CCLXXI. A NOTE ON PEROXIDASE SPECIFICITY.

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THE evidence for the view which has been held by Bach and others, that peroxidases can activate true organic peroxides, has never been very satisfactory. Such peroxides as had been found to act with peroxidases were those formed by replacing only one of the hydrogen atoms of hydrogen peroxide by an organic radical (*i.e.* peroxides of the formula $R.OOH$). More recent work (*e.g.* that of Wieland and Sutter [1930]) has tended to show rather that, though such peroxides can be utilised by peroxidases (although much less readily than hydrogen peroxide itself), substitution of both hydrogen atoms gives peroxides (of the formula R_2O_2) which are not acted on by peroxidase.

During the course of some experiments on milk peroxidase carried out some years ago the writer found that persulphates could be substituted for hydrogen peroxide in the guaiacum test for peroxidase. (This fact has already been briefly mentioned [Dixon, 1929] but no details have been published.) This observation has since been repeated with more than a dozen different preparations of milk peroxidase, with positive results in all cases. It has been found however that horseradish peroxidase is totally unable to activate persulphate, and completely negative results were obtained even with preparations of horseradish peroxidase which were many times as active towards H_2O_2 as those of the milk peroxidase. Table I gives the results of a typical experiment.

Table I.

Tube 1.	Guaiacum + persulphate		-
" 2.	" + milk peroxidase	+ persulphate	+ +
" 3.	" + boiled milk peroxidase	+ persulphate	-
" 4.	" + milk peroxidase	+ H_2O_2	+ + +
" 5.	" + horseradish peroxidase	+ persulphate	-
" 6.	" + horseradish peroxidase	+ H_2O_2	+ + + +

The milk peroxidase was prepared by Elliott's [1932, 1] modification of Thurlow's [1925] method, and a 10 % solution in dilute phosphate buffer at p_H 6.8 was used. The horseradish peroxidase solution was obtained by extracting with water scrapings of horseradish which had been well soaked in alcohol and adding a little phosphate buffer. 3 ml. of peroxidase solution were taken for each tube, and 3 drops of a freshly prepared tincture of guaiacum, followed by a few drops of a 1 % solution of potassium persulphate or a 0.1 % solution of H_2O_2 , were added. The strength of the blue colour after 3 minutes at room temperature is roughly indicated in the last column. (- means that no trace of blue colour could be detected.) On long standing tubes 1 and 3 also developed some blue colour, as persulphate very slowly oxidises guaiacum in the absence of peroxidase. It is essential to use freshly prepared tincture of guaiacum, as old solutions are very rapidly blued by persulphate in the absence of peroxidase.

The above experiment brings out a clear difference in specificity between the animal and the vegetable peroxidases, inasmuch as the former can activate persulphate while the latter cannot. The absence of colour from tube 5 cannot

be due to destruction of the horseradish peroxidase by the persulphate, for the subsequent addition of a trace of H_2O_2 immediately produced a blue colour equal to that in tube 6, showing that the peroxidase was still intact. The positive reaction in tube 2 cannot be due to traces of H_2O_2 in the persulphate solution, as the absence of colour from tube 5 shows that the solution used was completely free from such traces. This is perhaps the first time that a positive peroxidase reaction has been obtained with a peroxide of the type R_2O_2 (if we may so regard persulphate) where H_2O_2 was certainly absent.

Most "peroxidase reagents" cannot be used for the detection of the activation of persulphate, as they are instantaneously oxidised by persulphate alone. This is the case for example with benzidine, *p*-phenylenediamine and the "Nadi" reagent (see Table V). Catechol and guaiacol (methylcatechol) seem however to resemble guaiacum in not being oxidised by persulphate alone at an appreciable rate (at any rate no colour is produced), while they are readily oxidised by persulphate in presence of milk peroxidase with the production of yellow-brown and red-brown colours respectively. Pyrogallol is oxidised by persulphate alone, but sufficiently slowly to enable the accelerating effect of milk peroxidase to be clearly seen. The course of the reaction with these three reagents is shown in Tables II, III and IV. It will be seen that the activation of persulphate by milk peroxidase is clearly shown in all cases, while again completely negative results were obtained with horseradish peroxidase.

Table II.

Contents of tube	Colour after		
	5 mins.	10 mins.	20 mins.
Horseradish peroxidase + catechol + persulphate	—	—	—
Milk peroxidase + catechol + persulphate	+	+++	++++
Boiled milk peroxidase + catechol + persulphate	—	—	+
Catechol + persulphate	—	—	—

Table III.

Contents of tube	Colour after		
	2 mins.	4 mins.	8 mins.
Horseradish peroxidase + pyrogallol + persulphate	—	±	+
Milk peroxidase + pyrogallol + persulphate	++	+++	++++
Boiled milk peroxidase + pyrogallol + persulphate	—	±	+
Pyrogallol + persulphate	—	±	+

Table IV.

Contents of tube	Colour after	
	10 mins.	20 mins.
Horseradish peroxidase + guaiacol + persulphate	—	—
Milk peroxidase + guaiacol + persulphate	+	++
Boiled milk peroxidase + guaiacol + persulphate	—	—
Guaiacol + persulphate	—	—

All these experiments show that the milk and horseradish peroxidases are not equivalent, since one can use persulphate while the other cannot. Other clear differences between the two peroxidases have been shown by Elliott [1932, 2], who found that horseradish peroxidase could not bring about the oxidation of either nitrite or tryptophan by H_2O_2 , whereas the milk peroxidase could readily do so in both cases.

According to the fairly generally accepted view originally put forward by Kastle and Loevenhart [1903], peroxidases act by combining with H_2O_2 to form a complex peroxide which is "active" and able to oxidise such substances as guaiacum directly. This view is supported by the fact that many organic

peroxides, such as for example benzoyl peroxide ($\text{C}_6\text{H}_5\text{CO}$)₂O₂, are almost equivalent in their oxidising power to peroxidase + H₂O₂ (see below). The results of Wieland and Sutter mentioned above also appear to be what one would expect on this hypothesis. The activation of persulphate by milk peroxidase however seems difficult to explain in such a way.

Since haematin compounds in general have a peroxidase-like activity, and there is some reason to think that peroxidases themselves may be haematin compounds, experiments were made to see whether haematin resembles milk peroxidase in being able to activate persulphate. All attempts to demonstrate a catalytic action of haematin on the oxidation of guaiacum by persulphate were however unsuccessful, though it is of course possible that this was merely because the correct conditions were not found. Haematin itself seems to resemble horseradish peroxidase in being unable to catalyse this reaction. It was also found that haematin was unable to bring about the oxidation of nitrite by H₂O₂. Here again it resembles horseradish rather than milk peroxidase. (This is not however to be regarded as evidence that milk peroxidase is not a haematin compound. It may well be that certain other haematin compounds are able to catalyse these reactions. Preparations of milk peroxidase do in fact show a haematin spectrum [Elliott, 1932, 1]. The question of the haematin nature of horseradish peroxidase has recently been discussed critically by Elliott and Keilin [1934]. It is of interest in this connection that Keilin [1934] has shown that H₂O₂ forms a compound with methaemoglobin.)

It was mentioned above that benzoyl peroxide resembles peroxidase + H₂O₂ in its behaviour. Investigation showed that the parallelism is indeed very close, since it oxidises almost all the peroxidase reagents without the addition of peroxidase. Table V gives a summary of the results obtained. In this table

Table V.

	Guaiacum	Benzidine	Nadi reagent	<i>p</i> -Phenylenediamine	Guaiacol	Catechol	Pyrogallol	NaNO ₂
H ₂ O ₂ alone	—	—	—	—	—	—	—	—
H ₂ O ₂ + milk peroxidase	+	+	+	+	+	+	+	+
H ₂ O ₂ + horseradish peroxidase	+	+	+	+	+	+	+	—
Persulphate alone	—	+	+	+	—	—	+s	—
Persulphate + milk peroxidase	+	.	.	.	+	+	+	.
Persulphate + horseradish peroxidase	—	.	.	.	—	—	—	.
Benzoyl peroxide alone	+	+	+	+	—	+s	+s	+
Benzoyl peroxide + either peroxidase	.	.	.	.	—	.	.	.

+ denotes a markedly positive reaction, +s means that the reaction was quite definite but developed rather slowly, and — shows that the reaction was quite negative. As benzoyl peroxide is practically insoluble in water it was dissolved in dilute alcohol in order to carry out the tests. It will be noticed that nitrite is oxidised by benzoyl peroxide (the experiment was carried out with this, and not with benzoylhydrogen peroxide as stated by Harrison and Thurlow [1926, p. 227]). The disappearance of the nitrite is easily shown by means of the Griess-Ilosvay reagent.

Although the parallelism between benzoyl peroxide and H₂O₂ + peroxidase is almost complete, one case was found in which they definitely diverge. Guaiacol is oxidised by either milk or horseradish peroxidase + H₂O₂, and even by milk

peroxidase + persulphate, but benzoyl peroxide is unable to oxidise it. The reaction remains completely negative even after the addition of either of the peroxidases, showing that they are unable to activate benzoyl peroxide. Their inability to do so is not due to any toxic action of the benzoyl peroxide, as the subsequent addition of a little H_2O_2 immediately produced a strong positive reaction in both cases.

SUMMARY.

Persulphate is activated by milk peroxidase and can replace hydrogen peroxide in the system, although the velocity is then smaller. Horseradish peroxidase cannot activate persulphate.

Benzoyl peroxide is almost exactly equivalent to peroxidase *plus* hydrogen peroxide in its behaviour towards the peroxidase reagents, except in the case of guaiacol, which is oxidised by the peroxidase system but not by benzoyl peroxide.

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