# MINIREVIEW

# Physiologic Importance of Pyrroloquinoline Quinone (43218)

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*Abstract.* **Pyrroloquinoline quinone (PQQ, methoxatin) is a dissociable cofactor for a number of bacterial dehydrogenases. The compound is unusual because of its ability to catalyze redox cycling reactions at a high rate of efficiency and it has the potential of catalyzing various carbonyl amine reactions as well. In methylotrophic bacteria, PQQ is derived from the condensation of L-tyrosine with L-glutamic acid. Whether or not PQQ serves as a cofactor in higher plants and animals remains controversial. Nevertheless, a strong case may be made that PQQ and related quinoids have nutritional and pharmacologic importance. In highly purified, chemically defined diets, PQQ stimulates animal growth. Furthermore, PQQ deprivation appears to impair connective tissue maturation, particularly when initiated *in utero* and throughout perinatal development.** [P.S.E.B.M. 1991, Vol 197]

In 1967, Anthony and Zatman (1, 2) reported that the prosthetic group of alcohol dehydrogenase from *Pseudomonas* sp. M27 was unusual and not related to other known prosthetic groups. Subsequent reports confirmed this observation and identified additional methylotrophic bacteria, which contained dehydrogenase activity with unusual cofactor requirements (3–15). By the late 1970s, the cofactor was identified as an orthoquinone (6–9) and eventually was isolated as an acid-stable acetone adduct by Forrest *et al.* (6), who designated this cofactor as methoxatin, which is now most often designated as pyrroloquinoline quinone (PQQ) (Fig. 1).

## PQQ Chemistry

PQQ reacts readily with amines and amino acids (7, 16–25). The ability of PQQ to add to nucleophiles is in part related to the location of the pyridine nitrogen that is peri to C-5 carbonyl in PQQ (*cf.* Fig. 1). The redox capabilities of the dicarbonyl functional center and the ability to interact with primary amines provide PQQ with properties that are analogous to combining some of the best chemical features of riboflavin, ascorbic acid, and pyridoxal cofactors into one molecule. For example, one distinguishing chemical feature is the ability to carry out redox cycling so that picomole amounts of PQQ are capable of generating micromolar amounts of product (21–25).

Eckert *et al.* (16, 17) have studied the chemistry of PQQ, the trimethyl ester of PQQ, and related phenanthrene quinones. Environmental influences on redox processes by PQQ are important, since PQQ requiring methanol dehydrogenase often resides in lipophilic cell membranes (4). Depending upon solvent, PQQ catalyzes either one or two electron transfers. The more aprotic the solvent, the more likely one-electron transfers are favored. Reductions of PQQ, the PQQ triester, and phenanthrene quinones are thermodynamically and kinetically reversible (16, 17, 26, 27). The two-electron redox potential of PQQ at pH 7.0 is relatively high, +90 mV at pH 7.0 (4, 26).

NADH and NADPH can reduce PQQ to $PQQH_2$ under both aerobic and anaerobic conditions (27). With respect to the mechanism of electron flux from NAD(P)H to PQQ, PQQ-NAD(P)H systems require 1 mole of oxygen for the oxidation of 1 mole NAD(P)H at physiologic pH. Under anaerobic conditions, NAD(P)H reduction of PQQ to $PQQH_2$ occurs through

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the semiquinone form. PQQH and superoxide anion ($O_2^-$) are formed, possibly by the interaction of the intermediate semiquinone (PQQH) and/or NAD(P) with oxygen, during the oxidation of NAD(P)H. In the presence of oxygen, $PQQH_2$ and PQQH are easily oxidized to PQQ and $H_2O_2$ (17).

The spectral characteristics of PQQ, its semiquinone, ($PQQH^{\cdot}$), the PQ quinol ($PQQH_2$), and PQ dihydroquinol ($PQQH_4$) have been described by Duine and Jongejan (*cf.* (4) and references cited therein). The absorption spectra of PQQ are stable within pH 5–9, but change reversibly by altering temperatures. The fluorescent spectra of PQQ are more dependent on temperature; the high fluorescence intensity at low temperatures suggests a temperature-dependent equilibrium of fluorescent and nonfluorescent species of PQQ.

## PQQ Synthesis

The chemical synthesis of PQQ was first achieved by Corey and Tramontano (28). Later, Nijhuis *et al.* (29, 30) described the synthesis of PQQ by ring closure of ortho-substituted tertiary anilines. The synthesis scheme developed by Corey and Tramontano was adopted by Jongejan *et al.* (31), who used the approach to synthesize [3-$^{13}$C]PQQ and [3-$^{2}$H]PQQ.

Other routes to PQQ production have involved microbial synthesis and isolation (10, 32–38). For example, PQQ production by the methylotrophic bacterium W3A1 (methylophilus) has been described by McIntire and Weyler (35). They have defined growth conditions for two variants of the bacterium and found that W3A1 grows and secretes PQQ not only when grown on methanol, but also on methylamine and trimethylamine. PQQ has also been tentatively identified in a number of complex mixtures of plant and animal origin by Ameyama *et al.* (*cf.* (11) and references cited therein).

A biosynthetic precursor for PQQ synthesis in Methylobacterium AM1 (Pseudomonas AM1) has been elucidated by Houck *et al.* (38) using 1-$^{13}$C-labeled ethanol. The distribution of label assessed by NMR spectroscopy indicated that PQQ is synthesized from the amino acid glutamate and an amino acid derived from the shikimic acid pathway, most probably L-tyrosine. Since no intermediates of the shikimic acid pathway appear to be inserted directly into PQQ, it is now assumed that PQQ is most likely synthesized from condensation of L-tyrosine and L-glutamate in the manner originally proposed by Houck *et al.* (also *cf.* (39)).

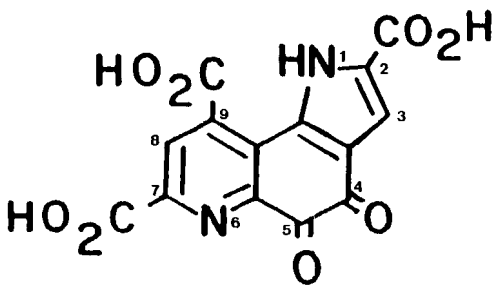


**Figure 1.** Pyrolloquinoline quinone (PQQ) or Methoxatin. The chemical designation is 4,5-dihydroxy-4,5-dioxo-*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid.

Genes that encode for putative enzymes involved in PQQ synthesis from *Acinetobacter calcoaceticus* have been described by Goosen *et al.* (36, 37). *A. calcoaceticus* contains a PQQ requiring glucose dehydrogenase. At least four genes appear to be involved in PQQ synthesis in this organism (36, 37, 40). Genes 3 and 4 are located on separate operons. A general method for the transfer of PQQ genes via bacteriophage-λ has also been described (41).

## PQQ as an Enzyme Cofactor

Ameyama *et al.* (10, 11) make a strong case that PQQ is a growth simulant for a number of bacteria, or alternatively serves as a bacterial vitamin-like cofactor. Apparently many strains of *Escherichia coli* do not produce PQQ and appear dependent on an exogenous source (4). The same is true for *Acinetobacter lwoffi*, *Azotobacter vinelandii*, Agrobacterium and Rhizobium species, all of which produce an apo-glucose dehydrogenase, which is dependent on PQQ for activity. Other enzymes that utilize noncovalently bound PQQ are the alcohol and aldehyde dehydrogenases of *Gluconobacter suboxydans* (4), *Pseudomonas testosteroni* (13), *Pseudomonas aeruginosa* (12), *Hyphomicrobium X* (9), and acinetobacter or rhizobium species (42).

However, controversy and doubt exist regarding whether or not PQQ serves as a covalently bound cofactor in prokaryotic organisms. For example, the putative cofactor in methylamine dehydrogenase of Pseudomonas AM1 has been proposed to be PQQ or a PQQ that exists in an "open form" composed of an indolequinone linked to a lysyl or glutamyl residue (43). However, McWhirter and Klapper (44) have recently obtained absorption spectra for the semiquinone forms of the methylamine dehydrogenase subunit, PQQ, and various PQQ-related *o*-quinones. All of the compounds generated spectra that differed substantially. They concluded that if the quinone in methylamine dehydrogenase and PQQ are related, there are a number of issues that need to be resolved, and the relationship is not obvious. Likewise, McIntire and Stults (45) have provided data on the structure and linkage of the cofactor of methylamine dehydrogenase. Although attempts were made initially to fit a quinoline quinone into methyl amine, reinterpretation and additional data now suggest that PQQ is not a part of the enzyme (William McIntire, personal communication). Obviously, this issue needs to be resolved, since much of what has been speculated regarding PQQ functions comes from the work on methylamine dehydrogenase

and related enzymes. For example, the concept of an open form of PQQ has been used by Van der Meer *et al.* (46) as a basis for proposing that PQQ may be the product of the indolequinone open form reacting with glutamate. This proposal is based on observations using *E. coli* in which the presence of a quinopyridoxoenzyme is reported, e.g., glutamic acid decarboxylase (46). Under conditions in which the glutamic acid decarboxylase is active, apoglucose dehydrogenase (one of the known PQQ requiring enzymes) is inactive and PQQ is not detected in cultures. Consequently, it is argued that since no PQQ is found in the media, PQQ associated with glutamic acid decarboxylase must be made as a posttranslational modification, and not by an independent pathway in *E. coli*. Although the evidence for this particular claim is indirect and weak, there is clearly the need to understand how PQQ-like cofactors arise and resolve the structures of potential quinone cofactors.

There is also now reassessment and reinterpretation of the role originally proposed for PQQ as a cofactor in plants and animals (3, 4, 47–58). As noted by Robertson *et al.* (59), virtually all of the data for a cofactor role of PQQ are based on the formation of adducts that employ conditions that are unfavorable for PQQ adduct formation. Using methods for PQQ detection that have been proposed by Duine's group, Robertson *et al.* (59) were not able to detect PQQ in dopamine-$\beta$-hydroxylase. Copper and ascorbate appear to be all that is needed for the functional activity of this enzyme. There is also now very clear evidence (60) that the quinone cofactor for plasma copper containing plasma amine oxidase is derived from a tyrosyl moiety, peptidyl 6-hydroxydopa, and is not PQQ (*cf.* section dealing with the Nutrition and Physiologic Importance of PQQ).

**PQQ Detection**

The recognition that PQQ is a probiotic or growth-stimulating factor for some bacteria has been important to the development of PQQ detection systems and assays. Growth of *P. testosteroni* is stimulated by the addition of PQQ to the culture medium (11, 33, 34). The use of apoenzymes that require PQQ, such as apoglucose dehydrogenase, whose activity is restored upon reconstitution with PQQ, has been of even more utility as the basis of an assay (34, 61–64). The glucose dehydrogenase of *E. coli* exists mainly in the apoform and is membrane bound. Ameyama *et al.* (61) and Geiger and Görisch (63) have used membranes of *E. coli* K-12 (ATCC 10798) in PQQ assays. Dokter *et al.* (65) have identified *Acinetobacter calcoaceticus* and Ameyama *et al.* (10, 11) have shown that mutants of *P. aeruginosa* synthesize an apoglucose dehydrogenase that may be utilized in PQQ assays.

Similarly, enzymatic determination of PQQ with a quinoprotein glycerol dehydrogenase has been proposed by Adachi *et al.* (66), who found that this enzyme is present almost completely as the apo-form in membranes of *Gluconobacter industrius*. When PQQ is incubated with the EDTA-treated membranes in the presence of $Ni^{2+}$, enzymatic activity is measured colorimetrically with ferricyanide as an electron acceptor. However, the sensitivity of this assay is low; linear responses are only obtained with PQQ concentrations ranging from 0.1 to 0.6 $\mu M$. Reconstitution to full enzymatic activity is also influenced by the redox state of PQQ, oxamine or oxazole complex formation, as well as interactions with compounds like ascorbate. Since PQQ adducts are easily formed at physiologic pH during tissue extraction or isolation from biologic materials, the use of enzymatic methods may result in an underestimation of PQQ. Accordingly, although Adachi *et al.* (66) demonstrated that PQQ amino acid adducts are better growth stimulators than authentic PQQ for many microorganisms, the same adducts may not cause stimulation of activity when added to preparations of glucose dehydrogenase.

Chromatography separation of PQQ and chemical detection methods have also been described (22–24, 67). Reduction of PQQ with sodium borohydride followed by oxidation with sodium periodate yields fluorescent products or, alternatively, reaction with phenylhydrazine has provided the basis for PQQ detection after chromatographic separation (67). However, the sensitivity of many of the methods described is poor (nano- to micromole range), and condensation reactions of phenylhydrazine with PQQ are complex (68). The use of phenylhydrazine to detect PQQ may be at best semiquantitative when using extracts or complex mixtures as starting materials (68, 69). For example, hydrazones are not the only possible products and, if chromatographed and ultimately detected at low pH, the recovery of product is low. An acid environment also promotes tauteromeric lactonization of PQQ (70).

For these reasons, several groups (47, 60) have utilized methods outlined by Paz *et al.* (22–24) for analysis of PQQ and PQQ-like cofactors. In the presence of potassium glycinate at pH 10, PQQ catalyzes redox cycling, which causes the efficient reduction of nitroblue tetrazolium to formazan. The assay is sensitive for PQQ in the picomolar range. It can function independently of the initial redox state of PQQ, and as long as PQQ is accessible to reagents, both bound and unbound forms of PQQ may be assayed. Although it has been stated that the redox-cycling assay is not suited for detection of PQQ in biologic samples (71), by adding sodium borate and metal chelating agents to assays, it is possible to reduce interfering side reactions. For example, the reduction of nitroblue tetrazolium by ascorbate is completely inhibited. A number of other

controls and simple chromatographic steps may also be introduced to improve specificity.

The high efficiency of redox cycling obtained with PQQ (up to $10^3$–$10^4$ cycles) may be due to the multiple negative charges on the PQQ molecule, which inhibit oxidative dimerization by charge repulsion. Picomolar concentrations of PQQ generate nano- to micromoles of product in redox cycling assays, whereas other reductants and quinones such as L-dopa, L-ascorbic acid, menadione, or tannins are required at substantially higher amounts to promote product formation. In the presence of amines, many quinone and dicarbonyl compounds easily polymerize to indigo and melanin-like products (68).

In this regard, however, it is essential to note that derivatives of 6-hydroxydopa are also unusual redox cycling catalysts (60). The hydantoin and quinoprotein peptides containing 6-hydroxydopa catalyze redox cycling at rates somewhat comparable to PQQ (60) and consequently must be separated from PQQ prior to any assay based on redox cycling.

Last, several investigators have reported the use of antibodies directed against PQQ as means of detecting free and peptide-bound or free PQQ (72).

### Nutritional and Physiologic Importance of PQQ

With the advances made in detecting PQQ and appreciation that a number of bacterial oxidoreductases utilize PQQ, there was encouragement to examine eukaryotic systems for quinoprotein, which utilized PQQ and related compounds. Within the mammalian enzymes, the copper-containing amine oxidases were considered the most likely candidates as quinoproteins (7, 15). This stemmed not only from the interest in PQQ, but a long-standing debate as to whether or not the copper-containing amine oxidases contained pyridoxal phosphate as a carbonyl cofactor (73).

Some of this work specifically centered around the possibility that lysyl oxidase, a copper-containing amine oxidase, is PQQ dependent (52, 58, 60). Lysyl oxidase catalyzes the initial steps in the covalent cross-linking of elastin and collagen by oxidatively deaminating peptidyl lysine to peptydyl $\alpha$-aminoadipic-$\delta$-semialdehyde (allysine) (73). This cross-linking step can be inhibited by a variety of ureides, hydrazines, and hydrazides *in vivo*, and by these and other carbonyl reagents *in vitro*, e.g., hydroxylamine (73, 74).

Williamson *et al.* (58) were among the first to provide data that supported PQQ as a possible carbonyl cofactor for lysyl oxidase. Comparison of resonance Raman spectra of phenylhydrazone derivatives of calf aorta lysyl oxidase showed strong similarities with similar derivations of bovine plasma monoamine oxidase. The absorption spectra of phenylhydrazones of lysyl oxidase peptides also closely resembled those of PQQ-phenylhydrazone and were different from the pyridoxal phosphate phenylhydrazones prepared under similar conditions. At about the same time, Van der Meer and Duine (52) provided additional observations that PQQ was a covalently bound cofactor of lysyl oxidase. They treated human placental lysyl oxidase with 2,4-dinitrophenylhydrazine, which appeared to result in spectral changes consistent with presence of PQQ and inhibition of enzyme activity. Furthermore, proteolytic degradation of the derivatized enzyme yielded only one single colored product, which was spectrally and chromatographically similar to the C-5 hydrazone of PQQ and 2,4-dinitrophenylhydrazine. Later Paz *et al.* (24) concluded that they had confirmed the presence of PQQ in lysyl oxidase, since hydrolysis of lysyl oxidase with pronase and subsequent analysis using the glycinate-nitroblue tetrazolium assay resulted in redox cycling. The recent observations by Janes *et al.* (60), however, that bovine plasma monoamine oxidase contains a 6-hydroxydopa as its quinone cofactor casts doubt that lysyl oxidase is also PQQ dependent, since much of what is known about the lysyl oxidase cofactor is based on its comparison to the cofactor in plasma amine oxidase (58).

If PQQ and pyridoxal phosphate are not involved directly as cofactors, how do these compounds influence lysyl oxidase activity? First, a case can be made that animals rendered vitamin $B_6$ deficient suffer from lathrytic-like signs and that the cross-linking of elastin and collagen is impaired (73–80). However, rather than acting as a lysyl oxidase cofactor, an alternative explanation for impaired cross-link formation is inferred from examining the cross-link profiles for collagens and elastin (80). The profiles resemble those observed for conditions such as homocystinuria (74, 75, 81). Homocystinuria can be a consequence of severe vitamin $B_6$ deficiency. With $B_6$ deficiency and homocystinuria, it is not lysyl oxidase activity, but it is the subsequent condensation reactions involved in the formation of stable cross-links that are altered (Fig. 2). Homocysteine

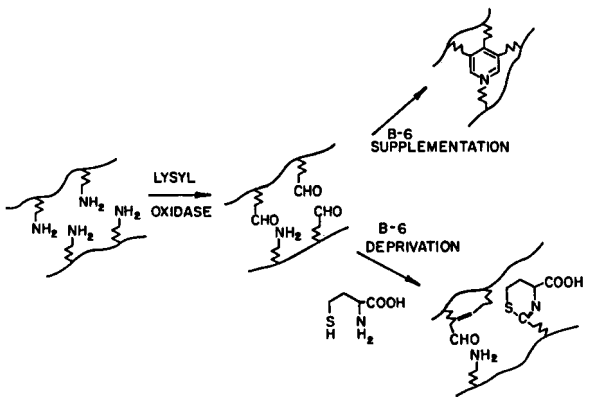


**Figure 2.** Potential products arising from the reaction of homocyteine with aldehydic functions in elastin or collagen as the result of vitamin $B_6$ deprivation.

blocks cross-link formation after the oxidative deamination of lysine residues in collagen and elastin by forming thiazine derivatives with aldehydic functions. Homocystinuria expressed genetically is also characterized by numerous deformities and defects in collagen and elastin maturation (74, 75, 81). Thus, it is possible to make a case for vitamin $B_6$ and impaired connective tissue protein cross-linking, but it is indirect at best.

What is the evidence for PQQ? When PQQ is omitted from chemically defined diets containing antibiotics, growth impairment is observed in mice and rodents, if the diet is introduced prior to neonatal development (47). A normal growth response is observed when PQQ is added to the deficient diet, or whenever PQQ or PQQ-like compounds are detectable in fecal samples or as a diet or water contaminant. Attempts to breed young female mice, when fed a PQQ-deficient diet for 8 to 9 weeks, result in either no litters or in pups that are immediately cannibalized at birth. Such responses or behaviors are not observed for female mice fed the PQQ-supplemented diet. Studies using $^{14}C$-labeled PQQ have also shown that PQQ is readily absorbed, chiefly from the large intestine (82). Signs of PQQ deprivation in mice include friable skin, general unfitness, and a hunched posture. Evidence of hemorrhage and diverticuli was also occasionally observed in the more severely affected animals. An interesting biochemical feature is that lysyl oxidase accumulation in skin is reduced in mice rendered PQQ deficient. Killgore *et al.* (47) observed only 30% of the normal values for lysyl oxidase using an enzyme-linked immunosorption assay. Consequently, if PQQ turns out not to be a cofactor for lysyl oxidase, it will remain beneficial to identify possible roles for PQQ. In this regard, a number of effects have been observed when PQQ is administered *in vivo*. Although free or protein-bound PQQ catalyzes the formation of active oxygen species, such as superoxide, administration of PQQ to fertile chicken eggs has been shown to protect against the formation of lens cataracts in developing embryos treated with hydrocortisone (83). Hydrocortisone causes a decrease in levels of reduced glutathione, which allows oxidative damage to occur in the lens. PQQ protects against this phenomenon. In addition, Watanabe *et al.* (84) have demonstrated that PQQ is protective *in vivo* against carbon tetrachloride, alcohol, or D-galactosamine-induced liver injury.

Whether or not PQQ is synthesized in higher organisms needs to be resolved. It is possible that PQQ-like compounds are only conditionally required. It is also possible that isomers related to PQQ may serve in some functional capacity; Janes *et al.* (60) have suggested a possible pathway for the synthesis of a pyrroloquinoid from 6-hydroxydopa as starting material. Peptides containing 6-hydroxydopa could also function as novel redox cycling agents (60).

## Conclusions

The biochemical importance of PQQ has only recently become apparent. PQQ-like compounds clearly influence the growth and metabolism of many prokaryotic organisms and may have a profound influence on mammalian metabolism. The chemistry of this quinone is novel and interesting. However, the literature on PQQ has been confusing and misleading. None of the PQQ requiring proteins described to date in mammals have withstood scrutiny when sensitive physical methods have been used to identify PQQ as covalently bound at the active center of enzymes. Furthermore, the nutritional work is highly dependent upon environments that are clean and free of PQQ-like contaminants. Although this is not unusual, e.g., to demonstrate a primary deficiency of biotin, vitamin K, vitamin $B_{12}$, some trace minerals, or folic acid requires an ultraclean environment (*cf.* (73) and (85) and references cited therein), the lack of evidence for an obvious enzymatic cofactor function makes interpretation of the nutritional studies on PQQ unclear. One possibility is that PQQ or PQQ-like compounds serve as oxidant scavengers. In this regard, vitamin E deficiency has been observed to produce symptoms similar to mild lathrysm (86). There is also an expansive literature, often ignored from a nutritional perspective, that focuses on the cathetins and bioflavonoids as beneficial to extracellular matrix maturation (87–95). The effects of PQQ could fit into this latter category.

Regardless, the novel chemical features of PQQ and its presence in the food chain excite interest. That compounds with the properties of PQQ may be of nutritional importance or pharmacologic benefit offers interesting areas for future investigation.

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An extensive review on pyrroloquinoline quinones and quinoproteins by Drs. Kagan and Gallop is currently in preparation for Physiological Reviews.

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