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# Quinone methide as a reactive intermediate formed during the biosynthesis of papiliochrome II, a yellow wing pigment of papilionid butterflies

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Mushroom tyrosinase and the recently identified, 4-alkyl-*o*-benzoquinone: 2-hydroxy-*p*-quinone methide isomerase were used to investigate the mechanism of biosynthesis of papiliochrome II pigment found in the yellow scales of the papilionid butterflies. Incubation of *N*-β-alanyldopamine (NBAD) and L-kynurenine with mushroom tyrosinase resulted in the formation of adducts tentatively characterized as NBAD quinone – L-kynurenine adducts. If quinone isomerase was included in this reaction mixture, the formation of two new products could be witnessed. These two products exhibited the same retention time and the same UV and visible spectral properties as those of papiliochrome II diastereoisomers. Since quinone isomerase catalyzes the conversion of quinones to quinone methides, the above studies indicate that papiliochrome II biosynthesis involves non-enzymatic and hence non-stereoselective condensation of enzymatically generated NBAD quinone methide with L-kynurenine.

Papiliochrome biosynthesis; *N*-β-Alanyldopamine quinone; *N*-β-Alanyldopamine quinone methide; Quinone methide sclerotization; Tyrosinase; Quinone isomerase

## 1. INTRODUCTION

Extensive studies carried out by Umebachi and his associates on the yellow wing pigments of the papilionid butterflies led to the discovery of a new group of pigments called papiliochromes in these organisms [1–3]. Umebachi identified different papiliochromes such as, papiliochrome II, M, and R, all of which contained dopamine, β-alanine and L-kynurenine as important structural constituents. Papiliochrome II is pale yellow, while papiliochrome M and R are deep yellow and reddish brown in color, respectively. Of these pigments, only the structure of papiliochrome II has been elucidated conclusively to be, $N_{ar}$-[α-(3-aminopropionylaminomethyl)-3,4-dihydroxybenzyl]-L-kynurenine [4,5]. Umebachi and his coworkers also demonstrated that papiliochrome II is a mixture of two optical isomers IIa and IIb, which exhibited opposite CD and ORD spectra [6] and both generated L-kynurenine and NBANE$^+$ upon acid hydrolysis [4]. Metabolic studies indicated that papiliochrome II is produced probably by the condensation of NBAD and L-kynurenine [7]. Accordingly, Yago [8] has demonstrated that a crude enzyme fraction from the left colleterial gland of the praying mantis, *Tenodera aridifolia sinensis* could catalyze the synthesis of papiliochrome II from NBAD and L-kynurenine and proposed that NBAD quinone methide is a transient intermediate formed during this reaction.

Our group has been engaged in the study of catecholamine metabolism for cuticular sclerotization in insects and discovered quinone methide sclerotization, a new mechanism in insects by which the freshly molted, soft and pale cuticle are hardened to form the tough exosketeton which protects the insects [9–11]. According to the quinone methide model, enzymatically generated quinone methides react non-enzymatically with cuticular components forming adducts by a 1,6-Michael type addition reaction. Initially, we proposed a direct two-electron oxidation of *N*-acyl dopamine derivatives as the route for the biosynthesis of these reactive compounds [9,10,12]. However, later we demonstrated that quinone methides are produced from catecholamine derivatives not directly but indirectly from the phenoloxidase generated quinones by the action of an *o*-quinone: *p*-quinone methide tautomerase [13,14]. Recently, we have reported the purification of this enzyme to apparent homogeneity and some of its physicochemical properties [15]. Using the purified quinone isomerase, we now present evidence that papiliochrome II can be biosynthesized from NBAD and L-kynurenine through the intermediacy of NBAD quinone and NBAD quinone methide.

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*Abbreviations:* NBAD, *N*-β-alanyldopamine; NBANE, *N*-β-alanylnorepinephrine

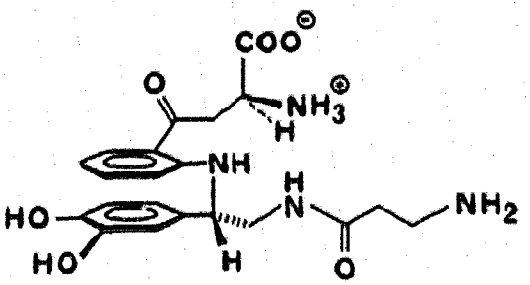

Fig. 1. Structure of papiliochrome II. Papiliochrome II is a mixture of two diastereoisomers of $N_{\omega}$-[α-(3-aminopropionylminomethyl)-3, 4-dihydroxybenzyl]-L-kynurenine. Only one structure is shown in the figure.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

NBAD was synthesized by coupling dopamine with *N*-α-*t*-butyloxycarbonyl-β-alanyl-*N*-hydroxysuccinimide ester in potassium borate, pH 9.0, under an atmosphere of nitrogen and deblocking the resultant conjugate with trifluoroacetic acid. After removal of acid, the slurry was concentrated by lyophilization and chromatographed on a Sephadex LH-20 column with 0.2 M acetic acid as the eluant. The fractions containing NBAD were pooled, lyophilized and used. NBANE was prepared similarly from norepinephrine. Papiliochrome IIa and IIb were generously donated by Dr Motoko Yago of Iwate Medical College, Morioka, Japan. Dopamine, norepinephrine, L-kynurenine, mushroom tyrosinase (specific activity 2200 U/mg), and *N*-α-*t*-butyloxycarbonyl-β-alanyl-*N*-hydroxysuccinimide ester were obtained from Sigma, St. Louis, MO. *o*-Quinone: *p*-quinone methide isomerase was purified from the hemolymph of *Sarcophaga bullata* as outlined recently [15].

### 2.2. *HPLC analysis*

HPLC analyses of reaction mixtures were carried out as outlined in an earlier paper [13]. Separations were achieved on a Beckman $C_{18}$ ultrasphere reversed-phase column (5 μm, 4.6 × 150 mm) using a step gradient. From 0 to 5 min, the elution was carried out with 100 mM sodium phosphate buffer, pH 3.3 in 10% acetonitrile at a flow rate of 1 ml/min. After 5 min, the acetonitrile concentration was raised to 40% (in 5 min) at the same flow rate. At the end of this period, the flow rate was increased to 1.5 ml/min for the next 5 min. Finally, the column was re-equilibrated with the starting buffer before next use.

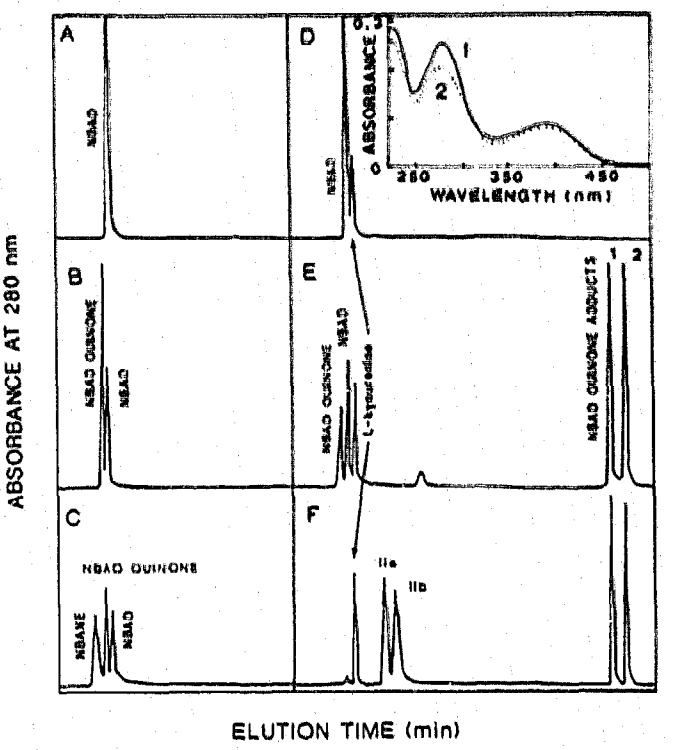


Fig. 2. HPLC analysis of NBAD — L-kynurenine reaction mixtures. A reaction mixture (1 ml) containing 2 mM NBAD, 2 mM L-kynurenine, 30 μg of mushroom tyrosinase and 0.5 U of quinone isomerase was incubated at room temperature for 5 min and 20 μl aliquots were subjected to HPLC analysis as outlined in section 2. (A) Control reaction containing NBAD only. (B) Control reaction containing NBAD and mushroom tyrosinase. (C) Control reaction containing NBAD, mushroom tyrosinase and quinone isomerase. (D) Control reaction containing NBAD and L-kynurenine. (E) Control reaction containing NBAD, L-kynurenine and mushroom tyrosinase. (F) Complete reaction. The retention times of NBANE, NBAD quinone, NBAD, L-kynurenine, papiliochrome IIa, papiliochrome IIb, NBAD-quinone L-kynurenine adduct 1 and NBAD-quinone L-kynurenine adduct 2 are 1.28, 1.81, 2.06, 2.34, 3.38, 3.81, 11.90, 12.48 min respectively. The minor product appearing at 4.91 min in chromatogram 2E was due to an unidentified compound.

*Inset:* The UV and visible spectra of NBAD quinone L-kynurenine adducts in water. Solid line, 11.90 min Peak; broken line, 12.48 min Peak.

## 3. RESULTS

Fig. 2 shows the HPLC analysis of the reaction mixtures containing NBAD, L-kynurenine, mushroom tyrosinase and quinone isomerase in different combinations. Incubation of NBAD with mushroom tyrosinase alone resulted in the appearance of NBAD quinone as the sole product (Fig. 2B). Inclusion of quinone isomerase in this reaction, produced an additional product identified as NBANE (Fig. 2C), in accordance with the recent findings [15].

If L-kynurenine was included in the tyrosinase-NBAD reaction mixture, formation of two new products eluting at 11.9 and 12.48 min could be witnessed (Fig. 2E). The UV and visible spectra of these products are shown in the inset of Fig. 2. These products were not formed if any one of the above three components of the reaction mixture were omitted indicating that they are adducts of NBAD quinone with L-kynurenine. Accordingly, even chemically synthesized NBAD quinone generated these compounds when allowed to react non-enzymatically with L-kynurenine (data not shown). From these studies and the well known reaction of quinones with amines [9–11], these adducts were tentatively identified as L-kynurenine adducts of NBAD quinone. The structure of these adducts are still under investigation.

If the purified *o*-quinone: *p*-quinone methide isomerase [15] was included in the above reaction mixture, the formation of both NBAD quinone and its kynurenine adducts were reduced and two new products eluting at 3.38 and 3.81 min were formed (Fig. 2F).

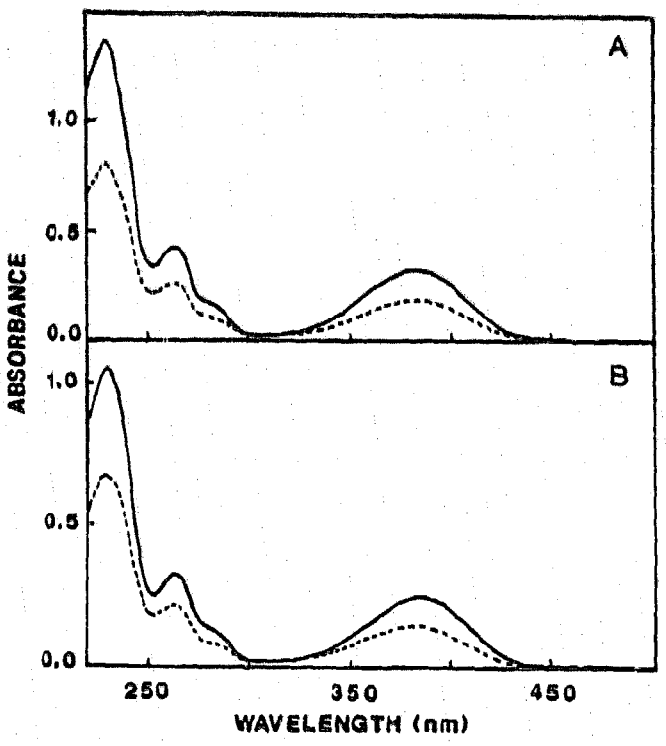


Fig. 3. Ultraviolet and visible spectra of (A) papiliochrome IIa and (B) papiliochrome IIb in water. Solid line, authentic sample supplied by Dr Yago of Iwate Medical College, Morioka, Japan; broken line, enzymatic products.

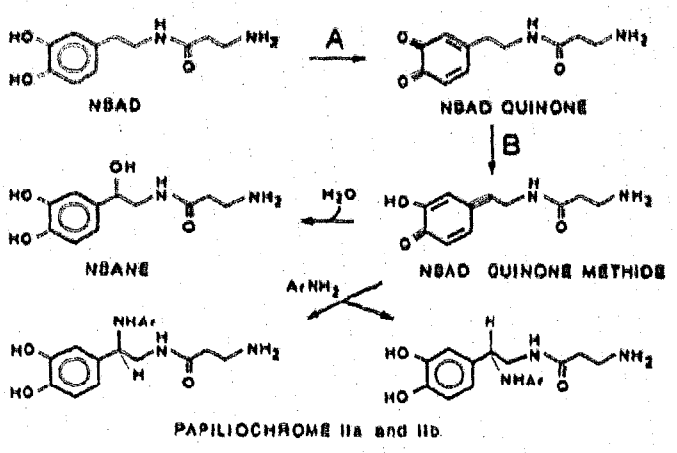


Fig. 4. Proposed mechanism for observed reactions. Mushroom tyrosinase (A) oxidizes NBAD to its quinone, *o*-quinone : *p*-quinone methide isomerase (B) transforms the NBAD quinone to NBAD quinone methide which reacts rapidly and non-enzymatically with water by a Michael-1,6-type addition reaction to produce NBANE. If, L-kynurenine is present in the reaction mixture, both NBAD quinone-L-kynurenine adducts and NBAD quinone methide-L-kynurenine adducts (papiliochrome IIa and IIb) are formed by Michael type addition reactions. The structure of quinone adducts are yet to be established.

These two products designated as IIa and IIb, were not formed if any one of the four components were omitted from the reaction mixture. Therefore, they were tentatively identified as NBAD quinone methide adducts of L-kynurenine (= papiliochrome II). In agreement with this conclusion, IIa and IIb exhibited the same retention times as those of authentic papiliochrome IIa and IIb provided by Dr Yago. In addition to co-chromatography (data not shown), the two enzymatic products also exhibited the same UV and visible spectra as those of authentic papiliochrome IIa and IIb (Fig. 3) confirming that they are indeed papiliochrome IIa and IIb.

## 4. DISCUSSION

The linking of NBAD and L-kynurenine observed during papiliochrome II biosynthesis in the yellow wing scales of butterflies obviously involves an enzyme catalyzed condensation. Given the fact that enzymes catalyze reactions with remarkable stereospecificity, the natural occurrence of papiliochrome II as a mixture of two diastereoisomers therefore calls for a non-stereoselective condensation and hence probably suggests a non-enzymatic reaction.

Recently, we discovered a new enzyme in insects which catalyzes the conversion of 4-alkylquinones to hydroxy-*p*-quinone methides [13-15]. We demonstrated that the unstable quinone methides formed during the quinone isomerase catalyzed reaction undergo non-enzymatic and hence non-stereoselective transformations to generate Michael-1,6-addition products [15]. For instance, *N*-acetyldopamine is tautomerized by the enzyme to its quinone methide analog which rapidly undergoes non-enzymatic reaction to form *N*-acetylnorepinephrine. If, methanol is included in the reaction mixture, *β*-methoxy *N*-acetyldopamine can be recovered as an additional product reflecting the non-enzymatic nature of the addition reaction. Finally, the *β*-methoxy *N*-acetyldopamine formed can be shown to be a racemic mixture attesting to the non-enantioselective nature of the reaction [15].

The quinone isomerase shows wide substrate specificity and attacks a number of compounds including NBAD [15]. Therefore, we made use of this enzyme to check whether quinone methide is a transient intermediate formed during papiliochrome II biosynthesis. Incubation of quinone isomerase with mushroom tyrosinase, NBAD and L-kynurenine resulted in the production of papiliochrome IIa and IIb in the reaction mixture. These results can be accounted for as follows: NBAD quinone produced by action of tyrosinase on NBAD is tautomerized by the quinone isomerase to NBAD quinone methide. The quinone methide thus formed undergoes non-enzymatic and hence non-stereoselective addition with L-kynurenine to generate a mixture of diastereoisomers of papiliochrome II.

Quinone methides have been characterized as reactive intermediates formed during (a) the oxidative metabolism of catecholamine derivatives and related compounds [16,17], (b) the bioreductive activation of antitumor antibiotics [18], (c) the biosynthesis of lignins [19], tannins [20], melanin [21] and neolignans [22], (d) the oxidation of butylated hydroxy-toluenes [23], and (e) the sclerotization of insect cuticle [9–11]. The present studies attest that they are also involved during the biogenesis of papiliochrome II pigments present in the yellow wing scales of butterflies.

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