Photosensitivity of Respiration in *Neurospora* Mitochondria

A PROTECTIVE ROLE FOR CAROTENOID

By Z. RAMADAN-TALIB and J. PREBBLE
Bedford College, Regent's Park, London NW1 4NS, U.K.

(Received 30 May 1978)

1. The effect of visible light on respiratory activity was studied in two strains of *Neurospora crassa*, one a wild-type strain able to synthesize carotenoid and the other an albino mutant lacking carotenogenic activity. Light had no effect on growth under the conditions studied, but inhibited respiration of hyphal suspensions, the degree of inhibition being dependent on the carotenoid content of the hyphae. 2. In studies of respiration of isolated mitochondria, three types of photosensitive site were detected. These were the flavo-protein dehydrogenases themselves, a site separate from the latter also associated with the dehydrogenase but re-activatable by treatment with a thiol reagent, and the respiratory quinone, ubiquinone. Cytochrome oxidase, previously reported as photosensitive from many sources, was not appreciably affected by light in these preparations. 3. The degree of inactivation of the respiratory quinone was dependent on the amount of carotenoid in the preparation, high concentrations of the pigment in the mitochondrial membranes providing substantial protection against the effect of light. 4. Separation of the inner and outer membranes of mitochondria showed that under conditions where carotenoid appears to protect quinone, significant amounts are found in the inner mitochondrial membrane, otherwise carotenoid is restricted to the outer membrane.

There have been few studies of the photosensitivity of respiratory systems using visible light. In eukaryotes, the cytochrome oxidase has been found to be photoinactivated in a wide range of systems (Ninnesmann *et al.*, 1970a,b; Epel & Butler, 1969), although Aggarwal *et al.* (1976b, 1978) reported inhibition by light of the respiratory system, but not the oxidase, in liver mitochondria. Photosensitizers, such as Toluidine Blue and flavin mononucleotide, have been shown to sensitize several respiratory activities to visible light (Codd, 1972; Prebble & Huda, 1973; Ascenzi & Cooney, 1975). In the absence of a photosensitizer, high light intensities inactivate respiration at three sites in *Micrococcus luteus* (*Sarcina lutea*) (Prebble & Huda, 1977). These have been identified as the respiratory quinone and two sites in the dehydrogenase complex, one of which is associated with a thiol group (Anwar & Prebble, 1977). The carotenoid of the membranes of non-photosynthetic bacteria has been shown to protect the organism from the lethal effects of visible radiation (Mathews & Sistrom, 1960; Mathews-Roth & Krinsky, 1970). In *Micrococcus luteus*, these pigments have also been shown to protect the respiratory quinone specifically while having no effect on the other photosensitive sites (Anwar & Prebble, 1977; Anwar *et al.*, 1977).

The purpose of the present study with *Neurospora crassa* was to investigate the photosensitivity of the

respiratory chain, to determine whether carotenoid is able to protect the respiratory system from inactivation by high light intensities and to examine the carotenoid content of the mitochondria. The latter issue arises from the work with *Phycomyces* by Riley & Bramley (1976), who suggested that the mitochondrial carotenoid found by Neupert *et al.* (1972) in *Neurospora* may be an artifact of preparation.

A preliminary report of this work has appeared (Prebble & Ramadan, 1976).

Materials and Methods

Cultures

Strains of *Neurospora crassa*, Shear and Dodge, were obtained from the Fungal Genetics Stock Centre, California State University, Humboldt Foundation, Arcata, CA, U.S.A. Strain FGSC 987 was used as the carotenogenic wild-type organism and FGSC 16, an albino mutant (locus al-2), was used as the carotenoidless strain (Barratt & Ogata, 1968). Stock cultures were maintained on malt/agar slopes.

Experimental cultures were grown from conidial suspensions. These were prepared from mycelia grown on Wainwright's (1959) agar medium (50 ml in 200 ml medical flats) in the light at 28°C for 7-9 days, which gave good conidial formation. Conidial sus-

pensions were used to inoculate 1-litre culture flasks containing 500 ml of modified Czapek–Dox medium (Oxoid Ltd., Basingstoke, Hants., U.K.). The flasks were incubated with shaking (approx. 70 oscillations/min) in a reciprocal shaker at 28°C in the dark or in the light (2.5 W/m²) as required for 2½ days.

Preparation of mitochondria

Unwashed mitochondria were prepared by the method of Hall & Greenawalt (1967) and were finally suspended in a sucrose (0.25 M)/EDTA (0.005 M) medium.

Inner and outer membrane fractions were prepared by the technique of swelling and shrinking used by Neupert & Ludwig (1971) for *Neurospora*. Before swelling and shrinking treatment, the mitochondrial suspension was centrifuged at 1500g for 15 min, filtered through glass wool and centrifuged at 27 000g for 20 min. The pellet was resuspended, filtered a second time, centrifuged and suspended in the swelling solution. After swelling, shrinking and gentle sonication (15 s, medium setting, with 150 W Ultrasonic disintegrator, MSE Scientific Instruments, Crawley, Sussex U.K.), membranes were separated on sucrose layers: 1.5 M- and 1.05 M-sucrose layers gave the best separation.

Irradiated treatments

Suspensions of hyphae or mitochondria were illuminated at 10°C in a water-jacketed cuvette having a 1 cm light-path and equipped with a stirrer. An air-cooled 1000 W quartz–halogen lamp was used as the light source. The light was passed through heat filters (5 mm Chance glass and 2 cm of circulating water) and an appropriate interference or coloured-glass filter. The temperature of the specimen cuvette was within 1°C of the circulating water system. Light intensities were measured with a thermopile (Zipp and Zonen, Delft, Holland) at the inside front face of the cuvette. Enzyme activities were measured at low laboratory light intensity (less than 0.2 W/m²) and 30°C on samples removed from the illumination cuvette. Non-irradiated controls were maintained under comparable conditions to irradiated preparations.

Measurement of growth rate

This was measured as the rate of movement of the growing mycelial front along a 2 cm glass tube containing a layer of Wainwright's agar medium (Ryan *et al.*, 1943).

Assay methods

DL-Kynurenine and menadione were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K. The succinate oxidase system was assayed polarographically at 30°C in a mixture of 25 mM-succinate and 50 mM-potassium phosphate

buffer, pH 7.0. Succinate dehydrogenase (succinate–phenazine methosulphate reductase, EC 1.3.99.1) was assayed by autoxidation of phenazine methosulphate with the oxygen electrode by using 25 mM-succinate, 0.5 mM-phenazine methosulphate, 50 mM-Na₂N₃ and 40 mM-potassium phosphate buffer, pH 7.0. Succinate–ubiquinone reductase was assayed spectrophotometrically at 30°C by using a 1 cm light-path and an incubation mixture containing 3 mM-succinate, 0.02 mM-ubiquinone-10, 0.03% (v/v) Triton X-100, 0.056 mM-2,6-dichlorophenol-indophenol, antimycin (1.3 µg/ml) and 50 mM-potassium phosphate buffer, pH 7.0 (Ziegler & Reiske, 1967). The reduction of dichlorophenol-indophenol was read at 600 nm and was linear with time. NADH oxidase was assayed with 0.15 mM-NADH either polarographically or spectrophotometrically by following the decrease in A₃₄₀. NADH dehydrogenase (EC 1.6.99.3) was measured spectrophotometrically by following changes in A₄₂₀ by using 0.15 mM-NADH, 1 mM-potassium ferricyanide and 50 mM-Na₂N₃. Menadione reductase assays were performed with 0.5 mM-menadione in the presence of 50 mM-Na₂N₃ and the autoxidation of menadione was measured polarographically. Cytochrome oxidase (EC 1.9.3.1) was assayed polarographically with 25 mM-ascorbate and either 0.05 mM-tetramethylphenylenediamine or 2.5 µM-mammalian cytochrome c. Kynurenine hydroxylase (EC 1.14.13.9) was assayed by the method of Okamoto (1970) by measuring the decrease in A₃₄₀ due to the oxidation of NADPH.

Ubiquinone was assayed by the method of Pumphrey & Redfearn (1960). Pellets were extracted with cold methanol and the quinone was then transferred to light petroleum (b.p. 40–60°C). The petroleum was evaporated to dryness under N₂ and the residue dissolved in 3 ml of ethanol. The ubiquinone concentration was estimated from the difference in A₂₇₅ before and after borohydride reduction by using $\epsilon = 12250 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Pumphrey & Redfearn, 1960). The same ethanolic solution was used for estimating ergosterol by its absorbance at 282 nm by using $\epsilon = 11500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Huber *et al.*, 1945; Hallermayer & Neupert, 1974). The carotenoid content of ethanolic solution was estimated by using the absorption coefficient for neurosporaxanthin ($A_{1\text{cm}}^{1\%} = 1714$; Aasen & Liaaen-Jensen, 1965), which is the major carotenoid present in such preparations of *Neurospora* (Harding *et al.*, 1969; Hallermayer & Neupert, 1974).

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Photosensitivity of growth and respiration

The linear rate of growth of mycelia on an agar medium along a horizontal 1 cm diameter tube was

about 3 mm/h in both the carotenogenic strain (FGSC 987) and the carotenoidless mutant (FGSC 16). The growth rate was not affected by irradiation with white light (210 W/m²) or blue light (385–495 nm, 80 W/m²) for up to 8½ h.

When suspensions of young hyphae were assayed for respiratory capacity with succinate as substrate, prior irradiation with either wide-band (385–495 nm, 150 W/m²) or narrow-band (460 ± 5 nm, 15 W/m²) blue light resulted in loss of activity. Carotenoidless hyphae grown in the dark were only slightly more sensitive than the wild-type (Table 1). Dark-grown cultures of the carotenogenic strain possess only low amounts of pigment (see Fig. 2), but carotenoid concentrations can be substantially enhanced by photoinduction of carotenoid synthesis (Harding *et*

al., 1969; Howes *et al.*, 1969). If cultures were grown in the light, the respiration of mutant hyphae was much more photosensitive than that of the carotenoid-containing strain.

Photosensitivity of respiration in mitochondrial suspensions

Since blue light was previously found to be effective in studies of bacterial respiration, similar wavelengths (385–495 nm) were used here. Succinate oxidation was more photosensitive than that of substrates whose oxidation is NAD⁺-dependent. Further, succinate oxidation was photoinactivated faster than NADH oxidation during irradiation for 30 min (Fig. 1). Comparison of the carotenogenic and carotenoidless strains showed that there was little

Table 1. *Effect of irradiation on the respiratory activity with succinate as substrate of young hyphal suspensions of Neurospora crassa grown in the light and dark*

Shake cultures were grown for 16 h, centrifuged (1500 g, 15 min) and resuspended in 0.05 M-potassium phosphate buffer, pH 6.8, to give a pipettable suspension. The hyphae were illuminated with wide-band (385–495 nm, 150 W/m²) or narrow-band (460 ± 5 nm, 15 W/m²) blue light at 10°C. Samples were assayed for their ability to oxidize succinate before and after illumination. Activity is expressed as µg-atoms of oxygen taken up/min per g dry wt. of mycelium.

Irradiation treatment		Carotenoid synthesis induced	Strain 987 (carotenogenic)		Strain FGSC 16 (albino)	
λ (nm)	min		Respiratory activity	Photoinactivation (%)	Respiratory activity	Photoinactivation (%)
385–495	90	—	2.66	20	2.19	26
385–495	90	+	2.38	13	2.04	26
460 ± 5	60	—	2.35	5	2.02	12
460 ± 5	60	+	1.76	0	2.24	12

Fig. 1. *Photoinactivation of respiratory activity in mitochondrial suspensions from carotenoid-containing (a) and albino (b) strains of Neurospora crassa*

Suspensions of mitochondria were irradiated with blue light (385–495 nm, 150 W/m²). Samples were removed for assay of succinate oxidase and NADH oxidase (□) activities after various periods of time. Succinate oxidase assays were carried out in the presence (●) and absence (○) of 0.5 mM-cysteine.

difference in the photosensitivity of their mitochondrial succinate oxidation. Treatment of mitochondria with 0.5 mM-cysteine after irradiation resulted in a partial restoration of succinate oxidase activity in both white and yellow strains. Proportionately, cysteine was less effective with longer irradiation times, suggesting that there were other sites in the chain that were also photoinactivated but not re-activated with cysteine. Dithiothreitol and glutathione were also able to re-activate respiratory activity after irradiation. *p*-Chloromercuribenzoate (0.15 mM) produced an inhibition of succinate oxidase comparable in magnitude with that produced by blue light at 150 W/m² for 10 min.

Location of photosensitive sites in the respiratory chain of mitochondria from dark-grown cultures

Since inactivation of NADH oxidase was less than that of succinate oxidase, even though the activity of the former was higher, a major site of inactivation in the succinate dehydrogenase complex might be expected. Table 2 shows the results of experiments in which enzyme activities have been measured after irradiation of mitochondrial suspensions with blue light as compared with non-irradiated controls. Inactivation of succinate-ubiquinone reductase after 5 min irradiation is almost as great as that of succinate oxidase. By contrast, succinate-phenazine methosulphate reductase gave only slight inactivation, indicating that the major photosensitive site, pre-

sumably a thiol group(s), probably lies in the dehydrogenase complex between the moiety reducing phenazine methosulphate and that reducing ubiquinone. As cysteine interferes with the succinate-ubiquinone reductase assay, it has not been possible to make a direct measurement of the repair.

Further irradiation increases the inactivation of succinate oxidase and of succinate-phenazine methosulphate reductase (Table 2), showing that the initial segment of the dehydrogenase complex is also photosensitive, although less so than the segment carrying the photosensitive thiol group. Inactivation of succinate-phenazine methosulphate reductase was always substantially less than that of the oxidase.

The NADH dehydrogenase appeared to possess a comparable photosensitive thiol group. The NADH dehydrogenase assayed as menadione reductase showed more inhibition than the oxidase (Table 2). Oxidase activity was completely restored by treatment with cysteine after irradiation.

Comparison of NADH-menadione reductase and NADH-ferricyanide reductase inactivations (Table 2) suggests that, as with the succinate dehydrogenase complex, there are also two photosensitive sites in this complex, one assayed with ferricyanide and less sensitive, and a sensitive thiol group plus the first site assayed with menadione.

Succinate-phenazine methosulphate reductase activity is high in relation to the succinate oxidase system (Table 2), but since the photoinactivations

Table 2. *Inactivation of various respiratory activities by blue light (385–495 nm, 150 W/m²)*

Shake cultures were grown in the dark for 2½ days. Mitochondria were prepared and assayed for enzyme activity before and after irradiation. Activities are expressed as nmol of substrate oxidized/min per mg of protein.

Enzyme activity	Irradiation time (min)	Strain 987 (carotenogenic)		Strain 16 (albino)	
		Activity	Inactivation by light (%)	Activity	Inactivation by light (%)
Succinate oxidase	5	28	24	27	29
	20		35		40
	45		49		67
Succinate-phenazine methosulphate reductase	5	32	3	33	3
	20		20		22
Succinate-ubiquinone reductase	5	34	20	32	22
Succinate oxidase + 0.5 mM-cysteine	5	18	5	27	4
	20		17		20
Succinate oxidase + 0.5 mM-cysteine + ubiquinone (50 µg/ml)	20	25	11	23	12
NADH oxidase	5	38	12	35	11
	15		19		19
NADH-menadione reductase	5	31	15	35	20
NADH-ferricyanide reductase	5	20	4	—	—
	15		11		—
NADH oxidase + 0.5 mM-cysteine	5	—	—	31	0

measured in our experiments are low, the initial step in the dehydrogenase complex will not be rate-limiting for the succinate-oxidation system after irradiation. It therefore seems likely that the photo-inactivation of succinate oxidase shown in Fig. 1 after cysteine treatment must be due to a further photosensitive component in the chain. Cytochrome oxidase, known to be photosensitive in other organisms, showed little or no inactivation. A dose of blue light sufficient to give 25% inactivation in yeast (Ninnemann *et al.*, 1970a) had no effect on ascorbate-tetramethylphenylenediamine oxidase and inactivated ascorbate-cytochrome *c* oxidase by only 8%, but decreased succinate oxidase activity by 49% in the wild-type.

However, the addition of the respiratory quinone ubiquinone, in addition to cysteine, did bring about a further substantial recovery of succinate oxidase activity after irradiation. The small residual inactivation of succinate oxidase seen after quinone and cysteine addition might be attributed to the inactivation associated with the succinate-phenazine methosulphate segment of the chain. The results of experiments with both carotenogenic and carotenoidless strains are similar, again suggesting that carotenoid under these conditions does not appreciably influence photosensitivity.

Role of carotenoid

Since in bacteria (*Micrococcus*) carotenoid protects the respiratory quinone from destruction by 450nm light (Anwar & Prebble, 1977), we have

examined the photosensitivity of the respiratory chain using a narrower band of radiation (460 ± 5 nm). Longer irradiation times were necessary to compensate for the low-energy transmission of the 460nm filter, but the dose response is similar to that for the wide-band filter system. Table 3 shows the effects of 460nm light on various enzyme activities which for dark-grown cultures were similar to those obtained with the wide-band blue filter. However, when light-grown cultures were used, the photosensitivity of particles from the two strains was clearly different. In the absence of carotenoid, succinate oxidase is about twice as photosensitive as in its presence. The high-sensitivity site of the dehydrogenase was affected similarly in both strains, but cysteine totally restored succinate oxidase activity in the carotenogenic preparation, whereas the addition of quinone was necessary in addition to cysteine to restore activity of mitochondria from the carotenoidless strain. Thus, under the conditions of this experiment, there appeared to have been an inactivation of the succinate dehydrogenase in both strains, but loss of quinone only in the absence of carotenoid.

Direct extraction of quinone from mitochondria shows that large doses of irradiation of the carotenogenic strain grown in the light results in a small loss of quinone but a moderate loss of carotenoid, whereas in the albino mutant there is a substantial loss of mitochondrial quinone as a consequence of irradiation. In dark-grown cultures, a small effect only on carotenoid is seen (Table 4).

Table 3. Inactivation by 460 ± 5 nm light of respiratory activity in mitochondrial suspensions

Suspensions of mitochondria prepared from 2½-day-old cultures of the carotenogenic strain (FGSC 987) and the carotenoidless mutant (FGSC16) were irradiated with 460 ± 5 nm light (15 W/m^2) for 85 min. Samples were assayed for enzyme activity before and after irradiation. Activities are expressed in nmol of substrate oxidized/min per mg of protein. Cultures were grown in the dark (non-induced for carotenoid synthesis) or in the light (induced for carotenoid synthesis).

Enzyme	Induction of carotenoid synthesis	Strain 987 (carotenogenic)		Strain 16 (albino)	
		Activity	Inactivation by light (%)	Activity	Inactivation by light (%)
Succinate oxidase	—	25	24	22	27
Succinate oxidase + cysteine (0.5mM)	—	25	16	21	14
Succinate oxidase + cysteine (0.5mM) + ubiquinone (50 µg/ml)	—	25	8	21	5
NADH oxidase	—	59	17	—	—
NADH oxidase + cysteine (0.5mM)	—	57	9	—	—
NADH oxidase + cysteine (0.5mM) + ubiquinone (50 µg/ml)	—	56	4	—	—
NADH-ferri cyanide reductase	—	—	—	20	0
Succinate oxidase	+	18	15	23	30
Succinate oxidase + cysteine (0.5mM)	+	18	0	23	13
Succinate oxidase + cysteine (0.5mM) + ubiquinone (50 µg/ml)	+	18	0	23	4

Table 4. *Effect of visible light on ubiquinone and carotenoid concentrations in suspensions of Neurospora mitochondria* Suspensions of mitochondria from 2½-day-old cultures of strain FGSC 987 (carotenogenic) and FGSC 16 (albino mutant) were irradiated for 40 min with filtered light (Chance glass filter OY13 transmitting wavelengths above 440 nm, 532 W/m²). Samples were taken before and after irradiation and assayed for ubiquinone (expressed as µmol/g of protein). Cultures were grown either in the dark (non-induced for carotenoid synthesis) or in the light (induced for carotenoid synthesis).

	Induction of carotenoid synthesis	Strain FGSC 987 (carotenogenic strain)		Strain FGSC 16 (albino mutant)	
		Content	Loss (%)	Content	Loss (%)
Ubiquinone	—	2.0	30	1.6	48
Carotenoid	—	81	52	—	—
Ubiquinone	+	1.7	11	2.2	41
Carotenoid	+	622	59	—	—

As noted above, the method of growth of the wild-type influences the amount of carotenoid found in hyphae, light and oxygen being the essential requirements for carotenogenesis (Harding *et al.*, 1969).

Although the light requirement is small, the oxygen concentration for maximum carotenogenesis was found to be greater than that obtaining in our shake cultures. Greater pigmentation was produced by exposing mycelial mats obtained from shake cultures to light and air on a window bench for 1–2 h. Results of experiments where varied carotenoid concentrations were tested for their ability to protect quinone are shown in Fig. 2. High concentrations of carotenoid substantially, though not completely, protected quinone, whereas concentrations below about 150 µg/g of mitochondrial protein did not protect quinone with maximal efficiency. The irradiation treatment also destroyed about 50% of the carotenoid in the wild-type strain. The proportion of pigment lost was more or less constant and quantitatively unrelated to the degree of quinone protection, thus leading to the conclusion that the processes of quinone and carotenoid destruction are not directly related.

To determine whether the loss of quinone was a simple consequence of photodestruction without the involvement of other factors, the loss of quinone consequent on irradiation of ethanolic solutions was measured under conditions similar to those applied to mitochondrial suspensions. In general, only about two-thirds as much quinone is lost in ethanolic solution as is lost from irradiated mitochondria. Allowing for absorbance of other components in the mitochondrial suspension, it would seem unnecessary to postulate the existence of a photosensitizer involved in sensitizing ubiquinone destruction.

Location of carotenoid in mitochondria

After separation of inner and outer membrane fractions the carotenoid of dark-grown cultures was found primarily in the outer-membrane fraction, and

Fig. 2. *Destruction by light of ubiquinone in mitochondrial preparations containing variable amounts of carotenoid* Each point represents the percentage loss of quinone consequent on irradiation of a mitochondrial preparation of known carotenoid content. Mitochondria from the carotenoidless mutant (FGSC16), dark grown wild-type (FGSC987) and light-grown wild-type with higher concentrations of carotenoid were illuminated with yellow light (OY13 Chance glass filter transmitting wavelengths above about 440 nm, 530 W/m²) for 40 min. Carotenoid and ubiquinone were estimated before and after irradiation. The carotenoid values represent those obtained before irradiation, which bleaches about 50% of the pigment. Since tests showed that ergosterol was not affected by the irradiation treatment, it was used as an internal standard to correct for variations in extraction procedure. The standard deviation for quinone concentrations in preparations from the white mutant containing no carotenoid is also shown.

the small amount found in the inner-membrane fraction appeared to correlate with the presence of

kynurenine hydroxylase (the outer-membrane marker) in the fraction (Table 5). The concentration of carotenoid in the two membranes can be estimated by solving the equations:

$$\frac{SD_I \cdot x}{SD_I + SD_{II}} + \frac{Kyn_I \cdot y}{Kyn_I + Kyn_{II}} = car_{IM}$$

$$\frac{SD_{II} \cdot x}{SD_I + SD_{II}} + \frac{Kyn_{II} \cdot y}{Kyn_I + Kyn_{II}} = car_{OM}$$

where subscripts I and II refer to the inner and outer membrane fractions respectively, SD being succinate dehydrogenase activity and Kyn the kynurenine hydroxylase activity. x and y are the carotenoid concentration in the inner and outer membrane fractions respectively and car_{IM} and car_{OM} are the estimated carotenoid contents of the inner and outer membrane. The use of enzymic markers rather than lipid markers such as ergosterol and ubiquinone is preferred in view of the report of Riley & Bramley (1976) that lipids may transfer between membrane fractions during fractionation procedures.

The application of the above equations to several preparations leads to the conclusion that in dark-grown cultures there is negligible carotenoid in the inner membrane and that the pigment is present in the outer membrane. Full induction of carotenoid synthesis results in a large increase in the concentration of pigment in the outer membrane, but also the appearance of appreciable amounts of pigment in the inner membrane. With preparations showing 190–200 μg of carotenoid/g of protein in the outer membrane, about 50 μg /g of protein is found in the inner membrane. If the carotenoid of the inner membrane arose simply by partition between inner and outer membrane during preparation of the material, larger concentrations should have been found in the inner membranes of non-induced preparations. We therefore conclude that the pigment in the inner membrane is not found in that fraction simply as a consequence

of a redistribution of carotenoid during preparative procedures.

Our results appear to be in agreement with those of Hallermeyer & Neupert (1974) since their washed mitochondria contained 110 μg of carotenoid/g of protein, 15 μg of ergosterol/mg of protein with a molar ratio of carotenoid/ergosterol of 1:171, and all of the carotenoid in the outer membrane. Our preparations of unwashed mitochondria had a similar molar ratio (10 μg of ergosterol/mg of protein, 75 μg of carotenoid/g of protein, molar ratio = 1:166) when prepared from dark-grown cultures and the carotenoid was also found only in the outer membrane.

Discussion

The studies of succinate oxidase (summarized in Scheme 1) lead to the conclusion that there are three sites that are sensitive to blue light. Further photosensitive sites are associated with the NADH dehydrogenase complex, which, like the succinate dehydrogenase complex, possesses a photolabile thiol group and probably a further photosensitive site nearer to the catalytic site for NADH. In succinate oxidase, the thiol group is the most photosensitive site. This pattern of photosensitivity closely resembles that described earlier for *Micrococcus luteus* (Anwar & Prebble, 1977), where the membrane-bound flavoprotein dehydrogenase, malate dehydrogenase, has similar photosensitivity to the succinate dehydrogenase described here. Photosensitivity of mitochondrial dehydrogenases has also been described in liver by Aggarwal *et al.* (1978). The failure to demonstrate photosensitivity in the cytochrome oxidase was unexpected in view of the work of Epel and others (Epel & Butler, 1969; Ninemann *et al.*, 1970a,b), but Aggarwal *et al.* (1976b, 1978) also failed to observe photosensitivity of the oxidase in rat liver mitochondria.

Table 5. Determination of carotenoid in inner and outer membrane fractions of *Neurospora* mitochondria

Inner and outer mitochondrial membranes were prepared by a modified method of Neupert & Ludwig (1971). Kynurenine hydroxylase activity (outer-membrane marker) and succinate dehydrogenase activity (inner-membrane marker) were assayed for each of the fractions. Mitochondrial membranes prepared from non-induced (dark-grown) cultures are compared with those from cultures which have been fully induced for carotenoid synthesis (grown in the light).

	Non-induced		Induced	
	Inner membrane	Outer membrane	Inner membrane	Outer membrane
Kynurenine hydroxylase (nmol of kynurenine reduced/ min per mg of protein)	3.0	12.2	0.8	18.0
Succinate dehydrogenase (μmol oxidized/h per mg of protein)	6.0	1.7	12.2	2.5
Carotenoid (μg /g of protein)	18.7	76.7	48.7	204

Scheme 1. *Photosensitive sites in the respiratory chain of Neurospora crassa*

The approximate sites of interaction of the respiratory system with added electron donors and acceptors are shown with dotted arrows. Photosensitive sites are indicated by λ .

The photosensitivity of isolated mitochondria matches, in its general features, that found in whole hyphae. Dark-grown cultures show only a slight effect of carotenoid on photosensitivity. The increased concentrations of carotenoid in light-grown cultures result in lower photosensitivity, presumably because carotenoid protects the quinone to a large extent. The consequences of strong irradiation for a mitochondrion in an intact hypha may be predicted as (i) a loss of ubiquinone, the rate of loss being dependent on the carotenoid concentration, (ii) a rapid turnover of carotenoid and possibly bleaching, (iii) a slow loss of flavoprotein dehydrogenase activity, (iv) a diminution of the mitochondrial thiol content. Loss of mitochondrial thiol groups has been shown to be a consequence of irradiation of rat liver mitochondria (Aggarwal *et al.*, 1976a). The photo-oxidation of thiol groups associated with the dehydrogenases, and their reduction by other mitochondrial thiol groups, such as glutathione (Jocelyn, 1975), may account for all or part of the loss of total thiol groups. Unimpaired growth in strong light, such as we have observed for carotenogenic and albino strains, may be possible simply because of adequate resynthesis of destroyed cell components. Nevertheless, although the presence of carotenoid does not affect growth in light, it does protect the viability of *Neurospora* conidia subjected to visible radiation (Blanc *et al.*, 1976). Carotenoid has also been shown to protect the viability of other

fungi (Goldstrohm & Lilly, 1965; Maxwell *et al.*, 1966; Maxwell & Chichester, 1971).

Riley & Bramley (1976) have concluded from experiments with *Phycomyces blakesleeanus* that carotenoids may transfer from one particle to another during fractionation procedures, which has led these workers to question the results of Hallermayer & Neupert (1974). We believe that carotenoid is present in the mitochondrion of *Neurospora crassa* for the following two reasons. Firstly we find carotenoid in the outer-membrane fraction of our mitochondria and also in the inner membrane when carotenoid synthesis is fully induced. Secondly we find that carotenoid protects ubiquinone from photodestruction in isolated mitochondria and that the pigment protects respiration in whole hyphae which have not been subjected to disruption. Since the quinone is located in the inner membrane, its protection against the effects of visible light could only be achieved by the presence of carotenoid also in the inner membrane or possibly by carotenoid in the outer membrane screening the quinone from photo-products produced extramitochondrially. The latter seems improbable, but we prefer the former for positive reasons. Our evidence quoted here does not seem to require any agent for quinone destruction other than the quinone itself. Secondly, in studies of a similar bacterial system, carotenoid is located in the same membrane as the quinone that it protects and, in

experiments with isolated carotenoid, it was found necessary to have the quinone in close proximity to the carotenoid (Anwar *et al.*, 1977). Further, studies of photodynamic action with Acridine Orange in *Sarcina* showed that carotenoid protected membrane enzymes, but failed to protect the DNA (Mathews-Roth, 1967).

References

- Aasen, A. J. & Liaaen-Jensen, S. (1965) *Acta Chem. Scand.* **19**, 1843–1853
- Aggarwal, B. B., Avi-Dor, Y. & Packer, L. (1976a) *Abstr. Int. Congr. Photobiol.* 7th, abstr. P125
- Aggarwal, B. B., Avi-Dor, Y., Tinberg, H. M. & Packer, L. (1976b) *Biochem. Biophys. Res. Commun.* **69**, 362–368
- Aggarwal, B. B., Quintanilha, A. T., Cammack, R. & Packer, L. (1978) *Biochim. Biophys. Acta* **502**, 367–382
- Anwar, M. & Prebble, J. (1977) *Photochem. Photobiol.* **26**, 475–481
- Anwar, M., Hasan-Khan, T., Prebble J. & Zagalsky, P. F. (1977) *Nature (London)* **270**, 538–539
- Ascenzi, J. M. & Cooney, J. J. (1975) *Photochem. Photobiol.* **21**, 307–311
- Barratt, R. W. & Ogata, W. N. (1968) *Neurospora Newsl.* **13**, 25–95
- Blanc, P. L., Tuveson, R. W. & Sargent, M. L. (1976) *J. Bacteriol.* **125**, 616–625
- Codd, G. A. (1972) *FEBS Lett.* **20**, 211–214
- Epel, B. L. & Butler, W. L. (1969) *Science* **166**, 621–622
- Goldstrohm, D. D. & Lilly, V. G. (1965) *Mycologia* **57**, 612–623
- Hall, D. O. & Greenawalt, J. W. (1967) *J. Gen. Microbiol.* **48**, 419–430
- Hallermayer, G. & Neupert, W. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 279–288
- Harding, R. W., Huang, P. C. & Mitchell, H. K. (1969) *Arch. Biochem. Biophys.* **129**, 696–707
- Howes, C. D., Batra, P. P. & Blakeley, C. F. (1969) *Biochim. Biophys. Acta* **189**, 298–299
- Huber, W., Ewing, G. W. & Kriger, J. (1945) *J. Am. Chem. Soc.* **67**, 609–617
- Jocelyn, P. C. (1975) *Biochim. Biophys. Acta* **369**, 427–436
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mathews, M. M. & Sistrom, W. R. (1960) *Arch. Mikrobiol.* **35**, 139–146
- Mathews-Roth, M. M. (1967) *J. Bacteriol.* **93**, 506–507
- Mathews-Roth, M. M. & Krinsky, N. I. (1970) *Photochem. Photobiol.* **11**, 419–428
- Maxwell, W. A. & Chichester, C. O. (1971) *Photochem. Photobiol.* **13**, 259–273
- Maxwell, W. A., Macmillan, J. D. & Chichester, C. O. (1966) *Photochem. Photobiol.* **5**, 567–577
- Neupert, W. & Ludwig, G. D. (1971) *Eur. J. Biochem.* **19**, 523–532
- Neupert, W., Ludwig, G. D. & Pfaller, A. (1972) in *Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E. & Siliprandi, N., eds.), pp. 559–576, Academic Press, New York
- Ninnemann, H., Butler, W. L. & Epel, B. L. (1970a) *Biochim. Biophys. Acta* **205**, 499–506
- Ninnemann, H., Butler, W. L. & Epel, B. L. (1970b) *Biochim. Biophys. Acta* **205**, 507–512
- Okamoto, H. (1970) *Methods Enzymol.* **17**, 460–463
- Prebble, J. & Huda, A. S. (1973) *Photochem. Photobiol.* **17**, 255–264
- Prebble, J. & Huda, A. S. (1977) *Arch. Microbiol.* **113**, 39–42
- Prebble, J. & Ramadan, Z. (1976) *Abstr. Int. Congr. Photobiol.* 7th abstr. P356
- Pumphrey, A. M. & Redfearn, E. R. (1960) *Biochem. J.* **76**, 61–64
- Riley, G. J. P. & Bramley, P. M. (1976) *Biochim. Biophys. Acta* **450**, 429–440
- Ryan, F. J., Beadle, G. W. & Tatum, E. L. (1943) *Am. J. Bot.* **30**, 784–799
- Wainwright, S. D. (1959) *Can. J. Biochem. Physiol.* **37**, 1417–1430
- Ziegler, D. & Rieske, J. S. (1967) *Methods Enzymol.* **10**, 231–235