Preparation of ^{14}C- and ^{3}H-Methoxyl-labelled Forms of Ubiquinone by Photochemical *O*-Demethylation and Subsequent Remethylation

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SUMMARY

Ubiquinone [ubiquinone (50), coenzyme Q_{10}, CoQ_{10}] undergoes O-demethylation to the 2- and 3-hydroxyubiquinones, reduction to the hydroubiquinone, and cyclization to the ubichromenol derivative when irradiated, in alcohol solution, with a sunlamp. It is likely that the ubichromenol and hydroubiquinone derivatives form via a common intermediate which is different from that involved in the formation of the hydroxyubiquinone derivatives. The mixture of hydroxyubiquinones, on methylation with ^{14}C- and ^{3}H-labelled methyl iodide or diazomethane, yields methoxyl-labelled ubiquinone, a material useful in the study of ubiquinone photodecomposition, metabolism, and biochemistry.

INTRODUCTION.

The removal of ubiquinone [ubiquinone (50), coenzyme Q_{10}, CoQ_{10}] from mitochondria by washing with pentane destroys their NADH oxidase and succinoxidase activities but these systems are again functional when ubiquinone is added back to the pentane-washed particles [1, 2]. This establishes not only a critical role for ubiquinone but also the possibility of removing the normal ubiquinone from mitochondrial or submitochondrial particles and adding back radiolabelled ubiquinone to facilitate quantitative titration and binding studies. Recent investigations with NADH oxidase inhibitors give additional impetus to such binding studies using radiolabelled ubiquinone. Rotenone-^{14}C and piericidin A-^{14}C, two potent NADH oxidase inhibitors, bind at the same specific site of submitochondrial particles, inhibit

NADH oxidase activity in direct proportion to the amount of inhibitor bound at this site, and give rise to a titer for the specific site which is either equivalent to or twice that of the NADH dehydrogenase content of the particles [3–5]. There is an obvious structural similarity between ubiquinone and piericidin A [6] which suggests that the aforementioned inhibitors possibly localize at a position(s) at the specific site(s) normally occupied by that portion of the endogenous ubiquinone normally involved in electron transport between NADH dehydrogenase and the cytochromes. For this reason, it is of interest to consider the titration properties of ubiquinone and the effect, if any, of piericidin A and rotenone on the titer of ubiquinone and, accordingly, to develop methods for the radiosynthesis of ubiquinone, particularly one which allows convenient preparation of both the ^{14}C- and ^{3}H-labelled compound. For example, the availability of ubiquinone-^{3}H allows competitive binding experiments between ^{14}C-labelled inhibitor and ^{3}H-labelled coenzyme, using the two isotopes to differentiate the inhibitor and coenzyme and, therefrom, the binding interactions involved.

Radiolabelled ubiquinone has been prepared from several labelled precursors by biosynthesis [7], which results in products with a low specific activity. Synthetic routes have been used to incorporate a ^{14}C-label in the first two carbon atoms of the side chain in CoQ_{10} [8, 9] and a ^{3}H-label on these carbons in the side chain or in the methyl group of the benzoquinone moiety of CoQ_9 [9]. Also, it has been suggested that an alkoxy exchange reaction should allow convenient labelling of the methoxyl groupings [10], an approach which, based on the present studies, also gives a product with a low specific activity. The route of radiosynthesis selected for the study described here involves photolytic *O*-demethylation of ubiquinone followed by methylation of the resulting hydroxyubiquinones with a ^{14}C- or ^{3}H-labelled reagent.

The photochemical reactions of coenzyme Q_7 and ubiquinone include *O*-demethylation to form approximately equal amounts of the 2- and 3-hydroxyquinone derivatives, and cyclization to form the chromenol derivatives [11-13]; reduction to the hydroquinone derivatives also occurs [12]. The results given below indicate that the chromenol and hydroquinone derivatives possibly form through the same intermediate but that a different intermediate is involved in hydroxyubiquinone formation. Methylation of the hydroxyubiquinone mixture is conveniently achieved, on a microscale, with either labelled methyl iodide or labelled diazomethane to give ubiquinone-methoxyl-^{14}C or -^{3}H of high specific activity.

The availability of methoxyl-labelled ubiquinone allows a quantitative reconsideration of the photochemical reactions of ubiquinone in respect to (1) the effect of air, oxygen, or nitrogen atmosphere, using a methoxyl-^{14}C preparation of ubiquinone for deriving the quantitative data, and (2) the products formed on photolysis of the hydroubiquinone derivative.

MATERIALS, METHODS, AND PRODUCTS.

Chemicals and analytical procedures.

Ubiquinone (melting point 48-49° C) was supplied by Merck, Sharp and Dohme Research Laboratories Division, Merck & Co., Inc., Rahway, N. J. The ubiquinone derivatives studied were those shown in Figure 1, which also gives the reaction schemes used for their preparation.

The ubiquinone derivatives were separated and purified by dissolving them (50 to 250 mg) in hexane (5 ml) and passing the solution through a chromatographic column of silicic acid (45 g) (100-mesh powder, analytical reagent for chromatography, Mallinckrodt Chemical Works, St. Louis, Mo.) prepared from a slurry of the acid in benzene, eluting with 250 ml of benzene-chloroform-acetic acid mixture (50 : 50 : 1), and collecting four fractions of approximately equal volume. Thin-layer chromatography (T.L.C.) utilized 20 × 20-cm silica gel F_{254} chromatoplates (precoated, 0.25 mm, Brinkmann Instruments Inc., Westbury, N. Y.) and the products were detected by their natural color, quenching of gel fluorescence when viewed under short wavelength ultraviolet light, formation of yellow spots on exposure to iodine vapors or when sprayed with 2 % (w/v) iodine in chloroform, or formation

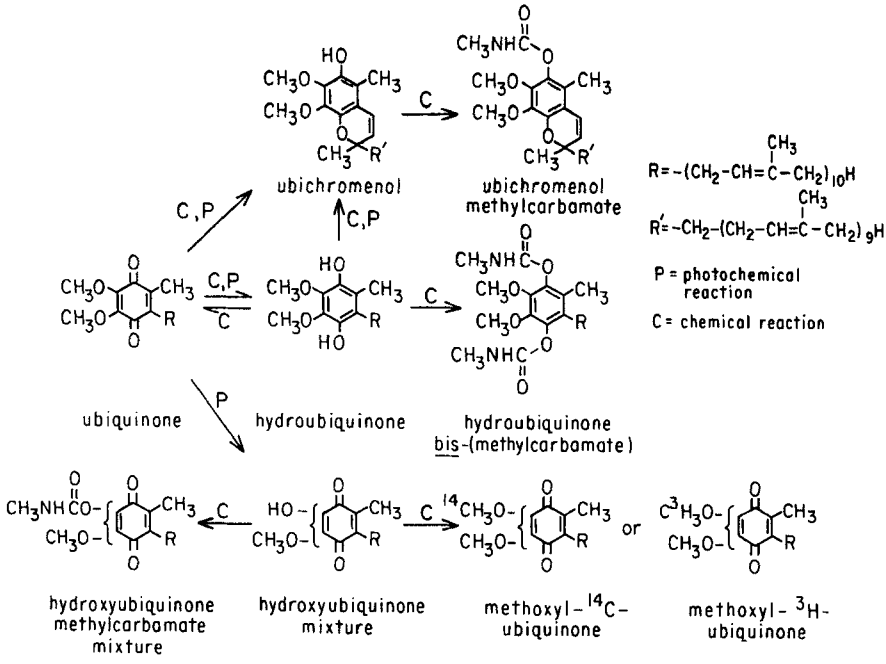

FIG. 1. Reaction schemes and names used.

of blue spots when sprayed with 10 % (w/v) phosphomolybdic acid in ethanol followed by heating for 10 minutes at 110° C. The radioactive products on the T.L.C. plates were detected by autoradiography, and quantitated by scraping the appropriate gel region free from the glass support and adding it directly to a scintillation vial for determination of radioactivity levels by direct scintillation counting. In the development of the T.L.C. plates, a single solvent system was not adequate to resolve the various ubiquinone derivatives; so, product purification was accomplished by development in one solvent system, scraping the desired gel region (found by viewing under normal or ultraviolet light) free from the glass support, extracting the gel with acetone, evaporating the acetone, and rechromatographing in a second solvent system capable of resolving the mixture. This was followed by a second acetone extraction of the desired gel region and, sometimes, by crystallization of the product. In all cases, the solvents were evaporated either under reduced pressure or by blowing a fine stream of nitrogen onto the solution at room temperature. Table I lists the solvent systems used and gives the T.L.C. R_f values of ubiquinone and its derivatives obtained with them.

Special difficulties were encountered in resolving ubiquinone, hydroubiquinone, and ubichromenol because they have similar R_f values in each solvent system and because hydroubiquinone slowly oxidizes on the T.L.C. plates to form ubiquinone. These difficulties were overcome by converting the mixture of compounds to their respective methylcarbamate derivatives (see Fig. 1). This was accomplished by dissolving the mixture (0.5 to 10 mg)

TABLE 1. Thin-layer chromatographic solvent systems and the respective R_f values for ubiquinone and its derivatives.

Compound	R_f values in indicated solvent system [a]				
	A	B	C	D	E
Ubiquinone	0.27	0.73	0.58	0.31	0.65
Hydroubiquinone	0.27	0.73	0.54	0.35	0.62
Hydroubiquinone *bis*-(methylcarbamate)	0.00	0.50	0.04	0.00	0.13
Hydroxyubiquinone	0.12	0.54	0.10	0.04	0.20
Ubichromenol	0.32	0.75	0.60	0.27	0.66
Ubichromenol methylcarbamate	0.03	0.67	0.30	0.04	0.49

[a] Composition of T.L.C. solvent mixtures :

A = hexane-ether-acetic acid mixture (79 : 20 : 1).
B = dichloromethane-ether-acetic acid mixture (50 : 50 : 1).
C = benzene-ether-hexane mixture (1 : 1 : 1).
D = benzene-chloroform mixture (1 : 1).
E = benzene-ether mixture (1 : 1).

in dry ether (1 ml) containing methyl isocyanate (0.1 ml) and triethylamine (0.05 ml), holding at 25° C for 12 to 24 hours, evaporating the solvent, and purifying the methylcarbamate by T.L.C. in solvent system E (Table I); sometimes, the recovered methylcarbamates were also recrystallized from hexane. [The *bis*-(methylcarbamate) derivative of hydroubiquinone is stable; so, it can be determined without loss by oxidation to ubiquinone.]

Melting points were determined with the Fisher-Johns melting point apparatus and were not corrected. Infrared spectra, determined with the Perkin-Elmer Model 456 grating infrared spectrophotometer on 5 % (w/v) solutions in chloroform, were obtained on all products and interpreted partially on the basis of the following bands (indicated as cm^{-1}) : ubiquinone = 1 645 and 1 610 (quinone), 2 920 (CH_2), 2 960 (CH_3), 1 260 and 1 100 (OCH_3); hydroxyubiquinone = 3 400 (OH), 1 640 and 1 615 (quinone); hydroxyubiquinone methylcarbamate = 1 640 and 1 615 (quinone), 3 390 (NH), 1 685 (C = 0); ubichromenol = 3 530 (OH); ubichromenol methylcarbamate = 3 460 (NH), 1 740 (C = 0); hydroubiquinone = 3 540 (OH); hydroubiquinone *bis*-(methylcarbamate) = 3 460 (NH), 1 740 (C = 0). Ultraviolet spectra, determined with the Bausch and Lomb Spectronic 505 ultraviolet spectrophotometer on 1 % (w/v) solutions in ethanol, also gave useful information [λ max. (ε)] : ubiquinone = 275 (159); hydroxyubiquinone = 277 (158); ubichromenol = 233 (219), 275 (84), 280 (82), 330 (32); ubichromenol methylcarbamate = 243 (90), 274 (90), 315 (33); hydroubiquinone = 288 (47); hydroubiquinone *bis*-(methylcarbamate) = 230 (44), 269 (6). [The only colored products are ubiquinone (yellow), hydroxyubiquinone (reddish-violet), and hydroxyubiquinone methylcarbamate (reddish-violet); the only noncrystalline product at 20° C is the ubichromenol.]

Photodecomposition procedure.

Ubiquinone and hydroubiquinone, as 0.5 to 1.3 % (w/v) solutions in ethanol or isopropanol, were photodecomposed by positioning the solution, in a Pyrex round bottom flask of appropriate size, above a 275-watt sunlamp (General Electric Co., Cleveland, Ohio) in such a manner that mild refluxing, from the heat of the lamp, was maintained for the photodecomposition period. In certain cases, oxygen or nitrogen was slowly bubbled through the reaction mixture during the period of exposure to light.

Ubichromenol and its methylcarbamate.

A solution of ubiquinone (40 mg) in triethylamine (5 ml) was heated in a 10-ml sealed ampoule for 2 hours at 100° C, the solvent was removed by evaporation, and the residue was separated by T.L.C. using solvent system A to obtain ubichromenol (73 %). Reaction with methyl isocyanate and T.L.C.

purification of the product with solvent system C gave ubichromenol methylcarbamate (86 %) (melting point 62-63° C).

Ubichromenol was also obtained by heating a solution of hydroubiquinone (30 mg) in triethylamine (2 ml) in a 5-ml sealed glass ampoule for 3 hours at 100° C, during which time the color changed from colorless to yellow to faint yellow. After evaporation of the triethylamine, separation of the products by T.L.C. in solvent system A, recovery of the major band from the gel by extracting with acetone, and treatment of the mixture with methyl isocyanate, the products isolated by T.L.C. using solvent system C were : ubiquinone (melting point 47-48° C) (13 %); ubichromenol methylcarbamate (melting point 58-59° C) (51 %); hydroubiquinone *bis*-(methylcarbamate) (melting point 95-96° C) (32 %).

Hydroubiquinone and its bis-*(methylcarbamate).*

Reduction of ubiquinone was accomplished with lithium aluminum hydride in anhydrous ether or, preferably, with zinc powder in acetic acid. Slow addition of ubiquinone (5 mg) in dry ether (1 ml) to a stirred suspension of lithium aluminum hydride (5 mg) in ether (1 ml) at 5° C, stirring for an additional 30 minutes, addition of water (0.1 ml) followed by 5 % hydrochloric acid (1 ml), extracting 3 times with ether, and drying the ether phase (sodium sulfate) gave hydroubiquinone (60 %). Alternatively, addition of zinc powder (10 mg) to a solution of ubiquinone (5 mg) in glacial acetic acid (1 ml) in a 5-ml round bottom flask, heating for 1 hour at 65-70° C with stirring, evaporation under nitrogen, and extraction of the residue with hexane gave hydroubiquinone (melting point 47-48° C) (90 %). Reaction of hydroubiquinone (8 mg) with methyl isocyanate and purification two times by T.L.C., using solvent system E, yielded hydroubiquinone *bis*-(methylcarbamate) (melting point 96-97 °C) (45 %).

Photolysis products of ubiquinone and hydroubiquinone.

Photolysis of ubiquinone (200 mg) in absolute ethanol for a 23-hour period under air resulted in a color change from yellow to red-orange and yielded at least 8 products, as detected with phosphomolybdic acid, on T.L.C. development with solvent system A (Table I). In addition to the ubiquinone-hydroubiquinone-ubichromenol mixture, and the hydroxyubiquinones, at least 3 unidentified products were evident at R_f values between these materials (i.e., R_f 0.15-0.25). The residue from evaporation of the ethanol was chromatographed on a silicic acid column to yield four fractions (considered here in the order of their elution). Fraction I consisted, on evaporation of the solvents, of colorless crystals (5 mg). The pale yellow residue from fraction II (43 %) was purified several times by T.L.C. in solvent system A to yield the ubichromenol (15 %), less than 1 mg of the hydroxyubiquinone isomer mixture,

and the remainder was ubiquinone and hydroubiquinone; an attempt to isolate hydroubiquinone by T.L.C. treatment, using solvent system C, was successful in resolving the products but the hydroubiquinone was oxidized in part to ubiquinone in the process. Fractions III and IV, purified by T.L.C. with solvent system D, gave the mixture of hydroxyubiquinones (melting point 42-43° C) (5 %) after recrystallization from hexane. Reaction of the hydroxyubiquinone mixture (8 mg) with methyl isocyanate followed by T.L.C. purification with solvent system B, yielded a mixture of the respective hydroxyubiquinone methylcarbamates (37 %) which had appropriate infrared spectral characteristics.

Photolysis of ubiquinone (25 mg) in isopropanol for 7 hours was done under nitrogen, in one experiment, and under oxygen, in another experiment. The residue from solvent evaporation was separated into 3 bands by T.L.C. using solvent system A. The lower band, light brown in color, possibly consisted of very polar photodecomposition products, but they were not identified; the intermediate purple band was the hydroxyubiquinone mixture, which was directly repurified by T.L.C. using solvent system C; the upper, slightly yellowish band, was a mixture (ubiquinone, hydroubiquinone, and ubichromenol) which was recovered, reacted with methyl isocyanate, and the methylcarbamates were isolated by T.L.C., with solvent system E. The yields of the isolated products were as follows : ubiquinone = 10 % with oxygen, 12 % with nitrogen; ubichromenol methylcarbamate = 23 % with oxygen, 33 % with nitrogen; hydroxyubiquinone = 2.9 % with oxygen, 2.5 % with nitrogen; hydroubiquinone *bis*-(methylcarbamate) = 0 % with oxygen, 16 % with nitrogen; unidentified products = 50 % with oxygen, 33 % with nitrogen. (It is evident that the ratio of hydroubiquinone to ubichromenol shifts to increase the amount of residual hydroubiquinone when nitrogen is used but that the hydroxyubiquinone yield remains largely unaffected.)

The products from photolysis of ubiquinone (24 mg) in isopropanol for 7 hours under nitrogen were converted to their methylcarbamate derivatives before T.L.C. resolution, first with solvent system E and then (for purification) with solvent system A. The compounds isolated, which were not completely pure, were : ubiquinone (2.5 %), ubichromenol methylcarbamate (23 %), hydroubiquinone *bis*-(methylcarbamate) (18 %), and a small amount of reddish-violet derivative, probably hydroxyubiquinone methylcarbamate. (This confirms the formation of hydroubiquinone in the photolysis of ubiquinone.)

The photolysis of hydroubiquinone (10 mg) in isopropanol for 7 hours under nitrogen produced little if any hydroxyubiquinone. The products were isolated by T.L.C., using solvent system A for direct resolution of the reaction mixture and solvent system E for separation of the methylcarbamates formed from the products found in the hydroubiquinone-ubichromenol region of the chromatogram obtained with solvent system A; they were the

following : ubiquinone (5 %), ubichromenol methylcarbamate (melting point 58-59° C) (42 %), and hydroubiquinone *bis*-(methylcarbamate) (18 %).

Methoxyl-^{14}C- and methoxyl-^{3}H-ubiquinone by methylation of hydroxyubiquinone.

The hydroxyubiquinone (9 mg), methyl-^{3}H iodide (0.25 mmole, 100 mCi/mmole) and anhydrous potassium carbonate (8 mg) in dry acetone (2 ml) were sealed in a 5-ml glass ampoule and heated at 65-70° C, with stirring, for 1 hour, the color changing from purple to yellow. The ampoule was cooled, opened, and the soluble portion of the reaction mixture was evaporated to dryness under nitrogen, extracted with hexane, and the hexane-soluble products were purified by T.L.C. in solvent system D to give ubiquinone-methoxyl-^{3}H (50 %) (100 mCi/mmole). In a similar experiment, methyl-^{14}C iodide gave ubiquinone-methoxyl-^{14}C (55 %) (5 mCi/mmole). Each product was similar to authentic natural ubiquinone in melting point (41-42° C), T.L.C. R_f values, and reaction properties [conversion to ubichromenol, ubichromenol methylcarbamate, hydroubiquinone, and hydroubiquinone *bis*-(methylcarbamate)] as ascertained by cochromatography studies, and the addition of authentic ubiquinone did not result in reduction in the specific activity through a series of recrystallizations.

In an alternative methylation procedure for the preparation of ubiquinone-methoxyl-^{3}H, tritiated water (50 µl) was equilibrated for 1.5 hour with diazomethane (0.3 mmole) in 1.0 ml of ethanol-ether mixture (1 : 3), hydroxyubiquinone (0.5 mg) was added to the solution which was held for 15 minutes at 25° C, and the products were purified by column and thin-layer chromatography. This procedure gave the desired labelled product, which had a specific activity related to that of the tritiated water used, but the yield was low (10 %).

Discussion.

Exposure of ubiquinone solutions in absolute ethanol or isopropanol to sunlamp irradiation through Pyrex results in photoreduction to the hydroubiquinone, photocyclization to ubichromenol, and photodemethylation to a mixture of hydroxyubiquinone isomers (Fig. 1). Product isolation and characterization is facilitated by the use of the crystalline methylcarbamate derivatives of the hydroxy compounds. Conversion of the hydroxyubiquinone isomer mixture to labelled ubiquinone (2-methoxyl-^{14}C- and 3-methoxyl-^{14}C mixture, and 2-methoxyl-^{3}H- and 3-methoxyl-^{3}H mixture) is a convenient method for radiosynthesis.

Irradiation of ubiquinone under nitrogen gives a somewhat different result than irradiation under oxygen. The hydroubiquinone formed by photoreduction does not extensively reoxidize to ubiquinone under nitrogen [when the hydroubiquinone is isolated as the *bis*-(methylcarbamate) to prevent reoxidation during isolation] but the hydroubiquinone does not accumulate

under oxygen because it converts to ubiquinone as quickly as it forms. The ubichromenol forms from both ubiquinone and hydroubiquinone under conditions of light irradiation in a nitrogen atmosphere; also, it forms from both ubiquinone and hydroubiquinone on heating with triethylamine. The yields of the mixture of hydroxyubiquinone derivatives from ubiquinone are not greatly different under oxygen or nitrogen but little, if any, of the hydroxyubiquinones are formed from the hydroubiquinone under nitrogen, suggesting that the hydroxyubiquinones arise from an intermediate formed from ubiquinone but not from hydroubiquinone.

Folkers and coworkers [12] consider that the photocyclization reaction involves a polar excitation state proceeding by elimination of a proton from the 1-position of the isoprenoid side chain to the intermediate which stabilizes by cyclization to form ubichromenol (Fig. 2, reaction A). An alternative possibility, which allows for both photoreduction and photocyclization, and is in accord with results found in recent studies on photorearrangement of other alkyl-*p*-benzoquinones [14] and the reaction of trialkylphosphites with quinones [15], is shown as reaction B in Figure 2. This reaction series is initiated by formation of a diradical which can either be reduced to the hydroquinone analog in the presence of a hydrogen donor (such as an alcohol) or, on prolonged irradiation, can result in electron migration through the mesomeric diradical in the quinone ring to form a dipolar transition state; the electron deficient oxygen atom is favorable for electrophilic addition to the double bond on abstraction of the allylic hydrogen and transfer of the hydrogen to the oxygen, forming the chromenol analog. Although it is not included in the present studies and is not shown in Figure 2, an allylic rearrangement to

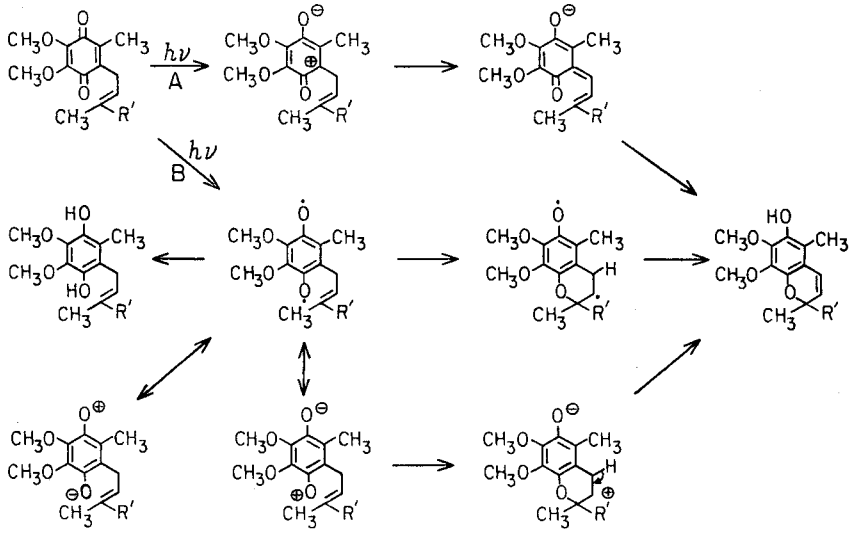

FIG. 2. Mechanisms proposed for photoreduction and photocyclization.

an isoubiquinone is known to occur on photodecomposition of CoQ_7 [16]; the formation of this product is consistent with the intermediates shown as reaction B in Figure 2. One possible mechanism for the photochemical *O*-demethylation (Fig. 3) involves the $n \rightarrow \pi^*$ transition at the oxygen atom of the methoxyl group followed by electron transfer to the quinone oxygen by an electromeric effect; this occurs equally at the two methoxyl groupings in ubiquinone so that equivalent amounts of 2- and 3-hydroxyubiquinones are formed.

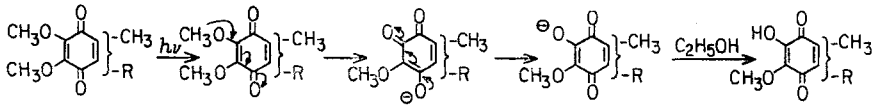

FIG. 3. Mechanism proposed for photodemethylation.

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