CLII. OXIDATIONS CATALYSED BY HORSE-RADISH- AND MILK-PEROXIDASES.

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IN a previous paper [Elliott, 1932] a method was described for discovering whether any substance was oxidisable by H_2O_2 under the influence of peroxidase catalysis, and it was shown that the range of substances whose oxidation was catalysed by milk-peroxidase was very limited. Very dilute H_2O_2 with the enzyme had no effect on the various fatty acids, fats, sugars, most amino-acids, and other possible metabolites tried. Other than those compounds used in ordinary tests for peroxidase, the only substances found to be oxidised were tryptophan and tyrosine, and the oxidation of nitrite, previously observed by others, was fully confirmed.

The peroxidase from horseradish is one of the most intensively studied of enzymes and yet very little has up to now been known of its activity as a catalyst of biological oxidations. This fact and the interest of comparing the activity of an enzyme of plant origin with the corresponding enzyme from an animal source, led me to apply to horseradish-peroxidase preparations the same method as utilised with the milk-enzyme. The results obtained indicated a few more experiments to be made with the milk-enzyme.

Preparation of horseradish-peroxidase.

For this work it was necessary to have an enzyme preparation free from reducing substances and catalase, but a highly concentrated enzyme was not required. The "crude" product of Willstätter and Stoll [1918] would probably have been suitable but its preparation is laborious and occupies several weeks. The following entirely different method was found to be very satisfactory.

1470 g. of horseradish were washed and without further treatment chopped up and passed through an ordinary meat-mincer. To the mince 2 litres² of distilled water were added and the mixture stirred frequently during 2 hours. The liquid was squeezed through a cloth, and another extraction was made with 2 litres of water in the same way. A third extraction was done with 2 litres allowing the mixture to stand overnight before straining off the liquid. Qualitative tests showed that the third extract was considerably less active

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² It has since been found better to make the 3 or 4 extractions with only one litre each time.

than the first two, and though a large amount of enzyme remained in the solid it was not economical to extract it further.

The combined extracts, 6400 cc. including juice, were centrifuged or filtered through fluted filter-papers, and the translucent liquid was then fully saturated with ammonium sulphate. This precipitated the enzyme completely, together with a considerable amount of insoluble impurity. The precipitate was separated by centrifuging or on Büchner funnels, and lixiviated with a little water (250 cc. in this example, but preferably less). The turbid solution was dialysed through animal (or parchment) membrane for 3 days with frequent changes of outside water, until salt-free. The fluid, which had doubled in volume during the dialysis, was filtered clear and twice its volume, 1000 cc., of denatured¹ alcohol was added. The precipitate which formed was collected by centrifuging and dried in a vacuum desiccator. To prevent the alcohol paste splashing during the drying, it was covered by a slanting large perforated disc.

470 mg. of dried and ground up powder were obtained, having a purpurogallin number of 25.3.

To the alcoholic liquid a further 2 volumes, 1000 cc., of alcohol was added and the precipitate collected and dried as before.

125 mg. of a powder of purpurogallin number 212 were obtained.

In another preparation carried out as above the whole four volumes of alcohol were added together and the product obtained weighed 410 mg. with purpurogallin number 95.2.

The material obtained by this method is not easily soluble, and for estimation and other purposes it is necessary to grind it up carefully to a paste with very little water before diluting.

The whole preparation, including the period of dialysis can easily be finished in one week.

Oxidations by peroxidase and H_2O_2 .

The method used was that described in the previous paper (pp. 16 and 19), the only change being that the experiments were made at 20° instead of at 37°.

Nitrite. It was previously shown that nitrite is quantitatively oxidised by H_2O_2 under the influence of milk-peroxidase catalysis; this oxidation was used as a test of the method, and hence nitrite was the first substance tried with the plant-enzyme. The remarkable result was obtained that horseradish-peroxidase is completely unable to catalyse the oxidation of nitrite.

Experiments carried out exactly as with milk-peroxidase showed no H_2O_2 usage at all, and, using very small amounts of nitrite, the Griess-Ilosvay test at the end of experiments showed no diminution in nitrite whatever. Experiments were made with the same result at room temperature and at 37°, at various hydrogen ion concentrations between p_H 5.7 and 7, using more H_2O_2 ,

¹ EtOH 96 %, MeOH 2.5 %.

or increasing the time allowed for the reaction, and with various preparations of the enzyme including a Willstätter adsorption product kindly given me by Dr H. Sutter.

The following is the summary of one experiment.

Flasks A and B each contained 20 cc. of a solution of 55 mg. of an enzyme preparation of P.Z.¹ 54, in phosphate buffer at p_H 6. A, contained 3 cc. of 3 mg./cc. sodium nitrite solution; B, 3 cc. of water. During 45 minutes at 20°, 10 cc. of 0.02 % H_2O_2 were run into each flask; after a further 15 minutes small samples tested with *p*-phenylenediamine showed by immediate coloration that both active enzyme and H_2O_2 were still present. On estimating the H_2O_2 remaining by the MnO_2 method in neutral solution, 0.64 cc. and 0.68 cc. of O_2 were liberated from the contents of flasks A and B respectively. No usage of H_2O_2 had therefore taken place. A similar experiment using only 0.5 cc. of nitrite solution, the H_2O_2 therefore being in excess, showed the same intensity of colour with the Griess-Ilosvay reagent after the experiment as a standard solution of the nitrite of the same dilution.

Qualitative tests showed that the enzyme was still active at the end of the experiments. In order to make sure that no inactivation at all was caused by the nitrous acid at the slightly acid reaction, the following experiment was made. To 5 cc. of a solution of 5 mg. of peroxidase of P.Z. 54 in phosphate buffer p_H 5.5, 2 cc. of nitrite solution 3 mg./cc. were added and the mixture kept for 1½ hours. On diluting and carrying out an estimation, the enzyme was found to have lost none of its activity.

Thurlow [1925] was also unable to show the oxidation of nitrite by plant-peroxidase but believed this to be due to reducing substances in her crude preparation reacting with the nitrate formed. My method excludes this possibility since it shows that not only does nitrite not disappear, but H_2O_2 is not used up.

The slight colour mentioned in the last paper as developing when H_2O_2 was added to nitrite and milk-peroxidase solution was again observed with the plant-peroxidase. It is not formed in the absence of nitrite or of enzyme and is therefore difficult to account for unless traces of nitrite are oxidised and the trace of nascent nitrate formed gives a xanthoproteic reaction. The colour deepens with ammonia.

It is necessary here to correct an error in my previous paper. I have found that in acid medium nitrous acid reduces H_2O_2 quantitatively, so that H_2O_2 cannot be estimated in acidified solutions containing nitrite. In Tables III, IV, V and VI of that paper the fact that no O_2 was liberated by MnO_2 in acidified solution in the presence of excess of nitrite is therefore meaningless. But in all those tables the qualitative tests recorded show that the H_2O_2 had all been used up before adding acid. The estimations in Table II showing the oxidation of nitrite were done in neutral solution, as also were the experiments on nitrite recorded here above. These estimations and all results and conclusions are therefore perfectly valid. Careful controls have shown that nitrite in neutral solution and in buffer as acid as p_H 5.5, does not affect the H_2O_2 estimation by MnO_2 .

¹ P.Z. (=Purpurogallin Zahl) is the usual abbreviation for Purpurogallin Number as defined by Willstätter and Stoll [1918].

Possible metabolites. Besides nitrite the two other substances found to be oxidised under the influence of milk-peroxidase catalysis were tryptophan and tyrosine. With the plant-enzyme tyrosine was found to be oxidised, but no sign of oxidation of tryptophan could be observed; we have here therefore another remarkable difference between the two enzymes. The following compounds of different types were tried with the plant-peroxidase preparation and found, as with milk-peroxidase, not to be oxidisable; *viz.* formate, acetate, lactate, oleate, glucose, fructose, glycerol, ethyl alcohol, acetaldehyde, glycine, glutamic acid, phenylalanine, histidine, brucine.

It was previously shown that dihydroxyacetone and phenylglyoxal are oxidisable by the very dilute H_2O_2 without peroxidase catalysis; the same can now be said of formaldehyde.

Tables I-V give the experimental details of some of the above results.

The amount of enzyme used in all experiments after Table I is about half of what was used in the previous paper, the smaller amount being used to economise with the enzyme preparations and to diminish the slight destruction of H_2O_2 by catalase. The experiments were all done under roughly the same conditions of H_2O_2 concentration, p_{H} and temperature as in the ordinary purpurogallin method of estimation. Calculating, therefore, from the rate of H_2O_2 usage in the purpurogallin method, this smaller amount of enzyme would be 2250 times enough to use up all the H_2O_2 in the time of the experiments if the enzyme worked equally rapidly with other reducing substrates. Insufficiency of enzyme, therefore, cannot account for the negative results obtained.

Table I.

Each flask contained 1.15 P.E.* of enzyme (*i.e.* 12 mg. of P.Z. 95) in 20 cc. phosphate buffer p_{H} 6.4, and the neutralised substrate in 5 cc. H_2O . 10 cc. of 0.02 % H_2O_2 were added over 45 minutes. Total time 1 hour before removing 1 cc. sample and adding acid. Temperature 20°.

	<i>p</i> -Phenylene- diamine 50 mg.	Tyrosine 50 mg.	Trypto- phan 50 mg.	Histidine- HCl 50 mg.	Controls	
	Blue	Yellow	-	-	Water	Water H_2O_2 all added together and acid at once
Colour formed	Blue	Yellow	-	-	-	
Sample { Colour with <i>p</i> -phenylene- diamine	.	-	++	++	++	.
Do. plus H_2O_2	.	++++	++++	++++	++++	.
H_2O_2 remaining at end. (cc. O_2 evolved by MnO_2)	0.06	0.0	0.67	0.66	0.57	1.61

* P.E. (=Purpurogallin Einheit) is the usual abbreviation for Purpurogallin Unit as defined by Willstätter and Pollinger [1923].

p-Phenylenediamine and tyrosine use up H_2O_2 and are therefore oxidised. Tryptophan and histidine are not oxidised. The enzyme is always still active at the end of the experiment.

Table II.

As for Table I except that each flask contained only 0.64 P.E. of enzyme, *i.e.* 6.7 mg. of P.Z. 95.

		Controls					
		Glycine 25 mg.	Glut- amic acid 50 mg.	Acetic acid 25 mg.	Sodium lactate 50 mg.	Alcohol 25 mg.	All H ₂ O ₂ added to- gether and acid at once
Sample	Colour with <i>p</i> -phenylene-diamine	++	++	++	++	++	+
	Do. <i>plus</i> H ₂ O ₂	++++	++++	++++	++++	++++	+
cc. O ₂ evolved by MnO ₂ at end		0.87	0.82	0.87	0.86	0.84	1.45

None of the above compounds is oxidised. The enzyme is active at the end of all experiments.

Table III.

0.63 P.E. of enzyme in each flask.

		Formic acid 25 mg.	Glucose 50 mg.	Form- aldehyde 30 mg.	Glycerol 40 mg.	Acet- aldehyde 40 mg.	Water
Sample	Colour with <i>p</i> -phenylene-diamine	++	++	(+)	++	++	++
	Do. <i>plus</i> H ₂ O ₂	+++	+++	++	+++	+++	+++
cc. O ₂ evolved by MnO ₂		0.92	0.80	0.14	0.86	1.16	0.85

Formic acid, glucose, glycerol, and acetaldehyde are not oxidised. The curious effect of rather greater liberation of O₂ in the presence of acetaldehyde than in its absence occurred also with milk-peroxidase. I have shown by experiments that acetaldehyde in acid solution yields no gas with MnO₂ nor is extra gas yielded on decomposing H₂O₂ when acetaldehyde is present. One can only assume that the aldehyde protects the H₂O₂ to some extent from the slight decomposition by catalase during the experiment.

Formaldehyde used up the hydrogen peroxide but separate experiments showed that this occurred to the same extent in the absence of any enzyme.

Table IV.

		Brucine 60 mg.	Fructose 90 mg.	Oleic acid 50 mg. in neutral emulsion	Tannin 100 mg. Dark green colour formed	Water
Sample	Colour with <i>p</i> -phenylene-diamine	++	++	++	-	++
	Do. <i>plus</i> H ₂ O ₂	+++	++++	++++	++	+++
cc. O ₂ evolved by MnO ₂ at end		0.66	0.63	0.62	0.0 In neutral solution	0.56

Table V.

Colour formed	Phenylalanine 50 mg.	Tyrosine 25 mg. powdered	Catechol 25 mg.	Gallic acid 40 mg.	Water
	-	Yellow	Deep yellow	Dark greenish brown	-
Sample { Colour with <i>p</i> -phenylenediamine	++	-	-	-	++
Do. plus H ₂ O ₂	++++	++++	++++	+++	++++
cc. O ₂ evolved by MnO ₂ at end	0.97	0.0	0.0	0.15	1.01

Phenylalanine is not oxidised while tyrosine, catechol, and gallic acid are.

Phenols. Tyrosine is acted upon by H₂O₂ and peroxidase and it has long been known that various other phenols are oxidised, the reactions with them having been used as tests for the enzyme [see Raper, 1928]. Experiments were made with a representative series of phenolic substances with the result that one may say that all substances containing a phenolic group are oxidisable by H₂O₂ under the influence of catalysis by both horseradish- and milk-peroxidase. Only one exception has been found, namely resorcinol, which, though oxidised by the plant-enzyme, completely inhibits the activity of the milk-enzyme; it is not oxidised itself and in small amount prevents the oxidation of iodide, benzidine, *p*-phenylenediamine, etc.

Table VI gives a summary of the observations on phenols. In this table under "qualitative" are given the observations in simple test-tube experi-

Table VI.

	Plant-peroxidase		Milk-peroxidase	
	Qualitative	Quantitative	Qualitative	Quantitative
Phenol	Yellow		Yellow	
o-Cresol	Greenish-yellow + ppt.		Greenish-yellow	
<i>p</i> -Cresol	White ppt.		-	+
<i>m</i> -Cresol	White ppt.	+ Faint yellow	-	+ Faint yellow
Vanillin	White ppt.	+ Faint yellow	-	+ Yellow
Tyrosine	-	+ Yellow	-	+ Yellow
Tyramine	-	+	-	+
Catechol	Yellow	+ Deep yellow	Yellow	
Resorcinol	Brownish-yellow		Inhibition	
Quinol	Pale yellow		Pale yellow	
Protocatechuic acid	Yellow		Yellow	
3:4-Dihydroxy-phenylalanine	Red-brown		Red-brown	
Adrenaline	Red		Red	
Pyrogallol	Yellow		Yellow	
Gallic acid	Green-brown	+ Colour	Green-brown	
Tannic acid	Dirty yellow	+ Colour	Dirty yellow.	+ Colour
			Enzyme largely precipitated with protein	
α -Naphthol	Magenta + ppt.	+ Colour	Magenta	+ Colour
β -Naphthol	White ppt.		-	+ Faint yellow

- signifies "nothing easily observable."

+ signifies oxidised.

ments. A number of the substances, especially with the milk-enzyme, showed no marked colour or precipitation reaction; these were therefore tested by the quantitative method of estimating H_2O_2 usage, and a number of the others were confirmed by this method. The results are shown under the heading "quantitative."

No attempt has been made to discover the products of reaction.

Tables VII–VIII give the details of some of these "quantitative" experiments.

Table VII.

Horseradish-peroxidase. 0.66 P.E. in each flask.

		Tyramine 40 mg.	Gallic acid 40 mg.	α -Naphthol 40 mg.	Vanillin 40 mg.	<i>m</i> -Cresol 40 mg.	Water
Colour formed		? Scarcely detect- able	Violet going at once green- brown	Light yellow with white ppt.	Faint yellow white ppt.	Light yellow milky white ppt.	—
Sample	Colour with <i>p</i> -phenylene- diamine	—	—	—	—	—	++
	Do. <i>plus</i> H_2O_2	+++	+++	+++	++++	++++	++++
cc. O evolved by MnO_2 at end		0.0	0.15 In neutral solution	0.0	0.0	0.0	0.81

Table VIII.

Milk-peroxidase. 0.7 P.E. in each flask.

		Tyr- amine 40 mg.	α -Naph- thol 40 mg.	β -Naph- thol 40 mg.	Vanillin 50 mg.	<i>m</i> - Cresol 50 mg.	<i>p</i> - Cresol 40 mg.	Tannic acid 40 mg.	Water
Colour formed		Scarcely detect- able	Ma- genta	Faint yellow	Faint yellow	Faint yellow	White opal- escence	Yellow- brown	.
Sample	Colour with <i>p</i> -phenylene- diamine	—	—	—	—	—	—	—	+++
	Do. <i>plus</i> H_2O_2	+++	+++	+++	+++	++	+++	(+)*	+++
cc. O_2 evolved by MnO_2 at end		0.0	0.0	0.0	0.0	0.0	0.0	0.39	0.96

* Considerable precipitation of enzyme had taken place.

Experiment showed that none of these substances took up H_2O_2 in the absence of enzyme; all of them are therefore oxidised under the influence of catalysis by the enzyme.

The determination of unused H_2O_2 in the experiments with tannin and gallic acid by the MnO_2 method had to be made in neutral solution since it was found that acid solutions of these substances, and of pyrogallol, evolve an uncertain amount of gas (oxygen?) when shaken with MnO_2 in the absence of any H_2O_2 .

Dilute iron solutions (0.1 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ or FeCl_3) with dilute H_2O_2 oxidised some of the above phenols slowly, but in no case was the reaction anything like as strong as with the enzyme solutions.

Pyrrole was tried (50 mg. and 15 mg. in the usual volume) and found not to be oxidised with milk- or horseradish-peroxidases. Its presence causes probably complete inhibition of the milk-enzyme and very great inhibition of the plant-enzyme. With pyridine also, some degree of inhibition of the activity of both enzymes was observed.

DISCUSSION.

The following is a list of the substances shown, in this and my last paper and by previous workers, to be oxidised by H_2O_2 under the catalysis of the peroxidases of milk and horseradish. The numerous compounds of various types tried and found not to be oxidised by the system are listed elsewhere.

Milk-peroxidase.

Iodide, nitrite, tryptophan.

Diamines such as *o*- and *p*-phenylenediamine, benzidine, *etc.*

Guaiacum and all phenolic substances (except resorcinol which inhibits the enzyme).

Horseradish-peroxidase.

Iodide.

Diamines such as benzidine, phenylenediamines, leuco-malachite green, *etc.*

Guaiacum and all phenolic substances.

Studies of these two very similar enzymes have brought to light a number of interesting differences between them which should have some bearing on theories of the mechanism of their actions. The difference in p_{H} range of activity mentioned in the last paper is probably a function of the accompanying colloidal material in the preparation; the inhibition by resorcinol of the milk-enzyme, but not of the plant preparation, may possibly also have something to do with the colloidal states of the two preparations. But the oxidations of nitrite and tryptophan by the one and not by the other seem to be real differences between the active systems. Also a glance at Table VI suggests that the products of oxidation are not always the same in the two cases. As to the specificity towards the oxidising substrate, Wieland and Sutter [1930] have studied the activation of various organic peroxides by horseradish-peroxidase and it would be interesting to see their work repeated with the milk-enzyme. Dixon [1929] has stated that milk- but not horseradish-peroxidase will use persulphate as oxidising substrate.

The theory of the mechanism of peroxidase reaction put forward by Mann [1931] could perhaps explain these differences by assuming differences in the specificity of the linkages combining the two substrates to the enzyme.

The present extension of my last paper has not thrown any further light on the place of peroxidase as a constituent of the respiratory mechanism. If a function is to be found for peroxidase it may be, as suggested before, that in its normal environment the enzyme is provided with substrates "activated" by other catalysts; its ready ability to form coloured oxidation products may be of importance, though the non-oxidation of, and inhibition by, pyrrole does not seem to support this in the case of animal tissues.

In view of the general reaction demonstrated with phenols, some revision of the present ideas concerning the interrelation of oxidases and peroxidases in plant tissues may be necessary.

SUMMARY.

1. The preparation of horseradish-peroxidase in fairly concentrated form is described. The method consists simply in extraction of the minced root with water, precipitation of the enzyme by saturation with ammonium sulphate, purification of the precipitate by dialysis and filtration, and finally fractional precipitation by alcohol.

2. Using the method described by Elliott [1932] it was shown that nitrite and tryptophan are not oxidisable by H_2O_2 with the plant-enzyme, which is therefore different from the milk-peroxidase.

3. The following substances have also been tried with the horseradish-peroxidase and found not to be oxidised: formate, acetate, lactate, oleate, glucose, fructose, glycerol, ethyl alcohol, acetaldehyde, glycine, glutamic acid, phenylalanine, histidine, brucine.

4. Formaldehyde, like dihydroxyacetone and phenylglyoxal as shown previously, is oxidised by very dilute H_2O_2 without enzyme.

5. Positive results with a representative series of phenols allow the conclusion to be drawn that all phenolic substances are oxidised by H_2O_2 with both horseradish- and milk-peroxidases.

6. To the above generalisation one exception was found. Resorcinol, though oxidised with the plant-enzyme, is not oxidised by milk-peroxidase the activity of which it inhibits.

7. Pyrrole is not oxidised with either enzyme and also has an inhibitory action which appears more complete with the milk-enzyme. Pyridine also has some inhibitory effect on the action of both enzymes.

It is a pleasure for me here to record my gratitude to Geheimrat H. Wieland for allowing me to work in his department, and for his kind interest in this work. I am also grateful to Sir F. G. Hopkins for his encouragement.

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