The Tautomerism of Quinones and the Question of Quinone Methide Intermediates in Oxidative Phosphorylation

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SUMMARY

A series of quinones, including vitamin K$_{1(10)}$, have been shown not to exhibit isotopic hydrogen exchange. This finding is strong evidence against both the hypothesis of quinone methide tautomerism and the suggested occurrence of quinone methide intermediates in oxidative phosphorylation and various chemical reactions.

The results of previous studies on duroquinone with the use of nuclear magnetic resonance had been interpreted in terms of hydrogen isotope exchange in basic solution. This interpretation has been shown to be mistaken. The nuclear magnetic resonance results have now been shown to be due to the formation of a free radical in basic solution. The radical was also detected with the use of electron spin resonance spectroscopy.

The relevance of these results to the mechanism of oxidative phosphorylation is discussed.

In 1935, Fuson (1) proposed that quinones with alkyl substituents on the ring might react in tautomeric quinone methide form, e.g. as shown in Scheme 1. This hypothesis has since been invoked to explain a large number of reactions, including the condensation of malonic ester with duroquinone (2) and the mechanism of oxidative phosphorylation (3-5). Stable quinone methides have been prepared and have been the subject of much past and current research. A review covering the chemistry of quinone methides up to 1964 has recently appeared (6). The isolation of several quinone methides suggests that the reversible tautomeric equilibrium shown above does in fact apply to certain quinones, but until recently no direct evidence has been offered for tautomerism.

There are three obvious lines of attack on the problem. (a) One can try to show by chemical (or preferably physical) means that a given quinone exists as a mixture of two forms, as may be shown for many keto-enol equilibria. (b) One can try to show that the rate of reaction of a quinone with a series of reagents is independent of the nature and concentration of the reagents at high reagent concentration. For example, the rate-determining step in the attack of halogens on acetone is the conversion of the keto to the enol form, although the latter has not been detected physically as a separate species (7). (c) One can try to show that the quinone undergoes hydrogen isotope exchange under conditions in which it is supposed to exhibit tautomerism. If, in addition, the rate of hydrogen exchange is the same as the rate of reaction of the quinone in a chemical reaction, strong support is given to the existence of tautomerism.

Of these three criteria, none has been satisfied for any quinone. No quinone has been shown to exist in two forms. Some quinone methides are stable, but they have not been obtained from the corresponding quinone; e.g. ortho-benzoquinone methide, which is stable at temperatures below about $-50^\circ$, is obtained by the pyrolysis of O-methoxymethylphenol (8).

In terms of Criteria b, no kinetic studies on the reactions of quinones have been interpreted in terms of a rate-determining step depending on the quinone alone.

The third criterion, hydrogen exchange, is the subject of this paper. In 1963 we attempted, and failed, to find hydrogen exchange in solutions of duroquinone. Recently it has been suggested on the basis of nuclear magnetic resonance studies (9) that such exchange does in fact occur, presumably via a reversible tautomeric equilibrium. These results were regarded as being strong evidence for the occurrence of quinone methides as intermediates in oxidative phosphorylation. We therefore repeated our earlier exchange studies, also studying vitamin K$_{1(10)}$, again with negative results. A preliminary description of the experiments has been published elsewhere, with a discussion of the relevance of the results to the mechanism of oxidative phosphorylation (10). We have now extended our studies to other quinones and have also examined the amination of duroquinones as described by Cameron, Scott, and Todd (11), since this reaction has been assumed to proceed via a quinone methide intermediate (Scheme 2).

In no case have we found hydrogen isotope exchange. Moreover, we find that the NMR results for duroquinone are evidence not for hydrogen exchange, as has been suggested (9), but for the formation of a free radical in basic solutions. This interpretation is supported by electron spin resonance studies.

The abbreviations used are: NMR, nuclear magnetic resonance; ESR, electron spin resonance.
Materials—Duroquinone, 2,3-dimethyl-1,4-naphthoquinone, and vitamin K$_1$(20) were obtained commercially. 2,3-Dimethyl-1,4-naphthoquinone-1-phosphate was prepared by the method of Andrews (12). D$_2$O (99.7%) was supplied by Norsk Hydro-elektrisk.

NMR spectra were recorded on a Varian A-60 Spectrometer at 23 ± 1°. ESR spectra were recorded on a Varian V-4502-12 spectrometer at 21 ± 1°.

Hydrogen Exchange of Duroquinone (1,2,3,4-Tetramethyl-benzoquinone)—Duroquinone (60 mg) was dissolved in 0.1 N acid (DCl) or 0.1 N alkaline (NaOD) solution in deuteriated methanol CH$_2$OD (5 ml). After 30 min, the solution was neutralized, the solvent was evaporated, and the duroquinone was recovered from the residue by sublimation. The NMR spectrum of a weighed amount of the sublimate in CCl$_4$ gave a peak due to the methyl group of duroquinone with an intensity indicating no significant deuteration exchange. We repeated the above experiment with the use of the conditions and concentrations given by Scott (9), but no deuteration exchange was found. In addition, when duroquinone (10 mg) is dissolved in alkaline solution (0.014 to 1.0 N NaOH) in methanol-d or normal methanol (0.5 ml), the methyl resonance broadens and appears to lose intensity, as described by Scott (9). This loss is illusory, however, since integration of the broadened peak shows that there is no decrease in the total area under the peak. Furthermore, the change in the spectrum is effectively instantaneous, and there was no change in the spectrum between 30 sec and 1 hour after adding NaOH or NaOD. The most significant and surprising result was that in both normal and deuterated methanol the change in the spectrum is completely reversible. Neutralization of the sodium hydroxide in the solutions by adding hydrochloric acid (or DCl) immediately results in the restoration of the methyl resonance to a narrow line with no loss of intensity. The changes in the spectrum in CH$_3$OH are illustrated in Fig. 1. The intensity of the methyl line in acidic or neutral solution was estimated both from its height and by integration. The intensity of the broadened line in alkaline solution could only be determined by integration. To avoid errors due to possible fluctuations in the radiofrequency field over the time of the experiments, the intensities in CH$_3$OH were measured relative to the intensity of one of the $^{13}$C satellites of the methyl peak of the solvent methanol. This line is convenient since it lies about 20 cps downfield from the duroquinone resonance and in our experiments was of comparable intensity. The above changes in the NMR spectrum of duroquinone are paralleled by changes in the ESR spectrum of the same solutions.

The ESR spectra of a 0.1 M solution of duroquinone immediately after addition of base shows an extremely broad line which in the absence of hyperfine structure cannot be interpreted. On standing, the spectrum changes slowly to the 13-line spectrum of the duroquinone-free radical. The measured hyperfine constant was 1.90 gauss compared to the value of 1.887 gauss given by Venkataraman and Fraenkel (15). The neutralization of a basic solution of duroquinone results in the complete destruction of free radical as ascertained by the absence of an ESR signal from the solution.

Hydrogen Exchange of 2,3-Dimethyl-1,4-naphthoquinone—The compound (0.5 g) was dissolved in a 1:1 mixture of D$_2$O and dioxan (15 ml). The exchange was studied at three different pH values: (a) in the solvent with no additional buffer; (b) at pH 8 to 9 in the presence of sodium bicarbonate; and (c) at pH 1 (DCl). The mixtures were kept at 100° in sealed tubes in the absence of light for 5 to 8 days. The solvent was then evaporated and the naphthoquinone was recovered from the residue by sublimation. The NMR spectrum of the sublimate in CCl$_4$ gave a single peak corresponding to the methyl group (at 2.2 ppm from tetramethylylene as an external reference) and a group of lines centered on 7.9 ppm due to the aromatic protons. The ratio of the integrated intensity of these lines was 3:2, indicating no deuteration exchange, since it is highly improbable that both the aromatic and methyl groups exchanged at the same rate.

Oxidation of 2,3-Dimethylnaphthoquinone-1-phosphate—This was carried out in D$_2$O solution at 25° by adding saturated solutions of bromine in D$_2$O. The oxidation is instantaneous (14, 15); the product, 2,3-dimethylnaphthoquinone, was extracted with petroleum ether, and after evaporation of the solvent the naphthoquinone was recovered by sublimation. Once again the NMR spectra was identical with that of the normal (undeteriated) compound.

Hydrogen Exchange of Vitamin K$_1$(20)—2-Methyl-3-phytyl-1,4-naphthoquinone (100 mg) was dissolved in methylene chloride (500 ml) containing 12 ml of a 0.1 N solution of HClOd in CH$_2$COOD. These conditions are those given by Maimont et al. (16) in their study of the side chain cyclization in vitamin K$_1$(20). After 4 hours at 4–5°, the solvent was removed. The NMR
spectrum of the residue was taken in CHCl₃. Examination of the peaks due to vitamin K₃ showed that the ratio of intensities of the peaks due to the a-methyl and the aromatic protons was the same as in the normal compound. The chemical shifts from tetramethylsilane were the same as those given by Mamont et al. (16).

2,3-Dimethyl-5,6-bis(piperidinomethyl)quinol—A solution of duroquinone (0.4 g) in a mixture of D₂O (5 ml) and freshly distilled piperidine (10 ml) was left at room temperature for 35 hours and then evaporated under vacuum. The residue was recrystallized from ethanol (m.p. 160-162°). The NMR spectrum of this product in CHCl₃ was identical with that of the compound prepared with H₂O, showing that there was no deuterium incorporation during the reaction in D₂O. The results are summarized in Table I.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>Temperature</th>
<th>Reaction time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol (0.014-1.0 n NaOH)</td>
<td>23°</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>Piperidine-d</td>
<td>23</td>
<td>35 hrs</td>
</tr>
<tr>
<td></td>
<td>Dioxane-D₂O</td>
<td>100</td>
<td>8 days</td>
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<tr>
<td></td>
<td>Dioxane-D₂O (pH = 9-9)</td>
<td>100</td>
<td>5 days</td>
</tr>
<tr>
<td></td>
<td>Dioxane-D₂O (pH = 1)</td>
<td>100</td>
<td>4 days</td>
</tr>
<tr>
<td></td>
<td>Br₂-D₂O (pH = 4)</td>
<td>25</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Methylene chloride CH₂COOD (0.1 m HClO₃)</td>
<td>4-5</td>
<td>4 hrs</td>
</tr>
</tbody>
</table>

DISCUSSION

The results show that when the above named quinones are dissolved in deuterated solvents under various conditions and then recovered from solution, no hydrogen isotope exchange can be detected by NMR spectroscopy. The behavior of duroquinone requires more comment. The results show that the changes in the NMR and ESR spectra of duroquinone following the addition of base are completely reversed by subsequent acidification and occur in both the presence and absence of deuterium in the solvent. The changes are therefore not due to hydrogen isotope exchange. They are, however, consistent with the presence of a free radical in basic solution. The addition of base results in the formation of a radical which, by taking part in an electron exchange reaction with duroquinone, results in a broadening of the NMR peak of this molecule. The subsequent neutralization of the solution results in the disappearance of free radicals and the simultaneous restoration of the broad NMR line to a narrow peak. The formation of the free radical by addition of base to an alcoholic solution of duroquinone has previously been reported by Brandon and Lucken (17), who suggest that the alkoxide ion is oxidized to the peroxy radical. The fate of the radical is unknown. If it dimerizes, this must be a slow reaction since a strong ESR spectrum is observed up to 24 hours after addition of base. Any extensive irreversible destruction of the radical would not be compatible with the NMR evidence that no significant loss of duroquinone occurs because of the successive addition of base and acid. The extremely broad electron paramagnetic resonance observed directly after the addition of base may have a number of explanations, e.g. fast electron exchange between duroquinone and its free radical or intermolecular dipolar relaxation effects. There might also be significant concentrations of other radicals beside the duroquinone radical ion. The nature of the reactions of duroquinone in alkaline methanol and the role of oxygen are not known, but,
Although of interest in themselves, they have no direct bearing on the question of isotopic hydrogen exchange. It is known that the addition of KOH to a saturated solution of duroquinone in ethanol gives a dimer of duroquinone, the structure of which is uncertain (18). This dimer is not formed in detectable amounts under the conditions of our experiments for the following reasons. We have prepared the dimer and examined its NMR spectrum in CDCl₃. Major resonance peaks occur at 1.3, 1.45, 2.05, 2.15 ppm from tetramethylsilane (as an external standard), and have relative integrated intensities of 1:1:2.85:2.1, respectively. These peaks are tentatively assigned to the seven methyl groups in the structure shown in Scheme 3. While no definite assignment is made here, it is probable that the peak at lowest field (2.15 ppm) is due to the methyl groups 2 and 3, since these are on a quinoid ring and have approximately the same chemical shift as the methyl groups of duroquinone. There are two small peaks at 2.55 and 2.9 ppm of equal intensity. Each of these peaks has an integrated intensity of about a third of the two high field lines. There also appears to be a smaller line at 3.2 ppm. It may be that these lines are due to magnetically inequivalent protons of the methylene group at position 9. There is also a small line at 4.3 ppm, but we hesitate to assign this to the —OH group of the structure shown above. In alkaline solutions of duroquinone in methanol at the concentration used to study hydrogen exchange, only one sharp resonance was observed. Also, the dimer had a melting point of 206–207 °C (18), while the material isolated from the dilute alkaline solution in methanol had a melting point of 110 °C, corresponding to duroquinone. The results for duroquinone are thus explicable in terms of the formation of a free radical in base and its destruction by acid. No significant hydrogen isotope exchange occurs.

We have not examined trimethyl(3-methyl-2-butenyl)-1,4-benzoquinone, the other compound stated to undergo hydrogen isotope exchange under basic conditions. Scott (9) states that, in methanol d containing 0.1 m NaOD, the peak due to the methyl group on the ring lost about 95% of its intensity in 20 min, whereas a control lost about 20% of its intensity in the same time. If the line broadening on addition of base to this compound depends on the concentration of base, as found for duroquinone (Fig. 1), it may be that the difference between the control and the experiment can be explained by a small difference in the base concentration. It would be advisable to repeat this experiment, isolate the quinone, purify it, and re-examine the NMR spectrum. It was also stated that in this compound there was no change in the (CH₃)₃C— and HO—NMR peaks of the isoprenoid chain during the time that deuterium exchange occurred in the 3-methyl group. It was concluded that ring closure is not necessary for quinone methide formation. It seems likely that in fact no exchange occurs in this compound and that the conclusions drawn are incorrect.

In the oxidative phosphorylation scheme suggested by Vilkas and Lederer (4, 5), the oxidation of quinol phosphates is indicated as proceeding through a quinone methide (Scheme 4). If so, a similar scheme might apply to oxidation in vitro, but our results show that, in the oxidation of 2,3-dimethynaphthoquinone-1-phosphate in D₂O solution with bromine, no incorporation of deuterium into the product quinone was found.

Cameron, Scott, and Todd (11) studied the amination of a number of methylated quinones and suggested that the reaction, which yields substituted quinones, may proceed via a quinone methide intermediate. The authors point out that such an intermediate was postulated by Smith and Deynes (19) in the reaction of duroquinone with sodium hydroxide. Once again, if a quinone methide molecule is reversibly formed in the amination of duroquinone, then, if amination occurs in a D₂O solution of piperidine-d₆, there should be incorporation of deuterium in the final product. However, as described under “Experimental Procedure and Results,” when this experiment was performed no deuterium was found in the product from NMR measurements. We conclude either that a quinone methide is not formed reversibly, or, if it is formed, that the rate of amination is far faster than the return to the normal duroquinone molecule.

Gardner, Suraizadeh, and Rand (20) have proposed that the displacement of the amine group of certain hydroxybenzyl derivatives proceeds via a transition state similar to a charge-separated quinone methide. Such a transition state might occur in the reactions of vitamin K. The possible tautomerism of vitamin K is of great biological interest. Vilkas and Lederer (4, 5) have presented evidence to show that a methide form of vitamin K₁(20) occurs in various reactions, for example, in the formation of a dimer, the addition of styrene and the addition of diphenyl ethylene (21). Since no hydrogen isotope exchange has been detected for vitamin K₁(20), it may be that a quinone methide-like transition state provides a better explanation of the results than the suggested tautomerism. Since purely chemical
evidence might be regarded as being inapplicable to processes in vivo, it is interesting that during oxidative phosphorylation there is no incorporation of tritium into ubiquinone-10 in rat liver mitochondria (22) or into benzoquinone in bovine heart mitochondria (23). Active photophosphorylation in chromatophore particles from Rhodospirillum rubrum is likewise unaccompanied by tritium incorporation into ubiquinone-10 (22). However, Gutnick and Brodie (23) found that, during oxidative phosphorylation in a system from Mycobacterium phlei, tritium is incorporated into vitamin K(1)(24). Incorporation only occurred in the presence of phosphate ion, and does not occur with other analogues of vitamin K(1)(z) which do not take part in a phosphorylation reaction, although they do participate in the oxidation path. Thus, tritium incorporation requires both oxidation and phosphorylation, and is not due to a simple tautomeric equilibrium. The position of the incorporated tritium is not known.2

Acknowledgment—We wish to thank Dr. C. Eden of the Hebrew University for help with the ESR measurements.

REFERENCES

2 Note—To resolve a possible contradiction between the results from the bacterial and mitochondrial systems, Snyder, Di Mari, and Rapoport (24) studied oxidative phosphorylation in a system from Mycobacterium phlei and found no incorporation of tritium from T2O medium into vitamin K(1)(25). Analogous results have been reported by Horth et al. (26).
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