PEROXIDASE IN RELATION TO BACTERIAL GROWTH WITH SPECIAL REFERENCE TO THE INFLUENZA BACILLUS

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The most interesting work of recent years on *Bacillus influenzae* has dealt with its growth requirements. A number of investigators (Davis, 1921 a and b; Fildes, 1921; Rivers and Poole, 1921; Thjötta and Avery, 1920–1921) have definitely shown that two distinct accessory food substances or factors are essential to the growth of true influenza bacilli in aerobic culture. Thjötta and Avery (1920–1921) designated the two separable factors as X and V respectively. Since the recognition and separation of the two factors, organisms have been described which require only one of the factors for their growth in aerobic culture. Rivers (1922, a, b, and c) reported that Friedberger's *Bacillus hemoglobinophilus-canis* requires the X-factor only, and that bacilli have been obtained from human cases which require the V-factor only. The latter were called *Bacillus parainfluenzae.*

The nature of the X and V factors and their rôle in the growth of the influenza bacillus has not been made clear. For the most part the cultivation of the influenza bacillus has been associated with the presence of peroxidase in the medium. Olsen (1921) states that media which supported its growth gave a positive guaiac or benzidine test for peroxidase. Fildes (1921) believes that peroxidase is a dominant factor in the growth of the influenza bacillus and appears to be identical with the X-factor. The work of Fildes (1924) in which the V-factor organism grew in symbiosis with the X-factor organism on ordinary media, indi-

cates that those organisms which require for growth one factor only in the culture medium, either X or V, synthesize the other factor. On this basis it was assumed that, if the X-factor were peroxidase, then the V-factor organism which does not require for growth the addition of X-factor to the medium would give a positive peroxidase test, whereas, the X-factor organism which requires the addition of X-factor and the true influenza bacillus which requires the addition of both factors to the medium would give a negative peroxidase test. After undertaking to establish the truth of this hypothesis and obtaining the results of this investigation, search of the literature revealed that Valentine and Rivers (1927) had found previously that the V-factor organism in mass gave a positive peroxidase test. Therefore, this investigation is merely an elaboration of the work begun by Rivers.

Kopp (1927–1928) published a paper on the growth requirements of the influenza bacillus in regard to the X and V factors in anaerobic culture. Under anaerobic conditions he found that the true influenza bacillus does not require the X-factor for growth, only the V-factor being essential. His results have been corroborated recently by the writer.[1] The growth of the influenza bacillus, anaerobically, on peroxidase-free media in the form of V-factor agar or broth, afforded a means of determining whether the organism produced peroxidase. Previous to Kopp's discovery there was no satisfactory way of testing the bacillus for peroxidase production since it was grown on media which themselves gave a positive peroxidase reaction.

In this study eight strains were employed. They included 6 strains (one of which was hemolytic) of true influenza bacilli, 1 strain (No. 2) of *Bacillus parainfluenzae* (V-factor organism) and 1 strain (No. 8) of *Bacillus hemoglobinophilus-canis* (X-factor organism). None of the strains grew on plain agar aerobically. All of the strains grew anaerobically on agar containing V-factor alone. The eight strains were grown anaerobically on V-factor agar for forty-eight hours at 37°C. and tested for the production of peroxidase by the benzidine method.

[1] Journal of Hygiene, in press.

Reagent. A large pinch of crystalline benzidine-hydrochloride was placed in a test tube with 3 cc. of glacial acetic acid. After most of the crystals had dissolved, an equal volume of hydrogen peroxide was added. A peroxidase-containing substance in contact with this milky white reagent turns blue.

TABLE 1

The production of peroxidase by true influenza and influenza-like organisms

STRAINS	HEMOLYSIS	FACTORS REQUIRED AEROBIC CONDITION	PEROXIDASE
1	+	X and V	−
2	−	V	+
8	−	X	−
27S	−	X and V	−
44	−	X and V	−
121	−	X and V	−
126	−	X and V	−
499	−	X and V	−

TABLE 2

Production of peroxidase by anaerobes as shown by the benzidine reaction

ANAEROBES	PEROXIDASE PRODUCTION
1. *B. aerofoetidis*	−
2. *B. bellonensis*	−
3. *B. bifermentans*	−
4. *B. botulinus* A	−
5. *B. botulinis* (European)	−
6. *B. fallax*	−
7. *B. oedematis*	−
8. *B. putrificus*	−
9. *B. sporogenes*	−
10. *B. tertius*	−
11. *B. tetanus*	−
12. *B. vibrion septique*	−
13. *B. Welchii*	−

A drop of the reagent was placed on the colonies produced by each strain. The true influenza bacilli and *Bacillus hemoglobinophilus-canis* gave negative reactions. *Bacillus parainfluenzae* gave a positive reaction, in which the individual colonies turned a

solid blue color. The parainfluenza organism exhibited the positive reaction for peroxidase on V-factor media both aerobically and anaerobically in successive transplants. Table 1 indicates the results obtained with each strain.

Avery and Morgan (1923–1924) found that an iron compound which gave the benzidine reaction for peroxidase when added to

TABLE 3

The production of peroxidase by aerobes

AEROBES	PEROXIDASE REACTION	AEROBES	PEROXIDASE REACTION
1. *Act. asteroides*	+	23. *B. subtilus*	+
2. *B. acidi-lactici*	+	24. *B. suipestifer*	+
3. *B. alkaligenes*	+	25. *B. typhosus*	+
4. *B. anthracis*	+	26. *B. xerosis*	+
5. *B. anthracoides*	+	27. *Br. melitensis*	+
6. *B. cereus*	+	28. *M. tetragenus*	+
7. *B. coli-communior*	+	29. *Myc. stercusis*	+
8. *B. coli-communis*	+	30. *Ps. fluorescens*	+
9. *B. diphtheriae*	+	31. *Sarc. aurantiaca*	+
10. *B. fuchsina*	+	32. *Sarc. lutea*	+
11. *B. Hofmanni*	+	33. *Sarc. ventriculi*	+
12. *B. lactis aerogenes*	+	34. *Sp. cholerae*	+
13. *B. mallei*	+	35. *Sp. Finkler-Prior*	+
14. *B. Morgani*	+	36. *Sp. Metchnikovi*	+
15. *B. mycoides-roseus*	+	37. *Sp. tyrogenum*	+
16. *B. paratyphosus* A	+	38. *Staph. albus*	+
17. *B. pestis-caviae*	+	39. *Staph. aureus*	+
18. *B. prodigiosus*	+	40. *Staph. citreus*	+
19. *B. proteus-vulgaris*	+	41. *Staph. zymogenes*	−
20. *B. pseudotetanicus*	+	42. *Str. bovis*	−
21. *B. pyocyaneus*	+	43. *Str. liquifaciens*	−
22. *B. rouge de Kiel*	+	44. *B. suisepticus*	−

bouillon supported the growth of obligate anaerobes in the presence of air through repeated transfers. In view of this fact and the results with the influenza bacillus and related organisms which suggest that the function of peroxidase is intimately connected with the ability of these strains to grow in the presence of air, several anaerobes were tested for peroxidase. They were transferred to fresh meat tubes and incubated for forty-eight hours at

37°C.; a drop of the meat culture was transferred by a capillary pipette to an infusion agar plate and streaked with a platinum loop. After seventy-two hours incubation in the anaerobic jar, the strains were tested for peroxidase by transferring a loop of the plate culture to a drop of the benzidine reagent. Of thirteen anaerobes tested, not one gave a positive reaction for peroxidase. The results are shown in table 2.

Since the influenza bacillus has been grown on agar medium in symbiosis with certain aerobic organisms it was decided to test a representative number of aerobes for the production of peroxidase. The tests were carried out in the manner described for anaerobes, i.e., a loop of culture, from plain or infusion agar medium, of each strain was transferred to a drop of the benzidine reagent. Of 44 aerobes tested only 4 were negative for peroxidase. Table 3 summarizes the results. Organisms such as the diphtheria bacillus and xerosis bacillus which, from reports of various investigators, enable the influenza bacillus to grow well in symbiosis on blood-free media were especially rich in peroxidase as revealed by the intensity of the color reaction with benzidine. It is of interest to note that Williams and others (1914) were unable to obtain the growth of influenza strains in the presence of streptococci on blood-free media. Table 3 shows the streptococci which were examined, negative in regard to peroxidase. The same may have been true for Williams' streptococci which failed to induce growth. Thus, the growth of influenza bacilli in association with aerobes does not eliminate the need for the presence of peroxidase.

Thjötta and Avery (1921) stated that banana contains the essential growth factors but fails to react positively to benzidine. If this is so, then it would be an exception to the rule that peroxidase is essential to the growth of the influenza bacillus in aerobic culture. Banana was tested in this study for peroxidase and surprisingly gave a positive reaction to benzidine. It appears then that the influenza bacillus has never been grown aerobically without the presence of peroxidase in the medium.

The question arises whether there is a functional difference between the action of the very stable peroxidase-like substance associated with an iron compound in hemoglobin, and the com-

paratively heat labile peroxidase found in vegetable tissues. Opinions differ in this regard.

Madelung is of the opinion that the activation of peroxides by hemoglobin is functionally not different from their activation by tissue peroxidases as there apparently exist in the tissues complex iron compounds which may be capable of conveying oxygen. On the other hand, Oppenheimer states, that for the present at least we must accept the active, thermo-labile peroxidases as ferments. The fact should be recognized that we can demonstrate very active peroxidases not containing manganese or iron. In the living tissues provisions would seem to be made that biological catalyzing agents, ferments, should do what under other conditions inorganic catalyzers are able to do.[2]

In view of the latter fact it would seem that the peroxidase-like activity of blood and of known chemical compounds which function as the X-principle in relation to the influenza bacillus is that of an inorganic catalyst, while the tissue peroxidases which function as the X-factor are analogous to organic catalysts, with both types of catalysts performing the same function.

DISCUSSION

Although it is definitely known that the influenza bacillus will grow anaerobically without the X-factor, which all evidence indicates to be peroxidase, the nature of its action in aerobic cultures still remains to be explained.

In Callow's work (1923) on catalase in bacteria, none of nine anaerobes tested gave off gas when treated with hydrogen peroxide, whereas, all of the aerobes investigated were shown to contain catalase under both aerobic and anaerobic conditions with the exception of the streptococci.

These results, as far as they go, show a striking parallelism to the peroxidase test carried out in the present study. Accordingly, the non-peroxidase-producing organisms fall into three main groups, (1) that represented by the anaerobes which fail to grow aerobically on solid media even though peroxidase has been added, (2) that represented by the influenza bacilli which grow aerobi-

[2] Quoted from von Fürth, Chemistry of Metabolism, Smith.

cally in liquid or on solid media upon the addition of peroxidase when the second factor is also present and, (3) that represented by certain streptococci which neither produce peroxidase nor require it for growth.

Callow states,

> According to Wieland's theory of respiration, water is decomposed by an oxido-reductase into (OH) and (H), the latter combining with the oxygen of the air to form hydrogen peroxide which is at once decomposed by the enzyme catalase into water and molecular oxygen. In the absence of catalase the hydrogen peroxide would accumulate and kill the cell. . . . . In the absence of air no hydrogen peroxide would be formed and anaerobes would be able to develop.

While growth of anaerobes aerobically on solid media in the presence of peroxidase does not occur, Avery and Morgan (1923–1924) have reported their growth through repeated transfers in broth containing an iron compound with the usual reactions of peroxidase. Callow (1923) on the other hand, obtained no conclusive evidence that anaerobes were able to grow in broth containing catalase prepared from bacteria, fat and yeast. The only difference recognized between the terms catalase and peroxidase seems to be that the former is thought to liberate molecular oxygen and the latter atomic oxygen from hydrogen peroxide.

Applying McLeod's and Gordon's theory of hydrogen peroxide production by bacteria, the difference in the growth requirements of the three groups mentioned would appear to involve its production and their sensitiveness to it. According to these authors, it may be supposed that all bacteria tend to produce hydrogen peroxide and that the production of catalase by the majority of bacteria, prevents the accumulation of hydrogen peroxide to which they are only slightly or moderately sensitive. On the other hand they state that the sensitiveness of anaerobes to hydrogen peroxide, which they produce as soon as oxygen is available, is so very great that they cannot tolerate minute traces even in the presence of added catalase which they lack, therefore they cannot grow or survive in the presence of oxygen. The influenza bacilli would apparently belong to a group whose sensitiveness

to hydrogen peroxide is not great enough to prevent growth under aerobic conditions when the missing peroxidase is artificially supplied in the medium. To quote McLeod and Gordon,

> Against the general application of such a theory is the group (three) which is apparently devoid of catalase [and peroxidase], "not particularly sensitive to hydrogen peroxide and have not been shown to produce hydrogen peroxide. An alternative supposition would be that the production of hydrogen peroxide is associated with a certain type or types of bacterial metabolism.

It could still be assumed that the third group produces hydrogen peroxide but in such minute amounts as not to be detected and to which its sensitiveness is negligible; in such a case catalase or peroxidase would not be required. However, we are still left in the dark as to the actual conditions.

Whatever definite rôle the X-principle of peroxidase plays in the growth of the influenza bacillus, it must be concerned in rendering harmless an otherwise harmful substance formed in the presence of atmospheric oxygen. It seems unlikely that molecular oxygen as such is directly harmful,—else how could peroxidase act to make it harmless?

CONCLUSIONS

1. *Bacillus influenzae*, hemolytic and non-hemolytic and *Bacillus hemoglobinophilus-canis* do not produce peroxidase. *Bacillus para-influenzae* produces peroxidase under both aerobic and anaerobic conditions.
2. Not one of 13 anaerobes tested, reacted positively to the benzidine test for peroxidase.
3. Of 44 aerobes tested, all but four strains, two of which were streptococci, gave a positive reaction for peroxidase.
4. Banana contains peroxidase as tested by benzidine.
5. All evidence appears to substantiate Fildes' view (1922) that the X-factor is associated and identical with peroxidase.

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