The Effect of Ultraviolet Light on Mitochondria

II. RESTORATION OF OXIDATIVE PHOSPHORYLATION WITH VITAMIN K1 AFTER NEAR-ULTRAVIOLET TREATMENT*

ROBERT E. BEYER

From the Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts

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The demonstration by Martius and Nitz-Litzow (4), and Martius (5), that vitamin K1 may play a functional role in oxidative phosphorylation in animals has led to an increased interest in the participation of vitamin K1 and related quinones in electron transport, oxidative phosphorylation, and photosynthetic phosphorylation. Since vitamin K1 is altered by the action of ultraviolet radiation (6), we have treated rat liver mitochondria with near-ultraviolet light in order to alter such intramitochondrial components. After such treatment, oxidative phosphorylation with succinate as substrate is dependent upon the addition of cytochrome c, and oxidative phosphorylation with glutamate as substrate is dependent upon the addition of cytochrome c and vitamin K1. The action of vitamin K1 appears to be specific in that a number of related compounds do not restore oxidative phosphorylation.

EXPERIMENTAL

Reagents—Common chemicals were of analytical reagent grade. Solutions were prepared in deionized water and adjusted to pH 7.4. The various coenzymes were procured from the Sigma Chemical Company; vitamin K1 was purchased from California Foundation for Biochemical Research; and d,α-tocopherol from Distillation Products Industries.

Mitochondrial Preparation—Rat liver mitochondria, washed twice, were prepared in 0.25 m sucrose essentially according to Schneider and Hogeboom (7). Homogenation was performed in a motor-driven glass homogenizer with a Teflon pestle. All operations before final incubation with substrate were carried out at 0-2°.

Irradiation Procedure—Mitochondria, at a density of 167 mg. equivalents per ml. (mitochondria derived from 167 mg. of fresh rat liver in 1 ml.) were irradiated with 14 General Electric, 18-inch BLB lamps1 for 60 minutes in a rotary irradiator previously described (8). Quartz tubes were employed for the mitochondrial irradiation and control mitochondria were treated identically except that the tubes were covered with aluminum foil during irradiation. After irradiation, the particles were reisolated by high speed centrifugation.

Preparation of Oil Emulsions—Emulsions of vitamin K1 and α-tocopherol, to be incubated with mitochondria, were prepared as follows. Stock ethanolic solutions of the vitamins were diluted and 1 ml. containing either 9 x 10^{-4} mg. of vitamin K1 or 8.0 x 10^{-2} mg. of α-tocopherol was pipetted into the chamber of a 9 kc Raytheon magnetrestractive oscillator and dried under a stream of nitrogen. 10 ml. of 2 per cent bovine crystalline serum albumin in 0.25 m succrose, pH 7.4, irradiated with a low pressure 15-watt General Electric germicidal lamp, were added and the mixture vibrated at full capacity for 15 minutes. The resulting emulsion is stable in the cold or frozen state for at least 2 weeks. All vitamin solutions were stored in low actinic glassware.

Incubation Procedure—The reisolated mitochondria were suspended in 0.25 m succrose and 1 ml. of either the solution containing a compound to be tested for restorative activity or succrose solution was added. The particles remained in the cold with occasional gentle agitation for 5 additional minutes. The mitochondrial suspension was then added to 10 ml. of a solution (0-2°) containing K2HPO4 (500 μmoles); glucose (600 μmoles); AMP (43 μmoles); KCl (1500 μmoles); and tracer P32, pH 7.4. Of this standard suspension, 1.5 ml containing 500 mg. equivalent of mitochondria was added to Warburg vessels which contained 0.2 ml of 20 per cent KOH in the center well fitted with a filter paper wick, either succinate or glutamate (30 μmoles) Mg++ (7.5 μmoles), and hexokinase (330 KM units). Incubation vessels contained 1.5 μmoles of DPN, 2 x 10^{-5} μmoles of vitamin K1, 2 x 10^{-3} μmoles of d,α-tocopherol, 8 x 10^{-2} μmoles of cytochrome c, and crystalline bovine serum albumin, 2 mg. per ml. when these components were added. KCl, 1.15 per cent, was added to make a volume of 2.0 ml of reaction mixture. Substrate and hexokinase were contained in the side arms and were tipped into the main compartment to initiate the reaction. No equilibration period was employed. The reaction proceeded at 31° for 20 minutes and was terminated by the addition of 0.2 ml of 5 M H2SO4.

Analyses—The μmoles of P32 esterified were determined on 0.1-ml aliquots of the Warburg vessel contents according to the isotope distribution method of Lindberg and Ernster (9). The isotope distribution method was checked against the Fiske and SubbaRow method (10) and yielded quantitatively equivalent, although more consistent, results. Oxygen consumption was

1 Over 93 per cent of the emission energy of these lamps is between 3200 and 3800 A with maximal emission at 3550 A.

2 The abbreviations used are: P, inorganic orthophosphate; Q275, a quinone isolated from beef heart mitochondria (12); P:O, ratio of μmoles of P, esterified to atoms of oxygen consumed; Amytal, 5-ethylisoamylbarbiturate.
measured by the conventional Warburg manometric technique, readings being obtained every 5 minutes for a period of 20 minutes. Since no equilibration period was employed, oxygen consumption for the first 5 minutes was obtained by extrapolation. This would tend to produce P:O ratios slightly lower than those which obtain when an equilibration period is used since phosphorylation probably proceeds more slowly during the first five minutes (during temperature elevation of the chilled mitochondrial suspension) than during subsequent periods and oxygen consumption for the first 5 minutes is taken as equal to the second 5-minute period.

Photochemical Breakdown of Vitamin K₁—The alteration of the vitamin K₁ molecule catalyzed by near-ultraviolet light was determined by irradiating absolute ethanol solutions of vitamin K₁ in quartz Beckman cuvettes placed 1 cm. from the surface of one General Electric 18-inch BLB lamp. Extinction loss was measured with a Beckman model DU spectrophotometer for 1 hour at 3280 and 3660 A after each 10 minutes of irradiation.

RESULTS

Irradiation Effect—When mitochondria were irradiated with near-ultraviolet light for varying periods of time, a loss of both the oxidative and phosphorylative ability with glutamate and succinate as substrates was observed (Table I). The data in Table I also reveal that oxidation and phosphorylation with glutamate as substrate were more sensitive to the radiation than were those from succinate. Also apparent was the greater sensitivity to irradiation of the phosphorylative system linked to both substrates, as compared to the oxidative system.

Restoration with Vitamin K₁—Inactivation of oxidative phosphorylation with succinate as substrate was partially reversed by the inclusion of cytochrome c to the mitochondrial system (Table II). Lower concentrations of cytochrome c than that reported did not appear to be as effective, and greater concentrations were at times somewhat less effective restorers of high P:O ratios. Vitamin K₁ plus cytochrome c did not significantly inhibit the control mitochondrial system with either succinate or glutamate as substrate. The addition of vitamin K₁ to the irradiated system with succinate as substrate had no effect on the P:O ratios when the system was either inhibited by near-ultraviolet treatment or restored by the addition of cytochrome c. Albumin was without restorative effect when either substrate was employed. With glutamate as substrate the addition of cytochrome c, DPN, or albumin did not result in restoration. The addition of vitamin K₁ alone partially restored the oxidation of glutamate and associated phosphorylations, the latter effect being of greater magnitude. Inclusion of cytochrome c plus vitamin K₁ restored both glutamate oxidation and electron transport phosphorylation to nearly original levels, resulting in nearly maximal P:O values.

Effect of Other Quinones—A number of other preparations were tested for restorative activity (Table III). Of those listed in Table III, only three appeared to have any influence on the radiation-inhibited system. Vitamin K₁ exhibited its usual restorative effect on oxidation and phosphorylation. Both vitamin K₁ diacetate and the Mycobacterium phlei extract, which has been shown by Brodie et al. (11) to contain a quinone-like compound and to be necessary for oxidative phosphorylation, were more sensitive to the radiation than were those from succinate. The addition of vitamin K₁ diacetate and the Mycobacterium phlei extract, which has been shown by Brodie et al. (11) to contain a quinone-like compound and to be necessary for oxidative phosphorylation, were more sensitive to the radiation than were those from succinate. The addition of vitamin K₁ diacetate and the Mycobacterium phlei extract, which has been shown by Brodie et al. (11) to contain a quinone-like compound and to be necessary for oxidative phosphorylation, were more sensitive to the radiation than were those from succinate.
Effect of Ultraviolet Light on Mitochondria. II

Vol. 234, No. 3

The effect of near-ultraviolet light on vitamin K1, exposure of vitamin K1 to near-ultraviolet light resulted in an extinction loss at the 3280 A peak as well as a loss of extinction at a shoulder near the irradiation source at 3600 A (Fig. 1). Other conditions as stated in Experimental section.

Effects of Inhibitors on Restored System—The effect of three metabolic inhibitors, Amytal, 2,4-dinitrophenol, and Dicumarol, was studied on the vitamin K1-restored oxidative phosphorylation system (Table IV). The restoration of succinate oxidation by cytochrome c, and glutamate oxidation by cytochrome c plus vitamin K1, was observed in the presence of 2,4-dinitrophenol. The rate of oxidation of each of these substrates upon restoration was not seriously affected by 2,4-dinitrophenol. Amytal, which is known to block completely electron transport from DPNH-dependent substrates over a phosphorylative pathway (14), inhibited electron transport with glutamate as substrate in the vitamin K1 restored system (Table IV). Amytal did not inhibit the cytochrome c-restored succinate oxidation nor associated phosphorylations. Dicumarol, a potent uncoupler of oxidative phosphorylation, which may act as a competitive inhibitor of vitamin K1 (15), also inhibited electron transport phosphorylation in the restored systems without seriously affecting the rate of oxidations (Table IV).

Discussion

Since the original suggestion by Martius and Nitz-Litzow (4) and Martius (5) that vitamin K1 may be a component of the oxidative phosphorylation system in animals, a number of reports, both theoretical and experimental, concerning a possible role for quinones in electron transport, oxidative phosphorylation, and photosynthetic phosphorylation have appeared. For example, Wessels (16) has postulated a role for vitamin K in photosynthetic phosphorylation on the basis of the demonstration that vitamin K antagonists such as Dicumarol, 2-methyl-1,3-naphthaquinone, phthiocol, and others, inhibit this reaction in chloroplasts. Wessels postulates the reduction of both carbonyl groups of vitamin K, a phosphorylation by P, in the 4-position, a labilization of the ester (O-P) bond upon reoxidation of the 1-position, and the subsequent transphosphorylation to ADP to form ATP. This cyclic reaction would involve the passage of 2 electrons from the hydroquinone to, presumably, an oxidized cytochrome. More recently, Arnon et al. (17) and Wessels (18) have provided experimental evidence for such a view by demonstrating a vitamin K1 (menadione) dependence of anaerobic photosynthetic phosphorylation. In bacterial systems, Wosilait and Nason (19) have reported the existence of a menadione reductase in Escherichia coli, and Weber and Brodie (20) have recently studied a menadione dependent cytochrome c reductase in M. phlei extracts. Brodie et al. (11) have also presented evidence for an obligatory role for vitamin K1 in coupled oxidative phosphorylation in extracts of M. phlei. In animal tissues, Martius and his colleagues have reported lowered efficiency of coupled oxidative phosphorylation in liver mitochondria isolated from vitamin K-deficient chicks (4) as well as the involvement of vitamin K1 in electron transport with DPNH as substrate in a pig mitochondrial system (21). Dallan and Anderson (22) have reported that uncoupling of phosphorylation from electron transport may be brought about in isolated rat mitochondria, nor was vitamin K1 which had previously been irradiated with near-ultraviolet light. d,a-Toecopheryl, reported to be involved in mammalian DPNH-cytochrome c reductase (13), was also not capable of restorative activity.

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liver mitochondria by treatment with far-ultraviolet light (2537 Å) and that the phosphorylation may be almost completely restored by the addition of vitamin K₁. Beyer (1, 2) has also presented preliminary data concerning the restoration of oxidation and phosphorylation in rat liver mitochondria by vitamin K₁ after inactivation by near-ultraviolet light. The phosphorylation of ADP accompanying the oxidation of menadiol (reduced vitamin K₂) by heart muscle sarcosomes has been observed by Colpa-Boonstra and Slater (23). The isolation of a quinone from beef heart mitochondria (12, 24) and from various tissues (25–27) has been reported and more recently this quinone has been characterized and synthesized (28–30). This substituted benzoquinone has been shown to be capable of undergoing reversible oxidation and reduction in the presence of mitochondria and substrate (12, 31) and thus appears to be involved in electron transport.

Subsequent to Wessels’ (16) hypothesis that vitamin K acts as a primary phosphate acceptor, Harrison (32) formulated a more generalized theory involving the phosphorylation of substituted hydroquinones which transfer phosphate to ADP during oxidation by cytochrome c. Grabe (33) has provided theoretical evidence from statistical mechanical calculations for such a view, whereby the removal of 2 α-electrons from the benzenoid ring by the cytochrome complex may bring about activation of the phosphoryl group on position 4. Clark et al. (34) have presented chemical evidence which indicates that such reactions are possible in vitro.

In the present work, near-ultraviolet light has been employed to study the role of light-sensitive compounds in electron transport and accompanying phosphorylations. Near ultraviolet light treatment of mitochondria resulted in a progressive loss of the ability of these particles to oxidize succinate and glutamate as well as to couple efficiently the phosphorylation of ADP to electron transport. The DPN-dependent glutamate oxidation and accompanying phosphorylations were more sensitive to this type of irradiation than the oxidation of succinate and phosphorylations accompanying succinate oxidation. Inhibition of the more resistant oxidative phosphorylation with succinate as substrate was reversed by the addition of cytochrome c to the incubation medium; however, cytochrome c alone did not have any appreciable restorative effect on phosphorylations accompanying the DPN-dependent transfer of hydrogen from glutamate to oxygen. The addition of vitamin K₁ alone to the glutamate system partially restored oxidation and phosphorylation, but the same addition to the mitochondrial systems with succinate as substrate was without effect. Both cytochrome c and vitamin K₁ were necessary for good restoration of oxidative phosphorylation with glutamate as substrate. These data indicate that a rate-limiting step in electron transport, before the point in electron transfer common to succinoxidase and glutamate oxidase, was inhibited by near-ultraviolet light treatment of mitochondria and that the inhibition before this common point may be reversed by vitamin K₁. Inhibition at the cytochrome level was common to both pathways, and thus the presence of cytochrome c was necessary for good restoration of both the succinate and glutamate systems.

A number of quinones other than vitamin K₁, as well as an extract of M. phlei exhibiting an absorption spectrum similar to vitamin K₁ (11), were tested as to their restorative activities in order to gain information on the specificity of vitamin K₁ in this irradiated system. Vitamin K₁ diacetate, which differs from vitamin K₁ in that the 1 and 4 position carbonyl groups are acetylated, was able to induce slight restoration. This is in agreement with Brodie et al. (11) who found vitamin K₁ diacetate to be somewhat restorative in a M. phlei system. A lipide extract of M. phlei, which, like vitamin K₁, restores oxidative phosphorylation from DPNH in M. phlei (11), was also capable of inducing slight restoration in the rat liver mitochondrial system. Q₁₅, a quinone which has been reported to participate in the succinoxidase system (12) was not active in restoring the system after treatment with light in the near-ultraviolet. α-Tocopherol, which has been reported (13) to be able to restore DPNH-cytochrome c reductase activity after extraction of the enzyme preparation with isooctane, was not active in our system. The absorption spectrum of vitamin K₁ was altered by near-ultraviolet light treatment, and after such alteration the vitamin was no longer capable of restoring electron transport with glutamate as substrate. Since it is generally stated that vitamin K₁ appears to be limited to green plants and vitamin K₂ to microbial systems (35), it does not seem reasonable to claim that vitamin K₁ is present in mammalian mitochondria. It is more probable that the vitamin K₁ molecule is structurally similar to a component of this system and thus may replace the mitochondrial component after its alteration by near-ultraviolet light.

Cooper and Lehninger (36) suggest that vitamin K₁ is not a component of the electron transport system but acts as an intermediate phosphate carrier between electron transport and the final phosphorylation of ADP. Such an interpretation would imply that upon alteration of the intermediate phosphate in a tightly coupled system electron transport would be limited by the lack of available phosphate acceptor. Since electron transport was restored by vitamin K₁ in the presence of 2,4-dinitrophenol with glutamate as substrate, the vitamin K₁ replaceable factor must function as an electron carrier in electron transport and not solely as an intermediate phosphate carrier. Dicumarol completely inhibits phosphorylation in the restored glutamate systems which is in agreement with Cooper and Lehninger (36) who have reported that Dicumarol blocks phosphorylations in the span β-hydroxybutyrate to oxygen and β-hydroxybutyrate to cytochrome c. These latter authors are in disagreement with Martius (5, 15) and Martius and Nitz-Litzow (4) who have offered the hypothesis that Dicumarol acts competitively with vitamin K in oxidative phosphorylation and that vitamin K functions in oxidative phosphorylation solely in the span DPNH to cytochrome c. Our finding that vitamin K₁ is able to restore oxidative phosphorylation in the glutamate pathway, but not in the succinate, as well as the finding that the vitamin K₁-restored system is sensitive to Amytal, indicates that the step restored by vitamin K₁ precedes the point common to electron transport from succinate and DPNH and is thus between DPN and cytochrome b. An alternative hypothesis to both those of Martius (5, 15) and Martius and Nitz-Litzow (4) and Cooper and Lehninger (36) is that quinones function as energy conservation sites in electron transport at all three phosphorylation sites, but that the quinones at each site differ from each other either structurally or in their linkage to adjacent electron carriers. The quinones may be so structurally related as to be inhibited in their phosphorylative roles by Dicumarol, while at the same time only one of the quinones, that replaceable by vitamin K₁, is sensitive to alteration in situ by near-ultraviolet light.
SUMMARY

Isolated rat liver mitochondria which have been irradiated with near-ultraviolet light partially lose the ability to oxidize succinate and glutamate and to carry out oxidative phosphorylation. Oxidative phosphorylation with succinate as substrate is restored by the addition of cytochrome c, and oxidative phosphorylation with glutamate as substrate is dependent upon the addition of cytochrome c plus vitamin K₁. Of a number of quinones tested, only vitamin K₁ acetate and a lipide extract of Mycobacterium phlei are capable of slight restoration. The inhibitors 2,4-dinitrophenol and Dicumarol uncouple all phosphorylations in the restored systems, and Amytal blocks electron transport in the vitamin K₁-restored system. These data indicate that near-ultraviolet light treatment of mitochondria alters a factor functioning in oxidative phosphorylation between diphosphopyridine nucleotide and cytochrome b which may be replaced by vitamin K₁.

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