XXV. STUDIES ON XANTHINE OXIDASE.

IV. RELATION OF XANTHINE OXIDASE AND SIMILAR OXIDISING SYSTEMS TO BACH'S OXYGENASE.

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IN two recently published papers, one by Haas and Hill [1923], the other by Haas and Lee [1924], the authors state the existence in milk not only of a substance which can reduce nitrates but of another substance which, in the presence of aldehyde and molecular oxygen, can oxidise nitrites to nitrates. They call this latter substance itate, and explain its activity in the following way. In the presence of itate the aldehyde reacts with atmospheric oxygen to form an aldehyde peroxide, the oxygen of which is activated by the peroxidase of milk and oxidises the nitrite. Since the nitrate reducing power of milk has been shown by Dixon and Thurlow [1924] to be due, not to a special substance with that characteristic property, but to the xanthine oxidase plus any one of its substrates, the question arose as to whether the nitrite oxidising power of milk might not be ascribed to the same system. A possible mechanism which would bring about the oxidation of nitrites in such a system would be a coupled reaction. The substrate of the xanthine oxidase, having been activated by the enzyme, is oxidised by molecular oxygen. During the course of this reaction active oxygen would be produced and might oxidise the nitrite. If this were true, then not only aldehyde but hypoxanthine and xanthine in the presence of milk and molecular oxygen should bring about the oxidation of nitrites. The following typical experiment showed that such was indeed the case.

In each of four tubes were placed 10 cc. of milk and 0.2 mg. NaNO_2 . To the first was added 1 mg. of hypoxanthine, to the second 1 mg. of xanthine, to the third 0.5 cc. of 10 % acetaldehyde. To the fourth no substrate was added. The tubes were aerated at room temperature for 45 minutes and then tested for nitrite by the addition of Griess-Ilosvay reagent, which gives a pink colour in the presence of nitrite. The intensity of this colour is proportional to the amount of nitrite present, and is indicated in the table by a varying number of + signs. A negative test therefore indicates that all the nitrite has been oxidised. The results are given in Table I.

If then, as this experiment indicates, the oxidation of nitrite is being in some way brought about by the ordinary oxidase of milk the reaction should

occur upon the addition of any substrate of the enzyme, but not upon the addition of a substance not activated by the enzyme. The reaction should be inhibited by the same substances which Dixon and Thurlow have shown to inhibit the activity of the xanthine oxidase. Table I shows that in the presence of hypoxanthine, xanthine, aldehyde and adenine, all of which are activated by the oxidase of milk, nitrites are oxidised. The amount of nitrite oxidised is roughly proportional to the velocity with which the substrates are oxidised by the enzyme, as is shown in the table by comparison of the reduction-time of methylene blue by the various substrates. In the presence of those substances which are not activated by the oxidase, *e.g.* uric acid, guanine and caffeine, nitrites are not oxidised. Table I also shows that the oxidation of nitrites is inhibited by the same substances as inhibit the oxidation of hypoxanthine.

The effect of varying concentration of substrate was then investigated. Increasing the hypoxanthine concentration gave the inhibition expected from the results given in the previous papers, whereas increasing the aldehyde concentration increased the velocity of oxidation of nitrite, which result was also obtained in the previous work. An experiment typical of many is given in Table I. These results left little doubt that xanthine oxidase was a constituent of the system which was responsible for the oxidation of nitrites.

Table I.

Each tube in the aeration experiments contained 10 cc. milk, 0.05 mg. NaNO_2 and the substance given in column 1. In each case enough buffer of p_H 7.6 was added to keep the volume constant. In the anaerobic experiments each tube contained 5 cc. milk, 0.5 cc. 1/5000 methylene blue and the substance given in column 1.

Substance added	Time of aeration	Nitrite remaining unoxidised	Reduction time of methylene blue
Control	∞	+++	∞
1 mg. hypoxanthine	45 mins.	—	2 mins.
1 mg. xanthine	45 "	+	4 "
0.5 cc. 10 % aldehyde	45 "	++	58 "
1 mg. adenine... ..	8 hrs.	+++	50 "
1 mg. uric acid	8 "	++++	∞
1 mg. guanine	8 "	++++	∞
1 mg. caffeine	8 "	++++	∞
1 mg. hypoxanthine	1 hr.	—	3 mins.
1 mg. hypoxanthine + 3 mg. uric acid	1 "	+++	15 "
1 mg. hypoxanthine + 2 mg. adenine	1 "	++++	68 "
1 mg. hypoxanthine + 2 mg. guanine	1 "	+++	15 "
1 mg. hypoxanthine + 2 mg. caffeine	1 "	+	3 "
0.6 mg. hypoxanthine	1 "	—	2 "
6 mg. hypoxanthine	1 "	++++	16 "
0.2 cc. 10 % aldehyde	2 hrs.	+++	50 "
1 cc. 10 % aldehyde... ..	2 "	—	10 "

If the oxidation of nitrites could be explained by the occurrence of a coupled reaction, the components of the system would be xanthine oxidase, substrate, nitrite and molecular oxygen. The system proved however not to be quite so simple, because, although whenever milk was used the nitrite was always oxidised, when the caseinogen-enzyme preparation described by Dixon and Thurlow was used, either with hypoxanthine or aldehyde, the nitrite was

never oxidised. There must therefore be present in milk some necessary component of the system not present in the enzyme preparation. It was thought that this might be the fat, which is removed from the caseinogen-enzyme preparation by extraction with ether. Dixon and Thurlow have shown that fat accelerates the oxidation of hypoxanthine, and it was thought that the oxidation of nitrites might be so slowed up by the removal of fat that no change in nitrite concentration could be observed within the time of an ordinary experiment. Boiled cream was therefore added to the caseinogen-enzyme preparation, but again no oxidation of nitrite occurred. It was therefore obvious that the fat was not the required factor.

According to Haas and Lee when milk is coagulated with rennin and the whey half-saturated with ammonium sulphate, the precipitate obtained can, in the presence of aldehyde, oxidise nitrites, whereas the filtrate cannot. The filtrate would of course contain no oxidase, because this is precipitated by half-saturation with ammonium sulphate. According to the experiments already given showing that xanthine oxidase is connected with the nitrite reaction, a filtrate containing no oxidase could not be expected to oxidise nitrites. On the other hand when the precipitate was carefully washed with half-saturated ammonium sulphate until it was free from all traces of the filtrate, it behaved just like the enzyme preparation—oxidising hypoxanthine and aldehyde but not nitrites. The only difference between this procedure with the precipitate obtained by half-saturation with ammonium sulphate, and the procedure of Haas and Lee was the thorough washing. The obvious conclusion was that the filtrate contained the unknown nitrite-oxidising factor, which had been separated from the xanthine oxidase by the precipitation, so that neither precipitate nor filtrate caused the oxidation of nitrites. The recombination of the two should again complete the system, and give the same results as milk. This was actually found to be the case, as is shown in the following experiment. The filtrate containing the nitrite-oxidising factor was then saturated with ammonium sulphate and the factor was found to be precipitated completely. Repeating this experiment, using aldehyde instead of hypoxanthine, gives exactly the same results. Typical experiments are given below.

Each tube contained 0.05 mg. NaNO_2 + 0.4 mg. Hx + the substance given in column 1. The tubes were aerated at room temperature for 3 hrs.

Substance added	Nitrite remaining unoxidised
5 cc. half-saturated filtrate + 5 cc. 7.6 buffer	... + + + +
5 cc. precipitate obtained by half-saturation + 5 cc. filtrate	... +
5 cc. precipitate obtained by half-saturation + 5 cc. buffer	... + + + +
5 cc. saturated filtrate + 5 cc. oxidase preparation	... + + + +
5 cc. precipitate obtained by saturation + 5 cc. oxidase preparation	... -
5 cc. precipitate obtained by saturation + 5 cc. buffer	... + + + +
5 cc. oxidase preparation + 5 cc. buffer	... + + + +

It can be seen from these experiments that oxidation of nitrite occurs only when the nitrite-oxidising factor is added to the xanthine oxidase.

The question then arises as to the nature of this factor which, in the presence of molecular oxygen, xanthine oxidase and one of its substrates, can bring about the oxidation of nitrites. Haas and Lee consider the possibility of its being peroxidase, but discard it because the filtrate obtained from whey half-saturated with ammonium sulphate gives a peroxidase test but will not oxidise nitrites. It has just been shown that the combination of this filtrate and the precipitate obtained by half-saturation with ammonium sulphate oxidises nitrites, and that it is the addition of the peroxidase-containing filtrate to the xanthine oxidase which brings about the oxidation. The filtrate from whey or milk completely saturated with ammonium sulphate does not give the peroxidase test. Neither does it, when added to xanthine oxidase and hypoxanthine, cause the oxidation of nitrites. In no case has it been possible to get a solution which contained the milk peroxidase and which, when added to xanthine oxidase and its substrate, failed to oxidise nitrite. Nor, on the other hand, has it been possible to get a solution which did not give the peroxidase test but did contain the nitrite oxidising factor. Both the milk peroxidase and the nitrite-oxidising factor are destroyed by boiling but neither is destroyed by acid, so that it seems more than probable that the unknown factor is peroxidase. The following scheme makes clear the separation of xanthine oxidase from peroxidase.

It has not been possible to substitute a vegetable peroxidase in the system. Preparations of the peroxidases of horseradish, malt and potato were tried, but they all contain reducing substances which reduce nitrate with such alacrity that it is impossible to test for the disappearance of nitrite. The artificial peroxidases of Dony-Henault [1908] were tried. That made from ferrous sulphate reduced nitrates and was therefore of no use. The other, made from manganese formate, when substituted for milk peroxidase, did not bring about the oxidation of nitrites.

An animal peroxidase was then made in the following way. Ox liver was perfused until as much blood as possible had been washed away. It was then extracted with water and an equal volume of alcohol added to dissolve the remaining haemoglobin. The precipitate after repeated washing with 50 % alcohol was stirred up in water. The method of Kasanski [1912] for removing catalase by pyrogallol was tried, but it was found difficult afterwards to obtain the peroxidase free from pyrogallol, the presence of which renders impossible

any comparison of pink colours obtained with the Griess-Ilosvay reagent. Owing to the presence of catalase it was impossible to get a negative test, *i.e.* to get all the nitrite oxidised, but in every case there was a distinct difference between the tube containing hypoxanthine and the control. An alkaline phosphate extract of washed ox muscle, which gave a faintly positive peroxidase test, was also tried a number of times. The results of these experiments are given below.

5 cc. liver peroxidase + 5 cc. oxidase preparation	+0.05 mg. NaNO ₂	+0.3 mg. Hx	++
5 cc. liver peroxidase + 5 cc. oxidase preparation	" "	—	++
5 cc. muscle extract + 5 cc. oxidase preparation	" "	+0.3 mg. Hx	++
5 cc. muscle extract + 5 cc. oxidase preparation	" "	—	+++

These experiments show that an animal peroxidase can be substituted for the nitrite-oxidising factor and that therefore the latter must be peroxidase.

The next question to be considered is the rôle played by the peroxidase in bringing about the oxidation of nitrites in the presence of the xanthine oxidase and one of its substrates. Haas and Lee state that itate causes aldehyde in the presence of molecular oxygen to form aldehyde peroxide, the oxygen of which, when activated by the peroxidase, oxidises nitrite. They were able to get a test for peroxide when milk containing aldehyde was aerated. The activation of the aldehyde, however, as has been shown in the preceding experiments, is not due to an unknown factor, but to the milk oxidase, since any substrate of that enzyme can take the place of the aldehyde in bringing about the oxidation of nitrite. Therefore, if peroxide is formed it must be in the course of the oxidation brought about by the xanthine oxidase. The system would then be as follows:

1. Xanthine oxidase + substrate (Hx, X, or Aldehyde) + O₂ → peroxide
2. Peroxide + nitrite-oxidising factor (peroxidase) + nitrite → nitrate

Haas and Lee observed that nitrites were oxidised by hydrogen peroxide and milk peroxidase, but not by hydrogen peroxide alone. These experiments have been confirmed and the same results have been obtained with a system composed of milk peroxidase, nitrite and hydrogen peroxide as with a system composed of milk peroxidase, nitrite, xanthine oxidase and hypoxanthine or aldehyde. Since the second of the reactions given above is thus shown to occur, it remains to show that the first also occurs. Experiments were carried out to determine whether peroxide is formed in the course of oxidation of hypoxanthine, xanthine and aldehyde by molecular oxygen. Several tests for peroxide were used. Tincture of guaiacum was made up fresh each day and a water extract of horseradish scrapings, which had first been soaked in alcohol and dried, was added when the test for peroxide was made in order to supply the necessary peroxidase. Another very sensitive test is that suggested by Kaiser and McMaster [1908], a mixture of solutions of ferric chloride and potassium ferricyanide. Titanium sulphate was used in several experiments, but the yellow colour caused by the presence of peroxide was obscured to a large extent by the precipitated caseinogen so that it was less easy to detect small amounts of peroxide with this reagent than with guaiacum or the combination

of ferric salts. The benzidine reagent could not be made to work in the presence of the caseinogen-enzyme preparation. A large number of experiments have been done in which a solution of caseinogen-enzyme preparation and hypoxanthine, xanthine or aldehyde has been aerated and tested at intervals for the presence of peroxide. Typical results are shown in Table II.

Table II.

In each experiment to 20 cc. caseinogen-enzyme preparation dissolved in p_H 7.6 phosphate buffer was added 1 cc. of substrate. The results are indicated by an arbitrary standard assumed for each test. The separate experiments are not comparable as far as the relation between time and amount of peroxide present is concerned, because, since various caseinogen-enzyme preparations were used, the activity of the enzyme was not the same in all cases.

Substrate	Test used	Time	Amount of peroxide present
Hypoxanthine	Guaiacum + horse-radish peroxidase	0	-(became blue on standing 10 mins.)
		10 mins.	++++
		20 "	++++
		40 "	+
		120 "	-
Adding more hypoxanthine		20 "	+
Hypoxanthine	$FeCl_3 + K_3Fe(CN)_6$	0	-
		20 "	+
		60 "	++
		90 "	+
Hypoxanthine	Titanium sulphate	0	-(became faintly yellow on standing 15 mins.)
		20 "	+
		60 "	+
		90 "	-
Xanthine	Guaiacum + horse-radish peroxidase	0	-(became blue on standing 10 mins.)
		10 "	+++
		20 "	++
		40 "	<+
		60 "	-
Adding more xanthine		20 "	+
Xanthine	$FeCl_3 + K_3Fe(CN)_6$	0	-(became blue on standing 20 mins.)
		20 "	+
		40 "	+
		60 "	+
Aldehyde	Guaiacum + horse-radish peroxidase	0	-
		15 "	<+
		30 "	++
		45 "	++
		60 "	-

These results have been obtained so many times that there seems to be no doubt that peroxide is formed in the oxidation of hypoxanthine, xanthine and aldehyde in the presence of molecular oxygen. The fact that the test is negative at the end of the reaction shows that the peroxide must be used up during the course of the reaction. This is explained by the observation of Wieland [1921] that hydrogen peroxide and various other peroxides act as hydrogen acceptors for activated hydrogen, forming water.

The destruction of peroxidase by peroxide was observed by Schellhase [1908]. If peroxide were being formed, one would expect to find the peroxidase

disappearing as oxidation occurred. The gradual destruction of the peroxidase during the course of oxidation of aldehyde in molecular oxygen was observed by Haas and Lee and has been confirmed by my own experiments. I have found that peroxidase is also destroyed during the course of oxidation of hypoxanthine by molecular oxygen. The xanthine oxidase is also destroyed during the course of the reaction with molecular oxygen but not in the anaerobic oxidation.

Still further evidence for the belief that a peroxide is formed in the aerobic oxidation of hypoxanthine, xanthine and aldehyde, and that it is the activated oxygen of this peroxide which brings about the oxidation of nitrite, is afforded by the results obtained when these substances are oxidised anaerobically. In anaerobic oxidations methylene blue takes the place of molecular oxygen as hydrogen acceptor so that a peroxide could not be formed. According to the theory advanced earlier in the paper nitrite should not be oxidised. The following experiment shows that in the anaerobic oxidation of hypoxanthine the oxidation of nitrite does not occur.

Contents of vacuum tube	Reduction time of methylene blue
5 cc. diluted milk + 0.5 cc. methylene blue + 0.2 mg. Hx + 0.1 cc. H_2O	5.5 mins.
5 cc. diluted milk + 0.5 cc. methylene blue + 0.2 mg. Hx + 0.1 mg. $NaNO_2$	6 „

If the nitrite were oxidised, the reduction time of methylene blue should be decreased, whereas the reduction time with and without nitrite is the same.

Finally, the results obtained with catalase show definitely that hydrogen peroxide is formed. The work of various authors has shown that in a system containing both peroxidase and catalase the hydrogen peroxide present distributes itself between the two. Therefore if it is really hydrogen peroxide that is being utilised by the peroxidase to bring about the oxidation of nitrites, the reaction ought to be retarded by catalase. Catalase was made according to the method of Batelli and Stern [1904], and the solution of it used was adjusted to p_H 7.6.

Contents of tubes	Nitrite remaining unoxidised
3 cc. oxidase preparation + 3 cc. peroxidase + 4 cc. catalase + 0.05 mg. $NaNO_2$ + 0.8 mg. Hx	+ +
3 cc. oxidase preparation + 3 cc. peroxidase + 4 cc. buffer p_H 7.6 + 0.05 mg. $NaNO_2$ + 0.8 mg. Hx	-

It can be seen that the reaction is inhibited to a marked degree by catalase. There seems to be no doubt therefore that in the course of aerobic oxidation of hypoxanthine, xanthine and aldehyde, hydrogen peroxide is formed.

The problem was now attacked from a different point of view. It has been shown that the oxidation of nitrite depends, not on the presence of peroxidase and a specific oxidisable substance like aldehyde, but on the presence of peroxidase and hydrogen peroxide. The hydrogen peroxide may be produced during the course of oxidation if the entire system is being considered, or it may be added to the peroxidase and nitrite. It might then be argued that if oxidising systems are at all comparable with one another it should be possible

to substitute for the xanthine oxidase and its substrate other oxidising systems, such as another oxidising enzyme and its substrate, or a substance with a readily oxidisable group like the —SH compounds. Succinic oxidase and its substrate sodium succinate were substituted in the system for xanthine oxidase and hypoxanthine. The succinic oxidase was made by Ohlsson's [1921] method. The following is a typical experiment:

Contents of tubes			Nitrite remaining unoxidised
5 cc. succinic oxidase + 5 cc. milk peroxidase	+ 0.05 mg. NaNO_2	+ 2.5 mg. sodium succinate	+
5 cc. succinic oxidase + 5 cc. 7.6 buffer	" "	" "	++
5 cc. succinic oxidase + 5 cc. milk peroxidase	" "	—	++++

This shows that succinic oxidase and sodium succinate behave like xanthine oxidase and hypoxanthine in the system. The succinic oxidase preparation gave a faint peroxidase test, which accounts for the fact that some nitrite is oxidised without the addition of milk peroxidase.

A solution of succinic oxidase and sodium succinate was aerated and tested without success for peroxide, probably because the peroxide is formed very much more slowly than in the case of hypoxanthine and is used as an oxidising agent as rapidly as it is formed. This is substantiated by the fact that the period of aeration of the succinic system must be about six times as long as that of the xanthine oxidase system with hypoxanthine before the same quantity of nitrite can be oxidised. The succinic oxidase preparation contains a large amount of catalase, and therefore no inhibition was obtained by the addition of more catalase. This result agrees with the curve obtained by Chodat and Pasmanik [1907] for the partition of hydrogen peroxide between peroxidase and catalase. They showed that the addition of catalase beyond a certain point caused no further inhibition of the peroxidase reaction.

The other type of oxidising system, namely that which does not require activation by an enzyme, was then studied. Xanthine oxidase and its substrate were replaced by cysteine, so that the system was now composed of cysteine, peroxidase and nitrite.

Contents of tubes	Nitrite remaining unoxidised
5 cc. cysteine + 5 cc. 7.6 buffer + 0.05 mg. NaNO_2	++++
5 cc. peroxidase + 5 cc. buffer + 0.05 mg. NaNO_2	++++
5 cc. cysteine + 5 cc. peroxidase + 0.05 mg. NaNO_2	—

Thus nitrite is oxidised when cysteine oxidises in the presence of peroxidase, and the inference can be made that during the oxidation of cysteine, peroxide is formed. Professor Hopkins (unpublished experiments) working with the titanium sulphate test was unable to show the presence of peroxide in oxidising cysteine solutions. This can be explained by the observation of Abderhalden and Wertheimer [1923] that cysteine is instantaneously oxidised by added hydrogen peroxide, so that if hydrogen peroxide were formed during the

oxidation of cysteine it would be removed from the solution as soon as produced. Thus the quantity of peroxide in the solution at any instant would be too small to give a positive test. The effect of catalase was then tried but with a negative result; that is, catalase does not inhibit the oxidation of nitrites in the cysteine system. This might be considered as evidence for the formation of an organic peroxide by cysteine. On the other hand it may be explained by the assumption that the peroxidase acts upon very small quantities of hydrogen peroxide more rapidly than does catalase, so that the effect of catalase is so small as to be unobserved.

Further evidence for the formation of peroxide in the oxidising cysteine solution is the fact that during the oxidation peroxidase is destroyed.

Finally, glutathione was used instead of cysteine.

Contents of tubes	Nitrite remaining unoxidised
2.5 cc. glutathione (25 mg.) + 2.5 cc. 7.6 buffer + 0.025 mg. NaNO_2	+ + + +
2.5 cc. glutathione (25 mg.) + 2.5 cc. peroxidase + 0.025 mg. NaNO_2	+

The above experiment shows that the glutathione system is similar to the others that have already been considered, in that during the course of oxidation of glutathione in the presence of peroxidase nitrite is oxidised, and therefore a peroxide must be formed during the oxidation.

Discussion.

The fact that a peroxide is formed during the oxidation of hypoxanthine, xanthine and aldehyde has been established above, and since such systems as oxidising cysteine, oxidising glutathione and oxidising succinate behave in the same way in bringing about the oxidation of nitrite when peroxidase is added, there is little doubt that in these systems also peroxide is formed. The question arises as to the nature of the peroxide. Is it an organic peroxide or hydrogen peroxide? Bach [1913] believed in the formation of an organic peroxide in some oxidations and of hydrogen peroxide in others. His experimental evidence [1905] for the formation of an organic peroxide was the fact that when he added catalase to a system composed of plant oxygenase, peroxidase and pyrogallol, the oxidation of the pyrogallol was not inhibited. Therefore since catalase destroys hydrogen peroxide but not organic peroxides, that formed by the oxygenase must be organic. Bach [1905] showed, however, that pyrogallol destroys catalase so that in such a system no inhibition could be expected. There seems therefore to have been no evidence that an organic peroxide was formed during the course of such oxidations. Haas and Lee suggest that the peroxide formed in the oxidation of aldehyde is organic, but this view is not based on experimental evidence. There are strong reasons, however, for believing that the peroxide concerned is hydrogen peroxide. In the first place it seems unlikely chemically that compounds such as hypoxanthine would form peroxides. No compounds of such a type are known. Secondly, the explanation of the activity of the enzyme is far more simple if it is assumed that hydrogen peroxide is directly formed. The enzyme can then

be considered as producing active hydrogen, a point of view which is generally assumed for enzymes of this type, and which makes the oxidation of hypoxanthine and aldehyde comparable to the oxidation of succinic acid. A general consideration of those systems which produce active hydrogen, and hence reduce methylene blue, shows that in every case the active hydrogen produced converts molecular oxygen to hydrogen peroxide [see Traube, 1893]. Armstrong and Colgate [1913] refer to a number of such systems, giving as typical examples:

1. The cathode in the electrolysis of dilute solutions of electrolytes. Any oxygen present is reduced to hydrogen peroxide by the active hydrogen produced.
2. When metals dissolve in acids, active hydrogen is produced which forms hydrogen peroxide with molecular oxygen.
3. Autoxidisable substances such as indigo white and hydrazobenzene during oxidation act as hydrogen donors, and the active hydrogen thus produced forms hydrogen peroxide with molecular oxygen.

Many other cases are known. In any system producing active hydrogen it would seem, then, that either of the following reactions might occur.

Thirdly, as has already been mentioned, milk peroxidase acts upon hydrogen peroxide to bring about the oxidation of nitrites. Fourthly, that it is hydrogen peroxide which is connected with the nitrite oxidation in the system of oxidising hypoxanthine or aldehyde is shown by the inhibition by catalase. Bach and Chodat [1903] have shown that catalase does not decompose organic peroxides. Fifthly, Kastle and Loevenhart [1901] have shown that organic peroxides blue guaiacum directly without peroxidase, whereas hydrogen peroxide blues guaiacum only in the presence of peroxidase. A solution of oxidising hypoxanthine does not cause the faintest blueing of guaiacum until peroxidase is added, whereupon, as already stated, a deep blue appears. Sixthly, it has been shown that succinic acid oxidising to fumaric acid behaves in a manner exactly similar to oxidising hypoxanthine. A consideration of the formulae of succinic and fumaric acids makes the belief in the formation of an organic peroxide during this oxidation untenable; for while it is just possible to view hypoxanthine as a peroxide, it is inconceivable that succinic acid can do anything but lose two hydrogen atoms, which with molecular oxygen would form hydrogen peroxide.

The peroxides in the cysteine and glutathione systems remain for consideration. The reason for assuming the formation of hydrogen peroxide rather than of an organic peroxide in the case of the —SH compounds is only that such a view makes it possible to explain the mechanism of their oxidation in

a manner more nearly comparable to that involved in the other oxidising systems which have been considered. It is impossible at the moment, however, as far as the —SH compounds are concerned, to make a definite assertion as to the nature of the peroxide formed. The case, however, for the direct formation of hydrogen peroxide rather than of an organic peroxide in the oxidation of hypoxanthine, xanthine, aldehyde and succinic acid seems well established.

We now come to a theoretical consideration of the results obtained. Bach maintained that a complete oxidising system consisted of two enzymes. The first was an easily oxidisable body which, with molecular oxygen, formed a peroxide and which he called oxygenase. The second was a peroxidase which acted upon the peroxide formed by the first enzyme, and the oxygen thus activated could bring about the oxidation of some other substance. Because of its properties, Bach believed the oxygenase to be an enzyme, although this point has been disputed by other workers. The Schardinger enzyme was, according to Bach, part of a quite different system. By means of water it reduced methylene blue in the presence of aldehyde, and was therefore called a reducing enzyme. As the same reactions have been shown by Morgan, Stewart and Hopkins [1922] to occur with molecular oxygen as acceptor as with methylene blue, the Schardinger enzyme, or rather aldehyde-oxidising enzyme (since in milk, as has been shown by Dixon and Thurlow, it is probably the same as the xanthine oxidase), can be considered as an oxidising enzyme. The Schardinger enzyme + aldehyde could then be considered as Bach's oxygenase except that the substance upon which the enzyme acts and not the enzyme itself would form the peroxide. Such a view would simplify the consideration of oxidations, as Bach's hydrolytic oxidation-reduction system could be thought of as comparable to his ordinary oxidising system. The experimental facts already given completely bear out such a conception, and explain why in some cases oxygenase seemed to behave like an enzyme, while in others it did not. Considering the oxygenase firstly from Bach's point of view that it is an easily oxidisable substance forming a peroxide, I have shown that in the oxidation of cysteine and glutathione, both easily oxidisable substances, a peroxide must be formed, because when the second part of Bach's system is added, namely peroxidase and another oxidisable substance, oxidation of the latter occurs. Cysteine and glutathione are therefore oxygenases, but cannot be considered as enzymes. But Bach's observation that in many cases the oxygenase is thermolabile and therefore behaves like an enzyme can be explained by the fact that oxygenase may not always be a single substance. In some systems it may be an oxidising enzyme and its substrate such as the xanthine oxidase and hypoxanthine, the aldehyde enzyme and aldehyde, or the succinic enzyme and succinic acid. I have shown that all these systems form peroxide, the oxygen of which in the presence of peroxidase can be used for further oxidations. Onslow [1920] has shown that in plants a catechol derivative and an oxidising enzyme form an oxygenase. She, however, refers to the enzyme as oxygenase, whereas by Bach's

definition the term oxygenase includes both enzyme and peroxide-forming substance.

The only difference in the oxidising systems which I have shown to be oxygenases is that in some cases the oxidation consists of a loss of hydrogen and in others of an addition of oxygen. In the latter case it is necessary to suppose water as a reacting part of the system. The essential mechanism involved, however, can be assumed on Wieland's theory of hydrogen activation to be the same in all cases. Morgan, Stewart and Hopkins suggest that hypoxanthine adds on water to form a hydrate, the hydrogen of which is activated in Wieland's sense by the enzyme and is transported, if the oxidation be anaerobic, to methylene blue, or, if the oxidation be aerobic, to molecular oxygen. I have shown that in the latter case hydrogen peroxide is formed. It might be assumed that the oxidation of —SH compounds is similar to the oxidation of the hypoxanthine hydrate. Warburg claims that —SH compounds can oxidise only in the presence of iron. The iron might be considered as activating the —SH group, thus rendering its hydrogen labile. Mr D. C. Harrison (unpublished experiments) has recently obtained some results which make this view highly probable. The activated hydrogen would reduce methylene blue or with molecular oxygen would form hydrogen peroxide. The action of iron on the —SH compounds would on such a view be analogous to that of the oxidising enzymes on their substrates; and the conception of oxidising systems would be somewhat simpler.

SUMMARY.

1. Hypoxanthine, xanthine and adenine can replace aldehyde in the nitrite-oxidising system in milk, termed itate by Haas and Hill.
2. The caseinogen preparation of xanthine oxidase + one of its substrates will not bring about the oxidation of added nitrite, but will do so if milk peroxidase is added. The complete system consists of xanthine oxidase; hypoxanthine, xanthine or aldehyde; milk peroxidase and nitrite.
3. The peroxidase of liver has been substituted for the milk peroxidase in the nitrite-oxidising system.
4. Peroxide is formed in considerable quantities when hypoxanthine, xanthine or aldehyde is oxidised by the xanthine oxidase in the presence of molecular oxygen.
5. The oxidation of nitrite in the presence of milk, hypoxanthine and molecular oxygen is retarded by catalase.
6. Nitrite is oxidised by the system succinic oxidase, succinic acid, animal peroxidase and molecular oxygen.
7. Nitrite is also oxidised during the autoxidation of the —SH compounds cysteine and glutathione, provided milk peroxidase is present.

8. These results lead to the conclusion that peroxide is formed during the aerobic oxidation of succinic acid, cysteine and glutathione.

9. In the cases of hypoxanthine, xanthine, aldehyde and succinic acid hydrogen peroxide is formed directly by the addition of active hydrogen to the oxygen molecule.

10. The systems xanthine oxidase + any one of its substrates, succinic oxidase + succinic acid, cysteine and glutathione are shown to be oxygenases as defined by Bach.

11. A preparation of animal peroxidase suitable for general use can be obtained by saturating with ammonium sulphate the filtrate obtained by half-saturating milk with ammonium sulphate.

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