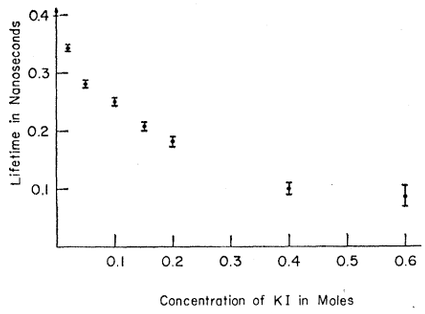

Fig. 2. Quenching effect of potassium iodide (*KI*) on the fluorescence decay of methylene blue (in water at room temperature) as determined with the mode-locked laser method.

blue exist, and although the quenching effect of potassium iodide on the fluorescence of methylene blue is very well known, decrease of τ could not be measured directly with previous instruments. Also, the value of τ for methylene blue in a mixture of ether, isopentane, and ethyl alcohol was measured to be 1.00 ± 0.05 nsec at room temperature which increased to 4.5 ± 0.5 nsec at 77°K. It should be noted that the convenience of instantaneous readout is particularly advantageous for measurements at cryogenic temperatures.

The calibration of the apparatus was checked by measuring the fluorescence decay of chlorophyll b and phycocyanin. The value of chlorophyll b (3.87 ± 0.05 nsec) is in good agreement with the reported value of 3.9 ± 0.4 nsec (*5*), whereas for phycocyanin our measurement (1.14 ± 0.01 nsec) corresponds to the lower limit of the reported value of 1.8 ± 0.6 nsec (*5*). The consistency and accuracy of these measurements appear to validate the values obtained for methylene blue.

Fluorescence decay time of chlorophyll a in *Chlorella pyrenoidosa* was determined by both direct measurement of decay and the phase-delay technique. Our photomultiplier had 1-nsec response, and therefore decay times had to be determined from the associated convolution integral (*6*). A value of 1.6 ± 0.2 nsec for τ in *Chlorella* (measured by direct method) is in fair agreement with that obtained by phase method (1.40 ± 0.05 nsec); these results provide evidence that the mode-locked laser method is applicable for both direct measurement of radiative decay as well as the phase-delay method of lifetime determination and is unique in this sense. Reported values for chlorophyll a in *Chlorella pyrenoidosa* range from 1.15 to 2.0 nsec (*5–7*).

In the presence of $5 \times 10^{-6} M$ of DCMU [3-(3,4-dichlorophyenyl)-1,1-dimethylurea], which inhibits photosynthesis, τ values ranged from 1.68 to 1.94 nsec in various cultures. Although these variations and those in unpoisoned *Chlorella* may be due to slight changes in the growing of algae, the critical environmental conditions responsible for these differences have not been established. These values are to be compared to 1.97 ± 0.03 nsec (*7*).

Both the inherent and the driven pulsating characteristics of lasers simplify measurements of short radiative decay. A medium-power laser emits in each pulse more energy per unit frequency (at the chosen wavelength) than any lamp suitable for lifetime measurement can. Much more precise direct oscilloscope observation of radiative decay is therefore possible than with flash excitation. Furthermore, the rise and fall time of laser pulses can be well below the nanosecond value whereas it is difficult or impossible to generate sufficient intensity for select band excitation on those time scales with conventional flash sources. As demonstrated here, it is possible to extend the range of decay measurements well below the nanosecond region of time scales. Continuous, high-repetition rate of emission of these pulses makes the approach suitable for the well developed, high-speed electronic sampling techniques and provides the convenience of instantaneous readout particularly useful for measurements at cryogenic temperatures (*8*).

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9. We thank Professor E. Rabinowitch for discussions on photosynthetic processes and Dr. E. C. Jordan for the support of this effort. Supported by the University of Illinois Research Board grant (CRR Grad EE Merkelo), the Research Corporation grant, and NSF grant GB7331.

23 October 1968

New Light-Sensitive Cofactor Required for Oxidation of Succinate by Mycobacterium phlei

Abstract. *Irradiation of the electron transport particles of* Mycobacterium phlei *with light at a wavelength of 360 manometers resulted in a loss of oxidase activities of succinate and the reduced form of nicotinamide adenine dinucleotide. The lesion in the two pathways caused by irradiation of the particles differs. The succinoxidase pathway was more labile to irradiation than the pathway linked to nicotinamide adenine dinucleotide. Restoration of succinoxidase activity (up to 50 to 60 percent) occurred on addition of a thermostable, water-soluble material obtained from* Mycobacterium phlei *cells or with an extract of mitochondria from boiled rat liver. Other known cofactors, such as flavine adenine dinucleotide, flavine mononucleotide, benzo- and naphthoquinones, as well as sulfhydryl agents, failed to restore succinoxidase activity after irradiation. Water-soluble material from* Mycobacterium phlei *appears to function between the flavoprotein and cytochrome b on the succinoxidase pathway. In contrast to the requirements for restoration of the pathway linked to nicotinamide adenine dinucleotide, restoration of succinoxidase does not occur with quinones or other cofactors such as flavine adenine dinucleotide.*

Exposure of the electron transport particles of *Mycobacterium phlei* to near-ultraviolet light (wavelength, 360 nm) resulted in a loss of the natural naphthoquinone and in the ability to carry out oxidative phosphorylation (*1*). Restoration of both oxidation and phosphorylation occurred with sub-

strates linked to nicotinamide adenine dinucleotide (NAD^+) by the addition of vitamin K_1, the naturally occurring naphthoquinone [vitamin K_9 (II H)] or closely related homologs. In contrast, neither naphtho- nor benzoquinones restored oxidative phosphorylation on succinate, indicating that a light-sensitive factor other than the known quinones is required for electron transport from succinate (*2*). Since knowledge of the components and sequence of carriers of the respiratory chain is necessary for an understanding of the bioenergetic process, a study was undertaken of the nature and role of this new light-sensitive factor required for succinoxidase activity.

Light (360 nm) exerted a differential effect on the succinate and NAD^+-linked pathways; results observed in particles exposed to light for various periods are shown in Fig. 1. Although both the succinate and NAD^+-linked pathways were labile to irradiation, the succinoxidase pathway was more sensitive than that observed with the generated reduced form of nicotinamide adenine dinucleotide (NADH). Up to 80 percent of the succinoxidase activity of the particles was lost after 5 minutes of irradiation, whereas only 20 percent of the NADH oxidase activity was lost during this period. The activity lost with NADH was restored by the addition of vitamin K_1; succinoxidase activity was restored only by addition of a preparation made from the supernatant fraction or the material obtained from boiled whole cells. The degree of restoration of succinoxidase activity by the factor was dependent on the amount of damage to the particles. Little or no restoration occurred with preparations in which the succinoxidase activity was completely destroyed and only 50 to 60 percent restoration occurred with preparations that had lost 80 percent of this activity. Length of exposure of the particles to light was thus very critical with respect to recovery of the activity lost on irradiation.

The factor required for restoration of succinoxidase activity in irradiated particles was prepared from the supernatant fraction (obtained after removal of the particles) from whole cells of *M. phlei* and from rat liver mitochondria. After fractionation with ammonium sulfate, the factor was found in the fractions obtained between 25 to 65 percent saturation; at this stage it was protein-bound and labile to short periods of irradiation. The factor required for restoration of succinoxidase activity was thermostable and water-soluble. The dialyzed ammonium sulfate fraction containing 150 m*M* KCl and 20 m*M* 2-mercaptoethanol was held in a boiling water bath for 10 minutes. After removal of the denatured protein the supernatant was capable of restoring succinoxidase activity; in the absence of the factor, 2-mercaptoethanol failed to restore succinoxidase activity.

Table 1. Effect of the factor on succinate oxidation by particles of *Mycobacterium phlei*. Reaction mixture in a final volume of 3.5 to 4.25 ml contained 150 μmole of tris-HCl buffer (*p*H 7.4), 30 μmole of $MgCl_2$, 40 μmole of succinate, and particles equivalent to 3 to 6 mg of protein. Additions when made were as follows: 0.5 ml of *M. phlei* factor or extract of boiled liver mitochondria, potassium cyanide ($2.3 \times 10^{-3}M$), and 2 μg of 2-*n*-nonylhydroxyquinoline *N*-oxide (NHQNO) per milligram of protein. Oxygen uptake (10^{-9} g-atom per minute per milligram of protein) was measured polarographically (*4*).

Additions	Oxygen uptake
Particles before irradiation	
None	62.0
Factor	62.0
Particles after 10 minutes of irradiation	
None	10.9
Factor	20.0
Factor + KCN	0.0
Factor + NHQNO	1.8
Extract of boiled liver mitochondria	17.8
Methanol-chloroform extract of *M. phlei*	10.9

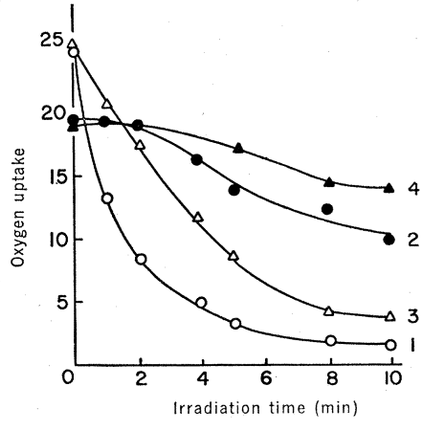

Fig. 1. Effect of time of irradiation on oxidation of succinate (curve 1) and generated NADH (curve 2) by *Mycobacterium phlei* particles. Restoration of succinoxidase by the factor and restoration of NADH oxidase by vitamin K_1 are represented in curves 3 and 4, respectively. Oxygen uptake (10^{-9} g-atom per minute per milligram of protein) was measured at 30°C by use of Clark's oxygen electrode (*4*).

In contrast to the protein-bound form, the factor obtained by boiling and removal of the protein was resistant to irradiation; at least 5 to 6 hours of irradiation (360 nm) was required to destroy the thermostable factor. Exposure of the protein-bound factor to light (60 to 90 minutes) prior to heating resulted in its loss, the destruction being more pronounced when the irradiation was carried out in 2-mercaptoethanol. In addition, the protein-bound factor was resistant to tryptic digestion. These results suggest that the binding of the factor to protein renders it more labile to irradiation damage than it is in its free form.

The absorption spectrum of the protein-bound and thermostable forms of the succinoxidase factor is shown in Fig. 2A. The spectrum of both materials was similar, major absorption peaks being at 257 and 405 nm with a shoulder at 385 nm. After irradiation the major peaks were unaffected, but the shoulder at 385 nm was lost. The addition of borohydride failed to affect the 275-nm peak; however, with hydrosulfite the peak at 405 nm was found to shift to 415 nm, and the shoulder at 385 nm disappeared. Aeration of the hydrosulfite-treated sample resulted in the reappearance of the shoulder at 385 nm and in a shift of the peak from 415 to 405 nm (Fig. 2B).

The activity and site of interaction of the factor was investigated with dyes which "tap" electrons at different sites in the succinoxidase pathway and with inhibitors. Irradiation (360 nm) of the particulate fraction resulted in a loss of succinoxidase and NAD^+-linked oxidation. The succinoxidase factor failed to restore NADH oxidation, whereas vitamin K_1 suspended in lecithin was capable of restoring 50 to 60 percent of the NADH oxidase activity. Although the succinoxidase factor was capable of restoring succinate oxidation to irradiated particles, neither flavine mononucleotide, flavine adenine dinucleotide, vitamin K_1, coenzyme Q, nor a methanol-chloroform extract of *M. phlei* replaced the water-soluble succinoxidase factor. The restored activity, like untreated preparations, was sensitive to 2 μg of 2-*n*-nonylhydroxyquinoline-*N*-oxide per milligram of protein and cyanide ($10^{-3}M$) (Table 1). A similar inhibition of activity was observed with the succinoxidase activity restored by the factor obtained from mammalian mitochondria.

Table 2. Effect of the factor on reduction of artificial electron acceptors by *Mycobacterium phlei* particles in the presence of succinate. Abbreviations: SDH, succinate dehydrogenase; PMS, phenazine methosulfate; DCIP, dichlorophenol indophenol; and MTT, thiazolyl blue tetrazolium.

Test system	Additions	SDH activity		Reduction of dyes‡		
		PMS*	PMS-DCIP†	Ferricyanide	MTT	DCIP
Particles	None	29.0	12.5	47.0	1.2	1.7
	Factor	29.0		47.0	2.3	3.4
Particles irradiated 10 minutes	None	29.0	12.6	47.0	0.17	0.8
	Factor	29.0			.60	2.8
	Irradiated factor				.16	0.8
	Extract of boiled liver mitochondria				.65	2.60

* Activity assayed by measurement of oxygen uptake (*5*) (10^{-9} g-atom per minute per milligram of protein). † Assayed spectrophotometrically (*6*) and expressed as nanomoles of dichlorophenol indophenol reduced per minute per milligram of protein. ‡ Expressed as nanomoles of dye reduced per minute per milligram of protein.

Previous studies indicated that the lesion caused by irradiation occurred between the flavoprotein and cytochrome b on the succinoxidase pathway (*3*). The thermostable form of the factor also appears to interact in this region of the chain, since the factor was not required for succinic dehydrogenase activity as assayed with phenazine methosulfate or phenazine methosulfate and dichlorophenol indophenol but was required for restoration of the reduction of dyes (thiazolyl blue tetrazolium and dichlorophenol indophenol) which "tap" electrons at the level of cytochrome c (Table 2). Succinic dehydrogenase was not lost after irradiation of the particles; however, such treatment resulted in an inhibition of the reduction of cytochromes b, c, and a + a_3. Addition of the succinoxidase factor was required for reduction of the cytochromes after irradiation. In a number of experiments the ability to restore reductase activity of dichlorophenol indophenol after irradiation was completely restored by the addition of the succinoxidase factor.

In conclusion, irradiation of *M. phlei* particles with light at 360 nm resulted in a loss of succinoxidase and NADH oxidase activities. In contrast to the lesion on the NAD^+-linked pathway, which appeared on the loss of the endogenous quinone, the lesion in the succinoxidase pathway occurred in a component that interacted between the flavoprotein and cytochrome b. The protein-bound factor was dissociated into a thermostable component that retained the ability to restore succinoxidase activity after irradiation of the particulate fraction. Although the spectrum of the component remains constant after purification, it is not known whether the active material exhibits absorption in the ultraviolet and visible regions. The chemical nature of the thermostable component requires further study.

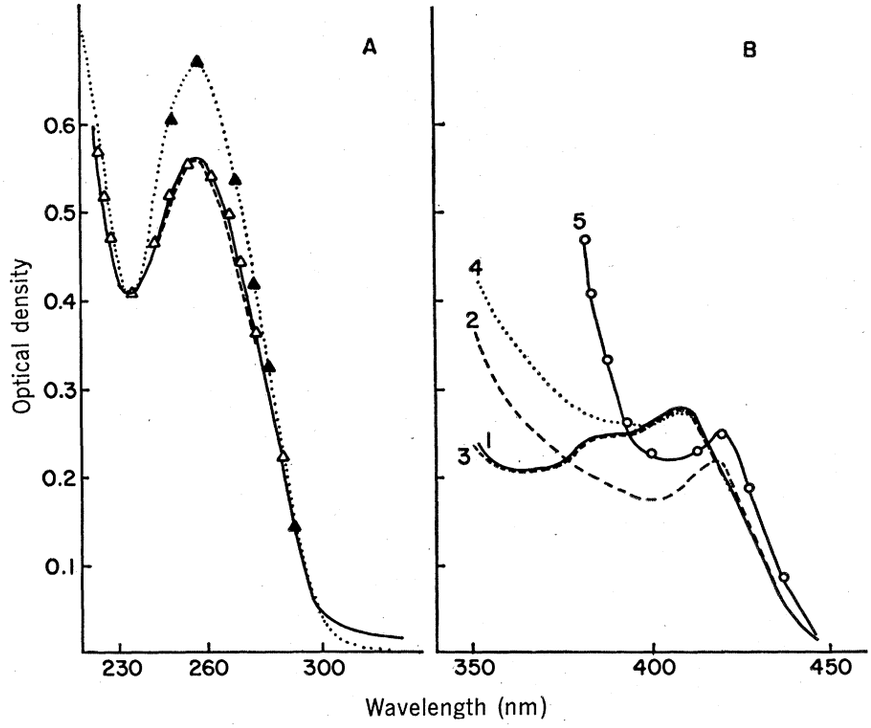

Fig. 2. Absorption spectra of the factor prepared from *Mycobacterium phlei* and extract of mitochondria from boiled rat liver. The preparations were diluted 100 fold with water for measurement of the absorption spectra. For measurement of optical density in the 350 to 450 nm region, the protein-bound form of factor was used and contained 5 mg of protein per milliliter. (A) *M. phlei* factor; solid triangles, *M. phlei* after borohydride reduction; dashed line, extract of mitochondria from boiled rat liver; and open triangles, the same extract after borohydride reduction. (B) Curve 1, *M. phlei* factor before irradiation; curve 2, *M. phlei* factor plus $Na_2S_2O_4$; curve 3, *M. phlei* factor plus $Na_2S_2O_4$ after aerial oxidation of $Na_2S_2O_4$; curve 4, *M. phlei* factor after 5 hours of irradiation; and curve 5, *M. phlei* factor plus $Na_2S_2O_4$ after irradiation.

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7. Supported by grants from NSF (GB-6257X), and NIH (AI 05637), a contract from AEC [AT (11-1)-113], and by the Hastings Foundation of the University of Southern California School of Medicine. This is the 39th paper in a series dealing with oxidative phosphorylation in fractionated bacterial systems. We thank Mrs. Hiroko Sakamoto and Miss Patricia Brodle for technical assistance. Dr. Murti is a visiting associate professor on leave from Central Drug Research Institute, Lucknow, India.

6 November 1968