CCXXV. THE NATURE OF THE ACTION OF TYROSINASE.

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CERTAIN oxidising enzymes, such as xanthine oxidase, can bring about oxidation of their substrates even in absence of oxygen, provided some other substance able to function as suitable hydrogen acceptor is present, such as methylene blue. It is probable that such oxidising enzymes do not exert their action on the oxygen or other hydrogen acceptor, but on the substrate and act by activation of hydrogen. Such enzymes Raper [1928] has called anaerobic oxidases. He has designated as aerobic oxidases such oxidising enzymes as have never been observed to bring about their action on their substrate in absence of oxygen, that is to say, the oxygen has not been replaced by any other hydrogen acceptor. It is concluded that such enzymes probably act by activation of oxygen. Enzymes which activate peroxide, such as peroxidase, are classed as aerobic oxidases, because they affect the oxygen potential (as do activators of oxygen) rather than the hydrogen potential of the system.

Tyrosinase is an enzyme which has never been known to exert any effect in absence of oxygen, with the exception of certain observations reported by McCance [1925]. He found that there was slight reduction of methylene blue by catechol or *p*-cresol in presence of glycine, which was considerably hastened by tyrosinase preparations. This he took to indicate that the action of tyrosinase is on the substrate, that it acts by hydrogen activation, and that this is also the method by which it acts aerobically. He found that methylene blue was also slowly reduced by tyrosine, but the reduction was not hastened by tyrosinase. This he attributed to the unsuitability of methylene blue as the hydrogen acceptor for this substrate.

Now it has been established by Raper and his collaborators that the chemical change undergone by a monohydric phenolic substrate such as phenol, *p*-cresol or tyrosine under the influence of tyrosinase in air is the formation of the corresponding catechol derivative, and that this is further oxidised to an *ortho*quinone before subsequent changes occur. It is difficult to understand how a single oxygen atom could be introduced between hydrogen and the nucleus as a result of mere activation of hydrogen. It is evidently necessary to postulate either addition of water to the nucleus, followed by dehydrogenation, or what is more probable, activation of the molecular oxygen. Experimentally, tyrosine has never been shown to be "dehydrogenated" by an inorganic catalyst. In view of the importance of McCance's results therefore an attempt to repeat his essential experiments has been carried out.

EXPERIMENTAL.

When an attempt was made to repeat the essential experiments reported by McCance, difficulty was experienced in eliminating last traces of oxygen. Even an electric pump left enough oxygen to colour *p*-cresol slightly in presence of

tyrosinase. In the present experiments the trace of air left after evacuation was washed out with nitrogen, the process being repeated several times. Nitrogen from the cylinder, however, contains oxygen. This was not removed adequately by passage through several wash-bottles containing alkaline pyrogallol solution, or by passage through a furnace containing reduced copper oxide, for *p*-cresol in a Thunberg tube repeatedly filled with such nitrogen was still slightly coloured by tyrosinase.

Eventually the process of Kautsky and Thiele [1926] for freeing nitrogen from oxygen, adapted as follows, was found to be effective. The apparatus consisted of (1) nitrogen cylinder, pressure gauge and regulator, (2) inverted aspirator containing alkaline sodium hydrosulphite solution, (3) wash-bottle containing 10 % KOH, (4) wash-bottle containing water, (5) 3-way tap, (6) 2-way tap, (7) electric pump and mercury manometer. Into the inlet of the aspirator was fixed a Berkefeld filter to break up the gas into fine bubbles as it entered the liquid. The outlet of the aspirator was covered with a lead disc through which passed glass tubing and was cemented together with white sealing-wax. All the glass ends were joined together with fairly flexible lead (compo) tubing, and the joints were cemented with white sealing-wax. The 2-way tap marked the end of the gas-tight part of the apparatus. All liquids to be used were well boiled to expel air and cooled overnight in a desiccator over pyrogallol solution into which alkali was spilt after evacuation. (Since *p*-cresol is very volatile in steam, the solution was made stronger than required and was always tested to make sure that plenty remained. To avoid contaminating other liquids, the *p*-cresol must be in a desiccator by itself.)

Before the apparatus was used, nitrogen was run through it for several hours. The substrate solution was placed in a Thunberg tube, the enzyme preparation in a small tube loose inside the Thunberg tube. The cylinder was turned nearly off, and the 3-way tap was turned to cut off the cylinder. The Thunberg tube was then cemented with white sealing-wax through lead tubing on to the 3-way tap. The pump was switched on and the tube evacuated. The 2-way tap was then shut, and the 3-way tap was opened to admit nitrogen. The process was repeated several times. The contents of the Thunberg tube were then allowed to boil to remove last traces of oxygen and to mix the enzyme and substrate. The tube was again filled with nitrogen several times.

The enzyme preparation was freshly prepared for each experiment as follows. Mealworms were fed on cream crackers for some time, then starved for a few days. Of these 5 g. were chloroformed, crushed in a mortar with 20 ml. water, pressed through muslin and centrifuged. The supernatant liquid was discarded, and the residue in the tube was rubbed up with another 20 ml. water and again centrifuged. Four lots of washings were discarded, and the final residue was suspended in 20 ml. water.

The series of tubes was filled as follows: (1) 1 ml. 0.5 % catechol in phosphate buffer p_H 8.0, (2) 1 ml. approximately 0.5 % *p*-cresol similarly, (3) 1 ml. saturated tyrosine similarly, (4) 1 ml. 0.5 % glycine similarly. To each of these tubes was added 1 ml. buffer. (5) 1 ml. catechol solution and 1 ml. glycine solution, (6) 1 ml. *p*-cresol and 1 ml. glycine, (7) 2 ml. buffer. To each tube was added 0.3 ml. *M*/1000 methylene blue in water.

In series I, in addition, 1 ml. water was added to each tube and no enzyme preparation. There was no reduction of the methylene blue in any tube after two days. Series II, as I, but incubated at 80°. Overnight, tubes 3 and 5 were partially reduced. After two days, 5 was completely reduced, 3 considerably, 1 partially; trace of reduction in 4. Series III (tubes 3, 5, 7 only) as II, but with

addition of a little solid thymol to each tube. Overnight, 3 and 5 were partially reduced. After two days, 3 and 5 were completely, 7 partially reduced. Series IV, only 0.5 ml. water was added, and 0.5 ml. enzyme preparation in the little tube. Tubes 4 and 6 were partially reduced overnight, completely after two days, when 3 and 7 were partially reduced. The tubes smelt unpleasantly when opened. Series V, as IV, but with addition of thymol. After two days, very slight reduction in 6 alone—faintly greenish blue—repeated and confirmed. Series VI, as IV, but incubated at 80°. Tube 6 was reduced in 40 mins. and tube 5 in 1½ hours. Overnight 1 and 3 were partially reduced. Series VII, as IV, but incubated at 80° with addition of thymol. After two hours, tubes 5 and 6 were completely reduced. After two days, all tubes were slightly paler. Series VI was repeated at 80° and at 60°, using the same preparation from mealworms. At 80°, tube 6 was reduced in ½ hour, tube 5 in ¾ hour; at 60°, tube 6 in 40 mins., tube 5 in 1 hour.

Table I.

	I	II	III	IV	V	VI	VII
	S	S 80°	S 80° T	S E	S E T	S E 80°	S E 80° T
(1) Catechol	—	+	—	—	—	++	++
(2) <i>p</i> -Cresol	—	—	—	—	—	—	+
(3) Tyrosine	—	++	++	x	—	++	++
(4) Glycine	—	?	+	x	—	—	+
(5) Catechol and glycine	—	++	++	—	—	++++	+++++ approx.
(6) <i>p</i> -Cresol and glycine	—	—	—	x	?+	+++++	+++++ approx.
(7) Buffer	—	—	+	x	—	—	+

S, substrate; 80°, incubated at 80°; T, with addition of a little solid thymol; E, enzyme preparation. ?+, slight reduction after a day or two; +, partial reduction after a day or two; ++, partial reduction overnight; ++++ to +++++, reduction complete in a few hours; x, bacterial development?

The results are summarised in Table I. They indicate that (1) catechol without any enzyme, on heating, slowly brings about a reduction of methylene blue, which is hastened in presence of glycine. This is probably a dehydrogenation of catechol to *ortho*quinone, which oxidises the amino-acid and is reduced back to catechol. The final liquid was brownish pink. In all other instances, reduction resulted in a colourless liquid. This is particularly noteworthy in the case of tyrosine, as it contrasts with the red colour deepening to black produced by the action of tyrosinase on tyrosine in air. (2) The very slow reduction of methylene blue by catechol is slightly hastened by incubation with the enzyme preparation. This may be due to the effect of amino-acids produced by proteolysis of the enzyme preparation, which contains a protease. (3) The slow reduction of methylene blue by tyrosine is not affected by the enzyme preparation. (4) In absence of antiseptic, bacteria probably develop in presence of the enzyme preparation and cause the reduction of methylene blue. (5) Thymol reduces methylene blue, though extremely slowly even at 80°. (6) Even at 80° the reduction of methylene blue by glycine is almost negligible and is not hastened by the enzyme preparation. (7) The reduction of methylene blue by catechol with glycine is very considerably hastened in presence of the enzyme preparation. (8) Reduction of methylene blue is even more rapid by *p*-cresol with glycine in presence of the enzyme preparation.

It is interesting to compare the quickest rate of reduction of methylene blue in these experiments with the oxygen uptake which accompanies the action of tyrosinase on catechol or *p*-cresol. The amount of methylene blue in each

Thunberg tube, 0.3 ml. $M/1000$, is equivalent to $3.36 \mu\text{l. O}_2$. The following mixtures as in Series IV, but without methylene blue, were placed in Barcroft micro-respirometers, with buffer in place of substrate solution in the control bottle in each case. Corresponding to tube 1 absorbed $229 \mu\text{l. O}_2$ in 15 mins.; to tube 2, $135 \mu\text{l.}$; to tube 5, $204 \mu\text{l.}$; to tube 6, $212 \mu\text{l.}$ Oxidation in presence of oxygen is thus of a totally different order of magnitude from the reduction of methylene blue, even when the latter is warmed at 80° .

Precautions were taken to avoid gross contamination with iron by use of freshly glass-distilled water and of phosphate buffer decanted from precipitated iron salt. No coloration whatever was observed on mixing catechol with glycine solution, such as was observed by McCance. The *p*-cresol used had been twice distilled and stored in a paraffin-waxed bottle, as otherwise *p*-cresol is liable to become contaminated with homocatechol.

The enzyme preparation used in these experiments contained no peroxidase, as determined by absence of coloration of guaiacol in presence of hydrogen peroxide.

DISCUSSION.

The essential observations made by McCance have been confirmed. The slight reduction of methylene blue by catechol, particularly in presence of glycine, is considerably hastened by a preparation of tyrosinase from mealworms. The effect of such a preparation in hastening the reduction of methylene blue by *p*-cresol with glycine is even more striking.

While the facts, however, are confirmed, there are certain obstacles in the way of ascribing the phenomenon to the action of tyrosinase.

(1) The definite slight reduction of methylene blue by tyrosine is not hastened by the tyrosinase preparation, although methylene blue evidently is a suitable acceptor for the dehydrogenation of tyrosine, since appreciable reduction of methylene blue is brought about by tyrosine alone. (But possibly some portion of the molecule other than the phenol group is concerned.)

(2) The rate of the oxidation which takes place with methylene blue as hydrogen acceptor is of the order one-hundredth or less of that of the oxidation brought about by the same tyrosinase preparation in air, although methylene blue will usually act as hydrogen acceptor for hydrogen in substrates not sufficiently activated for the hydrogen to be taken up by molecular oxygen, *e.g.* lactic and citric dehydrases.

(3) The optimum temperature for the phenomenon described is about 80° , whereas the optimum temperature for the action of tyrosinase normally is about ordinary temperature.

While there are these difficulties in the way of accepting the phenomenon as due to the tyrosinase in the mealworm preparation, no more satisfactory explanation is at present forthcoming. There is, too, other evidence in support of the suggestion that tyrosinase acts by activation of hydrogen in its substrates.

(a) Okuyama [1929] measured the hydrogen potentials developed when tyrosinase was mixed with various substances and concluded, in support of McCance's theory, that it acts by activation of hydrogen in its phenolic substrates, in presence of amino-acid. (The paper, however, contains a discrepancy, between figures 2_2 and 10_2 .)

(b) The action of tyrosinase on a monohydric phenolic substrate is hastened by hydrogen peroxide (even when the liquid is well aerated with oxygen), or by *ortho*quinone, or by a catechol derivative (which gives rise to hydrogen peroxide and *ortho*quinone). This may be due to the superior oxidation potential of

hydrogen peroxide and of *ortho*quinone over molecular oxygen as hydrogen acceptor [see Pugh, 1930].

(c) The same suggestion is supported by the inhibitory effect of substances which might be expected to reduce the oxidation potential. Pugh and Raper [1927] found that benzenesulphinic acid prevented the oxidation of phenol by tyrosinase, while permitting that of catechol. Again, the oxidation of a monohydric phenol is more strongly inhibited by sulphite or by cyanide than that of an *o*-dihydric phenol [Pugh, 1930]. Richter [1934] has shown that *o*-phenylenediamine, bisulphite, potassium iodide and aniline all inhibit the action of the enzyme on monohydric phenols at a concentration at which they still permit action on catechol. He ascribes this to removal of the *ortho*quinone normally produced, which he considers to play a specific part in the reaction; but it may be that the action is essentially one of lowering of the oxidation potential.

The question must be left open. There does, however, appear to be the possibility of co-ordinating all these phenomena by the hypothesis that tyrosinase activates hydrogen in its substrates, and that methylene blue, molecular oxygen, hydrogen peroxide and *ortho*quinone are all, in suitable circumstances, able to act as hydrogen acceptors for the action of tyrosinase.

SUMMARY.

McCance's observation that a preparation from mealworms hastens the reduction of methylene blue by a mixture of catechol or *p*-cresol with glycine has been confirmed. There are obstacles in the way of accepting the suggestion that this denotes the activation of hydrogen in its substrates as the mode of action of tyrosinase, but there is also certain supporting evidence, which is discussed.

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