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Investigation of the anomalous action of 5-hydroxyresorcinol on tyrosinase.

by

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Resorcinols have been much used as depigmenting agents in dermatology (Shimizu et al., 2000; Solano et al., 2006; Chang, 2009) but their mode of action has until recently been obscure. A series of studies of suicide inactivation of tyrosinase (Land et al., 2007; Land et al., 2008; Ramsden et al., 2009; Ramsden and Riley, 2010a; Ramsden and Riley, 2010b) demonstrated that the process requires monooxygenase (cresolase) activity and indicated the manner by which resorcinols act as inactivators of tyrosinase by altering the oxidation state of an active site copper atom (Ramsden and Riley, 2014a). A study of this process employing a series of substituted resorcinols gave results consistent with the proposed inactivation mechanism (Stratford et al., 2013). However, an unexpected exception was found in the case of 5-hydroxyresorcinol (5-HR) which, in contrast to other 5-substituted resorcinols, not only failed to exhibit an inactivating effect but, on the contrary, appeared to be an activator of tyrosinase. We report here the results of our investigations of this strikingly anomalous behaviour.

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In brief, we have shown that 5-HR (***1***) is not a substrate for tyrosinase, showing no oxygen utilisation, no alteration in spectral absorbance, no evidence of any product detectable by HPLC/MS, and no evidence of any diminution in tyrosinase activity on subsequent testing with known substrates such as 4-ethylphenol (EtP, ***2***) and 4-methoxyphenol (MOP). We have excluded the failure to act as a primary substrate due to a mechanism similar to that for hydroquinone, which involves a 1,2-benzoquinone to 1,4-benzoquinone tautomerism (Ramsden and Riley, 2014b). We have also demonstrated that 5-hydroxyresorcinol is not a secondary tyrosinase substrate since no oxidation of 5-hydroxyresorcinol takes place after tyrosinase activation by prior oxidation of 4-ethylphenol.

Experiments in which 5-HR-treated tyrosinase was exposed to EtP (***2***) showed the generation of a product absorbing at 450 nm not observed in the absence of 5-HR, associated with increased oxidation rate, diminished lag period and increased total oxygen utilisation. Similar results were obtained with MOP (see Table 1) implying that the enzyme is activated by a catechol. Since the formation of a catechol cannot be by a direct redox reaction with 5-HR, we propose that the 450 nm absorbing product is a compound derived from a 1,2-benzoquinone and 5-HR. We were able to confirm by pulse radiolysis using 4-ethylcatechol and 4-methoxycatechol that the 450 nm-absorbing product in the presence of 5-HR was due to the rapid formation of an addition compound (rate constants = 0.08 sec^{-1} and 0.13 sec^{-1}, respectively). This reaction did not occur under acidic conditions (pH 5.8) indicating that the reaction between 1,2-benzoquinones and 5-HR is base catalysed.

To identify the products of the addition reaction we examined the tyrosinase reaction system by HPLC/MS in the presence of equimolar 5-HR and EtP at pH 8.5 and showed the generation of five major products (see Table 2). On the basis of their molecular weights and spectral absorption data we propose the formation of the products **4**-**9** as set out in Scheme 1. The initial addition products undergo redox exchange reactions and the resulting 1,2-benzoquinone products undergo *ipso*-cyclisation to give 2,5-cyclohexadienones concordant with previous findings (Waiss et al., 1966; Crescenzi et al., 1991).

The reaction scheme (Scheme 1) shows the proposed reaction pathway which involves two addition reactions between 4-ethyl-1,2-benzoquinone (**3**) and 5-HR (**1**), and five redox exchange reactions with the 1,2-benzoquinone (**3**) generating 1,2-dihydroxy-4-ethylbenzene which acts as a catecholic substrate for tyrosinase. This accounts for the activation of *met*-tyrosinase, and hence the effect on the lag period (Ramsden and Riley, 2014a) and the additional oxygen utilisation. The calculated total oxygen stoichiometry according to the ratio of the products formed, based on the mean absorbances at 400 nm, is 1.35 moles of oxygen per mole of EtP which corresponds closely with the data obtained (Table 1).

We conclude that the 5-hydroxy group of 5-HR interferes with access to the active site of tyrosinase and further work may clarify the detailed molecular nature of this phenomenon. The major action of 5-HR is as a powerful trapping agent of 1,2-benzoquinones. Its observed influence in the presence of phenolic substrates on the characteristics of tyrosinase-catalysed oxidation, such as the lag period and maximum oxidation rate, is attributable to the formation of catecholic C-addition products that function as secondary activating substrates for tyrosinase and which cyclise to form a range of unusual 2,5-cyclohexadienenones. These results explain how 5-hydroxyresorcinol acts as an activator of tyrosinase by enabling the generation of a catecholic substrate in a manner similar to the formation of dopa by redox exchange with the natural internal addition product, cyclodopa. The demonstration of this extraneous activation pathway suggests that the biological regulation of melanogenesis is sensitive to the availability of compounds able to form reductive addition compounds with 1,2-benzoquinones.

Supplementary data associated with the experimental details of this work can be viewed in the online version at http://xxx

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Table 1. Summary of kinetic data from oxidation experiments.

Order of addition	substrate (nmol)	5-HR (nmol)	Oxygen uptake (nmol)	Lag period (secs)	Vmax (nmol min^{-1})
5-HR/EtP	400	400	515	24	143
EtP/5-HR	400	400	521	5	546
EtP/5-HR/5-HR/EtP	800	400	720	-	-
MOP	0	250	220	90	145
MOP/5-HR	250	250	310	-	-
MOP/5-HR/MOP	250	500	615	12	456

Table 2. Summary of data from HPLC/MS experiments.

Retention Time (mins)	*m/z* (M+1)	λ_{max} (nm)	Structure (see Scheme 1)	Peak area at 400 nm (%)
3.48	399	285	**5**	15
4.35	261	418	**4**	31
4.70	397	428	**6**	9
4.88	397	428	**7**	14
5.10	395	453	**8 + 9**	25

Scheme 1. Reactions between 5-HR (**1**) and EtP (**2**) in the presence of tyrosinase. The proposed identity of the products and their molecular weights are indicated. Redox reactions with 4-ethyl-1,2-quinone (**3**) are followed by *ipso*-cyclization. Compounds not observed by chromatography are indicated in square brackets.