Kinetic Analysis of Oxazolopyrroloquinoline Formation in the Reaction of Coenzyme PQQ with Amino Acids by Capillary Zone Electrophoresis

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Kinetics of the formation of oxazolopyrroloquinoline (OPQ) derivatives from coenzyme pyrroloquinoline quinone (PQQ) and three α-amino acids (glycine, serine and valine) was studied with the aid of capillary zone electrophoresis. Glycine and valine were, respectively, converted exclusively into the unsubstituted OPQ (OPQ1) and an OPQ with the valinyl residue (Val-OPQ2), while serine gave OPQ1 and Ser-OPQ2 under basic and acidic conditions, respectively. The OPQ formation exhibited a first-order dependence on each of PQQ and the amino acids. The OPQ1 generation from glycine is 45 times faster than that of Val-OPQ2 at pH 7.4. This result is in accord with an observation that PQQ spiked in bovine serum was converted predominantly into OPQ1. The mechanism of the OPQ formation is discussed in detail.

To date, the existence of three quinonoid redox cofactors, pyrroloquinoline quinone (PQQ), topa quinone (TPQ) and tryptophan tryptophyl quinone (TTQ), has been confirmed. TPQ is an amino acid-derived cofactor associated tightly with bovine plasma amine oxidase matrix through an amide linkage[1] and has been reported to be distributed in nature, not only in mammals.[2,3] TTQ is also an amino acid-derived cofactor, which is covalently bound to methylamine dehydrogenase from a soil bacteria.[4] On the other hand, PQQ has been confirmed as a non-covalently bound redox cofactor of several bacteria.[5,6] Therefore free PQQ is proposed to exist in mammalian bodies also as a 'vitamin-like' compound,[7] although the occurrence of PQQ as a cofactor of mammalian enzymes has not been proved yet. On the other hand, much attention has been attracted by several pharmaceutical activities of PQQ, for example, a therapeutic effect on cataracts[8–12] and a stimulative effect to produce nerve growth factor hormone (NGF) which is considered as a potent drug for Alzheimer's syndrome.[13] From these viewpoints, reliable detection techniques of PQQ and its related compounds in biological media have been required in various fields. However, the detection of free PQQ in mammalian fluids is very difficult because of its extremely low concentration, if any, and its high reactivities toward nucleophilic reagents such as amino acids.[14,15] Our previous work has shown that most of PQQ spiked in bovine serum is converted immediately into the unsubstituted OPQ (OPQ1).[16,17] Therefore, study of the formation of OPQs as derivatives of PQQ is very important in considering the occurrence and/or role of PQQ in mammals.

Our aim in this work is to discuss the OPQ formation in biological media from the kinetic point of view. Spectroscopic measurements would not be suitable for the present purpose because several amino acids (serine for example) generate more than one species of products in the reaction with PQQ and their absorption bands are overlapped with one another.[17] Some separation analysis is required. In our previous paper, we have emphasized the special feature of capillary zone electrophoresis (CZE) as a tool for separation analysis of PQQ and OPQs.[17] Thus, CZE is used in this paper for the first time to monitor the OPQ formation from glycine, serine and valine. The OPQ formation is discussed from the viewpoints of the mechanism and the biological and/or pharmaceutical significance.

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Experimental

Pyrroloquinoline quinone (PQQ) was obtained from Ube Industry (Tokyo, Japan) and used as received. All other chemicals used were of analytical grade.

Electrophoretic separations were performed in a fused silica tube (GL Science, Tokyo, Japan) with 0.05 mm i.d. and a column length of 600 mm with a separation length of 350 mm unless noted otherwise. All other details for CZE were the same as those described in a previous paper.[17]

The PQQ-spiked serum sample was pretreated for CZE analysis as reported in our previous paper.[17] The pretreatment of the sample was performed 48 h after the PQQ spiking.

Reaction solutions of amino acids and PQQ were prepared as follows. A PQQ solution (5.0×10^{-5} dm^3; 2.92×10^{-3} mol dm^{-3}) was added to 5.0×10^{-4} dm^3 of each of the buffer solutions containing amino acid at certain concentrations. The buffer solutions were 0.05 mol dm^{-3} phosphate buffer (pH 3, 6.5, 7.4) and 0.1 mol dm^{-3} borate buffer (pH 9.0). Incubations were performed at room temperature. These reaction solutions were sampled periodically (30 min–a few-hours interval depending on experimental conditions) and subjected to CZE without any pretreatments. The time lag due to the sample injection (*ca.* 10 s)[17] was negligibly short compared with the present kinetic time windows. For qualitative analysis, *o*-nitrobenzoic acid was used as an internal standard, which was mixed into the reaction solution before the PQQ addition. The detection wavelength was set at 249 nm. The standard deviation of the quantitative analysis of PQQ and OPQ1 was about 1% for standard samples under the present CZE conditions.

The reduction of PQQ with glycine was carried out under anaerobic conditions. An aliquot of PQQ solution was syringed into 5.0×10^{-2} mol dm^{-3} glycine solution to start the reaction (final concentration: 7.0×10^{-6} mol dm^{-3}). The buffer solutions used were made from 0.1 mol dm^{-3} phosphate ($6.5 < pH < 8.5$), 0.2 mol dm^{-3} tris(hydroxymethyl)aminomethane ($8 < pH < 9$), 0.1 mol dm^{-3} carbonate ($9 < pH < 11$) and 0.05 mol dm^{-3} phosphate ($pH > 11$). The generation of the reduced PQQ was monitored by rotating disk amperometry using a Yanagimoto P-1000 potentiostat, a Nikko Keisoku SC-5 motor-speed controller and a Nikko Keisoku RRDE-1 rotating disk motor. A gold disk electrode with a diameter of 2 mm was used as a working electrode. The electrode was set at potentials sufficiently more positive than the redox potential of PQQ.[18,19] The pseudo-first order rate constants were evaluated from the current-time curves, in which the oxidation current was converted into the concentration of the reduced PQQ using the Levich equation.[20]

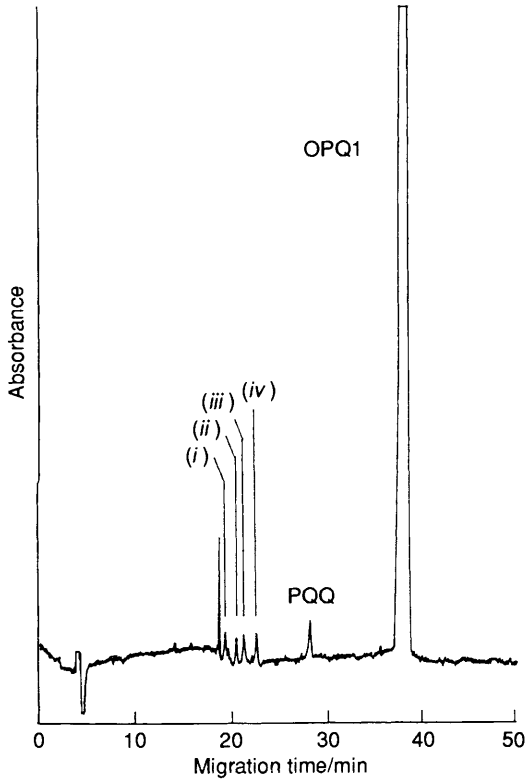


Fig. 1 Electropherogram of PQQ-spiked (1.0×10^{-4} mol dm^{-3}) bovine serum sample. Electrolyte solution, 0.01 mol dm^{-3} phosphate buffer (pH 7.5) containing 5% DMSO; capillary, 700 mm × 0.05 mm i.d. (550 mm effective length); separation voltage, 20 kV, current, 10 μA; injection (siphoning), 15 cm. 7 s; detection wavelength 420 nm; peaks (*i*)–(*iv*) can be assigned to OPQ2 from Tyr, Phe, Leu (or Ile) and Val, in turn.

Results and Discussion

Products' Analysis in PQQ-spiked Bovine Serum.—There exist many kinds of nucleophilic substances in serum at high concentrations. Therefore, PQQ can be converted into several derivatives, because of its high reactivity toward nucleophiles. Although only OPQ1 was detected in a PQQ-spiked (5.0×10^{-6} mol dm^{-3}) serum sample as described in our previous paper,[17] several other kinds of undetectable derivatives could be generated in trace amounts. In this work, the concentration of PQQ spiked in serum was increased up to 1.0×10^{-4} mol dm^{-3} and a 67-fold concentration was carried out in the sample preparation. Fig. 1 shows an electropherogram of the PQQ-spiked sample detected at 420 nm, in which the capillary length was 700 mm (separation length was 550 mm) and the electrolyte solution was 0.01 mol dm^{-3} phosphate buffer (pH 7.5) containing 5% (v/v) dimethyl sulfoxide (DMSO). The addition of DMSO was essential for good reproducibility to avoid adsorbing OPQs on the inner wall of the capillary.[16] More than 90% of the spiked PQQ was converted into OPQ1. In addition to the main peak of OPQ1 and the peak of the unreacted PQQ (3.7% compared with OPQ1), at least four minor peaks (numbered 1–4) are observed at about 0.3–0.4% each compared with OPQ1. The four peaks were assigned, respectively, to OPQ2s derived from tyrosine, phenylalanine, leucine (or isoleucine) and valine by the addition of the corresponding standard OPQs. Several other minor products were also detected at 249 nm (not shown). Judging from the peak height ratio at 249 and 420 nm,[15,17,21] they were not OPQ. The predominant formation of OPQ1 compared with OPQ2s seems to be governed mainly by the kinetics of the OPQ formation.

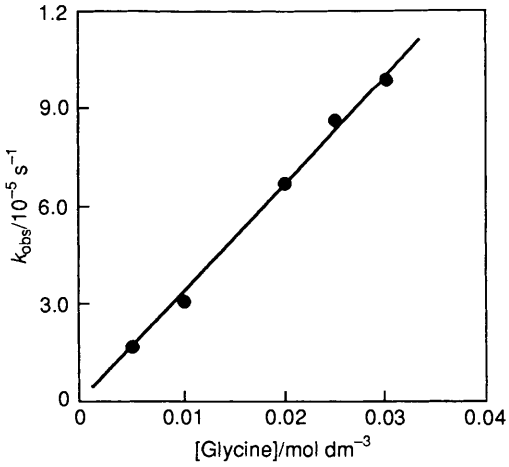


Fig. 2 Dependence of k_{obs} on the concentration of glycine at pH 6.5

Table 1 Second-order rate constants for OPQ generation[a]

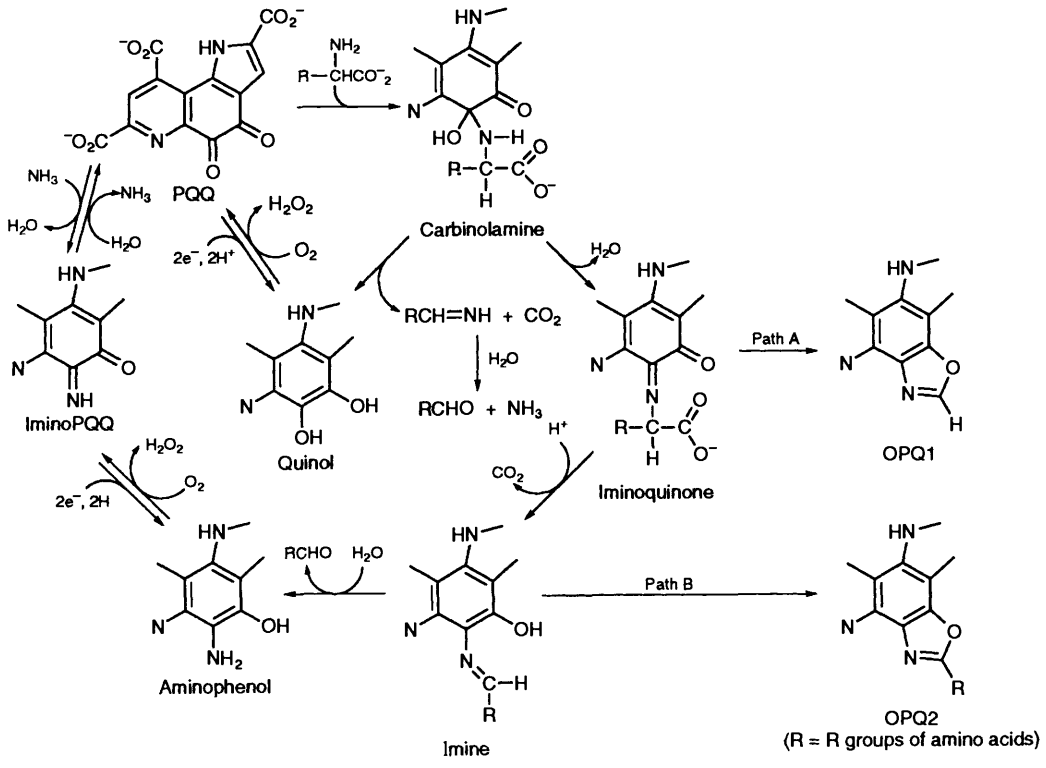
	pH 3.0	pH 6.5	pH 7.4	pH 9.0
OPQ1 Glycine	2.2×10^{-3}	3.1×10^{-3}	4.2×10^{-3}	2.3×10^{-2}
Serine	—	3.3×10^{-4}	1.4×10^{-3}	9.2×10^{-3}
OPQ2 Serine	1.2×10^{-3}	2.2×10^{-4}	—	—
Valine	5.0×10^{-4}	1.7×10^{-4}	9.4×10^{-5}	2.5×10^{-5}

[a] Values in dm^3 mol^{-1} s^{-1} at room temp.

Kinetics of OPQ Formation.—The reactions of PQQ with amino acids were carried out at room temperature under aerobic conditions. The time course of the PQQ disappearance and/or the OPQ generation was measured by CZE. In the presence of glycine, PQQ was converted exclusively into OPQ1. The OPQ1 formation proceeded at pseudo-first order in the concentration of PQQ at pH 6.5 in the presence of an excess of glycine ([glycine] = 5.0×10^{-3}–3.0×10^{-2} mol dm^{-3} ≫ $[PQQ]_0 = 2.92 \times 10^{-4}$ mol dm^{-3}). As shown in Fig. 2, a plot of the observed pseudo-first order rate constant of the OPQ formation (k_{obs}) *vs.* [glycine] gave a linear relationship. This result indicates that the reaction proceeds in first order in each of the concentrations of PQQ and glycine. Valine gave the corresponding OPQ2 (Val-OPQ2) exclusively. Serine gave OPQ1 at low pH, while it gave the corresponding OPQ2 (Ser-OPQ2) at high pH, predominantly. The OPQ formation from valine and serine also proceeded at first order in each of the concentration of PQQ and the amino acids.

Table 1 summarizes the second-order rate constants (k) of the OPQ formation with glycine, valine and serine at pH 3.0, 6.5, 7.4 and 9.0. The OPQ1 formation from glycine was increased with a rise in pH. The Val-OPQ2 formation was increased with decreasing pH. The formation of OPQ1 and OPQ2 from serine was increased under basic and acidic conditions, respectively. The rate constant of the OPQ1 formation from glycine is 45 times larger than that of the Val-OPQ2 formation at pH 7.4. The OPQ1 formation from serine is also faster than the Val-OPQ2 formation. These results suggest that the OPQ1 formation is inherently faster than the OPQ2 formation at biological pH, although the predominant (or exclusive) OPQ1 formation is restricted to glycine, tryptophan, threonine, serine and tyrosine.[17] This kinetic consideration explains well the finding of the predominant OPQ1 formation from PQQ spiked in bovine serum.

Mechanism of OPQ Formation.—Besides the OPQ formation, it is well known that amino acids are oxidatively decarboxylated with PQQ to yield their quinol and aminophenol forms (Scheme 1).[14,21–24] Under aerobic conditions, the



Scheme 1 Catalytic oxidation of amino acids with PQQ and the OPQ formation

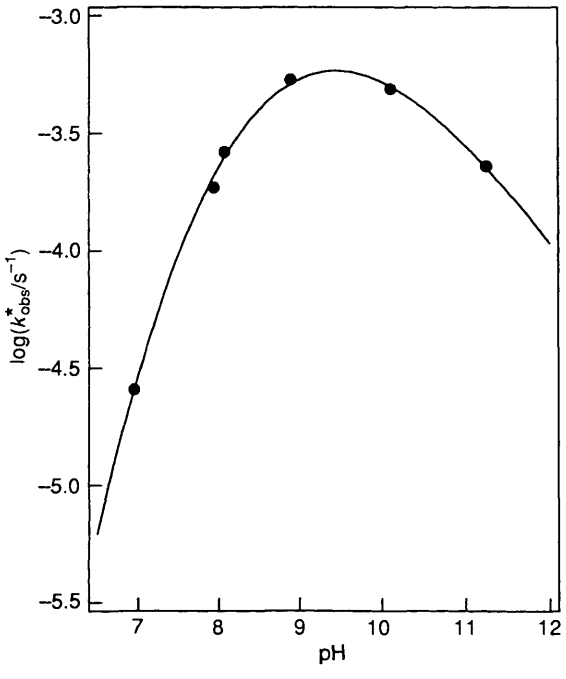


Fig. 3 pH Dependence of k^*_{obs} under anaerobic conditions

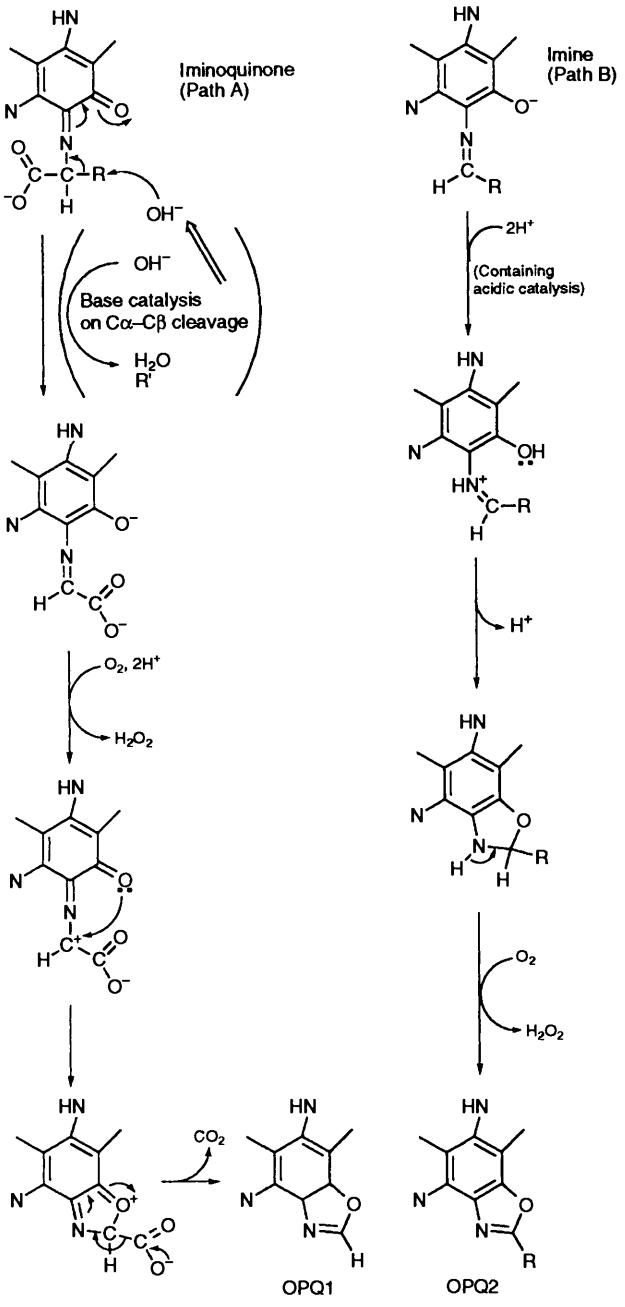
reduced PQQ is reoxidized to PQQ.[15] Itoh *et al.* discussed the kinetics of the reduction of PQQ with glycine under anaerobic conditions.[21] The PQQ reduction proceeds at first order in [PQQ] and at both second and first order in [glycine] as expressed by eqn. (1). The terms involving the second- and first-

$$d([\text{aminophenol}] + [\text{quinol}])/dt = [\text{PQQ}](k_1[\text{glycine}] + k_2[\text{glycine}]^2) \quad (1)$$

order dependence on [glycine] correspond to the quinol and the aminophenol formation, respectively (Scheme 1). Glycine works as a catalyst as well as a substrate in the quinol formation process.[21]

The OPQs are considered to be generated in side reactions of the catalytic oxidation of amino acids with PQQ.[15,21,22,25] The OPQ formation is irreversible and so OPQs are accumulated as final products during the redox cycling under aerobic conditions. As described above, the OPQ formation proceeds at first order in the concentration of amino acids. This result suggests strongly that OPQs are produced in by-path reactions of the redox cycling reaction *via* the aminophenol as shown in Scheme 1. The reaction between PQQ and amino acids proceeds *via* an ionic mechanism involving a carbinolamine-type intermediate which is dehydrated to give iminoquinone.[21] When C_α–C_β bond cleavage followed by decarboxylation from the iminoquinone intermediate occurs, OPQ1 is generated (path A). In contrast, when decarboxylation is facilitated in the iminoquinone, OPQ2 as well as aminophenol is produced *via* the corresponding imine (path B). The later pathway is a major one for most of the amino acids as reported in our previous paper.[17]

In the case of glycine, OPQ1 can be produced *via* path A or B of Scheme 1. Considering the proposal that the OPQ formation is a by-path of the redox cycling, we studied the kinetics of PQQ reduction with glycine under anaerobic and pseudo-first order conditions using rotating disk amperometry. In this experiment, aminophenol and quinol are simultaneously detected.[26] Fig. 3 shows a pH dependence of the pseudo-first order rate constant (k^*_{obs}). The remarkable increase in k^*_{obs} up to pH 9 is ascribed to an increase in the fraction of the conjugate base of glycine ($^-OOCCH_2NH_2$, pK_a = 9.6), which is a true nucleophilic substrate. The decrease in k^*_{obs} above pH 10 is attributable to a decrease in the fraction of the redox active PQQ as a true oxidant due to hydration (pseudo pK_a = 10.3).[18,25] The pH dependence of the OPQ1 formation from glycine resembles that of the PQQ reduction qualitatively. This supports the proposal concerning the by-path mechanism. The fact that the OPQ1 formation from glycine is much faster compared with serine at



Scheme 2 Proposed mechanism of the formation of OPQ1 and OPQ2

high pH is also explained by our kinetic result that k^*_{obs} of glycine is larger than that of serine (*ca.* five times larger at pH 9).

Duine *et al.* have revealed that the OPQ formation involves oxygen consumption.[15] Taking their findings into account, we propose detailed reaction pathways to generate OPQ1 and OPQ2, as shown in Scheme 2. The formation of Val-OPQ2 and Ser-OPQ2 was enhanced with decreasing pH. This suggests the involvement of an acid catalysis. At lower pH, the imine intermediate in the redox cycle will be protonated to give the corresponding imino cation, which will suffer a ring closure by nucleophilic attack of phenolic oxygen. The resultant intermediate will be oxidized to give OPQ2 (Scheme 2, path B).

For tryosine and serine, the predominant product is switched from the corresponding OPQ2 to OPQ1 with increasing pH. As in the case of Val-OPQ2, the OPQ2 formation from these two amino acids is slowed down with increasing pH. On the other hand, the OPQ1 formation will be enhanced by the base attack on a tyrosine or serine residue to cause C_α–C_β cleavages in the corresponding iminoquinone intermediate (Scheme 2, path A). The imine intermediate will be oxidized by oxygen, as in the case of di- or poly-phenols. The resultant iminoquinone will suffer ring closure by nucleophilic attack of the carbonyl group. Afterwards decarboxylation will follow to yield OPQ1. Thus path A overcomes path B at elevated pH. Threonine and tryptophan generate OPQ1 predominantly in the broad pH range.[17] The base-catalytic C_α–C_β cleavage model may not be applicable to these amino acids. Some structural factors might also govern the reaction pathway.

Conclusions

From the present kinetic studies and product analyses of the reaction of PQQ with amino acids, it has been revealed that PQQ is converted predominantly into OPQ1 during the redox cycling in mammalian fluids. Some OPQ2s are also generated, however, the conversion factor is much lower than that of OPQ1 because of the larger kinetic barrier. The result is very important in pharmaceutical aspects. As mentioned in the beginning, PQQ possesses biological and/or pharmacological activities. For example, addition of PQQ *in vitro* promotes the NGF production over 40 times in an NGF productive cell line.[13] However, the promoting effect was scarcely observed in the case of administration of PQQ *in vivo*.[13] This conflicting result may be explained by the finding that PQQ in serum is converted into several inactive species, especially OPQ1. For further discussion, detailed speciation of PQQ administrated into mammalian fluids will be required to identify a true species having biological activity. On the other hand, it is interesting that Ser-OPQ2 exhibits much effective growth stimulating activity for bacteria, compared with PQQ itself.[27] As described here, the *in vivo* generation of Ser-OPQ2 was not observed at least in bovine serum. Therefore, it seems to be important that PQQ is derivatized into much more stable forms before administration. Such derivatization will be also useful in the identification of a biologically active form of PQQ in mammals.

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Paper 4/01914H
Received 30*th March* 1994
Accepted 22*nd June* 1994