XXXII. THE HEAT-STABLE PEROXIDASE OF BACTERIA.

By ANNE BARBARA CALLOW.

From the Biochemical Laboratory, Cambridge.

Report to the Medical Research Council.

(*Received February 9th, 1926.*)

THE term peroxidase was originally applied to an enzyme of the higher plants that was capable of blueing guaiacum in the presence of hydrogen peroxide. Plant extracts that had been boiled gave no peroxidase reaction. Later work showed that certain iron-containing substances, such as haemoglobin, also gave the peroxidase reaction. Buckmaster found that these iron-containing substances gave the reaction equally well after they had been boiled, and he used the term pseudoperoxidase for this reaction [Buckmaster, 1908]. There are certain indications in the literature that bacterial peroxidase is also heat-stable, and the present investigation confirms this observation. In this respect therefore the peroxidase of bacteria resembles pseudoperoxidase, and differs from the peroxidase of the higher plants, which is destroyed by heat.

Detection of peroxidase. The following reagents were used for the detection of peroxidase in bacteria:

(i) 1 % solution of benzidine in 50 % alcohol;

(ii) 1 % solution of guaiacum in 95 % alcohol. The guaiacum was obtained from the inside of a lump of resin, boiled with alcohol in the presence of animal charcoal to remove peroxides, and then filtered.

In each case, two drops of the freshly prepared solution were mixed with the material to be tested in a white dish, and two drops of hydrogen peroxide (20 volumes) added. In both tests a blue colour indicates the presence of peroxidase.

Pure cultures of various aerobic bacteria, obtained from the National Collection of Type Cultures, were grown in Roux bottles of tryptic broth. Good growth of the anaerobe *B. sporogenes* was obtained as follows: 6-inch test-tubes, half full of glucose tryptic broth, were autoclaved, cooled rapidly, and inoculated at once with material from a similar glucose broth culture. Cultures were incubated at 37° for 18 hours, and then centrifugalised. The clear broth was decanted off, and the bacteria were ground up in a mortar with distilled water to wash away traces of broth. The emulsion was again

centrifugalised and the water decanted off. This process was repeated, and the bacterial mass was finally emulsified with enough distilled water to give a very thick suspension, which was tested for the presence of peroxidase. It was found that benzidine and guaiacum gave similar results. In all cases controls were carried out using the reagents without addition of bacteria. The guaiacum + H_2O_2 control gave a very slight blue colour when left for several hours, but the benzidine + H_2O_2 control gave no trace of blue colour. Another advantage of benzidine was that it generally gave a deeper blue colour with bacterial peroxidase than was obtained when guaiacum was used.

The following bacteria were investigated: *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. fluorescens*, *B. prodigiosus*, *B. proteus vulgaris*, *B. coli*, *B. paratyphosus* A, *B. paratyphosus* B, *B. subtilis*, *B. megatherium*, *Staphylococcus aureus*, *Streptococcus acidi lactici*, and the anaerobe *B. sporogenes* (Metchnikoff). All these bacteria were found to give the peroxidase reaction, although in the case of the *Streptococcus* and *B. sporogenes* the blue colour was very much less intense than with the other bacteria investigated. This is another example of the way in which *S. acidi lactici* differs from most aerobes and resembles the anaerobes [see Callow, 1923, 1924]. In all cases the peroxidase was stable to heat and, with the exception of the two organisms just mentioned, a very intense blue colour was given by bacteria that had been boiled in a Pyrex flask for an hour, cooled, and tested with benzidine + H_2O_2. The peroxidase was found to be remarkably stable, as shown by the following experiment. *B. faecalis alcaligenes* was grown in bulk, washed, and dried in a vacuum desiccator over sulphuric acid. The resulting dry powder was ground up with distilled water, the washings being removed after centrifugalisation. After being washed seven times, the bacterial mass was suspended in distilled water and boiled for 5 minutes. The water was removed as before, and the bacteria finally treated with 98 % alcohol and again dried. The resulting "thermostable residue" [see Hopkins and Dixon, 1922] gave the peroxidase reaction, as well as being positive to the nitroprusside test. This does not of course imply that the fixed SH group is responsible for the peroxidase reaction, and indeed this is highly improbable, because certain substances such as boiled egg-white which contain a fixed SH group give no peroxidase reaction.

In most bacteria the blue colour obtained with benzidine + H_2O_2 is actually very much deeper after the bacterial emulsion has been boiled than it was before. Sometimes, in fact, practically no blue colour is obtained with the fresh organism. Generally, with fresh bacteria, a blue colour appears which fades away after about half an hour, unlike the permanent blue colour that is obtained with boiled bacteria. Fresh, washed cultures of *B. faecalis alcaligenes*, etc., are also able to cause fading in the blue colour obtained with a weak solution of haemoglobin + benzidine and H_2O_2. Most bacteria contain large quantities of catalase, which may possibly interfere with the peroxidase reaction by decomposing the H_2O_2. But there appears to be some other factor in fresh bacteria that affects the peroxidase reaction because, although catalase

might possibly prevent the formation of the blue colour, there is no reason to suppose that it can destroy the blue colour that has already been formed in the peroxidase reaction. Moreover, the factor that causes the blue colour to fade appears to be less stable than catalase, and tends to disappear when emulsions of bacteria are kept, or dried, etc. The action of heat is shown by the following experiment. A suspension of fresh, washed *B. faecalis alcaligenes* gave with benzidine + H_2O_2 a very slight blue colour which faded completely in 10 minutes. After being kept at 83° for 1 minute, it gave a deep blue colour that was quite permanent.

It was found that the blue colour obtained in the peroxidase reaction could be removed by the action of certain reducing agents, such as the SH group (in neutralised thioglycollic acid). It appeared possible, therefore, that some heat-labile reducing system in bacteria might be responsible for the fading of the blue colour. The bacteria that were used for examining the fading of the blue colour were: *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. fluorescens*, *B. prodigiosus*, *B. proteus*, *B. subtilis*, and *Staphylococcus aureus*. Fresh young cultures of all these bacteria gave a rapidly fading blue colour with the peroxidase reagents, and in each case a very deep permanent blue colour was obtained after the organism had been boiled. It may be mentioned that the factor that causes fading was found in *S. acidi lactici*; fresh, washed material gave either a very slight fading blue, or no blue colour at all, but after boiling a distinct, although slight, permanent blue colour was obtained. Two reducing systems of bacteria were investigated to see whether they corresponded with the factor that causes the blue peroxidase colour to fade.

I. *Reduction of nitrates.* A most striking fact in connection with the peroxidase reaction of fresh bacteria, such as *B. faecalis alcaligenes*, etc., is that the fading of the blue colour does not take place if nitrates are present. This suggested that possibly there might be some connection between the removal of the blue colour and the reduction of nitrates by bacteria. Young cultures of bacteria were washed in the usual way, and mixed with distilled water to form strong, even emulsions. To 3 cc. of bacterial emulsion, 1 cc. of a 5 % solution of potassium nitrate was added. The mixture was placed in a water-bath kept at 60° for 3 minutes. It was then cooled, and tested for nitrites by two methods.

(1) Addition of potassium iodide, starch paste and sulphuric acid, which gives a blue colour with nitrites.

(2) The Griess-Ilosvay reagent, which gives a pink colour with traces of nitrites.

Certain bacteria were investigated in this way, and it was found, for instance, that *B. pyocyaneus*, *B. fluorescens*, *B. coli*, and *Staphylococcus aureus* reduced nitrates to nitrites under these conditions, but boiled suspensions of these bacteria were inactive. *B. faecalis alcaligenes* gave no trace of nitrite formation. In order to exclude the possibility of obtaining negative results by not taking enough material, a measure of the strength of suspension was

obtained as follows: 1 cc. was placed in a small tared centrifuge tube and centrifugalised. The water was decanted off, and the tube containing the bacteria was dried in a vacuum desiccator over sulphuric acid, and weighed. It was found that suspensions which contained 0·03 g. dry weight of bacteria per cc. gave a strong nitrate reduction with the bacteria mentioned above, but that even with 0·06 g. per cc. *B. faecalis alcaligenes* showed no trace of nitrite formation [cf. Quastel, Stephenson and Whetham, 1925; Quastel and Wooldridge, 1925].

II. *Reduction of methylene blue.* The reduction of methylene blue was studied in a way similar to that used for nitrates. A solution of methylene blue was added to washed suspensions of bacteria, which were then placed in a water-bath at 60°. The surface of the mixture was protected by a layer of olive oil to prevent re-oxidation of the leuco-methylene blue by air. The time taken for known suspensions of bacteria to reduce various concentrations of methylene blue was observed. Great difficulty was experienced in this part of the investigation. Since the reducing system was heat-labile, an attempt was made to obtain emulsions of bacteria that would reduce given solutions of methylene blue when kept for only 2 or 3 minutes at 60°. Unfortunately, strong emulsions of bacteria were capable of reducing only very weak solutions of methylene blue in such a short time. Finally, 0·1 cc. of *M*/5000 methylene blue was added to the bacterial suspension. The strength of the suspension was found as before, and the time taken for complete reduction of the methylene blue was noted. The following results are typical:

Organism	Number of washings	Approx. dry weight in g. of bacteria in suspension	Time in seconds for reduction of methylene blue solution
1. *B. faecalis alcaligenes*	2	0·023	90
2. *B. pyocyaneus*	2	0·030	80
3. *B. fluorescens*	2	0·016	100
4. *B. coli*	1	0·055	120
5. *B. megatherium*	1	0·037	100

Boiled emulsions of all these bacteria had no reducing power. The general conclusion from the results is that the bacteria which were found to reduce methylene blue, under the given conditions, all gave a fading blue colour with the peroxidase reagents. Further evidence on this point was obtained by observing the effect on the peroxidase reaction of certain substances which are known to affect the system in bacteria that is responsible for the reduction of methylene blue. The following substances were selected:

(1) *Neutral sodium formate.* This has been shown to accelerate the reduction of methylene blue by *B. coli*, *B. faecalis alcaligenes*, and *B. prodigiosus* [Quastel and Whetham, 1925; Quastel and Wooldridge, 1925].

(2) *Propyl alcohol and cyclohexanol.* These substances have been shown to retard reduction of methylene blue by *B. coli*, a small quantity of cyclohexanol producing complete inhibition [Quastel and Whetham, 1925].

The following experiment shows that the fading of the blue peroxidase colour is also accelerated by sodium formate, and retarded by propyl alcohol and cyclohexanol. A fresh, young culture of *B. faecalis alcaligenes* was washed and emulsified in water. Half of the emulsion was boiled for 5 minutes, and cooled. The table shows the substances added, and their effect on the fading of the blue peroxidase colour. In each case 1 cc. of bacterial suspension was used.

Condition of suspension	Substance added	Colour obtained on addition of benzidine and H_2O_2
1. Fresh	None	Slight blue which faded rapidly
2. Boiled	None	Deep permanent blue
3. Fresh	Neutralised sodium formate	No blue colour
4. Boiled	Neutralised sodium formate	Deep permanent blue
5. Fresh	2 drops of propyl alcohol	Deep permanent blue
6. Boiled	2 drops of propyl alcohol	Deep permanent blue
7. Fresh	2 drops of cyclohexanol	Deep permanent blue
8. Boiled	2 drops of cyclohexanol	Deep permanent blue

Cyclohexanol was added to No. 3, but still no blue colour appeared. This suggests that, as found by Quastel and Whetham with the methylene blue reducing system, cyclohexanol acts not as an oxidising agent, but as an inhibitor of the reducing system. The above eight experiments were repeated with *B. coli*, and similar results were obtained.

The function of peroxidase. Keilin's recent work [1925] is of great interest in connection with the function of the heat-stable peroxidase. He has found that the respiratory pigment cytochrome is common to animals, bacteria, yeast, and the higher plants, and he considers that "cytochrome and its derivatives are responsible, at least partly, for the peroxidase reaction in organisms." Moreover, he has shown that the aerobic bacteria which give a strong peroxidase reaction contain cytochrome, whereas *S. acidi lactici* and *B. sporogenes*, which give only a very slight peroxidase reaction, contain no cytochrome.

Summary.

1. A strong peroxidase reaction was given by *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. fluorescens*, *B. prodigiosus*, *B. proteus*, *B. coli*, *B. paratyphosus* A, *B. paratyphosus* B, *B. subtilis*, *B. megatherium* and *Staphylococcus aureus*.

2. *S. acidi lactici* and the anaerobe *B. sporogenes* gave only a very slight peroxidase reaction.

3. In all cases the peroxidase was stable to heat.

4. With fresh, young, washed cultures of many bacteria, the blue colour formed in the peroxidase reaction faded rapidly. After being boiled, all the bacteria examined gave a permanent blue colour.

5. The heat-labile factor responsible for the fading of the blue peroxidase colour showed certain points of resemblance to the methylene blue reducing system.

In conclusion, I gladly take this opportunity of thanking Sir F. G. Hopkins for the interest he has taken in this work.

REFERENCES.

Buckmaster (1908). *J. Physiol.* **37**, *Proc.* xi.
Callow (1923). *J. Path. Bact.* **26**, 320.
—— (1924). *Biochem. J.* **18**, 507.
Hopkins and Dixon (1922). *J. Biol. Chem.* **54**, 527.
Keilin (1925). *Proc. Roy. Soc. Lond.* B. **98**, 312.
Quastel, Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
Quastel and Whetham (1925). *Biochem. J.* **19**, 520.
Quastel and Wooldridge (1925). *Biochem. J.* **19**, 652.