Bioorganic & Medicinal Chemistry 20 (2012) 4364–4370

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

The influence of hydroquinone on tyrosinase kinetics

Michael R. L. Stratford [a], Christopher A. Ramsden [b,*], Patrick A. Riley [c]

[a] Gray Institute for Radiation Oncology & Biology, Department of Oncology, University of Oxford, Roosevelt Drive, Oxford OX3 7DQ, UK
[b] Lennard-Jones Laboratories, School of Physical and Geographical Sciences, Keele University, Staffordshire ST5 5BG, UK
[c] Totteridge Institute for Advanced Studies, The Grange, Grange Avenue, London N20 8AB, UK

ARTICLE INFO

Article history:
Received 4 April 2012
Revised 14 May 2012
Accepted 16 May 2012
Available online 24 May 2012

Keywords:
Tyrosinase
Hydroquinone
Quinone
Quintox mechanism
Oximetry

ABSTRACT

In vitro studies, using combined spectrophotometry and oximetry together with hplc/ms examination of the products of tyrosinase action demonstrate that hydroquinone is not a primary substrate for the enzyme but is vicariously oxidised by a redox exchange mechanism in the presence of either catechol, L-3,4-dihydroxyphenylalanine or 4-ethylphenol. Secondary addition products formed in the presence of hydroquinone are shown to stimulate, rather than inhibit, the kinetics of substrate oxidation.

1. Introduction

This paper reports our studies of the influence of hydroquinone on tyrosinase oxidation and discusses the significance of our observation that, in contrast to catechol, hydroquinone is neither a substrate nor a suicide inactivator of tyrosinase.

The generation of *ortho*-quinones has several important functions in living organisms[1] and is the main pathway to the generation of melanin, a pigment that is the predominant component of surface colouration of vertebrates.[2] The crucial enzyme involved in the synthesis of melanin is tyrosinase.[3,4]

Hydroquinone **3c** has been widely used for skin depigmentation but its use as a topical agent is not without risk. Its use is banned in the European Union and in 2006 the FDA revoked its previous approval for use in the USA. In the past it has sometimes been assumed that hydroquinone causes depigmentation by inhibiting tyrosinase and thus interfering with melanin formation as suggested in the literature[5,6] although the mechanism is not clear.[7] Catechols irreversibly inactivate tyrosinase: we have studied this inactivation in some detail and have proposed a mechanism (the Quintox mechanism) in which the bound substrate (a catechol) deprotonates leading to reductive elimination of Cu(0) from the active site.[8,9] In particular, tyrosinase (EC 1.14.18.1) contains two copper atoms at its active site and binds dioxygen to give oxy-tyrosinase (Scheme 1). This form of the enzyme catalyses *ortho*-quinone formation by oxidation of either phenols (monooxygenase activity) or catechols (oxidase activity). Tyrosinase is unique in having these two discrete modes of oxidation with subtly different mechanisms. Catechols are normally transformed by oxidase activity but they can also act as monooxygenase substrates of oxy-tyrosinase leading to reductive elimination of Cu(0) and inactivation (Scheme 1). Theoretically, hydroquinone might also be expected to lead to inactivation of tyrosinase by a similar mechanism but only if it is a monooxygenase substrate. In fact, and in contrast to catechol, we confirm that hydroquinone is neither a tyrosinase substrate nor a suicide inactivator.

2. Materials and methods

2.1. Materials

2.1.1. Oximetry

Incubations were made in a volume of 3.65 mL water or phosphate buffer at pH 6.3. The incubation mixtures for mass spectrometry used smaller total volumes and in most cases employed larger comparative amounts of enzyme as detailed below.

The simultaneous oximetry and spectrophotometry experiments were made using the method previously described.[10] Experiments were conducted at 24 °C using an apparatus consisting of a quartz cuvette (3.65 mL capacity) adapted to hold a Clark-type oxygen electrode, as described previously.[10] Spectrophotometric data were recorded using a Hewlett-Packard diode-array spectrophotometer (Model 8452A) and the oxygen uptake monitored using a Yellow Springs Instruments (Model 5300) polarimeter. Oxygen electrode tracings were converted to electronic form using

* Corresponding author.
E-mail address: c.a.ramsden@chem.keele.ac.uk (C.A. Ramsden).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.05.041

ScanIt (version 1.0) software (© van Baten & Baur, 2002). Kinetic analysis was conducted using the Origin software (version 7, OriginLab Corp., Northampton, MA, USA). Simulations were performed using an in-house computer model. Spectral changes were examined using the kinetic mode of the UV–vis Chemstation A0801(66) software (Agilent Technologies, Hannover, Germany).

The oxygen uptake was found to fit first-order kinetics from which the total oxygen utilisation (U_t) and the inactivation rate constant (k_2) were obtained in accordance with the equation:

$$U_t = \frac{k_1}{k_2} E_0(1 - \exp(-k_2 t))$$

where E_0 = the initial enzyme concentration and k_1 = the catecholase rate constant, as described by Land and co-workers.[8] For large t the solution is $U_t = (k_1/k_2)\ E_0$. Analysis of the oximetry data for first-order exponential decay (using Origin software) gave values for U_t and k_2. Values of k_1 were estimated from the best fit of the first-order equation to the oximetry data using an in-house computer model.

2.1.2. Hplc/mass spectrometry

Incubations were made at room temperature using 2 mL specimen tubes containing millimolar substrate(s) in deionised water to which 50 units of tyrosinase were added. Control incubations were made without tyrosinase.

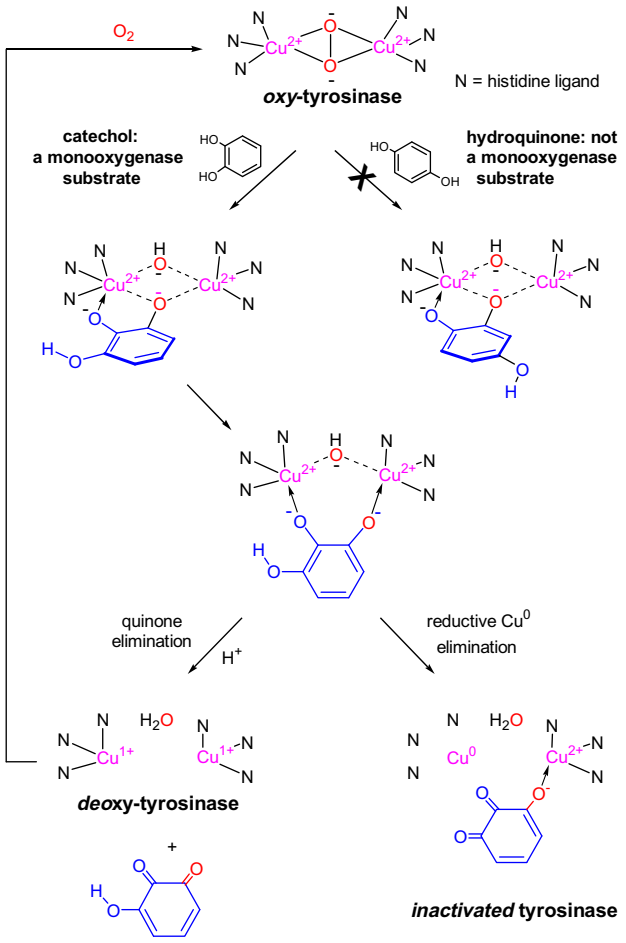
Separations were carried out using a Waters 2695 separations module (Waters, Elstree, UK) with an Atlantis dC18, 150 × 3.0 mm hplc column (Waters) maintained at 35 °C. The hplc eluents were: A, 10 mM formic acid; B, acetonitrile, with gradients of 5–70% B (catechol), 2–70% B (DOPA) and 20–85% B (4-ethylphenol) over 5 min. The flow rate was 0.5 mL min^{-1}, which was split after diode-array detection to give a flow rate of ~200 μl min^{-1} to the mass spectrometer. The eluent was monitored using a Waters 2996 diode-array detector (215–450 nm, 2.4 nm resolution) and a Waters Micromass ZQ mass detector (mass range 115–500 Da) in negative ion mode using electrospray ionisation, and using Waters Empower 2 software. The mass detector employed the following conditions: capillary voltage, 2.1 kV; cone voltage, 20 V; source temperature, 120 °C; desolvation temperature, 425 °C; desolvation gas flow, 450 L h^{-1}; cone gas flow, 100 L h^{-1}.

It should be noted that under these conditions all phenolic derivatives showed ions at M−1 whereas *ortho*-quinone derivatives lacking any acidic OH substituents showed ions at M+1, where M is molecular weight.

3. Results

Under the conditions employed in these experiments the autoxidation of hydroquinone was negligible and we confirmed that hydroquinone is not a primary substrate for tyrosinase, there being: (i) no spectrometric evidence of loss of substrate or product formation; (ii) no oxygen utilisation; and (iii) no evidence of products by hplc/mass spectrometry. However, in the presence of tyrosinase substrates we detected the formation of 1,4-benzoquinone and we compared the effect of equimolar hydroquinone on the tyrosinase-catalysed oxidation of three substrates, namely catechol, L-3,4-dihydroxyphenylalanine (dopa), and 4-ethylphenol. In all cases 1,4-benzoquinone was generated as shown by the strong absorbance at 246 nm in the final spectra of the products in comparison with the controls incubated in the absence of hydroquinone (Fig. 1).

The formation of 1,4-benzoquinone was at the expense of the initial *ortho*-quinone product of the primary substrates as shown by the diminution of absorbance at 400 nm (and at 304 nm in



Scheme 1. Catechol is a monooxygenase tyrosinase substrate and hydroquinone is not.

the case of dopa) which we interpret as evidence that 1,4-benzoquinone is formed by redox exchange of hydroquinone with the enzyme-generated *ortho*-quinone.

Oxygen uptake (Fig. 2) showed increased oxygen utilisation in all cases compared to the controls without hydroquinone. In the case of catechol this was partly accounted for by a decrease in the inactivation rate (k_2) derived from the first-order kinetic parameters and partly due to an increase in oxidation rate (k_1). In the case of dopa, as the formation of 1,4-benzoquinone decreased there was evidence of increased formation of dihydroxyindole **4b**, although indolequinone **4c** formation (λ = 473 nm) was little affected (Fig. 1).

In the case of 4-ethylphenol no inhibitory effect on the kinetics of oxygen utilisation in the presence of equimolar hydroquinone was observed and the formation of 1,4-benzoquinone was associated with a shortening of the 'lag-period'.[9] The half life of the lag-period was 7.55 ± 0.22 min in the presence of hydroquinone in comparison with 8.43 ± 0.25 min for the control.

Calculation of the effect of hydroquinone on the oxygen stoichiometry showed that, in the case of catecholic substrates (catechol and dopa), the control value was close to two moles product per mole oxygen and 1:1 for the monohydric phenol substrate, 4-ethylphenol, as expected (Fig. 3, open circles).

In the case of catechol oxidation in the presence of hydroquinone there was a marked discrepancy after about 600 nmol product had formed with increased oxygen utilisation without obvious formation of product, and with a final 1:1 stoichiometry, implying the generation of a monohydric phenol substrate. This was the

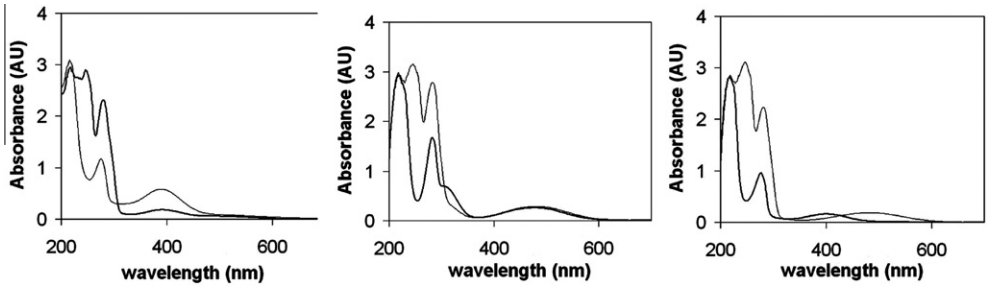


Figure 1. Final spectra of tyrosinase oxidation products from catechol, dopa, and 4-ethylphenol with (thin line) and without (heavy line) hydroquinone.

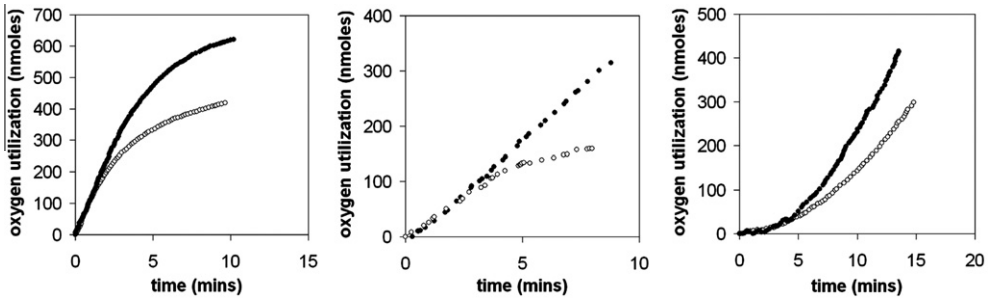


Figure 2. Comparative oxygen utilisation by catechol, dopa and 4-ethylphenol with (●) and without (○) equimolar hydroquinone.

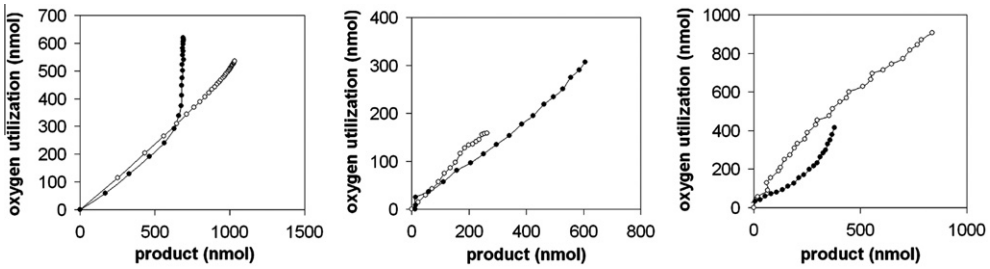


Figure 3. Comparative stoichiometry for catechol, dopa and 4-ethylphenol oxidation by tyrosinase with (●) and without (○) equimolar hydroquinone.

inverse of the stoichiometry of 4-ethylphenol in the presence of hydroquinone which began with a 2:1 slope but veered towards the 1:1 ratio at a later stage of the oxidation.

The hplc/mass spectrometry data relating to these incubations are summarised below. The structures identified in Table 1 are consistent with the products of the reactions proposed in Schemes 2–7.

The catechol oxidation products detected after 22 min incubation were identified as the compounds illustrated in Scheme 2 and the properties are summarised in Section 2 of Table 1. Not all the postulated intermediates were identified and we assume that in these cases the rate of subsequent steps in the sequence of reactions is rapid in comparison with the rate of formation. Consequently the steady-state concentration of these intermediates is too low for them to be identified in our system.

In the co-presence of hydroquinone **3c** the proposed reactions are summarised in Scheme 3. The 1,4-benzoquinone formed by redox exchange with *ortho*-quinone was not detected in the products separated after 33 min incubation and appears to react rapidly with water to form a hydroxylated derivative absorbing at about 266 nm, some of which is oxidised to the corresponding quinone **3a** (λ_{max} 378 nm). The remaining products appear to be formed by the addition of hydroquinone to *ortho*-quinone giving rise to a tyrosinase substrate **3b** which undergoes further oxidation to the *ortho*-quinone **3d** and cyclisation leading to the dibenzodioxin derivative **3e**.

The observed products of dopa **4a** oxidation after 72 min incubation consisted of only 5,6-dihydroxyindole **4b** and the corresponding quinone **4c** (see Scheme 4 and Section 4 of Table 1).

As summarised in Scheme 5, the equivalent incubation (33 min) in the presence of hydroquinone revealed several compounds derived from the vicarious oxidation of hydroquinone including the product **5a** formed by addition of hydroquinone to 1,4-benzoquinone, from which further products (**5b**–**e**) are formed by oxidation and cyclisation. In some cases the products eluted from hplc very closely together but could be separated by splitting the peaks, for example **3a** and **5c** at RT 5.0 and **5a** and **5b** at RT 5.2 min (Table 1, Section 5).

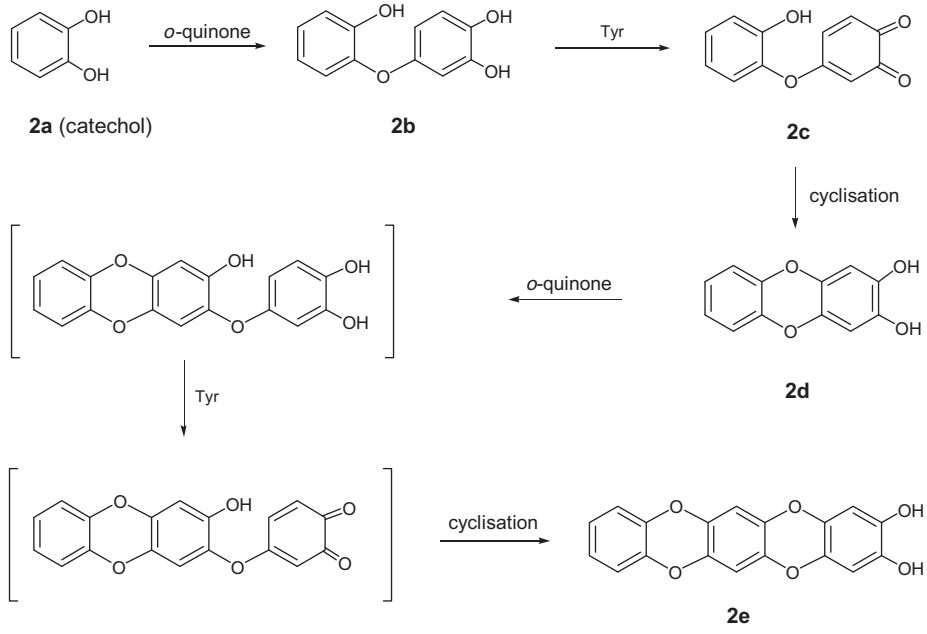
Scheme 6 shows that 4-ethylphenol **6a** is oxidised to the corresponding *ortho*-quinone **6b** which apparently undergoes rapid water addition leading either to the redox generation of 4-ethylcatechol **6c** or to an addition product with 4-ethyl-*ortho*-quinone **6b** which, following further oxidation, forms the cyclised product **6e**. Products are also formed by the addition of the catechol **6c** to 4-ethylquinone **6b** culminating in the formation of the derivative **6d**.

Scheme 7 shows that with hydroquinone present a set of reactions similar to those described in Scheme 3 yield the hydroxylated 1,4-benzoquinone **3a** and the addition product **7c**. A second set of products (**7a** and **7b**) are derived from the addition of hydroquinone to 4-ethylquinone **6b**.

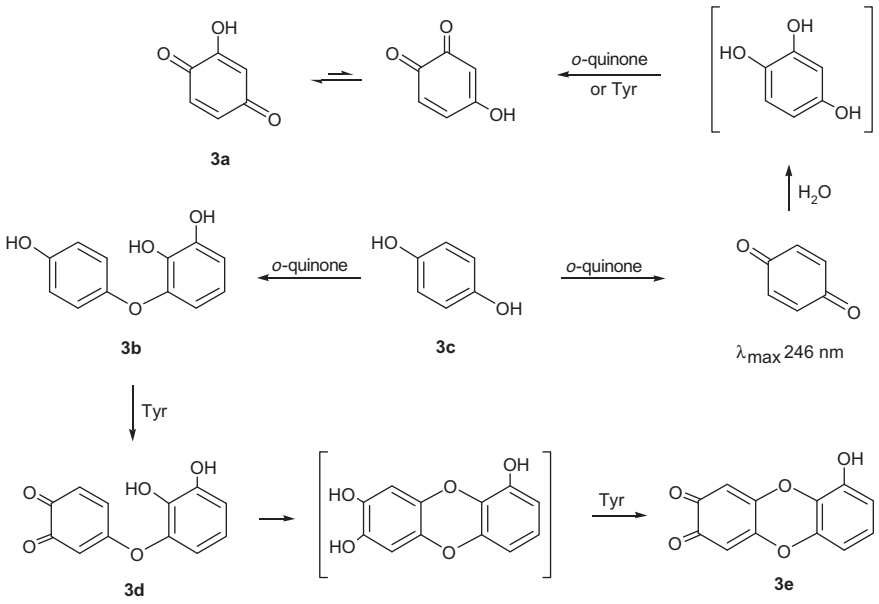
Table 1
Products of tyrosinase-catalysed oxidation with and without addition of hydroquinone

Substrate(s)	RT[a]	*m*/*z*	λ_{max}	Structure[b]
1. Hydroquinone	4.1	109 (M−1)	289	**3c** (Hydroquinone) no oxidation products
2. Catechol	4.2	109 (M+1)	388	*ortho*-Quinone
	5.3	109 (M−1)	275	**2a** (Catechol)
	6.0	217 (M+1)	~360	**2c**
	6.4	215 (M−1)	290	**2d**
	6.5	217 (M−1)	291	**2b**
	8.0	321 (M−1)	279	**2e**
3. Catechol + hydroquinone	4.1	109 (M−1)	289	**3c** (Hydroquinone)
	4.7	123 (M−1)	266/378	**3a**
	5.3	109 (M−1)	275	**2a** (Catechol)
	5.6	231 (M−1)	465	**3d**
	6.5	217 (M−1)	252	**3b**
	6.7	229 (M−1)	506	**3e**
4. Dopa	2.4	196 (M−1)	280	**4a** (Dopa)
	3.7	148 (M+1)	301/473	**4c**
	5.4	148 (M−1)	244/300	**4b**
5. Dopa + hydroquinone	2.5	196 (M−1)	280	**4a** (Dopa)
	3.6	148 (M+1)	296/480	**4c**
	4.5	109 (M−1)	290	**3c** (Hydroquinone)
	4.6	247 (M−1)	290	**5d**
	5.0	123/231 (M−1)	266/378	**3a** & **5c**
	5.2	217/245 (M−1)	301/464	**5a** & **5b**
	5.5	148 (M−1)	244/300	**4b**
	5.7	229 (M−1)	250/472	**5e**
6. 4-Ethylphenol	5.0	137 (M+1)	398	**6b**
	5.5	137 (M−1)	282	**6c**
	5.9	287 (M−1)	266	**6e**
	6.4	121 (M−1)	277	**6a** (4-Ethylphenol)
	7.4	407 (M−1)	270	**6d**
7. 4-Ethylphenol + hydroquinone	2.4	109/123 (M−1)	290	**3c** (Hydroquinone)
	2.6	123 (M−1)	367	**3a**
	3.4	229 (M−1)	245	**7c**
	4.6	259 (M−1)	432	**7a**
	5.4	137 (M−1)	282	**6c**
	6.3	121 (M−1)	277	**6a** (4-Ethylphenol)
	7.8	261 (M−1)	—[c]	**7b**

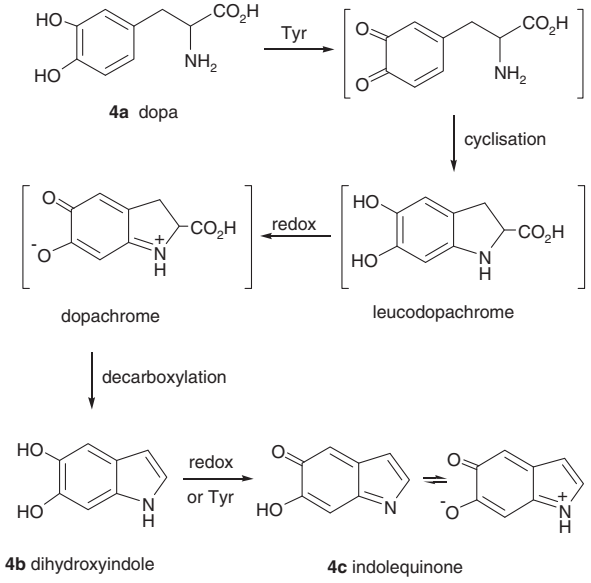
[a] Retention time in minutes.
[b] Product numbers correspond to the structures shown in Schemes 2–7.
[c] The sensitivity of the detector was insufficiently accurate to ascribe a peak absorbance.



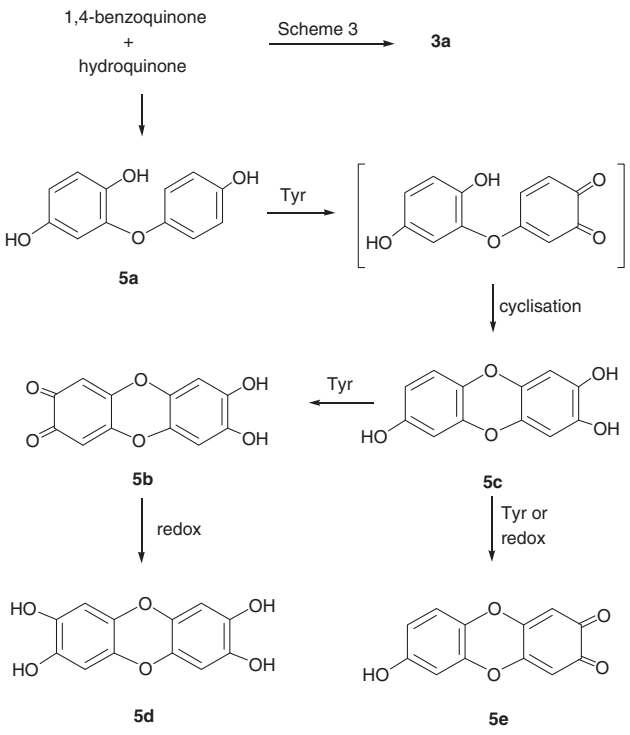
Scheme 2.



Scheme 3.



Scheme 4.



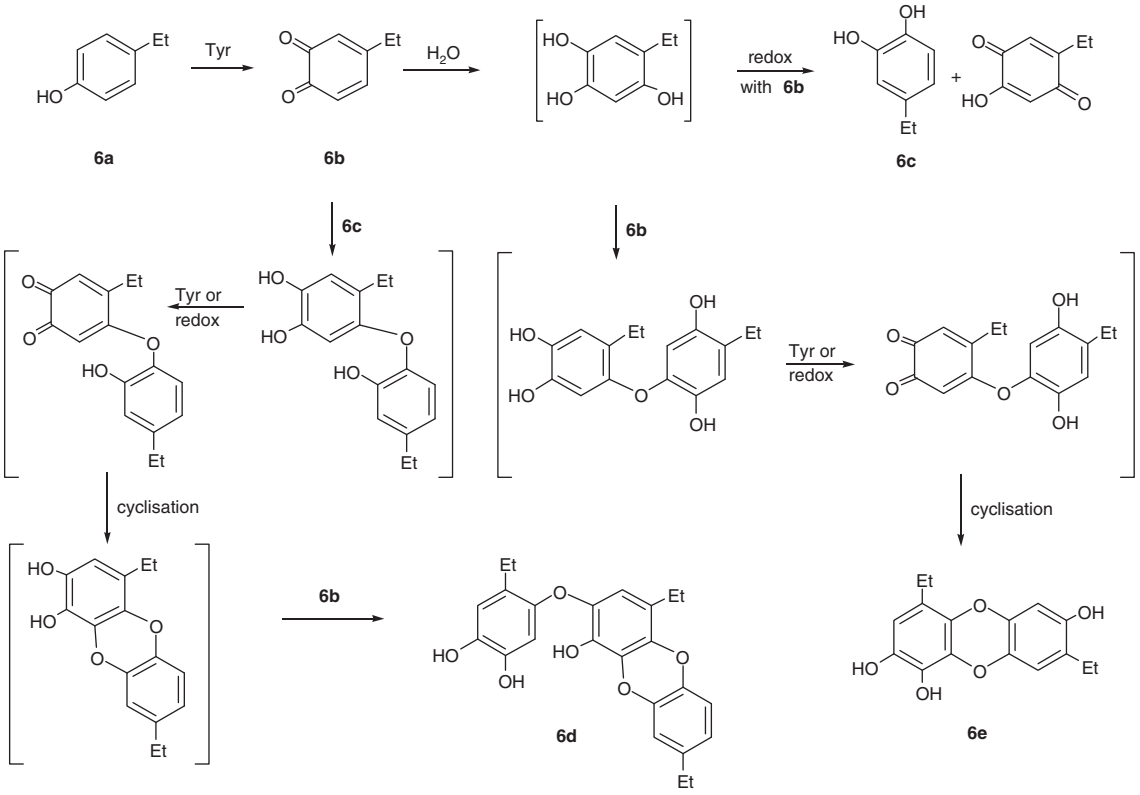
Scheme 5.

4. Discussion

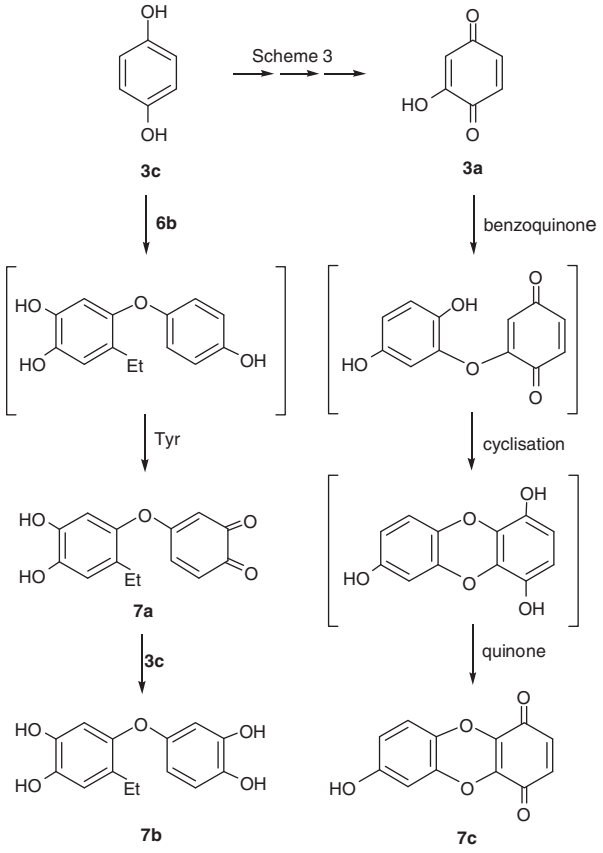
Our data confirm that hydroquinone is not a primary substrate for tyrosinase and is in agreement with earlier studies.[12–14] We found no evidence of monooxygenase action on hydroquinone, even after prolonged incubation with tyrosinase. We conclude that the presence of the *para*-hydroxy group prevents binding to the tyrosinase active site. This positional effect appears to be limited to the *para*-hydroxy function since other 4-substituted phenols with closely-related 4-substituents, such as alkyl, alkoxy and halo, are monooxygenase substrates exhibiting characteristic lag-period kinetics.[11,15] The lag-period kinetics result from the recruitment of met-tyrosinase[16] and may involve the formation of an activating catechol by the generation of an addition compound.

However, hydroquinone is oxidised in the presence of tyrosinase substrates, and we have demonstrated this vicarious oxidation in the presence of both phenolic and catecholic primary substrates.

The mechanism of the secondary oxidation of hydroquinone appears to be by a redox exchange between one or more of the *ortho*-quinone products (generated by tyrosinase-catalysed oxidation of a primary substrate) and hydroquinone to yield 1,4-benzoquinone. The redox potentials favour such a reaction[4,17] and comparison of the spectrophotometric data shows, in the presence of hydroquinone, the formation of 1,4-benzoquinone and a diminution of *ortho*-quinone formation (see Fig. 1).



Scheme 6.



Scheme 7.

Mass spectrometry confirmed the utilisation of hydroquinone and the formation of a number of oxidation products. Under the conditions of our experiments we detected a rapid hydroxylation of 1,4-benzoquinone due to water addition, possibly involving reactions with semiquinones formed by comproportionation of 1,4-benzoquinone and hydroquinone.[18] This pathway is consistent with the observed oxygen stoichiometry.

Examination of the products of tyrosinase-catalysed oxidation demonstrated the formation of adducts which we ascribe to reductive addition of hydroxy compounds to *ortho*-quinones. These products were demonstrated in the absence of hydroquinone and were mainly formed by reaction with residual substrate in the reaction mixture. In the co-presence of hydroquinone a range of addition products were detected and structures are shown in the accompanying Schemes.

An interesting consequence of the formation of such addition products is the generation of compounds that are potential substrates for tyrosinase, and we have evidence that they are formed and oxidised. In the case of catecholic compounds this oxidation could take place indirectly by redox exchange but this cannot be the case for phenolic adducts (e.g., structures **3b**, **5a** and **5c** in the reaction Schemes) for which we have evidence of oxidation. Such compounds can act as competitive monooxygenase substrates for tyrosinase and thus inhibit the auto-inactivation mechanism recently proposed.[8,9,19–21] Such a process is consistent with the kinetic data which show a diminished rate constant of inactivation.

5. Conclusions

We have found that under the in vitro conditions of our experiments, rather than inhibiting the enzyme, hydroquinone stimulates tyrosinase activity by the indirect formation of adducts that are able to act as secondary substrates. Thus, catecholic adducts diminish the lag-period of phenol oxidation, and phenolic adducts

interfere with the mechanism of auto-inactivation. The transformations summarised in Schemes 2–7 are novel and consistent with the known modes of reaction of *ortho*-quinones.[22]

Our investigations are in agreement with the conclusions of Penney and coworkers[23] and the data reviewed by Taieb and coworkers,[24] which conclude that the depigmenting action of hydroquinone is exerted by a cytotoxic mechanism and does not reflect any inhibitory effect on tyrosinase.

Acknowledgment

P.A.R. thanks the Director of the Gray Institute for access to laboratory facilities.

References and notes

1. Riley, P. A. The Evolution of Melanogenesis. In *Melanin; its Role in Human Photoprotection*; Zeise, L., Chedekel, M. R., Fitzpatrick, T. B., Eds.; Valdemar Publishers: Overland Park, 1995; p 11.
2. Prota, G. *Melanins and Melanogenesis*; Academic Press: San Diego, 1992.
3. Chang, T.-S. *Int. J. Mol. Sci.* **2009**, *10*, 2440.
4. Riley, P. A.; Ramsden, C. A.; Land, E. J. In *Melanins and Melanosomes: Biosynthesis, Biogenesis, Physiological, and Pathological Functions*; Borovansky, J., Riley, P. A., Eds.; Wiley-VCH Verlag GmbH & Co.: Weinheim, 2011; p 63.
5. Lerner, A. B.; Fitzpatrick, T. B. *Physiol. Rev.* **1950**, *30*, 91.
6. Palumbo, A.; d'Ischia, M.; Misuraca, G.; Prota, G. *Biochim. Biophys. Acta* **1991**, *1073*, 85.
7. Denton, C. R.; Lerner, A. B.; Fitzpatrick, T. B. *J. Invest. Dermatol.* **1952**, *18*, 119.
8. Land, E. J.; Ramsden, C. A.; Riley, P. A. *Tohoku J. Exp. Med.* **2007**, *212*, 341.
9. Ramsden, C. A.; Riley, P. A. *Arkivoc* **2010**, *i*, 260.
10. Cooksey, C. J.; Garratt, P. J.; Land, E. J.; Pavel, S.; Ramsden, C. A.; Riley, P. A.; Smit, N. P. M. *J. Biol. Chem.* **1997**, *272*, 26226.
11. Naish-Byfield, S.; Riley, P. A. *Pigment Cell Res.* **1998**, *11*, 127.
12. Adams, M. H.; Nelson, J. M. *J. Am. Chem. Soc.* **1938**, *60*, 2474.
13. Graubard, M. *Enzymologia* **1939**, *5*, 332.
14. Gregg, D. C.; Nelson, J. M. *J. Am. Chem. Soc.* **1940**, *62*, 2510.
15. Riley, P. A. *J. Theor. Biol.* **2000**, *203*, 1.
16. Riley, P. A. *Cell. Mol. Biol.* **1999**, *45*, 951.
17. Berger, St.; Rieker, A. In *The Chemistry of the Quinonoid Compounds*; Patai, S., (Ed.); John Wiley & Sons: London, 1974; p 163.
18. Eigen, M.; Matthies, P. *Chem. Ber.* **1961**, *94*, 3309.
19. Land, E. J.; Ramsden, C. A.; Riley, P. A.; Stratford, M. R. L. *Tohoku J. Exp. Med.* **2008**, *216*, 231.
20. Ramsden, C. A.; Stratford, M. R. L.; Riley, P. A. *Org. Biomol. Chem.* **2009**, *7*, 3388.
21. Ramsden, C. A.; Riley, P. A. *Arkivoc* **2010**, *x*, 248.
22. Ramsden, C. A. *Adv. Heterocycl. Chem.* **2010**, *100*, 1.
23. Penney, K. B.; Smith, C. J.; Allen, J. C. *J. Invest. Dermatol.* **1984**, *82*, 308.
24. Taïeb, A.; Cario-André, M.; Briganti, S.; Picardo, M. In *Melanins and Melanosomes: Biosynthesis, Biogenesis, Physiological, and Pathological Functions*; Borovansky, J., Riley, P. A., Eds.; Wiley-VCH Verlag GmbH & Co.: Weinheim, 2011; p 119.