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Oxidation of Chlorogenic Acid, Catechins, and 4-Methylcatechol in Model Solutions by Apple Polyphenol Oxidase

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The oxidation of chlorogenic acid (CG), (+)-catechin (CA), (−)-epicatechin (EC), and 4-methylcatechol (4MC) catalyzed by apple polyphenol oxidase (PPO) was followed between pH 4 and 5 by polarography, spectrophotometry, and HPLC. In the presence of cysteine, the stoichiometry O_2/phenol consumed was always close to 0.5 regardless of the time of measurement, the initial O_2/phenol ratio, and the phenols tested at pH between 4 and 5. In the absence of cysteine, the observed stoichiometries greatly varied with the preceding factors. Moreover, the *o*-quinone reactivities widely differed from one phenol to another. Two pathways have been proposed for the degradation of 4MC *o*-quinones. The first one, favored by acid pH, corresponds to an hydroxylation followed by a coupled oxidation of another molecule or *o*-quinone leading to the formation of 2-hydroxy-5-methyl-*p*-benzoquinone and the regeneration of 4MC. The second pathway corresponds to polymerization reactions which were favored by higher pH values. The same pathways have been observed with CG, although the polymerization reactions seemed to be dominant. Due to the high instability of EC *o*-quinones, this study was not carried out with EC. Nevertheless, the different pathways of *o*-quinone degradation and their relative importance which varied with the pH, the phenol, and the relative concentrations of the reactants (phenol, *o*-quinone, and O_2) could explain the variation in stoichiometry.

INTRODUCTION

Enzymatic browning in damaged fruits and vegetables is an important cause of quality and nutritional loss. This reaction results from the oxidation of endogenous phenols by polyphenol oxidase (EC 1.14.18.1; PPO) in the presence of oxygen (Vamos-Vigyazo, 1981). With *o*-diphenols as substrate, the so-called "catecholase" activity of the enzyme resulted in the primary formation of *o*-quinones with an accepted stoichiometry of 0.5 mol of oxygen consumed/mol of phenol degraded and *o*-quinone formed (Mayer and Harel, 1979; Mayer, 1987). Depending on the phenol, the *o*-quinones show great differences in stability and undergo subsequent reactions leading to dark-colored pigments. Moreover, the color intensities vary widely from one phenol to another (Lee and Jaworski, 1988; Rouet-Mayer et al., 1990). Variable amounts of oxygen and/or phenols can also be consumed during nonenzymatic degradations of *o*-quinones. Thus, in the literature, the ratios oxygen/phenol consumed and oxygen/*o*-quinone formed vary from 0.4 to more than 2 (Forsyth and Quesnel, 1957; Roberts, 1959; Mason and Peterson, 1965; Mayer et al., 1966; Pierpoint, 1966, 1969; Esterbauer et al., 1977; Singleton, 1987; Cheynier and Van Hulst, 1988; Cheynier et al., 1988; Rigaud et al., 1988). However, these results were based in many cases on the sole measurement of oxygen uptake, assuming that all the phenol was consumed at the end of the reaction (Forsyth and Quesnel, 1957; Roberts, 1959; Mason and Peterson, 1965; Pierpoint, 1966, 1969). Furthermore, when the amount of the remaining phenols was estimated, either the assay with the molybdate reagent was not sufficiently specific (Mayer et al., 1966) or the enzymatic reaction was stopped by sulfur dioxide (Cheynier and Van Hulst, 1988; Cheynier et al., 1988; Rigaud et al., 1988). In the latter case, according to the authors, SO_2 can cause a partial reduction of *o*-quinones to the original phenol, leading to an underestimation of the phenol really consumed. On the other hand, in the presence of a sufficient amount (higher than the original phenolic substrate) of cysteine (Roberts, 1959), glutathione (Mason and Peterson, 1965; Cheynier and Van Hulst, 1988), benzenesulfinic acid (Pierpoint, 1966), or 2-nitro-5-thiobenzoic acid (Esterbauer et al., 1977), the stoichiometry was always close to 0.5 mol of oxygen/mol of phenol. It was clear that *o*-quinones were trapped by these compounds and then could not enter in further side reactions.

The purpose of this paper was to examine the stoichiometry during the enzymatic oxidation of different phenols, chlorogenic acid (CG), (+)-catechin (CA), (−)-epicatechin (EC), and 4-methylcatechol (4MC).

MATERIALS AND METHODS

Materials. Apples from the variety Red Delicious picked at commerical maturity were used as an enzyme source. The PPO was 120-fold purified from the cortex in three steps: extraction, fractional precipitations by ammonium sulfate, and hydrophobic chromatography with Phenyl-Sepharose CL4B (Pharmacia) according to the method of Janovitz-Klapp et al. (1989). CG, CA, and EC were from Extrasynthèse (Genay, France), and all other chemicals were of reagent grade from Sigma (St. Louis, MO).

Methods. *Assay for PPO Activity.* PPO activity was assayed polarographically according to the method of Janovitz-Klapp et al. (1990b) using 4MC (20 mM) as substrate. Activity was expressed as nanomoles of oxygen consumed per second (nanokatals) in the assay conditions. The reaction was started by 50 μL of purified apple PPO (25 nkat).

Simultaneous Measurements of Oxygen Consumption, Phenol Degradation, Colored Products, and o-Quinone Formation. For

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each phenolic substrate at 0.8 mM, the assays were carried out in the presence of 0.2 mM vanillic acid in a total volume of 3 mL. The latter compound was used as internal standard in the subsequent quantitation by HPLC. Owing to its very weak inhibition properties (Janovitz-Klapp et al., 1990a), its effect on velocity was negligible.

Assay of Oxygen Consumption. Oxygen uptake was measured by Oxygraph K-IC (Gilson, Villiers-de-Bel, France) equipped with a Clark electrode fitted in a 3-mL jacketed cell at 30 °C. The initial oxygen concentration of air-saturated solutions, used to calibrate the Clark electrode, was determined according to the Winkler method as described by Green and Hill (1984) and found at 220 ± 5 μM.

Assay of Colored Products. The formation of colored products was followed by spectrophotometry at 390 nm for CA and at 420 nm for CG. This was carried out in a series of parallel experiments in the same conditions as those used for polarography.

Assay of Phenol and o-Quinone. For each time tested, 0.5 mL was withdrawn from the Oxygraph cell and immediately mixed with an equal volume of stopping solution containing 2 mM NaF. Sodium fluoride was shown to be a very strong inhibitor of apple PPO (Janovitz-Klapp et al., 1990a). In a first series of experiments at pH 4, the residual phenols and formed *o*-quinones in the free form were separated and quantified by reversed-phase HPLC using dual-wavelength detection (Beckman 167 UV–visible detector) at 280 and 390 nm for CA and at 300 and 420 nm for CG, respectively. The gradient method used for the separation was slightly modified from that described by Rouet-Mayer et al. (1990). The solvents used were (A) 2.8% AcOH (pH 2.6) and (B) MeOH; the column was equilibrated at a flow rate of 1 mL min^{-1} with 15% B. The gradient profile was 0–3.5 min, 15% B; 3.5–12 min, 15–60% B; 12–16 min, 60% B; and 16–18.5 min, 60–15% B.

In a second series of experiments at pH 4, 4.5, or 5, cysteine (2 mM) was incorporated in the NaF stopping solution to trap free quinones (Richard et al., 1991). The residual phenols and the *o*-quinones in the form of cysteinyl addition compounds were separated and quantified by HPLC (9010 pump and 9050 UV detector driven by a 9020 workstation from Varian) on 10-μL samples using isocratic conditions (Richard et al., 1991). The relative response factors of phenols and their respective cysteinyl addition compounds were determined by injection of pure compounds prepared according to the method of Richard et al. (1991). In this series, and to characterize the oxidation products of 4MC and CG, the stopping solution was also supplemented by ascorbic acid (2 mM) and an electrochemical detector (HP 1049A electrochemical detector from Hewlett-Packard) was also used in parallel with the UV detector. Then, to separate and quantify the secondary oxidation products, the HPLC column equilibrated with 90% solvent C and 10% solvent D was used. The gradient profile was 0–5 min, 90% C; 5–12 min, 90–50% C; 12–14 min, 50% C; and 14–16 min, 50–90% C. The two solvents were (C) 3% AcOH and (D) MeOH for 4MC or (C) water at pH 2.6 adjusted with 85% phosphoric acid and (D) acetonitrile for CG. The flow rate was at 1 mL min^{-1}.

RESULTS AND DISCUSSION

Oxidation of Chlorogenic Acid and (+)-Catechin by Apple PPO. The results obtained during phenol oxidation by purified apple PPO are given in Figures 1 and 2 for CG and CA, respectively. Simultaneous measurements of oxygen uptake (Figures 1A and 2A), phenol degradation (Figures 1B and 2B), colored products (Figures 1C and 2C), and *o*-quinones (Figures 1D and 2D) formations were carried out.

With CG, the phenol degradation (Figure 1B) closely followed the oxygen uptake (Figure 1A) in the initial phase of the reaction. However, there was still a slight consumption of phenol at the end of the reaction in the absence of oxygen. The browned product (Figure 1C) and *o*-quinone (Figure 1D) formations were maximum between 3 and 5 min of reaction and then steadily decreased, to almost zero in the latter case after 15 min. The observed

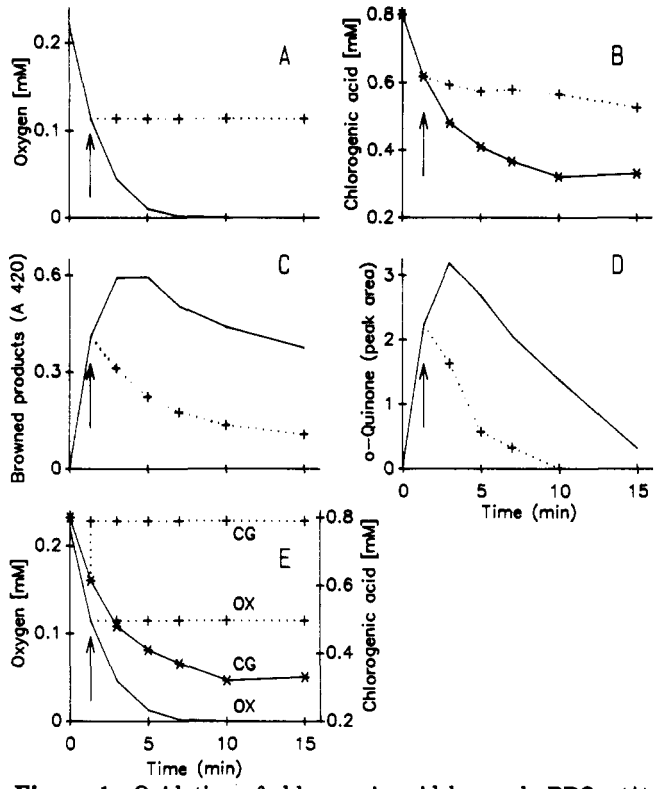

Figure 1. Oxidation of chlorogenic acid by apple PPO. (A) Oxygen uptake (polarography); (B) chlorogenic acid degradation (HPLC measurement); (C) browned product formation (spectrophotometry at 420 nm); (D) *o*-quinone formation (HPLC measurement); (E) oxygen uptake (polarography) and chlorogenic acid degradation (HPLC measurement). The arrow indicates the addition of the stopping solution containing NaF either alone (A–D) or with 2 mM ascorbic acid (E). Full lines are for the results obtained without addition of the stopping solution, and dotted lines are for the results obtained after addition of the stopping solution.

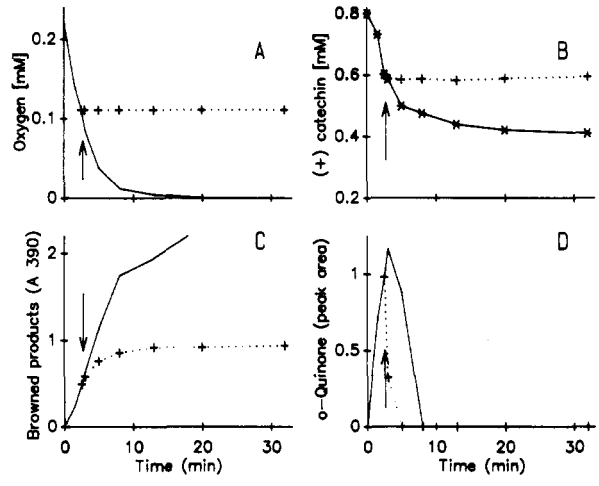

Figure 2. Oxidation of (+)-catechin by apple PPO. (A) Oxygen uptake (polarography); (B) (+)-catechin degradation (HPLC measurement); (C) browned product formation (spectrophotometry at 390 nm); (D) o-quinone formation (HPLC measurement). The arrow indicates the addition of the stopping solution containing NaF (A–D). Full lines are for the results obtained without addition of the stopping solution, and dotted lines are for the results obtained after addition of the stopping solution.

maximum corresponded roughly to the beginning of the anaerobic conditions. After the enzymatic reaction was inhibited by the NaF addition, the oxygen uptake was immediately stopped; a slight degradation of CG was apparent, whereas the absorbance at 420 nm and the quinone content decreased rapidly. When ascorbic acid

was incorporated in the NaF solution (Figure 1E), the CG concentration was almost restored to its initial value and remained constant.

With CA, a similar behavior was observed in the initial phase for the oxygen consumption (Figure 2A), the phenol degradation (Figure 2B), and the browned product (Figure 2C) and o-quinone (Figure 2D) formations. However, the initial rates of these phenomena were lower than with CG due to the specificity of apple PPO (Janovitz-Klapp et al., 1990b). Other differences were also apparent. First, the additional consumption of CA in anaerobic conditions was almost nil. Second, there was no maximum in the formation of colored compounds at 390 nm, although the o-quinone content increased and then decreased rapidly in the first few minutes of the reaction. Lastly, when the enzymatic reaction was stopped by NaF, no additional degradation of CA was observed; the o-quinone content fell rapidly, whereas the absorbance at 390 nm slightly increased.

The different behavior in the formation of colored products and o-quinones has been already observed by Rouet-Mayer et al. (1990). They indicated that at the first phase of the reaction the CA o-quinones were much less stable and less colored than those from CG, whereas in the second phase of the reaction the secondary products were darker than their source quinones for CA but lighter for CG.

The additional consumption of CG in anaerobic conditions after inhibition of PPO suggested a nonenzymatic degradation for CG. This way, in which oxygen was not consumed, was probably linked to the reactions of o-quinones and phenols. Such reactions involving o-quinones and their originating phenols have been already proposed (Gordon and Hamilton, 1973; Singleton, 1987; Cheynier et al., 1988), although the resulting dimers have never been formally characterized. These side reactions occurred simultaneously with the enzymatic reaction, and their importance was probably variable from one phenol to another depending on the stability of the o-quinone. Therefore, we decided to study the stoichiometry O_2 to phenol consumed during the oxidation catalyzed by apple PPO for CG and EC, two of the main phenols found in apple fruit, and to compare to that obtained with 4MC, which is one of the most often used substrate for PPO. This study was carried out as the enzymatic reaction proceeded at three pH values between 4 and 5 either in the absence or in the presence of cysteine.

Stoichiometry Oxygen/Phenol Consumed. At pH 4, in the first 3 min during which 80% of the available oxygen was consumed, the decrease in CG closely followed the oxygen uptake (Figure 3A). On 6 experimental values, the mean stoichiometry was equal to 0.50 ± 0.02 mol of O_2 consumed/mol of phenol degraded. This value is in agreement with the theoretical value (Mayer and Harel, 1979). The o-quinone formation deviated rapidly from the former curves since after 2 min, which was the maximum for o-quinones, they represented only 70% of the phenol consumed. This deviation indicated the presence of further reaction for o-quinone. After 3 min, the oxygen uptake slowed rapidly since all of the available oxygen was consumed, whereas the CG and its o-quinone were degraded further even in the absence of oxygen. After 15 min, the o-quinone content was almost zero and the overall stoichiometry decreased to 0.41 mol of O_2/mol of phenol (Table I). Thus, the partial stoichiometry between two successive experimental points remained constant at 0.5 in the first 2 min and then gradually fell to almost zero after 10 min of reaction.

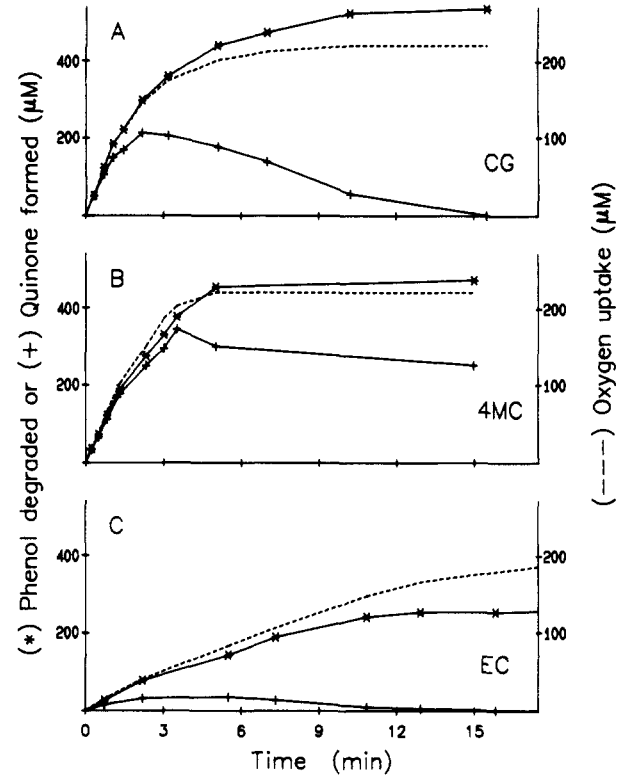

Figure 3. Enzymatic oxidation of three different phenols by apple PPO at pH 4. (A) Chlorogenic acid; (B) 4-methylcatechol; (C) (−)-epicatechin. (- - -) Oxygen consumed; (*) phenol degraded; (+) o-quinone formed.

A similar phenomenon was observed at pH 4.5 and 5 (Table I). However, during the initial period, the stoichiometry was found at 0.55 and 0.56 mol of O_2/mol of phenol, respectively, in the 0–120 and 120–180 μM oxygen cuts at both pH. In the second period (180–220 μM oxygen cut), the additional consumption of phenol together with a decrease of o-quinone and a small oxygen uptake led to an overall stoichiometry lower than 0.5 mol of O_2/mol of phenol. At these pH values, the partial stoichiometry between two successive assays was constant at 0.55 in the first 2 min and fell rapidly to almost zero after 5 min of reaction.

Similar results were obtained with 4MC since in the first period of the reaction the stoichiometry was found between 0.54 and 0.58 mol of O_2/mol of phenol with a small increase with pH between 4 and 5 (Table I). In the same way, the o-quinone formation showed a deviation from the oxygen uptake and the 4MC degradation after 3 min of reaction (Figure 3B). However, the 4MC o-quinones were more stable than those of CG since after 15 min of reaction there was more than 200 μM of remaining 4MC o-quinones, whereas it was almost zero with CG o-quinones. Moreover, the additional degradation of 4MC in the anaerobic phase of the reaction was less pronounced than that of CG. This can be obviously related to the lower reactivity of 4MC o-quinones toward 4MC compared to those observed with CG and its o-quinone.

The results obtained with EC were different. Whereas with CG and 4MC the overall stoichiometry O_2/phenol decreased at the end of the reaction to values lower than 0.5, the amount of oxygen consumed per mole of EC was always greater than 0.5 and increased gradually as the reaction proceeded (Table I). At the beginning of the reaction, when oxygen content was greater than 100 μM, the mean overall stoichiometry was close to 0.56, whereas it increased to 0.73 at pH 4, 0.77 at pH 4.5, and 0.79 at pH

Table I. Overall Stoichiometry of Mole of Oxygen Uptake per Mole of Phenol Degraded for CG, MC, and EC as a Function of the Amount of Oxygen Consumed at pH between 4 and 5[a]

pH		O_2 consumed 0–120 μM	O_2 consumed 120–180 μM	O_2 consumed 180–220 μM	final values O_2/phenol	final values % quinone[b]
			Chlorogenic Acid			
4	mean	0.51 (4)[c]	0.49 (2)	0.43 (3)	0.41	tr[d]
	SD	0.01	0.01	0.02		
4.5	mean	0.55 (3)	0.56 (3)	0.49 (3)	0.46	1
	SD	0.01	0.01	0.03		
5	mean	0.55 (3)	0.56 (2)	0.47 (3)	0.44	2
	SD	0.03	0.02	0.03		
			4-Methylcatechol			
4	mean	0.54 (3)	0.55 (2)	0.50 (3)	0.47	51
	SD	0.01	0.01	0.03		
4.5	mean	0.55 (3)	0.56 (2)	0.43 (4)	0.38	53
	SD	0.02	0.01	0.04		
5	mean	0.58 (3)	0.57 (2)	0.53 (4)	0.49	65
	SD	0.02	0.01	0.04		
			(−)-Epicatechin			
4	mean	0.56 (4)	0.65 (3)	0.71 (2)	0.73	0
	SD	0.03	0.04	0.02		
4.5	mean	0.54 (4)	0.68 (3)	0.74 (4)	0.77	0
	SD	0.03	0.04	0.03		
5	mean	0.58 (3)	0.70 (3)	0.78 (4)	0.79	0
	SD	0.01	0.03	0.01		

[a] Conditions: The phenol was initially set at 0.8 mM, and the final values of stoichiometry and *o*-quinone were measured after 15 min with CG and 4MC and after 20 min with EC. [b] Residual *o*-quinones in percent of degraded phenol. [c] Number of assays in the specified oxygen cut. [d] tr, traces.

5 when the oxygen content fell below 50 μM (Table I). Compared to CG and 4MC, the relative instability of the *o*-quinones of EC was confirmed since the observed maximum between 2 and 5 min was less than 35 μM, i.e., hardly 10% of the value obtained with 4MC (Figure 3C). Moreover, the additional consumption of EC was not observed in anaerobic conditions probably owing to the absence of *o*-quinones.

In the presence of cysteine at a concentration which was more than twice that of phenol, the stoichiometry was always close to 0.5 mol of O_2/mol of phenol whatever the pH tested between 4 and 5 and for all the phenolic substrate regardless of the time of measurement (data not shown). Moreover, the stoichiometry remained the same for CG and 4MC if the addition compound formed was considered instead of the phenol degraded. For EC, two addition compounds were formed in equal amounts (Richard et al., 1991) and the stoichiometry mole of O_2 consumed per mole of addition compounds (*cis*-2′-epicatechin + *cis*-5′-epicatechin) was still equal to 0.5 for the three pH values tested.

In the preceding experiments, the initial phenol concentration was always more than twice that of oxygen; therefore, the end of the reaction was always in anaerobic conditions. In a second series of experiments at pH 4.5, the initial concentration of phenol was largely decreased to obtain a plateau in the oxygen consumption, the value of which was not zero. For each phenol between 0 and 420 μM, the value of oxygen consumed to reach the plateau has been evaluated together with the remaining phenol and *o*-quinone (Table II). The measurement was performed at least 10 min after the plateau had been reached. For the three phenols tested, the oxygen uptake increased as the initial concentration in phenol was increased. For a given concentration in phenol, the plateau was independent on the amount of enzyme. Thus, when PPO activity was varied from 10 to 100 nkat mL^{-1} in the presence of 200 μM CG, the final value of the oxygen consumed remained constant at 155 ± 5 μM, whereas the time needed to reach the plateau largely decreased.

Regardless of the initial amount and the type of phenol, there was always a small amount of residual phenol 10 min after the plateau had been reached. However, large differences have been observed in the final values of stoichiometry (Table II). For CG, it was as high as 0.84 for low initial amount of CG and decreased to 0.59 when the CG content was increased to 370 μM. In this latter case, 90% of the available oxygen was consumed, conditions close to those observed after 3 min in the preceding experiments where the obtained ratio was 0.49 (Table I). However, the content of residual *o*-quinones was almost zero, whereas it was at a maximum in the first experiment. For 4MC, the ratio O_2/phenol consumed was close to 0.5 and increased to 0.56 when the conditions approached those of the first series of experiments, i.e., a quasi-anaerobiosis, whereas with EC the value decreased from 0.72 to 0.50 when initial EC content increased from 99 to 420 μM (Table II). Thus, the observed stoichiometries are dependent not only on the type of phenol but also on the relative proportions of oxygen and phenol. Secondary

Table II. Oxygen Uptake and Phenol Degradation during Oxidation by Apple PPO at pH 4.5 for Low Initial Concentrations of Phenol (CG, MC, and EC)[a]

	initial concn, μM	phenol consumed, μM	residual *o*-quinone, μM	oxygen consumed, μM	ratio O_2/phenol
chlorogenic acid	95	85	9	71	0.84
	120	105	10	86	0.82
	235	215	10	174	0.81
	290	250	12	195	0.78
	370	344	16	202	0.59
4-methyl-catechol	95	90	57	44	0.50
	194	188	91	90	0.48
	289	280	141	140	0.50
	385	361	197	202	0.56
(−)-epi-catechin	99	80	0	57	0.72
	189	170	0	113	0.66
	260	250	0	151	0.60
	306	300	0	164	0.55
	420	403	0	201	0.50

[a] Conditions: The initial amount of phenol was oxidized by 10 nkat mL^{-1} of apple PPO in a McIlvaine's buffer at pH 4.5, and the measurement was carried out 10 min after the plateau in oxygen consumption had been reached.

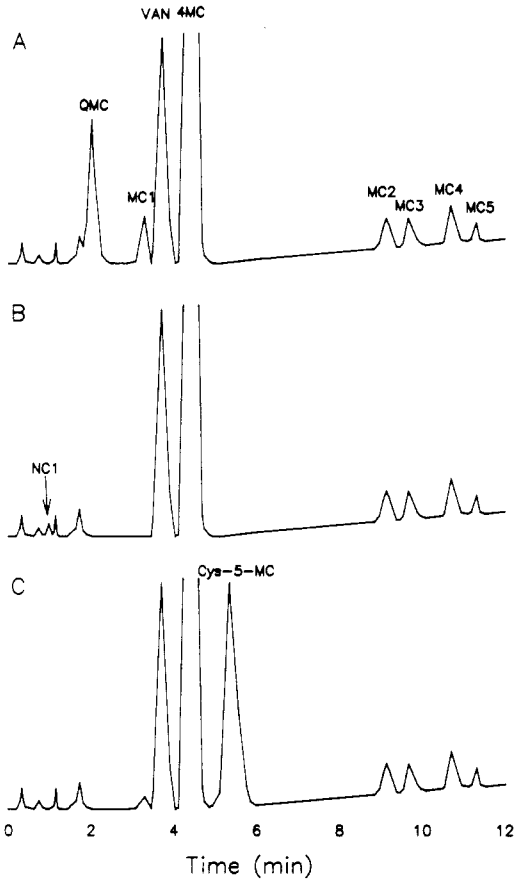

Figure 4. HPLC elution profile of the products formed by the PPO-catalyzed oxidation of 4-methylcatechol. Conditions: 2 mM 4MC was oxidized by 10 nkat mL^{-1} of apple PPO in a McIlvaine's buffer at pH 4.5. The reaction mixture was analyzed after 10 min of oxidation after the stopping solution was added. (A) Stopping solution with NaF alone; (B) stopping solution with NaF supplemented with ascorbic acid; (C) stopping solution with NaF supplemented with cysteine.

reactions involving *o*-quinone, phenol, and oxygen, which differ in intensity owing to variations in the relative reactant concentrations and *o*-quinone stabilities, could probably explain these differences. Therefore, the study of these secondary reactions was undertaken.

Oxidation Products of 4-Methylcatechol. The enzymatic oxidation of 4MC by apple PPO was monitored by HPLC at 280 nm. A typical chromatogram corresponding to 10 min of enzymatic oxidation is represented in Figure 4. Two main peaks (*o*-quinone QMC and MC_1) were eluted before 4MC followed by four less polar minor compounds, MC_2, MC_3, MC_4, and MC_5 (Figure 4A). When the detection was carried out at 390 nm, all six peaks were still observed but the value for MC_1 at 390 nm was much less significant than that of the five other peaks.

To characterize these peaks, the NaF stopping solution supplemented either with ascorbic acid (Figure 4B) or with cysteine (Figure 4C) was used. The results obtained after different enzymatic reaction times for the three stopping solutions are given in Table III. If the stopping solution with NaF alone is considered first (Table IIIA), the decrease in 4MC for the first 5 min was accompanied by an increase of all the other peaks, which was faster for QMC than for the other MC peaks. After 5 min, both QMC and 4MC decreased, whereas all five MC peaks increased. The addition of ascorbic acid to the stopping solution (Table IIIB compared to Table IIIA) resulted in a total loss of QMC and MC_1 and a relative increase of 4MC proportional to the QMC loss, whereas MC_2, MC_3, MC_4, and MC_5 were almost unaffected. Moreover, a new peak, NC_1, eluting at the beginning of the chromatogram (Figure 4B), was formed, the area of which was relative to the MC_1 loss. Lastly, the addition of cysteine led to a total loss of QMC and a partial loss of MC_1, whereas the other peaks were unchanged (Table IIIC compared to Table IIIA). A new peak, Cys-5-MC, eluting after 4MC, was also apparent (Figure 3C). It has been already demonstrated that Cys-5-MC corresponds to the adduct of QMC with cysteine (Richard et al., 1991). These results suggest, first, that MC_1 is a reaction product of QMC (Table IIIA) and, second, that it has a quinone structure (Table IIIB). The partial loss in MC_1 after the cysteine addition could be due to equal HPLC retention times for MC_1 and its adduct with cysteine since no new peak was observed in this case. The quinone structure of MC_1 was confirmed by using an electrochemical detector during the HPLC experiments (Rappaport et al., 1982; Brunmark and Cadenas, 1987) with stopping solutions containing NaF either alone or with ascorbic acid. Table IV shows that only 4MC and NC_1 were detected under mild oxidizing conditions, whereas QMC and MC_1 gave a peak under reducing conditions. The small peak detected for MC_1 under strong oxidizing conditions could be attributed to the presence of a reducing group (i.e., a phenolic group) in the molecule.

Although more stable than *o*-quinones from CG and EC, the 4MC *o*-quinones underwent further reactions (Mayer et al., 1966; Rouet-Mayer et al., 1990). Therefore, enzymatic oxidation of 4MC (2 mM) was stopped with NaF after 5 min and the QMC degradation at 0 °C and pH 4 was monitored during 2 h by HPLC to study the nonenzymatic transformation of QMC. An exponential decrease of QMC with a rate constant of 0.013 min^{-1} was observed together with an increase of MC_1 and 4MC. In the first 15 min, these increases were proportional to the loss in QMC, the molar ratio being close to 2 mol of QMC/mol of 4MC regenerated. After 15 min, these stoichiometries increased, indicating either that QMC was degraded further to other pathways with no formation of MC_1 and regeneration of 4MC or that QMC reacted with 4MC to form polymers (e.g., MC_3).

It is well-known that pH is of great importance in secondary reactions involving *o*-quinones (Dawson and Tarpley, 1963). Thus, during Dopa oxidation by mushroom tyrosinase, two pathways of Dopaquinone evolution have been elucidated: one is hydroxylation by addition of a water molecule, and the other is cyclization to give Dopachrome and polymeric melanins (Garcia-Carmona et al., 1982; Rodriguez-Lopez et al., 1991). According to these authors, the first reaction is favored by acid pH, whereas the latter is the sole reaction observed at greater pH values. The same phenomenon was shown with Dopamine with a pH limit of 6 (Garcia-Moreno et al., 1991). This prompted us to study the pH effect on the nonenzymatic evolution of QMC. This was done between pH 2 and 6 for QMC and 4MC (Figure 5) and between 2 and 4.7 for MC_1, MC_2, MC_3, and MC_4, and MC_5 (Figure 6). First, it was apparent that the more acid the pH, the more rapid the QMC degradation (Figure 5A). Second, a regeneration of 4MC was observed at pH lower than 4 and a degradation appeared at pH higher than 4.7 (Figure 5B). Third, MC_2, MC_4, and MC_5 were steadily increased with no pH effect, but the high acidity favored the MC_1 formation, whereas the reverse was observed for MC_3 (Figure 6), indicating competing pathways for the MC_1 and MC_3 formations.

Table III. Degradation of 4-Methylcatechol (2 mM Initial) and Formation of Its Oxidation Products during the Enzymatic Reaction and Effects of Adding Ascorbic Acid or Cysteine to the NaF Stopping Solution (10 nkat mL^{-1} of Apple PPO) at the Specified Reaction Time[a]

time, min	NC_1	QMC	MC_1	4MC	Cys-5-MC	MC_2	MC_3	MC_4	MC_5
				(A) Stopping Solution NaF Alone					
0				5.64					
2	0	0.462	tr[b]	4.52		0.060	tr	0.023	tr
5	0	0.825	0.061	3.20		0.116	0.023	0.093	0.024
10	0	0.722	0.186	3.10		0.139	0.106	0.186	0.093
15	0	0.660	0.290	2.85		0.278	0.116	0.209	0.139
25	0	0.340	0.480	2.25		0.325	0.174	0.255	0.162
				(B) Stopping Solution NaF + Ascorbic Acid (2 mM)					
0				5.64					
2	0	0	0	5.65		0.055	tr	0.024	tr
5	tr	0	0	5.32		0.104	0.023	0.090	0.023
10	0.035	0	0	4.97		0.128	0.128	0.162	0.104
15	0.068	0	0	4.51		0.255	0.104	0.186	0.151
25	0.116	0	0	3.19		0.290	0.162	0.232	0.174
				(C) Stopping Solution NaF + Cysteine (2 mM)					
0				5.64					
2	0	0	0	4.42	0.74	0.060	tr	0.022	tr
5	0	0	0.022	3.11	1.27	0.116	0.020	0.093	0.021
10	0	0	0.058	3.02	1.13	0.151	0.128	0.197	0.117
15	0	0	0.081	2.77	1.03	0.291	0.115	0.221	0.139
25	0	0	0.161	2.19	0.53	0.313	0.197	0.244	0.186

[a] Results are given in relative area to vanillic acid at 280 nm. [b] tr, traces.

Table IV. Electrochemical Detection of 4-Methylcatechol (4MC), QMC, MC_1, and NC_1 under Different Electrode Potential Conditions[a]

	solution A			solution B	
detection mode	4MC	QMC	MC_1	4MC	NC_1
UV 280 nm[b]	385 500	30 200	157 500	412 500	34 500
electrochemical[c]					
electrode potential, V					
oxidizing conditions					
0.2	tr	0	0	4 942	47 692
0.4	131 394	0	0	140 597	180 983
0.6	689 396	0	0	738 178	209 559
0.8	1 108 550	0	0	1 147 870	266 712
1.0	1 169 885	0	0	1 247 130	285 763
1.2	1 190 440	0	4 146	1 265 350	323 864
1.4	1 220 170	0	5 657	1 306 170	466 746
reducing conditions					
−0.2	0	56 378	218 149		

[a] 4MC (2 mM) was oxidized by 20 nkat mL^{-1} of apple PPO during 5 min, and the reaction was stopped by NaF either alone (solution A) or with ascorbic acid (solution B). [b] Arbitrary units (9050 UV detector from Varian). [c] Arbitrary units (HP 1049A electrochemical detector from Hewlett-Packard).

The spectra obtained for QMC, MC_1, NC_1, and MC_3 during HPLC using a diode array detector are given in Figure 7. The QMC spectrum showing a maximum at 400 nm and a shoulder at 255 nm (Figure 7A) is very close to that given by Dawson and Tarpley (1963) for the *o*-benzoquinone. The comparison between the absorbances at 280 and 400 nm led to an extinction coefficient of 1100 at 400 nm for QMC in the eluant. This value is lower than that obtained by Waite (1976), 1400, and close to those given by Rouet-Mayer et al. (1990), which ranged between 1200 and 1093. Two reasons could explain these differences. First, all of the published values were obtained in buffer solutions between pH 4 and pH 7, and, second, it was assumed either that all of the phenol was converted to stable *o*-quinones (Waite, 1976) or that *o*-quinones were the only entity reacting with ascorbic acid (Rouet-Mayer et al., 1990). Regarding our results, the first assumption seems unlikely, whereas the second one is more reliable, at least in the beginning of the enzymatic reaction. Two absorption maxima observed on the MC_1 spectrum (Figure 7B) showed a strong one at 265 nm and a weaker one at 400 nm. The hydroxyquinone (obtained after oxidation of 1,2,4-trihydroxybenzene) spectrum given by Dawson and Tarpley (1963) showed similar characteristics. The only maximum observed for NC_1 was at 290 nm (Figure 7C), consistent with a bathochromic effect of an additional phenolic group on a 4MC molecule. Moreover, this spectrum is very close to that of 1,2,4-trihydroxybenzene in the same eluant (not shown). Lastly, the MC_3 spectrum showed two maxima at 270 and 420 nm (Figure 7D).

To explain our results, two pathways of QMC degradation could be proposed (Figure 8). A first one corresponds to the hydroxylation of QMC by water (reaction A), leading to the 1,3,4-trihydroxytoluene (NC_1). This compound immediately reacts with another QMC to regenerate 4MC and give the 2-hydroxy-5-methyl-*p*-benzoquinone (MC_1) (reaction C). Such a pathway has been already proposed for Dopa (Rodriguez-Lopez et al., 1991), Dopamine (Garcia-Moreno et al., 1991), and 4MC in the presence of proline (Valero et al., 1988). The second pathway corresponds to the polymerization reaction (reaction D) as proposed by Singleton (1987) that gives products such as MC_2, MC_3, MC_4, and MC_5. The first pathway is mainly observed at acid pH (below pH 4), whereas the second one, although also present at acid pH, is favored by higher pH values (above pH 4). In the presence of active PPO, this scheme is further complicated. Since NC_1 and phenolic dimers have an orthodiphenolic

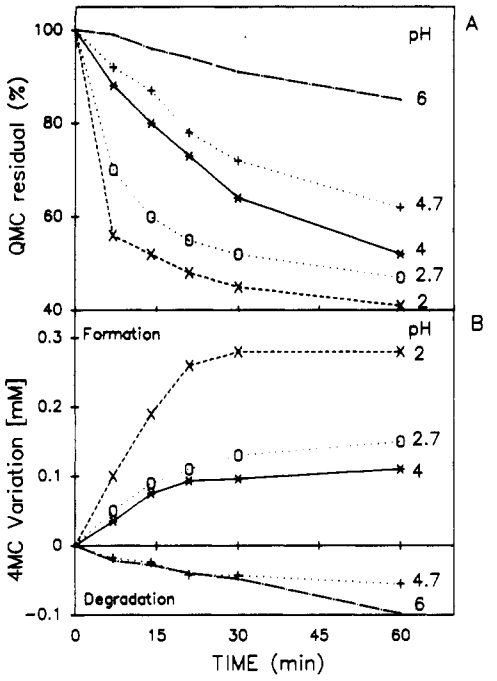

Figure 5. Effect of pH on the nonenzymatic degradation of 4-methylcatechol quinones (A) and 4-methylcatechol (B) at 0 °C. Conditions: 2 mM 4MC was oxidized by 20 nkat mL^{-1} of apple PPO in a McIlvaine's buffer at pH 4.5. After 5 min, the enzymatic reaction was stopped by NaF and the pH adjusted at different values between pH 2 and 6. During storage at 0 °C, aliquots of the solutions were periodically analyzed by HPLC as described under Materials and Methods.

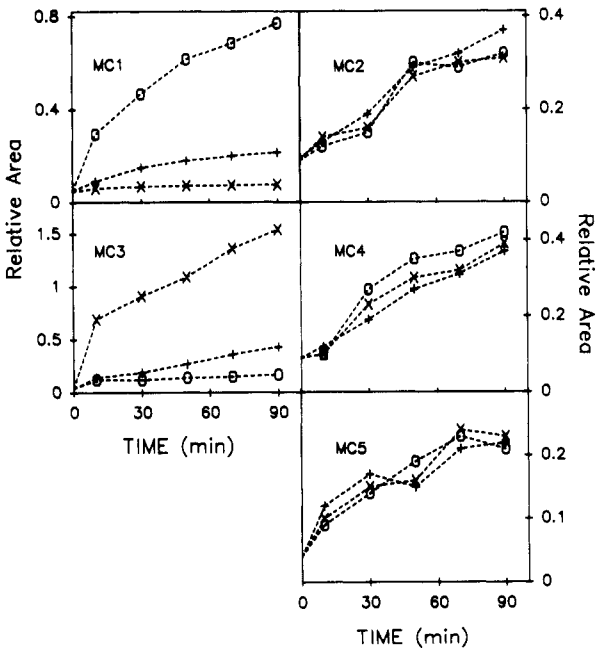

Figure 6. Effect of pH on the nonenzymatic formation of MC_1, MC_2, MC_3, MC_4, and MC_5 (oxidation products of 4-methylcatechol). Conditions: see Figure 5. (×) pH 4.7; (+) pH 4; (○) pH 2.

structure, their direct oxidation by PPO (reaction B) cannot be ruled out. Thus, NC_1 is more than likely a substrate for PPO. It has been shown by Palumbo et al. (1991) that 1,2,4-trihydroxybenzene was a substrate for mushroom tyrosinase, and we have observed that it was also a good substrate, almost equivalent to 4MC, for apple PPO (unpublished results). Likewise, as postulated (Singleton, 1987), dimers (or larger oligomers) could be good substrates for PPO (reaction E), although Cheynier

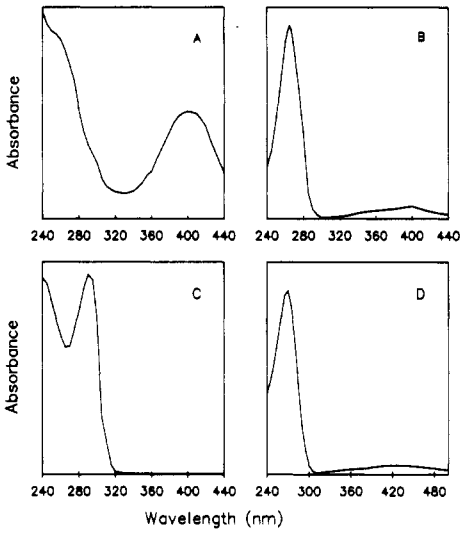

Figure 7. Spectra of the oxidation products of 4-methylcatechol. (A) *o*-Quinone of 4-methylcatechol; (B) MC_1; (C) NC_1; (D) MC_3. Conditions: 2 mM 4MC was oxidized by 20 nkat mL^{-1} of apple PPO in a McIlvaine's buffer at pH 4.5. After 5 min, the enzymatic reaction was stopped by NaF either alone (A, B, and D) or with ascorbic acid (C). The resulting solution was immediately injected (A–C) or after 2 h at 0 °C and pH 5 (D). The spectra were obtained using a Waters 990 diode array detector.

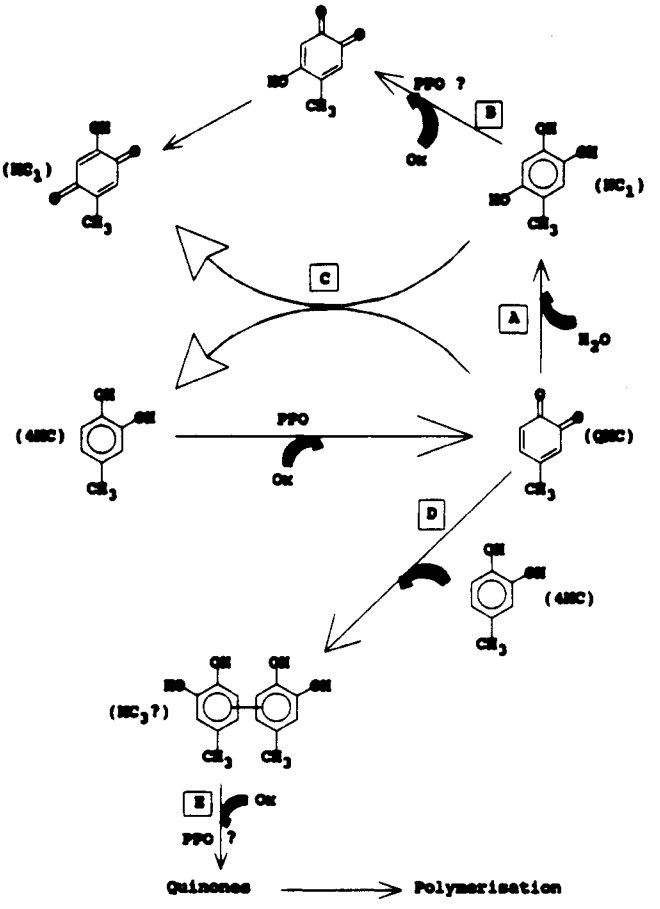

Figure 8. Proposed pathways for the formation of oxidation products from 4-methylcatechol. (Pathways labeled with PPO are for enzymatic reactions and others are for nonenzymatic reactions.)

and Ricardo da Silva (1991) indicated that procyanidin dimers and trimers were not substrates for the grape PPO. By this scheme, the stoichiometry of 2 mol of QMC degraded for 1 mol of 4MC regenerated is explained. The NC_1 and MC_1 spectra and their HPLC retention times are

consistent with a triphenol and its *p*-quinone. The exact structure of MC_3 remains to be elucidated. Nevertheless, due to the lack of ascorbic effect on the MC_3 peak, it probably has a dimeric (or more) hydroquinone structure.

The same experiments were carried out with CG. After 15 min of enzymatic oxidation, the HPLC chromatogram showed the presence of four components more polar than CG (including its *o*-quinone or chlorogenoquinone) and at least eight condensed products eluted after the original phenol, during the gradient (not shown). A similar behavior of QMC was also found for the nonenzymatic degradation of chlorogenoquinone. Thus, at pH 4 and 0 °C, the pseudo-first-order rate constant of degradation was found to be 0.035 min^{-1}, whereas the values 0.049 and 0.024 min^{-1} were observed at pH 3 and 5, respectively. Therefore, this confirms that, first, the chlorogenoquinones were less stable than QMC and, second, that again the stability decreased with decreasing pH. The addition of ascorbic acid or cysteine to the stopping solution permitted again the characterization of a triphenol (result of the hydroxylation of chlorogenoquinone by water) and its *p*-quinone. However, in comparison with 4MC, their formations were favored by acid pH and the polymerization pathway seemed to be more dominant. Moreover, the triphenol was always present in appreciable amounts during the enzymatic oxidation, meaning either that it was not a substrate for apple PPO (or at least a poor substrate) or that its reaction with chlorogenoquinone was slow.

The chromatogram of enzymatically oxidized EC during 10 min showed three minor peaks before the phenol and a great number (more than 10) of condensed products eluted during the gradient. There were almost no *o*-quinones since adding ascorbic acid or cysteine to the stopping solution did not modify the chromatogram. It was only in the very beginning of the enzymatic reaction that a small peak, more polar than EC, was lost upon addition of ascorbic acid, leading to a small increase of the phenol peak, or of cysteine, leading to the formation of two new peaks corresponding to the adducts of *o*-quinone with cysteine (Richard et al., 1991). Therefore, the great instability of *o*-quinones of EC was confirmed and made impossible for us to study its nonenzymatic degradation. Nevertheless, the polymerization pathway is more than likely the major one as illustrated by the great number of condensed products (Oszmianski and Lee, 1990; Rouet-Mayer et al., 1990).

Conclusion. We observed that, in the time range studied here, the oxygen consumption during enzymatic oxidation of phenols was only due to the enzyme action since, when NaF was added to the reaction medium, oxygen uptake was almost nil. Therefore, the obtained stoichiometry corresponded to reactions in which the PPO actively participated, probably through the formation of active oxygen species. Nevertheless, the different degradation pathways of *o*-quinone (Figure 8) can lead to different stoichiometries of oxygen to phenol consumed. Thus, the hydroxylation of *o*-quinones (reaction A) gives a 1:1 stoichiometry if either the triphenol is a substrate for the enzyme (reaction B) or its reaction with *o*-quinone (reaction C) is fast to regenerate the original phenol and give the *p*-quinone. The polymerization pathway (reaction D) gives a 1:4 stoichiometry if the dimers are not substrates of the enzyme. If they are, the value can vary from 2:4 to 3:4 accordingly as one or two ortho-diphenolic groups of the dimer are enzymatically oxidized into quinones (reaction E). Moreover, each mole of *o*-quinone which nonenzymatically condenses with a hydroquinone (a dimer or a larger oligomer) increases the observed stoichiometry since one oxygen atom was consumed for its formation from the phenol. Obviously, the value of stoichiometry approaches 1:2 as the size of the oligomer increases. Therefore, the observed stoichiometry is dependent on many factors. The nature of the phenol oxidized is determinant. Accordingly, it generates *o*-quinones with large differences in the relative importance of their degradation pathways. The secondary products formed may or may not be good substrates for the enzyme and may exhibit differences in their reactivities with primary *o*-quinones. The chemical conditions of the reaction can affect the stoichiometry by modifying the balance among the different pathways. Lastly, the ratio of enzyme to substrate concentration is also important since it greatly influences the relative rates among enzymatic and nonenzymatic reactions.

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