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REACTIONS OF 4-METHYL-*o*-BENZOQUINONE, GENERATED CHEMICALLY OR ENZYMATICALLY, IN THE PRESENCE OF L-PROLINE

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Key Word Index—Tyrosinase; 4-methylcatechol; 4-methyl-*o*-benzoquinone; L-proline.

Abstract—The reactions of 4-methyl-*o*-benzoquinone in the presence of L-proline were studied by rapid scanning spectrophotometry. It was concluded that it was transformed into 5-methyl-4-*N*-propyl-*o*-benzoquinone, according to the equation: 2(4-methyl-*o*-benzoquinone) + L-proline → 4-methylcatechol + 5-methyl-4-*N*-prolyl-*o*-benzoquinone. Rate constants for the reaction of 4-methyl-*o*-benzoquinone with water and L-proline have been measured.

INTRODUCTION

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, E.C. 1.14.18.1.) is widely distributed in nature and is mainly involved in the biosynthesis of melanins and other polyphenolic compounds. It catalyses both the *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones. The two enzymatic activities are commonly referred to as cresolase or monophenolase and catecholase or diphenolase activities, respectively.

Recent studies of melanin biosynthesis in mammals [1, 2] have established that *o*-dopaquinone-H^+ is always the direct product of the enzymatic action of tyrosinase on L-dopa, and that the formation of dopachrome is achieved by the cyclization of the molecule following a Michael intramolecular 1,4-addition.

Substrates of tyrosinase of plant origin are generally mono- or *o*-diphenols whose *o*-quinones cannot undergo a Michael intramolecular 1,4-addition and can only change into other more stable compounds by addition of water or an external nucleophile; the chemical pathways from these quinones and the factors which can have an effect on them are not well studied.

In the present paper, we report on the oxidation of 4-methylcatechol, a natural substrate of plant tyrosinases [3]. The fate of 4-methyl-*o*-benzoquinone generated by chemical and enzymatic oxidation from 4-methylcatechol at pH 6.0 in the presence of L-proline, which can react with the *o*-benzoquinone [4, 5], has been studied. In addition, the stoichiometry of the reactions of 4-methyl-*o*-benzoquinone and the rate constants for the addition of water and L-proline have been determined.

RESULTS AND DISCUSSION

4-Methylcatechol oxidation by periodate

It is well established that chemical oxidation of *o*-diphenol by sodium periodate leads to *o*-quinone production [6, 7].

Oxidation of 4-methylcatechol by sodium periodate was carried out at pH 6.0 in the presence of 0.64 mM L-proline at three different $[NaIO_4]/[4MC]$ ratios: 6, 1/6 and 1. With a six-fold excess of periodate, the oxidation of *o*-diphenol to *o*-quinone, with a λ_{max} at 400 nm, was nearly instantaneous (Fig. 1A). The peak at 400 nm was then shifted with time towards another one of λ_{max} 520 nm (Fig. 1A), that corresponded to the quinonic form of the addition product of L-proline to 4-methyl-*o*-benzoquinone [4]. Isosbestic points were seen at 377 and 426 nm. When the L-proline concentration was increased to 20 mM, no shift was observed in the isosbestic point at 377 nm. Graphical analysis of the recordings of Fig. 1A by the matrix method of Coleman *et al.* [8] established the presence of two absorbing species in solution (Fig. 1B): 4-methyl-*o*-benzoquinone and 5-methyl-4-*N*-prolyl-*o*-benzoquinone. The transformation of 4-methyl-*o*-benzoquinone into 5-methyl-4-*N*-prolyl-*o*-benzoquinone occurred with a 1:1 stoichiometry since the excess of sodium periodate prevented the accumulation of 4-methylcatechol in the medium.

When the concentration of sodium periodate was a sixth that of 4-methylcatechol, a set of recordings was obtained (Fig. 1C) which showed once again an initial maximum at 400 nm that was shifted with time towards a maximum at 520 nm. Two isosbestic points were seen at 370 and 449 nm. When the L-proline concentration was increased to 20 mM, no shift was observed in the isosbestic point at 370 nm. Graphical analysis of the spectra gave a good fit for two, kinetically related, absorbing species in solution (results not shown). Thus, although there are really four kinetically related species: 4-methylcatechol, 4-methyl-*o*-benzoquinone, 5-methyl-4-*N*-propyl-catechol and 5-methyl-4-*N*-prolyl-*o*-benzoquinone, the 5-methyl-4-*N*-prolyl-catechol concentration was too small to be detected by matrix analysis, and the equimolar amounts of 4-methylcatechol and 5-methyl-4-*N*-prolyl-*o*-benzoquinone formed at a constant rate from 2 mol 4-methyl-*o*-benzoquinone could not be differentiated by matrix analysis.

Finally, when the oxidation was carried out with an equimolar ratio ($[NaIO_4]/[4MC] = 1$), no isosbestic

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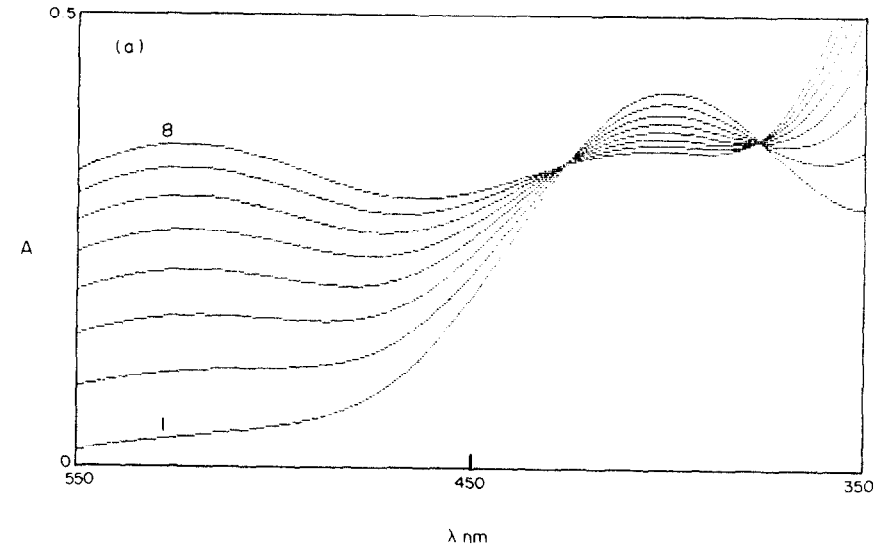
Abbreviations: 4MC; 4-methylcatechol, dopachrome; 2-carboxy-2,3-dihydroindole-5,6-quinone, dopaquinone; 4-(2-carboxy-2-aminoethyl)-1,2-benzoquinone.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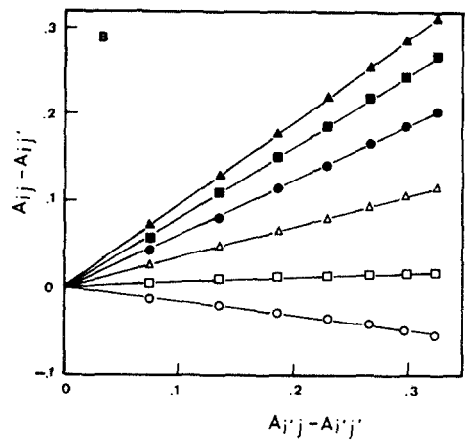
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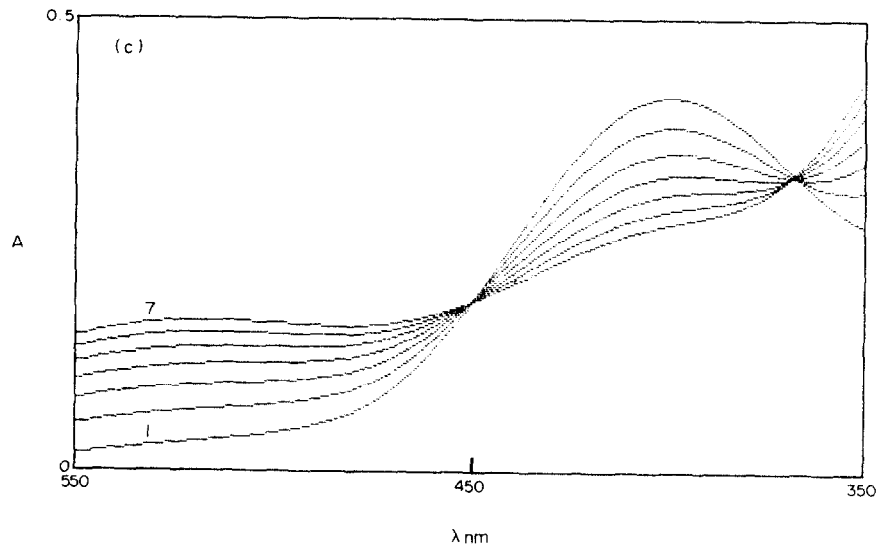
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Fig. 1. A, B, C.

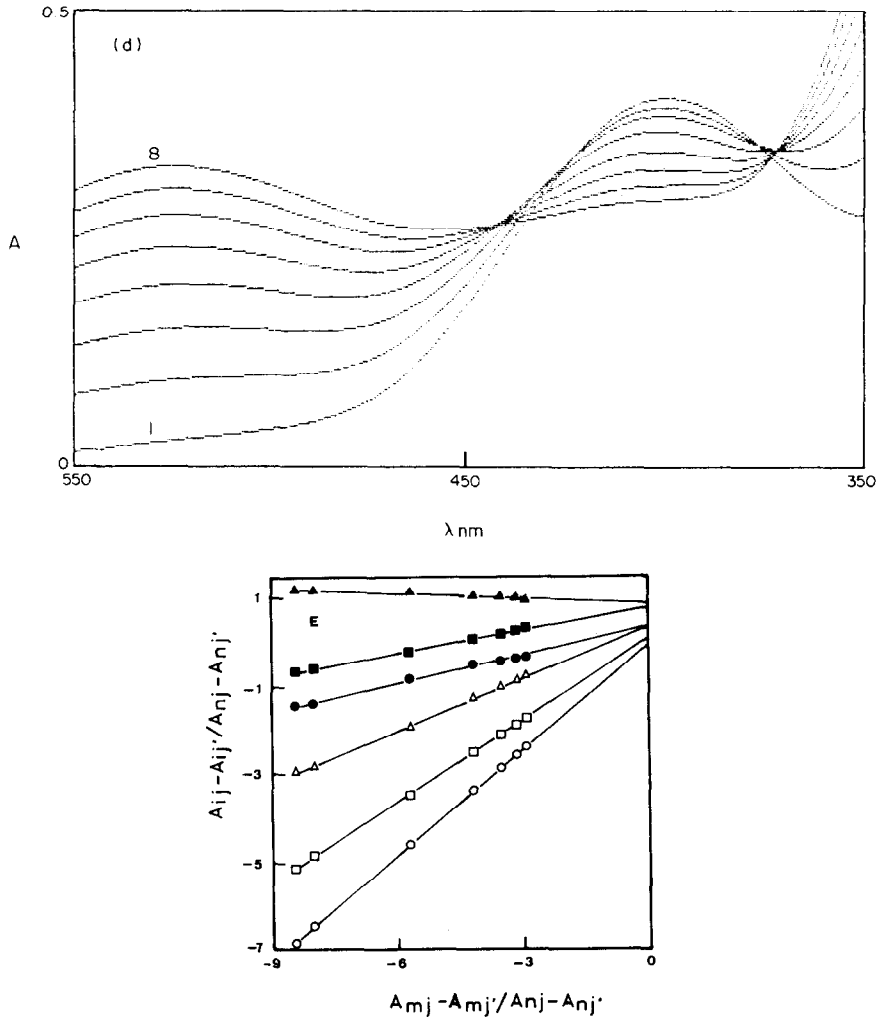


Fig. 1. Spectrophotometric recordings of the oxidation of 4-methylcatechol with sodium periodate in the presence of 0.64 mM L-proline. (A) 4-Methylcatechol (0.24 mM) oxidized with an excess of $NaIO_4$ (1.44 mM). Scan speed was up to 60 nm/min. (B) Graphical analysis of spectra recorded in Fig. 1A. The test for two species with restriction was applied. In this analysis, A_{ij} is the absorbance at wavelength i, obtained during tracing j, so that A_{21} is the absorbance at 430 nm obtained during the first recording of the absorption spectrum. Selected wavelengths were i' = 530 nm, i = ○ (410 nm), i = □ (430 nm), i = △ (450 nm), i = ● (470 nm), i = ■ (490 nm), i = ▲ (550 nm), being j' = first tracing. (C) 4-methylcatechol (1.92 mM) oxidized with sub-stoichiometric concentration of sodium periodate (0.32 mM). Scan speed was up to 120 nm/min. (D) 4-Methylcatechol (0.24 mM) oxidized with an equimolar ratio of sodium periodate (0.24 mM). Scan speed was up to 60 nm/min. (E) Graphical analysis of spectra recorded in Fig. 1D. The test for three species with restriction was applied. Selected wavelengths were m = 530 nm, n = 410 nm (two reference wavelengths), i = ○ (490 nm), i = □ (470 nm), i = △ (450 nm), i = ● (370 nm), i = ■ (430 nm), i = ▲ (390 nm), being j' = first tracing.

points appeared (Fig. 1D). Graphical analysis of the spectra showed the presence of three, kinetically related, absorbing species (Fig. 1E), which were considered to be 4-methylcatechol, 4-methyl-*o*-benzoquinone and 5-methyl-4-*N*-propyl-*o*-benzoquinone.

4-*Methylcatechol oxidation by tyrosinase*

The visible spectra of the products formed when 4-methylcatechol was oxidized by mushroom tyrosinase at pH 6.0 in the presence of L-proline is shown in Fig. 2A. An initial maximum at 400 nm gave rise to a band at 520 nm. No isosbestic points were present. Graphical analysis of the different recordings established the presence of three absorbing species in solution (results not shown), i.e. a similar situation to the one obtained when 4-methylcatechol oxidation by sodium periodate was performed under stoichiometric conditions.

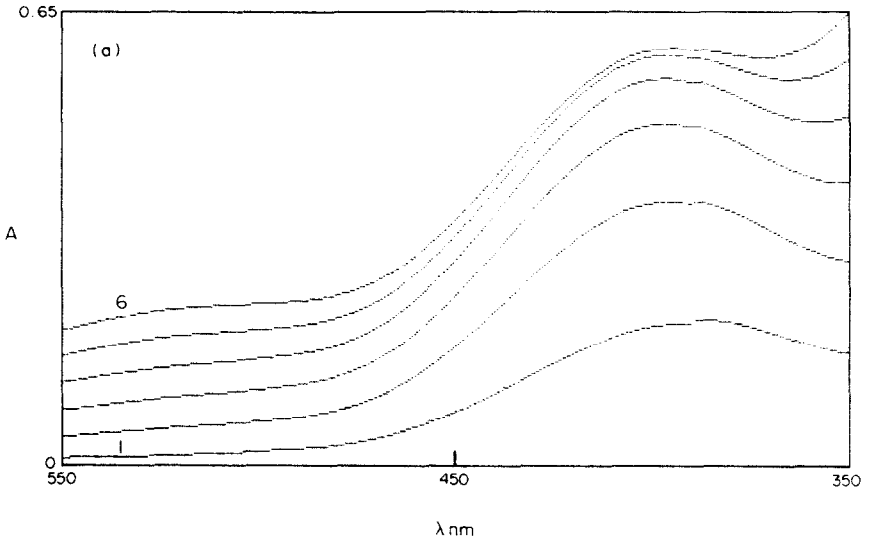
When 4-methylcatechol oxidation was carried out at pH 6.0 with a five-fold greater amount of tyrosinase (Fig. 2B), it was possible to obtain oxygen depletion in the assay medium within a few minutes from the start of the reaction. The first tracing was obtained when the enzymatic reaction was complete. So, all the spectra corresponded to the chemical transformation of *o*-quinone. As

can be seen, two isosbestic points were present at 370 and 449 nm. Graphical analysis for this last recording showed that there were two, kinetically related, species (results not shown). It can be seen that these isosbestic points are the same as those obtained when 4-methylcatechol oxidation was performed with insufficient sodium periodate (Fig. 1C).

Stoichiometry of 4-methylcatechol oxidation by periodate

When oxidation of 4-methylcatechol was carried out with an equimolar ratio of periodate in the presence of L-proline (Fig. 3), all the *o*-diphenol present in the reaction medium was rapidly oxidized to 4-methyl-*o*-benzoquinone (trace b, maximum 400 nm). If the reaction was followed until there was no further increase in absorbance, the existence of two compounds, 5-methyl-4-*N*-prolyl-*o*-benzoquinone (300 and 520 nm) and 4-methylcatechol with a maximum at 280 nm was evident (trace c). Furthermore, the addition of a new equivalent of periodate to the reaction mixture increased the absorbance at both 300 and 520 nm two fold (trace d). Two equivalents of periodate were therefore necessary to form one equivalent of 5-methyl-4-*N*-prolyl-*o*-benzoquinone.

The intercepts between traces b and c (Fig. 3) coincided with the isosbestic points obtained in Fig. 1C, while



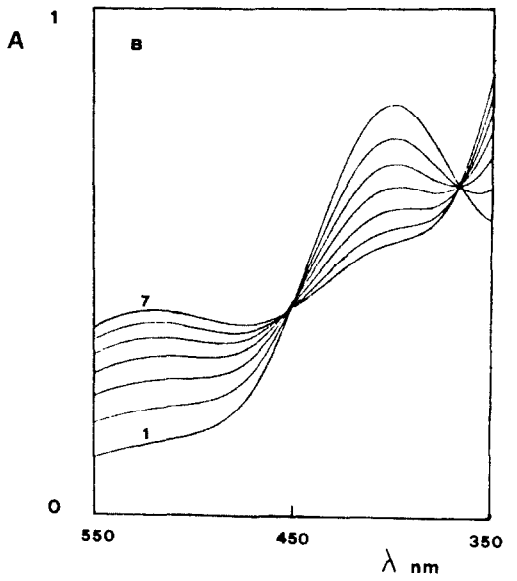


Fig. 2. (A) Spectrophotometric recordings for the oxidation of 4-methylcatechol (1.92 mM) by tyrosinase (8 μg/ml) in the presence of 0.64 mM L-proline. Scan speed was up to 120 nm/min. (B) Spectrophotometric recordings for the oxidation of 4-methylcatechol (1.92 mM) by tyrosinase (40 μg/ml) in the presence of 0.64 mM L-proline with oxygen depletion. Scan speed was up to 100 nm/min, at intervals of 30 sec; the first recording was started 20 sec after the start of the reaction.

the intercepts between traces b and d were the same as isosbestic points obtained in Fig. 1A.

The results obtained suggested a set of steps (Fig. 4), similar to those postulated for the oxidation of L-dopa by tyrosinase [2, 9–11]. According to this scheme, the addition of L-proline to 4-methyl-*o*-benzoquinone causes the accumulation of 4-methylcatechol (step 4). This fact is confirmed by the observation that the addition of a second equivalent of sodium periodate to the reaction medium doubles the amount of 5-methyl-4-*N*-prolyl-*o*-benzoquinone produced. Therefore it is possible to control the rate of accumulation of *o*-diphenol by means of the nucleophile concentration.

These experiments allowed us to conclude that the stoichiometric equation for the conversion of 4-methyl-*o*-benzoquinone into 5-methyl-4-*N*-prolyl-*o*-benzoquinone is 2(4-methyl-*o*-benzoquinone) + L-proline → 4-methylcatechol + 5-methyl-4-*N*-prolyl-*o*-benzoquinone.

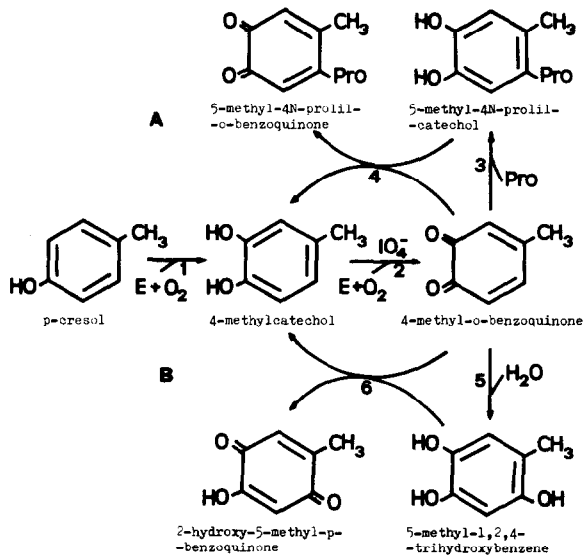


Fig. 4. Pathways proposed for the oxidation of 4-methylcatechol by sodium periodate or tyrosinase in the presence or absence of L-proline. (A) Addition of L-proline to 4-methyl-*o*-benzoquinone. (B) Addition of water to 4-methyl-*o*-benzoquinone.

Evaluation of rate constants for the chemical transformation of 4-*methyl*-o-*benzoquinone*

According to the Scheme postulated (Fig. 4), the chemical transformation of 4-methyl-*o*-benzoquinone into 5-methyl-4-*N*-prolyl-*o*-benzoquinone can be kinetically characterized by measuring at 520 nm the appearance of 5-methyl-4-*N*-prolyl-*o*-benzoquinone formed from 4-methyl-*o*-benzoquinone generated by chemical or enzymatic oxidation, since 2-hydroxy-5-methyl-*p*-benzoquinone does not absorb at this wavelength.

As the redox steps 2, 4 and 6 (Fig. 4) occur rapidly with either an excess or with sub-stoichiometric concentrations of sodium periodate [1], the rate limiting steps of the

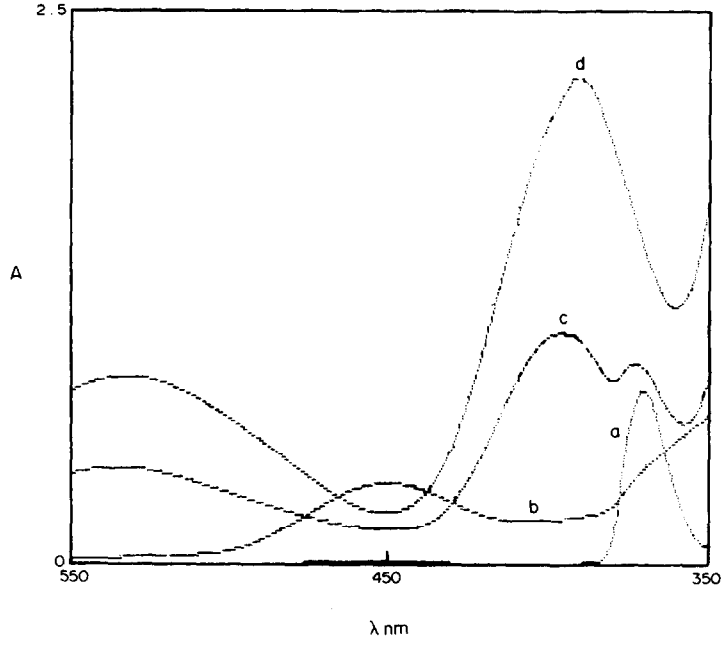
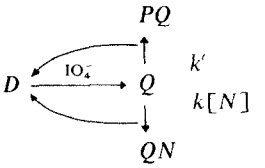


Fig. 3. Oxidation of 4-methylcatechol by sodium periodate in the presence of 2 mM L-proline. (a) 4-Methylcatechol (0.3 mM). (b) 4-Methylcatechol (0.3 mM) reacted with 0.3 mM sodium periodate, recording at the beginning of the reaction. (c) Recording at the end of the reaction, conditions as in (b). (d) 4-Methylcatechol (0.3 mM) reacted with 0.6 mM sodium periodate recording at the end of the reaction.

system should be 3 and 5, so the general Scheme can be simplified to:



where D = 4-methylcatechol, Q = 4-methyl-*o*-benzoquinone, PQ = 2-hydroxy-5-methyl-*p*-benzoquinone, QN = 5-methyl-4-*N*-prolyl-*o*-benzoquinone, $[N]$ = [L-proline], k' = pseudofirst-order rate constant of reaction of addition of water and k = second-order rate constant of addition of L-proline.

The following equations can be established:

$$d[Q]/dt = -2(k' + k[N])[Q] \quad (1)$$

$$d[QN]/dt = k[N][Q]. \quad (2)$$

The factor 2 appears because of the stoichiometry of transformation of 4-methylcatechol into 5-methyl-4-*N*-prolyl-*o*-benzoquinone with a substoichiometric concentration of sodium periodate. If the appearance of 5-methyl-4-*N*-prolyl-*o*-benzoquinone is followed with an excess of sodium periodate this factor does not appear.

Integrating Eqn 1, substituting it into Eqn 2 and again integrating, we obtain the equation for the accumulation with time of 5-methyl-4-*N*-prolyl-*o*-benzoquinone in conditions of insufficient sodium periodate:

$$[QN] = \frac{k[N][Q_0]}{2(k' + k[N])}(1 - e^{-2(k' + k[N])t}) \quad (3)$$

where Q_0 = initial 4-methyl-*o*-benzoquinone concentration, equivalent to the limiting reactive concentration, of 4-methylcatechol or sodium periodate. The accumulation of 5-methyl-4-*N*-prolyl-*o*-benzoquinone as measured by the absorbance at 520 nm follows first order kinetics with $k_{app} = 2(k' + k[N])$ with a sub-stoichiometric concentration of sodium periodate and $k_{app} = k' + k[N]$ with an excess of periodate. The variation of the k_{app}, calculated according to the Guggenheim equation [12], with the concentration of L-proline in the reaction medium is shown in Fig. 5A. The calculated k' and k values are $k' = 5 \times 10^{-4}\ sec^{-1}$ and $k = 2.2 \times 10^{-4}\ mM^{-1}\ sec^{-1}$ with a sub-stoichiometric concentration of sodium periodate, and $k' = 5 \times 10^{-4}\ sec^{-1}$ and $k = 2.5 \times 10^{-4}\ mM^{-1}\ sec^{-1}$ in the presence of an excess of sodium periodate.

On the other hand, for $t \to \infty$, accumulation of 5-methyl-4-*N*-prolyl-*o*-benzoquinone will correspond with a sub-stoichiometric concentration of sodium periodate:

$$[QN]_\infty = \frac{k[N][Q_0]}{2(k' + k[N])} \quad (4)$$

and in excess of sodium periodate:

$$[QN]_\infty = \frac{k[N][Q_0]}{k' + k[N]}. \quad (5)$$

The experimental results shown in Fig. 5B, agree with the corresponding equations.

EXPERIMENTAL

Mushroom tyrosinase (2000 units/mg), 4-methylcatechol and L-proline were purchased from Sigma Chemical Co.; all other chemicals were of analytical grade.

Spectra were recorded with scanning speeds depending on the reaction rates and, unless otherwise indicated, at intervals of 40 sec; the first recording was started 40 sec after initiation of the reaction.

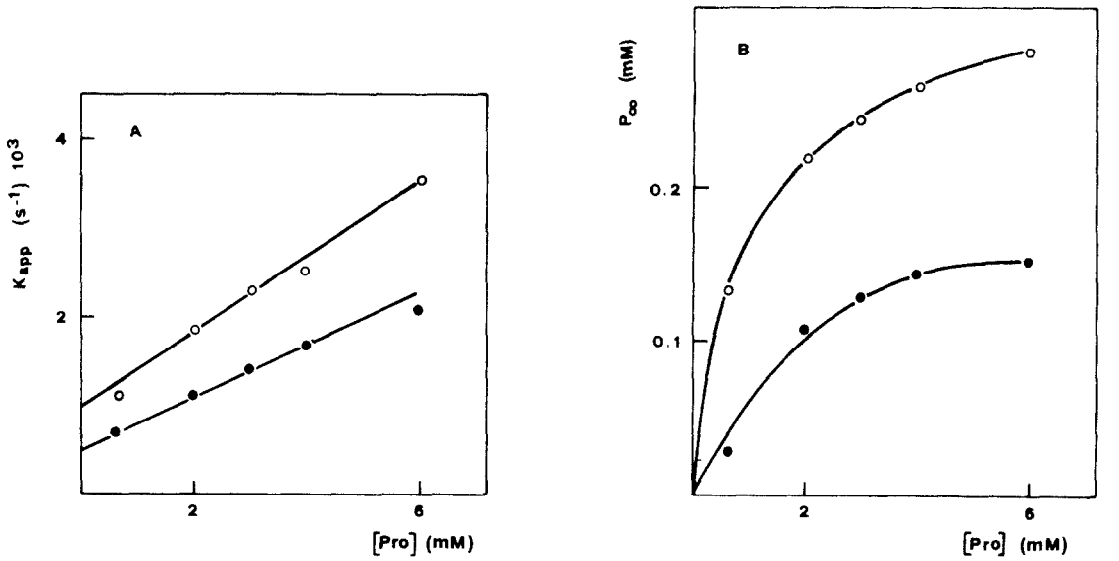


Fig. 5. (A) Variation of apparent rate constants of accumulation of 5-methyl-4-*N*-prolyl-*o*-benzoquinone with L-proline concentration in the presence of either an excess of periodate (●) or a sub-stoichiometric concentration of it (○). [4MC] = 0.32 mM, [IO_4] = 1.92 mM (●); [4MC] = 1.92 mM, [IO_4] = 0.32 mM (○); [L-proline] was varied in a range of 0–6 mM. (B) Variation of infinite product (5-methyl-4-*N*-propyl-*o*-benzoquinone) with L-proline in the presence of either an excess of periodate (○) or a sub-stoichiometric concentration of periodate (●). Conditions are the same as those used in (A).

The appearance of 5-methyl-4-*N*-prolyl-*o*-benzoquinone was measured at 520 nm, $\varepsilon = 3500\ M^{-1}\ cm^{-1}$. All measurements were taken in 10 mM Na-Pi buffer, pH 6.0, at 25°. Temperature was controlled by a Gilson bath and a digital Cole-Parmer thermistor with a SR ± 0.1°. Protein concentration was determined by the method of ref. [13].

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