CLVIII. THE MODE OF ACTION OF TYROSINASE.

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It is well known that tyrosinase in presence of molecular oxygen will oxidise phenol, *p*-cresol, *m*-cresol and tyrosine, which are all monohydric phenols. The action of tyrosinase on these phenols is specific and distinguishes this enzyme from laccase and from peroxidase. These latter either do not oxidise the above substances or do not oxidise them in the same way that tyrosinase does. Tyrosinase, as well as laccase and peroxidase, readily oxidises *o*-dihydric phenols such as catechol, homocatechol, or 3 : 4-dihydroxyphenylalanine. This ability of tyrosinase to oxidise both monohydric and *o*-dihydric phenols raises the question of the nature of tyrosinase: whether it consists of a single enzyme able to oxidise both these types of substance, or whether it is composed of two enzymes, one acting only on monohydric phenols, the other only on *o*-dihydric phenols.

That the latter view is unlikely is rendered probable by experiments described in this paper. A number of methods have been used in attempts to separate the two hypothetical enzymes, or to destroy the one without the other, but without success. In presence of either cyanide or sulphite, however, the action of tyrosinase on *p*-cresol was retarded to a greater extent than on catechol. Heating or storage has also brought out a difference between the action on monohydric and *o*-dihydric phenols. These differences do not necessarily indicate two different enzymes, but may be explicable in terms of the mode of action of the enzyme, as discussed later.

A further possibility is that tyrosinase consists of an enzyme which oxidises *o*-dihydric phenols, together with a trace of an *ortho*quinone, and that the latter is able to convert a monohydric into an *o*-dihydric phenol. The *o*-dihydric phenol thus produced would be converted into an *ortho*quinone by the enzyme, so that, once action had been started by the trace of *ortho*quinone, the reaction would proceed. This view has been put forward by Onslow and Robinson [1928].

The evidence on which this suggestion was based consisted principally in experiments in which the *ortho*quinone assumed to be present in the enzyme preparation was supposed to be selectively adsorbed by charcoal. The enzyme which remained acted on catechol but not on tyrosine, although it acted on tyrosine when a trace of catechol was added. A consideration of these experiments as described by Onslow and Robinson shows that an interpretation other

than that put upon them is possible. Onslow and Robinson treated an oxidase preparation from the potato with charcoal several times, and after three treatments obtained a preparation which showed no lessening in activity on catechol, but which acted scarcely at all on tyrosine or *p*-cresol unless catechol were added as activator. In these experiments, action on catechol was "instantaneous" until after the fourth treatment with charcoal, so that if action on catechol had been much quicker originally than after the third treatment, the fact would not have been apparent. Action on catechol fell off with further treatment with charcoal. It appeared therefore possible that action on *o*-dihydric as well as on monohydric phenol might be retarded by treatment with charcoal, owing to slight adsorption of the enzyme. The experiments of Onslow and Robinson have been repeated, with the modification that each sample was diluted before being tested, to avoid instantaneous action on catechol. It was found that action on *p*-cresol and on catechol fell off together.

It must be pointed out that if the view of Onslow and Robinson were correct, it should be possible to imitate the action of tyrosinase on monohydric phenols by means of an enzyme which oxidises *o*-dihydric phenols, by addition to it of a trace of an *ortho*quinone. The experiment has been tried, using the slug *Arion ater* as source of the enzyme; no action on monohydric phenols was observed, even on addition of a little homoquinone. Further, if the action of tyrosinase on *o*-dihydric phenols depended on conversion of monohydric to *o*-dihydric phenol by *ortho*quinone, *ortho*quinones should be able to bring about this change in absence of the enzyme. Attempts to show such a conversion by means of homoquinone did not succeed. The conclusion, therefore, seems warranted, that there is no satisfactory experimental evidence in favour of the view that tyrosinase has the constitution assigned to it by Onslow and Robinson.

Evidence has been brought forward by Raper [1926] and by Pugh [1929] that the action of tyrosinase on monohydric phenols is augmented by addition of a trace of *o*-dihydric phenol. The initial lag which can be observed when tyrosinase acts on tyrosine, *p*-cresol or phenol is absent if a little catechol or 3 : 4-dihydroxyphenylalanine is added. These observations show that the action of tyrosinase on those monohydric phenols which it attacks is initially accelerated by means of some product of its action on *o*-dihydric phenols. It has already been pointed out that there is no ground for the view that this is due to the oxidation of the monohydric phenol by an *ortho*quinone produced by action of the enzyme on the trace of *o*-dihydric phenol added. An additional difficulty in accepting this view is that 3 : 4-dihydroxyphenylalanine will act as activator, although the product of action of tyrosinase on it does not blue guaiacum, oxidise an external amino-acid, or form an anilinoquinone in presence of aniline, all of which reactions are given by a free *ortho*quinone, such as is produced from catechol.

It is possible, however, that an *ortho*quinone, acting as a peroxide, may increase the activity of the enzyme in some way. The possibility of a similar

action by hydrogen peroxide is also to be considered. It has been definitely established that the action of tyrosinase on *o*-dihydric phenols produces *ortho*-quinones; the simultaneous production of hydrogen peroxide is possible, but has not been proved. Onslow and Robinson [1926] demonstrated the production of hydrogen peroxide during the autoxidation of catechol, and also by the action of potato oxidase on catechol, which was confirmed by Platt and Wormall [1927]. Potato oxidase, however, contains both tyrosinase and laccase, so that it is not certain which of them produces hydrogen peroxide or whether both do. If hydrogen peroxide were produced by the action of tyrosinase, it might augment the action of the enzyme on monohydric phenols. It was shown by Martinon [1885] that phenol can be converted into catechol almost quantitatively by the action of hydrogen peroxide, in presence of a catalyst such as an iron or copper salt. It was conceivable that tyrosinase might serve as a catalyst in this reaction. Evidence is brought forward in this paper that both *ortho*quinone and hydrogen peroxide can accelerate the action of tyrosinase on monohydric phenols. This suggests that the mode of activation of tyrosinase by *o*-dihydric phenols is by peroxide formation. If we assume that hydrogen peroxide is produced when tyrosinase oxidises *o*-dihydric phenols to *ortho*quinones, then both products of action being peroxides can act as activators of the reaction.

Neither *ortho*quinone nor hydrogen peroxide appears to be able to activate the enzyme in absence of a suitable substrate, since preliminary treatment of the enzyme preparation with either of them did not hasten its activity. No evidence has been obtained that either *ortho*quinone or hydrogen peroxide can by itself convert monohydric to *o*-dihydric phenol in absence of the enzyme at the requisite rate to account for action on monohydric phenol. Nor is there any evidence that anything apart from molecular oxygen is necessary to enable tyrosinase to act on monohydric phenols.

Experimental.

Attempts to resolve tyrosinase into two enzymes.

The following attempts were made to separate tyrosinase into two enzymes able to act respectively on monohydric and on *o*-dihydric phenols. Mealworm tyrosinase, prepared by the method of Raper [1926] and filtered, was used. The activity of the preparations was tested by measuring the rate of oxygen absorption which accompanied action on *p*-cresol and on catechol in micro-respirometers of the Barcroft type. The left bottle contained 1 cc. 0·5 % *p*-cresol in phosphate buffer, p_H 7·0, or 1 cc. 0·5 % catechol in phosphate buffer, p_H 6·0, 1 cc. enzyme preparation, buffer to 3 cc. The right bottle contained 1 cc. enzyme preparation, buffer to 3 cc. Thymol was added, and sodium hydroxide was placed in the small tube fused into each bottle, to absorb carbon dioxide. Table I gives the results. Differential activity on *p*-cresol and catechol was not obtained after subjecting the enzyme to the modes of treat-

ment described in column 1 of the Table. Hence these methods provide no evidence of the existence of two separate enzymes in tyrosinase.

Table I.

Treatment	mm.^{3} O_2 absorbed in 1 hour: Untreated *p*-Cresol	Untreated Catechol	Treated *p*-Cresol	Treated Catechol
Dialysis	138	104	57	49
Heated at 75° for 10 mins.	152	127	25	21
In *N*/64 HCl for 40 mins.; neutralised	152	127	15	22
In *N*/32 NaOH for 40 mins.; neutralised	152	122	71	90
Precipitated by 60 % sat. $(NH_4)_2SO_4$; dissolved in 0·01 % ammonia	200	227	62	91
Precipitated by acetic acid at p_H 5·0; dissolved in 0·01 % ammonia	200	227 *	210	243 *
Precipitated by 50 % alcohol; dissolved in 0·01 % ammonia	200	227	189	223

* At p_H 7·0.

Sensitivity of tyrosinase to cyanide, using different substrates, was also measured in Barcroft micro-respirometers. Tyrosinase from mealworms, prepared by the centrifuge method described below, was used. It was found, working at p_H 7·0, that *M*/2500 KCN retarded action on *p*-cresol to the extent of about 68 %, on phenol 71 %, on catechol 47 %. Thus a distinct difference towards cyanide was exhibited between action of the enzyme on monohydric and on *o*-dihydric phenols.

Preparation of tyrosinase from mealworms. The following modification of the method of Raper [1926] for the preparation of tyrosinase from mealworms was adopted. About 100 g. mealworms are chloroformed and washed, then crushed with water acidulated with acetic acid to the extent of about 0·1 %. The pulp is strained through coarse muslin, and the residue again crushed with acidulated water. The process is repeated until the residue consists only of hulls, and the fluid measures about 300 cc. The fluid is centrifuged immediately. A creamy layer separates at the surface, and is poured away together with the liquid. The greyish residue is beaten up with water acidulated with acetic acid to the extent of about 0·01 %, altogether about 300 cc., and the product is again centrifuged. This washing is repeated twice more. The residue is transferred to a wide-mouth stoppered bottle with the aid of 300 cc. water, and is well shaken up with it. Then 1 % ammonia is added till a blue colour is just discerned on testing with absorbent red litmus paper. Chloroform is added as antiseptic, and the bottle is allowed to stand at room temperature for several days with occasional shaking. The product is centrifuged, then submitted to dialysis in running water for two days, and again centrifuged. The final volume is usually about 400 cc. Before the preparation was used in respirometer experiments, chloroform was removed from it *in vacuo*, and replaced by thymol.

Note on tyrosinase from mealworms. It is advisable to free the preparation from fat as it contains a lipolytic enzyme which otherwise causes fatty particles

to appear, and a diminution in alkalinity of the preparation. An alternative method of freeing it from fat to that described above is to dry the crushed mealworms, extract with ether, and dry again; treatment with alcohol, when followed by drying, inactivates the enzyme. Tyrosinase in mealworms appears to be associated with protein; it disappears during prolonged autolysis, and it is more readily extracted with dilute salt solutions than with pure water. Owing to the presence of a proteolytic enzyme, the preparation should be freshly dialysed for use in experiments in which amino-acids interfere; the enzyme is only partially precipitated by dialysis. The high figure obtained by Pugh and Raper [1927] for total oxygen absorption when tyrosinase acts on phenol was possibly due to use of a preparation in which proteolytic enzyme had been acting for some time; freshly prepared enzyme yields the figure 2·9 atoms of oxygen per molecule of substrate, in line with other monohydric phenols. The tyrosinase content of mealworms diminishes as they approach the pupal stage.

Attempts to resolve tyrosinase into enzyme and coenzyme.

An oxidase preparation was prepared from the potato as described by Onslow and Robinson [1928] and was treated with charcoal. The method of testing the preparations described by Onslow and Robinson was modified as follows. After each treatment with charcoal, 1 cc. liquid was removed and mixed with 5 cc. water. Of each of the diluted liquids 0·5 cc. was added to 2·5 cc. 0·2 % *p*-cresol, and 0·2 cc. to 2·5 cc. 0·2 % catechol, both in phosphate buffer, p_H 6·5. Comparison tubes were prepared containing 2·5 cc. buffer, and respectively 0·5 and 0·2 cc. of the diluted preparation. The times which elapsed before the first appearance of colour were noted.

Merck's medicinal charcoal.

	Original liquid	After 1st treatment	After 2nd treatment	After 3rd treatment	After 4th treatment	After 5th treatment
p-Cresol	1½ hrs.	2 hrs.	3 hrs.	4 hrs.	6 hrs. (faint)	Nil
Catechol	2 mins. 2¼ mins.	2¾ mins. 2¾ mins.	4 mins. 4 mins.	5½ mins. 5¾ mins.	10 mins. (faint) 10 mins. (faint)	Nil Nil

The final depth of colour diminished progressively in both series, as was likewise noted by Onslow and Robinson. Analogous experiments using Merck's animal charcoal yielded similar results to the above.

Experiments were also carried out with mealworm tyrosinase, prepared by the centrifuge method, in place of potato oxidase. This preparation was shaken for 15–20 mins. with Merck's charcoal, of which 0·1 g. was used for every 5 cc. The mixture was filtered, and the process repeated with the filtrate. After each treatment 1 cc. of the liquid was mixed with 9 cc. water. Of each of the diluted liquids 0·5 cc. was added to 2·5 cc. 0·2 % *p*-cresol, and 0·2 cc. to 2·5 cc.

0·2 % catechol, both in phosphate buffer, p_H 6·5. The liquid after the fifth treatment was tested undiluted.

Merck's medicinal charcoal.

	Original liquid	After 1st treatment	After 2nd treatment	After 3rd treatment	After 4th treatment	After 5th treatment
p-Cresol	1¼ hrs.	1½ hrs.	1¾ hrs.	2½ hrs.	10 hrs. (faint)	Nil
Catechol	2 mins. 2 mins.	2¼ mins. 2 mins.	2¼ mins. 2¼ mins.	3¼ mins. 3 mins.	12 mins. 10 mins.	35 mins. 35 mins.

Thus action on *p*-cresol and on catechol fell off together. Analogous experiments using Merck's animal charcoal yielded similar results.

Other adsorbents also failed to separate tyrosinase from any coenzyme which might enable it to act on monohydric phenols. Kaolin was heated with ammonia, then thoroughly washed till neutral to litmus. Of this 0·1 g. was used per 5 cc. mealworm tyrosinase. The mixture was shaken for 10 mins., centrifuged, and the process repeated with the supernatant liquid. A sample was taken after each treatment, and diluted 1 in 4. Of each of the diluted liquids 0·75 cc. was added to 2·5 cc. 0·2 % *p*-cresol in phosphate buffer, p_H 7·0. The diluted liquids were each further diluted 1 in 30, and 0·5 cc. was added to 2·5 cc. 0·2 % catechol in phosphate buffer, p_H 7·0.

	Original liquid	After 1st treatment	After 2nd treatment	After 3rd treatment	After 4th treatment
p-Cresol	⅝ hr.	1¼ hrs.	1⅝ hrs.	2½ hrs.	3¼ hrs.
Catechol	20 mins.	35 mins.	45 mins.	60 mins.	75 mins.

Analogous experiments using barium carbonate and calcium carbonate yielded similar results.

Alumina was prepared by dropping hot aluminium sulphate solution into an excess of hot 15 % ammonia slowly with shaking. Steam was then passed for 12 hours, and the product was washed by decantation till free from sulphate. It was found to adsorb the enzyme completely. The enzyme was only slightly eluted with dilute ammonia or with phosphate solution, p_H 8·0. A more satisfactory adsorbent was prepared by precipitation of magnesium sulphate solution with sodium carbonate solution. The product was filtered, washed three times, and used fresh from the filter. The enzyme was completely adsorbed with this, and could be eluted with phosphate solution. The product was dialysed to remove phosphate, and the process repeated. The liquids were tested by addition of 0·5 to 2·5 cc. 0·2 % *p*-cresol in phosphate buffer, p_H 7·0, and diluted 1 in 7 before adding 0·5 to 2·5 cc. 0·2 % catechol in phosphate buffer, p_H 7·0. The original liquids were further diluted 1 in 5.

	Original	First elution	Second elution
p-Cresol	30 mins.	36 mins.	2⅛ hrs.
Catechol	1¾; 1¾ mins.	2; 2 mins.	7; 7¼ mins.

Thus, even two adsorptions and elutions of the enzyme failed to alter the relative rates of action on *p*-cresol and catechol.

Dialysis was also without effect in this respect, and the dialysed preparation blued guaiacum slowly just as did the undialysed preparation. Partial inactivation of the preparation by heating, however, caused, with some preparations, an increase in rapidity of attack on *p*-cresol relative to action on catechol. Some dialysed and some undialysed preparation was heated at 65° for 10 mins. To 2·5 cc. 0·2 % *p*-cresol in phosphate buffer, p_H 7·0, or catechol in phosphate buffer, p_H 6·0, was added 0·5 cc. of each preparation, diluted 1 in 5 for testing on catechol.

	Unheated	Heated
Undialysed preparation		
p-Cresol	10 mins.	19 mins.
Catechol	2¼ mins.	6 mins.
Dialysed preparation		
p-Cresol	24 mins.	31 mins.
Catechol	5 mins.	10 mins.

It was suspected that a thermostable substance autoxidisable with production of hydrogen peroxide might be present in the preparation. The effect of aeration of freshly crushed mealworms on the relative rates of action on *p*-cresol and catechol was therefore determined. Some mealworms were ground up with water, and strained through muslin. The product was placed immediately in a desiccator which was then evacuated; a tube containing strong alkali was then upset into pyrogallol solution in the desiccator, to absorb the last traces of oxygen. Other mealworms were similarly crushed and strained, and a stream of air was drawn through the product for 1½ hours. Both preparations were then rapidly centrifuged, the intermediate layers were pipetted off, diluted 2 in 7, and 0·5 cc. added to 2·5 cc. 0·2 % *p*-cresol in phosphate buffer, p_H 6·5, then further diluted 1 in 8 and tested similarly on catechol.

	Aerated	Not aerated
p-Cresol	15 mins.	2¾ mins.
Catechol	6½ mins.	1⅓ mins.

Thus no detectable difference in relative rates of action on *p*-cresol and catechol was caused by aeration in this way.

Increase in age of a preparation was found to increase its rate of attack on *p*-cresol relative to catechol. Tyrosinase from mealworms was prepared by the centrifuge method, and some was dialysed, centrifuged and tested by adding 0·5 cc. enzyme diluted 1 in 50 to 2·5 cc. 0·2 % *p*-cresol in phosphate buffer, p_H 7·0, and 0·5 cc. enzyme diluted 1 in 200 to catechol similarly. The preparation was preserved with chloroform. After about six weeks, some was similarly dialysed and tested, the enzyme being diluted 1 in 10 for action on *p*-cresol, and 1 in 40 for catechol.

	First test	Second test
p-Cresol	2 hrs.	20 mins.
Catechol	2¾ mins.	2¾ mins.

Thus the preparation after standing attacked *p*-cresol six times as quickly as before relatively to its action on catechol. This observation may possibly account

for the fact that old material is not activated by heating, since it is already activated.

The activation of tyrosinase by an ortho*quinone.*

*Ortho*quinones are unstable substances, and are fairly readily decomposed by water. In order to investigate the effect of addition of *ortho*quinone on the action of tyrosinase on phenols, an *ortho*quinone in aqueous medium was required. Homoquinone is more stable than *o*-benzoquinone, and was therefore used for this purpose. Creosol was prepared from vanillin, and demethylated to homocatechol as described by de Vries [1909]. Of this homocatechol 3 g. was oxidised with silver oxide as described by Willstätter and Müller [1911], 100 cc. of ethereal solution containing the product being obtained. The ethereal solution was washed with water 20 times, 30 cc. water being used each time. The last two washings were combined, filtered, and used as an aqueous solution of homoquinone. This homoquinone water blued guaiacum strongly, yielded a red colour with glycine solution, no colour with ferric chloride, and none with titanium sulphate (after several extractions with purified ether). On standing, the homoquinone water became much paler. After 48 hours it produced no colour with guaiacum, or with glycine, but gave a strong ferric chloride reaction, though (after extraction with ether) it gave no colour with titanium sulphate. It was shown by Willstätter and Pfannenstiel [1904] that *o*-benzoquinone is decomposed by water with almost quantitative production of catechol. It is probably an analogous change which takes place in homoquinone water.

Homoquinone water added to tyrosine solution produced a pink or red colour, which later faded to very pale yellow. No subsequent darkening occurred. It is suggested that a red substance is produced by action of an external *ortho*quinone on tyrosine, different from that formed by condensation of the *ortho*quinone, produced by action of tyrosinase on tyrosine, with its own amino-group. This would explain the following observation made by Onslow and Robinson [1928]. When they allowed potato oxidase to act on 1 cc. saturated tyrosine in phosphate buffer, p_H 7·0, action being activated by addition of 1 cc. 0·0005 % catechol, a red colour first produced faded, then the liquid darkened slightly, but the reaction never increased in intensity. They concluded that some additional factor to those needed for the early stages of oxidation was required for melanin formation. This conclusion is very improbable, since it was shown by Raper and Wormall [1923] that the red substance produced by action of tyrosinase on tyrosine forms melanin in air without any enzyme or other addition.

The effect of homoquinone water on the action of tyrosinase on monohydric phenols was examined as follows. The following mixtures were placed in four test-tubes (1) 2·5 cc. 0·2 % *p*-cresol in phosphate buffer, p_H 7·0, 1 cc. water, and 0·5 cc. tyrosinase from mealworms prepared by the centrifuge method, freshly dialysed and centrifuged, diluted 1 in 16 with water, (2) 1 cc. homoquinone water freshly prepared replaced 1 cc. water in (1). Comparison

tubes were prepared in which 2·5 cc. buffer, p_H 7·0, replaced the *p*-cresol solution in (1) and (2). In a second set of test-tubes, phenol replaced *p*-cresol, and the tyrosinase was diluted only 1 in 2. All tubes were duplicated, with identical results. The times which elapsed before appearance of colour were as follows. *p*-Cresol (1) 50 mins.; (2) 15 mins. Phenol (1) 2 hrs. 20 mins.; (2) 1 hr. 20 mins. Thus *p*-cresol and phenol were more rapidly attacked by tyrosinase in presence than in absence of homoquinone water. The tests were repeated after the homoquinone water had stood for 24 hours, and 48 hours, with identical results. Thus the same degree of activation was observed after all tests for homoquinone had become negative. Assuming that homocatechol had been produced by decomposition of homoquinone by water, it would have been reconverted rapidly to homoquinone by action of the enzyme.

The action of tyrosinase on catechol was not activated by homoquinone water. The tubes contained (1) 2·5 cc. 0·2 % catechol in phosphate buffer, p_H 7·0, 1 cc. water, and 0·5 cc. tyrosinase diluted 1 in 150; (2) 1 cc. homoquinone water replaced 1 cc. water in (1). Comparison tubes were prepared in which buffer replaced catechol solution. In both (1) and (2) colour appeared after 2½ mins. When 1 cc. homoquinone water was mixed with 2·5 cc. 0·2 % catechol in water or phosphate buffer, the mixture became paler much more quickly than without catechol, and the guaiacum reaction disappeared much more quickly. This is probably due to a combination of *ortho*quinone with *o*-dihydric phenol such as has been suggested by Szent-Györgyi [1925].

In order to determine whether *ortho*quinone could convert monohydric to *o*-dihydric phenol, 1 cc. homoquinone water was mixed with 2·5 cc. 0·2 % *p*-cresol or phenol in phosphate buffer, p_H 7·0, and allowed to stand for 3 hours. The guaiacum reaction still persisted. To these mixtures was then added 0·5 cc. tyrosinase, diluted 1 in 16 for action on *p*-cresol, and the time which elapsed before appearance of colour was noted (1). The same addition was made to freshly prepared mixtures of substrate solution with homoquinone water (2), and with water (3). Tubes were prepared for comparison, in which buffer replaced substrate solution. *p*-Cresol (1) 20 mins; (2) 20 mins.; (3) 45 mins. Phenol (1) 15 mins; (2) 15 mins.; (3) 35 mins. Hence homoquinone water alone did not convert monohydric to *o*-dihydric phenol.

There was the possibility that the whole phenomenon of initial lag when tyrosinase acts on monohydric phenol might be due to the presence in the enzyme preparations of some substance inhibitory to the action of tyrosinase on monohydric but not on *o*-dihydric phenol, and oxidisable by *ortho*quinone produced by action of the enzyme on the *o*-dihydric phenol. If this explanation were correct, preliminary treatment of the enzyme preparation with homoquinone water should activate the enzyme by eliminating the initial lag of action on monohydric phenol. In order to test this, 12·5 cc. mealworm tyrosinase prepared by the centrifuge method were shaken frequently with 5 cc. homoquinone water for half an hour. The mixture was then extracted three times with its own volume of ether, and the remaining ether was boiled

off *in vacuo*. A control preparation was treated with water in place of homoquinone water, and similarly extracted. The test solutions were (1) 2·5 cc. 0·2 % *p*-cresol and (2) 2·5 cc. 0·2 % catechol, both in phosphate buffer, p_H 6·5, while a third tube contained buffer for comparison. To each was added 0·5 cc. enzyme preparation diluted 1 in 10. The times which elapsed before appearance of colour were as follows. Treated with homoquinone water (1) 2½ hrs.; (2) 1 min. Treated with water (1) 2½ hrs.; (2) 1 min. Thus preliminary treatment of the enzyme preparation with homoquinone water did not facilitate action on monohydric phenol.

The activation of tyrosinase by hydrogen peroxide.

It was shown by Bach [1906] that addition of hydrogen peroxide accelerates the action of tyrosinase on tyrosine. Later [1908, 1] he concluded that this effect was greater the older the material. Curves given by him in a succeeding paper, however [1908, 2], show that insufficient tyrosine was present to allow for greater action than that produced by the fresh preparation alone. In contradiction to Bach, Chodat [1907] found that the action of tyrosinase on tyrosine was retarded by hydrogen peroxide. It was then shown by v. Fürth and Jerusalem [1907] that hydrogen peroxide caused activation or retardation according as it was present in small or large quantity. In the present experiments this has been confirmed. Hydrogen peroxide was found to have a retarding effect on the action of tyrosinase in buffered solution without causing any detectable change in p_H, provided the enzyme were present in sufficiently small quantity. If the hydrogen peroxide, however, were present in small quantity relative to the amount of enzyme, it caused activation.

The effect of hydrogen peroxide on the action of tyrosinase on monohydric phenols and on catechol was examined as follows. In the initial experiments the concentration of hydrogen peroxide was the same, and the enzyme was diluted for action on catechol and *p*-cresol, so that coloration took place in a measurable time. Conical flasks were used, in order to ensure adequate air supply. They were divided into sets of three, A, B and C. In the first set, flask A contained 25 cc. 0·2 % phenol solution in phosphate buffer, p_H 6·5, 1 cc. water and 1 cc. mealworm tyrosinase prepared by the centrifuge method, freshly dialysed and centrifuged. In flask B, 1 cc. 1 % hydrogen peroxide replaced 1 cc. water in flask A. Flask C contained 25 cc. phenol solution, 1 cc. water, and 1 cc. 1 % hydrogen peroxide. In the other sets of flasks, phenol was replaced by *p*-cresol, and the enzyme was diluted 1 in 10, or by saturated tyrosine in phosphate buffer, p_H 7·0, or by 0·2 % catechol in phosphate buffer at p_H 6·5, and the enzyme diluted 1 in 10. Control flasks were prepared for comparison, containing plain buffer in place of substrate solution. The times which elapsed before appearance of colour were as follows. Phenol A 4 hrs. (faint); B 35 mins. *p*-Cresol A 2½ hrs.; B 25 mins. Tyrosine A 1 hr. (faint); B 15 mins. Catechol A 3 mins.; B 15 mins.; C 2½ hrs. Catechol in buffer alone acquired colour next day. In the other flasks no colour appeared for several

days, after which a pinkish colour appeared in the phenol and *p*-cresol C flasks, and the tyrosine C flask darkened slightly.

It appeared from these results as if the action of tyrosinase on monohydric phenols were accelerated, and on *o*-dihydric phenol retarded, by hydrogen peroxide. The concentration of hydrogen peroxide was then diminished proportionally to the dilution of the enzyme, and slight initial activation of action on catechol was detected. It was suspected that this might be due to increased aeration owing to breaking up of hydrogen peroxide by catalase in the mealworm preparation; the experiments were therefore repeated in large boiling-tubes, through which a current of oxygen, washed through water, was passed. The same results were obtained as in conical flasks. Action on catechol was compared with that on the corresponding monohydric phenol, phenol itself. In tube (1) were placed 40 cc. 0·2 % phenol in phosphate buffer, p_H 7·0, 4 cc. water, and 2 cc. enzyme preparation. In tube (2) 4 cc. 10-volume hydrogen peroxide replaced water in tube (1). In tubes (3) and (4) catechol replaced phenol in tubes (1) and (2) and both enzyme and hydrogen peroxide were diluted 1 in 100. Comparison tubes were prepared in which buffer replaced substrate solution. The times which elapsed before appearance of colour were as follows. Tube (1), 1 hr. Tube (2), less than ½ min. Tube (3), 3 mins. Tube (4) acquired colour after ½ min., but did not proceed with the rapidity of tube (3). After 5 mins., (3) and (4) were equally deeply coloured, then (3) gradually became deep yellow, while (4) became paler, and after 15 mins. was practically colourless. If, however, sufficient enzyme were present to produce sufficient colour, action of tyrosinase on phenol, catechol and *p*-cresol in presence of hydrogen peroxide was seen to produce a red colour in place of the yellow colour without hydrogen peroxide.

Action of tyrosinase on tyrosine was also compared with that on 3 : 4-dihydroxyphenylalanine. Various dilutions of enzyme and of hydrogen peroxide were used, identical in the two series. Colours appeared in the tubes with tyrosine very much more slowly than in those with 3 : 4-dihydroxyphenylalanine, otherwise the rates of appearance of colour and the fading observed were analogous to those described above for phenol and catechol. Slight fading of the tyrosine tubes containing hydrogen peroxide as well as of the dihydroxyphenylalanine tubes containing it was also observed after several days. H. Onslow [1917] working with tyrosinase from rabbits, activated with hydrogen peroxide, observed a similar fading. The above experiments suggest that the action of tyrosinase on monohydric phenols is more activated by means of hydrogen peroxide than is its action on *o*-dihydric phenols. The nature of the activation of action on catechol is not clear, but such activation needs to be obtained with an enzyme free from catalase before any deductions can be drawn from it.

It was shown as follows that the augmentation of the action of tyrosinase on monohydric phenols by means of hydrogen peroxide is not due to oxidation of inhibitory substances by the hydrogen peroxide. Mealworm tyrosinase

prepared by the centrifuge method was treated with an equal volume of 1 % hydrogen peroxide. It was allowed to stand for a few hours with occasional shaking, until no further bubbles came off. The product was diluted 1 in 5. A second sample of the same mealworm tyrosinase, to which no hydrogen peroxide had been added, was diluted 1 in 10. The test solutions were (1) 2·5 cc. 0·2 % *p*-cresol, (2) and (2 *a*) 2·5 cc. 0·2 % catechol, all in phosphate buffer, p_H 6·5. To each was added 0·3 cc. of the preparation, except to (2 *a*), to which 0·1 cc. was added. Tubes were prepared for comparison, containing buffer in place of substrate solution. The times which elapsed before appearance of colour were as follows. Treated with hydrogen peroxide (1) 2 hrs.; (2) $\frac{2}{3}$ min.; (2 *a*) $2\frac{1}{4}$ mins. Not treated (1) $1\frac{1}{2}$ hrs.; (2) $\frac{1}{2}$ min.; (2 *a*) $1\frac{3}{4}$ mins.

If hydrogen peroxide is the effective substance in the conversion of monohydric to *o*-dihydric phenol by tyrosinase, its action must be catalysed in some way. This was shown as follows. To 2·5 cc. saturated tyrosine in phosphate buffer, p_H 7·0, was added (1) 1 cc. water; (2) 1 cc. 0·006 % H_2O_2; (3) 1 cc. water; (4) 1 cc. 0·1 % H_2O_2. Similar mixtures were prepared using 0·2 % *p*-cresol and 0·2 % phenol in place of tyrosine. After 4 hours, the following additions were made: (1) 1 cc. 0·006 % H_2O_2; (2) 1 cc. water; (3) 1 cc. 0·1 % H_2O_2; (4) 1 cc. water. Comparison tubes were prepared containing buffer in place of substrate solution. To each tube was then added 0·5 cc. mealworm tyrosinase and the times required for appearance of colour were noted. Tyrosine (1) and (2) 45 mins.; (3) and (4) 10 mins. *p*-Cresol (1) and (2) 10 mins.; (3) and (4) 3 mins. Phenol (1) and (2) 2 hrs.; (3) and (4) 12 mins. Some tyrosine was recrystallised from phosphate buffer, p_H 7·0, four times and 20 cc. of each liquid was tested by addition to it of 1 cc. 1 % hydrogen peroxide. After a few days all tyrosine flasks to which hydrogen peroxide had been added were distinctly brownish. *p*-Cresol, twice distilled in a stream of carbon dioxide, as used in the work described in this paper, was similarly tested. To 20 cc. 0·2 % *p*-cresol was added 1 cc. 1 % hydrogen peroxide. After a few days a pink colour developed. Controls without hydrogen peroxide were uncoloured. Thus although hydrogen peroxide may be able to convert monohydric to *o*-dihydric phenol, it does not do so at the rate at which this conversion is brought about in presence of tyrosinase.

The lag of action of tyrosinase on a monohydric phenol can be not only diminished as already shown, but, by means of sulphite, can be lengthened. To a series of tubes containing 2·5 cc. 0·2 % *p*-cresol in phosphate buffer, p_H 6·5, was added (1) 1 cc. and (2) 0·5 cc. 0·005 % sodium sulphite solution in phosphate buffer, p_H 6·5. Another tube (3) contained no sodium sulphite. For each tube a comparison tube was prepared, containing buffer in place of *p*-cresol solution. Each mixture was made up to 4·5 cc. with buffer, and 0·5 cc. tyrosinase was added, diluted 1 in 10. Tube (3) acquired colour after 1 hour, tube (2) after 4 hrs., tube (1) a faint trace of colour next day. The action of tyrosinase on catechol was also retarded by sulphite, but to a less extent. Sulphite of concentration 0·005 % had no detectable effect. Using

0·2 % catechol in phosphate buffer, p_H 6·5, and 0·05 % sodium sulphite solution, tube (3) acquired colour after 1½ mins., (2) 6 mins., (1) 20 mins.

Discussion.

Experiments described in this paper show that there is no evidence that tyrosinase can be obtained in a form which does not act on monohydric phenols in absence of some coenzyme or activator. The failure of attempts made to obtain it in such a form appears to indicate that tyrosinase in presence of molecular oxygen is able to act on the monohydric phenols which it attacks without any coenzyme being necessary. This conclusion is borne out by the fact that neither *ortho*quinone nor hydrogen peroxide without any catalyst is able to convert monohydric to *o*-dihydric phenol at the observed rate of oxidation of monohydric phenols by tyrosinase. Such a conclusion does not preclude the possibility of the existence of another enzyme able to oxidise only *o*-dihydric phenols, and corresponding to the "oxygenase" of Onslow [1920]. It is found that a preparation obtained from a large black slug, *Arion ater*, by grinding it with sand, and extracting with phosphate buffer, p_H 8·0, causes oxidation of catechol and of 3 : 4-dihydroxyphenylalanine, but not of monohydric phenols, even on addition of homoquinone water or hydrogen peroxide. Happold [1930] has found that certain bacteria contain enzymes able to cause oxidation of catechol and not of the monohydric phenols attacked by tyrosinase. These facts suggest that there is present in tyrosinase preparations a specific enzyme which causes the oxidation of certain monohydric phenols to take place, as well as the less specific oxidation of *o*-dihydric phenols.

The action of tyrosinase on monohydric phenols is, however, hastened by some product of its action on *o*-dihydric phenols, and both *ortho*quinone and hydrogen peroxide seem able to act in this way. The fact that 3 : 4-dihydroxyphenylalanine will serve as activator, although its *ortho*quinone does not yield the usual reactions of an *ortho*quinone, suggests that hydrogen peroxide is produced by the action of tyrosinase, and is the activator in this instance. With catechol it appears possible that both *ortho*quinone and hydrogen peroxide may serve as activators. Any source of either of these peroxides would therefore cause activation. Experiments described in this paper show that there is present in the tyrosinase preparations used some substance able to hasten the action of tyrosinase on monohydric phenols, which is not removed by dialysis, is relatively stable to heating and to storage, and is more affected by cyanide and by sulphite than is tyrosinase itself. The retarding effect of sulphite is explicable as counteracting the effect of peroxides. The fact that this activator is found in old preparations in large quantity relative to the amount of enzyme, and that peroxidase was found by Pugh [1929] to increase the activity only of an old preparation of tyrosinase from mealworms, suggests that the activator may be of the nature of the precursor of a peroxide. Nevertheless, tyrosinase can occur and act in absence of a peroxide such as is necessary for the action of peroxidase. This is shown by the following con-

siderations. It is found that pieces of banana skin cause oxidation of tyrosine and of *p*-cresol, but not of guaiacol until hydrogen peroxide is added. This shows that no peroxide is present, although both peroxidase and active tyrosinase are present. Hence tyrosinase does not require such a peroxide to enable it to act on monohydric phenols, and does not consist of a peroxide together with a specific peroxidase. Molecular oxygen appears to suffice for the action of tyrosinase, although the action may be hastened by a peroxide.

SUMMARY.

1. Attempts to separate tyrosinase into two enzymes have yielded negative results.

2. Attempts to separate tyrosinase into enzyme proper and a coenzyme which would be responsible for its action on monohydric phenols have yielded negative results.

3. Moderate heating, or storage, of certain tyrosinase preparations increases their activity on monohydric relative to *o*-dihydric phenols. Cyanide or sulphite has the contrary effect.

4. The action of tyrosinase on monohydric phenols is hastened by water containing homoquinone.

5. The action of tyrosinase on monohydric phenols is hastened by hydrogen peroxide in small concentration. Slight initial activation of action on *o*-dihydric phenol has also been observed; its cause is uncertain.

6. There is no evidence that tyrosinase alone in presence of molecular oxygen is not able to oxidise monohydric phenols.

7. The initial acceleration of the action of tyrosinase on monohydric phenols by traces of *o*-dihydric phenols appears to be due to peroxide formation, either hydrogen peroxide or both hydrogen peroxide and *ortho*quinone being responsible.

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