II. MILK PEROXIDASE.

ITS PREPARATION, PROPERTIES, AND ACTION WITH H_2O_2 ON METABOLITES.

WITH A METHOD FOR DETERMINING SMALL AMOUNTS OF H_2O_2 IN COMPLEX MIXTURES.

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It is generally believed that peroxidases are almost universally present in the tissues of animals and widely distributed in plants and bacteria, but though there is a large literature concerning them, it has not yet been shown experimentally that they serve any physiological function. It is even doubted whether their specific oxidising substrate, hydrogen peroxide, ever occurs significantly in normal living matter, since no positive test in such material has been obtained. But it is certain that H_2O_2 is formed by certain oxidising systems separated from living matter; thus H_2O_2 has been shown to be formed in the aerobic oxidations promoted by xanthine oxidase, aldehyde oxidase, succinic oxidase, tyramine oxidase, and the catechol oxidase of potatoes, and also in the oxidation of cysteine and glutathione [Thurlow, 1925; Kodama, 1926; Hare, 1928; Onslow and Robinson, 1926], and the work of M'Leod and Gordon [1922] and of Avery and Neill [1924, 1925] makes it clear that hydrogen peroxide is produced in bacterial metabolism.

Thus it seems likely that H_2O_2 could be a normal tissue product. The difficulty in detecting it in normal tissues may be quite easily accounted for. Peroxidase acts at extraordinarily low concentrations of H_2O_2, and if reducing substrates are present, the H_2O_2 would be removed by this mechanism as soon as it is formed; also reduced glutathione present in the tissues would use up H_2O_2, and Keilin [1930] has shown that reduced cytochrome-*c* is readily oxidised by H_2O_2. Finally any accumulation would be destroyed by catalase which appears to be present in all tissues.

Dakin [1905–1909] showed that many biological substances could be oxidised by H_2O_2, in some cases with iron catalysis, to give products similar to those met with in studies of intermediary metabolism. This, and the fact that H_2O_2 formed by aerobic oxidising systems could be made to perform secondary oxidations [Thurlow, 1925; Harrison and Thurlow, 1926; Szent-

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Györgyi, 1928; Wieland and Rosenfelt, 1930] suggested that hydrogen peroxide might be an important biological oxidising agent. In Thurlow and Harrison's work haemoglobin was oxidised in the secondary stage to methaemoglobin, and, with iron as catalyst, lactate and β-hydroxybutyrate were oxidised. But the only oxidations they observed to be catalysed by peroxidase were those of nitrite and an ether-soluble substance, presumed to be a fat, present in the milk from which the enzyme itself was prepared, and only present in summer. Szent-Györgyi [1928] demonstrated the oxidation by H_2O_2 and peroxidase of phenolic substances, such as adrenaline, which in turn oxidised the hexuronic acid found by him in plants and the adrenal cortex of animals, and he claimed to have shown a connection between this system and the glutathione sulphydryl-disulphide system.

The presence of peroxidase is detected by colour reactions with H_2O_2 and a large variety of organic compounds, but the only substances of biological interest known to be oxidised by this enzyme and H_2O_2 are adrenaline (Szent-Györgyi) and the rather doubtful ether-soluble substance of Harrison and Thurlow mentioned above. Thus, though peroxidase is so widely distributed, with H_2O_2 quite possibly a regular metabolite, there is no direct evidence for its having any physiological function except for the complicated, and limited, scheme of Szent-Györgyi.

It was felt that more knowledge of the powers of peroxidase with H_2O_2 as an oxidising agent for substances of metabolic interest might throw light on its possibilities as a biological mechanism. The main object of the work to be described has therefore been to see what possible metabolites can be oxidised by H_2O_2 and an animal peroxidase under more or less physiological conditions.

Batelli and Stern [1908] claimed to have shown the oxidation of formate, with evolution of CO_2, by H_2O_2 with various tissues, especially liver, the catalyst responsible being thermolabile. However, their methods were crude; H_2O_2 was used in concentrations far greater than could occur in normal tissue, and probably sufficient to destroy any true peroxidase; the formate concentration was also very high, and the p_H was unknown but was certainly kept very low in order to diminish destruction of the hydrogen peroxide by catalase. Using a refined method with a more purified enzyme preparation in proper conditions their results have not been confirmed by me.

Preparation of the enzyme.

The peroxidase of milk was chosen as being a true peroxidase of animal origin, easily prepared as a soluble solid free from other enzymes, especially catalase.

Thurlow [1925] used a preparation of this enzyme obtained by mixing milk with an equal volume of saturated ammonium sulphate solution, filtering the precipitated caseinogen and fat, and then saturating the filtrate with ammonium sulphate. The lactalbuminous precipitate thus obtained gave

fairly active solutions and was used in her experiments. Her method is here modified in view of the following observations.

If, instead of a fresh ammonium sulphate solution, a filtrate (containing lactose *etc.*) from a previous preparation was used for the half-saturation of milk, the peroxidase activity was deposited entirely on the caseinogen precipitate leaving the filtrate inactive. Similar results are obtained when using a fresh ammonium sulphate solution if a few extra crystals of the salt are added as well. Thus a slight excess of crystalloidal matter is sufficient to determine the loss of all activity in the filtrate, and by the Thurlow method quite a considerable amount of the enzyme is lost on the caseinogen. (The enzyme can be washed off the caseinogen precipitate again by a one-third saturated solution of ammonium sulphate.) For the purpose of this work, however, it was not permissible to add to the milk only the minimum amount of salt required to precipitate the caseinogen, as it was very necessary to deposit the catalase and this is only accomplished on approaching half-saturation [mentioned by Raudnitz, 1901].

If solid ammonium sulphate is added gradually to the active filtrate free from caseinogen and fat, it is found that the enzyme is deposited entirely on the first protein precipitate formed so that activity disappears quite sharply from the liquid phase. Saturation with the salt is therefore not necessary, as it simply brings down more inactive protein.

Below is described a typical example of the new preparation, which has the following advantages. By adding solid ammonium sulphate instead of a saturated solution, the volumes are kept smaller and a considerable economy of the salt is effected. If the correct amount of ammonium sulphate is used, the resulting preparation contains a negligible amount of catalase. By adding only just sufficient salt in the final precipitation of the enzyme, the amount of protein accompanying the enzyme is diminished to about one-third, and far less ammonium sulphate is present in the preparation than when the liquid has been saturated.

To 8 litres of fresh milk 2000 g. of solid ammonium sulphate were added. (The amount of salt to be added was always determined by adding various amounts of the salt to 50 cc. samples of the milk to be used, and, by qualitative tests[1], finding the greatest amount of the salt that could be added without appreciably diminishing the peroxidase activity of the filtrate. As mentioned above, if too much salt is added the peroxidase is lost on the caseinogen, if too little, then the preparation will be contaminated with catalase.) On filtering through large fluted filter-papers, 7350 cc. of a clear filtrate were obtained. To the filtrate 1240 g. of ammonium sulphate were added. (By filtering small samples during the addition and testing the activity of the filtrates this amount was found to be sufficient to precipitate the enzyme completely.) On filtering, and drying the precipitate between filter-papers and in a vacuum desiccator, 20·3 g. of the active solid were obtained.

[1] In all qualitative tests for peroxidase it was found best to add the H_2O_2 in 0·02 % solution.

The inactive filtrate was then saturated with ammonium sulphate by the addition of a further 1600 g.; the dried lactalbumin precipitate of 43 g. was completely inactive and was used for certain control experiments to be described later.

The milk had a "purpurogallin number" of 0·0225 and therefore contained 180 units of peroxidase in the 8 litres, and the filtrate free from caseinogen and fat had a purpurogallin number of 0·010 and therefore contained 73·5 units. (If it were not necessary to get rid of small amounts of catalase, a much higher yield could be obtained by adding less salt.) The solid preparation had a purpurogallin number of 2·21, and contained therefore in the 20·3 g., 44·9 units corresponding to a yield of 25 % of the activity of the milk.

Preparations of more than twice the strength of the above have been obtained by the same method.

The estimations of peroxidase activity were made by the method of Willstätter and Stoll [1918]. In estimating the activity of milk 0·9 cc. of the milk was required, which caused a thick emulsion to be formed in the ether extraction; this emulsion was best broken by small additions of alcohol. With the other enzyme preparations the emulsion could usually be broken by the addition of sodium chloride.

Properties of the enzyme.

A solid preparation when kept in the ice-chest loses its activity very slowly; for example, in 6½ months at 0–5° the activity of one sample dropped from a purpurogallin number of 3·6 to 2·4.

The enzyme is active over a wide range of hydrogen ion concentration. In the alkaline range from p_H 8 to nearly 10 the activity is diminished but recovers immediately on neutralising; there is still a trace of activity at p_H 10 though the enzyme is almost completely and irreversibly destroyed. In the acid range, between p_H 4·2 and 3·8 a certain amount of precipitate is formed and the activity is diminished; between p_H 3·6 and 3·2 the precipitate redissolves and all activity disappears. On bringing these acid solutions to about p_H 7 and allowing to stand overnight, the activity is regenerated to some extent; this interesting observation is worth further study.

It will be noted that the peroxidase from milk is more sensitive to acidity than that from horse-radish. In consequence it is impossible to use the malachite green method of Willstätter and Weber [1926] for estimating activity, as this method necessitates working at p_H 3·5 to keep leuco-malachite green in solution, and at this acidity milk peroxidase is quite inactive.

Up to p_H 7 this peroxidase oxidises benzidine to give a blue colour which usually changes to brown. In more alkaline solutions the benzidine is oxidised directly to the brown substance. *p*-Phenylenediamine gives a blue colour throughout the range of activity of the enzyme.

In the experiments on the preparation of the enzyme, it was noticed that whenever a clear active solution was obtained a slight but distinct brown colour was present, while inactive solutions were considerably less coloured; a concentrated solution of the solid preparation was strongly coloured. The

solid on reduction and treatment with pyridine gave a haemochromogen spectrum similar to, but not the same as, that given by a horse-radish preparation when treated in the same way. The indications are that some coloured haematin body is connected with the enzymic activity, and this question is being studied further.

Effects of sulphydryl compounds on tests for peroxidase.

The inhibitory effect of very small amounts of H_2S on the activity of peroxidases is well known. It was found that various other sulphydryl compounds interfere with the test when using benzidine, tincture of guaiacum, or *p*-phenylenediamine as substrate for the peroxidase and H_2O_2. Solutions of egg- or milk-albumin, which have been denatured by boiling in alkali and then neutralising, give a nitroprusside test for —SH, and when added to a peroxidase solution prevent the blue colour forming with H_2O_2 and benzidine or guaiacum; the formation of colour with *p*-phenylenediamine is noticeably slowed. Neutral solutions of cysteine and reduced glutathione have the same effect. In boiled milk the enzyme is destroyed and a slight positive nitroprusside test is given; adding boiled milk to fresh milk will cause some interference with the peroxidase test using benzidine, guaiacum or *p*-phenylenediamine.

However, the action of these sulphydryl substances is not apparently an inhibition of the enzyme, as is that of H_2S, but is due to their reducing action on the coloured products of the oxidations. Whereas 0·1 cc. of $M/100$ H_2S solution will inactivate 5 cc. of a solution of the enzyme, only some delay in the colour formation from *p*-phenylenediamine is caused by 2 cc. of $M/100$ cysteine solution. Guaiacum is more sensitive, 0·4 cc. causing a delay; and benzidine is very sensitive, 0·2 cc. of the cysteine solution completely preventing the formation of the blue colour. It is found that addition of —SH compounds to benzidine blue immediately decolorises it; guaiacum blue is fairly rapidly decolorised. The effect on *p*-phenylenediamine blue is scarcely noticeable for some hours.

The reaction with pyrogallol is not affected by cysteine or denatured protein, and it was found that purpurogallin is not decolorised by —SH compounds.

Estimation of hydrogen peroxide.

As the chief method of attacking the problem was to see whether hydrogen peroxide was used up in the presence of peroxidase and the substances in question, it was necessary to devise a method for estimating small quantities of H_2O_2 in the presence of proteins and other substances which would interfere with the usual methods. It was decided to make use of the catalytic decomposition of H_2O_2 by manganese dioxide, and to measure the oxygen evolved.

Two 100 cc. Erlenmeyer flasks were fitted with rubber stoppers through which passed glass tubes connected by pressure tubing with a Haldane

evolution manometer [Haldane, 1920] to measure 1 or 2 cc. of gas. One of the glass tubes was so made that a short test-tube could be suspended from it by means of a platinum hook. The suspended tube contained about 0·2 g. of manganese dioxide powder and the flask in which it was hung contained the liquid the H_2O_2 content of which was to be measured. The other flask contained an equal volume of water and was connected up as temperature compensator. The two flasks were clamped close together in a tank so that they could be steadily shaken while up to their necks in water at room temperature. They were shaken gently for 20 minutes or more until equilibrium was reached on the manometer. The manometer was then set, and the suspended tube was jerked into the liquid and that flask vigorously shaken by hand for about 5 minutes. The two flasks were then again shaken steadily until equilibrium was reached, and the volume of gas evolved was read off on the manometer.

Table I. *Gas evolved on shaking various solutions with MnO_2.*

In all cases the total volume was made up to 35 cc. 10 cc. of the hydrogen peroxide solution (0·02 %) give theoretically 0·66 cc. of O_2 at 15° on catalytic decomposition.

H_2O_2 solution cc.	20 % H_2SO_4 cc.	*M*/15 phosphate buffer cc.	Other solutes	Gas evolved cc.
10	—	—	—	0·79 0·81 0·72
10	—	20	—	1·16 1·12 1·20
10	—	8	—	1·16
10	3	—	—	1·32
10	—	—	1 g. lactalbumin	1·16
10	3	20	—	1·30
10	—	20	1 g. lactalbumin	1·23 1·09
10	3	20	1 g. lactalbumin	1·30
10	—	—	0·4 g. peroxidase preparation	1·02
10	—	20	0·4 g. peroxidase preparation	1·16
10	3	20	0·4 g. peroxidase preparation	1·30
10	—	—	1 g. egg-albumin	0·83
10	—	—	0·5 g. gelatin	0·77
10	—	—	Albulactin	0·94 0·95 0·93
10	—	—	0·5 g. Na_2SO_4	0·83
10	—	—	1 g. $(NH_4)_2SO_4$	0·90
5	—	—	—	0·43
2·5	—	—	—	0·18
5	3	—	—	0·70
5	—	20	—	0·63
2·5	—	20	—	0·33
5	3	20	1 g. lactalbumin	0·68
5	—	—	1 g. lactalbumin	0·62

The reaction of MnO_2 with hydrogen peroxide is not always a simple catalytic decomposition of H_2O_2, and it was found that the presence of various substances affected the reaction, all causing evolution of O_2 to be in excess of the amount required by the equation, $H_2O_2 = H_2O + \frac{1}{2}O_2$. For instance,

when the O_2 evolved according to this equation should be 0·7 cc., the presence of phosphate caused the production of 1·2 cc. of gas, though variations in the concentration of phosphate did not affect this amount. Lactalbumin had a similar effect, and other proteins and salts also caused increased evolutions of gas. In the presence of sulphuric acid 1·3 cc. were evolved, and this amount was not affected by other substances in solution as well. These results are illustrated in Table I. It is probable that these substances cause the reaction $MnO_2 + H_2O_2 = MnO + H_2O + O_2$ to take place to some extent; with sulphuric acid manganous sulphate would be formed.

In all cases it appears that the evolution of O_2 remains proportional to the amount of H_2O_2 initially present, when solutions containing the same solutes but varying amounts of H_2O_2 are estimated. Thus the method is suitable, and very convenient, for estimating roughly and comparatively very small amounts of H_2O_2 (in these experiments, a maximum of 2 mg. H_2O_2 in 35 cc.) in a mixed solution, provided the other substances in solution are the same in each case. Rough absolute values for H_2O_2 concentrations could be obtained by standardising against known H_2O_2 solutions containing the same solutes. It is recommended that sulphuric acid should be added to the liquid before estimating. As is seen in Table I, the greatest yield of gas is then obtained and it appears not to be affected by other solutes.

When estimating H_2O_2 in an acid solution it is very necessary to use MnO_2 that has been previously thoroughly washed with acid to remove traces of carbonate that are often present.

Oxidations by peroxidase and H_2O_2.

The method used consisted simply in slowly adding dilute hydrogen peroxide to a solution containing both peroxidase and the substance under experiment. After a suitable time the H_2O_2 was estimated by the manganese dioxide method. If at the end of the experiment there was as much H_2O_2 remaining as in a control without the substance, it could be concluded that the substance was not oxidised. If, however, there was a disappearance of peroxide (and a further control without the enzyme but with the substance showed no uncatalysed usage of H_2O_2) it was concluded that the enzyme *plus* the substance under experiment were using up H_2O_2, *i.e.* oxidation was occurring.

From the work of Bach [1904], Willstätter and Weber [1926], and Mann [1931] on the peroxidase of horse-radish, it is known that excess of H_2O_2 inhibits the activity of the enzyme and, in larger amounts, destroys it. It was therefore necessary to work with very low concentrations of H_2O_2. In the method used the maximum concentration of H_2O_2 was 2 mg. in 35 cc. ($1{\cdot}4 \times 10^{-3}$ *M*), and if the peroxide was used up at all rapidly its concentration would not exceed 0·1 mg. in 35 cc. (or 7×10^{-5} *M*). With such concentrations Willstätter and Weber, and Mann, with the vegetable peroxidase

and pyrogallol, leuco-malachite green, or guaiacol, found their enzyme quite active. Destruction of enzyme was found not to be significant. Mann showed, with the vegetable peroxidase, that very high concentration of the reducing substrate had an inhibitory effect. The effect was not very large, but nevertheless the concentrations of substances to be tested were always made small, though more than sufficient to use up all the H_2O_2 if they were being oxidised by it.

Nitrite.

Before making experiments on substances of more biological interest, it was decided to see whether the method to be used would give clear-cut results with nitrite, since this substance is known to be oxidised by peroxidase and H_2O_2 [Haas and Lee, 1924; Thurlow, 1925].

The following solutions were used.

A 10 % solution of a Thurlow[1] peroxidase preparation in water or phosphate buffer, adjusted roughly with dilute sodium hydroxide to p_H 7.

A 0·02 % solution of hydrogen peroxide. (Diluted from Merck's "perhydrol.")

A solution of sodium nitrite containing 1 mg. $NaNO_2$ per cc.

The following seven experiments were made in 100 cc. Erlenmeyer flasks, A to G.

Flask A contained 20 cc. peroxidase solution and 8 cc. nitrite solution. While the flask was kept at 38–40° in a bath and gently shaken, 10 cc. of the hydrogen peroxide solution were slowly added from a burette fixed in the stopper of the flask during 45 minutes, and the flask was kept warm for a further 45 minutes. It was then cooled and connected with the estimation apparatus and the hydrogen peroxide remaining estimated. No O_2 was evolved showing that the H_2O_2 had all been used up, the nitrite added having been more than the equivalent of the hydrogen peroxide.

The procedure was the same in the remaining six experiments, the contents of the flasks varying as follows:

B. Only 3 cc. nitrite solution, *plus* 5 cc. water.

C. No nitrite, 8 cc. water.

D. Same as B, but 10 cc. of water were added instead of the H_2O_2 solution.

E. Same as B, but the peroxidase solution was first inactivated by heating to 85–90° for 7 minutes.

F. Peroxidase solution of only half the usual strength was used and only 1 hour instead of 1½ hours was allowed.

G. The same enzyme preparation was used after it had lost most of its activity on standing 6 weeks at room temperature. 2·5 cc. nitrite solution.

The results of these experiments are summarised in Table II. A further control experiment showed that H_2O_2 and manganese dioxide did not oxidise

[1] This experiment was done before the improved preparation was devised. Similar results were later obtained with much more dilute solutions of the stronger preparations.

the nitrite. Similar experiments using half the strength of H_2O_2 and less nitrite gave similar results.

In A, B and F, when nitrite was being oxidised a yellow colour developed; in none of the other experiments did it appear, and it is probable that it is due to a xanthoproteic reaction given by the nitrate formed, as addition of ammonia was found to deepen the colour to orange.

Table II. *Oxidation of nitrite by H_2O_2 and peroxidase.*

Exp. No.	Characteristic	H_2O_2 unused cc. O_2 evolved by MnO_2	Griess-Ilosvay test at end for nitrite	Xantho-proteic (?) colour formed during exp.
A	Excess nitrite	0·02	+ + + +	+ +
B	Low nitrite	0·34	−	+
C	No nitrite	1·14	−	−
D	No H_2O_2	0·0	+ + + +	−
E	Heat-inactivated enzyme	0·96	+ + + +	−
F	Half amount of enzyme. Less time. (Low nitrite)	0·37	+	+
G	Age-inactivated enzyme	0·75	+ · + +	−

Possible metabolites.

Having shown that the method gives clear results with nitrite, various substances of metabolic interest were tried. In each series of experiments, especially when new enzyme preparations were being used, an experiment with nitrite was also done in order to make sure that the conditions were correct.

In the following experiment formate, glycine, glucose and oleic acid were tried.

Six 100 cc. Erlenmeyer flasks, each fitted with a 10 cc. burette fixed in the stopper, were set up. Each contained 20 cc. of an aqueous 2 % solution of a peroxidase preparation of purpurogallin number 4·3, roughly adjusted with $N/10$ soda to p_H 7. In addition 5 cc. of the following solutions were added to the respective flasks: 5 mg./cc. sodium formate, 5 mg./cc. glycine, 5 mg./cc. glucose, 10 mg./cc. oleic emulsion, 3 mg./cc. sodium nitrite, and distilled water. The "oleic emulsion" was prepared by dissolving oleic acid in the equivalent amount of NaOH solution and then adding acid till the reaction was about p_H 7·8. The p_H of the peroxidase solution was not appreciably affected by 5 cc. of this emulsion.

During 45 minutes' gentle shaking in a bath at 38–40°, 10 cc. of 0·02 % hydrogen peroxide were added in 0·5 cc. lots every 2½ minutes from the burette attached to each flask. The flasks were allowed to stand another 20 minutes in the bath and then a 1 cc. sample was withdrawn from each flask, before adding 3 cc. of 20 % sulphuric acid. The addition of acid stabilised the remaining hydrogen peroxide which was estimated by the manganese dioxide method at room temperature as soon as convenient.

To the 1 cc. samples a couple of drops of *p*-phenylenediamine solution

were added. If colour developed it showed that both H_2O_2 and peroxidase activity were still present. If no colour formed, a little peroxide solution was added as well to see whether the enzyme was still active.

A seventh flask contained the peroxidase solution, the whole 10 cc. H_2O_2 solution was run in at once and the acid was added immediately without the 65 minutes in the water-bath.

The results of these experiments are summarised in Table III.

Table III.

		Formate	Glycine	Glucose	Oleic acid	Nitrite	Water	Acid added before exp.	Theory for 10 cc. H_2O_2 solution
1 cc. sample	Colour formed on addition of *p*-phenylene-diamine	+	+ +	+	+ +	−	+		
	plus H_2O_2	+	+ +	+	+ +	+ +	+		
H_2O_2 remaining at end. cc. O_2 evolved by MnO_2		0·68	0·57	0·58	0·57	0·00	0·62	1·29	0·68

In the control with immediate addition of acid it is seen that 1·29 cc. of gas were evolved instead of the theoretical 0·68 cc. This has been remarked on above. The difference between this control and the experimental flask containing only water and no reducing substrate shows the amount (1·29 − 0·62 = 0·67 cc.) of H_2O_2 which has been destroyed during the experiment by traces of catalase.

In the presence of excess nitrite we see again that the H_2O_2 has been completely used up, but with none of the other substances tried has there been any diminution of the H_2O_2 below that found in the water control. The qualitative tests also show that the enzyme is active at the end of the experiment in all cases, and that H_2O_2 has disappeared in the presence of nitrite but not in the presence of the other substances.

It can therefore be concluded that formate, glycine, glucose and oleic acid are not oxidised by peroxidase and H_2O_2, at least in conditions in which nitrite is quantitatively oxidised, and such substances as benzidine, pyrogallol and *p*-phenylenediamine are readily oxidised.

The result with formate is in contradiction to the finding of Batelli and Stern [1908] with liver and other tissues. They measured the extra CO_2 produced by formate oxidation. The above experiment with formate was repeated using a partially evacuated flask so that the peroxide solution could be added, and acid run in at the end of the experiment, without letting gas escape. After adding acid, samples of the gas in the flask were withdrawn into a Haldane gas analysis apparatus and the CO_2 estimated. No more CO_2 was found in the gas after an experiment with formate than in a control without.

In Tables IV–VII are given results of experiments carried out exactly similarly to those summarised in Table III.

For the results shown in Table IV the solutions used were: peroxidase preparation same as for Table III but the solution here used contained only 1·6 % of the solid, *p*-phenylenediamine 10 mg./cc., tryptophan 10 mg./cc. adjusted to p_H 7, sodium nitrite 3 mg./cc., stearic emulsion containing 5 mg./cc. stearic acid, prepared similarly to the oleic emulsion. 5 cc. of each were used, the tyrosine was added as 100 mg. finely ground crystals with 5 cc. water, the reaction fluid thus becoming saturated with this slightly soluble substance.

Table IV.

		p-Phenylene-diamine	Trypto-phan	Stearic acid	Tyrosine	Nitrite	Water
Sample	Colour with *p*-phenylene-diamine	.	+ +	+	−	−	+
	plus H_2O_2	.	+ +	+	+ +	+ +	+
Colour formed during experiment		Dark	Yellow-brown	.	Yellow-brown	Yellow	.
H_2O_2 remaining at end. cc. O_2 evolved by MnO_2		0·0	0·15	0·70	0·00	0·00	0·70

Here the H_2O_2 has been used up by nitrite, *p*-phenylenediamine, tryptophan and tyrosine, the last three giving coloured products.

The results in Table V were obtained with a 1·6 % solution of a new enzyme preparation of purpurogallin number 4·9. Only 50 mg. of powdered tyrosine were used. The tryptophan, phenylalanine and glycerol solutions each contained 10 mg. per cc.

Table V.

		Nitrite	Water	Tyrosine	Trypto-phan	Phenyl-alanine	Glycerol
Sample	Colour with *p*-phenylene-diamine	−	+ +	−	−	+ + +	+
	plus H_2O_2	+ + + +	+ +	+ + + +	+ + +	+ + +	+
Colour formed during experiment		Yellow	.	Brown	Brown	.	.
cc. O_2 evolved by MnO_2		0·00	0·60	0·00	0·04	0·53	0·43

This confirms the results with tyrosine and tryptophan; phenylalanine and glycerol gave negative results.

For the results in Table VI the peroxidase solution contained 1·2 % of the same preparation as in Table V. 5 cc. each of the following solutions were used: acetaldehyde 5 mg./cc., sodium acetate, histidine and phenylglyoxal each 10 mg./cc.

No colour was formed (except with nitrite) and acetaldehyde, acetate and histidine are evidently not oxidised. Phenylglyoxal has used up H_2O_2, but this is further discussed below.

Table VI.

		Nitrite	Water	Acet-aldehyde	Acetate	Histidine	Phenyl-glyoxal
Sample	Colour with *p*-phenylene-diamine	–	+ +	+ + +	+ +	+ + + +	Yellow
	plus H_2O_2	+ + +	+ +	+ + +	+ +	+ + + +	Yellow
cc. O_2 evolved by MnO_2		0·00	0·50	0·62	0·50	0·45	0·08

A 1·6 % solution of the same enzyme preparation as in Tables V and VI was used for the experiments of Table VII. 5 cc. of each of the following solutions were used in the respective experiments: ethyl alcohol 5 mg./cc., sodium lactate 10 mg./cc., dihydroxyacetone 10 mg./cc., an emulsion of triolein containing 10 mg./cc., and phenylglyoxal 10 mg./cc.

Table VII.

		Ethyl alcohol	Lactate	Dihydroxy-acetone	Triolein	Phenyl-glyoxal
Sample	Colour with *p*-phenylene-diamine	+	+	Yellow	+	Yellow
	plus H_2O_2	+	+	.	+	.
cc. O_2 evolved by MnO_2		0·53	0·49	0·26	0·43	0·0

No colour was formed in any of these experiments. Dihydroxyacetone and phenylglyoxal react with *p*-phenylenediamine giving a coloration which obscures the peroxidase reaction. Alcohol, lactate and triolein are apparently not oxidised. Dihydroxyacetone, as well as phenylglyoxal, uses up H_2O_2, but this is further discussed below.

Besides those substances appearing twice in the above Tables, the experiments with formate, glucose, glycine, phenylalanine, histidine and oleic acid were all repeated with different enzyme preparations with the same negative results.

A single experiment with β-hydroxybutyrate also gave a negative result. The experiments with phenylglyoxal and dihydroxyacetone were both done twice; they use up H_2O_2, but, as will be shown, this is not due to peroxidase activity.

Of all the substances tried above the only ones to take up H_2O_2 were tyrosine, tryptophan, phenylglyoxal and dihydroxyacetone; also *p*-phenylene-diamine which was tried as a further test of the method. It was necessary to see whether these oxidations were catalysed by peroxidase or whether the oxidation would take place in the absence of the enzyme. This possibility was controlled by making experiments in exactly the same way as before but without the enzyme; to make the conditions in the controls resemble those in the experiments as closely as possible the completely inactive lactalbumin preparation mentioned above was used in place of the enzyme preparation. Table VIII summarises the results of these controls, together with a test to

make sure that delay in estimating the H_2O_2 in acidified solutions does not appreciably affect the results. The lactalbumin used, unlike the enzyme preparation, contained no traces of catalase so that larger amounts of H_2O_2 are left at the end of the experiments.

Table VIII.

cc. O_2 liberated by MnO_2.

Water	Water (estimated after standing overnight)	Tryptophan	Tyrosine	Dihydroxyacetone	Phenylglyoxal	*p*-Phenylenediamine
1·34	1·27	1·33	1·33	0·24	0·04	0·28

It is seen that there is no trace of peroxide usage by tyrosine or tryptophan in the absence of peroxidase, nor was any colour formation whatever observed during the addition of H_2O_2. The H_2O_2 usage and colour formation with these substances in the presence of peroxidase must therefore have been catalysed by the enzyme.

p-Phenylenediamine, as would be expected, was oxidised and used up H_2O_2 to some extent without the enzyme. The enzyme of course accelerates the oxidation greatly. Dihydroxyacetone and phenylglyoxal both used up H_2O_2 to the same extent as in the presence of the enzyme; their oxidation by H_2O_2 is therefore not catalysed by peroxidase.

Discussion.

A representative selection of substances which are of interest in animal intermediary metabolism has thus been subjected to the influence of hydrogen peroxide and milk peroxidase, under conditions which are approximately optimal for the working of the enzyme and in which nitrite is quantitatively oxidised by the system.

With the following substances, probably concerned in fat, carbohydrate and protein metabolism respectively, no oxidation could be obtained:

Formate	Glucose	Glycine
Acetate	Glycerol	Phenylalanine
Oleate	Acetaldehyde	Histidine
Stearate	Lactate	
Triolein	Ethyl alcohol	
β-Hydroxybutyrate		

Oxidised by H_2O_2 without catalysis by the enzyme were:

Dihydroxyacetone Phenylglyoxal

Only two substances were found to be oxidised by H_2O_2 with peroxidase catalysis, namely,

Tryptophan Tyrosine

Thus the attempt to find any general activity of the system which might throw light on the physiological function of peroxidase has failed. It is possible that the enzyme from milk may differ fundamentally from peroxidases found in active tissues; but this seems unlikely since it is a very active enzyme and will oxidise all the substances which are used in the ordinary qualitative tests for other animal peroxidases, and in general seems likely to have the same properties as they have.

In view of the reported wide distribution of peroxidases it is difficult to dismiss them as serving no important physiological function, and it is not yet necessary to do so. The indophenol oxidase which is now very widely believed to be the central enzyme of tissue respiration, activates oxygen, but not directly to oxidise the various organic substrates, as it needs both an intermediate carrier, cytochrome, and the activation of the organic substrates by dehydrogenases [Keilin, 1929]. It is possible that peroxidase may be simply a counterpart to the indophenol oxidase, using H_2O_2 instead of O_2 as oxidising substrate, and needing the activation by other enzymes of most of its organic reducing substrates, and possibly the mediation of cytochrome as well. Cytochrome is oxidised by H_2O_2 alone [Keilin, 1930]; whether this oxidation is accelerated by peroxidase is not yet known. The system known as "succinic oxidase" has been shown [Fleisch, 1924; Keilin, 1929] to consist of the complete system, dehydrogenase + cytochrome + indophenol oxidase. It has been claimed [Thurlow, 1925] that this system with succinic acid and oxygen produces H_2O_2, and if this is so it is probable that H_2O_2 is formed in all oxidations in which indophenol oxidase is concerned. Hence the collaboration of peroxidase with indophenol oxidase may be advantageous to the efficient use of oxygen.

It is interesting to note that the two substances, tryptophan and tyrosine, found to be oxidised by peroxidase and hydrogen peroxide both give coloured products, as do all the non-biological organic substances which are oxidised by the system. It would however be premature to suggest pigment formation as a function of the enzyme.

A study of the powers of a vegetable peroxidase as a catalyst of oxidations by H_2O_2 is now in progress.

Summary.

1. A preparation of milk peroxidase by fractional precipitation with ammonium sulphate is described, whereby a crude but considerably concentrated preparation is obtained practically free from catalase.

2. The activity of the preparation degenerates very slowly in the refrigerator at 0 to 5°. The enzyme is active from about p_H 4 to nearly p_H 10. A colour appears to be associated with the enzyme, and haemochromogen bands can be obtained.

3. Compounds containing the —SH group, such as cysteine, glutathione, and denatured proteins, interfere with tests for peroxidase with benzidine,

guaiacum, and to some extent with *p*-phenylenediamine. This appears not to be an inhibition of the enzyme as with H_2S, but seems to be due to the reduction of the coloured reaction products by —SH compounds.

4. A method of estimating H_2O_2 in very small amounts (1 mg. in 35 cc.) by measuring the evolution of O_2 by manganese dioxide is described. The volume of gas obtained is affected by the presence of proteins, phosphate, acid, *etc.*, but in given conditions it is proportional to the H_2O_2 initially present.

5. Conditions were devised by which nitrite could be quantitatively oxidised by hydrogen peroxide with peroxidase, as shown by the usage of H_2O_2 and the disappearance of nitrite.

6. Under similar conditions tyrosine and tryptophan were found to be oxidised to coloured products.

7. Under the conditions in which nitrite is quantitatively oxidised, no oxidation by H_2O_2 and peroxidase of the following substances was obtained: formate, acetate, oleate, stearate, triolein, ethyl alcohol, glucose, glycerol, acetaldehyde, β-hydroxybutyrate, lactate, glycine, phenylalanine, histidine.

8. Dihydroxyacetone and phenylglyoxal are oxidised by very dilute hydrogen peroxide without peroxidase.

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