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DAMAGE TO MITOCHONDRIAL ELECTRON TRANSPORT AND ENERGY COUPLING BY VISIBLE LIGHT

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Summary

The effect of treating mitochondria with visible light above 400 nm on electron transport and coupled reactions was examined. The temporal sequence of changes was: stimulation of respiration coupled to ATP synthesis, a decline in ATP synthesis, inactivation of respiration, increased ATPase activity and, later, loss of the membrane potential. Loss of respiration was principally due to inactivation of dehydrogenases. Of the components of dehydrogenase systems, flavins and quinones were most susceptible to illumination, the iron-sulfur centers were remarkably resistant to being damaged. Succinate dehydrogenase was inactivated before choline and NADH dehydrogenase. Redox reactions of cytochromes and cytochrome *c* oxidase activity were unaffected.

Inactivation was O_2-dependent and prevented by anaerobiosis or the presence of substrates for the dehydrogenases. Light in the range 400—500 nm was most effective and the presence of free flavins greatly enhanced inactivation of all of the above mitochondrial activities. This suggests that visible light mediates a flavin-photosensitized reaction that initiates damage involving participation of an activated species of oxygen in the damage propagation.

Introduction

The exposure to visible light of microorganisms, higher plants, and animal cells in culture results in inhibition of growth and respiration [1]. Our laboratory has reported [2] that human diploid cells on exposure to 400—700-nm light lose their capacity to proliferate. Furthermore, electron microscopic

Abbreviations: FCCP, *p*-trifluoromethoxy(carbonylcyanide)phenylhydrazone; CAT_{16}, 4-(cetyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

observation of four types of cells in culture indicated the presence of numerous swollen mitochondria in the illuminated cells [3].

Chance et al. [4] have shown that the absorption spectrum of whole cells is qualitatively similar to that of isolated mitochondria. Thus, visible light absorption by cells may involve mitochondrial flavins, iron-sulfur centers or hemes as endogenous photosensitizers. Indeed, our previous studies [2] indicated that maximum photokilling of WI-38 cells occurs by illumination in the wavelength region of maximum absorption by flavins. In a preliminary study we reported that exposure of mitochondrial inner membranes to visible light in the presence of exogenous flavins accelerated the rate of, but did not change appreciably the pattern of, inactivation of flavin-linked dehydrogenases and oxidases [5]. In the present study we have investigated the temporal sequence of changes induced by visible light (above 400 nm) absorbed by endogenous photosensitizers in the mitochondrial inner membrane. Most of the components of the electron transport system (flavin enzymes, quinones, cytochromes and iron-sulfur centers) and of the ATPase-ATP synthetase system were examined.

Methods

Preparations.

Mitochondria were isolated from rat liver according to established procedures [6], in a medium of 0.25 M sucrose, 1 mM Tris and 1 mM EDTA (pH 7.4). For the last two centrifugations, the mitochondria were washed in 0.25 M sucrose and finally resuspended in this medium at a concentration of 80 mg protein/ml. Submitochondrial preparations were prepared by sonicating mitochondria at 20 kHz in 30 mM phosphate buffer (pH 7.4) during 30-s periods for a total of 2 min. Unbroken mitochondria were removed by centrifugation at 8500 $\times g$ for 10 min, and the supernatant was then centrifuged down at 100 000 $\times g$ for 60 min. The submitochondrial preparation was obtained by resuspending the pellet in 30 mM phosphate buffer.

Illumination.

Incubation of dark and light treated samples (10 ml at 20 mg protein/ml in 0.25 sucrose) was carried out in 50 ml conical flasks at 10°C in a specially-designed shaking water bath. The light source was a battery of 250-W quartz iodide lamps (General Electric) and the net light intensity, as measured by a YSI-Kettering (Model 65) radiometer using Kodak neutral density filters, was 300 mW/cm^2 at the sample level. The samples were located at a distance of 20 cm from the light source which was covered by a 400-nm cut-off filter, Corning Glass No. 3389, used to eliminate ultraviolet radiation. The flasks were shaken constantly during illumination. Aliquots were withdrawn at various time intervals and assayed at room temperature.

Respiration.

Respiration was assayed at mitochondrial protein concentrations of 3 mg/ml in a Rank Oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). The basic reaction medium consisted of 240 mM sucrose, 2 mM KH_2PO_4, 2 mM KCl and 5 mM Tris · HCl buffer (pH 7.4). Addition of various substrates (final

concentration of 5 mM) was made with a syringe and rates were recorded. All substrates were adjusted to pH 7.4 with a Tris buffer; durohydroquinone solutions were prepared in dimethylformamide each day before use. The rate of oxygen uptake was expressed as μatoms of oxygen consumed/min per mg protein. Respiratory control ratio was calculated from the rate of respiration with substrate plus ADP over the rate without ADP.

ATP synthesis and hydrolysis.

The standard assay medium for measurement of ATP synthesis contained 4 mM Tris · HCl buffer (pH 7.4), 10 mM potassium phosphate buffer (pH 7.4) containing $^{32}P_i$ ($2 \cdot 10^6$ cpm/μmol), 100 mM KCl, 8 mM $MgCl_2$, 0.1 mM EDTA neutralized by Tris (pH 7.4), 20 mM glucose, 0.5 mg of hexokinase/ml, 1 mM ADP, 5 mM glutamate and 5 mM malate in a final volume of 3 ml at 25° C. The experiment was initiated by the addition of mitochondria to a final concentration of approx. 6 mg protein/ml. At the exact moment at which the sample went anaerobic the reaction was stopped by the addition of 0.3 ml of 50% (w/v) trichloroacetic acid. After centrifugation (5000 × *g* for 10 min), 1 ml of the supernatant was removed and the amount of $^{32}P_i$ esterified determined by the method of Avron [7]. The P : O ratio was calculated as the ratio between the total amount of phosphate esterified to the total amount of oxygen used in the time interval between the addition of mitochondria and the addition of trichloroacetic acid.

ATP hydrolysis was determined from the amount of phosphate released in a reaction medium consisting of 250 mM sucrose, 0.25 mM $MgCl_2$, 2 mM KCl, 10 mM Tris · HCl (pH 7.4) and 0.1 mg mitochondrial protein in a total volume of 2 ml. The reaction was initiated by the addition of ATP (6 mM at pH 7.4) and carried out at 30° C for 20 min, after which it was stopped by the addition of 0.1 ml of 40% (w/v) ice-cold trichloroacetic acid; the sample was centrifuged (5000 × *g* for 10 min) and the amount of phosphate in the supernatant determined by the method of Lindberg and Ernster [8]. Specific activity was expressed in nmol of phosphate released/min per mg protein.

Transmembrane potential.

Changes in membrane potential in mitochondrial suspensions were estimated by following fluorescence changes with the cyanine dye, 3,3′-dipropylthiodicarbocyanine iodide at 620 nm excitation/670 nm emission in a Perkin Elmer MPF 44-A spectrofluorometer using a slit width of 5 nm [9]. The complete reaction medium contained 240 mM sucrose, 10 mM Tris · HCl (pH 7.4), cyanine dye at 13 nmol/mg protein and 0.75 mg mitochondrial protein in a final volume of 3 ml. Changes in the fluorescence intensity were recorded after the successive additions of 5 mM glutamate plus 5 mM malate for the energized condition and 0.6 μM *p*-trifluoromethoxy(carbonylcyanide)phenylhydrazone (FCCP) for the deenergized condition.

Spin label assay.

The reduction of the spin label 4-(cetyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide (CAT_{16}) by electron transport from various substrates was followed by a Varian E-109 spectrometer as previously

described [10]. CAT_{16} is an analogue of cetyl trimethyl ammonium bromide which partitions totally in the membrane; it was synthesized by Dr. R.J. Mehlhorn of our laboratory. The reaction medium used was identical to that used for assaying fluorescence changes above but using a submitochondrial preparation protein concentration of 6 mg/ml and a spin label concentration of approx. 15 nmol/mg protein.

Spectrophotometric assays.

Succinate, choline and NADH dehydrogenase activities were determined at room temperature using a Cary-14 spectrophotometer. Succinate dehydrogenase activity was measured by the phenazine methosulfate mediated reduction of 2,6-dichlorophenol indophenol as in [11] except that a fixed phenazine methosulfate concentration was used. The assay mixture contained 50 mM phosphate buffer (pH 7.8), 0.16 mM 2,6-dichlorophenol indophenol, 0.2 mM phenazine methosulfate, 2 mM KCN and 1 μg antimycin A in a final reaction volume of 1.0 ml. The reaction was followed at 600 nm by the addition of 10 mM succinate in the control. Choline dehydrogenase was measured as above but with 10 mM choline chloride as substrate.

NADH dehydrogenase was measured by following the reduction of $K_3Fe(CN)_6$ at 420 nm as in [12]. The assay mixture contained 4 mM Tris · HCl buffer (pH 7.8), 0.75 mM KCN, 1.33 mM $K_3Fe(CN)_6$ and 1 μg antimycin A in a final reaction volume of 1 ml. The reaction was started by the addition of 0.045 μmol of fresh NADH.

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

Determination of Fe-S centers.

EPR spectra of iron-sulfur centers were obtained using a Air Products Co. Heli-Tran LTD-3-110 liquid helium refrigeration system with a Varian E-109 spectrometer. Samples in quartz tubes of 3.0-mm internal diameter were frozen in liquid nitrogen prior to measurements. Samples of submitochondrial preparations at a protein concentration of 20 mg/ml were prepared both unreduced for measurement of center S-3, and reduced with 5 mM dithionite and 5 mM NADH for 2 min at 20° C for measurements of signals due to other iron-sulfur centers. Further reduction with excess dithionite did not increase the size of the EPR signals.

Determination of flavins.

Both bound and free flavins were determined spectrofluorometrically according to the procedure of Singer et al. [13]. Acid extractable flavins were isolated by precipitation of submitochondrial preparations with 5% trichloroacetic acid. One half of the supernatant was neutralized immediately with 2 M $KHCO_3$ to pH 7.4, while the other half was incubated at 38° C for 20 h and then neutralized with 2 M $KHCO_3$ to pH 7.4. Acid non-extractable (covalently bound) flavins were isolated from the trichloroacetic acid pellet by proteolytic hydrolysis [13]. Fluorescence of flavins was determined at 375 nm excitation/526 nm emission with a slit width of 5 nm.

Results

Oxygen uptake by mitochondria was recorded with substrates which enter the electron transport chain at various points. The coupled respiration of NAD^+ and flavin dehydrogenase-linked substrates (State 4) increased by as much as 3-fold during the first 5—7 h of illumination, almost reaching the value of ADP-stimulated rate (Fig. 1A). This indicates that the mitochondria were becoming uncoupled. Subsequently the rate of respiration decreased, becoming almost completely inhibited after 12 h of illumination. Under the same conditions the ADP-stimulated respiration (State 3) decreased steadily from the start of light treatment without showing the initial stimulatory phase. The activity of dark controls remained essentially unchanged during the 12 h of incubation. The State 4 respiration with durohydroquinone as substrate did not decrease significantly; however, State 3 respiration decreased (Fig. 1B). Cytochrome oxidase activity (substrate : ascorbate + TMPD) was not significantly affected by light exposure. The respiratory control ratio (State 3/State 4), an index of electrochemical coupling due to oxidative phosphorylation across the inner membrane

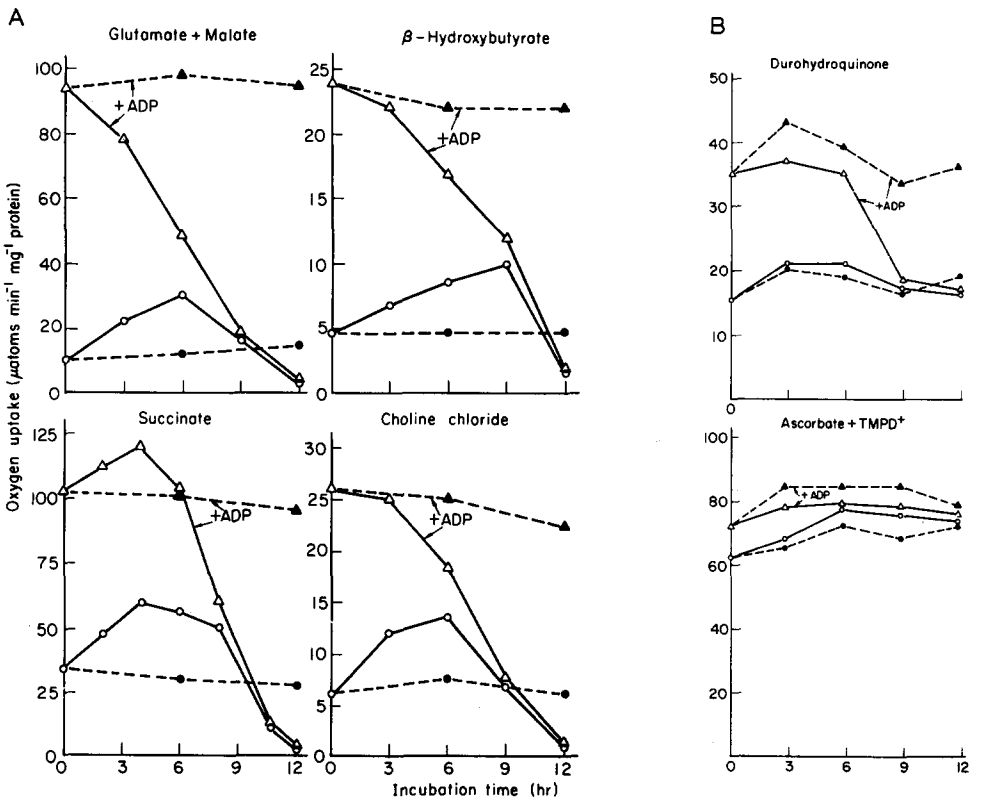

Fig. 1. Effect of time of illumination on mitochondrial respiration. (△———△, ○———○) represent illuminated samples and (▲———▲, ●———●) represent dark controls. A. Natural substrates reacting with dehydrogenases. B. Artificial substrates reacting with ubiquinone and cytochrome oxidase. Where indicated, 1 mM ADP, 1 mM durohydroquinone or 1 mM ascorbate + 0.1 mM TMPD were present.

was the first parameter to change; it declined rapidly during the first 3—6 h of illumination. These results suggest that during the first 6 h of illumination the mitochondria become uncoupled. To confirm this it was decided to assay directly ATP synthesis and hydrolysis.

ATP synthesis and hydrolysis

The rate of ATP synthesis showed (Fig. 2) a steady decline over 12 h of illumination. At 12 h of illumination the P : O ratio with glutamate plus malate as substrate was less than 1. It can be seen that the decline in the respiratory control ratio preceded that of the P : O ratio. A progressive stimulation of ATPase activity was observed during the first 6 h of illumination remaining more or less at this maximal level thereafter (Fig. 2). The maximum stimulation of ATPase activity observed was similar to that measured in the presence of the uncoupler FCCP in the non-illuminated mitochondria.

Transmembrane potential

A more direct measure of electrochemical coupling due to ion permeability across mitochondrial membranes is afforded by partitioning of charged dye cations. Mitochondria were energized either by succinate or by glutamate plus malate addition, and the fluorescence quenching of the cyanine dye, 3,3′-dipropylthiodicarbocyanine iodide, was followed. This measure of transmembrane potential remained unaffected during the first 6 h of illumination but thereafter rapidly declined (Fig. 2).

Destruction of various respiratory components

Dehydrogenases. Illumination for 12 h caused 90% inactivation of succinate dehydrogenase activity. Under these conditions 65% of choline dehydrogenase and only 40% of NADH dehydrogenase activity was destroyed (Fig. 3).

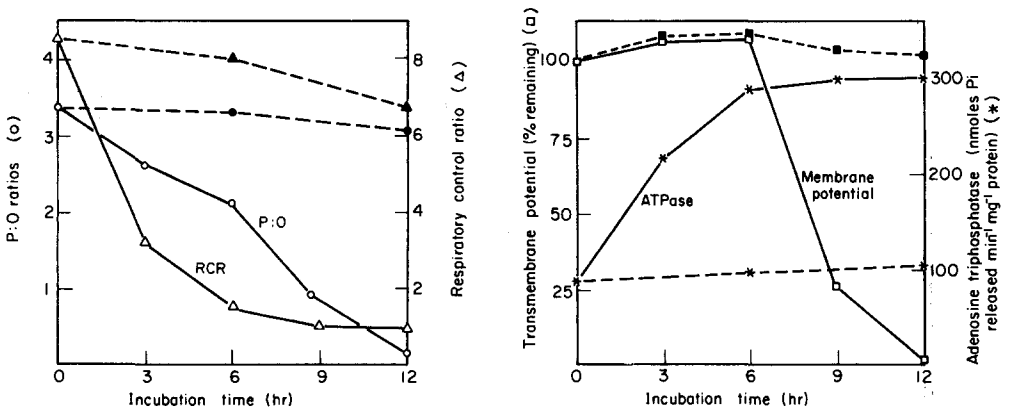

Fig. 2. Effect of time of illumination on coupled respiration, oxidative phosphorylation, and electrochemical coupling. Solid lines represent illuminated and dotted lines dark control samples. Glutamate + malate was used as substrate. RCR (respiratory control ratio).

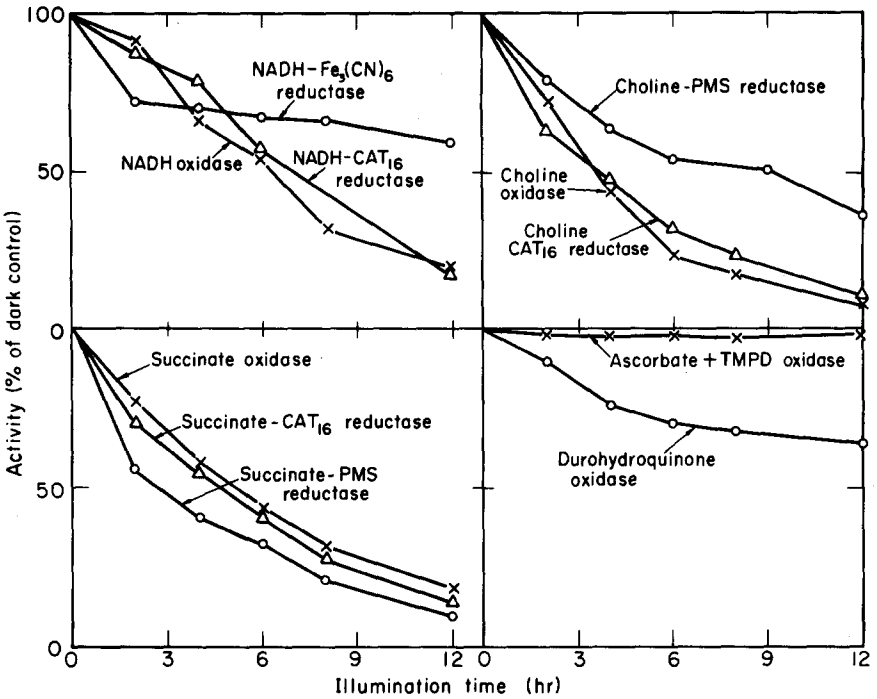

Fig. 3. Comparison of the effects of illumination time on dehydrogenase, CAT_{16} reductase and oxidase activities in submitochondrial preparations. PMS (phenazine methosulfate).

Although the rates of inactivation of various dehydrogenases were different, the rates of inactivation of the corresponding oxidase activities were all similar. Approx. 80% of the activity of the succinate, choline and NADH oxidases was lost in 12 h of illumination.

Quinones. The spin label CAT_{16} accepts electrons from the ubiquinone part of the electron transport chain [10]. CAT_{16} reductase activity with different substrates was inactivated in parallel with the oxidase activity which implies that illumination leads to inactivation of ubiquinone. Other evidence indicating that the ubiquinone region of the respiratory chain is damaged by visible light is that oxidation of durohydroquinone, a direct electron donor to the quinone region, was inactivated by 35% over a 12 h period (Fig. 3).

This conclusion is also supported by the rates of inactivation of NADH → O_2, NADH → CAT_{16} and NADH → $K_3Fe(CN)_6$. Those parts of the electron transport chain that involve ubiquinone, namely NADH → O_2 and NADH → CAT_{16}, were inactivated at the same rate, while NADH → $K_3Fe(CN)_6$, which does not require ubiquinone, was inactivated at a slower rate. This indicates that after exposure to light, damage to ubiquinone is probably the rate limiting step in the flow of electrons from NADH to CAT_{16} or to oxygen. A similar situation holds for choline oxidation. In the case of succinate the rates of inactivation of succinate → O_2, succinate → CAT_{16} and succinate → phenazine methosulfate were identical indicating that the rate of inactivation of succinate dehydrogenase was $\geqslant$ to that of the ubiquinone.

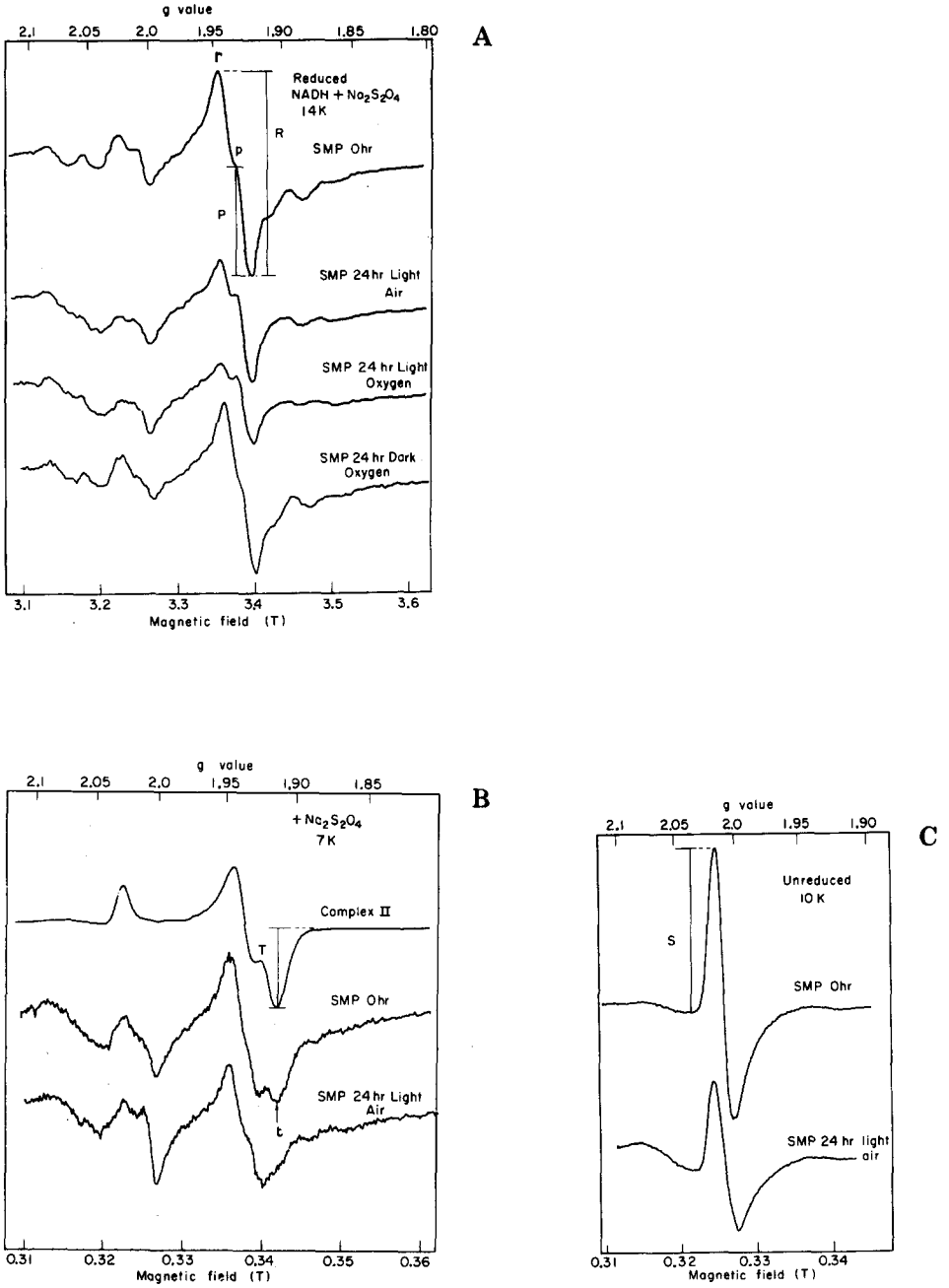

Fig. 4. EPR spectra of submitochondrial preparations (SMP) showing the effects of illumination on iron-sulfur centers. A. Samples reduced with NADH and dithionite measured at 14 K. B. Dithionite reduced samples at 7 K. C. Unreduced samples at 10 K. Samples under oxygen were in sealed flasks which were purged with O_2 for 2 min through an air-tight rubber stopper at the start of experiment and again after 12 h. Other conditions of measurements were: microwave power, 10 mW; frequency, 9.14 GHz; modulation amplitude, 1 mT; frequency, 100 KHz. The features P, R, S and T recorded in Table I were measured as shown.

Iron-sulfur centers. EPR spectra of reduced rat liver submitochondrial preparations, and the effects of illumination treatment are shown in Fig. 4. The spectra of reduced iron-sulfur centers of mitochondria are now well characterized [14]. We use the nomenclature of Ohnishi [15], by which the prefixes N- and S- refer to centers in Complexes I and II, respectively.

At 14 K, signals due to the Rieske Fe-S center and the outer membrane iron-sulfur center are saturated out and the principal signals observed are those due to NADH : ubiquinone reductase (Complex I), succinate dehydrogenase (Complex II) and electron-transfering flavoprotein dehydrogenase. These signals overlap to give the composite spectrum of Fig. 4A. Illumination under air of submitochondrial preparations up to 12 h had only a very small effect on the spectrum of the Fe-S centers. Further illumination of submitochondrial preparations for periods up to 24 h under air or oxygen did however give rise to selective loss of certain features of the spectrum. By contrast, incubation for 24 h in the dark under oxygen caused a smaller, nonspecific loss of all of the EPR signals; this loss was less pronounced under air (not shown). The selective loss in the iron-sulfur centers is indicated by the change in shape of the features around $g = 1.94$. The height of the derivative peak, labeled r, relative to the shoulder, p, was drastically reduced.

R is a measure of the general iron-sulfur signal intensity. It contains contributions from centers N-1a, N-1b, N-3, N-4, S-1, S-2 and the electron transfering flavoprotein dehydrogenase. Table I summarizes the effect of illumination on the sizes of the two signals, *P* and *R*, measured as shown in Fig. 4A. The feature *P* at $g = 1.93$ was much less affected than *R*. This feature (*P*) is normally due to center N-2. However, modification of center N-2 was occurring upon illumination because a loss of the peak at $g = 2.05$, which is also characteristic of this center, was observed (Fig. 4A).

At 7 K, spectra of several of the centers became saturated with microwave power, and a negative feature around $g = 1.91$ labeled t became more prominent (Fig. 4B). None of the centers of Complex I has a *g* value at this position [16]. The most likely origin of this feature is center S-2 of succinate dehydrogenase (Complex II). A spectrum of dithionite-reduced purified beef heart Complex II (a kind gift from Dr. T.P. Singer) is included for comparison. At

TABLE I

SUMMARY OF VARIOUS ILLUMINATION TREATMENTS ON THE FEATURES OF IRON-SULFUR EPR SPECTRA

Where indicated samples contained 50 mM succinate, 25 μg/ml antimycin A and 10 mM KCN; a further 50 mM succinate was added after 12 h. Where present FMN was 20 μg/ml. Signals were measured as shown in Fig. 4 and are expressed as percentage of dark controls. Less than 10% of the signal intensity was lost in the dark controls during the 24-h incubation.

Conditions	Illumination (h)	Feature			
		R	P	S	T
Air	24	71	88	57	65
Oxygen	24	51	76	21	37
Air with succinate + antimycin A + KCN	24	95	100	—	92
Air with FMN	4	79	96	55	47

this temperature the signal is principally due to reduced S-2. For Table I, the signal was measured by the depth below the baseline (feature *T*).

Center S-3 of succinate dehydrogenase can be identified unequivocally since it gives an EPR signal around $g = 2.01$ in the oxidized state (in contrast the other centers can only be observed in the reduced state). This signal S-3 is observed at temperatures below 20 K (Fig. 4C). The soluble "HIPIP-type" protein in mitochondria which gives a similar signal [17] is known to be lost in submitochondrial preparations. On illumination this signal decreased in size and also changed slightly in line shape, indicating that the environment of the center was slightly modified. To assess the size of this signal, the height of the derivative peak at $g = 2.02$ from the baseline was measured as illustrated. This avoids interference from free radicals.

Table I shows that the features of the EPR spectra were destroyed at different rates. *P*, the signal at $g = 1.93$, was relatively stable. Features *S* and *T*, attributed to succinate dehydrogenase were relatively labile.

Under conditions when some components are saturated, and when "passage" conditions are likely to arise as at 7 K and 10 mW, EPR signals may be distorted. Accurate quantitative evaluations in such a multicomponent system are difficult to obtain. Our results do however show that a significant change in EPR spectra only occurs after long periods of illumination (>12 h).

Endogenous cytochromes and flavins. Cytochromes as determined from their absorption spectra were inactivated by illumination at a slower rate than the other chromophores in mitochondria (not shown). No significant loss of durohydroquinone reducible cytochromes *b*-562, *b*-568, *c*-550, or *a*-605 was observed after 12 h illumination.

However, exposure of submitochodrial preparations to visible light caused considerable destruction of flavins. The acid-extractable FAD and FMN and covalently bound FAD were destroyed at similar rates. About 50% of each of the above types of flavins were lost over 12 h of illumination.

Factors influencing the rate of inactivation

Wavelength dependence. Several filters of broad bandwidth were used; this was necessary to obtain measurable rates of inactivation of succinate dehydrogenase at identical conditions of treatment. Light-intensity compensated rates of inactivation were as follows: 350—600 nm = 1.5; 430—500 nm = 1.1, and 580—800 nm = 0.05, relative to the rate in regular white light which was taken as unity.

Exogenous sensitizers. Inactivation of succinate oxidase in submitochondrial preparations was enhanced by the exogenous addition of flavins and several other photosensitizers (Fig. 5). Cytochrome *c* was ineffective. FMN and riboflavin were more effective than FAD. Within 30 min of illumination, riboflavin at 20 μM caused destruction of 90% of succinate oxidase activity. Added FMN caused considerable destruction of the iron-sulfur EPR signals within 4 h (Table I).

Oxygen concentration. Inactivation of succinate dehydrogenase in submitochondrial preparations was also enhanced by incubating the samples under 100% oxygen during the illumination treatment: 81% inactivation was observed after 3 h under 100% oxygen whereas only 55% inactivation occurred under

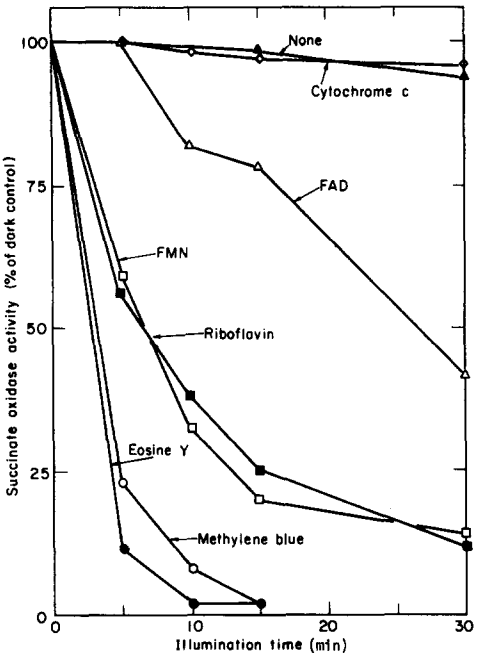

Fig. 5. Effect of various exogenous photosensitizers on the rate of inactivation of succinate oxidase in illuminated submitochondrial preparations. Cytochrome *c* was at 100 μM concentration; other substances were at 20 μM.

TABLE II

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

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

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

* 100% corresponds to activity at zero time.

TABLE III

PROTECTION OF VARIOUS DEHYDROGENASES AND UBIQUINONE AGAINST LIGHT DEPENDENT INACTIVATION BY VARIOUS RESPIRATORY SUBSTRATES AND INHIBITORS

Incubation conditions (8 h)	Residual activity (percent of dark control)	
	CAT_{16} reduction	Dehydrogenase
1. Protection by succinate	NADH → CAT_{16}	Succinate dehydrogenase
a. None	40	30
b. Succinate + Antimycin A	85	88
c. Succinate + thenoyl trifluoroacetone	30	90
2. Protection by choline	Choline → CAT_{16}	Choline dehydrogenase
a. None	17	50
b. Choline + Antimycin A	72	70
c. Choline + amytal	15	75
3. Protection by NADH	NADH → CAT_{16}	NADH dehydrogenase
a. None	36	70
b. NADH + Antimycin A	56	77
c. NADH + Rotenone	21	73

20% oxygen or air. Oxygen also enhanced the destruction of some of the features of the iron-sulfur EPR spectra (Table I). No significant inactivation of any respiratory component was observed under a 100% N_2 atmosphere (Table II).

Respiratory chain substrates and inhibitors. In submitochondrial preparations the sites of interaction of substrates are on the outer exposed surface of the membrane. In this system the protective effect of substrates can be directly measured without consideration of substrate penetration or transport. When succinate was present during illumination, succinate dehydrogenase was completely protected against inactivation. The electron transport inhibitors KCN and antimycin A were added to prevent succinate respiration during the 8-h illumination period; these had no effects in the absence of succinate. Partial protection of choline dehydrogenase was also observed when illumination was carried out in the presence of choline (Table III). However, little protection of NADH dehydrogenase was afforded by NADH. Protection of ubiquinone was observed when illumination was carried out in the presence of succinate plus antimycin A, which keeps ubiquinone in the reduced state. No protection of ubiquinone was observed in the presence of succinate plus thenoyl trifluoracetone when ubiquinone would be in the oxidized state.

Succinate plus antimycin A and KCN protected all the features of the iron-sulfur EPR spectra (Table I) even though we would not expect all the centers of Complex I to be in the reduced state.

Discussion

Our results indicate that visible light in the presence of oxygen inactivates the electron transport and energy coupling mechanisms in the inner membrane of mitochondria.

Electron transport

The respiratory chain shows a pattern of inactivation by visible light in the following order of sensitivity: succinate dehydrogenase $\geqslant$ ubiquinone > choline dehydrogenase > NADH dehydrogenase > iron-sulfur centers. The cytochromes do not seem to be affected.

The high sensitivity of succinate dehydrogenase may arise from the covalent binding of the flavin moiety to a histidyl residue of the enzyme [18] which is essential for activity. Bound or free flavin-sensitized photo oxidation of histidine is well known [19]. In NADH and choline dehydrogenase the flavin is not covalently bound to histidine which may account for their relatively lower sensitivity to visible light. A previous report [20] has also shown that the NADH dehydrogenase (Complex I) of beef heart mitochondria is less sensitive to blue light than the succinate dehydrogenase of Complex II.

Succinate and NADH-ubiquinone reductases are complex enzymes containing a number of electron transferring groups, i.e. flavin, iron-sulfur centers and possibly protein-bound ubiquinone. The activity of enzymes of this type may depend on the integrity of all their components, making their rate of inactivation more rapid than the rate of loss of any constituent; it is not surprising then to find, for example, that after 12 h of illumination the 90% loss of activity of succinate dehydrogenase (Fig. 3) is greater than the loss of bound FAD (50%) or centers S-2 and S-3 ($\approx$20%). The contribution of the iron-sulfur centers may be relatively more significant in NADH dehydrogenase.

The inactivation of ubiquinone by near ultraviolet light has been reported [21] and it has also been shown that pure ubiquinone is 4—8-times more sensitive to visible light than ubiquinone in the cells [22]. Several polar photoproducts of ubiquinone have been separated on thin layer chromatography.

The NADH and choline oxidases follow the same inactivation curves as the NADH-CAT_{16} and choline-CAT_{16} reductases, respectively (Fig. 3) while the corresponding NADH-$Fe(CN)_6$ and choline-phenazine methosulfate reductase get inactivated much more slowly as a function of illumination. Since CAT_{16} accepts electrons from ubiquinone [10] these results may indicate a rapid inactivation of the ubiquinone. Succinate oxidase and succinate-phenazine methosulphate or -CAT_{16} reductase follow roughly the same rates of rapid inactivation, making it difficult to distinguish the effect on the ubiquinone pool.

Previous studies of Ninneman et al. [23] have shown the selective destruction of cytochrome a_3 and inactivation of succinate oxidase in beef heart mitochondria by blue light in the wavelength range 330 nm $< \lambda <$ 540 nm (200 mW/cm^2) when illuminated at low protein concentrations ($\approx$0.2 mg/ml). Succinate dehydrogenase activity was not reported. By contrast, in this study white light with wavelengths $\lambda >$ 400 nm (300 mW/cm^2) was used and the mitochondria illuminated at protein concentrations 100-fold higher. Thus, the conditions employed in this investigation enable us to detect earlier stages of visible light inactivation which involve flavin photodestruction. Cytochrome destruction is probably a later event.

The stability of the iron-sulfur centers and in particular S-3 is somewhat surprising; in purified succinate dehydrogenase this center is rapidly destroyed unless succinate is present [24].

The evidence for flavins appearing as the endogenous photosensitizers for

the primary processes of inactivation of the respiratory chain is as follows.

a. All the inactivated dehydrogenases are flavin enzymes. This is consistent with the sensitivity to light of purified flavin enzymes [25].

b. All types of endogenous flavins of the mitochondria were gradually lost during illumination.

c. The addition of exogenous flavins greatly enhanced the rate but did not change the pattern of inactivation of the flavin enzymes, iron-sulfur centers and ubiquinone.

d. The wavelength dependence for inactivation indicates that the major inactivation occurs where flavins absorb.

e. Reduction of flavins during illumination by incubation of mitochondria in the presence of substrates and electron transport inhibitors protects against inactivation; this is expected since flavins in their reduced state absorb much less visible light.

Photosensitization by other components of the respiratory chain appears to be of secondary importance, at least until the flavins have been destroyed. For example, the rate of inactivation of the respiratory chain did not seem to be affected by the presence of exogenous added cytochrome *c* which absorbs light very strongly around 415 nm or even by the depletion of mitochondrial cytochrome *c*.

In the presence of substrates and respiratory inhibitors both the dehydrogenases and the ubiquinone will be protected if kept in the reduced state (in the presence of antimycin A) during illumination. In the presence of thenoyl trifluoroacetone, Rotenone or Amytal which inhibit electron flow before the ubiquinone site, the succinate, NADH and choline dehydrogenases respectively will be protected, but not the ubiquinone. This may be due to the fact that the reduced species of flavins and quinones absorb much less visible light than their oxidized species. It is possible that the binding of substrate to the active site may also help to stabilize the protein. Ray and Koshland [26] have reported that substrate binding to phosphoglucomutase protects various amino acids of the active site against methylene blue-sensitized damage.

In addition to visible light and a photosensitizer, the inactivation of respiratory functions may also require the presence of oxygen. Inactivation did not occur under nitrogen and was significantly enhanced by increasing the oxygen concentration in the gas phase. This may indicate the likely involvement of some activated species of oxygen, either superoxide radical, singlet oxygen or the hydroxyl radical, species that are known to cause damage to membrane proteins and lipids [27]. It is well known that eosine Y and methylene blue are photogenerators of singlet oxygen [28] and also that flavin-dependent photochemical reactions generate both singlet oxygen and superoxide O_2^- [29]. In the presence of EDTA and light, flavins will generate mainly O_2^- [30]. Eosine Y and methylene blue dramatically accelerate the light-dependent inactivation of succinate dehydrogenase (Fig. 5). Also, EDTA protects and purified superoxide dismutase does not prevent inactivation of the succinate dehydrogenase (data not shown). On the other hand, the lack of inactivation under anaerobic conditions may simply reflect the fact that the flavins are in their reduced state and will not absorb light, so that the dehydrogenases will not be inactivated.

Energy coupling

The inactivation of energy transducing functions of the mitochondria appears concomitantly with the inactivation of flavoprotein dehydrogenases (at approximately the same rate as the inactivation of the succinate dehydrogenase). Loss of energy coupling is shown by a decline in respiratory control ratio, ATP synthesis and transmembrane potential and an increase in ATP hydrolysis (Fig. 2). It is interesting to note that the P : O ratio and the respiratory control ratio decrease very significantly and lipid peroxidation increases during the first hours of illumination [3] while the ability of the mitochondrial membrane to maintain electrochemical gradients decreases only after approx. 6 h of illumination: this suggests specific effects on the ATPase-ATP synthetase protein complex or its coupling to the electron transport chain, and that even though ionic gradients can still be formed they can no longer be used for making ATP. Thus, in future studies we plan to clarify the role of lipids and identify the nature of the free radical involved in damage propagation so as to be able to develop a strategy for protection.

Note added in proof (Received February 21st, 1978)

A paper by Anwar and Prebble [31] has appeared recently which shows that the photo inactivation of the respiratory chain in *Sarcina lutea* (*Micrococcus luteus*) can be ascribed mainly to an inactivation of flavin containing dehydrogenases and the destruction of menaquinone (vitamin K-2).

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