Biochimica et Biophysica Acta, 387 (1975) 451–460

BBA 46936

TWO MECHANISMS OF NEAR-ULTRAVIOLET LETHALITY IN *SACCHAROMYCES CEREVISIAE*: A RESPIRATORY CAPACITY-DEPENDENT AND AN IRREVERSIBLE INACTIVATION

F. FONG*, JULIUS PETERS**, CRELLIN PAULING and R. L. HEATH

Department of Biology, University of California, Riverside, Calif. 92502 (U.S.A.)

(Received December 2nd, 1974)

SUMMARY

Near-ultraviolet irradiation of actively growing yeast cells leads to cell death by two distinct mechanisms. The first type of cell death is evident after low doses of near-ultraviolet light ($3 \cdot 10^4$ ergs $\cdot$ mm^{-2}) and is due to a reversible inactivation of the respiratory capacity of the cell. In studies with yeast mitochondrial membranes the quinones were identified as the site of inactivation by determining the relative levels of the following oxidase activities after irradiation: exogenous NADH, endogenous NADH (via isocitrate dehydrogenase), succinate, and D-lactate oxidases. A second type of cell death is caused after high doses ($1.8 \cdot 10^5$ ergs $\cdot$ mm^{-2}) and is irreversible. The mechanism of this inactivation is unknown.

INTRODUCTION

Irradiation with near-ultraviolet light (320–380 nm) is lethal to bacteria [1]; however, the mechanism of killing is distinct from that of far ultraviolet light (200–300 nm) [1–4]. Sublethal doses of near-ultraviolet light inactivate oxidative energy metabolism in *Escherichia coli* with vitamin K and coenzyme Q being the most labile components of the respiratory chain [5–7]. In addition to the inactivation of the quinones in *Mycobacterium phlei*, the photodestruction of a water-soluble "succino-oxidase factor" occurs [8]. Quinones have also been shown to be the primary near-ultraviolet light target in the inhibition of oxidative phosphorylation in rat liver mitochondria [9] and in *Rhodopseudomonas palustris* [10].

Saccharomyces cerevisiae was chosen for this study because of the extensive biochemical information which is available in this eucaryotic system. In this study we distinguish two types of near-ultraviolet lethality in yeast. One is reversible and is detectable when irradiated cells are plated with non-fermentable carbon sources. The other is irreversible, and requires relatively higher energy doses than the reversible lethality. The reversible lethality is shown to be related to the photodestruction of

* Present address: Department of Biology, Brandeis University, Waltham, Mass. 02154, U.S.A.
** Present address: Department of Medical Microbiology, California College of Medicine, University of California, Irvine, Calif. 92664, U.S.A.

the electron transport system, as examined in vivo and in vitro with a variety of electron donors.

MATERIALS AND METHODS

Organisms and culture conditions. *S. cerevisiae*, haploid strain X174, was obtained from H.L. Roman. Cultures were grown at 30 °C in defined medium containing, per l: 10 g yeast nitrogen base (Difco) and 5 g glucose. Viability was determined by plating appropriate dilutions in triplicate on yeast-peptone medium [11] containing either glucose (2 %), glycerol (2 %), or sodium lactate (0.8 %) as the carbon and energy source.

Respiration deficient mutants, petites, were identified by small colony formation on yeast-peptone-glucose medium and confirmed by the inability to form colonies on yeast-peptone-lactate medium [11].

Isolation of the mitochondrial fragments. Cells were grown in the yeast-peptone medium with the following modifications: omission of agar and reduction of the glucose to 5 g · l^{-1}. The cells were and washed resuspended in 25 ml of grinding medium containing: 0.3 M mannitol, 0.02 M sodium phosphate buffer (pH 7.0), 10^{-4} M disodium EDTA, and 0.4 % bovine serum albumin. The cells were then lysed with 35 g of fine glass beads using a Bronwill MSK tissue homogenizer [12], which results in approx. 75–80 % cell breakage as estimated by light microscopy.

The crude homogenate was centrifuged at 2000 x *g* for 10 min to pellet the cell debris, intact cells and beads. The 10 000 x *g* (10 min) pelleted fraction yielded only a small percentage of the total NADH oxidase activity. Thus the remaining supernatant was centrifuged at 100 000 x *g* for 60 min to pellet the remaining mitochondrial and microsomal membranes. This pelleted membrane fraction was resuspended in a solution containing: 0.3 M mannitol, 0.04 M KCl, 0.03 M MgCl, 0.01 M sodium phosphate buffer (pH 6.5). As the absolute levels of oxidase activity varied between experiments, studies on the relative photosensitivity of the different oxidase activities were performed on the same membrane preparation.

Respiration studies. The irradiated cells were harvested by centrifuging at 2000 x *g* for 5 min, and resuspending in 3 ml of fresh defined medium. The oxygen uptake was measured polarographically at 30 °C in a 3-ml stirred cell (Yellow Springs Instrument Model No. 53, Ohio, U.S.A.), with a Clark type oxygen electrode coupled to a recorder.

Irradiation of the membrane suspensions were with 10 μl of Antifoam B to control foaming during the experiment. Substrate-stimulated respiration was initiated with 30 μmol of either sodium succinate, sodium isocitrate, or calcium lactate, or 0.45 μmol of NADH [13,14].

NADH-flavin reductase assay. Flavin reduction was followed by measuring the absorbance changes of the membrane suspension at 465–510 nm at room temperature in an Amino-Chance dual beam spectrophotometer as described by Bragg and Hou [6]. The reaction was initiated with 0.1 mM NADH.

Protein was determined according to Lowry et al. [15].

Irradiation conditions. Exponential phase cells at a density of $3 \cdot 10^6$– $4 \cdot 10^6$ cells per ml were irradiated in a water-jacketed 3-ml cuvette at 25 °C by two Phillips HPW 125 W black-light lamps (365 nm maximum). The maximum dose rate was

$3 \cdot 10^3$ ergs $\cdot$ mm$^{-2} \cdot$ min^{-1}, with lower dose rates achieved by increasing the distance between the lamps and the cuvette. Dose rates were determined by ferrioxalate actinometry [16].

The Antifoam B was purchased from Sigma Chemical Co., and the coenzyme Q-10 (B-grade) from Calbiochem.

RESULTS

Whole cell studies

Fig. 1 illustrates the survival obtained upon irradiation of yeast cells with near-ultraviolet light, with aliquots plated on glucose-, glycerol-, or lactate-supplemented yeast-peptone medium. The major difference in the survival curves is the duration of the initial lag period where there was no loss of viability: 10 min for the lactate- or glycerol-supplemented media, and 50 min for the glucose-supplemented medium.

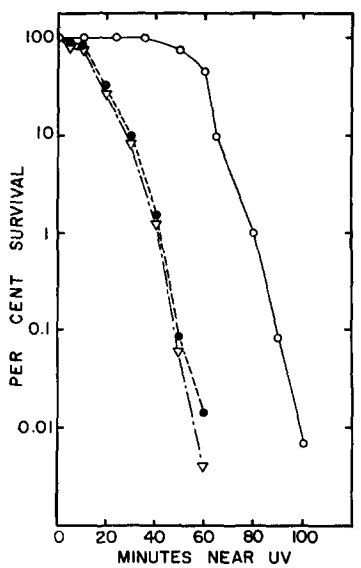

Fig. 1. Colony-forming ability of irradiated yeast. Cells were irradiated in glucose-containing medium, sampled and plated on either glucose- (○—○), lactate- (● - - -●) or glycerol- (▽- - -▽) supplemented media as described in Materials and Methods.

Cultures of this strain spontaneously give rise to 1–2 % petite colonies. The possibility that the reduction in the initial lag period observed on non-fermentable medium is caused by the near-ultraviolet light induction of respiratory mutants was eliminated by showing that the proportion of petites did not change as a function of dose (Table I).

We have determined that the reduction in the initial lag period observed on glycerol- or lactate-supplemented media is the result of a metabolic lesion by showing that this reduction could be reversed by an intervening period in glucose-supplemented medium (Fig. 2). A culture was irradiated to produce 0.1 and 100 % survival on yeast-peptone-lactate and yeast-peptone-glucose media, respectively. An aliquot

TABLE I

FREQUENCY OF RESPIRATORY MUTANTS AFTER IRRADIATION

Irradiation and plating methods were as described in Materials and Methods.

Irradiation (min)	Total numbers of colonies	Numbers of petites	Petites (%)
0	908	12	1.3
20	717	7	0.9
40	867	13	1.5
60	864	16	1.8

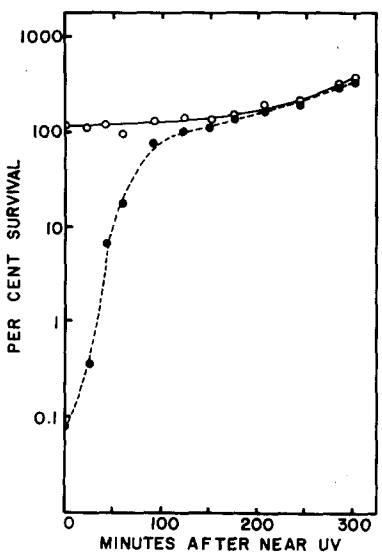

Fig. 2. Time course of recovery following irradiation of cells. Cells were irradiated, sampled and incubated with glucose medium and plated, as described in Materials and Methods. Cells plated on lactate-supplemented medium (●- - -●) or glucose-supplemented medium (○—○).

was then incubated at 30 °C in the dark with aeration in the defined medium containing 0.5 % glucose, and at intervals the viable count determined on either lactate-or glucose-supplemented yeast-peptone medium. Rapid recovery of colony forming ability on lactate-supplemented medium occurred after 120 min of incubation. During this period there was no proliferation of the remaining number of cells as determined by the number of colonies in the controls.

The cell suspension was either aerated or maintained under N_2 during the irradiation period to determine the oxygen dependence of the two types of near-ultraviolet-induced lethality. The survival after a given dose under aerobic and anaerobic irradiation conditions is compared in Fig. 3. In this normalized presentation a horizontal line would indicate complete oxygen dependence, whereas a 45 ° line would show oxygen independence. Under these conditions the ability of near-ultraviolet light to kill cells on glucose-supplemented plating medium was oxygen

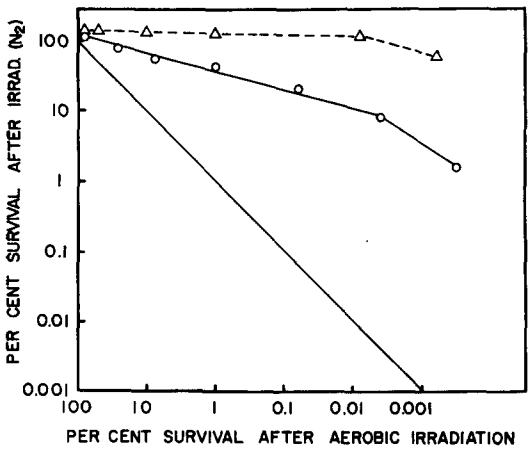

Fig. 3. Aerobic and anerobic irradiation of cells plated with glucose- or glycerol-supplemented media. Cells were irradiated aerobically or anerobically in glucose-containing medium, sampled and plated as described in Materials and Methods. Cells plated on glucose-supplemented medium (△- - -△), or glycerol-supplemented medium (○—○). Hypothetical oxygen-independent process is shown by solid line.

dependent. There was no significant loss in viability in anaerobically treated cells as compared to aerobically treated cells (0.001 % survival). The loss of viability on glycerol-supplemented plating medium was oxygen dependent as well, but to a lesser extent, in that a dose under anaerobic conditions produced 0.1 % survival whereas under aerobic conditions it produced 0.001 % survival.

The near-ultraviolet lethality observable only with non-fermentable carbon substrates indicates alterations in the respiratory capacity of the irradiated cells. Indeed, the endogenous respiration of the cells declined as a function of dose, leading ultimately to a complete loss after 90 min of irradiation (Fig. 4).

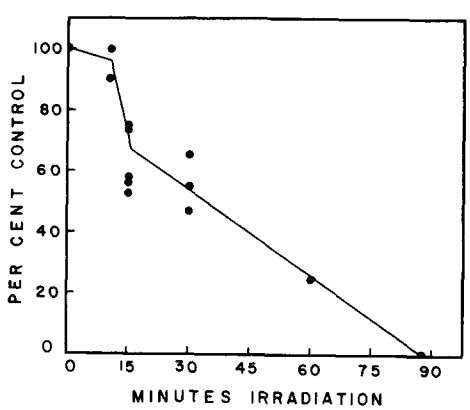

Fig. 4. Endogenous respiration in yeast cells after irradiation. Each point represents a separate culture. Control rates were determined from aliquots of the same culture but held aerobically at 25 °C untreated.

Mitochondrial studies

The nature of near-ultraviolet-induced respiratory changes can be best studied with mitochondrial membranes. Substrates are thought to enter the electron transport system of the mitochondria according to the following scheme (Fig. 5). Thus by using isocitrate, succinate, NADH or lactate as electron donors we can determine which portions of the electron transport system may be damaged by near-ultraviolet irradiation.

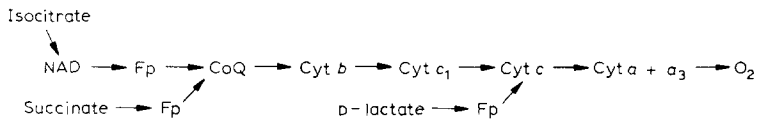

Fig. 5. Electron transport system of yeast mitochondria. Adapted from Chance et al. [20]. Fp, flavoprotein; CoQ, quinones; Cyt, cytochrome.

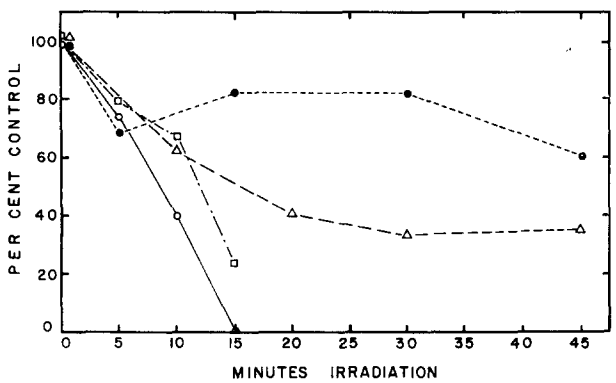

Fig. 6. Substrate-stimulated respiration of mitochondrial activities after irradiation. Respiration rates were measured as described in Materials and Methods. Control rates were as follows, in μmol O_2 consumed/min per mg protein: succinate (○—○), 3.38; NADH (△- - -△), 19.6; isocitrate (□- - -□), 2.56; and D-lactate (●- - -●), 3.38. Approx. 0.6 mg protein were used per assay.

Mitochondrial membranes were irradiated aerobically, and the different substrate-stimulated oxidase activities were measured (Fig. 6). After 15 min of near-ultraviolet at the maximum dose rate, no succino-oxidase activity remained whereas 80 % of the D-lactate oxidase, 50 % of the exogenous NADH oxidase, and 25 % of the endogenous NADH oxidase (via isocitrate) activities remained. The exogenous NADH oxidase activity remained at 40 % of its control rate with 30–45 min of additional irradiation. D-Lactate oxidase, though, declined to 70 % of its control rate after 45 min of irradiation. Control rates in all cases were determined by aerating the membrane suspension at 25 °C for the appropriate time period before assaying. Very little loss in control activity occurred after even 45 min of aeration.

If quinones are destroyed exogenously added quinones should restore the lost activity. As Table II shows, irradiation doses producing 50 or 100 % loss of succino-oxidase activity could be restored nearly completely when incubated for 5 min with 3 μM CoQ-10 and 15 μM Brij 58. On the other hand, the losses of NADH

TABLE II

RESTORATION OF INACTIVATED OXIDASE ACTIVITIES BY INCUBATION WITH QUINONES

Respiration rates were measured as described in Materials and Methods. After irradiation, the membrane suspension was incubated with 3 μM coenzyme Q-10 (Ubiquinone 50) with 15 μM Brij 58, or with Brij 58 alone, for 10 min at 30 °C prior to respiration measurements. Dose rate: $3 \cdot 10^3$ ergs $\cdot$ mm^{-2} $\cdot$ min^{-1}.

Substrate	Irradiation (min)	+Near-ultraviolet O_2 consumed (% control)	+Near-ultraviolet, CoQ-10, O_2 consumed (% control)
Succinate	10	50	100
	20	0	84
NADH	5	79	75
	5	80	79

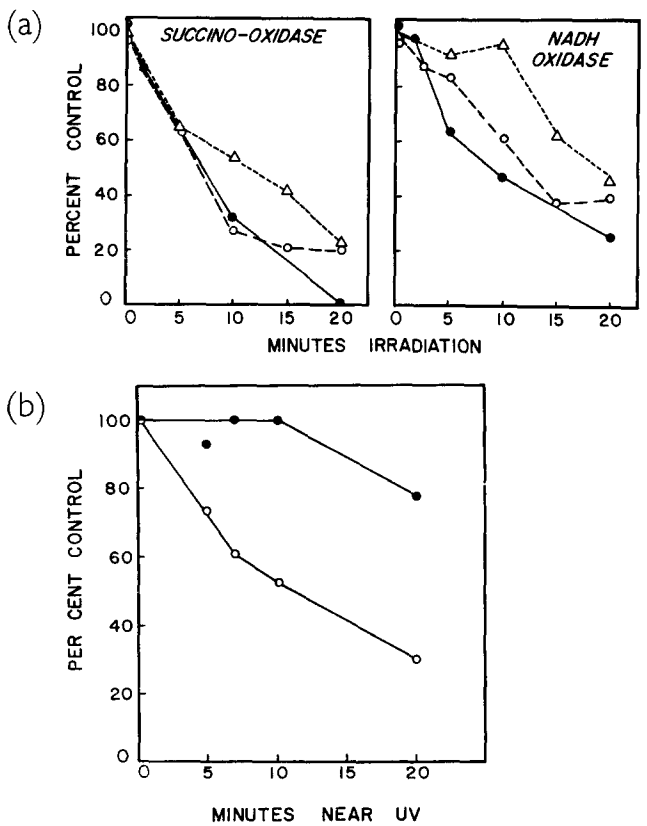

Fig. 7. (a) Intensity dependence of inactivation of succino- and NADH-oxidase activities. Membrane suspensions were irradiated with three different intensities and oxidase activities were assayed as described in Materials and Methods. Intensities used: $3 \cdot 10^3$ ergs $\cdot$ mm^{-2} $\cdot$ min^{-1} (●—●); $9 \cdot 10^2$ ergs $\cdot$ mm^{-2} $\cdot$ min^{-1} (○- - -○); and $4.5 \cdot 10^2$ ergs $\cdot$ mm^{-2} $\cdot$ min^{-1} (△- - -△). Control rates were as in Fig. 6. (b) Intensity used was $4.5 \cdot 10^2$ ergs $\cdot$ mm^{-2} $\cdot$ min^{-1}. ●—●, NADH oxidase; ○—○, succino-oxidase. Control rates were as in Fig. 6.

oxidase could not be restored under similar conditions. There was not a direct inactivation of the NADH-flavin reductase since no loss in its activity could be observed as measured by dual beam spectrophotometry [6].

By varying the intensity of irradiation, differential sensitivity of succino- and NADH oxidase activities was found. Fig. 7a shows that at three different intensities ($3 \cdot 10^3$, $9 \cdot 10^2$, and $4.5 \cdot 10^2$ ergs $\cdot$ mm$^{-2} \cdot$ min^{-1}) the inactivation of succino-oxidase activity was equivalent. But for the NADH oxidase activity, the two higher intensities produced similar inactivation rates, whereas at the lowest intensity there was a distinct lag period prior to any loss in activity (Fig. 7b).

Approx. 35–40 % of the exogenous NADH oxidase activity appeared to be near-ultraviolet resistant, as seen in Fig. 6. This activity apparently is not entirely of mitochondrial origin as seen in Table III. When comparing the proportion of near-ultraviolet-resistant oxidase activity in the membranes of decreasing sedimentation coefficient the pelleted membrane fraction after 100 000 x *g* centrifugation had greater amounts of this near-ultraviolet-resistant oxidase than in the 10 000 x *g*- pelleted fraction.

TABLE III

SUBCELLULAR LOCALIZATION OF NEAR-ULTRAVIOLET-RESISTANT OXIDASE-ACTIVITY

Membrane fractions were isolated, irradiated and assayed for oxidase activity as described in Materials and Methods. Membrane suspensions were irradiated for 45 min ($3 \cdot 10^3$ ergs $\cdot$ mm$^{-2} \cdot$ min^{-1}). The values are expressed as (μmol O_2 consumed/min per mg protein).

Membrane fraction (oxidase activity)	Control	+ Near-ultraviolet	Control (%)
10 000 × *g* (15 min)			
Succinate	2.4	0	0
NADH	14.9	1.3	8.8
100 000 × *g* (60 min)			
Succinate	2.6	0	0
NADH	16.7	3.5	21.0

DISCUSSION

Near ultraviolet-irradiated yeast cells lose viability by two distinct mechanisms. One type of cell death (low dose lethality) is manifested after $3 \cdot 10^4$ egs $\cdot$ mm^{-2}, and only when the irradiated cells are plated with a non-fermentable carbon energy source. The second mechanism of cell death (high dose lethality) is detectable after $1.8 \cdot 10^5$ ergs $\cdot$ mm^{-2}, and when the cells are plated on a fermentable carbon and energy source. During low dose lethality viability is reduced to about 0.1 % of the controls before there is any significant loss of colony forming ability by the high dose lethality (Fig. 1). This difference in survival obtained between cells plated on lactate- or glucose-based media is not due to the induction of respiration deficient mutants; the frequency of petites is not appreciably affected by doses up to $1.8 \cdot 10^5$ ergs $\cdot$ mm^{-2} (Table I). Furthermore, the low dose lethality is entirely reversible with an intermediate period of incubation with glucose- containing medium (Fig. 2). These results suggest that low dose lethality is the consequence of a metabolic lesion.

In *E. coli* [2] and in *Salmonella typhimurium* [19] near-ultraviolet light-induced lethality has been shown to be greatly enhanced by oxygenation. We have found essentially the same phenomenon with yeast; the high dose lethality was oxygen dependent, whereas the low dose lethality exhibited only partial oxygendependence (Fig. 3). This differential oxygen requirement is consistent with other evidence suggesting that different mechanisms are involved in low and high dose lethality.

Since low dose lethality appears to be the result of a reversible metabolic defect that is expressed only on a non-fermentable-plating medium, the respiratory capacity was suspected as the target. When actively growing cells were irradiated with doses sufficient to only produce the low dose-reversible lethality, there is a marked decrease in the endogenous respiration (Fig. 4).

Near-ultraviolet irradiation has been found to initially inhibit the coupled oxidative phosphorylations [18,19], and secondarily to destroy the quinone pools [5,10]. We were unable to determine if oxidative phosphorylations were more sensitive than the quinones to near ultraviolet light, as the oxidase activities in our membrane preparations never showed any ADP-stimulated activity. Using the appropriate electron donors to the yeast mitochondrial electron transport system [20], we were able to show the inactivation of quinone-related electron transfers. Substrates which donate electrons at redox levels higher than the quinones all showed greatest sensitivities to near-ultraviolet light (Fig. 4). D-Lactate which donates electrons at the level of cytochome *c* [17], shows a very slow loss in lactate oxidase activity that is probably from the destruction of cytochromes as reported in *E. coli* [7]. These results are consistant with studies on other biological systems, in that the quinone pools are the most near-ultraviolet-labile component of the electron transport chain [10, 19, 21].

In the rat liver mitochondria [9] and in *R. palustris* [10] the succino-oxidase activity was less sensitive to near-ultraviolet light than the NADH oxidase activity. Our results suggest the reverse situation in yeast. Both endogenous and exogenous NADH oxidase activities were less sensitive than the succino-oxidase activity (Fig. 6). Furthermore, with three different intensities of irradiation there was little difference in the inactivation of succino-oxidase activity suggesting that even the lowest intensity was still dose saturating. The inactivation of the NADH oxidase activity showed no differences at the two higher intensities, but at the lowest intensity there was no detectable loss in activity during the first 10 min (Figs 7a and 7b). This differential sensitivity could be caused by varying quinone pool sizes utilized by each oxidase or different absorption characteristics of the quinone environment in each oxidase activity.

Differences in the membrane environment of the NADH and succino-oxidase activities have been well studied in terms of hydrophobicity [22, 23]. In our studies Brij 58 was used to solubilize the CoQ-10, and this was sufficient to restore the succino-oxidase but not the NADH oxidase activities following irradiation. The lost NADH oxidase activity could not be explained by a denaturation of the NADH-flavin reductase. Thus this inability to restore activity may represent the precise phospholipid requirement as reported by Lenaz et al. [22].

A substantial portion of the NADH oxidase activity (35–40 %) appears to be near-ultraviolet resistant. In *M. phlei* and *Micrococcus lysodeikticus* [21] approx. 5 % of the NADH oxidase activity was near-ultraviolet resistant. This resistance

suggests an electron transport chain in which quinones are not a necessary component. The non-quinone-mediated pathways have been well established in rat liver mitochondrial outer membranes [24], and more recently in mung bean mitochondria [25], however, its existence in yeast is unresolved [13, 23, 25]. We have shown that greater percentages of this near-ultraviolet-resistant pathway are present in a more slowly sedimenting membrane fraction and may be representative of this particular non-quinone electron transport chain.

ACKNOWLEDGEMENTS

This research was supported in part by Grant No. AI-07798 from U.S.P.H.S. and Intramural Research Grants from the University of California. The authors wish to thank Dr Salomon Bartnicki-Garcia for the use of the Bronwill cell homogenizer.

REFERENCES

1 Hollaender, A. (1943) J. Bacteriol. 46, 531–541
2 Webb, R. B. and Lorentz, J. R. (1970) Photochem. Photobiol. 12, 283–289
3 Peak, M. J. (1970) Photochem. Photobiol. 12, 1–8
4 Ferron, W. L., Eisenstark, A. and MacKay, D. (1972) Biochim. Biophys. Acta 277, 651–658
5 Kashet, E. R. and Brodie, A. F. (1962) J. Bacteriol. 83, 1094–1100
6 Bragg, P. D. and Hou, C. (1968) Can. J. Biochem. 46, 631–641
7 Bragg, P. D. (1971) Can. J. Biochem. 49, 492–495
8 Murti, C. R. K. and Brodie, A. F. (1969) Science 164, 302–304
9 Beyer, R. (1959) J. Biol. Chem. 234, 688–692
10 King, M. T. and Drews, G. (1973) Biochim. Biophys. Acta 305, 230–248
11 Ogur, M. and St. John, R. (1956) J. Bacteriol. 72, 500–504
12 Thompson, E. D., Bailey, R. B. and Parks, L. W. (1974) Biochim. Biophys. Acta 334, 116–126
13 VonJagow, G. and Klingenberg, M. (1970) Eur. J. Biochem. 12, 583–592
14 Mackler, B. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds), Vol. X, pp. 251–253, Academic Press, New York
15 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
16 Hatchard, C. G. and Parker, C. A. (1956) Proc. R. Soc. Lond. Ser. A, 235, 518–536
17 Gregolian, C. and D'Alberton, A. (1964) Biochem. Biophys. Res. Commun. 14, 103–114
18 Eisenstark, A. (1970) Mutat. Res. 10, 1–6
19 Brodie, A. F. and Ballintine, J. (1960) J. Biol. Chem. 235, 226–231
20 Chance, B., Ernster, L., Garland, P. B., Lee, C. P., Light, P. A., Ohnishi, T., Ragan, C. I. and Wong, D. (1967) Proc. Natl. Acad. Sci. 57, 1498–1505
21 Fujita, M., Tshikawa, S. and Shimazono, N. (1966) J. Biochem. Tokyo 59, 104–114
22 Lenaz, G., Castelli, A., Littarru, G. P. and Bertoli, E. (1971) Arch. Biochem. Biophys. 124, 407–416
23 Ohnishi, T., Sottocasa, G. and Ernster, L. (1966) Bull. Soc. Chim. Biol. 48, 1189–1203
24 Sottocasa, G., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Biol. Chem. 32, 415–437
25 Douce, R., Manella, C. A. and Bonner, Jr, W. D. (1973) Biochim. Biophys. Acta 292, 105–116