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PQQ: Biosynthetic Studies in *Methylobacterium* AM1 and *Hyphomicrobium* X Using Specific ^{13}C Labeling and NMR.

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Abstract

Using ^{13}C labeling and NMR spectroscopy we have determined biosynthetic precursors of pyrroloquinoline quinone (PQQ) in two closely related serine-type methylotrophs, *Methylobacterium* AM1 and *Hyphomicrobium* X. Analysis of the ^{13}C-labeling data revealed that PQQ is constructed from two amino acids: the portion containing N-6,C-7,8,9 and the two carboxylic acid groups,C-7'and 9', is derived-intact -from glutamate. The remaining portion is derived from tyrosine; the phenol side chain provides the six carbons of the ring containing the orthoquinone, whereas internal cyclization of the amino acid backbone forms the pyrrole-2-carboxylic acid moiety. This is analogous to the cyclization of dopaquinone to form dopachrome. Dopaquinone is a product of the oxidation of tyrosine (via dopa) in reactions catalyzed by monophenol monooxygenase (EC 1.14.18.1). Starting with tyrosine and glutamate, we will discuss possible biosynthetic routes to PQQ.

Introduction

Pyrroloquinoline quinone (PQQ, 2,7,9-tricarboxy -1H-pyrrolo[2,3-f] quinoline 4,5 dione) was first recognized as a cofactor for the pyridine nucleotide independent bacterial dehydrogenases (Duine and Frank, 1981 and references therein). These quinoproteins represent a novel class of dehydrogenases distinct from the well-known pyridine nucleotide and flavoprotein dehydrogenases (Duine et al., 1986 and references therein). Recent studies indicate that PQQ is a cofactor for several well-known metallo enzymes including: bovine serum amine oxidase (EC 1.4.3.6) (Lobenstein-Verbeek et al.,1984), porcine kidney diamine oxidase (EC 1.4.3.6) (Meer et al., 1986), human placental lysyl oxidase (EC 1.4.3.13) (Meer and Duine, 1986), dopamine β hydroxylase (EC 1.14.17.1) (Meer et al.,1988) and soybean lipoxygenase 1 (EC 1.13.11.12) (Meer and Duine, 1988). The presence of PQQ in higher organisms raises the question of how this novel compound is biosynthesized and whether or not this compound or a related analogue is a vitamin. While the bacterial genes for the biosynthesis have been cloned from *Acinetobacter calcoaceticus* (Goosen et al.,1988) and expressed in *E. coli* (Dr N. Gossen personal communication), until recently none of the biosynthetic precursors or intermediates had been identified. This manuscript reviews two recent reports of the biosynthetic precursors of PQQ in two closely related serine-type methylotrophs. Independent studies were carried out on *Methylobacterium* AM1 by the Los Alamos group (Houck et al., 1988) and on *Hyphomicrobium* X by the Delft group (Kleef and Duine, 1988).

Materials and Methods

Methylobacterium AM1 (*Pseudomonas* AM1, ATCC 14718) was cultured in a standard mineral medium (Breadsmore etal, 1982) with methanol (0.5%) or ethanol (0.5%) as the carbon source. In experiments with ^{13}C-enriched carbon sources, the organism was cultured in a stirred tank fermentor (10% inoculum) until the methanol (or ethanol) was exhausted (24-48 h). *Hyphomicrobium* X was cultivated on mineral medium (Kleef and Duine, 1988) with [^{13}C]methanol added as a carbon and energy source. This medium was supplemented with tyrosine or phenylalanine (0.27 g/l, natural abundance ^{13}C).

In both systems, PQQ was isolated from the clarified culture broth in several chromatographic steps (anion exchange followed by one or two reverse phase chromatography steps) (Duine and Frank, 1980 and Ameyama et al., 1984) and analyzed for ^{13}C enrichment by NMR spectroscopy. The yield of PQQ was typically 1 mg/l of culture broth. Mixtures of amino acids were obtained from protein hydrolysates (Patter et al., 1969) and analyzed by GC-MS (White and Rudolph, 1978) or separated for NMR analysis by ion exchange chromatography (Hirs et al., 1954).

For NMR analysis, PQQ was dissolved in 2.3 ml d_6 DMSO and placed in 10 mm NMR tubes. Spectra were obtained at 50.3 MHz on a Bruker AM200 WB spectrometer (Los Alamos) or at 100.5 MHz on a Varian VXR 400 S spectrometer (Delft). Chemical shifts are referenced to tetramethylsilane. Chemical shifts of a natural abundance sample (17.2 mg/ml in d_6-DMSO at 25C) were determined using PQQ obtained from Fluka Chemical Co. ^{13}C NMR spectra were obtained at 25 C using a 45° pulse and with the ^{1}H decoupler gated off for 10 s to minimize NOE effects. ^{13}C Enrichments were determined from the relative integrals of ^{13}C NMR resonances which were obtained by Lorentzian line shape analysis and normalized to the enrichment at C 8 which was determined by ^{1}H NMR analysis. Data were not corrected for partial T_1 saturation effects.

Table 1

Chemical Shift Assignments and ^{13}C Enrichments of PQQ

Carbon	δ, ppm	^{13}C Enrichment (atom % ^{13}C) From [1-^{13}C]Ethanol	[2-^{13}C]Ethanol
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]

Results and Discussion

Summarized in Table I are the one-to-one assignments for the fourteen ^{13}C NMR signals from natural abundance PQQ (Fig. 2a) which were determined from the ^{1}H-^{13}C coupling patterns ($^{1}J_{CH}$ and $^{3}J_{CH}$) and carbon-carbon correlations. These data agree with the partial assignments made by Duine and coworkers (1981). Carbon-carbon couplings were observed using a sample of [U-^{13}C]PQQ (90+ % ^{13}C) isolated from cultures grown on [^{13}C]methanol (99.7%). The complete assignment was achieved by selecting for one-bond ^{13}C coupling interactions ($^{1}J_{C,C}$=55 Hz) in ^{13}C COSY experiments (Bax et al., 1981a and Bax and Freeman, 1981b).

^{13}C-Labeling Studies in Methylobacterium AM1-- During growth on methanol, *Methylobacterium* AM1 derives essentially all of its carbon from the methanol (Anthony, 1980); therefore, it is impossible to extract information pertinent to the biosynthesis of PQQ from experiments using [^{13}C]methanol as the sole carbon and energy source. A more useful labeled precursor is ethanol because, as described below, one can determine which carbons in PQQ are derived from C-1 and/or C-2 of ethanol. Ethanol is assimilated into four-carbon compounds by the action of malate synthase (Dunstan et al.,1972a and 1972b); three-carbon compounds are produced by decarboxylation of oxalacetic acid or by the action of serine transhydroxymethylase. Therefore, four-carbon compounds derived from malate should be labeled at C-1 and C-4 by [1-^{13}C]ethanol and at C-2 and C-3 by [2-^{13}C]ethanol. Three-carbon compounds such as pyruvate will be labeled only at C-1 by [1-^{13}C]ethanol and at C-2 and C-3 by [2-^{13}C]ethanol. These predicted labeling patterns were confirmed by examining the label distribution in amino acids isolated from cultures that contained [1-^{13}C]ethanol (Table II). Aspartate derived in two steps from malate was labeled predominantly at C-1 and C-4 by [1-^{13}C]ethanol; alanine derived from pyruvate was labeled only at C-1 by [1-^{13}C]ethanol. Becaase two carbon units are incorporated directly into glutamate C-5 and C-4, their labeling probabilities are correlated. Thus [1-^{13}C]ethanol labels glutamate C-5 essentially without dilution; glutamate C-4 is unenriched and not subject to the background scrambling (12-17%) observed in other carbons derived osteasibly from ethanol C-2. Alternately, [2-^{13}C]ethanol yields glutamate highly enriched at C-4 (77%) and unenriched at C-5 (<3%). This is consistent with published radiolabeling data (Dunstan et al., 1972a) and is characteristic of organisms that have an incomplete TCA cycle (Walker et al., 1987). [1-^{13}C]Ethanol labels the phenol ring of tyrosine at C-3' and C-4' yielding a NMR spectrum that exhibits $^{1}J_{C,C}$ coupling; this labeling pattern is identical to that observed in tyrosine isolated from *E. coli* cultured on [1-^{13}C]lactate (LeMaster and Cronan, 1982). The adjacent labeling of C-3' and C-4' of tyrosine arises from the C-1 to C-1 joining of two trioses in gluconeogenesis and is diagnostic of compounds that arise from the shikimate pathway.

Table II

Labeling of Amino Acids in *Methylobacterium* AM1 by [1-^{13}C]Ethanol

Amino Acid	^{13}C Enrichments From [1-^{13}C]Ethanol (atom % ^{13}C)						
	C-1	C-2	C-3	C-4	C-5		
Alanine	58	11	15				
Aspartate	68	17	17	68			
Glutamate	52	13	13	2	2		
	C-1	C-2	C-3	C-1'	C-2'(C-6')	C-3'(C-5')	C-4'
Tyrosine	70	17	17	17	18(18)	61(17)	61

The ^{13}C enrichments in PQQ biosynthesized from [1-^{13}C]ethanol based on analysis of NMR intensities are summarized in Table I. C-1 of ethanol labels predominantly the three carboxylates (C-2',7' and 9') and carbons 5, 5a and 9a. The predominantly singlet character of the carboxylates indicates that they are incorporated into positions in which their neighbors arise from C-2 of ethanol. Carbons 5, 5a and 9a each yield three resonances which are the combination of a singlet from singly labeled species and doublet ($^1J_{C,C}$=60 Hz) from species labeled at C-5 and C-5a or C-9a and C-5a. The [1-^{13}C]ethanol labeling experiment coupled with the obvious structural homologies provide a working hypothesis for the biosynthetic origins of PQQ (Fig. 1). We propose that glutamate provides N-6 and carbons 7', 7, 8, 9 and 9', while the remaining nine carbons and N-1 are donated by an amino acid from the shikimate pathway, most likely tyrosine.

The precursors were identified by comparing the selective ^{13}C labeling patterns in PQQ with those observed in amino acids. In PQQ, C-1 of ethanol significantly labels C-7' (59%) and C-9' (>99%), but not C-9 (<2%); similarly, C-2 of ethanol labels PQQ at C-7 (64%), C-8 (61%) and C-9 (76%), but not C-9'. These labeling patterns are essentially identical to those observed in glutamate (Table II).

The incorporation of C-1 of ethanol into C-2', 5, 5a, and 9 of PQQ is equivalent to its incorporation into C-1, 3' and 4' of tyrosine. The adjacent labeling evident from the high degree of ^{13}C coupling at C-4' and C-3' in tyrosine is also observed in the orthoquinone-containing ring in PQQ. While tyrosine C-3' and C-5' are chemically

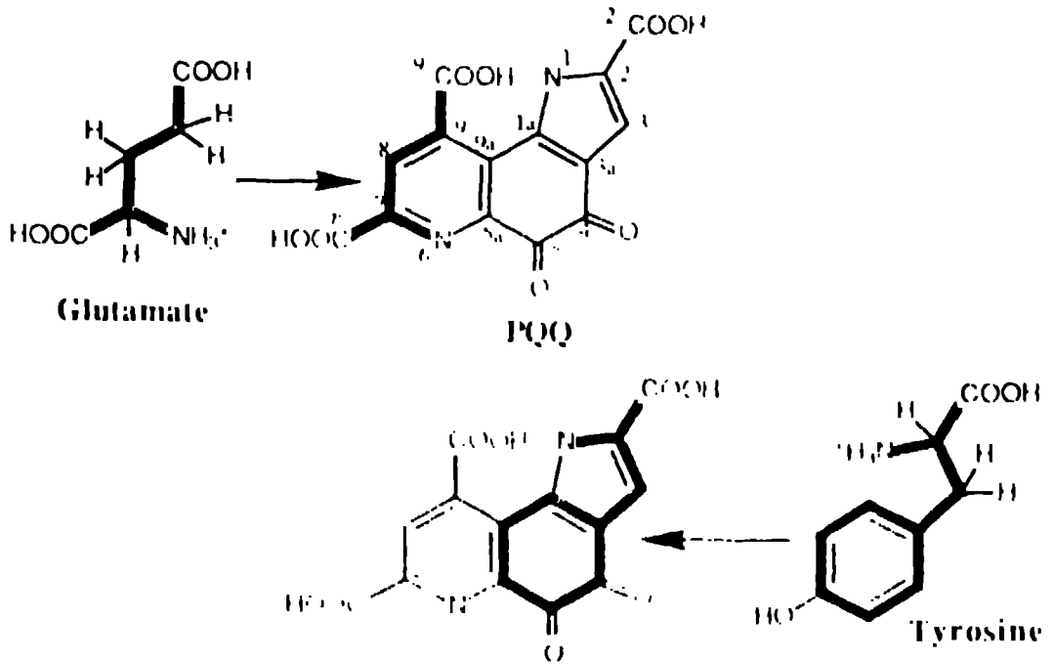

Figure 1: Proposed Biosynthetic Precursors for PQQ

equivalent, they are biosynthetically inequivalent because the aromatic ring is a product of asymmetric synthesis via the shikimate pathway (Haslam, 1974). Therefore, tyrosine C-3' arises from ethanol C-1, and C-5' from ethanol C-2. PQQ derived from [1-^{13}C]ethanol has adjacent ^{13}C labeling (doublets) at C-5a and C-5, or C-5a and C-9a. This labeling implies that the orthoquinone containing ring arises from a symmetric compound (C_2 axis through C-1' and C-4') and predicts that C-5 and C-9a will be labeled equivalently and to an intermediate extent by both C-1 and C-2 of ethanol. Indeed, [2-^{13}C]ethanol labels C-5 and

C-9a, but not C-5a. This symmetric labeling pattern rules out indole as a precursor for that portion of PQQ containing the orthoquinone and pyrrole rings.

Direct evidence for the incorporation of tyrosine -- To demonstrate directly that tyrosine is a precursor of PQQ, L-[3',5'-$^{13}C_2$]tyrosine (Walker et al., 1986) was added to *Methylobacterium* AM1 cultures (0.5 mM) growing on methanol (0.5%). PQQ isolated from this culture was examined by ^1H and ^{13}C NMR spectroscopy. The ^{13}C spectrum (Fig. 2) indicates that L-[3',5'-$^{13}C_2$]tyrosine labels PQQ at C-5 and C-9a as predicted by the biosynthetic model (Fig. 1). Because C-9a is vicinally coupled to H-8, ^1H NMR analysis can be used to determine the ^{13}C enrichment at C-9a. Under these culture conditions, PQQ was labeled at C-9a (and C-5) to an enrichment of 63%. The resonances for C-5 and C-9a are doublets as a result of geminal ^{13}C-^{13}C coupling, proving that the phenol group of tyrosine is incorporated intact into the ring of PQQ containing the orthoquinone.

The results outlined above prove that tyrosine provides the six carbons of the orthoquinone-containing ring. To examine the possibility that internal cyclization of the tyrosyl backbone forms the pyrrole-2-carboxylic acid moiety, *Methylobacterium* AM1 was cultured on a mixture of [3',5'-$^{13}C_2$]tyrosine (50%) and [3-^{13}C]tyrosine (50%). The ^{13}C NMR spectrum of PQQ isolated from the culture filtrate contained only three resonances corresponding to C-5, C-9a and C-3. ^{13}C enrichments were determined by ^1H NMR. The equal incorporation of ^{13}C at C-3 and C-9a (41% and 42%, respectively) indicates that tyrosine is incorporated intact into PQQ.

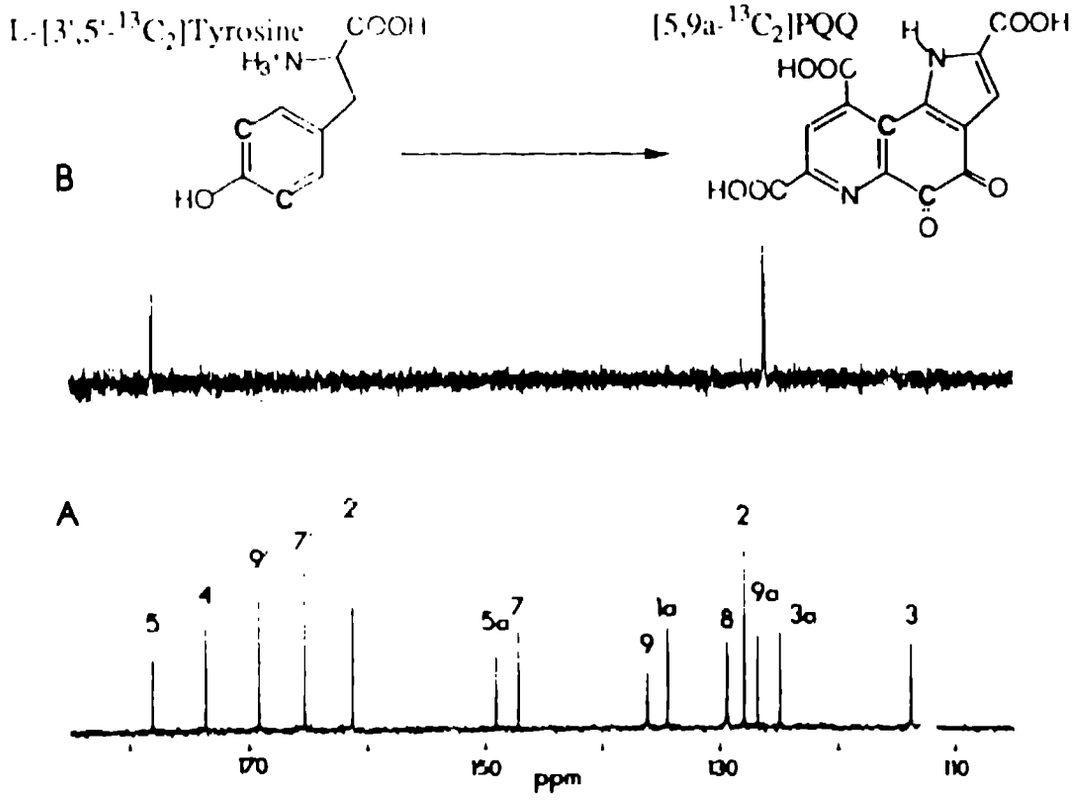

Figure 2) ^{13}C NMR spectrum of PQQ (A) and of [5,9a-$^{13}C_2$]PQQ (B) derived biosynthetically from L-[3',5'-$^{13}C_2$]tyrosine

^{13}C Labeling studies in Hyphomicrobium X -- A simple but efficient approach to establish the intact incorporation of precursors is the replacement method. The direct incorporation of amino acids into protein of *Hyphomicrobium* X was determined by culturing the organism in a medium that contained [^{13}C]methanol as a growth substrate and was supplemented with unlabeled amino acids. Amino acids were isolated from protein hydrolysates and the dilution of the label from [^{13}C]methanol was determined by mass spectrometry. On administration of phenylalanine, 98% of this amino acid was incorporated, and no other unlabeled amino acids were found (results not shown), indicating that *Hyphomicrobium* X is not able to synthesize L-tyrosine from L-phenylalanine by the action of L-phenylalanine 4-monooxygenase (EC 1.14.16.1). Most probably, these amino acids are both synthesized from a common precursor, namely prephenic acid (Haslam, 1974 and Gorsich, 1987). On administration of L-tyrosine, this amino acid was incorporated for 94%, whereas no other amino acids were found unlabeled in the hydrolyzed protein.

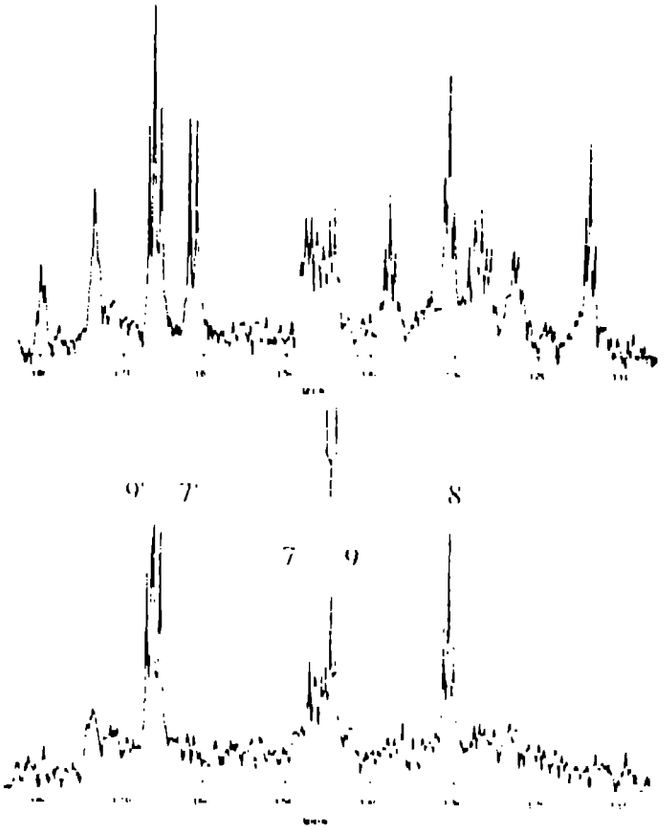

Figure 3. ^{13}C NMR spectra of [U-^{13}C]PQQ (top) and PQQ isolated from *Hyphomicrobium* X cultures grown in the presence of [^{13}C]methanol supplemented with L-tyrosine (bottom)

This replacement approach was used to examine the biosynthesis of PQQ. When PQQ was purified from the culture medium of *Hyphomicrobium* X, grown in the presence of ^{13}C-methanol plus L-phenylalanine, no significant changes in ^{1}H and ^{13}C NMR spectra were found as compared to the spectra of [U-^{13}C]PQQ, indicating that L-phenylalanine is not incorporated into the PQQ skeleton. However, PQQ isolated from cultures that were supplemented with tyrosine yielded ^{1}H and ^{13}C NMR spectra that were clearly altered. The ^{1}H NMR resonance from H-3 collapsed to a singlet (7.15 ppm); H-8 (8.64 ppm) was a doublet (168 Hz) as a result of one-bond C-H coupling. Thus C-3 had been totally replaced with ^{12}C (<5% ^{13}C) and must have been derived from tyrosine; C-8 was enriched

with ^{13}C (94%) and was derived from the labeled methanol. Inspection of the ^{13}C-NMR spectrum (Fig. 3, bottom) shows that signals only at δ 130.7 (C-8), 144.7 (C-9), 146.5 (C-7), 165.8 (C-7'), and 166.8 ppm (C-9') are present. The C-9 resonance is collapsed to a triplet due to the absence of coupling with C-9a. The absence of signals from C-2, C-2', C-3, C-3a, C-4, C-5, C-5a, C-9a, and C-1a indicates that these carbon atoms are derived from tyrosine. These results provide definitive proof that the carbon skeleton of tyrosine is incorporated intact into PQQ; the phenol side chain provides the six carbons of the ring containing the orthoquinone, whereas internal cyclization of the amino acid backbone forms the pyrrole-2-carboxylic acid moiety. Unfortunately, the replacement technique could not be used to test the potential of glutamate as a precursor for the remaining carbons (C-7,7',8,9 and 9') of PQQ in *Hyphomicrobium* X because glutamate added to the culture medium of *Hyphomicrobium* X did not significantly dilute label from [^{13}C]methanol in glutamate isolated from protein hydrolysates.

Possible routes of PQQ biosynthesis -- As demonstrated from the results obtained in experiments on *Methylobacterium* AM1 and *Hyphomicrobium* X, PQQ arises from the condensation of glutamate and tyrosine (Fig 1). A possible route for the biosynthesis of PQQ is diagrammed in Figure 4. In this route, tyrosine or some derivative of tyrosine is oxidized to dopachrome in a reaction catalyzed by a monophenol monooxygenase-like enzyme(EC 1.14.18.1, tyrosinase). Glutamate could form a Schiff base with dopaquinone. The cyclization of the tyrosine backbone to form the pyrrole ring could occur by a Michael-type addition analogous to the known non-enzymatic cyclization of dopaquinone to form dopachrome (Canovas, 1982). Alternatively, dopachrome may be an intermediate in the biosynthesis of PQQ. Efforts to assay tyrosinase in *Methylobacterium* AM1 and have thus far failed. In addition, no sequence homology exists between tyrosinase genes from *Streptomyces glaucescens* (Huber et al., 1985) or *Neurospora crassa* (Lerch, 1982) and the PQQ biosynthesis genes of *Acinetobacter calcoaceticus* (Dr. N. Gossen, personal communication).

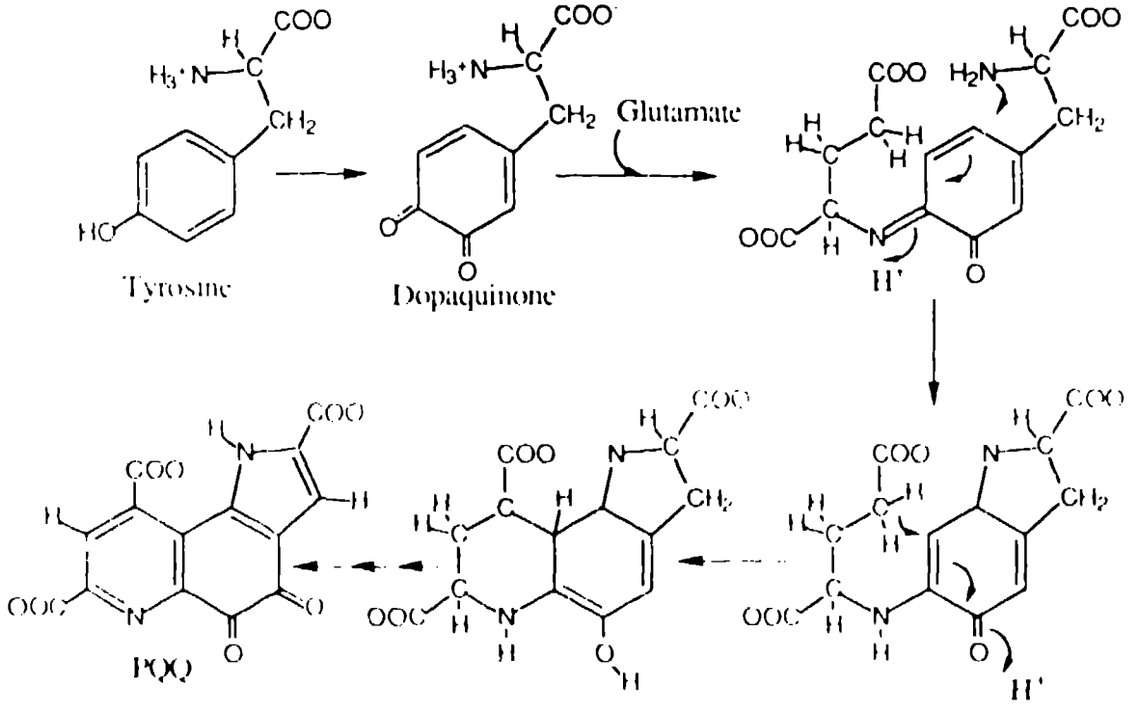

Figure 4) Possible route of PQQ biosynthesis.

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