CXXIX. THE ACTION OF INHIBITORS ON THE CATECHOL OXIDASE OF POTATOES.

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ATTEMPTS have been made to classify the phenolases into groups such as the *catechol oxidases, tyrosinases,* and *laccases* [Raper, 1928], but the classification is made difficult by the lack of satisfactory methods of characterising the enzymes [Onslow and Robinson, 1928].

The present work was undertaken with the object of testing whether the catechol oxidases of different origin behave similarly towards inhibitors, or whether they show any marked differences of behaviour which might be used as a further guide in classifying these enzymes.

Catechol oxidases are found widely distributed in plants, fungi and insects. In the animal kingdom, a phenolase specific for 3 : 4-dihydroxyphenylalanine ("Dopa oxidase") and a phenolase of horse's heart muscle [Wieland and Lawson, 1931] have been investigated. Other systems effecting the oxidation of adrenaline and phenolases present in melaniferous tumours have been described. Summaries of the literature are given by Raper [1928], Onslow and Robinson [1928], Hizume [1924] and Oppenheimer and Kuhn [1931].

Resorcinol was chosen as a suitable inhibitor, since preliminary experiments with the catechol oxidase of potatoes showed that the inhibition with resorcinol appeared to be of a simple competitive type, as it could be reduced by increasing the substrate concentration.

EXPERIMENTAL.

Catchechol oxidase of potatoes. The catechol oxidase of potatoes (called tyrosinase by Raper [1928]) was prepared by the method of Szent-Györgyi [Keilin, 1928] and was further purified by adsorption on aluminium phosphate at p_H 6 and elution with phosphate buffer at p_H 8. The aluminium phosphate [Tsuchihashi, 1923] was added to the enzyme solution until most of the enzyme was adsorbed. (This was shown by centrifuging off a small sample of the clear solution and testing for catechol oxidase by shaking in a test-tube with a few drops of 2 % catechol.) After adsorption, the aluminium phosphate was centrifuged off and washed twice with distilled water, and the enzyme was eluted by adding disodium hydrogen phosphate solution until the mixture was at p_H 8. About 50 % of the enzyme was lost in the process of adsorption, but the resulting preparation, which was nearly colourless, appeared to be very pure, and could be kept for several weeks in the ice-chest without any considerable loss of activity.

This preparation brought about a very rapid oxidation of catechol and its derivatives, but the system was found to be unsuitable for making kinetic measurements as the reaction came to a standstill after the first few minutes owing to the inhibition of the system by one of the reaction products (Fig. 1).

This auto-inhibition was probably due to the o-quinone formed in the reaction

$$C_6H_4(OH)_2 + O_2 \rightarrow C_6H_4O_2 + H_2O_2$$
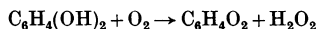
since it was greatly reduced by adding to the system substances such as aniline or o-phenylenediamine which readily combine with o-quinones. Other substances found to be effective in decreasing the auto-inhibition were m- and p-phenylene-diamine, glycine and alanine (Fig. 1).

For the purpose of making kinetic measurements with inhibitors $M/20$ aniline was therefore added to the system, which in the presence of $M/125$ catechol, and $M/50$ phosphate buffer p_H 6·8, gave a regular and reproducible reaction velocity. The anilinoquinone formed during the reaction separated out as a red precipitate.

Enzyme strength. As an arbitrary unit of the enzyme strength the amount of enzyme was chosen which in the presence of $M/125$ catechol, $M/50$ phosphate buffer p_H 6·8, and $M/20$ aniline (total volume 5 ml.) gave an oxygen uptake of 100 μl. in 10 minutes when shaken in air in a Barcroft apparatus at 20°. This enzyme unit is referred to as Catechol Unit or C.U. throughout the text.
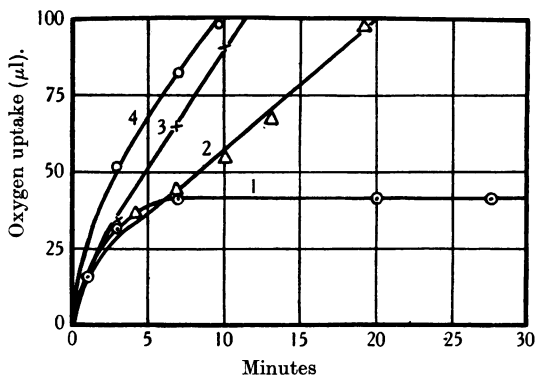

Fig. 1.

Fig. 2.

Fig. 1. In each experiment $M/125$ catechol, $M/150$ phosphate buffer p_H 6·8, and 0·9 C.U. potato catechol oxidase. Total volume 5 ml. Temperature 20°. 1. No addition. 2. $M/10$ glycine. 3. $M/20$ aniline. 4. 0·4 % o-phenylenediamine.
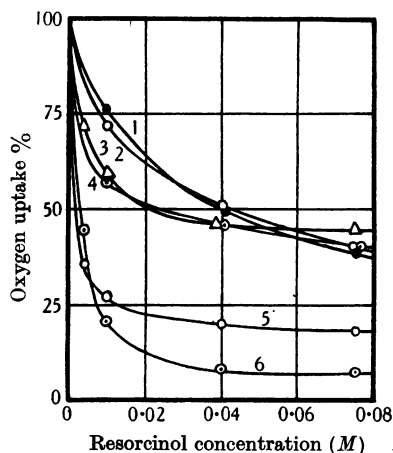
Fig. 2. In each experiment $M/125$ catechol, $M/50$ phosphate buffer p_H 6·8, $M/20$ aniline. Total volume 5 ml. Temperature 20°. 1. *Polyporus hispidus*. 2. *Tenebrio molitor*. 3. Potato. 4. *Agaricus campestris*. 5. *Sambucus nigra*. 6. *Syringa vulgaris*.

Inhibition by resorcinol.

The enzyme preparations used for comparison were prepared by the alcohol extraction method of Onslow and Robinson [1928] from fresh specimens of *Syringa vulgaris* (lilac), *Sambucus nigra* (elder), *Agaricus campestris* (mushroom), and *Polyporus hispidus*. The preparation from *Tenebrio molitor* (mealworm) was purified by repeated precipitation with alcohol and dialysis.

The enzymes were all used at approximately the same strength (0·3 C.U./ml.) under the conditions described for determining the enzyme strength of the

potato catechol oxidase, and the oxygen uptake in the presence of a series of concentrations of resorcinol was measured. The oxygen absorbed between the third and the thirteenth minutes was taken for comparison. The oxygen uptake plotted against the inhibitor concentration gave the curves shown in Fig. 2.

The inhibition curves obtained in this way appeared to depend very little on the degree of purity of the enzyme, since the curves obtained with crude potato juice agreed closely with the curves for the highly purified enzyme.

It can be seen at once from the curves (Fig. 2) that the catechol oxidases from different sources are not identical, but show marked specific differences in their behaviour. It is noticeable that the curves for *Syringa vulgaris* and *Sambucus nigra*, neither of which contains tyrosinase, lie together. While the usual methods of characterising enzymes, such as the measurement of p_H optimum or thermolability, must depend largely on the nature of the accompanying colloids, the characteristic selective inhibition of the phenolases by an inhibitor such as resorcinol probably depends more on the specific nature of the active centres of the enzymes.

Other inhibitors.

Apart from the inhibition by H_2S and HCN described by Keilin [1928] the catechol oxidase of potatoes is comparatively resistant towards narcotics and the usual enzyme poisons. The action of a number of substances was investigated by measuring the oxygen uptake of the potato enzyme at p_H 8 and 20° in their presence.

Of these substances phloroglucinol, *p*-nitrophenol, α-naphthol, dimethylaniline, thiolacetic acid, cysteine, salicylaldehyde and benzyl alcohol acted as inhibitors, while the following substances had little or no effect: urethane, methylurethane, methyl, ethyl, amyl and octyl alcohols, glycerol, glucose, pyridine, piperidine, diethylamine, urea, *o*-cresol, salicylic acid, *p*-quinone, quinhydrone, guaiacol, phenolphthalein, formaldehyde, acetaldehyde, phenylglyoxal, diacetyl, vanillin, sodium cholate, sodium *n*-hexanoate, sodium *n*-octanoate, fluorescein, haematin, *p*-rosaniline, methylene blue, picric acid, sodium pyrophosphate, uric acid, amygdalin, benzonitrile and quinine sulphate. The substances tested were brought to p_H 8 by addition of dilute sulphuric acid or sodium hydroxide before being tested.

It was observed that the addition of glycine or aniline to the system reduced the inhibition by benzyl alcohol, resorcinol and phloroglucinol, but this phenomenon was not further investigated.

Secondary oxidation of o-*phenylenediamine.*

It has been shown qualitatively that the *o*-quinones formed by the oxidation of catechol derivatives can effect the secondary oxidation of gum guaiacum, the "Nadi Reagent" and certain amino-acids [Szent-Györgyi, 1925].

o-Phenylenediamine was found to be oxidised in a similar manner, and some experiments were carried out to show the concentration of catechol derivative required for the reaction to take place.

$M/125$ *o*-phenylenediamine (freshly recrystallised) underwent no measurable oxidation on shaking for 8 hours with the potato catechol oxidase at p_H 6·8. On the addition of 0·02 mg./ml. of adrenaline or dihydroxyphenylalanine to

the system, an easily measurable absorption of oxygen took place, and the solution darkened in colour owing to the oxidation of the o-phenylenediamine (Fig. 3).
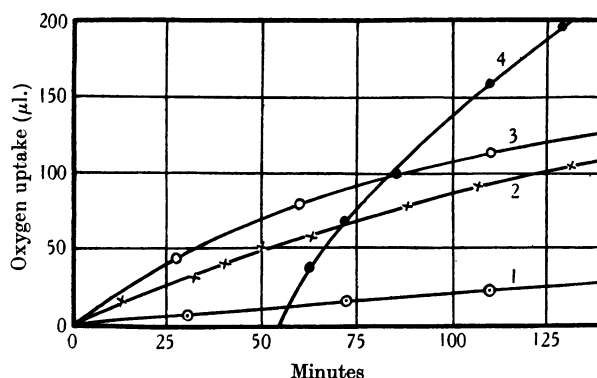

Fig. 3. In each experiment $M/125$ o-phenylenediamine, $M/50$ phosphate buffer p_H 6·8, and 0·54 c.u. potato catechol oxidase. Total volume 5 ml. Temperature 20°. 1. 0·02 mg./ml. adrenaline. 2. 0·1 mg./ml. adrenaline. 3. 0·06 mg./ml. dihydroxyphenylalanine. 4. 0·2 mg./ ml. dihydroxyphenylalanine (dihydroxyphenylalanine in 4 added after 54 minutes).

Secondary oxidation of thiolacetic acid.

Since —SH compounds are known to be present in tissues which contain the catechol oxidase, it seemed probable that this system would be able to bring about the secondary oxidation of —SH compounds to the disulphides.

Attempts to demonstrate this oxidation by adding a solution of thiolacetic acid were unsuccessful, since the catechol oxidase was almost completely inhibited by —SH compounds. The —SH concentration was therefore reduced by adding to the system an aldehyde such as formaldehyde, with which —SH compounds form dissociable complexes [Pirie and Pinhey, 1929]:

$$R.CHO + R'.SH \rightleftharpoons R.CH(OH).SR'$$
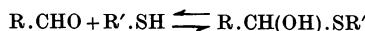
It was found that the addition of formaldehyde greatly reduced the inhibition of the catechol oxidase and allowed a rapid oxidation of the thiolacetic acid to take place. The following figures give the results of four experiments at 20°. Total volume 5 ml. in each experiment.

Enzyme, 14·6 c.u./ml. (ml.)	—	0·3	0·3	0·3
$M/10$ phosphate buffer, p_H 6·0 (ml.)	1	1	1	1
$M/50$ Na thiolacetate (ml.)	1·5	1·5	1·5	1·5
30 % formaldehyde (ml.)	0·3	0·3	—	0·3
$M/5$ catechol (ml.)	0·2	—	0·2	0·2
Time of experiment (mins.)	240	164	270	120
Oxygen absorbed, μl.	0	0	94	343
Thiolacetate ($N/50$ iodine)	1·5	1·5	1·0	0·1

The catechol and formaldehyde present interfered with the direct iodimetric estimation of the thiolacetic acid at the end of the experiment. The procedure adopted was to precipitate the thiolacetic acid with lead acetate at p_H 5, centrifuge off and wash the precipitate with water. On treating with potassium iodide and sulphuric acid, the precipitate was decomposed. The lead iodide was centrifuged off and washed, and the thiolacetic acid in solution was titrated directly with $N/50$ iodine.

Oxidation of monohydric phenols.

In addition to effecting the oxidation of catechol derivatives, preparations of the catechol oxidase of potatoes also bring about the oxidation of monohydric phenols, such as *p*-cresol, *m*-cresol, phenol and tyrosine. The reaction is specific, since phenols such as guaiacol, *o*-cresol, salicylic acid, thymol, α-naphthol and in general those phenols which on oxidation are likely to give quinol derivatives rather than catechol derivatives, are not attacked [Onslow and Robinson, 1925; 1926; 1928; Pugh and Raper, 1927].

Raper [1928] and Pugh [1930] hold the view that one enzyme only is involved in the oxidation both of catechol derivatives and of monohydric phenols.

Onslow and Robinson [1928] observed that the rate of oxidation of monohydric phenols was greatly increased by the addition of a catechol derivative. They concluded that the oxidation of the monohydric phenols is a secondary oxidation, effected by a reaction product of the catechol oxidase system. In her earlier papers Onslow concluded that the intermediate substance which oxidises the monohydric phenol is an organic peroxide, but later she held that it might be either hydrogen peroxide or an *o*-quinone. She assumed that a second enzyme might catalyse the secondary oxidation of the monohydric phenols.

Pugh [1929] has shown that the addition of a trace of a catechol derivative or of an *o*-quinone also accelerates the rate of oxidation of monohydric phenols with the phenolase preparation from mealworms, but she does not accept Onslow and Robinson's explanation as to the mechanism. She has also shown that the oxidation of monohydric phenols is accelerated by the addition of a trace of hydrogen peroxide, but this might be due to the formation of *o*-quinone by the partial oxidation of the monohydric phenol by the hydrogen peroxide, catalysed by peroxidase present in the enzyme preparation.
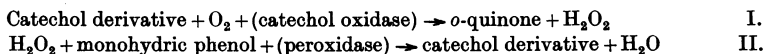
Onslow and Robinson [1928] claimed that they had effected a partial separation of the system into two enzymes, but this was disputed by Pugh [1930].

Acceleration by catechol derivatives. In the present work Onslow and Robinson's colorimetric experiments on the effect of catechol derivatives on the oxidation of monohydric phenols have been confirmed and extended by quantitative measurements of the oxygen uptake, using the phenolase system of the potato. *p*-Cresol was used as a substrate in preference to tyrosine, in which the initial oxidation is followed by a series of reactions connected with the amino-acid grouping, which introduces an unnecessary complication.

The oxidation of *p*-cresol in the presence of crude potato juice was very rapid and showed a regular oxygen uptake. With the purified enzyme, on the other hand, the rate of oxidation was initially very slow, but increased autocatalytically to a high value (Fig. 4).

The initiation of the reaction was strongly accelerated by the addition of boiled potato juice, or of a trace of a catechol derivative (potato juice contains catechol derivatives) such as adrenaline, dihydroxyphenylalanine or catechol. The acceleration produced by even 0·4 mg./litre adrenaline could be measured.

As a possible explanation of the action of catechol derivatives Onslow and Robinson suggested that the hydrogen peroxide formed by their oxidation might cause the secondary oxidation of the monohydric phenols:

$$\text{Catechol derivative} + O_2 + (\text{catechol oxidase}) \rightarrow o\text{-quinone} + H_2O_2 \qquad \text{I.}$$
$$H_2O_2 + \text{monohydric phenol} + (\text{peroxidase}) \rightarrow \text{catechol derivative} + H_2O \qquad \text{II.}$$

On this view the oxidation of the monohydric phenol by hydrogen peroxide would be catalysed by peroxidase present in the enzyme preparation and would

give rise to a catechol derivative which would in turn be oxidised by the catechol oxidase. The process would be continuous and might be regarded as a type of chain reaction.
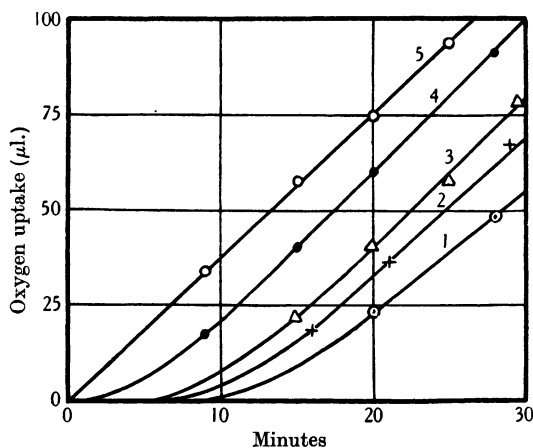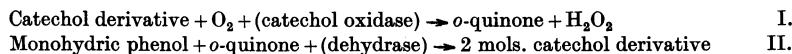

Fig. 4. In each experiment $M/125$ p-cresol, $M/50$ phosphate buffer p_H 6·8, and 2·4 c.u. potato enzyme. Total volume 5 ml. Temperature 20°. 1. No addition. 2. $M/250$ glycine. 3. 0·4 mg./litre adrenaline. 4. 0·5 ml. boiled potato juice. 5. 8 mg./litre adrenaline.

Against this view it was observed that the specimens of potato catechol oxidase purified by adsorption were almost free from peroxidase. On testing for peroxidase with gum guaiacum and hydrogen peroxide the enzyme solution gave only a faint blue coloration after standing for 10 minutes.

It was further observed that the addition of 0·3 % sodium pyrophosphate or saturation with pyrrole, which very strongly inhibit vegetable peroxidase [Elliott, 1932], had no appreciable effect on the rate of oxidation of p-cresol. It must be concluded that in the oxidation of monohydric phenols the peroxidase system is not primarily concerned.

The autocatalytic nature of the curve with the purified enzyme could be explained if during the purification a trace of inhibitor were accidentally introduced, and the reaction velocity slowly increased as the inhibitor was oxidised by the o-quinone produced during the reaction. This possibility was rendered improbable by the fact that the enzyme had the same properties when further purified by different methods, *e.g.* by adsorption, dialysis or precipitation with alcohol. Further, it was observed that when the enzyme was "activated" by the addition of a trace of catechol, it was found to be no longer activated if allowed to stand until the o-quinone formed had decomposed.

Inhibition of the oxidation of monohydric phenols. The most probable explanation of the action of catechol derivatives appeared to be Onslow and Robinson's suggestion that the monohydric phenols are oxidised by the o-quinones formed:

$$\text{Catechol derivative} + O_2 + (\text{catechol oxidase}) \rightarrow o\text{-quinone} + H_2O_2 \qquad \text{I.}$$
$$\text{Monohydric phenol} + o\text{-quinone} + (\text{dehydrase}) \rightarrow 2 \text{ mols. catechol derivative} \qquad \text{II.}$$

Here again the reaction would be continuous, since reaction II regenerates the catechol derivative required for reaction I.

Attempts to test this view by showing that a trace of o-quinone could be added in the place of the catechol derivative were inconclusive, since o-quinones are very unstable in aqueous solution, and after standing only for a few minutes

the solution contained an appreciable amount of catechol derivatives [Willstätter and Müller, 1911]. The addition of sodium β-naphthoquinonesulphonate, an o-quinone derivative which is relatively stable in aqueous solution, produced only a slight acceleration.

This view of the action of o-quinones was therefore tested by adding to the system substances which combine with o-quinones. It was found that the oxidation of p-cresol or tyrosine was completely inhibited by 0·5 % o-phenylene-diamine, $M/1000$ sodium bisulphite, 1 % potassium iodide or $M/10$ aniline for a period of over 5 hours, although the catechol oxidase was still active, since a rapid oxygen uptake occurred on adding catechol.

This inhibition by substances that combine with o-quinones gives additional evidence that o-quinones play an essential part in the oxidation of monohydric phenols. The view that one enzyme catalyses the direct oxidation of both catechol derivatives and monohydric phenols by molecular oxygen [Raper, 1928; Pugh, 1930] does not explain the extreme sensitivity of the latter, but not the former, reaction to traces of o-quinones and to substances which combine with o-quinones.

The instability of the o-quinones in solution makes it difficult to obtain direct experimental proof, but the oxidation potentials of the o-quinones are sufficiently high for the oxidation of the monohydric phenols, and the available evidence is consistent with the view that this oxidation takes place.

SUMMARY.

1. Catechol oxidase preparations from *Syringa vulgaris*, *Sambucus nigra*, *Agaricus campestris*, *Polyporus hispidus*, *Tenebrio molitor* and potato were compared by testing the extent to which they were inhibited by varying concentrations of resorcinol. Preparations of different origin showed marked specific differences in their behaviour. An improved method was obtained for preparing stable preparations of the catechol oxidase of potatoes.

2. Conditions were found under which the catechol oxidase system of potatoes effected the secondary oxidation of o-phenylenediamine and of thiol-acetic acid.

3. Measurements of the rate of oxidation of p-cresol by the catechol oxidase of potatoes showed the reaction to be strongly accelerated by substances which produce o-quinones, and strongly inhibited by substances which combine with o-quinones. The reaction would therefore appear to be essentially a secondary oxidation in which the formation of an o-quinone is involved, rather than a direct oxidation of p-cresol by the enzyme.

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