CLXXX. THE ACTION OF TYROSINASE ON PHENOLS.

WITH SOME OBSERVATIONS ON THE CLASSIFICATION OF OXIDASES.

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It has been known for many years that tyrosinase will bring about the oxidation of certain phenols, and the reaction with *p*-cresol has been used by Chodat [1912] as an effective means of identifying the enzyme. In the presence of amino-acids the reaction with *p*-cresol produces a series of brilliant colour changes, and in addition deamination of the amino-acid takes place. It was shown by Happold and Raper [1925], and almost at the same time by Robinson and McCance [1925], that the deamination was not due, as Chodat thought, to a direct action of tyrosinase on the amino-acid, but to the intervention of the phenol in the reaction. Happold and Raper [1925] brought forward evidence that *ortho*-quinones were first produced from those phenols which were attacked by tyrosinase, and that the deamination produced in the system, tyrosinase-phenol-amino-acid, was due to action of the *o*-quinone thus produced on the amino-acid.

It was shown at that time that *o*-benzoquinone would bring about the deamination of alanine and glycine under the same conditions as to reaction of the medium and temperature, which prevailed in the enzyme action. This evidence has been extended in the present paper by showing that *o*-homoquinone behaves in a like manner towards glycine. Additional indirect evidence of the formation of *o*-quinones from phenols by the action of tyrosinase has been put forward by Raper [1927] in explaining the action of this enzyme on tyrosine. In the series of changes which take place in this reaction, and which result finally in the production of melanin, it seems very probable that the 3.4-quinone of phenylalanine is one of the intermediate products. It has been shown by Onslow and Robinson [1925] that the system *p*-cresol-tyrosinase, and by Szent-Györgyi [1925] that *o*-quinone, turns tincture of guaiacum blue, and since the product of the action of tyrosinase on catechol

and certain other phenols will turn guaiacum blue it has been suggested by the latter that *o*-quinones are produced by the action of the enzyme on these phenols.

Since all this evidence is indirect it seemed desirable to establish it more securely, if possible, by the isolation of compounds of *o*-quinone from the reaction system. *o*-Benzoquinone is a very unstable substance and can be prepared only in dry ether, and *o*-homoquinone is not much more stable. It seemed likely therefore that these substances, if produced by the action of tyrosinase on phenol or *p*-cresol, would have only a transient existence, and that to isolate them some substance must be used to withdraw them from the sphere of reaction. Aniline, upon which tyrosinase has no action, was found to be suitable since it forms with quinones insoluble anilinoquinones. Benzenesulphinic acid, which has been shown by Hinsberg and Himmelschein [1896] to combine with *o*-benzoquinone, was also tried instead of aniline with the hope of being able to isolate such a compound from the products of the action of tyrosinase on phenol and on catechol. It was however successful only with catechol.

The anilinoquinones produced, as described above, by the action of tyrosinase on phenol, catechol, *p*-cresol, *m*-cresol, and homocatechol in presence of aniline, have been isolated, and identified by synthesis. It has also been shown by the same method that peroxidase and hydrogen peroxide acting on catechol and on homocatechol produce *o*-benzoquinone and *o*-homoquinone respectively. It seems, therefore, to be clear that the first stages in the action of tyrosinase on those monohydric phenols on which it acts are the introduction of a second hydroxyl group in the *o*-position to the first, thus producing a catechol derivative, and then oxidation of this to the corresponding *o*-quinone. Peroxidase and hydrogen peroxide also bring about the latter reaction.

Not all phenolic substances which are attacked by tyrosinase give rise to anilinoquinones when the enzyme acts on them in the presence of aniline, but *m*- and *p*-methoxyhydroxybenzene do so. Tyrosine and pyrogallol are examples of substances which, though oxidised by the enzyme, do not yield anilino-compounds under these conditions. There is good evidence, however, that in both these instances *o*-quinones are produced, but, owing to further reactions which these undergo, *i.e.* the formation of indole derivatives from tyrosine [Raper, 1927], and of purpurogallin from pyrogallol [Willstätter and Heiss, 1923], combination with aniline does not take place.

By means of the microrespirometer, some indication has been obtained of the quantitative relations which hold between the amounts of oxygen taken up and of phenols oxidised. The results show that in those instances in which it has been proved that *o*-quinones are formed, the oxygen taken up is greater than the amount required for the formation of the *o*-quinone. The enzyme preparation used in these experiments contained catalase, so that the excess oxygen uptake over and above that which is required for the

formation of the *o*-quinone, cannot be accounted for simply by the formation of hydrogen peroxide as represented, for example, in the reaction

in which oxygen is acting as a hydrogen acceptor. It would appear that the *o*-quinones take part in some further reaction or reactions which involve oxidation. The nature of these has not yet been discovered.

The results described in this paper together with those obtained by other workers in this field during the past few years suggest that the hypothesis as to the nature of the "direct oxidases" which was put forward by Bach and Chodat [1903] requires revision. The "direct oxidases" are usually detected by the fact that they will turn guaiacum tincture blue without the addition of hydrogen peroxide or other peroxides. This distinguishes them from peroxidases or "indirect oxidases" which only give this reaction when peroxides are added. Bach and Chodat believed that peroxidases were an essential component of the "direct oxidases," and that the reason why the latter turned guaiacum blue directly was that a second component was present which in presence of air produced a peroxide. Thinking that this second component was enzyme-like in its action they named it oxygenase. But we now know that peroxidase is not an essential component of the "direct oxidases." It has been shown by Szent-Györgyi [1925] that *o*-quinone will turn guaiacum blue, and in the present paper proof is presented that *o*-quinones are produced by the action of tyrosinase on certain phenols, some of which, *e.g.* catechol and some of its derivatives, are known to be present in plants. Furthermore, thermostable catalysts known as polyphenolases have been prepared from plant products by Euler and Bolin [1908] and Wieland and Fischer [1926], which oxidise dihydric phenols to quinones with the formation of hydrogen peroxide. In the presence of catechol or some catechol derivative and even in the absence of peroxidase, these catalysts would cause the direct blueing of guaiacum because of the production of *o*-quinones. Since these systems give the guaiacum reaction it has therefore ceased to be an accurate guide to the presence of a "direct oxidase" of the kind postulated by Bach and Chodat. Altogether four different oxidation systems have now been shown to give the "direct oxidase" reaction.

(1) Peroxidase + an autoxidisable substance which produces hydrogen peroxide or an organic peroxide in air. Gallagher [1923] has found a system of this type in the potato, the autoxidisable substance being a lipin or a mixture of lipins. It seems likely that many unsaturated substances, *e.g.* terpenes or unsaturated fats or fatty acids would behave likewise. Bertrand's laccase probably comes into this group.

(2) Peroxidase + an oxidoreductase and its substrate. An example of this system is that investigated by Thurlow [1925] in her studies of the xanthine oxidase of milk, which, when acting on xanthine or hypoxanthine in presence of air, gives rise to the production of hydrogen peroxide.

(3) Peroxidase + a polyphenolase with a polyphenol as substrate. Euler and Bolin [1908] have found a system of this type in *Medicago sativa*. Their polyphenolase was thermostable and was apparently a mixture of calcium salts of organic acids. Its action could be imitated by a mixture of calcium glycollate and manganese acetate. If the polyphenolic substrate is a catechol derivative, the peroxidase is not necessary to give the "direct oxidase" reaction, since the *o*-quinone produced by the action of the polyphenolase would blue guaiacum by itself. In this group would come also the thermostable polyphenolase isolated by Wieland and Fischer [1926] from *Lactarius*. This was different in composition from that obtained by Euler and Bolin but exerted the same type of action.

(4) Tyrosinase + one of its substrates (tyrosine, 3, 4-dihydroxyphenylalanine and pyrogallol excepted). This is the system described in the present paper.

It is clear from the composition of these various systems that the term "direct oxidase" is a misnomer if, by it, we wish to denote only one particular oxidising enzyme or oxidation system, and it would be better to abolish it. The "direct oxidase" reaction is essentially a test for the presence of those oxidases and thermostable oxidation catalysts which will bring about oxidation only in the presence of oxygen or peroxides, and the authors suggest that they be called *aerobic oxidases*. These agents are different from the oxidoreductases, which will act in the absence of oxygen provided a hydrogen acceptor is present, and which may be called *anaerobic oxidases*. The oxidoreductases are believed to act by activating hydrogen, but whether this is also true of the aerobic oxidases is at present uncertain. Wieland and Fischer [1926] suggest that the polyphenolase which they have investigated acts by activating hydrogen and not by activating oxygen in the sense that certain metallic ions are supposed to do. Their suggestion of the mechanism is that the catalyst, polyphenol and molecular oxygen unite to form an unstable compound which then breaks down, giving the quinone and hydrogen peroxide. Whether tyrosinase acts in this way is not yet decided in view of the lack of experimental evidence; but Willstätter and Weber [1926] explain the action of peroxidase by the formation of a compound between the enzyme and hydrogen peroxide in which the oxygen is more active than in hydrogen peroxide itself. Although it is clear therefore that the aerobic oxidases act only in the presence of oxygen or a peroxide, it is by no means decided whether they activate oxygen or hydrogen.

In addition to the hypothesis regarding the constitution of the "direct oxidases" which was put forward by Bach and Chodat, an alternative scheme has been suggested by Onslow [1920]. It is that the oxygenase of Bach and Chodat is really a mixture of an enzyme proper—to which Onslow applies the name oxygenase—and a catechol derivative. This view was based essentially on the separation from plants that give the "direct oxidase" reaction, of an alcohol-insoluble enzyme and an alcohol-soluble substance which gave

the typical green colour with ferric chloride which is characteristic of catechol derivatives. Neither of these alone gave a definite "direct oxidase" reaction, but when combined gave it very markedly. The results described in the present paper, however, show that tyrosinase has all the properties ascribed by Onslow to oxygenase and the system she describes is really that of tyrosinase with a catechol derivative. The polyphenolases of Euler and Bolin and of Wieland and Fischer would also give Onslow's oxygenase reaction. There is no evidence, therefore, that this is a new enzyme, and it seems unnecessary to retain the term oxygenase in the sense used by Onslow.

Nor does this term convey a precise meaning in the sense in which Bach and Chodat first employed it, because, as shown above, it may represent a different component in each one of the four known "direct oxidase" systems.

It is suggested, therefore, that in view of the present state of our knowledge, oxidases should be provisionally divided into an aerobic and an anaerobic group. Those of the former act only in the presence of oxygen or a peroxide, and comprise peroxidase, tyrosinase, and the thermostable catalysts known as polyphenolases. Those of the latter include the known oxidoreductases, and the thermostable catalysts which act in conjunction with glutathione. They will act anaerobically provided a suitable hydrogen acceptor is present.

EXPERIMENTAL.

The enzyme used in this investigation was prepared from mealworms as described by Raper [1926]. In experiments in which enzyme was used, chloroform (unless otherwise stated) was added as antiseptic. The p_H of solutions was adjusted by means of phosphate buffer.

The isolation of anilino-o-quinones.

o-Quinones being highly reactive and unstable substances, it was not expected that it would be possible to isolate them as such from solutions of phenols which had been submitted to the action of tyrosinase. Attempts were therefore made to remove them from the sphere of reaction by introducing aniline into the system and isolating them as anilinoquinones. It was found that by the action of tyrosinase on phenol in presence of aniline a bright red precipitate was obtained, which crystallised from ether as bright red needles having the characteristics of an anilinoquinone. Tests showed that a maximum yield of this deposit was obtained at p_H 6.5-7.0. The experiments were therefore carried out at p_H about 6.7. The action of tyrosinase in presence of aniline was investigated first of all on phenol and on catechol, then on the cresols and on homocatechol.

Controls. A preliminary control showed that aniline in phosphate buffer at p_H 6.7 is unchanged by tyrosinase. Phenol in buffered aniline water remained unchanged. Catechol became slowly pink, and after some time gave a reddish brown precipitate, this being due presumably to autoxidation to

o-benzoquinone which then reacts with the aniline. In presence of hydrogen peroxide the oxidation was more rapid. *p*-Cresol and *m*-cresol in buffered aniline water remained unchanged. Homocatechol underwent fairly rapid oxidation, a red substance and some tar being produced by reaction with aniline. The oxidation was hastened by hydrogen peroxide, but in no case was the product obtained nearly so rapidly or definitely as when enzyme was present.

Buffered solutions of phenol and catechol became darkened by the action of tyrosinase, and catechol was also darkened by the action of peroxidase and hydrogen peroxide. Buffered solutions of *p*-cresol, *m*-cresol, and homocatechol by the action of tyrosinase, and of homocatechol by the action of peroxidase and hydrogen peroxide, became orange-yellow, orange-brown, or reddish brown.

Action of tyrosinase on phenol in presence of aniline.

6 g. phenol and 12 cc. aniline were dissolved in about 1.5 l. of buffer solution, and 120 cc. tyrosinase solution were added. The liquid was placed in two 2 l. flasks, and gently aerated with air drawn through a wash-bottle containing chloroform and water. In a few minutes a red colour developed, the liquid clouded, and later red crystalline needles separated out. After two days the precipitate was filtered off on fluted filters, washed with 1 % HCl to remove excess aniline, then with water, and dried *in vacuo*. In cases where the filtrate was still reddish coloured, a further yield was obtained by acidification with 1 % HCl, or precipitation with CaCl_2 and 1 % NH_4OH , or by extraction with ether and evaporation of the ether. The total dried substance was extracted in a Soxhlet apparatus with acetone or light petroleum. The latter was slower in its solvent action. On evaporation of the solution bright red needles separated out. The product was purified by recrystallisation from acetone or light petroleum, or alternatively, by solution in ether and extraction with 0.02 *N* NaOH, the aqueous liquid being acidified with 2 % HCl, and the red precipitate filtered off and crystallised from acetone. Yield, 4.5 g. Properties: bright red needles; m.p. 193° ; soluble in organic solvents; soluble in concentrated sulphuric acid, giving an olive green colour, becoming red on dilution by reprecipitation; soluble in alkali and alkali carbonate, giving a purplish red solution, reprecipitated by acid.

Analysis. Found: C, 74.58 %; H, 5.08 %; N, 9.57 %.

Calculated for dianilino-*o*-benzoquinone, $\text{C}_{18}\text{H}_{14}\text{O}_2\text{N}_2$:

C, 74.48 %; H, 4.83 %; N, 9.66 %.

Action of tyrosinase on catechol in presence of aniline.

2 g. catechol, 4 cc. aniline, and 40 cc. tyrosinase solution were made up to nearly a litre in buffered solution, and aerated. Most of the precipitate was obtained the first day. The product was treated in the same way as that

obtained from phenol. Yield, 1.75 g. Properties: identical with those of the product obtained from phenol; m.p. 193°. Mixed m.p. with the product obtained from phenol, 193°.

Analysis. Found: N, 9.61 %.

Calculated for $C_{18}H_{14}O_2N_2$: N, 9.66 %.

*Action of peroxidase and hydrogen peroxide on catechol
in presence of aniline.*

As was expected, catechol is oxidised by peroxidase and hydrogen peroxide in presence of aniline with production of the same substance as is obtained by the action of tyrosinase on phenol and on catechol in presence of aniline. The peroxidase used was prepared by slicing horseradish into chloroform water, avoiding long exposure to air, and rejecting the outer portions. Only preparations were used which remained practically unchanged by aniline water in presence of hydrogen peroxide. 2 g. catechol and 4 cc. aniline were made up to nearly a litre, about 300 cc. peroxidase and 70 cc. 1 % hydrogen peroxide were added. A red precipitate appeared very rapidly. The product was treated in the same way as that obtained from phenol. Yield, 1.15 g. Properties: identical with those of the product obtained from phenol; m.p. 193°. Mixed m.p. with the product obtained from phenol, 193°; with that from catechol by tyrosinase, 192.5°.

Analysis. Found: N, 9.63 %.

Calculated for $C_{18}H_{14}O_2N_2$: N, 9.66 %.

Synthesis of dianilino-o-benzoquinone.

o-Benzoquinone in ethereal solution was synthesised by the method of Willstätter and Müller [1908] using 2.5 g. catechol. The ethereal solution was poured into a solution of 6 cc. aniline in 1700 cc. water, and shaken. A red precipitate separated out, which was filtered off. It was treated in the same way as the product obtained from phenol. Properties: identical with those of the products obtained from phenol and catechol; m.p. 193.5°. Mixed m.p. unchanged.

Analysis. Found: C, 74.47 %; H, 5.05 %; N, 9.67 %.

Calculated for $C_{18}H_{14}O_2N_2$: C, 74.48 %; H, 4.83 %; N, 9.66 %.

This proves the identity of the products obtained by enzyme action with dianilino-*o*-benzoquinone, and indicates therefore that *o*-benzoquinone is produced from phenol and from catechol by the action of tyrosinase, and from catechol by the action of peroxidase and hydrogen peroxide.

Dianilino-*o*-benzoquinone has been synthesised by Kehrmann and Cordone [1913] by oxidising catechol with silver oxide in glacial acetic acid solution containing aniline, the product being poured into water. The properties described are identical with the above.

The reactions taking place in the experiments described above may therefore be represented as follows:

Action of tyrosinase on p-cresol in presence of aniline.

6 g. *p*-cresol, 15 cc. aniline, and 100 cc. tyrosinase solution were made up to about a litre in buffered solution and aerated. The liquid became reddish, then a brownish red precipitate appeared. The major part of the substance remained too finely divided to be filtered off, so it was coagulated, or else extracted, as described for the filtrate in the case of phenol. The product was washed, dried, and extracted and crystallised by means of acetone. Some tarry substance was produced besides the anilinoquinone. The extraction was therefore fractionated; the tar, being exceedingly soluble in acetone, was removed in the earlier portions of the extract. Yield, about 1 g. of crystals. Properties: brownish red needles; m.p. 202°; soluble in organic solvents, but much less soluble in light petroleum than the product obtained from the lower homologue; soluble in concentrated sulphuric acid, giving a brownish crimson colour, becoming brown then yellow on dilution; insoluble in alkali.

Analysis. Found: C, 79.15 %; H, 5.55 %; N, 10.92 %.

Calculated for dianilino-homoquinoneanil, $\text{C}_{25}\text{H}_{21}\text{ON}_3$:

C, 79.15 %; H, 5.54 %; N, 11.08 %.

Action of tyrosinase on m-cresol in presence of aniline.

6 cc. *m*-cresol, 15 cc. aniline, and 100 cc. tyrosinase solution were made up to about a litre in buffered solution, and aerated. The product was treated in the same way as that obtained from *p*-cresol. Yield, less than 1 g. owing to the tarry nature of part of the extract. Properties: identical with those of the product obtained from *p*-cresol; m.p. 201.5°. Mixed m.p. unchanged.

Analysis. Found: C, 78.75 %; H, 5.57 %; N, 11.01 %.

Calculated for $\text{C}_{25}\text{H}_{21}\text{ON}_3$: C, 79.15 %; H, 5.54 %; N, 11.08 %.

Action of tyrosinase on homocatechol in presence of aniline.

The homocatechol used was prepared from creosol by the method of de Vries [1909], the creosol being prepared from vanillin. 1 g. homocatechol, 3 cc. aniline, and 40 cc. tyrosinase solution were made up to 150 cc. in buffered solution and aerated. The action was rapid and the yield remarkably good—about 0.9 g.—very little tar being obtained. Properties identical with those of the product obtained from *p*-cresol; m.p. 202°. Mixed m.p. with synthetic product (*vide infra*), 202°.

Analysis. Found: N, 10.80 %.

Calculated for $\text{C}_{25}\text{H}_{21}\text{ON}_3$: N, 11.08 %.

*Action of peroxidase and hydrogen peroxide on homocatechol
in presence of aniline.*

0.5 g. homocatechol and 1.5 cc. aniline were dissolved in about 100 cc. buffer solution, and 90 cc. peroxidase and 18 cc. 2 % H_2O_2 added. The liquid became red and clouded, but no precipitate appeared. It was extracted with ether, the extract was dried over anhydrous sodium sulphate, filtered, and the filtrate evaporated, crystals being obtained which were recrystallised from acetone. Properties: identical with those of the product obtained from *p*-cresol; m.p. 201° . Mixed m.p. with the product obtained from *m*-cresol, 201° .

Analysis. Found: N, 11.04 %.

Calculated for $\text{C}_{25}\text{H}_{21}\text{ON}_3$; N, 11.08 %.

Synthesis of dianilinohomoquinoneanil.

The following method was found to obviate the production of tar. Homoquinone in ethereal solution was synthesised by the method of Willstätter and Müller [1911], using 6 g. homocatechol. The solution was dropped slowly into a solution of 20 cc. aniline in water, mechanically stirred. The liquid became purplish, then almost black. On standing overnight, it became cloudy and brick red, and deposited a tarry solid. The lumps of solid matter which had separated were washed with a little ether and crystallised from acetone. A further yield was obtained by extracting the liquid with ether. This extract was dried over anhydrous sodium sulphate, filtered, and the filtrate evaporated on the water-bath, then left to stand in the vacuum desiccator, when crystals were deposited which were recrystallised from acetone. Total yield, about 2.5 g. Properties: identical with those of the products obtained from *p*-cresol, *m*-cresol, and homocatechol; m.p. 202.5° . Mixed m.p. unchanged.

Analysis. Found: C, 79.01 %; H, 5.67 %; N, 11.35 %.

Calculated for $\text{C}_{25}\text{H}_{21}\text{ON}_3$: C, 79.15 %; H, 5.54 %; N, 11.08 %.

The synthesis of dianilinohomoquinoneanil was also carried out by oxidising homocatechol with silver oxide in glacial acetic acid in presence of aniline, the product being poured into water. A method was also used analogous to that of Jackson and Koch [1898] for the preparation of dianilinoquinoneanil; homoquinone with aniline in chloroform was boiled on the water-bath for 10 minutes. The substances obtained by both these methods were identical with that obtained by the action of an ethereal solution of homoquinone on aniline water.

The identity of the products obtained by enzyme action with dianilinohomoquinoneanil is thus proved, and this indicates that homoquinone is produced from *p*-cresol, *m*-cresol, and homocatechol by the action of tyrosinase, and from homocatechol by the action of peroxidase and hydrogen peroxide.

The reactions taking place in the experiments just described may therefore be represented in a precisely similar manner to those with phenol (p. 1377).

The action of tyrosinase on other phenols.

The action of tyrosinase was tried on a number of other phenolic substances alone and in the presence of aniline. The following observations were made at p_H 6.5 or 7.0 after a period of 1 day, the phenols used being at concentrations of about 0.2 % or 0.1 % or, if only very slightly soluble, in saturated solution.

Substrate used	Substrate in buffer alone	Substrate in buffer with tyrosinase	Substrate in buffer with tyrosinase and aniline water	Substrate in buffer with aniline water
Quinol* (p_H 7.0)	Pink	Dark brown	Yellow	Yellow
" (p_H 6.0)	Faintly pink	"	"	"
Pyrogallol	Pale yellow	Darkened Brownish ppt. giving purpuro-gallin reaction with ammonia	Darkened Brownish ppt. No anilinoquinone	Pale yellow
<i>m</i> -Methoxyhydroxybenzene	Nil	Reddish brown	Bright red ppt. of anilinoquinone	Very faintly coloured
<i>p</i> -Methoxyhydroxybenzene	Nil	Reddish brown	Bright red ppt. of anilinoquinone	Very faintly coloured
<i>p</i> -Aminophenol	Brown ppt.	Brown ppt. Darkened	Brown ppt. Darkened	Brown ppt.
Tyrosine	Nil	Dark brown	Brown. No anilinoquinone	Nil

* The oxidation of quinol appears to be inhibited unless the solution is dilute and the enzyme very active. A similar phenomenon may possibly occur with guaiacol. The matter has not yet been fully investigated. The yellow colour with quinol and aniline is due to an addition compound.

No reactions were observed with the following: resorcinol, phloroglucinol, guaiacol, *o*-cresol, orcinol, creosol, eugenol, *iso*-eugenol, *o*-, *m*-, and *p*-nitrophenol, and thymol. Vanillin and salicylaldehyde reacted as aldehydes with aniline, the other tests being negative.

Thus the only further substances found to give rise to anilino compounds were *m*- and *p*-methoxyhydroxybenzene, which were attacked apparently in an analogous manner to *m*- and *p*-cresol. In presence of aniline bright red substances were obtained, having the properties of an anilinoquinone: they were soluble in concentrated sulphuric acid, giving a brownish colour, reprecipitated on dilution; soluble in alkali, giving a purplish red solution, reprecipitated on acidification.

Action of tyrosinase on catechol in presence of benzenesulphinic acid.

The addition product of *o*-benzoquinone with benzenesulphinic acid was obtained by Hinsberg and Himmelschein [1896] by the oxidation of catechol in presence of benzenesulphinic acid.

An attempt was made to obtain this compound by the action of tyrosinase on phenol, and also on catechol, in presence of benzenesulphinic acid. The attempt was successful in the case of catechol, but not in that of phenol. *o*-Dihydroxydiphenylsulphone (m.p. 165°) was found to be very feebly soluble in benzene, in which phenol, catechol, and benzenesulphinic acid are all readily soluble. The product of action of tyrosinase on catechol in presence of benzenesulphinic acid was isolated by making use of these facts.

0.2 g. catechol and 0.3 g. benzenesulphinic acid were dissolved in water, and the p_H adjusted with phosphate buffer to 6.0–6.5. The solution was made up to 100 cc., 3 cc. tyrosinase solution were added, and the liquid was allowed to stand for a few days, with frequent shaking. It was then extracted with ether and the ether evaporated. The residue was boiled under reflux with benzene for a minute or two and the white residue filtered off. This was dissolved in boiling water, from which it separated as crystals on cooling. The crystals were dried in a desiccator over concentrated sulphuric acid *in vacuo*, water of crystallisation being thereby removed. Properties: white powder, m.p. 164°; mixed m.p. with *o*-dihydroxydiphenylsulphone, unchanged; aqueous solutions of both gave a blue-green colour with ferric chloride, changed to red by sodium carbonate.

Several attempts, including the use of increased aeration, were made to obtain the same substance by the action of tyrosinase on phenol in presence of benzenesulphinic acid; but in every case the residue on evaporation of the ether was readily entirely soluble in benzene in the cold. Thus there is no evidence that tyrosinase will oxidise phenol in presence of benzenesulphinic acid. It is possible that a higher oxygen potential is required for the oxidation by tyrosinase of phenol than of a catechol, and that the reducing action of benzenesulphinic acid prevents this potential from being reached. But this can be determined only by further experiment.

Deamination of glycine by homoquinone.

The method used was essentially that described by Happold and Raper [1925], a funnel being used to run in the solutions, and homoquinone taken in place of *o*-benzoquinone. 0.2 g. solid homoquinone dissolved in ether was run into 0.5 g. glycine in buffer at p_H 7.0. The reaction mixture was aerated gently for a day, any ammonia escaping in this process being absorbed in $N/20$ H_2SO_4 . The whole of the ammonia produced was finally driven over into the absorption bottles by running into the reaction bottle 1.5 g. sodium carbonate in water, the stream of air being then continued for several hours.

The amount of acid neutralised by ammonia produced during the reaction

corresponded to 0.06 g. homoquinone, *i.e.* 30 % of the homoquinone used, the reaction

$\text{CH}_3 \cdot \text{C}_6\text{H}_3\text{O}_2 + \text{NH}_2 \cdot \text{CH}_2 \cdot \text{COOH} + \text{H}_2\text{O} = \text{NH}_3 + \text{CO}_2 + \text{H} \cdot \text{CHO} + \text{CH}_3 \cdot \text{C}_6\text{H}_5\text{O}_2$
being assumed. Another experiment gave 34 %.

The homoquinone does not, therefore, yield the equivalent of ammonia. This may be due partly to combination of the ammonia and formaldehyde to give hexamethylenetetramine, and partly to other reactions which lead to the production of highly coloured substances, since during aeration the liquid in the reaction bottle became deeply coloured, being blue by transmitted, red by reflected light. It was similar to a liquid obtained by the action of tyrosinase on *p*-cresol in presence of glycine (Chodat's reaction). Both on addition of alkali became reddish, and on addition of acid blue.

Measurements of oxygen absorption.

In most cases colour changes in the solution, and in some cases the formation of an anilinoquinone in presence of aniline, serve to determine whether a given phenol is oxidised by tyrosinase or not. In certain cases recourse was had to a microrespirometer of the Barcroft type, in which carbon dioxide was absorbed. The following pairs of mixtures were shaken side by side. Respirometer I. Left: the phenol used; phosphate buffer; tyrosinase. Right: buffer; tyrosinase. Respirometer II. Left: the phenol used; buffer. Right: buffer alone. Any autoxidation taking place could thus be detected. At the commencement of each experiment, the apparatus was equilibrated by shaking for 5 minutes with the taps open; the results obtained are therefore minimum values, since in some cases a small amount of oxygen would be absorbed during equilibration. The quantity of phenol used was 1 or 2 mg. Solid phenylurethane or thymol was added to each flask as anti-septic, the enzyme used being freed from chloroform *in vacuo*. The activity of the enzyme at the end of the experiment was tested by addition of *p*-cresol to the left flask of Respirometer I.

The amounts of oxygen absorbed by phenols attacked by tyrosinase were measured in this way, and calculated in terms of atoms of oxygen per molecule of phenol, some indication being thus obtained of the extent of oxidation taking place. The following substances were examined in the respirometer,

Substrate	p_{H}	Atoms of oxygen absorbed per molecule of substrate
Phenol ... \ ...	7.6	3.4 +
<i>o</i> -Cresol ... \ ...	7.6	Nil
<i>m</i> -Cresol ... \ ...	7.0	2.9
<i>p</i> -Cresol ... \ ...	7.6	2.6
" ... \ ...	7.8	3 \ Robinson and
Catechol ... \ ...	7.8	2 \ McCance
Resorcinol ... \ ...	7.0	Nil
Quinol ... \ ...	6.0	3.0
Pyrogallol ... \ ...	6.0	1.5
<i>m</i> -Methoxyhydroxybenzene (slightly impure)	7.0	2 +
<i>p</i> -Methoxyhydroxybenzene	7.0	3.0

with the results stated. Two complementary results obtained by Robinson and McCance [1925] are included.

p-Aminophenol hydrochloride, brought to p_H 6.0 with NaOH, and buffered, showed autoxidation, but a definite increase in oxygen absorption in presence of tyrosinase. Thymol was also tested, its absorption being nil.

The figure 1.5 for pyrogallol was obtained after deduction of slight absorption due to autoxidation. The absorption of three atoms of oxygen per two molecules of pyrogallol corresponds to the formation of purpurogallin.

The experiments of Robinson and McCance [1925], in which slight oxygen absorption was obtained with resorcinol in presence of tyrosinase and a delayed but considerable absorption when glycine was also present, were repeated, but no confirmation of their results was obtained. In most cases the enzyme was inactivated by resorcinol after a few days, but in a case in which it remained active no absorption was observed.

Catechol, *m*- and *p*-cresol, *m*- and *p*-methoxyhydroxybenzene all show an absorption per molecule of one atom of oxygen more than that corresponding to formation of *o*-quinone. Phenol shows an even greater absorption. This appears to indicate that some further reaction involving oxidation occurs. In absence of some substance with which they combine, the *o*-quinones appear to undergo further change almost at once. They are found merely in traces (as judged by the aniline reaction) when the action of the enzyme on the phenol has been allowed to go to completion.

SUMMARY.

1. Anilino-*o*-quinones produced by the action of tyrosinase on several phenols in presence of aniline have been isolated. The substances obtained from phenol and catechol were identical. The substances obtained from *m*-cresol, *p*-cresol, and homocatechol were also identical. This indicates clearly that *o*-quinones are produced by the action of tyrosinase on these phenols.

2. Peroxidase and hydrogen peroxide, acting on catechol or homocatechol, also produce *o*-quinones.

3. Further evidence indicating the formation of *o*-quinones consists in the isolation of a sulphinic acid derivative of *o*-benzoquinone by the action of tyrosinase on catechol in presence of benzenesulphinic acid.

4. Homoquinone deaminates glycine in the same manner as does *o*-benzoquinone.

5. *m*- and *p*-Methoxyhydroxybenzene were the only phenols, in addition to those mentioned above, which were found to give rise to anilinoquinones.

6. The oxygen absorbed during the oxidation by tyrosinase of several phenols has been measured, indications being obtained with those phenols giving rise to anilinoquinones that, in absence of aniline, some further reaction involving oxidation occurs beyond formation of the *o*-quinone.

7. The "direct oxidase" theory of Bach and Chodat is criticised in the light of the above results, and a new classification of oxidases into aerobic and anaerobic oxidases is suggested.

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