ON THE NATURE AND MODE OF ACTION OF THE COPPERPROTEIN, TYROSINASE

II. EXCHANGE EXPERIMENTS WITH RADIOACTIVE COPPER AND THE FUNCTIONING ENZYME

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SUMMARY

1. The exchange reaction between functioning tyrosinase and radiocupric ions has been studied.

2. In general, high catecholase enzymes incorporated much more radioactive copper during catalysis of the oxidation of *o*-dihydric phenols than of monophenols.

3. High cresolase enzymes retained little radioactivity in all experiments.

4. The copper exchange data support the suggestion of two distinct activity sites in tyrosinase; *i.e.* catecholase and cresolase activity centers. It appears that the copper at cresolase activity sites is non-exchangeable. The magnitude of exchange of the copper atoms at catecholase activity sites seems to depend on the number of *o*-dihydric phenol molecules oxidized.

5. The catecholase sites of tyrosinase appear to be little or not at all involved in the oxidation of monophenols.

6. On the basis of the exchange experiments it is suggested that the oxidation of a monophenol by tyrosinase may not proceed via an *o*-dihydric phenol.

INTRODUCTION

In the previous paper[1], exchange experiments involving radioactive ^{64}Cu and resting (non-functioning) tyrosinase were described. Copper, not protein bound, was removed from these systems by means of ion exchange resins. It was found that the small amount of radio-copper incorporated into the enzyme, in the absence of substrate, decreased as the purity of the enzyme increased. These results were explainable in terms of two types of copper sites (active and non-exchangeable; inactive and exchangeable) in the impure and/or partially inactivated enzyme.

The present paper describes the results of similar exchange experiments involving radioactive ^{64}Cu and the copper of the catalytically functioning enzyme.

The substrates commonly used in studies of tyrosinase are *p*-cresol and catechol.

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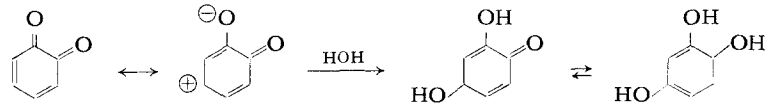
Preliminary experiments showed that the cation exchange resin Amberlite IR-100 quantitatively removed ionic copper from acetate buffered aqueous solutions of these compounds. However, their aerobic oxidation as catalyzed by tyrosinase produces insoluble melanin-like pigments. These precipitates rapidly clogged the resin columns to such an extent that further passage of solution through the columns was impossible. This difficulty was overcome by employing 4-*tert.*-butylphenol, 3,4-dimethylphenol, and the corresponding substituted catechols as the substrates in this investigation. The reasons why these substrates were most satisfactory are given below.

The tyrosinase catalyzed oxidation of both mono- and *o*-dihydric phenols involves the formation of the corresponding *o*-benzoquinones. The stability of these *o*-quinones in the aqueous media is markedly dependent upon their structure. Furthermore, the amount of oxygen consumed and the amount of precipitate formed are dependent on the stability of the quinone. The complete enzymic oxidation of *p*-cresol (presumably *via* the corresponding catechol and thence 4-methyl-benzoquinone-1,2, which is unstable) requires 3 atoms of oxygen/mole of *p*-cresol under optimum conditions[2]. The oxidation of catechol via the unstable *o*-benzoquinone requires 2 atoms of oxygen/mole of substrate[2]. However, as can be seen in Table I, the enzymic oxidation of 4-*tert.*-butylphenol, 3,4-dimethyl phenol, and the corresponding catechols, requires 1 atom less of oxygen than the oxidation of *p*-cresol and catechol. The oxidation of each of these phenols and catechols resulted in a stable yellow solution (stable quinone) and no precipitate was produced.

The instability of *o*-benzoquinone in water, which corresponds to its ability to produce insoluble pigments, has been explained in terms of a reaction between the quinone and water to produce hydroxyhydroquinone as a transitory intermediate in the pigmentation reaction[2].



The hydroxyhydroquinone is not detectable in the system because it is very rapidly oxidized by another molecule of the *o*-benzoquinone to produce catechol and the unstable hydroxyquinone. When the 4,5 positions of the original *o*-benzoquinone are occupied by methyl groups, or when one of these positions is occupied by the bulky *tert.*-butyl group (which sterically hinders the position ortho to it), the water reaction is suppressed and the substituted quinone is relatively stable in dilute aqueous solution[5]. As a result, the enzymic oxidation of the corresponding phenols or catechols results in one less atom of oxygen uptake (see Table I) and no production of melanin-like precipitate*. In this connection the observation of CUSHING[6] that 4-*n*-butylphenol consumes 3 atoms of oxygen/mole on enzymic oxidation is of interest. It

* In his review, *The Structure of Melanins*, MASON[3] has argued against the water reaction in favor of a direct polymerization of *o*-benzoquinones. His arguments, however, are not convincing, primarily because they do not account for the fact that *o*-benzoquinone is relatively stable in anhydrous media. When water is added to an anhydrous ether solution of *o*-benzoquinone (previously stable for a considerable period) the quinone rapidly disappears and the typical catechol melanin-like precipitate forms. This fundamental role of water in the *o*-quinone instability is readily followed spectrophotometrically[4].

points up the fact that the *n*-butyl group does not have the steric effect of the *tert.*-butyl group.

In addition to the 4-*tert.*-butyl- and 3,4-dimethylphenols and catechols, the dipeptide L-tyrosyl-L-alanine was found to be a satisfactory substrate for tyrosinase in the exchange experiments. While tyrosine is sparingly water soluble, absorbs 4 atoms of oxygen and forms an insoluble melanin on enzymic oxidation, L-tyrosyl-L-alanine is water soluble, also absorbs 4 atoms of oxygen during complete enzymic oxidation, but gives a dark purplish colored solution (no precipitate).

TABLE I

TOTAL OXYGEN UPTAKE OF THE SUBSTRATES USED IN THE TYROSINASE COPPER EXCHANGE EXPERIMENTS

The oxygen absorption was determined in Warburg respirometers using flasks of 30 ml capacity; shaking rate was 120 oscillations/min; Temperature, 25°, pH 5.7. Total reaction volume was 5.0 ml, consisting of 2.0 ml of 0.1 *M* acetate buffer, 1.0 ml (5 mg) of gelatine solution, 1.0 ml (1 mg) of aqueous substrate solution, 0.5 ml of high catecholase enzyme (containing a minimum of 60 cresolase units *i.e.* a large excess of enzyme), and 0.5 ml of distilled water. In the case of 4-*tert.*-butylphenol, which is only slightly soluble in water, the system contained 10% ethanol.

Substrate	*Total O_2 consumed per 1 mg (cmm)*	*Total O_2 uptake in atoms per mol of substrate*
4-*tert.*-butyl phenol	148	1.99
3,4-dimethyl phenol	174	1.91
4-*tert.*-butyl catechol	64	0.96
4,5-dimethyl catechol	77	0.95

On passing 10 mg of 4-*tert.*-butylbenzoquinone -1,2 and 50 μg of cupric ions through an Amberlite column, a colorless effluent resulted in which no copper could be detected. In other words, the quinone apparently remained on the resin column but did not interfere with the removal of cupric ions by ion exchange.

EXPERIMENTAL

Tyrosinase

The various enzyme samples were obtained from the common mushroom, *Psalliota campestris*. The methods of preparation, estimation of copper content, protein content, and enzymic activities have been described elsewhere[1].

The exchange experiments

The preparation and use of the radioactive ^{64}Cu solutions, the counting procedure, and the use of Amberlite IR-100 as a resin for removing cupric ion from the enzyme have been described previously[1]. The exchange experiments were set up and run in the same manner as the resting enzyme experiments[1] except that various amounts of substrate were added to the reaction mixture at zero time. In each experiment the substrate was completely oxidized as established by manometric and spectrophotometric measurements[5]. All functioning enzyme exchange data were corrected by subtracting from the actual measurements the percent radio-copper incorporation of the resting enzyme exposed to ^{64}Cu for the corresponding time interval[1].

Materials

Catechol, *p*-cresol, and ascorbic acid solutions were prepared and used as described previously[1]. The 4-*tert.*-butylphenol (Distillation Products Industries) and the 3,4-dimethylphenol (Kahlbaum A.G.) were re-crystallized from copper-free water. Their melting points (99–100° and 62–63°, respectively) checked with the published records[7,8]. The 4-*tert.*-butylcatechol and 4,5-dimethylcatechol had been prepared earlier in these laboratories by ROTH[9]. A sample of L-tyrosyl-L-alanine ($\alpha_D^{23} = +7.84°$) was kindly supplied by Drd. E. F. ERLANGER AND D. M. KIRSCHENBAUM of the Department of Biochemistry, Columbia University. The synthesis of 4-*tert.*-butyl-benzoquinone-1,2 is described elsewhere[5].

RESULTS

Exchange of enzyme copper during the oxidation of 4-tert.-butylcatechol

The incorporation of radioactive ^{64}Cu into the enzyme was found to depend on the amount of substrate employed and on the type of tyrosinase preparation used (Table II and Fig. 1). The maximum amount of substrate which could be used was 4 mg/5 ml total volume. At higher concentrations the solutions become turbid within a short time, indicating the beginning of precipitate formation and decreased stability of the 4-*tert.*-butyl-benzoquinone -1,2 (see ref. 5).

TABLE II

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING ITS CATALYTIC ACTION ON VARYING AMOUNTS OF 4-*tert.*-BUTYL CATECHOL

Reaction time, 50 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Type of enzyme	*Enzyme preparation No.*	*Cu in enzyme µg*	*Cu in effluent µg*	*mg of substrate*	*% in effluent of the original*		*% radioactivity incorporated into the enzyme*
					catecholase activity	*cresolase activity*	
High catecholase	C305D2	3.7	3.1	0.01	—	—	0
	C305D2	3.7	3.4	0.1	86	79	0
	C305D2	3.7	3.1	0.5	—	—	11.5 ± 1.2
	C305D2	3.7	3.2	1.0	—	—	28.1 ± 1.7
	C305D2	3.7	3.2	3.0	89	74	27.5 ± 1.7
High cresolase	C307D	3.7	3.7	0.2	—	—	0
	C307D	3.7	3.4	0.6	—	—	3.7 ± 1.5
	C307D	3.7	4.2	1.0	—	—	5.8 ± 1.7
	C307D	3.7	4.0	4.0	64	—	15.4 ± 1.6

Table II and Fig. 1 show that at any given substrate concentration the high catecholase preparation showed a higher copper exchange than the high cresolase preparation.

Although the radiocopper incorporation recorded with the high catecholase preparation at both 1.0 mg and 3.0 mg of substrate is about 28 %, indications were found that a figure close to the theoretical maximum of radioactivity in the enzyme, *i.e.* 50 %, would have been attained had it been possible to go to higher substrate concentrations. Namely, an indirect but considerable extension of the amount of substrate was achieved by oxidizing 1.0 mg of 4-*tert.*-butylcatechol with tyrosinase

in the presence of 12 mg of ascorbic acid under the conditions of the exchange experiments. During this reaction the enzyme incorporated 45 % of the radioactivity of the comparison standard. Since tyrosinase is a very poor catalyst for the oxidation of ascorbic acid*, one can expect that the 4-*tert.*-butyl-benzoquinone -1,2 formed was continuously reduced to 4-*tert.*-butylcatechol by the ascorbic acid until all the ascorbic acid had been consumed by oxidation to dehydroascorbic acid.

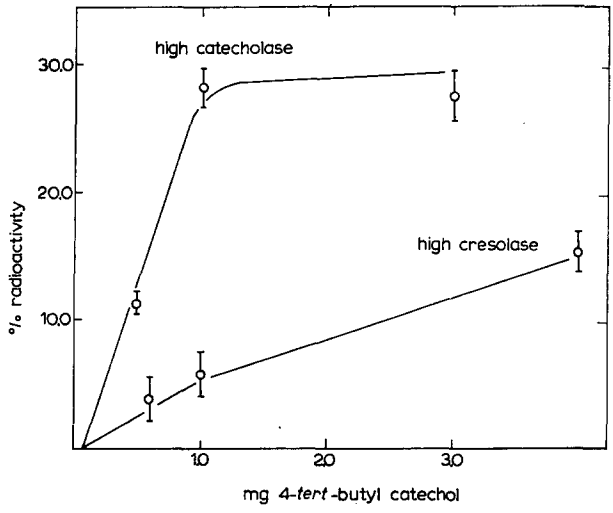


Fig. 1. Exchange with 4-*tert.*-butyl catechol as the enzyme substrate. Dependance of the exchange on the substrate concentration and enzyme type.

Exchange of enzyme copper during the oxidation of 4,5-dimethylcatechol

The findings are summarized in Table III. Only a high cresolase enzyme preparation was tested. The results were comparable to those obtained with a similar enzyme using 4-*tert.*-butylcatechol as the substrate.

TABLE III

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING ITS CATALYTIC ACTION ON VARIOUS AMOUNTS OF 4,5-DIMETHYL CATECHOL

Reaction time, 50 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

High catecholase enzyme preparation No.	*Cu in enzyme* μg	*Cu in effluent* μg	*mg of substrate*	*% in effluent of the original*		*% radioactivity incorporated into the enzyme*
				catecholase activity	*cresolase activity*	
C312.2	4.5	4.7	1.0	106	54	28.4 ± 2.0
C312.2	4.5	4.3	1.0	—	—	30.8 ± 1.9
C312.2	4.5	4.6	2.0	—	—	34.7 ± 3.2

* The large amount of tyrosinase which had to be used in each of the exchange experiments was found to catalyze the oxidation of ascorbic acid very slowly. This rate of oxidation was comparable to the rate of oxidation of ascorbic acid catalyzed by a similar amount of gelatine and cupric ions. The latter was determined by Dr. R. J. MAGEE and Miss N. BENHAMOU in these laboratories.

*Exchange of enzyme copper during the oxidation of 4-*tert.*-butyl-phenol*

Because of the very limited solubility of 4-*tert.*-butylphenol in water, this substrate was used in the form of 1 ml of an aqueous solution containing 10 % ethanol and 1.0 mg of the phenol. A preliminary study of control systems established that the small amount of alcohol thus introduced into the system did not inactivate the enzyme or alter the exchange of the resting enzyme. Due to the substrate solubility difficulties not more than 1.0 mg of 4-*tert.*-butylphenol could be introduced.

Before the tyrosinase catalyzed oxidation of monohydric phenols gets under way a lag period is observed, the length of which depends on the particular substrate. The lag time can be eliminated by a trace of *o*-dihydricphenol. Under the conditions of the exchange experiments the length of the lag period with 4-*tert.*-butylphenol was about 25 min. The addition of 0.01 mg of 4-*tert.*-butylcatechol, an amount which had not produced copper exchange by itself (see Table II), eliminated the lag time completely[5]. The time necessary for the complete oxidation of the 4-*tert.*-butylphenol could thus be kept to a minimum.

The results of the exchange experiments are presented in Table IV.

TABLE IV

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING ITS CATALYTIC ACTION ON 1.0 MG OF 4-*tert.*-BUTYL PHENOL

0.01 mg of 4-*tert.*-butyl catechol added as primer. Reaction time of 240 to 260 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Type of enzyme	*Enzyme preparation No.*	*Cu in enzyme* μg	*Cu in effluent* μg	*% in effluent of the original*		*% radioactivity incorporated into the enzyme*
				catecholase activity	*cresolase activity*	
High catecholase	C304D2	3.5	3.1	49	39	2.7 ± 1.3
	C304D2	3.5	3.2	—	—	4.2 ± 1.3
	C304D2	3.5	3.0	—	27	0.5 ± 1.8
	C304D2	3.5	3.2	—	—	0.5 ± 1.5
	C305D	3.7	3.3	53	57	0.8 ± 0.8
	C305D	3.7	3.7	—	—	1.9 ± 0.8
High cresolase	C306D	2.2	—	—	55	0

TABLE V

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING ITS CATALYTIC ACTION ON 1.0 MG OF 3,4-DIMETHYL PHENOL

Reaction time, 250 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Type of enzyme	*Enzyme preparation No.*	*Cu in enzyme* μg	*Cu in effluent* μg	*% in effluent of the original*		*% radioactivity incorporated into the enzyme*
				catecholase activity	*cresolase activity*	
High catecholase	C312-2	4.5	4.8	—	—	15.4 ± 1.6
	C312-2	4.5	4.0	108	56	15.3 ± 2.1
	C313-1	3.6	3.8	91	76	15.2 ± 1.4
	C313-1	3.6	3.4	98	72	14.8 ± 1.4
High cresolase	C307D	3.7	3.3	51	80	4.5 ± 1.2
	C307D	3.7	3.7	—	—	5.5 ± 1.2

It will be noted that the amount of radiocopper incorporated into the enzyme was small, regardless of whether a high cresolase or a high catecholase preparation was used.

Exchange of enzyme copper during the oxidation of 3,4-dimethylphenol

In the presence of the large amount of enzyme used, the oxidation started immediately and the use of a primer was not necessary. The results, summarized in Table V, show that the high catecholase enzyme showed an approximately three times higher incorporation of radiocopper than the high cresolase enzyme.

Exchange of tyrosinase copper during the oxidation of D-*tyrosyl*-L-*alanine*

This dipeptide showed the same oxygen uptake as tyrosine (4 atoms/mole). Therefore, the oxidation probably followed a pathway similar to that proposed for tyrosine[8]. The results of a duplicate exchange experiment with this substrate are presented in Table VI.

TABLE VI

PERCENT RADIOACTIVITY INCORPORATED INTO TYROSINASE DURING THE OXIDATION OF 2.0 MG OF L-TYROSYL-L-ALANINE

Reaction time, 100 min. The amount of ^{64}Cu involved was the same as the copper content of the enzyme.

Enzyme preparation No (high catecholase)	*Cu in effluent μg*	*Cu in emzyme μg*	*% radioactivity incorporated into the enzyme*
C305D	3.7	—	32.6 ± 1.9
C305D	3.7	3.4	34.4 ± 2.0

DISCUSSION

The exchange experiments could not be carried out at the optimum substrate concentration for tyrosinase for the following reasons: (a) In order to have a measurable quantity of copper in the column effluents relatively large amounts of enzyme had to be employed; and (b) the amounts of substrates which could be used were limited by their low solubilities in water. Nevertheless, interesting speculations on the mode of action of tyrosinase can be based on the results obtained.

It should be remembered here that the data presented in Tables I–VI have been corrected by subtracting from the measured count the radioactivity incorporated into the resting enzyme (absence of substrate) under identical conditions[1]. As in the case of the resting enzyme[1], the copper exchange data obtained with catalytically functioning tyrosinase cannot be explained on the basis of enzyme inactivation. For example, the inactivation of the high catecholase enzyme C305D2 was in the same range whether 0.1 mg or 3.0 mg of 4-*tert.*-butylcatechol was oxidized (Table II) but the incorporation of radiocopper into the tyrosinase in these two cases was very different (0 % and 27.5 %, respectively).

The oxidation of 4-*tert.*-butylcatechol by a high catecholase enzyme resulted in a considerable incorporation of radiocopper into the protein when at least 1.0 mg of substrate was used. A parallel oxidation with a high cresolase preparation gave a

much lower exchange (Table II). These findings are in line with the view that the monophenolase and *o*-dihydric phenolase activities of tyrosinase involve two different and distinct sites on the protein. The cresolase site cannot bring about the oxidation of a catechol (and the catecholase site cannot oxidize monohydric phenols). The high catecholase enzyme presumably had many more catecholase activity centers than the high cresolase preparation, even though the amount of enzyme copper involved was the same in both cases. The high catecholase enzyme, because it had more active catecholase sites, showed the highest extent of exchange with ^{64}Cu during the oxidation of 4-*tert.*-butylcatechol.

Regardless of the type of enzyme preparation involved, the oxidation of approx. 0.1 mg of 4-*tert.*-butylcatechol did not produce any measurable exchange of copper. In this case the ratio of substrate enzyme to molecules was approx. 300/1 while at the optimum substrate concentration this ratio is 1,000,000/1. Thus it may be that only a few of the catecholase activity centers participated in the oxidation of 0.1 mg of this substrate, and only a fraction of these actions may have led to the incorporation of radiocopper.

The experiments with 4,5-dimethylcatechol (Table III) supported the data obtained with 4-*tert.*-butylcatechol. For the same type of enzyme (high catecholase) and similar amounts of substrate (1.0 mg) the level of radiocopper incorporation into tyrosinase was comparable. This confirms the interpretation that the type of enzyme and total number of *o*-dihydric phenol molecules acted upon decide the magnitude of exchange.

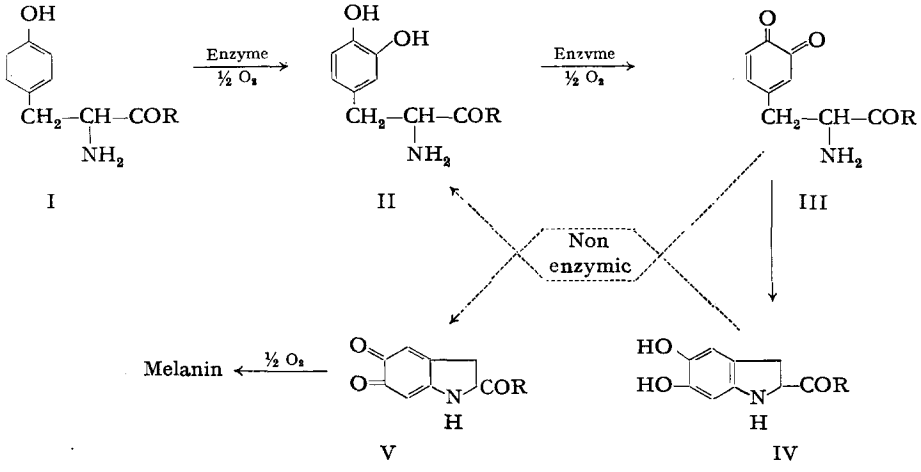
During the tyrosinase catalyzed oxidation of 1.0 mg of 4-*tert.*-butylphenol (Table IV); little, if any, radioactive copper was incorporated into the enzyme, regardless of the type of tyrosinase used. In contrast, the oxidation of 1.0 mg of 3,4-dimethylphenol produced considerable copper exchange when a high catecholase preparation was used, and only small exchange when a high cresolase preparation was used (Table V). However, even in the case of the high catecholase enzyme catalyzed oxidation of 3,4-dimethylphenol, the copper exchange was only about one half of that observed when the same amount of 4,5-dimethylcatechol was the substrate. It seems attractive to ascribe the differences in copper exchange observed with the two monophenols as tyrosinase substrates to the fact that the 3,4-dimethylphenol is much more rapidly oxidized than 4-*tert.* butylphenol. Yet, a high cresolase preparation which oxidized 3,4-dimethyl phenol at least as fast, or faster, than a high catecholase enzyme, showed only a fraction of the radiocopper incorporation of the high catecholase enzyme. Thus, differences in rates of substrate oxidation do not appear to account for the exchange observations. Similar arguments seem to speak against the possibility that differences in the rates of oxidation of mono- and dihydric phenols account for the differences in copper exchange found.

It is known that the tyrosinase copper is necessary for cresolase activity. All experiments, so far, with the high cresolase enzyme resulted in a much lower exchange of copper than those involving high catecholase enzyme. These observations suggest that the copper atoms at the cresolase activity centers are non-exchangeable, *i.e.* only copper atoms at catecholase sites can be exchanged against ^{64}Cu. It also appears that catecholase activity centers are little or not at all involved in the oxidation of monohydric phenols or their oxidation product(s). At first, the relatively high exchange observed during the oxidation of 3,4-dimethylphenol by a high catecholase

enzyme does not seem to fit the preceding suggestion. An attractive explanation for this "anomalous" case can be derived from a study by KENDAL[7]. He found that low concentrations of 3,4-dimethylphenol competitively inhibited the oxidation of 4,5-dimethylcatechol. Other similar substrate pairs (*e.g. p*-cresol and homocatechol) did not show this effect. KENDAL suggested that the "specific catecholase center" of the enzyme has a much greater affinity for 3,4-dimethyl phenol than for the other mono- or dihydric phenols. In our experiments, it appears possible that 3,4-dimethyl phenol formed many more complexes with catecholase activity centers when a high catecholase rather than high cresolase enzyme was used. If the exchange of copper is dependent on the formation of such complexes, our finding of higher exchange with high catecholase enzyme than with the high cresolase preparation when the dimethyl phenol was oxidized is understandable.

Anaerobic experiments might help to test the above suggestion. If complexing of the phenolic substrates with "catecholase sites" can indeed produce exchange, the presence of oxygen and the simultaneous oxidation of substrate may not be required.

The radiocopper incorporation into a high catecholase enzyme observed when it catalyzed the oxidation of the monohydric phenol, L-tyrosyl-L-alanine was of the same magnitude (Table VI) as that found with dihydric phenol as the substrate. This result disagrees with data obtained with 4-*tert.*-butyl phenol but can be explained by a consideration of the probable pathway of oxidation of the dipeptide. Presumably, L-tyrosyl-L-alanine (I; R = $NHCH(CH_3)COOH$) follows the oxidative reactions established for tyrosinase itself (I; R = OH) by RAPER[8]. As indicated in the reaction scheme below, an *o*-dihydric phenol (II) may form in this system by a non-enzymic reaction. Raper actually reacted synthetic dopaquinone (III; R = OH) with 5,6-dihydroindole-2-carboxylic acid (IV; R = OH) and found that dopa (II; R = OH) and hallachrome (V; R = OH) were formed. Thus it is quite possible that an *o*-dihydric phenol (II; R = $NHCH(CH_3)$–COOH) formed nonenzymically when L-tyrosyl-L-alanine was oxidized by tyrosinase. The considerable incorporation of ^{64}Cu into the enzyme during this reaction may be due to the oxidation of II (R = –NH–$CHCH_3$–COOH) at catecholase activity centers.



The results of the exchange experiments described above merit a reconsideration of the pathway of the tyrosinase catalyzed oxidation of monophenols. The current view that the oxidation of mono- and *o*-dihydric phenol involves two different sites on the enzyme, (with copper being essential to both activities) does not influence any evaluation as to whether a catechol is or is not the first product of the monophenol oxidation. Whether a catechol is the first intermediate of such oxidation at a monophenolase activity site and then migrates to a catecholase site for further oxidation to a quinone, or whether the production and oxidation of the catechol occurs at the same site, the oxidation of an equivalent amount of the corresponding catechol would be involved. Consequently, one would expect the same degree of exchange with ^{64}Cu during the enzymic oxidation of a monophenol and the corresponding *o*-dihydric phenol. In general, however, the experimental data indicated that considerable copper exchange occurred during the tyrosinase catalyzed oxidation of an *o*-dihydric phenol and little exchange took place during the oxidation of monohydric phenols. Therefore, the possibility is suggested that the oxidation of monohydric phenols by tyrosinase may not involve the corresponding *o*-dihydric phenols *per se* as an intermediate. Rather the monophenol might be directly converted to the corresponding *o*-benzoquinone.

KUBOWITZ[10] established that the copper bound to tyrosinase is reduced from the cupric to the cuprous state during the oxidation of *o*-dihydric phenols. Several authors[11–13] have suggested that the monophenolase activity of tyrosinase involves cuprous copper. These hypotheses were based on the observed elimination of the induction period apparent in monophenol oxidation by small amounts of reductants such as catechol and ascorbic acid. MASON[13] believes that a hydroxylation of monophenol to *o*-dihydric phenol occurs and produces an oxidation of cuprous to cupric enzyme copper. Again, the exchange experiments described here generally showed considerable radioactive copper incorporation into tyrosinase during the oxidation of *o*-dihydric phenols and minor copper exchange during the oxidation of monophenols. These findings are difficult to reconcile with MASON's hypothesis unless cupric ions cannot reach the enzyme activity sites. A configuration accessible to oxygen molecules and substrate but not to cupric ions would be required.

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REFERENCES

1 H. DRESSLER AND C. R. DAWSON, *Biochim. Biophys Acta*, 45 (1960) 508.
2 J. M. NELSON AND C. R. DAWSON, *Advances in Enzymology*, 4 (1944) 99.
3 H. S. MASON, *Pigment Cell Growth* (Proc. 3rd. Conf. Biol. Normal and Atyp. Pigment Cell Growth) 1953, p. 277.
4 W. B. TARPLEY, *Dissertation*, Columbia University, 1951.
5 H. DRESSLER AND C. R. DAWSON, to be published.
6 M. L. CUSHING, *J. Am. Chem. Soc.*, 70 (1948) 1184.
7 L. P. KENDAL, *Biochem. J.*, 44 (1949) 443.
8 H. S. RAPER, *Biochem. J.*, 20 (1936) 735; 31 (1937) 2162.
9 L. J. ROTH, *Dissertation*, Columbia University, 1944.
10 F. KUBOWITZ, *Biochem. Z.*, 296 (1938) 443; *Biochem. Z.*, 299 (1938) 32.
11 J. DOSKOCIL, *Collection Czechoslov. Chem. Communs.*, 15 (1950) 614.
12 H. S. MASON, W. L. FOWLKS AND E. PETERSON, *J. Am. Chem. Soc.*, 77 (1955) 2914.
13 H. S. MASON, *Nature*, 177 (1956) 79.