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THE CONSERVATION OF OXIDATIVE ENERGY IN PHOSPHATE-FREE SYSTEMS. FORMATION OF ACYL ANHYDRIDES *VIA* THE OXIDATION OF HYDROQUINONE MONOCARBOXYLIC ESTERS

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SUMMARY

1. The free energy released by the oxidation of hydroquinone monoesters or of 6-hydroxychroman-2-ones, using *N*-bromosuccinimide in glacial acetic acid as oxidant, is trapped and conserved by the formation of acyl anhydrides. For example, by oxidation of 2-methyl-1,4-naphthohydroquinone-1-acetate, acetic anhydride is formed in at least 98 % yield.

2. Such observations with model compounds suggest a cyclic scheme for energy conservation in mitochondria, in which ubihydroquinone is postulated to esterify with a protein carboxyl group (without prior carboxyl activation); the resulting ester is oxidized and a labile acid anhydride (or isoenergetic acyl function) is generated within the protein matrix. Reaction of the acid anhydride with inorganic phosphate and, subsequently, with ADP, provides a pathway for the oxidative generation of ATP, by way of a nonphosphorylated high energy intermediate.

3. That the proposed esterification step is feasible in a protein matrix is supported by the demonstration of a facile and complete conversion of coumarinic acid to coumarin at pH 4.

INTRODUCTION

In contrast to the mechanistic features of substrate level oxidative phosphorylation, those of respiratory chain oxidative phosphorylation have yet to be elucidated. In the latter process, the free energy made available by the reduction of molecular oxygen to water is utilized to generate at least three equivalents of ATP. At the present time, among the major unsolved biochemical problems are the understanding and duplication of the pathways by which "energy-rich" species are generated in the course of oxidation, *i.e.*, the mechanisms for the conservation of free energy in labile covalent bonds.

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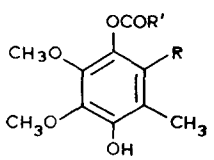
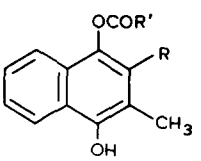
Perhaps the simplest approach depends on the demonstration of analogous conservation pathways by use of organic model compounds, which may or may not be related to those actually present in the mitochondrion. In previous efforts, energy conservation has been demonstrated in the oxidation of thiolesters[1], thioethers[2], arylthioethers[3], hydroquinone monophosphates[4,5], alkyl thiophosphates[6], and phosphate adducts of immonium bases[7]. The subject of energy conservation in model systems has been reviewed recently[8,9]. For an organic reaction to be considered an acceptable model, at least two conditions should be met: (a) there should exist a pathway for the facile reductive regeneration of substrate from its oxidation product and (b) the formation of substrate should not require a sizeable free energy input. For few of the cited model systems would it be possible to demonstrate turnover and participation in a cyclic process.

Convincing evidence has appeared, in studies with mitochondrial fragments and coupling factors[10,11], that an "energy-rich" species is generated prior to the incorporation of inorganic phosphate. Such evidence has prompted us to establish a third condition and to search for model systems which not only will fit into a cyclic scheme but will conserve free energy independently of phosphate. For example, the generation of acid anhydrides in the course of carboxylate-catalyzed oxidation of sulfides by iodine fulfills these conditions, since the resulting sulfoxides can be reduced readily back to sulfides[12].

The compounds selected for study in the present work* were chosen on the basis of their resemblance to the reduced forms of naturally occurring quinones which are believed to function in respiratory chain oxidative phosphorylation. Thus, 5,7-dibromo-6-hydroxychroman-2-one (III) and 2-methyl-1,4-naphthohydroquinone-1-acetate (VIII) can be considered to be analogues of monoesters of ubihydroquinone (I) and of reduced vitamin K (II), respectively.



I



II

R=lipid side-chain

EXPERIMENTAL

5,7-*Dibromo-6-hydroxychroman-2-one* (*III*)

Dihydrocoumarin (Aldrich Chemical Co.) was hydroxylated by Elbs persulfate oxidation[15]. The crude product was purified by treatment with Norit followed by column chromatography on silica gel and elution with ethyl acetate, a 22% yield of 6-hydroxychroman-2-one being obtained. To a solution of 10.25 g (0.063 mole) of 6-hydroxychroman-2-one in 125 ml warm glacial acetic acid was added a solution of 22.25 g (0.125 mole) *N*-bromosuccinimide (recrystallized from glacial acetic acid) in

* A preliminary report of this work has been published[13]; see also ref. 14.

250 ml warm acetic acid. The resulting golden yellow solution was kept at 45° for 30 min, diluted with 1 l of water and the mixture refrigerated. The buff-colored powder which separated (13.3 g, 62 %) was decolorized by passage through a silica gel column in ethyl acetate solution. The eluate was concentrated and the residue recrystallized from 250 ml glacial acetic acid to give 9.3 g of colorless material, m.p. 188–90° (lit. 182–184°) (ref. 16). An additional crop (1.8 g, m.p. 186–190°) was obtained by concentration of the mother liquors. (Found: C, 33.45; H, 1.87; Br, 49.43. $C_9H_6Br_2O_3$ requires C, 33.57; H, 1.88; Br, 49.63 %.)

N-(4-Bromophenyl)-2,4-dibromo-3,6-dioxo-1,4-cyclohexadien-1-propionamide (V)

To a suspension of 483 mg (1.5 mmoles) of III in 35 ml acetic acid was added 2.58 g (15 mmoles) of purified *p*-bromoaniline. The mixture was stored for 24 h at room temperature with exclusion of light. After removal of the solvent by lyophilization, the colorless residue was dissolved in ethyl acetate and the solution washed with 5 % hydrochloric acid and with water in an inert atmosphere. The organic layer was dried over magnesium sulfate, decolorized with a small amount of Norit, and then stirred for 1 h with 600 mg anhydrous sodium sulfate and 600 mg silver oxide. The solids were removed and the bright yellow filtrate concentrated to a small volume. Addition of methanol gave 220 mg of an orange crystalline precipitate which was recrystallized from a small volume of ethyl acetate. The yellow-orange quinone darkened at 180° and decomposed at 188–91°; infrared absorption in methylene chloride at 2.91 μ (NH), 5.94 μ and 6.04 μ (amide and quinone carbonyls); ultraviolet absorption maxima (acetonitrile) 250, 283 and 500 mμ (ε 19500, 11700 and 500, respectively). (Found: C, 36.86; H, 2.13; N, 2.86; Br, 48.41. $C_{15}H_{10}NO_3Br_3$ requires C, 36.61; H, 2.05; N, 2.85; Br, 48.73.)

Oxidation of III in acetic acid and trapping of the anhydride formed

To a stirred solution of 483 mg (1.5 mmole) III in 20 ml acetic acid (purified by distillation from potassium permanganate and dried by distillation from anhydrous copper sulfate) at 45° was added a solution of 267 mg (1.5 mmole) *N*-bromosuccinimide and 820 mg fused sodium acetate in 15 ml purified acetic acid*. The resulting bright yellow solution was kept at room temperature for 30 min. Dilution of an aliquot of the reaction mixture with acetonitrile and examination of its ultraviolet spectrum revealed that the original peak at 296 mμ had been displaced to 288 mμ, characteristic of a 2,6-dibromo-*p*-quinone[17] (Fig. 1).

To the reaction mixture was added a solution of 258 mg (1.5 mmole) purified *p*-bromoaniline in 7.5 ml benzene**. After 30 min storage at room temperature, the amber solution was lyophilized. The residue was dissolved in ethyl acetate and the solution washed with four 15-ml portions of 5 % hydrochloric acid and with six 15-ml portions of 5 % sodium bicarbonate. The red ethyl acetate solution was dried over magnesium sulfate and concentrated to a dark brown oil. Trituration of the oil with *n*-hexane yielded 600 mg of a dark brown solid. The material was transferred, in ethyl acetate solution, to a preparative thin-layer plate (silica gel GF) and the plate developed with ethyl acetate, using *p*-bromoacetanilide and V as markers. Appropriate bands were eluted with ethyl acetate and the extracts dried.

* Sodium acetate was added to neutralize the hydrogen bromide liberated in the reaction.

** The use of excess *p*-bromoaniline led to complex reaction with the dibromoquinone.

p-Bromoacetanilide was identified by m.p., ultraviolet and infrared spectra. The yield of anilide recovered from the plate was 62 % of theory, assuming this to be the only anilide formed. Under the same reaction conditions, *p*-bromoaniline and acetic acid alone formed a negligible amount of the anilide. The dibromoanilide (V) crystallized very slowly from cold methanol. The yield was low, probably due to partial destruction on the thin-layer plate. The ultraviolet and infrared spectra were identical to those of an authentic sample. Because of its poorly defined m.p. and low recovery, the compound was further identified by its mass spectrum. High resolution mass spectral measurements (obtained on an A.E.I. high resolution mass spectrometer, model MS-9) indicated base peaks at 488.820134, 490.817544, 492.814953 and 494.816474; theory for $C_{15}H_{10}NO_3Br_3$ requires 488.821233, 490.819263, 492.817293 and 494.815323.

When *N*-iodosuccinimide was substituted for *N*-bromosuccinimide and the reaction carried out as described above, *p*-bromoacetanilide was isolated in 34 % yield.

Oxidation of VIII in acetic acid

To a stirred solution of 324 mg (1.5 mmoles) 2-methyl-1,4-naphthohydroquinone-1-acetate (VIII)[18] in 15 ml purified acetic acid containing 80 mg fused sodium acetate was added a solution of 534 mg (3.0 mmoles) *N*-bromosuccinimide in 15 ml acetic acid. The resulting bright yellow solution was kept at room temperature for 30 min. Dilution of an aliquot to spectral concentration with acetonitrile revealed that the original peak at 300 mμ had disappeared and had been replaced by peaks at 245, 251, 280 and 333 mμ (Fig. 2). This spectrum is characteristic of 2-bromo-3-methyl-1,4-naphthoquinone (IX)[19].

To the reaction mixture was added a solution of 258 mg (1.5 mmole) *p*-bromoaniline in 7.5 ml benzene The mixture was worked up as described above for the oxidation of III. Preparative thin-layer chromatography, employing benzene as the developing solvent, resulted in the isolation of 2-bromo-3-methyl-1,4-naphthoquinone in 74 % yield and *p*-bromoacetanilide in 98 % yield. Both products were identified by infrared and ultraviolet spectra, mixed m.p. and combustion analysis. Upon repetition of the experiment in the absence of *N*-bromosuccinimide, no *p*-bromoacetanilide could be detected by thin-layer chromatography.

Hydrolysis and relactonization of coumarin (X)

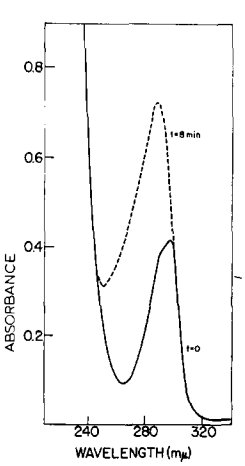
The rate of hydrolysis of coumarin ($7.5 \cdot 10^{-5}$ M) was measured spectrophotometrically at 25° in 0.0067 M potassium hydroxide (containing 10 % acetonitrile). Ultraviolet spectra were recorded with time (Cary spectrophotometer, model 14) and the rate constant for hydrolysis was calculated using the absorbance at 280 mμ (Fig. 3) and the first order rate equation. The rate of ring closure was determined in a similar fashion, after acidification of the above solution to pH 3.9 with glacial acetic acid. The infinity spectrum was identical to that of the starting material, coumarin. In both cases, strict obedience to the first order rate law was observed (Fig. 4).

RESULTS

Oxidation studies

Oxidation of 5,7-dibromo-6-hydroxychroman-2-one (III) by *N*-bromosuccinimide (or *N*-iodosuccinimide) in glacial acetic acid leads to the formation of the

dibromoquinone anhydride, IV. This species has been inferred by ultraviolet spectroscopy (Fig. 1) and trapped by *p*-bromoaniline. Thus, addition of one equivalent of *N*-bromosuccinimide in acetic acid to an acetic acid solution of the lactone (III) results in the immediate appearance of a yellow color and an absorption maximum at 288 mμ; the spectrum is quite similar to that of 2,6-dibromobenzoquinone[17]. The reaction appears to be quite rapid, since the spectrum remains constant after the first few minutes (Fig. 1). Addition of 1 equivalent of *p*-bromoaniline to the reaction solution leads to the formation of both *p*-bromoacetanilide and the quinone anilide, V.



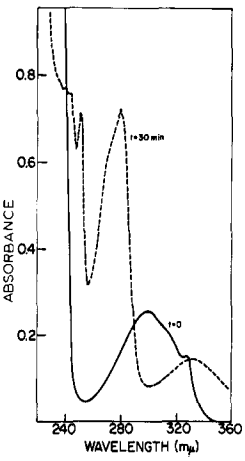


Fig. 1. Spectral changes resulting from the oxidation of 5,7-dibromo-6-hydroxychroman-2-one (III) by *N*-bromosuccinimide. Original reaction mixture in glacial acetic acid was diluted with acetonitrile for spectral examination (final concentration, 10^{-4} M). ———, spectrum of III prior to addition of oxidant; - - - - - - -, spectrum 8 min after addition of oxidant. At the concentrations used, the absorbances of *N*-bromosuccinimide and of succinimide are negligible.

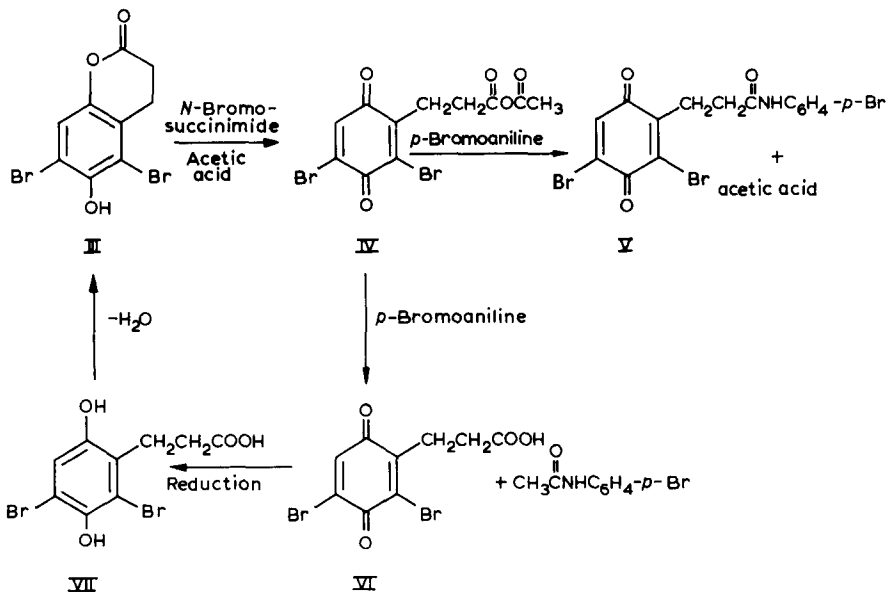
Fig. 2. Spectral changes resulting from the oxidation of 2-methyl-1,4-naphthohydroquinone-1-acetate (VIII) by *N*-bromosuccinimide. Original reaction mixture in glacial acetic acid was diluted with acetonitrile for spectral examination (final concentration, $5 \cdot 10^{-5}$ M). ———, spectrum of VIII prior to addition of oxidant; - - - - - - -, spectrum 30 min after addition of oxidant.

These products can be isolated by preparative thin-layer chromatography and have been identified by comparison with authentic samples. Attack by the nucleophile at either carbonyl of the mixed anhydride is competitive. Since the p*K* values of the corresponding carboxylic acids should be similar, a lack of selectivity in anilide formation might be anticipated; however, greater steric accessibility to the acetate carbonyl may account for the moderate preponderance of *p*-bromoacetanilide (62 %).

In a similar fashion, acetic anhydride and 2-bromo-3-methyl-1,4-naphthoquinone (IX) are formed during the oxidation of 2-methyl-1,4-naphthohydroquinone-1-acetate (VIII) by *N*-bromosuccinimide (2 equivalents) in acetic acid*. The acetic anhydride can be trapped as *p*-bromoacetanilide in 98 % yield by the addition of *p*-bromoaniline. The formation of 2-bromo-3-methyl-1,4-naphthoquinone (IX) can be detected by ultraviolet spectroscopy (Fig. 2) prior to the addition of the trapping

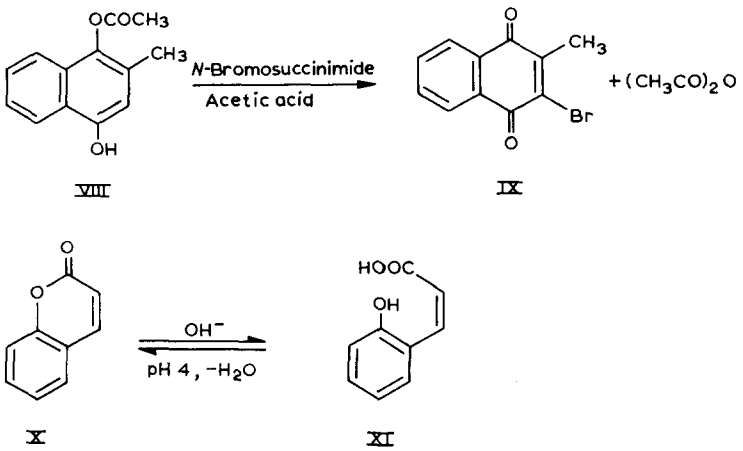
* Preliminary experiments indicate that acyl halides may similarly be generated in the presence of halide ion.

agent and can be isolated from the final reaction mixture in 74 % yield by preparative thin-layer chromatography.



Lactonization studies

The alkaline hydrolysis of coumarin (X) leads to the formation of the dianion of coumarinic acid (*cis-o*-hydroxycinnamic acid, XI). In 0.0067 M potassium hydroxide (10 % acetonitrile, apparent pH 11.8), hydrolysis of the lactone proceeds with a half-time of 4.3 min at 25° (first order $k = 0.161$ min^{-1}). Coumarinic acid is stable only as its salt; efforts to isolate the free acid lead, invariably, to recovery of coumarin exclusively[20]. Acidification of the alkaline solution to pH 3.9 with glacial acetic acid results in ring closure to regenerate the lactone, the reaction proceeding with a half-



time of 26.6 min at 25° (first order $k = 0.0261$ min^{-1}). Equilibrium is sufficiently in favor of lactonization that the infinity spectrum is identical to that of the starting lactone. The kinetics of ring-opening and cyclization may be followed by absorbance changes at 280 mμ (Fig. 3), both reactions obeying strict first-order kinetics (Fig. 4).

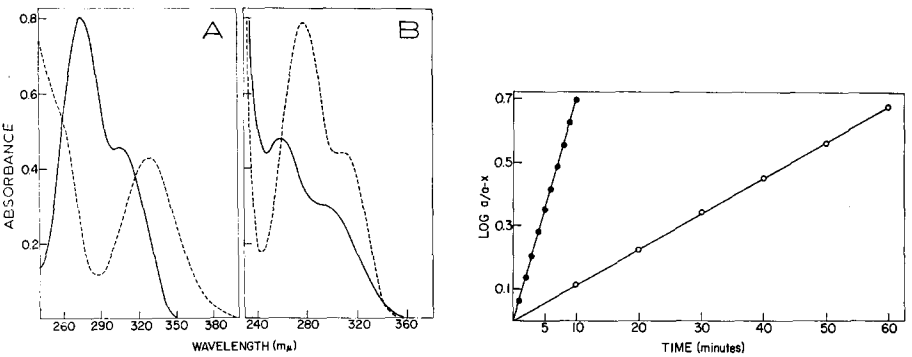
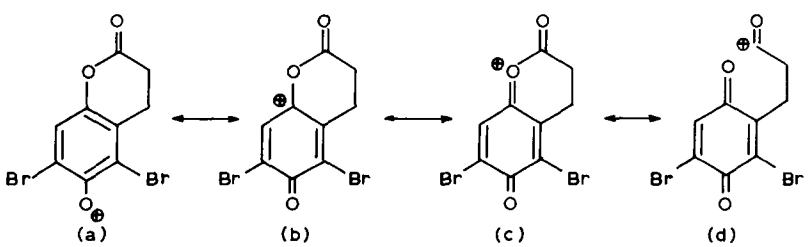


Fig. 3. A. Spectral changes resulting from the alkaline hydrolysis of coumarin (X), $7.5 \cdot 10^{-5}$ M in 0.0067 M KOH (10% acetonitrile): ———, coumarin (X); - - - - - - -, dianion of coumarinic acid (XI). B. Spectral changes resulting from the lactonization of coumarinic acid at pH 3.9: ———, coumarinic acid, immediately following acidification of dianion solution to pH 3.9; – – – –, coumarin, resulting from relactonization.

Fig. 4. ●—●, rate of hydrolysis of coumarin (X) in 0.0067 M KOH at 25°; ○—○, rate of formation of coumarin from coumarinic acid (XI) at pH 3.9 (25°).

DISCUSSION

The above examples clearly demonstrate the coupling of an exergonic oxidative process with an endergonic chemical process. These reactions proceed extremely rapidly under mild conditions and give rise to highly reactive chemical species, such as acid anhydrides. Although we have no information on the detailed mechanism of



XII

the reaction, a variety of pathways may be postulated, involving attack of acetic acid or of acetate ion on the electron-deficient species created by oxidation (XII). Anhydride formation may result from the formation and collapse of a tetrahedral intermediate or by direct solvolysis of a transient acylium ion (XIId). We hope to obtain additional information in future studies.

These findings lead us to propose the cyclic process of Fig. 5 as a scheme by which ubiquinone or vitamin K may function in the second ATP formation of respiratory chain oxidative phosphorylation. The process is initiated by NADH reduction of ubiquinone to ubihydroquinone. The lipophilic side-chain of the co-factor is assigned the role of binding the compound to mitochondrial protein and creating an orientation such that a phenolic hydroxyl is brought into proximity with a protein carboxyl group. We postulate that, under the unimolecular and sterically constrained conditions of protein–substrate interaction, ester formation can occur between the phenolic hydroxyl and protein carboxyl without *any* need for prior activation of the carboxyl group (see below). Oxidation of such a hydroquinone ester, bound to the protein, can occur with nucleophilic participation of a neighboring group on the protein, such as carboxyl, sulfhydryl or imidazole. In each case, the free energy of oxidation would be conserved in a labile acyl linkage within the protein matrix*. It is then apparent that the charged mitochondrion could not release an energy-rich species into extracting media. Subsequent reaction of acyl-X with inorganic phosphate would lead to protein-bound acyl phosphate which could, in turn, phosphorylate ADP.

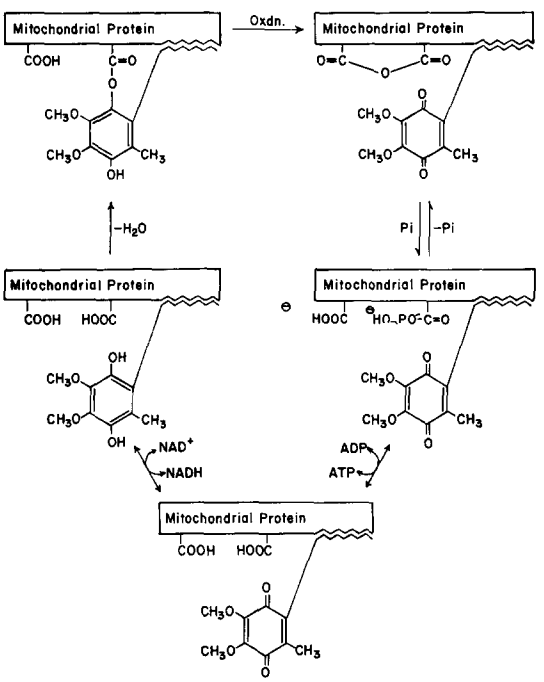


Fig. 5. Hypothetical scheme for an oxidative phosphorylation cycle.

The latter reactions are, of course, well-known to occur both *in vivo* and *in vitro*. Nor is there lack of precedent for activation of protein carboxyl groups[21]. Reduction of the ubiquinone released in the oxidative process permits the cycle to begin again. The above scheme accommodates fully the various findings on oxygen exchange in

* For additional proposals, see refs. 28 and 29.

mitochondrial systems[22] and the absence of exchange of ubiquinone oxygen[23] or hydrogen[24].

We must now face the question of whether it is thermodynamically realistic to postulate ester formation between a phenolic hydroxyl and a protein carboxyl group. Although equilibrium between a carboxylic acid and an alcohol lies well on the side of ester formation, only traces of ester are formed in the corresponding bimolecular system containing a phenol[25]. This difference is understandable in view of the pK differences between the two types of hydroxyls. Since an intramolecular model is a more valid test for the enzymatic system than is the corresponding bimolecular reaction[26], the answer to the question is more properly sought in a study of intramolecular esterification. The demonstration of rapid and complete lactonization of coumarinic acid (XI) at pH 4 provides the needed evidence that such a postulate is entirely reasonable on a chemical basis. Furthermore, earlier studies suggest that the rate of cyclization will be enhanced considerably by a decrease in the polarity of the medium[27]. A lipid environment in the mitochondrion would hardly deter such a process. The reductive conversion of the quinonepropionic acid (VI) to the hydroquinonepropionic acid (VII) is facile and complete; since the conversion of VII to III is relatively slow in aqueous media at 25°, we are currently investigating models which are more favorable for cyclization and which are more analogous to ubiquinone.

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