The Reaction of Coenzyme PQQ with Hydrazines

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The reaction of coenzyme PQQ with hydrazines, which are known to be inhibitors of quinoprotein amine oxidases, has been investigated *in vitro*. Only the redox reaction is observed in the reaction with phenylhydrazine, methylhydrazine, *N,N*-dimethylhydrazine, and *N,N'*-dimethylhydrazine. However, the PQQ adduct formation occurs concomitantly in the reaction with 4-nitrophenylhydrazine, and only hydrazone formation is observed in the reaction with 2,4-dinitrophenylhydrazine [(**3**) and (**4**), respectively]. Kinetic studies reveal that the order of the redox reactivity is phenylhydrazine ≃ methylhydrazine > *N,N'*-dimethylhydrazine ≫ *N,N*-dimethylhydrazine, which does not correlate with the two-electron redox potentials of these hydrazines. The reactivity of the PQQ model compounds [PQQ, PQQTME, and (**5**)–(**10**)] in the oxidation of methylhydrazine is also examined, the relative reactivity seems to be related to the reactivity of the quinone functional group toward nucleophilic addition (hydration), but does not reflect the two-electron redox potentials. These results suggest that reduction of PQQ with hydrazines proceeds *via* covalent addition of hydrazines to the quinone to form a carbinolamine type intermediate followed by electron flow from the nitrogen of hydrazines into the quinone moiety.

Copper-containing amine oxidases have been found to catalyse the oxidative deamination of primary amines [equation (1)][1] in a wide range of eukaryotic organisms such as mammals, plants, and fungi.[1]

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + H_2O_2 + NH_3 \quad (1)$$

The enzymes contain a second prosthetic group which interacts strongly with the carbonyl reagents (such as hydrazine derivatives) to be inactivated. Most researchers have proposed a role for pyridoxal phosphate as the cofactor, though there has been no conclusive evidence identifying the cofactor as a pyridoxal derivative.[1] In 1984, two independent groups reported that bovine plasma amine oxidase may contain pyrroloquinoline quinone (PQQ, 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid) as the possible cofactor.[2,3] Duine and his co-workers[3] developed the method for PQQ detection using a hydrazine by which a hydrazone or an azo adduct of PQQ was isolated after extensive proteolysis and identified by HPLC analysis.[4] However, details of such inhibition reactions are still disputable. Bruice and his co-workers reported briefly the reaction between PQQ analogues and NH_2NH_2 and proposed the ionic mechanism (addition–elimination).[5]

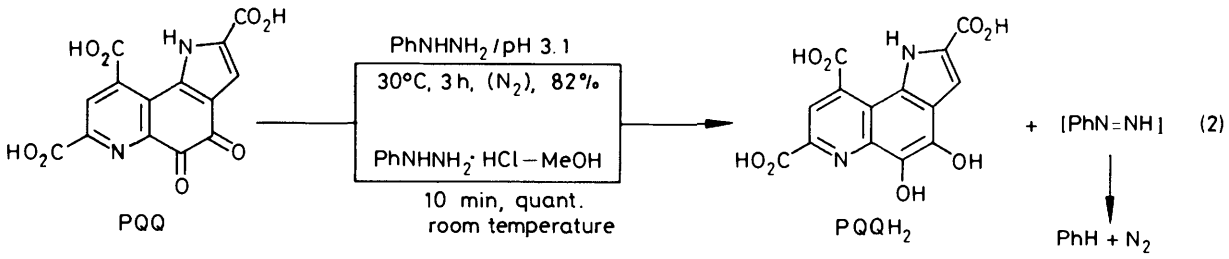
We have studied the reaction of coenzyme PQQ with amines as a model reaction of the amine oxidase, and demonstrated the effective oxidative deamination of amines and the ionic mechanism through an important carbinolamine intermediate.[6] In this paper, the reaction of PQQ with hydrazines is examined in order to elucidate the inhibition mechanism of amine oxidase. The chemical behaviour of PQQ toward hydrazines is very interesting not only from the view point of quinoprotein inhibition but also from the organic chemist's point of view. There have been a few reports concerned with the oxidation of hydrazines by quinones but the mechanism is still not well understood.[7]

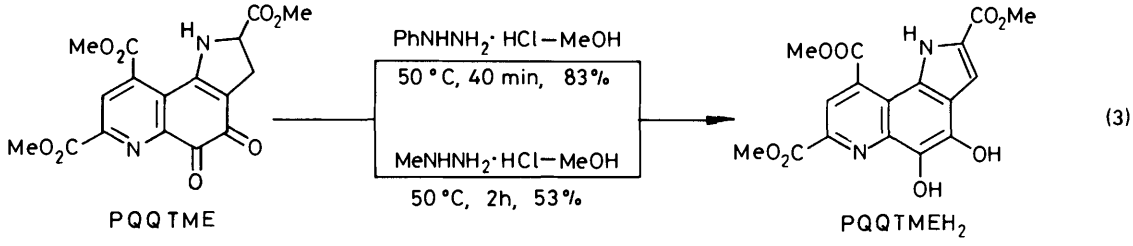
Results

Product Analysis.—When PQQ was treated with a tenfold excess of $PhNHNH_2$ in an aqueous buffer solution at pH 3.1 under anaerobic conditions, reduced PQQ (quinol form; $PQQH_2$) was obtained in 82% yield [equation (2)].[8] Formation of phenyldiazene (PhN=NH) was also confirmed by detecting its decomposition products such as benzene, biphenyl, *etc.* by HPLC and GC analysis.[9] The redox reaction also took place in the reactions of PQQ and phenylhydrazine hydrochloride, the trimethyl ester of PQQ (PQQTME) and phenylhydrazine, and PQQTME and methylhydrazine hydrochlorides in MeOH to yield the corresponding quinol ($PQQH_2$ and $PQQTMEH_2$) as the sole isolable product [100, 83, and 53%, respectively, equations (2) and (3)]. However, adduct formation was only observed when a simple *o*-quinone such as phenanthrenequinone was employed under identical conditions to yield a mixture of hydrazone (**1**) and azo (**2**) tautomers [68%, equation (4)].[10] In the reaction with 4-nitrophenylhydrazine hydrochloride, however, 5-hydrazone (**3**) was concomitantly formed with the generation of $PQQTMEH_2$ [equation (5)]. The structure of this adduct was well characterized by ^1H NMR, IR, and mass spectra. 5-Hydrazone adduct (**4**) formation proceeds predominantly in the reaction with 2,4-dinitrophenylhydrazine hydrochloride under the same conditions [equation (6)].

Kinetic Studies.—The reaction of PQQ with $MeNHNH_2$ was studied kinetically under pseudo-first-order conditions (at 30 °C, μ = 0.5 with KCl) in which total $MeNHNH_2$ concentration (4.0×10^{-3}–1.0×10^{-2} mol dm^{-3}) greatly exceeded PQQ concentration (4.0×10^{-5} mol dm^{-3}) (Figure 1). Monitoring the absorption at 300 nm established a short phase lag followed by the first-order appearance of $PQQH_2$ (Figure 2). The reaction was also first-order in total hydrazine concentration (Figure 3).

From buffer dilution studies (0.1–0.4 mol dm^{-3} at pH 4.7 and 5.7), it could be concluded that the oxidation of $MeNHNH_2$ by PQQ is not subject to buffer catalysis. In Figure 4 is shown the pH–rate profile in which levelling off at around pH 7.5 (pK_a^{app})





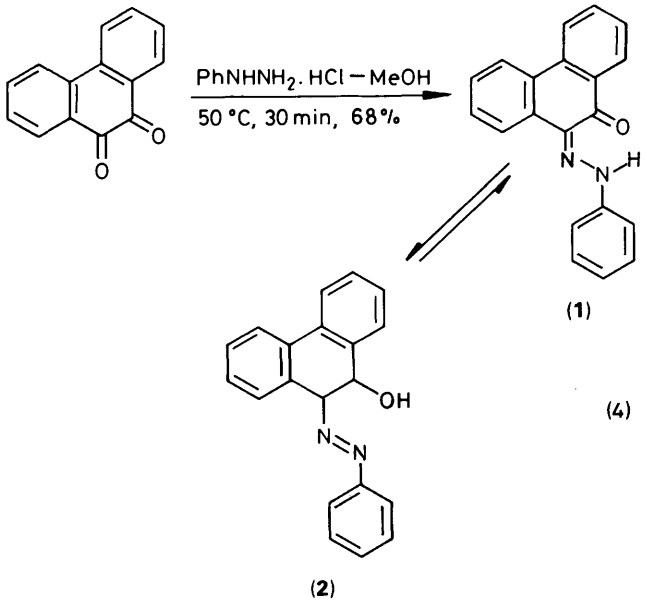


Table 1. The second-order rate constants for the oxidation of hydrazines by PQQ.[a]

Substrate	Kinetic pK_a	$E_{1/2}$ *vs.* SCE[b]	k_2 (dm^3 mol^{-1} s^{-1})
$PhNHNH_2$	4.3 (5.27)[c]	−0.36	5.9×10^2
$MeNHNH_2$	7.5 (7.87)	−0.38	6.0×10^2
MeNHNHMe	7.0 (7.52)	−0.39	1.3×10^2
Me_2NNH_2	6.3 (7.21)	−0.44	6.8
NH_2NH_2	— (7.95)	−0.22	10[d]
$PhCH_2NH_2$	— (9.45)	–	1.5×10^{-1}[d]

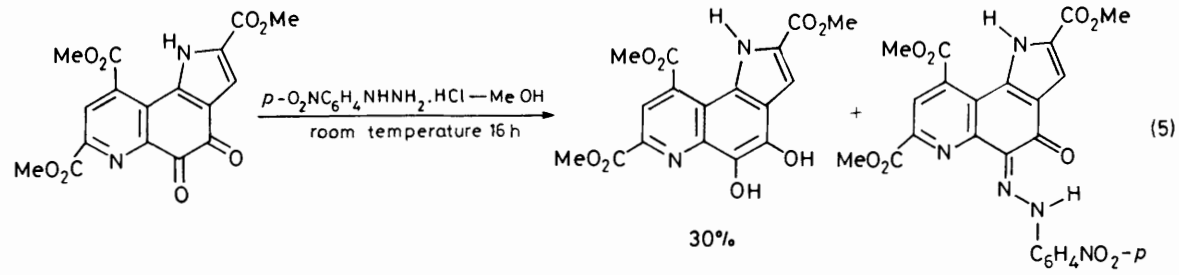
[a] [PQQ] = 4.0×10^{-5} mol dm^{-3} [Substrate] = 4.0×10^{-3}, 0.1 mol dm^{-3} acetate, phosphate, and carbonate buffers (μ = 0.5 mol dm^{-3} with KCl), 30 °C, anaerobic conditions (N_2). [b] Polarographic half-wave potential at pH 13.0.[12] [c] Reported pK_a values[11] are shown in parentheses. [d] Second-order rate constant at pH 10.1.

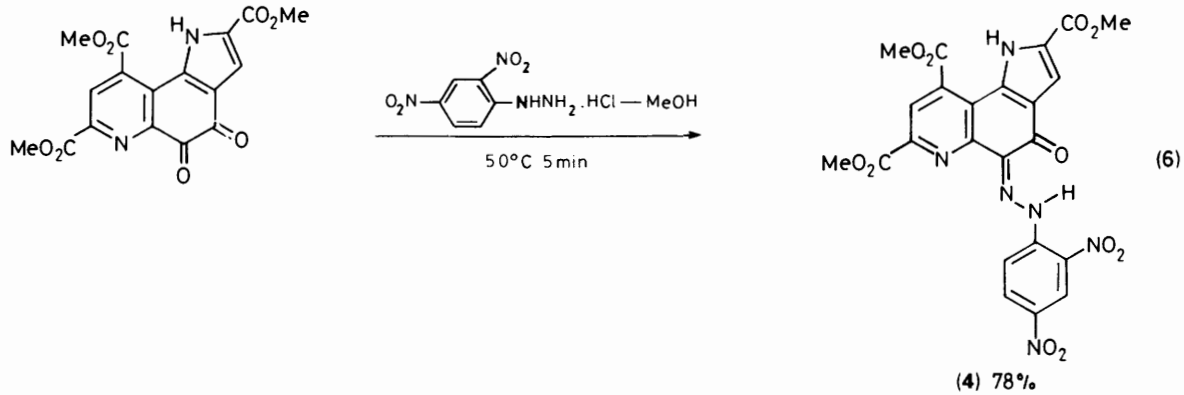
is observed. This value is comparable to the reported pK_a of $MeNHNH_2$,[11] indicating that the free hydrazine is an active species. It seems as if there is another plateau at pH < 2. This may be attributed to the protonation of the carboxy groups of PQQ which cause variation in the reactivity of PQQ. Simulation of the pH–rate profile by use of the kinetic equation (7) gives the second-order rate constant k_2 of 6.0×10^2 dm^3 mol^{-1} s^{-1} which is larger than that for the reaction of benzylamine with PQQ by three orders of magnitude.[6]

$$k_{obs} = k_2[MeNHNH_2] = k_2 \frac{K_a}{K_a + a_H}[MeNHNH_2]_T \quad (7)$$

In the kinetic studies with $PhNHNH_2$, MeNHNHMe, and Me_2NNH_2 only a redox reaction was observed in the pH range investigated (4.7–9.4). The same phenomenon was observed in the reaction with NH_2NH_2 at pH 10.0, but the reaction was somewhat complicated at pH < 7. The final absorption spectrum of the reaction with NH_2NH_2 at pH 6.7 resembled that of $PQQH_2$, but showed a broad shoulder around 370 nm, and this shoulder did not disappear upon exposure to air, indicating that the adduct (maybe the hydrazone) formation occurred competitively. The reduction by $PhNHNH_2$, $MeNHNH_2$, and MeNHNHMe followed pseudo-first-order kinetics up to six half-lives, whereas the reduction by Me_2NNH_2 followed consecutive first-order kinetics. The second-order rate constants k_2 obtained similarly to these hydrazines are listed in Table 1 together with their two-electron redox potentials.[12] $PhNHNH_2$ reacted almost as fast as $MeNHNH_2$, while MeNHNHMe and Me_2NNH_2 were less reactive (5-fold and 90-fold differences, respectively) compared with $MeNHNH_2$. It was found that the rate of reduction by these hydrazines did not reflect their two-electron redox potentials.

Reactivity of PQQ Models.—The reactivities of PQQ models in the oxidation of $MeNHNH_2$ are shown in Table 2. The kinetic studies were performed at pH 6.5 (μ = 0.2 with KCl, at 30 °C). In Table 3 the second-order rate constants k_2 are listed together with their two-electron redox potentials ($E_{1/2}$ *vs.* SCE at pH 6.8). The $E_{1/2}$ values of PQQ and (7) are reported by





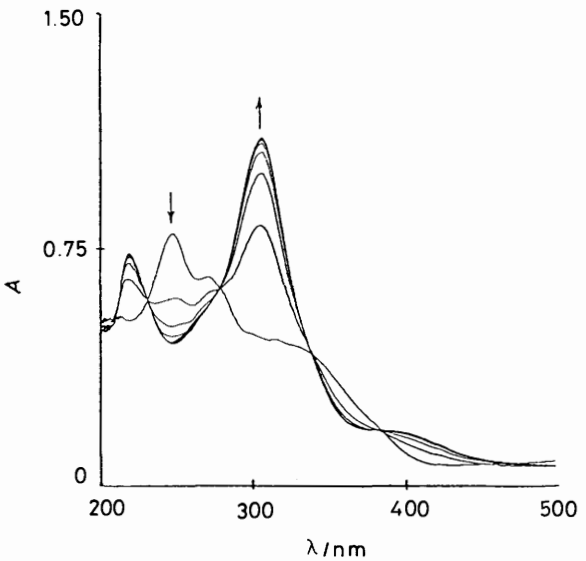


Figure 1. Spectroscopic change along the progress of the reaction of PQQ with $MeNHNH_2$. [PQQ] = 4.0×10^{-5}, [$MeNHNH_2$] = 4.0×10^{-3} mol dm^{-3}, 0.1 mol dm^{-3} acetate buffer (pH 4.7, μ = 0.5 with KCl), 30 °C, anaerobic conditions (N_2).

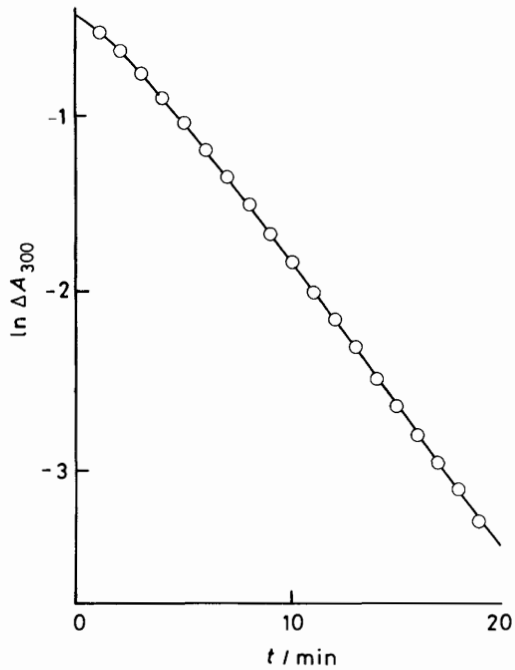


Figure 2. Pseudo-first-order plot for the oxidation of $MeNHNH_2$ by PQQ.

Bruice and co-workers[5] and these are very close to our results. 9-Decarboxy PQQ (**5**), 7-decarboxy PQQ (**6**), and 7,9-didecarboxy PQQ (**7**) showed less reactivity compared with PQQ itself though their $E_{1/2}$ values were quite similar (around −165 mV *vs.* SCE). On the other hand, the model compounds (**9**) and (**10**) showed much higher reactivity in spite of their lower redox potentials.

Duine and co-workers reported that hydration of the PQQ quinone group at the 5-position could be detected spectrophotometrically in an aqueous solution,[13] and that the characteristic features of the hydrated form lack the quinonoid n–π^* transition around 475 nm (very broad and weak) and a shoulder around 270 nm, but have two absorption maxima at around 330 and 360 nm (Table 4). Absorption maxima of PQQ models in an aqueous solution at pH 6.8 are also listed in Table 4. It was found that the spectra of (**9**) and (**10**) at pH 6.8 showed the characteristic features of the hydrated form mentioned above, whereas such characteristic features were not observed in the spectra of (**5**), (**6**), and (**7**), or are rather similar to that of the non-hydrated form. In other words, (**9**) and (**10**) are hydrated to a greater extent than the decarboxylated models, (**5**), (**6**), and (**7**) in a neutral aqueous solution.

These results suggest that the quinone carbonyl carbons of (**9**) and (**10**) are more reactive to nucleophilic addition than those

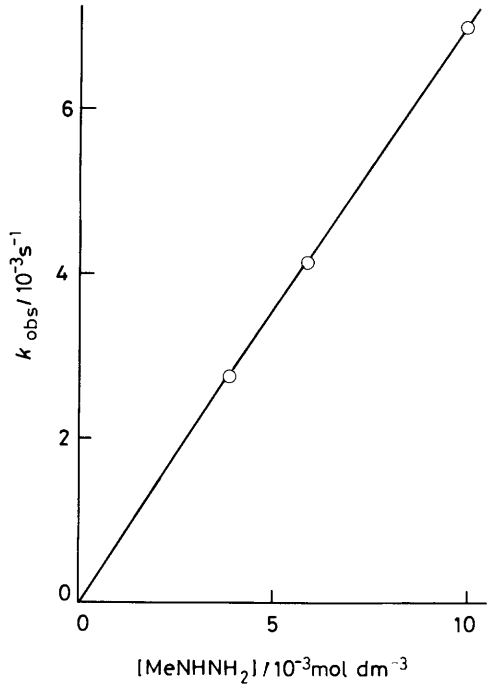


Figure 3. Pseudo-first-order rate constants (k_{obs}/s^{-1}) *vs.* $MeNHNH_2$ concentration (mol dm^{-3}).

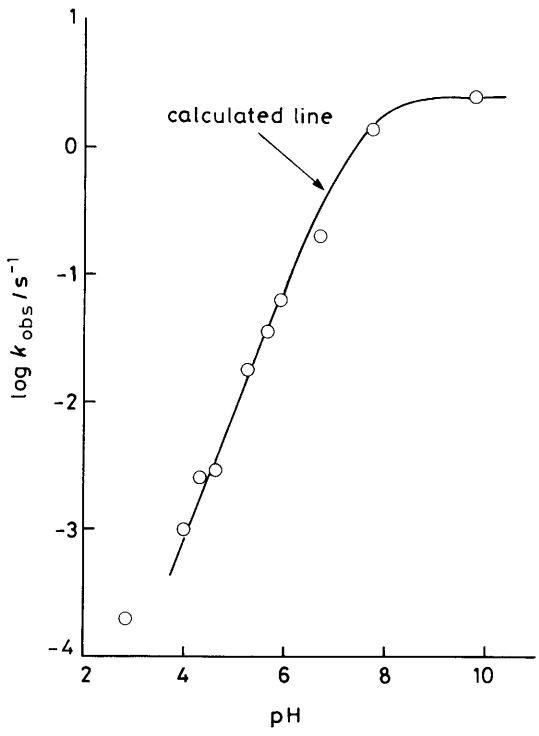


Figure 4. The pH–rate profile for the oxidation of $MeNHNH_2$ by PQQ.

of (**5**), (**6**), and (**7**). Thus model compounds which are more reactive toward nucleophilic addition could show higher reactivity in the reduction by methylhydrazine.

Discussion

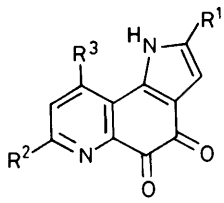
The results of the product analysis and the kinetic studies mentioned above indicate that the reduction of PQQ may proceed by formation of the carbinolamine type intermediate (**a**) followed by electron flow from nitrogen of hydrazines into the quinone as shown in the Scheme. If such electron flow is not fast enough, dehydration from the intermediate (**a**) predominantly proceeds to give the 5-hydrazone or azo adduct. Electron-withdrawing substituents would retard such electron flow as in the case of 4-nitrophenylhydrazine and 2,4-dinitrophenylhydrazine to give the corresponding adducts, while electron-donating substituents such as methyl or phenyl facilitate the reduction of PQQ. Both the redox reaction and the adduct formation are observed in the case of NH_2NH_2. The pyrroloquinoline quinone structure of PQQ is favourable for the redox reaction because only hydrazone adduct formation was observed in the case of phenanthrenequinone. Conjugation between C-4 quinone carbonyl and the pyrrole nucleus may help such electron flow in the stage of intermediate (**a**). The relatively small reactivity of MeNHNHMe compared with $MeNHNH_2$ may be due to the steric hindrance for the initial formation of the carbinolamine type intermediate. The slow rate of the reduction by Me_2NNH_2 might be explained by the fact that the formation of unstable oxidation product (**b**) (Me_2N^+=NH) is less favoured. The consecutive first-order kinetics observed in the reduction by Me_2NNH_2 indicates that the reaction proceeds in a stepwise manner (addition–elimination). In fact, some kind of saturation phenomenon was observed in the plot of the rate *vs.* Me_2NNH_2 concentration when the reaction was examined at higher concentrations of Me_2NNH_2 ($>10^2$ mol dm^{-3}). The formation of reduced PQQ could not occur from the hydrazone since the hydrazone is stable enough and is not reduced in the presence of excess hydrazine. A similar ionic mechanism has been proposed by Bruice and co-workers.[5]

The present results are different from those of Duine and co-workers. They reported that the 5-hydrazone of PQQ was formed in a slow reaction at higher O_2 concentration and the azo adduct was formed in a fast reaction with $PhNHNH_2$·HCl in MeOH.[4] In their findings, these adducts (hydrazone and azo adducts) were stable in water-containing solvents and did not interconvert, while these adducts were quite unstable in Me_2SO and were transformed into PQQ. In the present study, only the redox reaction was observed and such adducts were not obtained in the reaction with $PhNHNH_2$·HCl in MeOH [equation (2)]. The adducts obtained in the reaction with 4-nitrophenylhydrazine and 2,4-dinitrophenylhydrazine were so stable, both in an aqueous solution and Me_2SO, that the transformation of these adducts into PQQ was not observed at all. Spectroscopic studies using IR, UV–VIS, and NMR have shown that these kind of adducts between quinones and hydrazines are in azo–hydrazone tautomerism. The equilibrium between azo and hydrazone forms is dependant on several factors, such as solvent, substituents, and ring size.[10] Thus, tautomerism of azo–hydrazone in the case of PQQ and phenylhydrazine derivatives needs further investigation.

Experimental

PQQ and the trimethyl ester of PQQ (PQQTME) were prepared according to the reported method.[14] 9-Decarboxy PQQ (**5**), 7-decarboxy PQQ (**6**), and 2,7-didecarboxy PQQ (**7**) were synthesized by methods described before.[15] Model compounds (**8**), (**9**), and (**10**) were synthesized by modifying Corey's method.[16] All hydrazines and 4-nitrophenylhydrazine hydrochloride and 2,4-dinitrophenylhydrazine hydrochloride were obtained commercially and were purified by distillation under N_2 or by recrystallization. Phenylhydrazine and methylhydrazine hydrochlorides were prepared by treatment of these

Table 2. PQQ model compounds.



Model	R^1	R^2	R^3
PQQ	$-CO_2H$	$-CO_2H$	$-CO_2H$
PQQTME	$-CO_2Me$	$-CO_2Me$	$-CO_2Me$
(5)	$-CO_2H$	$-CO_2H$	$-H$
(6)	$-CO_2H$	$-H$	$-CO_2H$
(7)	$-CO_2H$	$-H$	$-H$
(8)	$R^1 = R^2 = R^3 = -CONMe_2$		
(9)	$R^1 = R^2 = R^3 = -CONHC(CO_2H)HMe$		
(10)	$R^1 = R^2 = R^3 = -CONHC(CO_2H)HCH_2CHMe_2$		

Table 3. Reactivity of PQQ model compounds in the reaction with $MeNHNH_2$.

Model	$E_{1/2}$/mV *vs.* SCE[a]	k_2/dm^3 mol^{-1} s^{-1}[b]
PQQ	−164	6.0×10^2 (1.0)[c]
PQQTME	−40	2.5×10^3 (4.2)
(5)	−165	1.8×10^2 (0.3)
(6)	−165	2.9×10^2 (0.5)
(7)	−164	0.7×10^2 (0.1)
(8)	−134	2.7×10^3 (4.5)
(9)	−218	4.4×10^3 (7.3)
(10)	−219	4.6×10^3 (7.7)

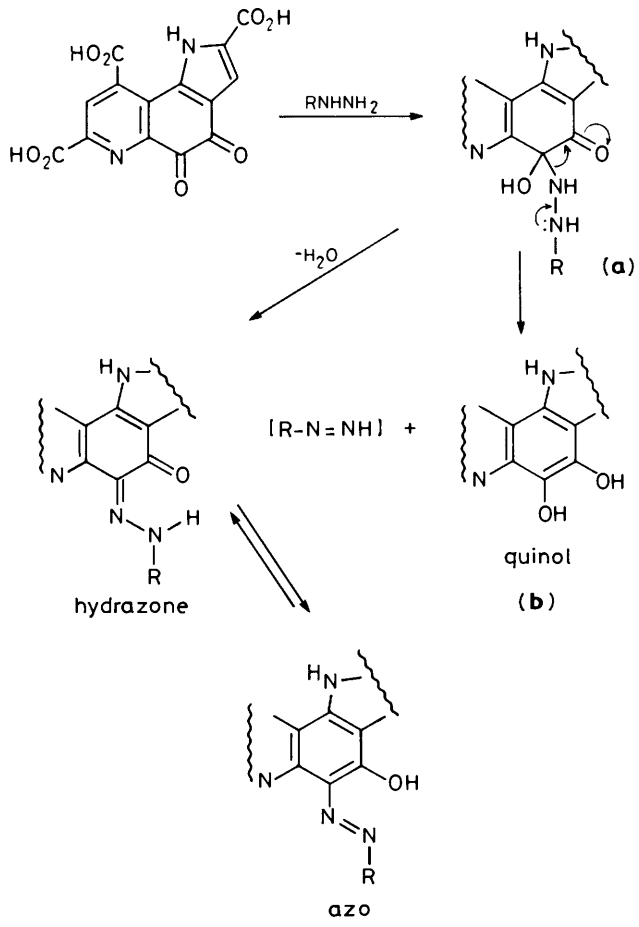
[a] In 0.1 mol dm^{-3} phosphate buffer (pH 6.8), determined by cyclic voltammetry. [b] [Model] = 4.0×10^{-5} mol dm^{-3}, [$MeNHNH_2$] = 3.5–6.4 × 10^{-4} mol dm^{-3}, anaerobic conditions (N_2), 30 °C, 0.2 mol dm^{-3} phosphate buffer (pH 6.5, μ = 0.5 with KCl). [c] Relative rates are shown in parentheses.

Table 4. Absorption maxima of PQQ model compounds.

Model	λ_{max}/nm[a]
PQQ	248, 270, 329
hydrated[b]	244, 328, 360[c]
non-hydrated[b]	254, 276, 328
PQQTME	255, 284, 366
(5)	246, 276, 316
(6)	245, 270, 320
(7)	271, 304
(8)	248, 274, 326
(9)	251, 327, 355[c]
(10)	251, 324, 365[c]

[a] At pH 6.8 in 0.1 mol dm^{-3} phosphate buffer. [b] Calculated values of λ_{max} of hydrated and non-hydrated PQQ.[13] [c] Quinonoid n–π^* transition around 475 nm was absent.

hydrazines with concentrated aqueous HCl solution in methanol and purified by recrystallization. Ultraviolet and visible absorption spectra were recorded on a Shimadzu UV-265 spectrophotometer equipped with a temperature-controlled cell holder, Shimadzu TCC-260. Values of pH were determined on a Horiba pH meter F-8. ^{1}H NMR spectra were recorded on a JEOL FT-NMR JNM-FX90Q (90 MHz) and a JEOL FT-NMR GSX-270S (270 MHz) spectrophotometers. Mass spectra were obtained on a JEOL JNX DX 303 HF mass



Scheme.

spectrophotometer. The redox potentials of PQQ models [PQQ, PQQTME, and (5)–(10)] were measured by cyclic voltammetry using a Hokuto Denko HA-301 potentiostat, a Hokuto Denko HB-104 function generator, a GC working electrode, a Pt auxiliary electrode, and an SCE as the reference.

Kinetics.—The kinetics of the reduction of PQQ with hydrazines were performed in an aqueous buffer solution (μ = 0.5 with KCl) at 30 °C under anaerobic conditions. Typically, aqueous buffer solution (1.5 cm^3) containing a hydrazine (8.0×10^{-3} mol dm^{-3}) was mixed with an aqueous buffer solution (1.5 cm^3) containing PQQ (8.0×10^{-5} mol dm^{-3}) in a Thunberg cuvette. Both solutions were degassed by bubbling N_2 (99.999%) through them for 30 min prior to reaction. The progress of the reaction was followed by monitoring of the appearance of the peak due to reduced PQQ at 300 nm.

Reaction of PQQ with phenylhydrazine hydrochloride. PQQ (10.6 mg, 32.1 μmol) was dissolved in dry MeOH (1 cm^3) and a slight molar excess of phenylhydrazine hydrochloride was added. The mixture was stirred for 10 min at 50 °C and then the solvent was removed under reduced pressure to give $PQQH_2$ quantitatively which was identified by comparison with the spectra of the authentic sample.[8]

Reactions of PQQTME with phenylhydrazine hydrochloride and methylhydrazine hydrochloride. PQQTME (10 mg, 26.9 μmol) was dissolved in dry MeOH (20 cm^3) and phenylhydrazine hydrochloride (fivefold excess of PQQTME) was added. The mixture was stirred at 50 °C for 40 min. The resulting precipitates were collected by centrifugation, washed (MeOH), and dried *in vacuo* to yield $PQQTMEH_2$ (83% yield) which was identified by comparison with the spectra of an

authentic sample.[8] Reduction of PQQTME with methylhydrazine hydrochloride was performed in the same manner (53% yield).

Reaction of PQQTME with 4-nitrophenylhydrazine hydrochloride. PQQTME (10 mg, 26.9 μmol) was treated with a slight excess of 4-nitrophenylhydrazine hydrochloride in dry MeOH (20 cm^3) at 50 °C for 6 h. The resulting dark-brown precipitate was collected by centrifugation, washed (MeOH), and dried *in vacuo* to yield a 1:1 mixture of $PQQTMEH_2$ and the 5-hydrazone of PQQTME (total 66%). The 5-hydrazone of PQQTME (**3**): m.p. (decomp.) > 300 °C; δ_H(270 MHz; $CDCl_3$) 3.99, 4.17, 4.19 (3 H, each s, OCH_3), 7.61 (1 H, d, 3-H, *J* 2.4 Hz), 7.82 (2 H, d, *J* 9.0 Hz), 8.34 (2 H, d, *J* 9.0 Hz), 8.66 (1 H, s, 8-H), and 12.56 (1 H, br s, 1-H); *m/z* 508 ($M^+ + 1$).

Reaction of PQQTME with 2,4-dinitrophenylhydrazine hydrochloride. PQQTME (10 mg, 26.9 μmol) was treated with a slight excess of 2,4-dinitrophenylhydrazine hydrochloride in dry MeOH (20 cm^3) at 50 °C for 5 min. The resulting red–brown solid was collected by centrifugation, washed (MeOH), and dried *in vacuo* to yield 12.7 mg (78%) of the 5-hydrazone of PQQTME (**4**): m.p. (decomp.) > 292 °C; δ_H(270 MHz; $CDCl_3$) 4.00, 4.13, 4.18 (3 H, each s, OCH_3), 7.66 (1 H, d, 3-H *J* 2.2 Hz), 8.58 (1 H, dd, *J* 9.2 and 2.6 Hz), 8.75 (1 H, d, *J* 9.2 Hz), 8.82 (1 H, s, 8-H), 9.23 (1 H, d, *J* 2.6 Hz), 12.90 (1 H, br s, 1-H), and 16.86 (1 H, br s); *m/z* 553 ($M^+ + 1$).

Reaction of phenanthrenequinone with phenylhydrazine hydrochloride. Phenanthrenequinone (100 mg, 480 μmol) and $PhNHNH_2$·HCl were stirred in MeOH (40 cm^3) at 50 °C for 30 min. The resulting red–brown solid was collected by centrifugation, and dried *in vacuo* to yield 97.8 mg (68%) of the adduct [azo–hydrazone tautomer (**1**) and (**2**)]; m.p. > 167–168 °C; δ_H(90 MHz; $CDCl_3$) 7.09–7.83 (9 H, m), 8.16–8.54 (4 H, m), and 11.61 (1 H, br s, OH); δ_C(90 MHz; $CDCl_3$) 116.5, 122.8, 122.9, 125.3, 126.8, 127.1, 127.4, 127.9, 128.3, 128.8, 129.6, 130.9, 132.3, 133.2, 136.2, 142.7 (C=N), and 178.9 (C=O); ν_{max}(KBr) 1 616, 1 598, 1 570, and 1 504 cm^{-1}; *m/z* 298 (M^+) (Found: C, 80.55; H, 4.7; N, 9.35. Calc. for $C_{20}H_{14}N_2O$: C, 80.51; H, 4.73; N, 9.39%).

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