et al (1966) and Hasegawa et al ~~Christensen and Maxwell~~ (1969) presented evidence that carotenoid

pigments did not protect the cells against lethal photosensitisation. It must be pointed out, however, that the action spectrum for this phenomenon shows a maximum in the ultraviolet with only a small effect at 410 nm. Thus it is not clear that this type of photosensitisation should be considered a photodynamic action.

Several mechanisms have been proposed, whereby cells may protect themselves from light and O_2. The idea that carotenoids may serve as light-filtering pigments was suggested by Goldstrohm and Lilly (1965) working on the fungus Dacryopinax spathularia. They found that carotenoid filters increased the survival of dark-grown cells when exposed to high light intensity. Greater protection occurred when the light passed through thicker carotenoid filters. Vail and Lilly (1968) have demonstrated that the carotenoids in this fungus are present in the cell wall fractions where they could act as a light filter, screening out potentially harmful radiation.

Calvin (1955) suggested that carotenoids could function as protective agents by serving as preferred substrates. In this case O_2 the actual causative agent of photosensitised oxidation, can interact with a suitable acceptor to form an oxidised product which presumably results in photodynamic action. If the acceptor were a carotenoid pigment then it would form an oxidised carotenoid. Sistrom et al (1956) suggested that epoxides might be formed across the double bonds in carotenoids. Krinsky (1966) has proposed that this mechanism accompanied by a dark regeneration of oxidised carotenoid to its original acceptor state might explain the protective action of carotenoids. The excited singlet state of oxygen cannot be involved since Foote et al (1970b) have presented evidence that only one out of a thousand carotenoid molecules involved in the quenching of $^1O_2$ undergoes a chemical oxidation and it appears to be primarily a

transfer of excitation energy.

The mechanism of quenching the triplet sensitiser by carotenoids was first described by Fujimori and Livingstone (1957) and has been amply confirmed by Mathis (1969) and others.

Foote and Denny (1968) argued that the protective action of carotenoids against photosensitised oxidations could not be explained by the ability of carotenoids to quench triplet sensitisers. This reaction is diffusion controlled and would compete with $^1O_2$ formation from the triplet sensitiser. Since both reactions occur at the same rates the carotenoids could only protect if their local concentration greatly exceeded that of O_2.

Foote and his collaborators (1968,1970a,1970b) have now demonstrated the ability of a variety of carotenoids to quench the singlet state of oxygen ($^1O_2$) effectively without the pigment suffering chemical change, and therefore to break the series of reactions which would normally lead to photosensitised oxidations.

They proposed a direct transfer of energy from $^1O_2$ to β-carotene to yield the carotenoid triplet (^3CAR) plus ground state triplet oxygen ($^3O_2$) where ^1CAR represents the ground state of carotenoid

$$^1O_2 + {}^1CAR \longrightarrow {}^3CAR + {}^3O_2$$

This could only occur if the triplet energy level of β-carotene was near or below $\Delta G'$ for $^1O_2$ of 22.5 K cal/mole. The exact values of the triplet states of β-carotene or other carotenoids are still uncertain.

3. MITOCHONDRIAL RESPIRATORY CHAINS

The electron transfer chain of the mitochondrion consists of a number of oxidation reduction components that transfer electrons primarily from succinate and reduced NADH to oxygen.

This chain includes at least two flavoprotein enzymes (the succinate and NADH dehydrogenases), non-haem iron proteins (several separate species), Co-enzyme Q (coQ_{10}) and several cytochromes including b, c, C, a and a_3. All of these components absorb visible light which could in turn cause photochemical oxidation in the cell. For example, riboflavins and porphyrins may catalyse photodynamic effects (Burchard and Dworkin,1966; Politzer et al,1971). It has been suggested that singlet oxygen may be formed during illumination by energy transfer from a triplet sensitiser. It has already been noted above that cytochrome a_3 is destroyed by light in isolated mitochondria (Ninnemann et al,1970). Quinones have also been proposed as endogenous photosensitisers by Barran et al (1974). Nevertheless, there have been relatively few investigations of the photosensitivity of respiration.

4. CONCLUSION

Carotenoids play an important role in the protection of cells against photodynamic killing.

It is known that the respiratory chain is present in the mitochrondrial membrane of N.crassa (Hall and Greenawalt, 1964, 1967). Also Neupert et al (1971) have reported that carotenoid pigment is located in the outer membrane of N.crassa mitochondria. Although effects of light on N.crassa have not so far been observed, it is desirable to re-examine photosensitivity in this organism particularly in view of the evidence discussed above for other fungi and bacteria.

It is possible that the primary lethal event due to visible light could involve the enzymes of the respiratory chain, many of which absorb visible light. So far no investigations have been made on the photosensitivity of N.crassa mitochondria. I have studied, therefore, the effect of visible light on the respiratory chain of isolated mitochondria of this fungus without using an exogenous photosensitiser. The role of carotenoids in protecting mitochondrial membrane enzymes and the relation of carotenoid location to the sensitive site has also been examined.

N.crassa has been chosen for this study for many reasons. It is easy to culture and there is a great difference in the content of the carotenoids in dark and light-grown cultures. It is also easy genetically to obtain white carotenoidless mutants. Finally, Neurospora has previously been found to be a good source for mitochondria.

II MATERIALS AND METHODS

MATERIALS AND METHODS

1. Strains of Neurospora crassa

Cultures of N.crassa were obtained from the Fungal Genetics Stock Centre, California State University, Humboldt Foundation, Arcuta, California, U.S.A. FGSC 987 was used as the wild-type pigmented strain and a white albino mutant (Locus al-2), FGSC 16, was used as the carotenoidless strain.

2. Culture Methods

a) Preparation of conidia

Stock cultures of pigmented and non-pigmented strains of N.crassa were grown on malt agar slopes. A loopful of conidia from these agar slopes was used for sub-culturing Wainwright's agar medium (1959) in flat bottles containing 50 ml. The conidial inocula were spread evenly over the agar surface in the bottles which were incubated at 28°C in the light. The formation of mature conidia of N.crassa requires 7-9 days.

Wainwright's medium per litre:

Na K tartrate 4 H_2O	5 g
$NaNO_3$	3 g
KH_2PO_4	3 g
Mg $SO_4.7H_2O$	0.5 g
K_2SO_4	0.3 g
NaCl	0.1 g
$CaCl_2$	0.1 g
Biotin	0.5 µg
Trace elements	10 ml
Sucrose	20 g
Agar	20 g
pH adjusted to 7.0	

Solution of trace elements:

	per litre
$Na_2B_4O_7.10H_2O$	0.0088 g
$(NH_4)_6\ MO_7O_{24}.4H_2O$	0.0064 g
$FeCl_3.6H_2O$	0.096 g
$Zn\ SO_4.7H_2O$	0.88 g
$CuCl_2$	0.027 g
$MnCl_2.4H_2O$	0.0072 g

b) Growth of mycelia from conidia

Culture media were prepared by dissolving 33.4 g of Czapek Dox powder (Oxoid liquid medium modified, Oxoid Ltd.,London) in a litre of distilled water. About 500 ml were used in 2 litre culture flasks for growing the mycelia. The flasks were autoclaved at a pressure of 15 lb per square inch for 15 min. Conidia suspensions were prepared by adding 50 ml sterile distilled water containing 3 drops of silicone antifoam as a wetting agent, to each conidial bottle. The bottles were shaken by hand to obtain the largest number of conidia in suspension. The suspension was allowed to stand for four minutes to sediment hyphal fragments. Culture flasks were inoculated by aseptically transferring about 50 ml of the conidial suspension. The flasks were incubated with shaking (about 70 oscillations/min) for 2.5 days at 28°C in the light or dark as required.

3. Preparation of Mitochondria

The hyphae were grown and mitochondria prepared by the method of Hall and Geenawalt (1967). A 2.5 day pigmented or non-pigmented culture of N. crassa was harvested by filtration on a Buchner funnel and washed with cold distilled water to give mycelial mats (for maximum pigmentation for the light-grown cultures the mats were put in the window in the light and open to the air). About 120 ml of the

blending medium at pH 7 (sucrose 0.25 M and EDTA 0.005 M) plus the mycelial mat from one 500 ml culture and about 100 g of glass beads (60 mesh) were homogenised at a maximum speed for 30 sec in a Warring blender. The ratio of liquid volume to beads and the grinding time were carefully standardised to obtain a satisfactory preparation. After homogenisation the contents were allowed to sediment for a few minutes. The unsedimented material was decanted into centrifuge bottles (250 ml) kept in ice. The sedimented material was washed twice with cold blending solution to remove trapped cellular components and the washes were added to the decanted liquid. The mitochondria were isolated from the decanted liquid by differential centrifugation. The first spin was at 1,500 g for 15 minutes at $4^{o}C$. The resulting supernatant was filtered through glass wool to remove contaminating cell wall fragments, and centrifuged at 14,000g for 30 minutes at $4^{o}C$. The final mitochondrial pellets were suspended in about 15 ml of blending medium (for each flask) using a hand homogeniser. The mitochondrial preparation was kept cold.

4. Illumination Apparatus

The apparatus, fitted with 1000 watt quartz halogen bulb, reflector and lenses, was designed by the Department. The perspex cuvette (together with light filter) was water-jacketed and connected to a temperature controlled water bath at $10^{o}C$. The cuvette had a 1 cm light path.

To prevent sedimentation of the mitochondrial preparation during illumination the cuvette was equipped with a small motor-operated, variable speed stirrer. The apparatus was fitted with photo-electric cell to measure the intensity of light. Using the violet wide-band interference filter, the apparatus gave an intensity of about 150 W/m^2 at the front face of the preparation.

5. Illumination of Mitochondrial Suspensions

Mitochondrial suspensions were diluted to give reasonable enzyme activity and a known optical density. A 1 in 5 dilution of the mitochondrial suspension gave an optical density of 0.2-0.4 at 600 nm. The suspensions were divided into two portions and placed in two perspex cuvettes jacketed by a constant temperature water. While one sample was being illuminated the other control was kept in the dark under identical conditions. Both control and illuminated samples were kept stirred. After irradiation the samples were kept in ice.

6. Experiments on Growth and Respiration of Mycelia

a) Tube method for growth rate

The growth tubes consisted of about 12 cm length of pyrex glass tubing, 2 cm in diameter. Terminal segments 3.5 cm. in length were bent up at an angle of 45°. The openings of the tubes were stoppered with cotton plugs, covered with tin foil and oven sterilised for 30 minutes at $160^{\circ}C$. About 10 ml of sterile Wainwright's agar medium was added to the tubes to half fill the horizontal portion. The agar was allowed to set with the tube in the horisontal position. A loopful of conidia from pigmented or non-pigment N.crassa agar slopes was used to incubate one end of the growth tubes. The tubes were incubated at $28^{\circ}C$ for 12 h in the dark to initiate the growth. Subsequently half the tubes were kept in the dark and the other half were illuminated as follows, both sets being at the same temperature:

(i) Using a 300 W slide projector, the growth tubes were placed at a distance of about 30 cm from the light source and illuminated for 8 h at an intensity of 200 W/m^2.

(ii) Using the illumination apparatus described above with only heat filters, the growth tubes were placed in the light beam. The incident light intensity was 270 W/m^2.

(iii) Using the violet interference filter (385-495 nm) for 6 h with the illumination apparatus, growth tubes were illuminated at 80 W/m^2.

The position of the mycelial frontiers were recorded at intervals of time.

b) Measurement of mycelial respiration

The mycelial shake cultures of pigmented or non-pigmented N.crassa were harvested after 16 h by direct centrifugation at 1,500 g for 15 min at $4^{o}C$. The mycelial pellet was washed in 0.25 M sucrose, 0.005 M EDTA, pH 7 and resuspended in sucrose-EDTA solution using a hand glass homogeniser gently so as not to break the mycelia. The optical density was measured (a 1 in 5 dilution of the suspension gave an extinction of 0.2-0.4 at 600 nm). The mycelial suspensions were divided into two portions and put in two perspex cuvettes jacketed by constant temperature water. While one sample was illuminated with violet light, the other (control) was kept in the dark under identical conditions. Both control and illuminated samples were kept stirred by bubbling air every 5 minutes using a Pasteur pipette in place of the electrical stirrer. (The mycelium was found to gather round the electrical stirrer).

7. Polarographic Assay Methods

a) Oxygen electrode

Succinoxidase, succinate dehydrogenase, succinate-menadione reductase, cytochrome oxidase and NADH oxidase were measured on a Bechman 39550 oxygen electrode assembly. The oxygen sensor was designed specifically for the measurement of oxygen dissolved in samples using the Bechman 100801 field Lab TM oxygen analyser. The analyser was connected to a Bryan's chart recorder, model 27000 with variable chart speeds via a zero suppressor and scale expander.

The oxygen electrode was daily calibrated with distilled water saturated with oxygen at 30°C. The scale was calibrated at 7.78 ppm (i.e. 7.78 parts of O_2 in 1 million parts of fluid) at 30°C.

The volume of the reaction vessel (cuvette) was 1.28 ml. Thus 0.5 ppm per minute was equivalent to $0.5 \times 10^{-6} \times 1.28 \times \frac{10^6}{16} = 0.04$ µg atoms of oxygen uptake per minute. Enzyme assays were repeated until reproducible rates were obtained.

b) Succinoxidase and oxidation of citric acid intermediates

0.5 ml of mitochondrial preparation was added to 0.5 ml of 0.1 M substrate, pH 7 and 1.0 ml of phosphate buffer 0.05 M, pH 7 and brought to 30°C in a constant temperature water bath. After aeration with the whirl-mixer, oxygen uptake was assayed in the electrode assembly and expressed as µg atoms of oxygen uptake per minutes per mg protein (or per 1 ml of mitochondrial preparation). Where sulphydryl reagents were used, 0.1 ml of cysteine 0.01 M in buffer pH 7 (or 0.1 ml of GSH 0.0025 M in buffer pH 7 or 0.1 ml DTT 0.005 M in buffer pH 7) was added to the incubation mixture in place of 0.1 ml of phosphate buffer.

Where the inhibitor PCMB was used, 0.1 ml 0.003 M in buffer was added in place of an equivalent volume of buffer.

Where reconstitution of respiration with ubiquinone was tested, 10 mg of ubiquinone was dissolved in 1 ml ethyl alcohol, 0.1 ml of this ubiquinone solution was mixed in 0.9 ml phosphate buffer and sonicated. 0.1 ml of this solution was added to assay mixtures in place of an equivalent volume of buffer.

c) Succinate dehydrogenase (King 1967)

The succinate dehydrogenase activity was assayed with the oxygen electrode through the auto-oxidation of phenzine methosulphate (PMS). The assay mixture normally contained 0.5 ml mitochondrial preparation, 0.1 ml PMS (0.01 M in neutralised buffer), 0.1 ml azide (1 M), 0.5 ml

substrate (0.1 M pH 7) and 0.8 ml phosphate buffer 0.05 M pH 7. The enzyme assay was carried out as described above. The activity was expressed as ug atoms oxygen uptake per minute per mg protein.

d) Succinate-menadione reductase

The activity was measured with the oxygen electrode by auto-oxidation of reduced menadione (vitamin K_3). The assay mixture was composed of 0.5 ml mitochondrial preparation, 0.5 ml substrate 0.1 M, pH 7, 0.1 ml menadione 0.01 M (0.01722 gm of menadione dissolved in 10 ml ethyl alcohol), 0.1 ml azide 1 M and 0.8 ml phosphate buffer 0.05 M, pH 7 to a total volume of 2 ml. The activity was measured and expressed as above.

e) Cytochrome oxidase (Wharton & Griffiths, 1962)

The activity was measured as oxygen consumption with the oxygen electrode. The reaction required an electron donor system which consisted of either a combination of cytochrome c and its chemical reductant ascorbate or TMPD and ascorbate. The reduction rate of cytochrome c by the reductant is assumed to be higher thant the oxidation rate by cytochrome oxidase and oxygen.

The assay mixture for ascorbate-TMPD oxidase contained 0.5 ml of mitochondrial preparation, 0.1 ml ascorbate 0.05 M in buffer pH 7, 0.1 ml TMPD (Tetramethylphenylene diamine) 0.001 M in buffer pH 7 and the volume was made up to 2 ml with phosphate buffer 0.05 M pH 7.

The assay mixture for ascorbate-cytochrome c oxidase contained 1 ml mitochondrial preparation, 0.3 ml ascorbate 0.05 M in buffer, 0.3 ml cytochrome c 0.02% in buffer and 0.4 ml phosphate buffer 0.05 M pH 7. A blank was prepared with ascorbate and cytochrome c but without mitochondrial preparation. The enzyme activity was assayed and expressed as above.

Where preparations were treated with lipoxygenase, 12.0 mg of lipoxygenase was dissolved in 40 ml of mitochondrial preparation (half the volume of the preparation). The mixture was allowed to stand for 2.5 h in the dark at room temperature before assay or illumination.

f) NADH oxidase

The NADH oxidase activity was measured with the oxygen electrode. The assay mixture was composed of 0.5 ml mitochondrial preparation, 0.3 ml of 0.1%NADH in buffer (added immediately before putting the sample in the electrode) and 1.2 ml of phosphate buffer 0.05 M pH 7. The activity was measured and expressed as above.

8. Spectrophotometric Assays

A Bechman model D.B.spectrophotometer was used with cuvettes having a 1 cm light path. The reactants were equilibrated at 30°C before mixing. Enzymes assays were repeated until reproducible rates were obtained.

a) Succinate-ubiquinone reductase (Ziegler & Rieske 1967)

The rate of reduction of CoQ_{10} (ubiquinone) by succinate was determined indirectly by measuring the rate of reduction of 2,6-dichlorophenol indophenol (DCPIP) which was reduced by added CoQ_{10}.

Reduction of DCPIP was measured spectrophotometrically by a loss of absorption at 600 nm.

The reaction mixture contained 0.4 ml of mitochondrial preparation in buffer (added immediately prior to the assay), 0.01 Triton X-100 10% (to enable CoQ_{10} to equilibrate with the endogenous CoQ_{10} located in the lipid layer), 0.01 ml CoQ_{10} 5 mg/1 ml ethanol, 0.1 ml antimycin 4 mg/100 ml ethanol, 0.1 ml substrate 0.1 M in buffer pH 7 and the volume made up to 3 ml with phosphate buffer 0.05 M pH 7. After temperature equilibration at 30°C (for 2 min) the reduction in absorption was read at 600 nm against a blank which contained all the

components except mitochondria. The loss of extinction at 600 nm was linear with time. It has been noted that the rate of reduction of DCPIP without adding CoQ_{10} was slower than the rate of succinate-ubiquinone reductase.

The activity was expressed as umoles DCPIP reduced per minute per mg protein

$$\frac{A\ 600\ nm}{W \times 7.00}$$

where A 600 nm is the change in extinction at 600 nm per minute and W is the protein (mg) in the 3 ml of assay mixture.

b) NADH oxidase

NADH oxidase activity was assayed spectrophotometrically using an incubation mixture containing 0.5 ml mitochondrial preparation in buffer, 0.3 ml of 0.1% NADH in buffer pH 7 (added immediately prior to the assay) and the volume of the mixture was made up to 3 ml with phosphate buffer 0.05 M pH 7. After temperature equilibration at 30°C for 2 min the mixture was placed in a cuvette of 1 cm optical path against a blank prepared without mitochondrial preparation. The decrease in extinction at 340 nm was measured. The activity was expressed as umoles of NADH per minute per mg protein

$$\frac{A\ 340\ nm}{W \times 6.22}$$

where W is the protein (mg) in the 3 ml incubation mixture.

c) NADH dehydrogenase

NADH dehydrogenase activity was assayed spectrophotometrically at 420 nm. The assay mixture contained 0.2 ml mitochondrial preparation, 0.3 ml ferricymide 0.01 M in buffer, 0.1 ml azie 1.0 M and 2 ml of 0.05 M phosphate buffer pH 7 to bring the mixture to 3 ml. After temperature equilibration at 30°C (for 2 min) 0.4 ml of 0.1%

NADH solution in buffer was added immediately before assay in a cuvette of 1 cm optical light path. The blank contained the incubation mixture without enzyme.

The activity was expressed as μmoles NADH oxidised per minute per mg protein

$$\frac{\text{A 420 nm per minute}}{1 \times 2 \times \text{mg protein in cuvette}}$$

where A 420 nm is the change in absorbance at 420 nm.

d) Kynurenine hydroxylase

The enzyme activity was assayed spectrophotometrically by the method of Okamoto (1970) by measuring the decrease of absorbance of NADPH at 340 nm in the presence of L-kynurenine. The incubation mixture contained 1 ml membrane (mitochondrial outer membrane) in phosphate buffer, 0.6 ml Tris-acetate buffer 0.5 M pH 8.1, 0.1 ml KCl 0.3 M pH 7, 0.1 ml of NADPH 0.0042 M in buffer pH 7, 0.02 ml of 0.03 M L-kynurenine in buffer pH 7 (either kynurenine or NADPH was added immediately prior to the assay) and the volume made up to 3 ml with phosphate buffer 0.05 M pH 7.

Where the homogenate (the filtrate after the second centrifugation in the mitochondrial preparation, since mitochondria failed to give reasonable rates) was used, 2.0 ml was taken and the assay completed as above.

After temperature equilibration at 30°C, the mixture was placed in a cuvette of 1 cm optical path against a blank which contained all the components except mitochondria.

The reduction in absorption was read at 340 nm. The molar extinction coefficient of 3-hydroxy kynurenine and kynurenine at 340 nm have been determined to be 2200 and 3290 respectively. A theoretical decrease of absorbance at 340 nm of 0.207 (based on the extinction coefficient of NADPH) is obtained when 0.1 μmole of

kynurenine is converted to 0.1 μmole of NADPH under the standard assay conditions.

Where the inhibitor hydrogen peroxide (H_2O_2) was used, 0.1 ml of 0.1% H_2O_2 was added in place of an equivalent volume of buffer and incubated for 10 minutes.

9. Protein Estimation

The protein was estimated by the Folin and Lowry method (Lowry et al,1951).

10. Assay of Ubiquinone in Mitochondria

Ubiquinone was assayed by the method of Pumphrey and Redfearn (1960).

Mitochondria were prepared as described above and the mitochondrial pellet resuspended in phosphate buffer 0.05 M pH 7. This suspension was divided into two parts. One part was illuminated and the other (control) kept in the dark. Samples were removed for protein estimation and the residual volumes measured. Both suspensions were centrifuged at 14,000 g for 30 min at 4°C. The resultant pellets were extracted by rapid addition of about 4 ml of cold methanol. The pellet was evenly suspended in the methanol and the mixture transferred to a 15 ml glass-stoppered test tube. About 5 ml of light petroleum (b.p.40-60 redistilled) was added to the methanol mixture and shaken rapidly for 30 sec. The tubes were allowed to stand for 1 min so that the phases separated. The upper light petroleum layer was transferred to another 15 ml glass-stoppered test tube. The methanol solution was extracted twice more with petroleum. The combined light petroleum extracts were treated with about 4 ml of 95% v/v methanol and the mixture was shaken for about 30 sec. This step was repeated twice to ensure the removal of the interfering lipid (e.g.phospholipid). After separation of the layers, the light petroleum solution was

evaporated to dryness under nitrogen. The residue was dissolved in 3 ml absolute ethanol and the spectrum was measured between 230 and 320 nm. The presence of ubiquinone was indicated by an absorption maximum at 275 nm. The ubiquinone was reduced by the addition of a small crystal of sodium borohydride (approx. 0.5 mg). The spectrum was determined after a fixed time (3 min) over the same wavelength range. The absorption maximum shifted from 275 to 290 nm and decreased in intensity giving a spectrum characteristic of ubiquinol. From the decrease in extinction at 275 nm (E 275 nm) with a correction for changes in light dispersion at 320 nm (E 320 nm), the concentration of ubiquinone in the lipid extract was calculated using the molecular extinction coefficient for the difference in absorption of the oxidised and reduced forms of ubiquinone ($E_{ox} - E_{red})_{275nm} - (E_{ox} - E_{red})_{320\ nm} = 12.25\ mM^{-1}\ cm^{-1}$ (Hemming,1958).

The ubiquinone concentration was expressed as μmoles/g protein of the mitochondrial extraction and was calculated from the equation

$$\mu\text{moles ubiquinone/g protein} = \frac{3 \times (E_{275nm} - E_{320nm}) \times 100}{12.25 \times \text{mg protein}}$$

11. Ergosterol Estimation

Ergosterol was extracted with the ubiquinone. Ultra-violet spectra were recorded. The content of ergosterol was estimated from the extinction at 282 nm from the equation

$$\mu\text{moles ergosterol/g protein} = \frac{E_{282nm} \times \text{total vol. of ethanol extract} \times 10^3}{11.5 \times \text{mg protein/ml}}$$

12. Carotenoid Estimation in Mitochondria

The carotenoid was extracted with the ubiquinone by the method described above. The visible absorption spectrum was recorded in ethanol between 600 to 380 nm and showed a maximum at about 477 nm. The extinction coefficient for carotenoid in a 1 cm cell at the wave-

length of maximal absorption in ethanol was assumed to be

$$E^{1\%}_{1cm} = 2500$$

The amount of carotenoid was calculated from the equation

$$\mu g \text{ carotenoid/g protein} = \frac{E_{(477nm)} \times \text{total vol. of ethanol extract} \times 10^3}{0.25 \times \text{mg protein/ml}}$$

where E is the absorption maximum at 477 nm.

13. Carotenoid Extraction and Determination in Mycelia

The N.crassa mycelia were prepared as described above. After filtration on a Buchner funnel the mycelium was divided into two samples and freeze-dried overnight. The samples were weighed, slightly wetted with distilled water, extracted with 4 ml of acetone and 4 ml methanol. About 5 ml of light petroleum (b.p.40-60 redistilled) was added, shaken, and the phases allowed to separate. The top light-petroleum layer was transferred to a glass-stoppered flask, evaporated to dryness under nitrogen and the residue dissolved in 3 ml of absolute ethanol. The visible spectrum of this solution was recorded spectrophometrically at between 600 and 380 nm. The carotenoid content was calculated as above.

14. Preparation of Outer and Inner Mitochondrial Membrane Fractions

The N.crassa mitochondrial membranes were separated according to the modified technique of swelling, shrinking and sonication used by Neupert and Ludwig (1971) and first applied to Neurospora mitochondria by Cassady and Wagner (1968). The principal of the method essentially depends on the outer membrane being permeable to both water and sucrose while water but not sucrose penetrates the inner membrane. In hypotonic media the increased internal pressure in the matrix compartment stresses and breaks the outer membrane. Unfolding of the cristal surface, however, allows free expansion of the inner membrane without breakage. In hypertonic solution, sucrose enters the

intra-membrane compartment and shrinks the matrix compartment.

The mycelia of N.crassa pigmented dark-grown and light-grown were prepared from 20 half-litre cultures. The mitochondria were obtained by the usual method. The resultant mitochondrial pellets were gently resuspended in the blending medium using a hand glass homogeniser and centrifuged again at 1,500 g for 15 min at 4°C. The supernatant was strained through glass wool to remove any cell wall fragments and centrifuged at 27,500 g for 20 min at 4°C. The pellets were resuspended in the blending medium using the hand homogeniser and filtered through glass wool before a further centrifugation at 27,500 g for 20 min at 4°C. The sedimented mitochondria were suspended in 60 ml Tris-phosphate 10 mM pH 7.6 (swelling solution) and homogenised gently and then allowed to stand for 30-45 min at 0°C. Homogenisation was repeated before addition of 20 ml of shrinking solution (2.0 M sucrose, 8 mM ATP, 8 mM $MgCl_2$ neutralised with KOH to pH 7.6). Shrinking was manifested by changes in the turbidity of the suspension. After a further 10 min the suspension was sonicated (Ultrasonic Disintegrator, 150W, MSE Scientific Instruments Ltd., medium setting) in 20 ml aliquots for 15 sec while cooled in an ice-bath. The sonicated suspensions were centrifuged for 60 min at 70,700 g at 4°C. The combined pellets were resuspended in 18 ml of blending solution, homogenised with the glass homogeniser and 6 ml were applied to the top of a discontinuous sucrose density gradient layered in a 25 ml centrifuge tube and consisting of 7 ml of 1.5 M sucrose in 10 mM Tris-HCl pH 7.5 in the bottom of the tube followed by 7 ml of 1.05 M sucrose in 10 mM Tris-HCl pH 7.5. Three tubes were centrifuged at 95,000 g for 1.5 h at 0°C using a swing-out head. After centrifugation two bands and a sediment could be distinguished, a bright yellow band in the 0.45 M sucrose layer, a

dark yellow to pink band at the interface between the 1.05 M and 1.5 M sucrose layers, and a tightly packed brown pellet. The two upper fractions were removed, diluted about 5 times with Tris-HCl 10 mM pH 7.5 and centrifuged at 70,700 g for 60 min at 0°C. The pellets were resuspended in 15 ml phosphate buffer 0.05 M pH 7.0. The contamination of the outer membrane fractions (FI) by inner membrane (FII) was calculated using equation

$$\frac{S.D._{FI}}{S.D._{FI} + S.D._{FII}}$$

where S.D. is the succinate dehydrogenase activity.

Contamination of Fll fraction by FI fraction is given by

$$\frac{Kyn._{FII}}{Kyn._{FII} + Kyn._{FI}}$$

where Kyn is the kynurenine hydroxylase activity.

The levels of carotenoid in the inner membrane were calculated by solving the simultaneous equations

$$\frac{Q_I \cdot x}{Q_I + Q_{II}} + \frac{Kyn._I \cdot y}{Kyn._I + Kyn._{II}} = car_I$$

$$\frac{Q_{II} \cdot x}{Q_I + Q_{II}} + \frac{Kyn._{II} \cdot y}{Kyn._I + Kyn._{II}} = car_{II}$$

where x and y are the carotenoid contents of inner and outer membranes and car is the observed carotenoid content of the fractions, Q is the quinone content and Kyn. is the kynurenine hydroxylase activity.

III RESULTS

RESULTS

I. INTRODUCTION

Visible radiation is inhibitory or lethal to a wide variety of prokaryotic and eukaryotic micro-organisms. They contain pigments (such as flavins, quinones and porphyrins of various types including cytochromes) which absorb visible light and may then be involved in photochemical reactions. In some cases, cells have been shown to possess mechanisms which protect them from visible radiation.

Carotenoids which occur in plant, animal, and bacterial systems, have been implicated in protection against the photodynamic effect of light (Krinsky,1971). For example, in bacteria, carotenoids with a minimum of nine conjugated double bonds can prevent lethal photosensitisation (Mathews-Roth et al,1974). Specifically, certain carotenoids quench the excited states of singlet oxygen (Foote et al, 1968). This quenching may protect against photosensitisation by preventing the formation of lipid peroxides and free radicals which could lead to membrane damage (Anderson and Krinsky,1973). The protective function of carotenoids in relation to the respiratory chain has been investigated in detail in S.lutea (Prebble and Huda, 1973; Anwar,1975). The effect of light on the N.crassa mycelium and on the respiratory activity of isolated N.crassa mitochondria will be described here, and such photoprotective mechanisms of carotenoids as may also be at work in this organism. In particular, the following aspects of photosensitivity in this fungus have been studied:

(i) The effect of light on the growth and respiratory activity of the pigmented and non-pigmented mycelium.

(ii) The sensitivity to visible light of the respiratory chain of isolated mitochondria from the pigmented and carotenoidless mycelium. An attempt has been made to identify those sites in the chain which are

particularly photosensitive using different substrates, artificial electron acceptors and respiratory chain inhibitors.

(iii) The role of carotenoids in protection of the photosensitive sites of the chain.

(iv) The location of carotenoids in relation to the sensitive sites of respiration.

2. THE EFFECT OF LIGHT ON THE GROWTH RATE AND RESPIRATION OF NEUROSPORA CRASSA MYCELIA

(a) Effect of light on the growth rate of mycelia

The rate of growth in the pigmented wild-type strain (FGSC 987) and in the non-pigmented white mutant (FGSC 16) of Neurospora crassa was determined by measuring the progression of mycelial frontiers growing on agar media within horizontal glass tubes. The tube method is particularly useful in studying the growth rate of rapidly growing organisms such as N.crassa. A rather remarkable property is the regularity of the colony frontier, a feature which is shared by many filamentous fungi.

The results presented in Table 1 suggested that mycelia grown on agar media have been only slightly inhibited by light. In most cases, this inhibition was not regarded as significant in either strain even when a high intensity of white light was used and even when this irradiation was continued for several hours.

Many studies have attempted to answer the question as to whether the growth rate is limited by the rate of energy production or whether the limitation is due to the rate of biosynthetic processes. Demonstration of an inhibitory effect of light on growth might be possible if the rate of metabolic processes were reduced by using low concentrations of nutrients. The agar media was diluted to one tenth of the original concentration in order to slow down the growth rate.

Table 1. Effect of white light and violet light on the growth rate of pigmented and non-pigmented Neurospora crassa

Expt.	Illumination time (hr)	Light source used	Pigmented		Percentage inhibition	Non-pigmented		Percentage inhibition
			Control	Illuminated		Control	Illuminated	
1	6.0	White light (210.0 W/m^2)	2.90	2.90	0	3.04	3.04	0
	8.30		3.27	3.13	4.2	3.15	3.00	4.7
2	6.30	White light (270.0 W/m^2)	2.84	2.88	-1.4	3.08	3.12	-1.2
3	6.0	Violet light (385-495 nm, 80.0 W/m^2)	3.07	3.00	2.2	2.76	2.65	3.9
4*	5.0	Violet light (385-495 nm, 80.0 W/m^2)	2.35	2.39	- 1.7	3.01	3.48	- 15.6

The growth rate is expressed in mm/hr. Measurements of linear growth were made on cultures grown in 2 cm tubes on agar (Wainwright's medium)

* organism grown on diluted medium, one tenth dilution of the original medium concentration

However, the reduced nutrient level did not affect the rate of linear growth. Irradiation with violet light (150 W/m^2) of young mycelia grown on diluted media resulted in activation of the growth rate of the mycelia particularly in the non-pigmented strain.

Experiments were performed with two pairs of cultures (two white and two yellow) with and without illumination, treated under the same conditions. The illuminated wild-type strain showed photo-induction of carotenoids. It is possible that the mycelia need a higher light intensity than was used in these experiments to reduce the growth rate. The failure to demonstrate an inhibitory effect of light on the rate of growth of pigmented or non-pigmented strains of N.crassa is consistent with the previous findings of Ryan et al (1943). In a recent paper, Sargent and Briggs (1976) have stated that only limited inactivation by visible light in the absence of exogenous photo-sensitisers was observed in both pigmented and albino strains of N.crassa.

(b) Effect of light on respiration of young mycelia

The wild-type pigmented strain of N.crassa produces carotenoid pigments when grown in the light much more than when grown in the dark. The respiratory capacity of irradiated freshly prepared young hyphal suspensions was measured as oxygen uptake. Young mycelia were used for easy and efficient pipetting. The experiments for pigmented and white strains were performed in pairs under the same conditions. The optical density for both preparations was the same.

The results obtained are shown in Tables 2 and 3. Table 2 shows that irradiation of dark grown mycelia with light of wavelengths 460 ± 5 nm (15 W/m^2) for 60 minutes caused about 12.3% inactivation in the non-pigmented strain and only slight inhibition (4.6%) in the wild-type. Since it is difficult to measure less than 5 per cent

Table 2. Effect of light on succinoxidase activity of pigmented and non-pigmented dark-grown young mycelia of N.crassa

Expt.	Illumination time (min)	Wavelength used (nm)	Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
			Control	Illuminated		Control	Illuminated	
1	60	460 ± 5	2.35	2.24	4.6	2.02	1.77	12.3
2	21	385 - 495	4.87	4.50	7.5	3.70	3.34	9.7
3	90	385 - 495	2.66	2.13	19.9	2.19	1.60	26.9
4	105	385 - 495	3.51	2.69	23.3	3.22	2.45	23.9

Activities are expressed in µg atoms O_2 uptake/min/ g dry weight mycelia. Shake-cultures were harvested after 16 h incubation in the dark and suspended in phosphate buffer. The mycelia were illuminated at 10°C using an intensity of light of 15 W/m^2 (460 nm) and 150 W/m^2 (385-495 nm)

The optical density of samples: Expt.3 = 0.78, Expt.4 = 0.15

Table 3. Effect of light on succinoxidase activity of pigmented and non-pigmented light-grown young mycelia of N.crassa

Expt.	Illumination time (min)	Wavelengths used (nm)	Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
			Control	Illuminated		Control	Illuminated	
1	60	460 ± 5	1.76	1.76	0	2.24	1.97	12.0
2	60	385 - 495	2.70	2.40	11.0	2.90	2.20	24
3	90	395 - 495	2.38	2.06	13	2.04	1.49	26
4	105	395 - 495	1.74	1.30	25	1.34	0.79	41

Activities are expressed in µg atoms O_2 uptake/min/g dry weight mycelia. Shake-cultures were harvested after 16 h incubation in the light and suspended in phosphate buffer. The mycelia were illuminated at 10°C using a light intensity of 15 W/m^2 (460 nm) and 150 W/m^2 (385-495 nm). For succinate oxidase assays 1 ml of the mycelial preparation was used

The optical density of samples: Expt.3 = 0.65, Expt.4 = 0.2

photo-inhibition, a higher light intensity (violet light) was used. Illumination, using violet light (150 W/m^2) for 21 minutes caused 7.5% and 9.7% inhibition in pigmented and white strains respectively. Increasing the dose of irradiation increased the percentage inactivation of respiration measured as succinoxidase activity in both strains. It can be seen from the results in Table 2 that the white mutant was only slightly more photosensitive than the dark-grown wild-type mycelia which possess low levels of carotenoids (Fig.2). Table 3 shows appreciable differences between pigmented and white strains. Illumination of light-grown mycelia with 460 $\pm$ 5 nm light for 60 minutes caused almost no inhibition in the pigmented strain and about 12.0% inactivation in the white mutant. Irradiation with violet light caused photo-inhibition in the non-pigmented strain about twice that of the wild-type for the same period of illumination. However, the respiration of light-grown pigmented mycelia which contain high levels of carotenoids (Fig.2) was much less photosensitive than the white mutant strain also grown in the light.

3. STUDIES ON THE EFFECT OF VIOLET LIGHT ON THE RESPIRATORY CHAIN OF NEUROSPORA CRASSA MITOCHONDRIA

The electron-transfer chain of the mitochondrion consists of a regular array of a number of oxidation reduction components that transfer electrons primarily from succinate and reduced NADH to oxygen. This chain includes at least two flavoprotein enzymes (the succinic and NADH dehydrogenases), several cytochromes (including a, a_3, b, c_1 and c), non-haem iron-proteins (several separate species), Coenzyme Q and copper. The inhibitory effects of visible light on the respiratory electron-transport chain have been studied by several workers (Mathews-Roth,1967; Epel and Butler,1970; Barran et al,1974; Ninnemann,1974; Anwar,1975).

In the prokaryote, Sarcina lutea, there is evidence suggesting that a major site of action of visible radiation is the respiratory chain (Prebble et al,1976). In eukaryotic organisms, Ninnemann et al (1970b) found that oxygen uptake by isolated beef-heart mitochondria with succinate as substrate was inhibited by irradiation with blue light.

As shown above, violet light caused damage to the respiration of young hyphae of N.crassa which may include damage to the respiratory apparatus itself. Since the mitochondrion houses the respiratory system, the effect of violet light has been tested on respiration of N.crassa mitochondria.

(a) Effect of violet light on the oxidation of various substrates by mitochondria from pigmented and white strains

The mitochondrion is a structure found in the cells of all aerobic, eukaryotic organisms. In general, the coupled oxidative reactions catalysed by the mitochondrion are integral with the citric acid cycle. The electrons can be derived from citric cycle substrates or from any of a considerable list of oxidisable substrates that play no direct part in the cycle.

The effect of violet light for 10 minutes on the rate of oxidation of various substrates by mitochondria from the pigmented and white strains was measured. In Table 4 it can be seen that there was substantial photo-inactivation of succinoxidase after 10 minutes illumination. Inactivation of succinoxidase was slightly less in mitochondria from pigmented than white mutant strains. Succinate oxidation was more photosensitive than the oxidation of the other substrates whose metabolism involved NAD-linked dehydrogenases more directly. However, the mitochondrial preparation oxidised succinate much more actively than other intermediates of the citric acid cycle.

Table 4. Effect of violet light on the oxidation of various substrates by mitochondria from pigmented and non-pigmented dark-grown strains of N.crassa

Substrates	Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
	Control	Illuminated		Control	Illuminated	
Succinate	.047	.030	36	.033	.020	39
Glutamate	.015	.013	13	.015	.012	20
Malate	.018	.015	17			
Citrate	.013	.011	15			
Oxaloacetate	.014	.013	7			
Pyruvate	.010	.008	20			
DL-Aspartate	.012	.012	0			
Isocitrate	.012	.011	8			
α-Ketoglutarate	.018	.013	28			

Activities are expressed in μg atoms O_2 uptake/min/1 ml mitochondrial preparation. The mitochondria were illuminated at 10°C using light intensity 150 W/m^2 for 10 minutes. Substrate assays 1 ml of mitochondrial preparation was used. The optical density of the suspension of the pigmented preparation was 0.61 and for the non-pigmented was 0.65, equivalent to approximately 0.6 mg protein/ml and 0.65 mg protein/ml respectively. The values shown are representative of two experiments.

(b) Location of the sites of photo-inactivation in the respiratory chain

(i) Photo-inactivation of succinoxidase

The effect of violet light (150 W/m^2) on succinoxidase activity was therefore studied in detail without an exogenous photosensitiser in mitochondria from pigmented and non-pigmented N.crassa strains. Irradiation of isolated mitochondria for 5 minutes partially inactivated succinoxidase of both strains (Table 5). This suggested that photodynamic action involving the respiratory system can occur in the presence of an endogenous photosensitiser which is already present in the membranes and which becomes active at high light intensities. Increasing the dose of illumination from 5 to 45 minutes increased the percentage of inactivation of succinoxidase from 19% to 56% in the pigmented strain and from 22% to 58% in the white mutant (Table 5). There was only a slight difference between photo-inhibition of mitochondrial respiration of the pigmented and the white mutant strains grown in the dark. After illumination with violet light for 5-10 minutes the photo-inhibition of succinoxidase was about 20-30% which was considered suitable for further study of the effect of light on the mitochondrial respiratory chain.

Some variation between the results from different preparations has been found (Tables 6 and 7). This could possibly result from biochemical differences between different preparations of mitochondria or from differences in the mitochondrial structure and integrity or from differences in coupling.

(ii) Photo-inactivation of succinate dehydrogenase (succinate-PMS reductase and succinate-menadione reductase)

Dyes such as phenazine methosulphate (PMS), methylene blue (MB), 2,6-dichlorophenolindophenol (DCPIP) and ubiquinone-dichlorophenol indophenol,etc., tap the electron transport pathways at different oxidation-

Table 5. Effect of time of illumination using violet light on succinoxidase activity of mitochondria from pigmented and non-pigmented dark-grown cultures of N.crassa

Time (min)	Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
	Control	Illuminated		Control	Illuminated	
5	.057	.046	19	.064	.050	22
10	.057	.040	30	.064	.044	31
15	.057	.035	39	.064	.038	41
20	.057	.032	44	.064	.033	49
30	.057	.030	47	.064	.030	53
45	.057	.025	56	.064	.027	58

Activities are expressed in μg atoms O_2 uptake/min/1 ml mitochondrial preparation. The mitochondria were illuminated at 10°C at a light intensity of 150 W/m^2. 1 ml sample of the mitochondrial suspension was removed at intervals of time from the illumination apparatus and assayed for activity. Preparation was used with an optical density of 0.87 (pigmented) and 0.83 (non-pigmented) corresponding to a protein content of 0.8 mg/ml and 0.75 mg/ml respectively. The values shown are representative of two experiments

reduction levels. PMS and ubiquinone-DCPIP (in the presence of Triton X 100) accept electrons at the dehydrogenase level and proved to be superior to ferricyanide, MB and DCPIP. Therefore these dye systems have been used for the assay of succinate dehydrogenase activity (the natural acceptor is presumably ubiquinone). The activity of succinate-PMS reductase of N.crassa mitochondria irradiated with violet light (150 W/m^2) for 5 minutes can be seen in Tables 6 and 7. Succinate dehydrogenase (succinate-PMS reductase) showed very small light sensitivity (about 3%) for mitochondria from both pigmented and white strains.

Menadione (Md) is a naphthoquinone which acts as an artificial electron acceptor in the succinate-menadione reductase system. The succinate-Md reductase activity was inhibited to a slightly greater extent by violet light than the PMS reductase when irradiated for 5 minutes (Tables 6 and 7). The inactivation of succinate-PMS reductase in mitochondria from both pigmented and non-pigmented dark-grown strains was similar. It can be seen that carotenoids present in the wild-type strain have shown no protection in these assays of the dehydrogenase site.

(iii) Photo-inactivation of the succinate-ubiquinone reductase

Ubiquinone was admitted to be a possible member of the respiratory chain after Crane et al (1957) isolated it as an oxidisable/reducible quinone from beef-heart mitochondria. Various studies show that ubiquinone functions at an early stage in electron transport from the dehydrogenase to oxygen. According to the oxidation reduction potential Morel et al (1961) placed ubiquinone at about the same potential level as cytochrome b.

It can be seen from the results in Tables 6 and 7 that the magnitude of depression of the succinate-ubiquinone reductase activity

Table 6. Effect of violet light (5 minutes) on dehydrogenase complexes and oxidases of mitochondria from the pigmented dark-grown strain of N.crassa

Preparation	Enzyme systems	Pigmented		Percentage inactivation
		Control	Illuminated	
1	Succinoxidase	.021	.016	24
	Succinate-PMS reductase	.032	.031	3
	Succinate-menadione reductase	.011	.010	9
	NADH oxidase	.058	.051	12
	NADH-menadione reductase	.081	.069	15
2	Succinoxidase	.020	.015	25
	Succinoxidase + cysteine (0.5 mM)	.018	.017	5
	Succinate-ubiquinone reductase	.034	.027	20

Samples of irradiated mitochondria were assayed by the methods shown in Chapter II. All values are representative of at least 4 mitochondrial preparations. Activities are expressed in μg atoms O_2 uptake/min/1 ml mitochondrial preparation except the succinate-ubiquinone reductase which is expressed as μ moles DCPIP reduced/min/1 ml mitochondria. The mitochondria were illuminated at $10^{\circ}C$ using a light intensity of 150 W/m^2

The optical density for preparation 1 was 0.4 and for preparation 2 0.45 corresponding to a protein content of about 0.5-0.55 mg/ml respectively.

Table 7. Effect of violet light (5 minutes) on dehydrogenase complexes and oxidases of mitochondria from non-pigmented dark-grown strain of N.crassa

Preparation	Enzyme system	Non-pigmented		Percentage inactivation
		Control	Illuminated	
1	Succinoxidase	.027	.019	30
	Succinate-PMS reductase	.033	.032	3
	Succinoxidase + cysteine (0.5 mM)	.027	.026	4
2	Succinoxidase	.015	.011	27
	Succinate-menadione reductase	.015	.014	7
	NADH oxidase	.035	.031	11
	NADH-menadione reductase	.035	.028	20
3	Succinoxidase	.016	.011	31
	Succinate-ubiquinone reductase	.032	.025	22
	NADH oxidase	.035	.031	11
	NADH oxidase + cysteine (0.5 mM)	.031	.031	0

Samples of irradiated mitochondria were assayed by the methods shown in Chapter II. Values are representative of at least 10 mitochondrial preparations. Activities are expressed in µg atms O_2 uptake/min/ml mitochondrial preparation except the succinate-ubiquinone reductase which is expressed as µ moles DCPIP reduced/min/1 ml mitochondria. The mitochondria were illuminated at 10°C with violet light (385-495 nm, 150 W/m^2)

The optical density for preparation 1 was 0.45, preparation 2, 0.4 and preparation 3 0.4, corresponding to a protein content of about 0.5-0.6 mg/ml protein.

was much more than that of succinate-PMS reductase and succinate-Md reductase. This suggests the possibility of another site of photo-inactivation on the electron-transport chain beyond the flavoprotein (succinate-PMS reductase) and prior to the level of ubiquinone. These results also show no appreciable difference of activities between mitochondria isolated from pigmented and white dark-grown strains.

(iv) Restoration of succinoxidase activity by cysteine

In an attempt to find another site of inactivation it was observed that cysteine could restore the depleted succinoxidase activity. This suggested that sulphydryl groups of proteins involved in the respiratory chain were also affected by illumination. Tables 6 and 7 show that in 5 minutes illumination using violet light (150 W/m^2) succinoxidase was inactivated to about 25% in pigmented and 30% in white strains. Most of the original activity was restored by the addition of freshly prepared cysteine. Therefore a sulphydryl group may be the second site of the inactivation.

(v) Inactivation of the NADH oxidase

The NADH oxidase of N.crassa mitochondria was very active. Compared with succinoxidase, it was less photosensitive when irradiated with violet light for 5 minutes (Tables 6 and 7). Its inactivation might be accounted for in the NADH dehydrogenase inactivation since the NADH-menadione reductase system was found to be more sensitive than the NADH oxidase in the pigmented and non-pigmented strain (Tables 6 and 7). The NADH oxidase activity was totally restored by the addition of freshly prepared cysteine in the non-pigmented strain (Table 7).

(vi) Summary

The results of irradiation of N.crassa mitochondria with violet light (150 W/m^2) for 5 minutes show a partial inactivation of succinoxidase of both strains. The inactivation can be accounted for

as succinate-ubiquinone reductase but is not seen in the succinate-PMS reductase. Photo-inhibition can be located between the site reducing PMS and that reducing the quinone. NADH oxidase is less photosensitive and its inactivation may be accounted for in the NADH dehydrogenase complex (NADH-menadione reductase). Repair of the photosensitive sites in succinoxidase and in NADH oxidase are almost totally achieved by cysteine treatment suggesting that photo-oxidation of an -SH group is involved and in succinate oxidation is presumably between the site reducing PMS and that reducing ubiquinone.

(c) Irradiation of mitochondria using violet light for 8.5 minutes

The succinoxidase activities of mitochondria isolated from the pigmented dark-grown strain were depressed when illuminated with violet light (150 W/m^2) for 8.5 minutes more than when illuminated for 5 minutes. Succinoxidase was inhibited by about 32-33% (Table 8). With increasing time of illumination succinate dehydrogenase (succinate-PMS reductase) photosensitivity was also increased as was the succinate-Md reductase. Similarly NADH oxidase and NADH-Md reductase inhibtion was also bigger. The NADH-ferricyanide reductase showed only slight inactivation although the NADH-Md reductase was more substantially affected (Table 8).

(d) Restoration of succinoxidase activity with sulphydryl reagents after inhibition by violet light

The essentiallity of the sulphydryl groups of succinate dehydrogenase has been reported for some bacterial systems. It has been found that there are sulphydryl groups, probably attached to non-haem iron protein (Kurup and Brodie,1967; Kaback,1972). In beef-heart mitochondria many sulphydryl groups have been shown to be associated with the purified succinate dehydrogenase (Pagani et al, 1974).

Table 8. Irradiation of mitochondria from the pigmented strain for 8.5 minutes

Preparation	Enzyme Activities	Pigmented		Percentage inactivation
		Control	Illuminated	
1	Succinoxidase	.0150	.0100	33
	NADH oxidase	.0115	.0100	13
	* NADH ferricyanide reductase	.0148	.0140	5
2	Succinoxidase	.028	.019	32
	Succinate-PMS reductase	.038	.033	13
	NADH oxidase	.021	.017	19
	NADH-menadione	.033	.028	15
3	Succinoxidase	.025	.017	32
	Succinate-menadione reductase	.006	.005	17
4	Succinoxidase	.021	.014	33
	NADH oxidase	.015	.012	20
	NADH-ferricyanide reductase	.020	.019	5

Samples of irradiated mitochondria were assayed by the methods shown in Chapter II. Activities are expressed in μg atoms O_2 uptake/min/1 ml mitochondria. All values are representative of at least 4 mitochondrial preparations. The mitochondria were illuminated at 10°C using a light intensity 150 W/m^2. For each assay 1 ml mitochondrial preparation was used.

* The activities were measured spectrophotometrically as reduction of ferricyanide at 420 nm and expressed as μmoles NADH oxidised/min/ml mitochondria.

The optical density for mitochondrial preparations was 0.3, 0.4, 0.35 and 0.35 corresponding to a protein content of about 0.35, 0.45, 0.4 and 0.4 mg/ml respectively.

(i) Effect of cysteine

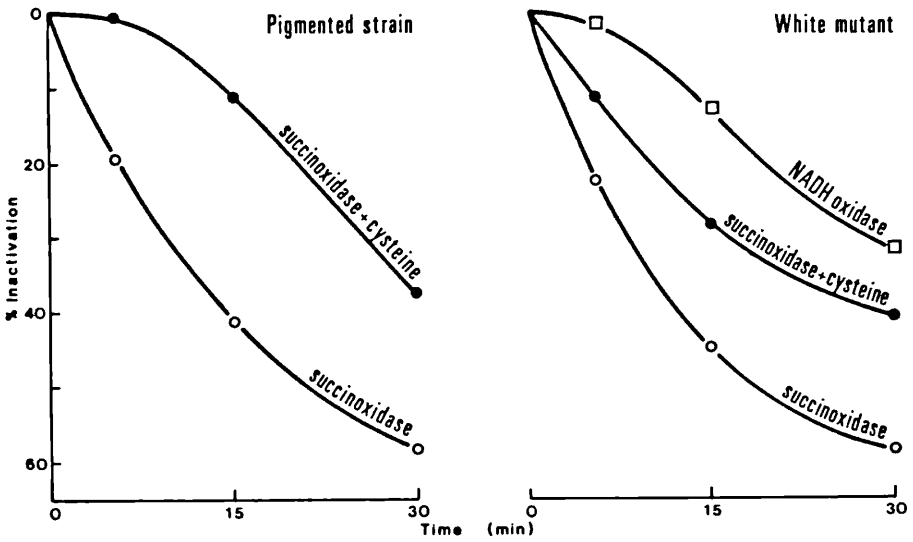
As stated earlier, the photo-inhibition of respiration depends markedly on the intensity of light and the period of illumination, that is, the dose of irradiation.

Results given in Figure 1 show that illumination with violet light (150 W/m^2) for 5 minutes produced an inactivation. Increasing the dose of irradiation to 15 and 30 minutes increased the inactivation of succinoxidase in mitochondria from the dark-grown strain. The proportion of inactivation was not repairable by cysteine rapidly increases with bigger light doses. Therefore, it seems probable that the remaining inhibition which cysteine did not repair is due to the irrepairable effect on flavoproteins or other photosensitive sites.

Similar experiments were carried out using mitochondria isolated from the dark-grown non-pigmented strain. The results in Figure 1 show that when the irradiation time using violet light (150 W/m^2) was prolonged to 15 and 30 minutes the inhibition of succinoxidase activity was increased in a similar way to the pigmented strain. Addition of cysteine could not totally restore the original activity. It seems clear that cysteine in the non-pigmented strain did not repair as much damage of succinoxidase as it did in the pigmented strain, especially after 5 and 15 minutes illumination. This may be explained by suggesting the presence of other sites of photo-inactivation which are partially protected by carotenoids in the pigmented strain.

After 30 minutes illumination the effect of cysteine was almost the same in both strains. Figure 1 also shows that NADH oxidase activity in mitochondria from the non-pigmented strain was less photosensitive than succinoxidase. Like succinoxidase, when the illumination time was prolonged to 15 and 30 minutes the activity was progressively depressed.

Figure 1



Photoinactivation of Respiration by Blue Light
(385-495 nm. 150 w/m²)

Samples of irradiated mitochondria were assayed for activity of succinoxidase, NADH oxidase and succinoxidase in the presence of 0.5 mM cysteine by the methods shown in Chapter II

(ii) Effect of other sulphydryl reagents

Other sulphydryl reagents such as dithiothreitol (DTT) and glutathione (GSH) have been tested for their ability to restore succinoxidase activity in mitochondria from pigmented and non-pigmented strains after illumination with violet light (150 W/m^2) for 10 minutes. The results in Table 9 showed that the effects of DTT and GSH resemble the effect of cysteine.

Vinogradov et al (1975) reported the irreversible inhibition of a highly reactive site by para-chloromercuribenzoic acid (PCMB). They presumed that this reactive site was responsible for electron transfer between the dehydrogenase and the respiratory quinone (iron-sulphur complex, or part of it). The effect of PCMB has been examined and it was found to inhibit the dark rate of succinoxidase more than violet light for 10 minutes (Table 9).

(e) Photosensitivity of the succinate-phenazine methosulphate reductase site

Previous experiments had shown that irradiation of N.crassa mitochondria for short times (5 minutes) using violet light (150 W/m^2) resulted in only a very small loss (about 3%) of succinate-PMS reductase activity in both strains whereas after 8.5 minutes irradiation, the photo-inhibition increased to 13% in the pigmented strain.

Furthermore, these investigations indicated that the photo-inactivation of succinate dehydrogenase increased with bigger irradiation doses. Illumination of mitochondria from the pigmented strain with violet light for 15-30 minutes caused the succinate-PMS reductase activity to decline by 14 and 26% respectively (Table 10). An almost similar loss of activity was observed in succinate-PMS reductase of mitochondria from the non-pigmented strains (Table 11).

Table 9. Restoration of succinoxidase activity by sulphydryl reagents after photo-inactivation by violet light

Enzyme systems	Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
	Control	Illuminated		Control	Illuminated	
Succinoxidase	.013	.009	31	.015	.010	33
Succinoxidase + cysteine (0.5 mM)	.012	.011	8	.015	.014	7
Succinoxidase + DTT (0.25 mM)	.013	.0123	5	.015	.014	7
Succinoxidase + GHS (0.125 mM)	.012	.011	8	.014	.012	14
Succinoxidase + PCMB (0.15 mM)	.0083	-	36	.0095	-	37

Values are representative of at least 2 mitochondrial preparations. Activities expressed in µg atoms O_2 uptake/min/mg protein. Optical density for samples: pigmented = 0.11, non-pigmented = 0.125

The mitochondria were illuminated at $10^{o}C$ with light intensity of 150 W/m^2 for 10 minutes.

The assay mixture contained 0.5 ml succinate 0.1 M in buffer and 1 ml mitochondrial preparation

Thus the presence of carotenoids did not protect the dehydrogenase against the photo-inactivation in agreement with comparable studies of a flavoprotein malate dehydrogenase in the bacterium Sarcina lutea by Anwar (1975).

(f) Restoration of succinoxidase activity by cysteine and ubiquinone

Earlier work showed that violet light has an inhibitory effect on the electron transport chain of N.crassa mitochondria. The results in Tables 12 and 13 show that the respiratory chain beyond cytochrome c towards oxygen is not affected by violet light and that the light inhibition must lie on substrate side of the cytochrome c.

Furthermore, having found that the loss of activity in succinoxidase was bigger than the loss of activity in succinate-ubiquinone reductase (Table 7), it seemed possible that ubiquinone could be a target for photo-inactivation.

It was of interested, therefore, to determine whether ubiquinone (Coenzyme Q_{10}) was being destroyed by violet light in N.crassa. Brodie and Ballantine (1960) found that 360 nm light destroyed menaquinone in M.phlei which was evidenced by the loss of respiratory activity and oxidative phosphorylation.

Prebble et al (1976) have reported that quinone (menaquinone) was the third photosensitive site in the respiratory chain of Sarcina lutea using violet light and it also protected by the presence of carotenoid.

Phillips et al (1970) could achieve restoration of respiration in Mycobacterium phlei after irradiation with 360 nm light by the addition of vitamin K and quinones which structurally resemble vitamin K, the respiratory quinone in this organism. However, Lester and Crane (1959) reported that N.crassa mitochondria possesses CoQ_{10} ubiquinone.

It was of interest, therefore, to determine whether ubiquinone (CoQ_{10}) was being destroyed by violet light in N.crassa. As shown

Table 10. Effect of violet light on the oxidation of succinate and NADH by mitochondria from the pigmented dark-grown strain of N.crassa

Enzyme systems	Percentage inactivation			
	15 min	20 min	30 min	45 min
Succinoxidase	33	35	44	51
Succinate-PMS reductase	14	-	26	-
Succinoxidase + cysteine (0.5 mM)	12	17	27	-
Succinoxidase + cysteine (0.5 mM) + ubiquinone (50 µg/ml)	8	11	18	-
NADH oxidase	19	-	27	40
*NADH-ferricyanide reductase	11	-	-	-

All values are representative of at least 4 mitochondrial preparations. The mitochondria were illuminated at 10°C using violet light (150 W/m^2). Activities are expressed in µg atoms O_2 uptake/min/1 ml mitochondrial preparation

* The activities were measured spectrophotometrically as the reduction of ferricyanide at 420 nm and expressed as µmoles NADH oxidised/min/ml mitochondrial preparation.

The optical density of the preparation was 0.15 corresponding to a protein content of about 0.3 mg/ml

Table 11. Effect of violet light on the oxidation of succinate and NADH by mitochondria from the non-pigmented dark-grown strain of N.crassa

Enzyme systems	Percentage inactivation			
	15 min	20 min	30 min	45 min
Succinoxidase	38	40	53	67
Succinate-PMS reductase	16	-	28	-
Succinoxidase + cysteine (0.5 mM)	16	20	30	45
Succinoxidase + cysteine (0.5 mM) + ubiquinone (50 µg/ml	10	12	9	25
NADH oxidase	19	-	30	47
*NADH-ferricyanide reductase	11	-	-	-

All values are representative of at least 4 mitochondrial preparations. The mitochondria were illuminated at 10°C using violet light (150 W/m^2). Activities are expressed as µg atoms O_2 uptake/min/1 ml mitochondria

* The activities were measured spectrophotometrically as reduction of ferricyanide at 420 nm and expressed as µmoles NADH oxidised/min/ml mitochondrial preparation.

The optical density of the preparation was 0.13 corresponding to a protein content of about 0.25 mg/ml.

above the results in Table 10 indicate that cysteine could not repair the loss in succinoxidase activity with a prolonged illumination time up to 30 minutes using violet light. Addition of CoQ_{10} plus cysteine to a pigmented preparation irradiated for 15 minutes was found to restore succinoxidase activity to some extent. Further, the loss of activity due to longer irradiation up to 20 or 30 minutes could be partially restored by adding CoQ_{10} plus cysteine (Table 10).

Furthermore, in mitochondria from the non-pigmented strain, the restoration of succinoxidase activity by cysteine is slightly different compared to the pigmented strain especially at 15 minutes (Table 11). Increasing the illumination time up to 45 minutes increased the proportion of inactivation which cysteine could not repair. The degree of restoration of activity after addition of CoQ_{10} was achieved in the white mutant. It restored relatively more of the succinoxidase activity. Cysteine plus CoQ_{10} was less efficient in restoring lost activity in mitochondria irradiated for 45 minutes (Table 11). The reason why cysteine did not repair succinoxidase activity totally after longer illumination times could be due to inactivation of succinate-PMS reductase and CoQ_{10} destruction.

From the results above, it may be concluded that even in the pigmented strain, illumination with violet light destroyed CoQ_{10} sufficiently to affect electron transport and therefore activity was restored by addition of the quinone. The same illumination doses applied to the white mutant destroyed CoQ_{10} more effectively and the addition of ubiquinone partially restored a greater proportion of the succinoxidase activity. Since the addition of CoQ_{10} gave a partial restoration of the lost activity, ubiquinone must be a site of photo-inactivation in the N. crassa respiratory chain. The residual inactivation

of succinoxidase after treatment with cysteine and ubiquinone may be attributed to photosensitivity of the succinate-PMS site.

(g) NADH dehydrogenase photosensitivity

NADH dehydrogenase is the primary dehydrogenase which accepts electrons directly from NADH and passes them through the electron transport chain. Artificial electron carriers such as ferricyanide were used to measure its activity.

The natural electron acceptor is Coenzyme Q. As shown earlier, when mitochondria from both strains are exposed to violet light for various periods, there was a progressive inactivation of NADH oxidase with time (Figure 1).

The results obtained in Table 10 show that up to 19% of the NADH oxidase activity was lost after 15 minutes illumination with violet light while NADH dehydrogenase (assayed as NADH-ferricyanide reductase) was inactivated by 11%. Similar results were obtained with mitochondria from the white mutant (Table 11).

Although both the succinate and NAD linked pathways were labile to irradiation, the succinoxidase pathway was more sensitive than the NADH pathway.

(h) Photosensitivity of cytochrome oxidase

Cytochrome oxidase is the enzyme which catalyses the terminal oxidation of reduced cytochrome by oxygen. The photosensitivity of cytochrome oxidase has been demonstrated in several eukaryotes (Epel and Butler,1970). Ninnemann et al (1970a) and Ninnemann (1974) reported an effect of visible light on the cytochrome oxidase in beef-heart mitochondria. However, Anwar (1975) did not find an effect of visible light on cytochrome oxidase in the prokaryote S.lutea despite the presence in this organism of cytochrome a_3 (Erickson and Parker,1969), the photosensitive site in the oxidase

of eukaryotes.

In studies of the respiratory chain, artificial electron donors have long been used. By this means segments of the respiratory chain can be examined. For studies of the effect of light on the segment between cytochrome c and oxygen, tetramethyl-para-phenylene diamine (TMPD) was introduced as a mediator of electrons between ascorbate as electron donor and cytochrome c/cytochrome oxidase.

Several experiments were performed to examine the effect of violet light on the photosensitivity of the cytochrome oxidase using two methods of assay. The rate of oxidation of ascorbate-TMPD was unaffected by violet light in mitochondria from both the pigmented and non-pigmented dark-grown strains (Tables 12 and 13). Even with prolonged illumination up to 30 minutes, the percentage photo-inhibition of cytochrome oxidase (as ascorbate-TMPD oxidase) was zero. However, the experiments with TMPD were not carried further because of the relatively high level of auto-oxidation of ascorbate-TMPD. With an electron donor system consisting of cytochrome c and ascorbate the oxidase was only slightly inhibited with violet light, even when illuminated for up to 45 minutes. No appreciable differences between mitochondria from pigmented and white mutant strains were observed (Tables 12 and 13).

Mammalian cytochrome c was used in these experiments. The failure to find a significantly large effect of light on the N.crassa cytochrome oxidase without added sensitiser agrees with studies on S.lutea where the oxidase was found to be insensitive to light (Anwar,1975).

Cytochrome oxidase exists as a tightly bound form with lipid in the mitochondria. West (1968) found that lipoxygenase could promote the photosensitivity of cauliflower bud mitochondria. This lipid-oxidising enzyme was also added to Neurospora mitochondria. This treatment

Table 12. Effect of different doses of violet light on cytochrome oxidase activity of mitochondria from the pigmented dark-grown strain of N.crassa

Enzyme systems	Percentage inactivation			
	15 min	20 min	30 min	45 min
Succinoxidase	26	35	41	49
Ascorbate-TMPD oxidase	0	-	0	-
Ascorbate-cytochrome c oxidase	-	7	7	8
Ascorbate-cytochrome c oxidase + lipoxygenase*	-	23	29	31

Values represent at least 4 mitochondrial preparations. Activities are expressed in μg atoms O_2 uptake/min/1 ml mitochondrial preparation. The mitochondria were illuminated at 10°C using violet light (150 W/m^2). Samples were removed at intervals of time for enzyme assay by methods described in Chapter II. For the assay mixtures 1 ml mitochondrial preparation was used. The optical density for the preparation was 0.15 which corresponds to a protein content of about 0.3 mg/ml.

* The mitochondrial suspension was treated with lipoxygenase (crystalline enzyme 0.3 mg/ml) for 2.5 h before irradiation with violet light.

Table 13. Effect of different doses of violet light on cytochrome oxidase activity of mitochondria from the non-pigmented dark-grown strain of N.crassa

Enzyme systems	Percentage inactivation			
	15 min	20 min	30 min	45 min
Succinoxidase	31	40	59	67
Ascorbate-TMPD oxidase	0	-	0	-
Ascorbate-cytochrome c oxidase	-	5	7	8
Ascorbate-cytochrome c oxidase + lipoxygenase*	-	28	31	-

All values represent at least 4 mitochondrial preparations. Activites are expressed in μg atoms O_2 uptake/min/1 ml mitochondrial preparation. The mitochondria were illuminated at 10°C using violet light (150 W/m^2). Samples were removed at intervals of time for enzyme assay by methods described in Chapter II. For the assay mixtures 1 ml mitochondrial preparation was used. Optical density for the preparation was 0.15 which corresponds to a protein content of about 0.3 mg/ml.

* The mitochondrial suspension was treated with lipoxygenase (crystalline enzyme, 0.3 mg/ml) for 2.5 h before irradiation with violet light

rendered the oxidase photosensitive. Results obtained in Tables 12 and 13 show that when the oxidase was incubated with lipoxygenase substantial amounts of inactivation were observed. Up to 31% inhibition was found after treatment with lipoxygenase when the illumination was continued for 45 minutes in the pigmented strain (Table 12). Almost similar results were obtained for mitochondria from the white strain (Table 13). Thus there are no great differences between the white and pigmented strains.

4. EFFECT OF 460 nm LIGHT ON THE RESPIRATORY ACTIVITY IN MITOCHONDRIA

As shown above, there are several possible sites for violet light (385-495 nm, 150 W/m^2) damage in the respiratory chain of the N.crassa mitochondria, including one in the flavoprotein region, a sulphydryl group associated with the succinate-ubiquinone reductase and ubiquinone.

In bacteria (S.lutea) our laboratory has found that the photosensitivity of a flavoprotein dehydrogenase (assayed with PMS) decreases rapidly with the increase in incident wavelength above 440 nm. Since the flavoproteins absorb light in the near ultra-violet and blue regions of the spectrum, it was presumed that when the 460 light was used the flavoproteins would be less affected and the major photosensitive site after cysteine repair might be only ubiquinone. At the same time, use of near ultra-violet light, which is known to excite ubiquinone strongly would be avoided. Therefore, a 460 $\pm$ 5 nm filter (giving 15 W/m^2 incident energy) was chosen and the effect of these wavelengths on succinoxidase and NADH oxidase were examined.

Succinoxidase in mitochondria from the pigmented strain irradiated with 460 nm light for 35 up to 85 minutes, declined in activity by up to 24% (Table 14). It was concluded that with diminished incident energy a duration of illumination of 85 minutes gave a measurable

Table 14. Effect of 460 nm light on succinoxidase activity in mitochondria from pigmented N.crassa strain

Irradiation time (min)	Pigmented		Percentage inactivation
	Control	Illuminated	
35	.021	.0190	10
55	.021	.0180	14
70	.021	.0170	19
85	.021	.0160	24

Activities expressed in μg atoms O_2 uptake/min/mg protein

The mitochondria were illuminated at 10°C with 460 $\pm$ 5 nm light, 15 W/m^2. At intervals of time samples were removed for enzyme assay

Table 15. Inactivation of NADH oxidase and NADH dehydrogenase by 460 nm light in *N. crassa* mitochondria from dark-grown cultures

		Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
		Control	Illuminated		Control	Illuminated	
*	NADH oxidase	0.040	0.035	12	0.062	0.055	11
**	NADH-ferricyanide reductase	0.017	0.017	0	0.02	0.02	0

The mitochondria were illuminated at 10°C using 460 ± 5 nm light of 15 W/m^2 for 85 minutes.

After irradiation samples were removed for assay. The values shown are representative of two preparations

* The activities are expressed as μ moles NADH oxidised/min/mg protein (measured spectrophotometrically at 340 nm)

** The activities are expressed as μ moles NADH oxidised/min/mg protein (measured spectrophotometrically as ferricyanide reduction at 420 nm)

inactivation in succinoxidase, useful for further studies.

The same dose of irradiation used with NADH oxidase produced 12 to 11% inhibition in pigmented and non-pigmented preparations (Table 15). The inactivation of NADH dehydrogenase as assayed as NADH-ferricyanide reductase was zero for both cultures (Table 15). Thus NADH oxidase was less sensitive than succinoxidase when 460 nm light was used; NADH dehydrogenase appeared insensitive.

(a) Ubiquinone and restoration of succinoxidase activity

Results obtained in Table 16 indicated that irradiation of mitochondrial suspensions from both pigmented and white dark-grown cultures with 460 nm light led to an inactivation of the succinoxidase which was not completely reversed by cysteine. Addition of ubiquinone plus cysteine substantially reduced the photo-inhibition of succinoxidase, suggesting that ubiquinone was inactivated by 460 nm light.

When both strains were grown in the light (resulting in a high level of carotenoids in the wild-type) irradiation of mitochondria resulted in a smaller inhibition of succinoxidase in the wild-type, which was reversed by the addition of cysteine (Table 17). However, the restoration of succinoxidase activity in the white mutant was different. The loss in succinoxidase activity after adding cysteine decreased to 13% which recovered further to 4% when ubiquinone plus cysteine was added.

This suggests that the carotenoid pigments were associated with the protection of the ubiquinone in the wild-type strain grown in the light.

(b) Restoration of NADH oxidase activity by ubiquinone and cysteine after photo-inactivation by 460 nm light

Irradiation of mitochondria from the pigmented dark-grown strain with 460 nm light for 85 minutes resulted in a loss of about 17% of

Table 16. Effect of 460 nm light on respiratory activities in mitochondria from pigmented and non-pigmented dark-grown N.crassa

Enzyme systems	Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
	Control	Illuminated		Control	Illuminated	
Succinoxidase	.025	.019	24	.022	.016	27
Succinoxidase + cysteine (0.5 mM)	.025	.021	16	.021	.018	14
Succinoxidase + cysteine (0.5 mM) + CoQ_{10} (50 µg/ml)	.025	.023	8	.021	.020	5
NADH oxidase	.059	.049	17			
NADH oxidase + cysteine (0.5 mM)	.057	.052	9			
NADH oxidase + cysteine (0.5 mM) + CoQ_{10} (50 µg/ml)	.056	.054	4			

Activities expressed in µg atoms O_2 uptake/min/mg protein. Values are representative of at least 6 mitochondrial preparations. The mitochondria were illuminated at 10°C with 460 ± 5 nm light (15 W/m^2) for 85 minutes. Samples were removed for enzyme assay by the methods shown in Chapter II

Table 17. Effect of 460 nm light on succinoxidase activity in mitochondria from pigmented and non-pigmented light-grown N.crassa strains

Enzyme systems	*Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
	Control	Illuminated		Control	Illuminated	
Succinoxidase	.018	.0152	15	.023	.016	30
Succinoxidase + cysteine (0.5 mM)	.018	.018	0	.023	.020	13
Succinoxidase + cysteine (0.5 mM) + CoQ_{10} (50 µg/ml)	.018	.018	0	.023	.022	4

Activities expressed in µg atoms O_2 uptake/min/mg protein. Values are representative of at least 6 mitochondrial preparations. The mitochondria were illuminated at 10°C with 460 ± 5 nm light (15 W/m^2) for 85 minutes. Samples were removed for enzyme assay by the methods shown in Chapter II.

* The control carotenoid content was 471.7 µg/g dry weight mycelium while the carotenoid content after illumination was 223 µg/g dry weight mycelium

the NADH oxidase activity (Table 16). This photo-inactivation in the NAD^+ linked pathway was less than that in succinoxidase pathway. However, treatment with cysteine led to a decrease in the inhibition to 9%. Addition of CoQ_{10} plus cysteine restored the lost activity further (Table 16). Thus the NAD respiratory pathway also shows photosensitive sites which are restorable by cysteine and ubiquinone addition.

5. SUMMARY OF STUDIES ON PHOTOSENSITIVITY OF RESPIRATION

The growth rate of the N.crassa mycelium on agar is not significantly affected by light in either strain, whereas irradiation of young hyphae suspension shows an inhibitory effect on respiration. The respiratory chain of isolated mitochondria showed photosensitivity in several sites using violet light (150 W/m^2).

The succinoxidase pathway was more labile to irradiation than the NADH pathway. Reversal of photo-inhibition by cysteine suggests that photo-oxidation of a sulphydryl group was involved in both pathways.

Succinate-PMS reductase, succinate-Md reductase and NADH-ferricyanide reductase showed photosensitivity when longer irradiation times were used.

Cytochrome oxidase was only slightly photosensitive even with larger doses of irradiation. A brief treatment with lipoxygenase rendered the oxidase photosensitive.

After irradiation of dark-grown preparations of mitochondria with 460 nm light, succinoxidase and NADH oxidase was not completely repaired by cysteine. Addition of ubiquinone plus cysteine, very substantially reduced the photosensitivity of the succinoxidase and NADH oxidase, suggesting that ubiquinone is photo-inactivated. The complete restoration of activity by cysteine in light-grown preparations

suggests protection of ubiquinone by carotenoid.

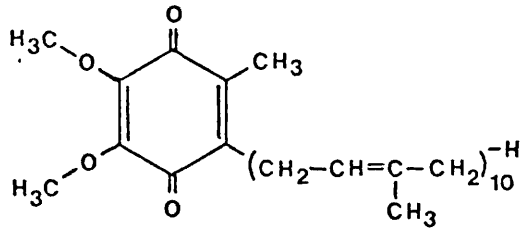
6. DESTRUCTION OF MITOCHONDRIAL UBIQUINONE BY LIGHT AND ITS PROTECTION BY CAROTENOIDS

In earlier experiments it was observed that the presence of carotenoids afforded some protection against photo-oxidation in mitochondria from the wild-type light-grown strain. A site beyond the flavoprotein dehydrogenase was inhibited by 460 nm light in the white strain, and protected in strongly pigmented preparations. Evidence presented earlier suggests that this site is the respiratory ubiquinone.

Therefore mitochondrial extracts have been estimated for ubiquinone, ergosterol and carotenoid content before and after illumination.

The ubiquinones are lipid-soluble substituted benzoquinones which yield ubiquinols on reduction with borohydride. In many tissues the ubiquinone has 10-isoprenoid-unit side chains and this ubiquinone is found in a wide range of vertebrate and invertebrate species (Lester and Crane,1959).

The structure of CoQ_{10} is



Ubiquinone has an absorption spectrum with a maximum of 275 nm in ethanol. The ubiquinol shows a much reduced absorption at 275 nm and a maximum at 290 nm. Using the molar extinction coefficient for the difference in absorption of the oxidised and reduced forms, the

ubiquinone concentration may be estimated. The carotenoid pigment was estimated from the absorption of the extract at 477 nm. The main pigment has been identified as neurosporaxanthin on the basis of its absorption spectrum maximum at 477 nm. It is the main acidic carotenoid in N.crassa (Neupert and Ludwig,1971; Harding et al,1969). The ergosterol content was calculated from the U.V.spectrum using the extinction at 282 nm (Hallermayer and Neupert,1974). In Sarcina lutea the protective action of the carotenoid pigments was effective only when bacteria were exposed to visible light and this protective mechanism was ineffective at shorter wavelengths (Mathews and Krinsky, 1965). Furthermore, Sargent and Briggs (1976) did not observe any difference in the sensitivity of N.crassa wild-type and albino conidia to ultra-violet radiation. Therefore, tests were made with light 460 ± 5 nm (15 W/m^2). However, it was found that the preparation required illumination for a long time (110 minutes) to get a measurable inactivation in the respiratory chain (Table 18).

The percentage of photodestruction of ubiquinone in the pigmented culture illuminated with 460 nm light was about 2%, while the same illumination dose caused about 21% destruction of ubiquinone in the white mutant grown under the same conditions. The level of ergosterol was diminished by about 10% in both cultures, whereas the carotenoid levels in pigmented culture decreased by about 25% due to illumination.

To economise in time an OY13 orthochromatic colour-glass filter was used, giving zero transmission at 400 nm, significant transmission at 440 nm, and a peak transmission of 75% from 500 nm, this filter cuts out the ultra-violet and violet regions of white light. With this filter only 40 minutes illumination was used.

Results presented in Table 19 show that measurement of ubiquinone levels in mitochondria isolated from N.crassa pigmented and white

Table 18. Effect of 460 nm light on extracted ubiquinone, ergosterol and carotenoid of mitochondria from dark-grown N.crassa cultures

	Control	Illuminated	Percentage inactivation
Pigmented			
Ubiquinone (μ moles/g protein)	1.79	1.76	2
Ergosterol (μ moles/g protein)	27.1	24.5	10
Carotenoid (μg/g protein)	55.8	41.8	25
Non-pigmented			
Ubiquinone (μ moles/g protein)	1.14	0.90	21
Ergosterol (μ moles/g protein)	10.7	9.6	10

The ubiquinone, ergosterol and carotenoid were extracted from mitochondria after illumination at $10^{o}C$ with 460 $\pm$ 5 nm light (15 W/m^2) for 110 minutes. Both control and illuminated samples were treated identically. The quinone, ergosterol and carotenoid were estimated spectrophotometrically (see Chapter II)

dark-grown cultures illuminated for 40 minutes with yellow light (OY13 filter) resulted in a loss of borohydride-reducible quinone. This light treatment also reduced carotenoid levels by about 50%. The level of ergosterol was unaffected by the light treatment. However, it can be seen from these results that the photo-destruction of quinone in the pigmented strain was less than in the white mutant, even though grown in the dark to minimise the amount of carotenoid in the pigmented strain.

Similar experiments were carried out using the wild-type and white strains grown in the light (resulting in high carotenoid levels in the wild-type). It was found that the high concentration of carotenoid protected ubiquinone from photo-destruction by yellow light (Table 20). The percentage of photodestruction for ubiquinone in the pigmented light-grown strain was about 11%, whereas the same illumination dose caused about 41% destruction of ubiquinone in the white mutant grown under the same conditions. The relationship between the concentration of the mitochondrial carotenoid and the percentage photodestruction of ubiquinone by yellow light (440 + nm) is shown in Figure 2. The percentage photodestruction of extracted quinone after illumination for 40 minutes was plotted against the mitochondrial carotenoid content for different preparations using pigmented and white mutant strains grown in the dark and in the light, under varying conditions of aeration. Each point represents one mitochondrial preparation. The relationship obtained shows that when the carotenoid level was zero (white mutant preparations), the photo-destruction of ubiquinone was about 49%. In the wild-type (987) preparation grown in the light, where the carotenoid concentration was above 250 µg/g protein, the ubiquinone destruction was only about 6 to 10%. Thus, this demonstrates that the greater the carotenoid

Table 19. Effect of yellow light (440 + nm) on the ubiquinone, ergosterol and carotenoid content of mitochondria from dark-grown cultures of N.crassa

	Control	Illuminated	Percentage inactivation
Pigmented			
Concentration of extracted ubiquinone (μ moles/g protein)	2.0	1.4	30
Concentration of extracted ergosterol (μ moles/g protein)	28.5	28.6	0
Concentration of extracted carotenoid (μg/g protein)	96	46	52
Non-pigmented			
Ubiquinone (μ moles/g protein)	1.6	0.82	48
Ergosterol (μ moles/g protein)	22.0	23	0

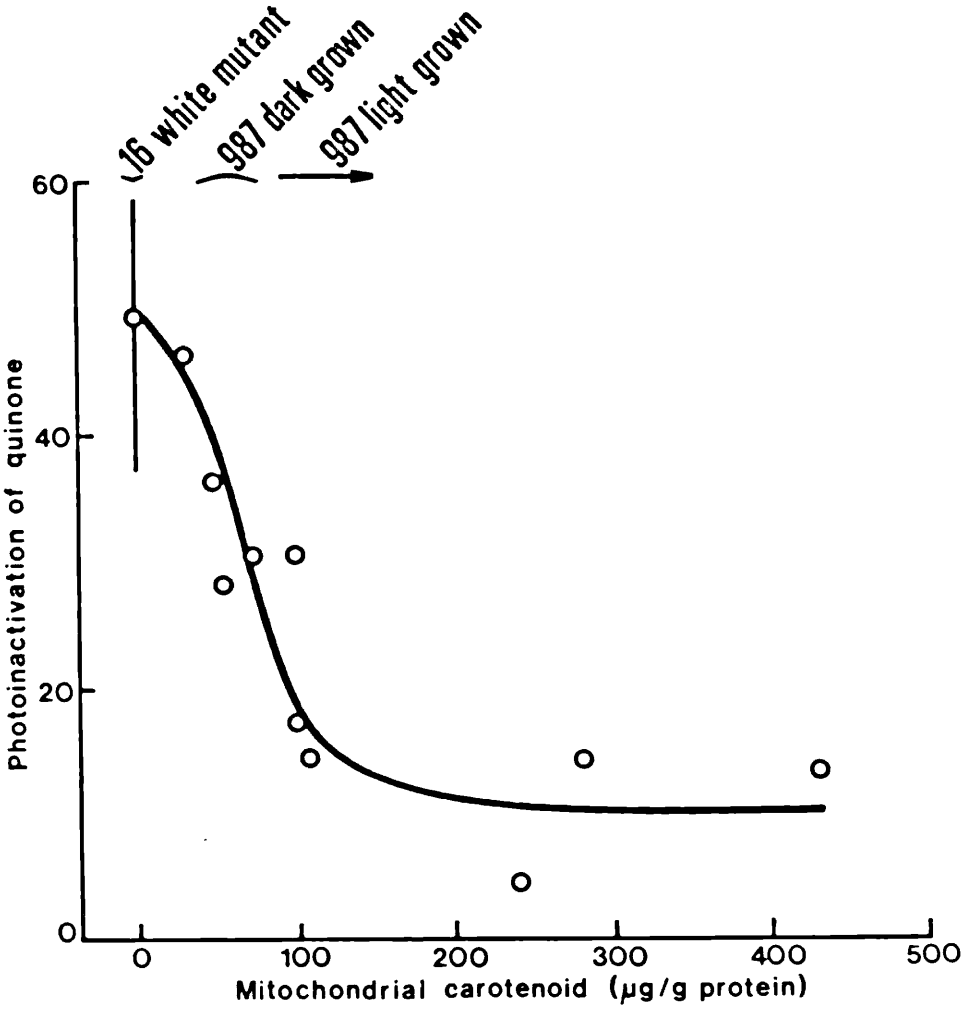
The ubiquinone, ergosterol and carotenoid were extracted from mitochondria after illumination at 10°C with OY13 filter (532 W/m^2) for 40 minutes and estimated spectrophotometrically as described in Chapter II

Table 20. Effect of yellow light (440 + nm) on ubiquinone, ergosterol and carotenoid of mitochondria from light-grown N.crassa cultures

	Control	Illuminated	Percentage inactivation
Pigmented			
Ubiquinone (μ moles/g protein)	1.68	1.50	11
Ergosterol (μ moles/g protein)	54.0	53.5	1
Carotenoid (μg/g protein)	429.0	174.0	59
Non-pigmented			
Ubiquinone (μ moles/g protein)	2.2	1.3	41
Ergosterol (μ moles/g protein)	77.0	76.0	1

The ubiquinone, ergosterol and carotenoid were extracted from mitochondria after illumination at $10^{o}C$ with an OY13 filter (440 + nm) (532 W/m^2) for 40 minutes and estimated spectrophotometrically as described in Chapter II

Figure 2



Destruction of Ubiquinone by Blue Light

Destruction of membrane bound ubiquinone by yellow light

Mitochondria were illuminated with yellow light (OY 13 filter, 440 + nm, 532 W/m^2) for 40 minutes. Carotenoid and ubiquinone were estimated before irradiation and after irradiation by the methods described in Chapter II. Each point represents an independent mitochondrial preparation. The ubiquinone levels were corrected for losses by assuming that the ergosterol levels in control and irradiated preparations should be identical. A standard deviation for the photo-destruction of quinone in mitochondria from the white mutant (FGSC 16) is also shown.

content of mitochondria up to about 120 µg carotenoid/g protein, the smaller the photodestruction of ubiquinone.

7. THE EFFECT OF LIGHT ON PURE UBIQUINONE

A considerable number of natural products are found to be effective sensitisers for photo-oxygenation reactions involving singlet oxygen in biological systems. Some typical examples are chlorophyll, riboflavin, porphyrins (e.g.protoporphyrin) and vitamin A.

Ubiquinone may absorb light itself or be sensitised by other components in the respiratory chain or on the membrane. Therefore the photosensitivity of pure ubiquinone was tested and compared with that of ubiquinone bound in the mitochondrion.

Thus, pure ubiquinone in ethanol was subjected to 460 ± 5 nm light (15 W/m^2) for various periods of time. The results in Table 21 show a steady loss of ubiquinone levels with increasing illumination times up to 100 minutes. However, the loss was comparatively small.

The results in Table 22 demonstrate that illumination using violet light (150 W/m^2) causes a substantial loss in the level of pure ubiquinone, even when the illumination time was not more than 30 minutes.

The results in Table 23 show that the destruction of pure ubiquinone in ethanol illuminated with yellow (440 + nm) light (532 W/m^2) is less than when irradiated with violet light. The destruction of ubiquinone is about 29% with an illumination time of 40 minutes. From these results and the studies on the photosensitivity of respiratory membrane-bound ubiquinone, it appears that pure ubiquinone in ethanol is less photosensitive than the respiratory ubiquinone estimated as an electron carrier in the respiratory chain or by extraction from the mitochondria. There could be many reasons for this difference such as the nature of the solvent, the differences

Table 21. Effect of 460 nm light on ubiquinone in ethanol

Time of illumination (min)	Ubiquinone (Q_{10}) (µg/ml ethanol)	Percentage destruction
Control	2.45	-
30	2.33	4
40	2.30	6
60	2.27	7
80	2.23	8
100	2.22	9

Pure ubiquinone in ethanol was illuminated at 10°C with 460 ± 5 nm with light (intensity = 15 W/m^2) and the quinone estimated spectrophotometrically as described in Chapter II

Table 22. Effect of violet light (385-495 nm) on ubiquinone in ethanol

Time of illumination (min)	Ubiquinone (Q_{10}) (µg/ml ethanol)	Percentage destruction
Control	2.5	-
5	2.28	9
10	2.08	16
15	2.00	20
20	1.72	31
30	1.48	41

Pure ubiquinone in ethanol was illuminated at 10°C with violet light (385-495 nm, intensity = 150 W/m^2) and the quinone estimated spectrophotometrically as described in Chapter II

Table 23. Effect of yellow light (440 + nm) on ubiquinone in ethanol

Time of illumination (min)	Ubiquinone (Q_{10}) (µg/ml ethanol)	Percentage destruction
Control	1.15	0
10	1.02	11
20	0.97	16
30	0.87	24
40	0.82	29

Pure ubiquinone in ethanol was illuminated at 10°C with a light intensity of 352 W/m^2 (440 + nm) and the quinone estimated spectrophotometrically as described in Chapter II

in concentration (the pure ubiquinone in ethanol was probably more dilute than that in the membrane), or the presence of an additional photosensitiser in the mitochondrial membrane.

8. The Location of Carotenoid in Relation to the Sensitive Site of the Neurospora Respiratory Chain

It has been shown that ubiquinone can be the cellular site of lethal photo-inactivation by visible light, and that the destruction of ubiquinone can be prevented by the presence of carotenoid pigments in light-grown cultures of N.crassa. However, it is believed that the location of carotenoids in relation to the photosensitive site is important in protection against photodynamic action (Mathews-Roth 1967). Neupert and Ludwig (1971) and Hallermayer and Neupert (1974) found the presence of substantial amounts of carotenoid only in the outer mitochondrial membrane of Neurospora mitochondria.

In contrast, the ubiquinone is found exclusively in the inner mitochondrial membrane together with all other components of the respiratory chain (Sottocasa and Sandri,1968).

Furthermore, as shown by Harding et al (1969), Rau et al (1968) and Rau (1969), the formation of carotenoid pigments in N.crassa is dependent on light and oxygen. So, if the production of carotenoids exerts a protective function against endogenous photosensitisers (Mathews and Sistrom,1960; Mathews,1963) the following question can be posed. Are the newly synthesised carotenoids (after photo-induction) deposited on the outer mitochondrial membrane, or are they added to the inner membrane to be close to the sensitive site in order to protect against photo-oxidation? Therefore, N.crassa mitochondrial outer membranes were separated from the inner membranes to determine whether there were any changes in the intramitochondrial distribution of the pigments as a result of photo-induction of carotenoid synthesis.

The separation of the two membranes was carried out using a modified combined technique of swelling, shrinking and sonication, followed by centrifugation through a discontinuous sucrose density gradient, first applied to Neurospora mitochondria by Cassady and Wagner (1968). The gradient was divided into two fractions. Fraction I (top of gradient) (outer membrane), and Fraction II (lower part of the gradient) (inner membrane) were collected for enzyme assay and analysis. Kynurenine hydroxylase, succinate dehydrogenase and ascorbate-cytochrome c oxidase were measured as described in Chapter II. The final pellets of fraction I and II were extracted and assayed for carotenoid, ergosterol and ubiquinone.

The data for the separation of the inner and outer membranes using mitochondria from dark-grown and light-grown strains are graphically illustrated in Figure 3. It should be noted that the amount of material recovered in both fractions depends critically upon the time of sonication (sonication intensity). The contamination of the outer membrane fraction by inner membrane was calculated using the equation given in Chapter II.

The results seen in Figure 3 indicate that kynurenine hydroxylase activity which serves as an outer membrane marker (Cassady and Wagner, 1968) was concentrated in Fraction I (outer membrane) and was low in Fraction II (inner membrane) in both pigmented light and dark-grown strains.

In contrast to kynurenine hydroxylase activity, succinate dehydrogenase which is known to be tightly bound to the inner membrane was concentrated in Fraction II, and was low in Fraction I in both strains. A similar distribution was obtained for ascorbate-cytochrome c oxidase activity (Table 24). Figure 3 clearly shows that ubiquinone, also an inner membrane marker, displays the same distribution pattern

as succinate dehydrogenase (succinate-PMS reductase), which was completely different from that of kynurenine hydroxylase.

It can be concluded from these data that since ubiquinone is localised primarily in the inner mitochondrial membrane fraction together with the components of the respiratory chain, such as succinate dehydrogenase, the inner membrane has been separated from the outer membrane to a very considerable degree.

The carotenoid distribution between inner and outer mitochondrial membranes has been tested, by extraction of the pellets obtained from Fractions I and II. Fraction I at the top of the gradient was red orange whereas Fraction II, the lower, was dark brown whether the original cultures were grown in the light or in the dark.

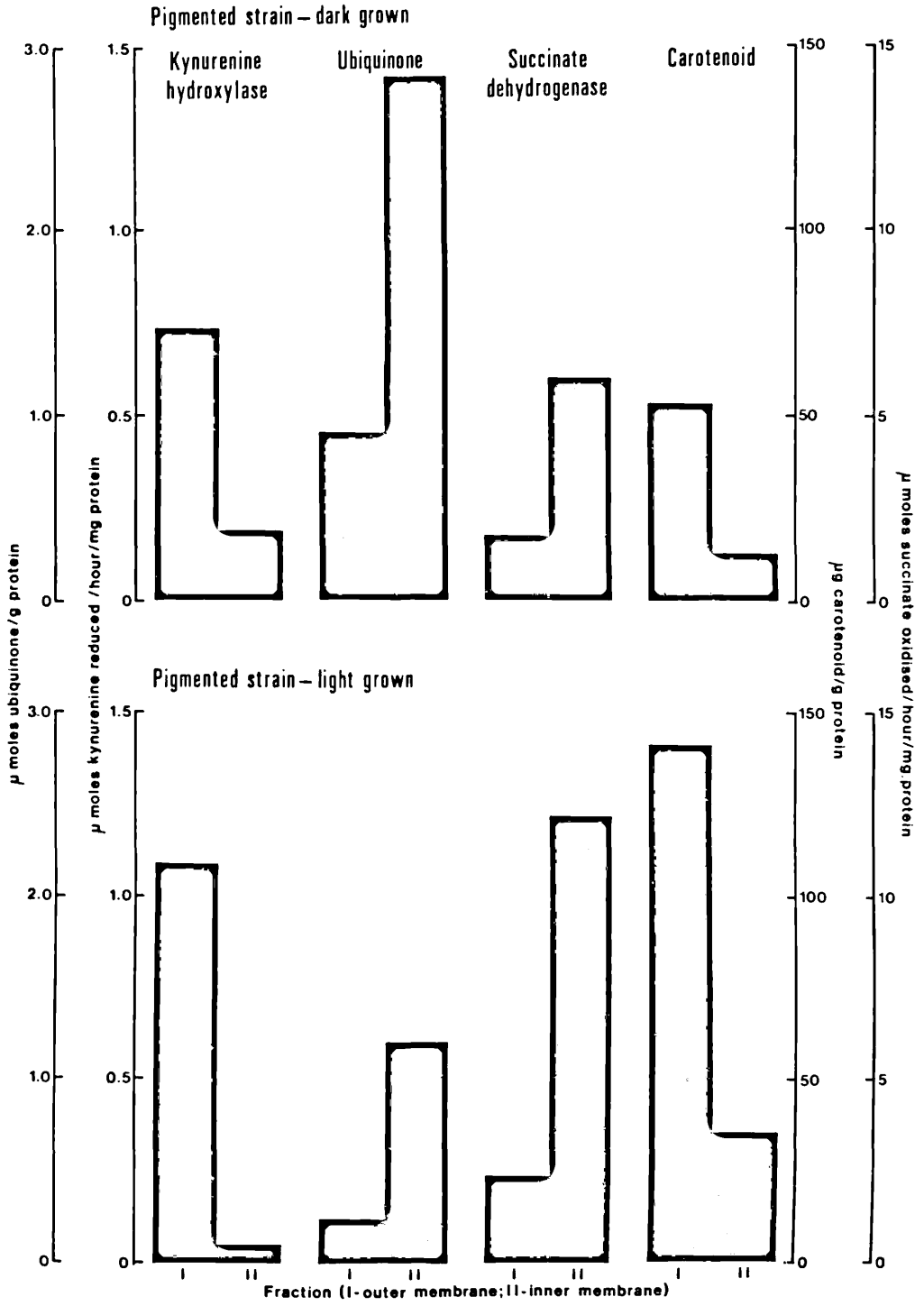
The pigment can be readily extracted and estimated by the method in Chapter II.

Figure 3 shows that in the dark-grown pigmented strain of N.crassa where carotenoid levels were low, probably all the pigment was located in the outer membrane, about 40-60 µg carotenoid/g protein, whereas in the light-grown pigmented strain, the carotenoid levels are increased to about 140 µg carotenoid/g protein. Now significant amounts of carotenoid are found in the inner mitochondrial membrane estimated at about 40 µg carotenoid/g protein.

Ergosterol, another lipid component of N.crassa mitochondria, was examined. This was located in the outer mitochondrial membrane. The ergosterol content has been estimated and it follows the same pattern as carotenoid in preparations from dark-grown cultures (Tables 24).

Thus it may be concluded that in dark-grown Neurospora, where carotenoid levels are low, probably all the pigment is in the outer

Figure 3



Distribution of Carotenoid Between Inner and Outer Membranes

The distribution of enzyme activities, carotenoid and ubiquinone between the inner membrane (Fraction II) and the outer membrane (Fraction I) is shown. The membranes were prepared, the enzymes assayed and the lipids estimated as described in Chapter II. The data shown are representative of at least five preparations of each strain.

Table 24. Distribution of cytochrome c oxidase and ergosterol between inner and outer mitochondrial membranes of pigmented strains

	Fraction I (outer membrane)	Fraction II (inner membrane)
Dark-grown cultures		
Cytochrome c oxidase	0.1	2.1
Ergosterol	30	11
Light-grown cultures		
Cytochrome c oxidase	0.66	2.69
Ergosterol	30	12

Cytochrome c oxidase is expressed as μ moles O_2 uptake/min/mg protein. Ergosterol is expressed as μ moles/g protein. The methods for separation of the membranes and assay of markers are described in Chapter II

mitochondrial membrane. In light-grown mycelia, carotenoid levels in the outer membrane are increased but significant amounts are found in the inner mitochondrial membrane.

9. Inactivation of Kynurenine Hydroxylase by Violet Light and Hydrogen Peroxide

The kynurenine hydroxylase activity and, in dark-grown cultures, the carotenoid, have been found to be concentrated in the outer mitochondrial membrane fraction of N.crassa. Recently, Zobnina et al (1975) found that Mycobacterium carotenum showed greater resistance to the effects of hydrogen peroxide (H_2O_2) than its white (carotenoid-free) mutant. Furthermore, there are indications that living systems may produce singlet oxygen internally. For example in the enzyme peroxidase-catalase system. This oxygen, which could be quenched by carotenoid, reacts with substances to initiate chain reactions which are not possible with normal oxygen (Foote,1968; Politzer et al,1971). Therefore it was of interest to investigate the resistance of kynurenine hydroxylase, the outer membrane enzyme, to the effect of H_2O_2 and violet light in pigmented and white mutant strains of N.crassa. The results obtained with homogenates of both strains are shown in Table 25.

Illumination of homogenates using violet light for 20 minutes resulted in a loss of kynurenine hydroxylase activity of about 18% in the dark-grown pigmented strain and 44% in the white mutant grown under the same conditions. When both strains were grown in the light, a similar result for photo-inactivation of kynurenine hydroxylase activity was obtained. Thus light inhibited and carotenoid protected kynurenine hydroxylase activity but there was almost no difference between dark-grown and light-grown preparations.

Table 25. Inactivation of kynurenine hydroxylase by violet light and hydrogen peroxide in N.crassa homogenates

Enzyme systems	Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
	Control	Illuminated		Control	Illuminated	
Dark-grown						
Kynurenine hydroxylase	2.78	2.29	18	2.30	1.30	44
Kynurenine hydroxylase + H_2O_2 (0.003%)	1.96	-	30	1.49	-	35
Light-grown						
Kynurenine hydroxylase	2.26	1.86	18	2.75	1.5	45
Kynurenine hydroxylase + H_2O_2 (0.003%)	2.12	-	6	1.80	-	34

Kynurenine hydroxylase was measured spectrophotometrically as described in Chapter II and the activity is expressed as μ moles hydroxykynurenine produced/hr/mg protein. The mitochondria were illuminated at 10°C using violet light (385-495 nm) of 150 W/m^2 for 20 minutes or treated with H_2O_2 for 10 minutes. The assay mixtures contained 1 ml mitochondria

Table 26. Inactivation of NADH oxidase by violet light and H_2O_2 in mitochondria from dark-grown strain

Enzyme systems	Pigmented		Percentage inactivation	Non-pigmented		Percentage inactivation
	Control	Illuminated		Control	Illuminated	
* NADH oxidase	0.040	0.036	10	0.062	0.055	11
NADH oxidase + H_2O_2 (0.003%)	0.030		25	0.042		32

The mitochondria were illuminated at 10°C using violet light of 150 W/m^2 for 15 minutes or treated with H_2O_2 (0.003%) for 10 minutes

* After irradiation the NADH oxidase was measured spectrophotometrically at 340 nm and the activity expressed as μ moles NADH oxidised/min/mg protein

The exposure of homogenates to 0.1% of H_2O_2 for 10 minutes caused the inhibition of kynurenine hydroxylase activity by about 30% in the pigmented dark-grown culture, while the loss of activity is decreased to about 6% in the light-grown strongly pigmented culture. Meanwhile the loss of activity was about 35% in white dark-grown cultures and nearly the same when this strain was grown in the light.

Irradiation of mitochondria using violet light for 15 minutes resulted in the inactivation of NADH oxidase (the inner membrane enzyme) by 10 or 11% for pigmented and non-pigmented strains respectively (Table 26) whereas exposure of mitochondria to H_2O_2 for 10 minutes caused inactivation of NADH oxidase by about 25% for the pigmented strain (dark-grown) and 32% for the white one (Table 26).

It can be concluded that the levels of carotenoid in the inner membrane for dark-grown cultures is not enough to protect NADH oxidase in the inner membrane.

IV DISCUSSION

DISCUSSION

1. PHOTOSENSITIVITY OF RESPIRATION

The present study has demonstrated that the growth rate of N.crassa mycelia on agar was not significantly affected by light, whereas irradiation of a young hyphal suspension showed an inhibitory effect on respiration. The exposure of N.crassa mitochondria to high intensity violet light without added photosensitiser resulted in the inactivation of several inner membrane enzyme activities. The succinoxidase system which has been studied in detail showed two sites of photo-inhibition in the flavoprotein dehydrogenase complex when a long time of illumination was used.

The first of these was the succinate dehydrogenase as assayed by PMS or menadione, while the second was assayed with menadione or ubiquinone-DCPIP as electron acceptor, was more sensitive than the PMS site.

The second site could be repaired by the addition of cysteine after short periods of illumination, suggesting the photo-inactivation of sulphydryl groups.

Cytochrome oxidase was almost insensitive to light even with long times of irradiation but it became sensitive after lipoxygenase treatment.

However, these sites of inactivation do not seem to be protected by carotenoids as yellow and white preparations showed the same degree of photo-inhibition of succinate dehydrogenase and of cytochrome oxidase after lipoxygenase treatment.

The third site of photo-inhibition was shown to be the respiratory ubiquinone as it was found that ubiquinone could repair the loss of succinoxidase activity in white mutant and pigmented dark-grown preparations.

Extraction after illumination showed that ubiquinone was inactivated strongly in the white and pigmented dark-grown cultures but not in light-grown preparations which indicated that the photosensitivity of this site was affected by the presence of carotenoids. Separation of inner and outer mitochondrial membranes indicated the presence of carotenoids in the outer membrane of dark-grown cultures, but a considerable amount was found in the inner membrane of light-grown cultures.

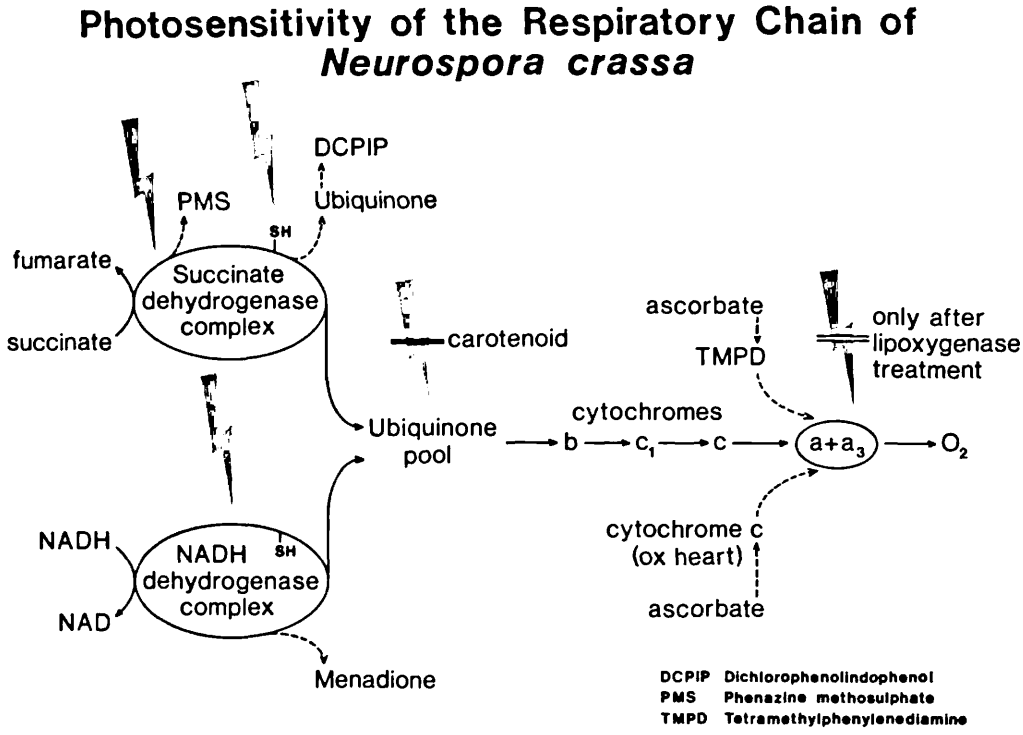
NADH oxidase was much less sensitive than succinoxidase. The flavoprotein dehydrogenase for NADH was also found to be sensitive to light and not protected by the presence of carotenoids. The photoinhibition of the NAD^+-linked pathway was also shown to be partly due to the loss of the endogenous ubiquinone. The photosensitivity of the Neurospora mitochondrial respiratory chain is summarised in Figure 4.

2. SUCCINOXIDASE AND NADH OXIDASE PHOTO-INACTIVATION

Maxwell and Chichester (1971) using Rhodotorula glutinis have suggested that the membrane-bound enzymes of mitochondria in the absence of added photosensitiser were the likely lethal site of photodamage. Investigations have been conducted to determine the possible sites of damage in the N.crassa mitochondrial respiratory chain. However, the photochemical processes due to visible light are complex involving many sites of photo-inactivation.

The succinoxidase pathway differs from that of NADH oxidase in response to illumination. Although both were labile to irradiation, the succinoxidase was more sensitive than NADH oxidase. However, since the succinoxidase pathway converges with the NAD^+-linked chain at the ubiquinone level, the difference in photosensitivity lies on the substrate side of the ubiquinone. In other words, succinate

Figure 4


Photosensitivity of the Respiratory Chain of
Neurospora crassa
DCPIP
PMS
Ubiquinone
SH
fumarate
succinate
Succinate dehydrogenase complex
carotenoid
ascorbate
TMPD
only after lipoxygenase treatment
Ubiquinone pool
cytochromes
b
c1
c
a+a3
O2
NADH
NAD
NADH dehydrogenase complex
SH
cytochrome c (ox heart)
ascorbate
Menadione
DCPIP Dichlorophenolindophenol
PMS Phenazine methosulphate
TMPD Tetramethylphenylenediamine

dehydrogenase differs in photosensitivity from that of the NADH dehydrogenase complex. Experiments using violet light (150 W/m^2, 5 minutes) inhibited succinoxidase and NADH oxidase to about the same extent as 460 nm light (15 W/m^2, 80 minutes). The dose in the two cases is of the same order of magnitude.

The results in Table 4 showed that the oxidation of the citric acid cycle (TCA) intermediates other than succinoxidase were less sensitive. Succinate is oxidised by a flavoprotein dehydrogenase and utilises a different respiratory pathway from that associated with NADH. However, the other oxidations of TCA intermediates are catalysed via endogenous NADH. Therefore, the photosensitivity of the oxidation of these intermediates is related to NADH photosensitivity which was less than succinoxidase.

3. SITES OF PHOTO-INACTIVATION

(a) Succinate dehydrogenase complex

There are two centres of reaction for PMS on succinate dehydrogenase. This idea was revived by Rossi et al (1970) who proposed that the presence of CoQ_{10} is required for the normal function of one of the two reaction sites of PMS.

Recently, Vinogradov et al (1975), using heart muscles, claimed the existence of two active redox centres in solubilised succinate dehydrogenase, the activity of centre 2 was much more labile than the activity of succinate-PMS reductase centre 1 in the dehydrogenase.

In N.crassa, succinate-PMS reductase was only photosensitive when larger doses of irradiation with violet light were used. The succinate-PMS reductase inhibition (site 1) is assumed to occur in centre 1, the succinate dehydrogenase flavoprotein itself.

A second site of inactivation was obtained in succinate-ubiquinone reductase which may be associated with centre 2, presumably the iron-

sulphur complex or part of it.

However, the effect of light observed with assays involving the ubiquinone, will therefore be the combined effects of the two sites in the dehydrogenase complex, since it was difficult to estimate the second site without including the first. Succinate-ubiquinone reductase showed a greater depression of activity after illumination for 5 minutes using violet light than PMS reduction which was only slightly photosensitive. Thus, photo-inactivation can be located between the site reducing PMS and that reducing ubiquinone. The observation of Rossi et al (1970) on the relation of CoQ_{10} content to activity in the PMS assay may indicate that the second reaction site of the dye is with the CoQ_{10} pool rather than at the flavoprotein itself. However, ubiquinone destroyed by light in this experiment was replaced by ubiquinone added externally to serve as an electron acceptor.

Illumination produced almost the same effect on PMS reduction in pigmented and white mutant preparations, showing that carotenoids did not protect the flavoprotein site. NADH-ferricyanide reductase also showed inactivation, suggesting that the NADH flavoprotein dehydrogenase was also sensitive to violet light, but less sensitive than the succinate one.

(b) The sulphydryl group

The involvement of sulphydryl groups in the respiratory chain has been reported by several workers (Gautheron,1973; Pagani et al, 1974). In particular, it has been suggested that they are associated with the non haem-iron protein which has been shown to be a component of the soluble malate-menadione reductase enzyme in Mycobacterium phlei (Brodie and Kurup,1967).

Aggarwal, Avi-Dor and Packer (1976) showed that illumination decreased

by approximately 40% the level of total sulphydryl groups in a rat liver sub-mitochondrial preparation.

Brodie and Murti (1969) were able to restore succinoxidase and NADH oxidase activities in M.phlei by adding a soluble factor prepared from the supernatant fraction from whole cells of M.phlei and from rat liver mitochondria. This supernatant could contain cysteine residues, cysteine or possibly glutathione. The presence of glutathione in rat liver mitochondria has been confirmed by Jocelyn (1975). In N.crassa it was observed that cysteine could repair the loss in succinoxidase activity after a small dose of violet light in pigmented and white mutant cultures under certain conditions (it was difficult to repair the loss in succinate-ubiquinone reductase activity by cysteine since it interfered with DCPIP in the assay).

Tests with other sulphydryl reagents such as glutathione and dithiothreitol reversed the light effect. This confirmed that sulphydryl groups could be considered as the second photosensitive site in the dehydrogenase complex.

The effect of PCMB (0.003 M) and violet light treatment for 10 minutes on the activity of succinoxidase were almost similar. However, PCMB is known to block the SH group between flavin and quinone (Vingradov et al,1975). From this it was concluded that restoration of enzyme activity with sulphydryl reagents may have involved similar sulphydryl groups presumably associated with centre 2 of the dehydrogenase complex.

Increasing the time of irradiation beyond 5 minutes increased the inhibition of succinoxidase and NADH oxidase (Fig.1), but cysteine did not totally reverse all the photo-inhibitory effect. It could be concluded that other photosensitive sites such as CoQ_{10} and PMS

were involved. In bacteria (Sarcina lutea), it has been found that the photosensitivity of flavoprotein dehydrogenases decreased rapidly with increase in incident wavelength above 440 nm (Huda,1970). In N.crassa, with 460 nm light for 85 minutes, NADH oxidase was not completely repaired by cysteine. Since 460 nm light did not affect the NADH-ferricyanide reductase, a further photosensitive site beyond the dehydrogenase but within the NADH-ubiquinone complex is suggested.

(c) Cytochrome oxidase

Ninnemann and Epel (1970a,1970b) have shown the photosensitivity of cytochrome oxidase in eukaryotes (yeast and beef heart). In N.crassa mitochondria this respiratory enzyme has been found to be only slightly photosensitive even after using violet light for 45 minutes (40×10^7 ergs cm^2 sec). Similar conclusions were reported by Aggerwal et al (1976) who found that cytochrome oxidase activity of rat liver mitochondria remained unchanged after exposure to visible light. The photosensitivity of cytochrome oxidase observed by Ninnemann and Epel is unlikely to be due to differences in the light used, since they used a high intensity source of blue light (up to 2.5×10^6 ergs cm^2 sec). Cytochrome oxidase is known to be tightly bound to lipid in mitochondria and mitochondrial lipids are known to be rich in unsaturated fatty acids (Bartly,1964). West (1968), using cauliflower mitochondria and a lower light intensity than I used, found that cytochrome oxidase became sensitive after lipoxygenase treatment.

In N.crassa also, cytochrome oxidase became sensitive after lipoxygenase treatment. This could be due to protection by a mechanism associated with poly-unsaturated fatty acids (such as linoleic acid). Alternatively, lipoxygenase treatment could change the conformation of the cytochrome oxidase complex. This change could bring together a photosensitiser and a photosensitised substrate. Prior to the change

the photosensitiser would be too far from the substrate to excite it. After treatment, light absorbed by the photosensitiser would excite the substrate leading to a photochemical reaction. The change in the unsaturated lipid content brought about by lipoxygenase might be enough to do this. Also lipoxygenase could catalyse the hydroperoxidation of polyunsaturated fatty acids which could lead to membrane damage (Anderson and Krinsky,1973).

(d) Ubiquinone

Involvement of quinone in the respiratory chain in micro-organisms has been well established. Lester and Crane (1959) reported that N.crassa mitochondria possess CoQ_{10} (ubiquinone). Brodie et al (1970) found that after loss of endogenous quinone addition of menaquinones which structurally resembled the quinone of M.phlei restored the electron transport by the same pathway as the natural quinone. As noted above, in N.crassa repair by cysteine of the photosensitive site in succinoxidase after using large doses of violet light is incomplete, suggesting that othe respiratory components such as the PMS site and probably ubiquinone are also photosensitive.

Addition of ubiquinone and cysteine reduced the photo-inhibition of succinoxidase (Table 11). Using 460 nm light for 85 minutes succinoxidase and NADH oxidase were not completely repaired by cysteine, but addition of ubiquinone and cysteine very substantially reduced the photo-inhibition of both enzyme systems. Thus, since ubiquinone restored the depleted activity which cysteine could not restore, the photo-inactivation must also involve the loss of endogenous ubiquinone which was an integral part of the electron transport chain.

Ubiquinone was required for restoration of succinoxidase activity only in the white mutant and dark-grown pigmented preparations, light-

grown wild-type preparation with higher carotenoid levels were almost completely repaired by cysteine, suggesting protection of ubiquinone by the presence of a high concentration of carotenoids.

Kashket and Brodie (1962) have demonstrated that quinones (benzoquinone Q8 and naphthoquinone K_2C_{45}) of E.coli were the most light sensitive respiratory components they examined using 360 nm light. Brodie and Murti (1969) concluded that the lesion on the NADH pathway of M.phlei irradiated with 360 nm light was caused by the loss of the endogenous quinone. In N.crassa the photosensitivity of membrane-bound ubiquinone was further demonstrated when ubiquinone was extracted from mitochondrial suspensions of pigmented and white mutant cultures after illumination with yellow light. The formation of high levels of carotenoids in N.crassa is dependent on light (Harding et al,1969) and oxygen (Rau,1969). The pigmented light-grown cultures were used after further exposure to a low light intensity and air. The data for ubiquinone extraction showed that illumination by high intensity light resulted in the loss of borohydride-reducible endogenous ubiquinone. This destructive effect decreased with increasing carotenoid concentration (Fig.2).

Thus, it was concluded that the third site of photosensitivity in the succinate and NADH oxidising respiratory chain of N.crassa is the ubiquinone which can be protected against photodestruction by the presence of carotenoid which is synthesised in response to light and oxygen.

Illumination with yellow light also reduced the carotenoid concentration in the light-treated preparation to about 50% of that in the dark-control preparation. Maxwell et al (1966) suggested that the loss in colour of the carotenoid pigment in R.glutinis cells using gas laser light was due to photo-oxidation of the pigment. However,

all the experiments described above were apparently carried out without a photosensitiser. Barran et al (1974) suggested that quinones are a possible candidate as endogenous photosensitisers and their illumination can inactivate amino acids transport. Since pure ubiquinone in ethyl alcohol was also photosensitive to 460 nm and violet light it can be concluded that photodestruction of ubiquinone need not require a separate sensitiser.

Hallemayer and Neupert (1974) found carotenoid only in the outer membrane of N.crassa mitochondria, but they did not mention whether their cultures were grown in light and enough oxygen to give high carotenoid levels or not. As ubiquinone is exclusively in the inner membrane, its photoprotection by carotenoid presumably requires the presence of the pigment in the inner membrane especially since it is believed that the intracellular location of carotenoid in relation to the photosensitive site is important in protection against photodynamic action (Mathews,1967; Mathews et al,1974). However, the carotenoid distribution between inner and outer mitochondrial membranes was re-examined using ubiquinone as inner and kynurenine hydroxylase as outer membrane markers.

From the data it was found that in dark-grown Neurospora, where carotenoid levels were low, all the pigment was in the outer membrane, in agreement with Hallemayer and Neupert (1974). In light-grown preparations, particularly where the mycelial mats were exposed after growth to light and open air, the carotenoid levels in the outer membrane increased, but significant amounts were found in the inner membrane (Fig.3). This may explain an important factor in the differences in the photosensitivity which has been observed in the pigmented light-grown preparation and the dark-grown one.

Therefore, with lower concentrations of carotenoids located in

the outer mitochondrial membrane in dark-grown pigmented preparation, ubiquinone and carotenoid molecules did not interact sufficiently to facilitate the transfer of the energy absorbed by ubiquinone (the sensitive site) to carotenoid molecules.

In light-grown cultures some carotenoid occurred in the inner membrane sufficiently close to the sensitive site to protect it against photosensitisation. Transfer of the excitation energy absorbed by ubiquinone to carotenoid molecules could result in the protection of ubiquinone but also could result in a simultaneous photo-oxidation or bleaching in the carotenoid. Following a period of illumination the carotenoid concentration might be reduced to a level which did not afford protection against visible light. Further, since carotenoid protected ubiquinone but did not protect either photosensitive site in the dehydrogenase complex, the ubiquinone cannot be acting as a sensitiser for the dehydrogenase photoreactions.

4. EFFECT OF VIOLET LIGHT AND HYDROGEN PEROXIDE ON KYNURENINE HYDROXYLASE ACTIVITY

In N.crassa carotenoids seemed to protect the activity of kynurenine hydroxylase, the outer membrane enzyme, against the effect of violet light. The difference in the photo-inhibition of the enzyme was very large in pigmented and white mutant preparations. But there was almost no difference between the dark-grown and light-grown pigmented preparations.

This is consistent with the fact that the carotenoid which appeared to protect kynurenine hydroxylase is always in the outer membrane of the wild-type, whether light or dark-grown.

Zobnina et al (1975) have shown that Mycobacterium carotenum had a greater resistance to the effect of hydrogen peroxide (H_2O_2) than did its white carotenoidless mutant.

In Neurospora the exposure of homogenates to H_2O_2 greatly affected the kynurenine hydroxylase activity in pigmented dark-grown and white mutant preparations whereas in the light-grown pigmented preparation the loss in the enzyme activity is considerably decreased. The presence of catalase was observed in both the pigmented and the white mutant preparations. This enzyme is known to decompose H_2O_2 and thereby to protect a cell from its highly toxic effect.

The pigmented light-grown culture with high levels of carotenoid in the outer membrane showed a lesser inactivation of kynurenine hydroxylase activity H_2O_2 than did pigmented dark-grown preparations with lower carotenoid in the outer membrane. It can be concluded that protection by carotenoid requires a threshold level of the pigments to be effective as a protective agent in the outer membrane.

5. EFFECT OF VISIBLE LIGHT ON RESPIRATION AND GROWTH OF YOUNG MYCELIA

(a) Respiration

The fact that 15-20 times as much radiation is required to produce the same inactivation in succinoxidase of hyphae as in isolated mitochondria, suggested that the hyphae possess protective mechanisms against damage by visible radiation. The thick resistant wall of this organism may provide some protection by serving as a filter between the cells and the source of light used. It also suggested a continuous adequate repair during radiation. Judis (1961) showed that thiols protected yeast against photodynamic killing. Recently, Jocelyn (1975) has confirmed the presence of glutathione in rat liver mitochondria and have also shown that glutathione diffuses from the suspended particles in the presence of phosphate; respiratory inhibitors inhibited the diffusion.

In the case of N.crassa in vivo it seems likely that the sulphydryl groups associated with succinate-ubiquinone reductase which has been

shown to be the second photosensitive site, is repaired by intra-mitochondrial sulphydryl groups.

Also, in mitochondria, it has been shown that carotenoid protected the ubiquinone site from destruction by visible light. This conclusion could apply to whole mycelia. The effect of light on succinoxidase activity in the white mutant and dark-grown pigmented cultures of young hyphae was bigger than the effect on pigmented light-grown culture. This confirms the involvement of carotenoids in protection of the succinoxidase system, especially the ubiquinone site, in vivo.

Carotenoids in light-grown cultures did not protect the succinoxidase activity totally from the effect of light. This could be explained by the involvement of another photosensitive site which is not protected by the presence of carotenoids such as site 1 (the PMS reductase centre) in the dehydrogenase complex. It has been shown that carotenoids did not protect this site in mitochondria.

(b) Growth

In agreement with other workers (Ryan et al,1943), I have failed to find any effect of high intensity light (270 W/m^2) on growth. However, this could be because the method used was not the best way to estimate growth since only linear extension was measured.

6. CONCLUSION

Light and oxygen are needed for synthesis of carotenoids in N.crassa, especially those with a greater degree of unsaturation.

Therefore, since carotenoid biosynthesis occurs when the conditions for photodynamic sensitivity are present, the existence of a well controlled regulatory mechanism for carotenoid synthesis may be postulated as a protective adaptive method to protect light sensitive components of the respiratory chain such as ubiquinone which appears to play an essential role as a physiological electron carrier.

Three sites in the respiratory chain of N.crassa mitochondria have been identified as sensitive to visible light, but only one, ubiquinone, is protected by carotenoids. It is concluded that ubiquinone may be the main site of the lethal action of light. Thus, the presence of carotenoids above a threshold level can provide protection to some of the inner and outer mitochondrial membrane enzymes against photodestruction.

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