TYPES OF BACTERIA ON BLOOD AND CHOCOLATE AGAR AND THE IMMEDIATE CAUSE OF THESE TYPES

EINAR LEIFSON

Department of Pathology and Bacteriology, Johns Hopkins University, Baltimore, Maryland

Received for publication March 30, 1932

The action of certain bacteria on blood agar is so striking and constant a phenomenon that it has come to be of considerable importance in diagnosis. This is especially true in the case of the cocci. The effect on blood agar of bacteria other than the cocci has been described from time to time but no systematic study of a large number of types seems to have been made. Paulson and Brown (1931) have recently described colonies of colon bacilli of the alpha, beta, and gamma types.

The present paper deals with the effect on blood agar of some 260 strains of Gram-negative bacilli and 14 selected strains of various types of streptococci and pneumococci. The probable cause of the green coloration produced by some of these bacteria has been determined, and the various factors which influence the production of the green coloration have been investigated. A study has also been made of the effect on chocolate agar of both bacilli and cocci. It must be emphasized at this time that the types of bacterial colonies in blood agar about to be described are all deep colonies. Many deep colonies of the gamma (non-hemolytic) type may be considerably more hemolytic as surface colonies.

The appearance of different types of colonies of streptococci in blood agar has been described by a number of investigators and especially by Brown (1919) in whose paper will be found a

1 The writer wishes to express his appreciation to Dr. J. Howard Brown for many helpful suggestions, and kindly criticism.
EINAR LEIFSON

review of the literature up to that time. The three distinct types of deep colonies of streptococci in blood agar are now well known, as are also the designations alpha, beta, and gamma given by Smith and Brown (1915). A fourth type, delta, was described by Bryant (1925), but the difference between this type as described by Bryant, and the alpha type seems to have been too slight to gain general recognition. Tunnicliff (1930) has found that some beta streptococci will give a green coloration on chocolate agar while others will not. From the literature referred to and the data which will be presented in this paper, we may now recognize two distinct kinds of bacteria which produce the alpha type of colony. The beta streptococci may also be divided into two types. A brief description of these types follows:

1. The gamma type produces no visible change of the corpuscles of a blood agar plate. None of the strains studied produced any visible effect on chocolate agar. This does not exclude the possibility that such strains exist, however. There are also a number of bacteria, both cocci and bacilli, which produce deep colonies of the gamma type but whose surface colonies are hemolytic. Staphylococci of this type are often found.

2. The beta type produces clear zones of hemolysis around the deep colonies in a blood agar plate. Occasionally bacteria are found which produce deep colonies having beta-like zones of partial hemolysis. These have been designated as alpha prime by Smith and Brown (1915). They are perhaps either to be considered as atypical beta types or atypical alpha. On chocolate agar some streptococci of the beta type produce a green coloration which according to McLeod and Gordon (1922) is due to the production of hydrogen peroxide. Others produce no visible effect. The former of these may be designated beta-H₂O₂ positive and the latter beta-H₂O₂ negative. More exhaustive investigation is needed to show whether the production of hydrogen peroxide by some streptococci of the beta type is sufficiently constant and clear-cut to be of classificatory significance.

3. The alpha type generally produces a green coloration around the deep colonies in a blood agar plate. In case of the bacilli of the alpha type the green coloration is very pronounced and the
accompanying hemolysis rather slight. Streptococci and pneumococci of the alpha type often produce a barely perceptible amount of green with considerable hemolysis. In many cases only microscopic examination of the colonies will determine their type. For a detailed description of the microscopic appearance of the alpha type the reader is referred to the monograph by Brown (1919). All of the strains of alpha streptococci and pneumococci studied produced a green coloration on chocolate agar. None of the alpha bacilli studied produced any green coloration on chocolate agar. Subsequent discussion will show that the two types of alpha bacteria, as determined by chocolate agar, differ fundamentally physiologically as well as morphologically in all cases which we have studied. The alpha bacilli produce a green coloration of blood agar by virtue of the hydrogen sulphide which they produce. The alpha cocci change hemoglobin to hematin and also produce hydrogen peroxide which reacts with hematin to give a green coloration. Rather than introduce a new name it

| TABLE 1 |
|-----------------|---------------------------------|---------------------------------|
| Types of bacterial colonies in blood and on chocolate agar | BLOOD AGAR | CHOCOLATE AGAR |
| TYPE | | |
| Gamma | No visible effect | No visible effect |
| Alpha-H₂S positive | Large green zone with little if any hemolysis. Appearance due to the production of H₂S, and subsequent oxidation by air of the sulfhemoglobin formed | | |
| Alpha-H₂O₂ positive | Green zones with partial hemolysis. Appearance due to the formation of hematin, and H₂O₂ which react to form the green coloration | Green coloration. Due to interaction of hematin and H₂O₂ |
| Beta-H₂O₂ negative | Large zones of complete hemolysis. No green | No visible effect |
| Beta-H₂O₂ positive | Large zones of complete hemolysis. No green | Green coloration. Due to interaction of hematin and H₂O₂ |
was decided to refer to the one group of alpha bacteria as alpha-
\( \text{H}_2\text{S} \) positive, and to the other as alpha-\( \text{H}_2\text{O}_2 \) positive. The term
alpha as here used is purely descriptive of the appearance of the
deep colonies in blood agar. In a report of this work given before
the last meeting of the Society of American Bacteriologists the
term delta was used for what we now call alpha-\( \text{H}_2\text{S} \) positive.

THE CAUSE OF THE GREEN COLORATION

The alpha-\( \text{H}_2\text{O}_2 \) positive type

The bacteria which produce this type of colony are often re-
ferred to as methemoglobin producers. This designation has
come about perhaps from the work of Cole (1914) and Blake
(1916) who showed that some green-producing streptococci and
pneumococci generally produce methemoglobin when blood is
added to a broth culture. Lyall (1914) found that some green-
producing streptococci produce little if any methemoglobin while
others produce considerable. Our own observations confirm
those of Lyall. The green-producing bacilli which we have desig-
nated as alpha-\( \text{H}_2\text{S} \) positive do not seem to produce demonstrable
amounts of methemoglobin. From these observations it is clear
that there is no general correlation between the production of
methemoglobin and a green coloration of blood agar. The sug-
gestion is made that the term "methemoglobin producers" as re-
ferring to green producing cocci should be discontinued. McLeod
and Gordon (1922) found a perfect correlation between the pro-
duction of hydrogen peroxide and a green coloration of heated
blood agar. These observations have been abundantly confirmed,
especially by the exhaustive work of Avery and Neill (1924), Neill
and Avery (1924, 1925), and Neill (1925). Although such oxidiz-
ing agents as sodium perborate will also produce a green colora-
tion of heated blood agar, hydrogen peroxide is most likely re-
sponsible for the green coloration produced by bacteria in heated
blood agar. The inference seems to have been made by some that
hydrogen peroxide is responsible for the green coloration produced
by bacteria in blood agar as well as in chocolate agar. Hydrogen
peroxide, however, does not produce a green coloration of blood
agar and cannot, therefore, alone be the cause of the green colora-
tion. Hagan (1925) suggested that the simultaneous production of acid and of hydrogen peroxide might be the cause of the green coloration. The amount of acid however, in combination with hydrogen peroxide, which is necessary to produce a green coloration is far in excess of that generally produced by these bacteria in blood agar. This explanation is therefore not very probable. Ruediger (1906) found that certain hemolytic streptococci would produce a green coloration in blood agar if a fermentable carbohydrate (glucose) was added. He concluded that the green coloration was due to the action of some acid on the erythrocytes of the blood. No such acid is known, however, and this explanation is not very probable.

When blood is treated with acid, sodium nitrite, or heated to 70°C. or above, the hemoglobin is broken down and hematin is formed. Hematin will react with hydrogen peroxide to give a green coloration but hemoglobin will not. Blood which has been treated with potassium ferrocyanide does not react with hydrogen peroxide to give a green coloration. This may be taken to show that hydrogen peroxide does not react with methemoglobin to give a green coloration. Any bacteria, therefore, which produce hydrogen peroxide will give a green coloration on blood agar which has been heated or treated with chemicals in such a way that hematin is produced, but will not necessarily give a green coloration on whole blood agar or on methemoglobin agar. The exact mechanism of the production of hematin from hemoglobin is still somewhat obscure. For a detailed discussion of this subject the reader is referred to the work of Avery and Neill. According to these authors the hemoglobin is first changed to methemoglobin by means of oxidizing enzymes. The methemoglobin is further changed by enzymes to form hematin. From the work of Lyall (1914) and also from our own observations it seems that in some cases methemoglobin is not formed to an appreciable extent. This does not prove that methemoglobin is not always an intermediary product in the change of hemoglobin to hematin, but rather that in some cases it is too transitory to be detected. The change of hemoglobin to methemoglobin and hematin takes place only under aerobic conditions and consequently
no green coloration is produced by the alpha cocci under anaerobic conditions.

The cause of the hemolytic zone around the colonies of alpha cocci is somewhat obscure. In some ways it would seem to be due to an agent distinct from that which causes the green coloration. Six strains of alpha streptococci and pneumococci, for example, produced a distinct hemolysis but no green on a synthetic blood agar the composition of which is given later (see fig. 4, plate 1). On the other hand, 5 of the 6 cocci when grown under strictly anaerobic conditions produced no hemolysis and no green, while one produced both hemolysis and a green coloration. Those alpha strains which are gamma under strictly anaerobic conditions become somewhat hemolytic when the oxygen tension is slightly increased, but no green coloration is apparent until the oxygen tension is increased still further. The beta type of hemolysis does not seem to be influenced by the oxygen tension. From these data it seems that the production of hemolysis and of a green coloration by alpha cocci are not independent phenomena, but that the two are caused by the same agent. Hemolysis may be a manifestation of a slight injury to the erythrocytes and may be brought about in a variety of ways. For example, if part of a blood plate is heated on a steam bath a wide zone of hemolysis appears between the zones of chocolate agar and of unchanged blood. Hemolysis may be brought about also by the action of acids, hydrogen peroxide, etc. From these data it seems most likely that hemolysis produced by the alpha type of cocci is due to very much the same substances which produce the green coloration, and is associated with a slight injury of the erythrocytes.

To summarize the course of the formation of the alpha-H$_2$O$_2$ positive type of colony: The oxyhemoglobin is first reduced to hemoglobin. In some way, as yet poorly understood, the hemoglobin is changed either first to methemoglobin and then to hematin and globin, or the hematin may be formed directly from the hemoglobin. The hematin reacts with the hydrogen peroxide formed by the bacteria to produce a green pigment. Both of these reactions take place only in the presence of air. Due, per-
haps, to hydrogen peroxide or some other substance produced by the bacterial metabolism, some of the erythrocytes are slightly injured and a zone of partial hemolysis is formed around the colony.

The alpha-\( \text{H}_2\text{S} \) positive type

The alpha-\( \text{H}_2\text{S} \) positive type has been distinguished from the alpha-\( \text{H}_2\text{O}_2 \) positive type by its failure to produce a green coloration on chocolate agar, and by its large zone of green coloration on blood agar, with little or no hemolysis. All green-producing bacilli studied belong to the alpha-\( \text{H}_2\text{S} \) positive type. The alpha type of bacilli produces no hydrogen peroxide, and neither met-hemoglobin nor hematin from hemoglobin. From the study of some 260 strains of Gram-negative bacilli a perfect correlation was found between the formation of a green coloration on blood agar and the production of hydrogen sulphide. Investigation of the effect of hydrogen sulphide on blood showed that it reacts with hemoglobin to form purple sulfrhemoglobin. Sulfrhemoglobin has a strong absorption band at about 620\( \mu \mu \). This absorption band may be distinguished from that of methemoglobin by the addition of ammonium hydroxide which causes the methemoglobin band to disappear but leaves the sulfrhemoglobin band intact. Carbon monoxide will cause a shift of the sulfrhemoglobin band to about 610\( \mu \mu \) but has no effect on the methemoglobin band. Sulfrhemoglobin is easily oxidized either by air or by oxidizing agents such as sodium perborate to form a green compound. Sulfrhemoglobin is rather difficult to demonstrate in blood cultures of alpha bacilli unless substances such as cysteine or thiosulphates are present. Blood plates exposed aerobically to hydrogen sulphide turn green immediately and no sulfrhemoglobin can be detected in the plates. Neither can any sulfrhemoglobin be detected in the green zones around the colonies of alpha bacilli. The green compound does not seem to have a characteristic spectrum. One typical strain of an alpha colon bacillus grown on blood agar plates under anaerobic conditions for forty-eight hours produced no green coloration. Exposure of these plates to air in the refrigerator did not lead to the production of a green coloration, but exposure at room
Compounds such as cysteine and sodium thiosulphate greatly stimulate the production of a green coloration by alpha bacilli.

Hydrogen sulphide does not produce a green coloration of chocolate agar, nor does it react with hematin to give a green coloration. Neither do alpha bacilli, in our experience, ever produce a green coloration on chocolate agar.

The effect of hydrogen sulphide on blood has been known for some time. Harnach (1899) studied the effect of hydrogen sulphide on blood and noted the development of a green coloration in the presence of air. V. d. Bergh (1905) found that the blood of some of his enteritis patients showed a typical sulhemoglobin spectrum. Addition of blood to bouillon cultures of the specific organisms gave, after twenty fours of incubation, a typical sulhemoglobin spectrum. He concluded that the sulhemoglobin in the blood of his patients was caused by hydrogen sulphide formed by the organisms in the intestine.

The data presented above seem to the writer to be adequate proof that the alpha type of colony, produced by the bacilli investigated, results from the interaction of hydrogen sulphide and hemoglobin to form sulhemoglobin. The sulhemoglobin thus formed is quickly oxidized to form a green compound. Any hemolysis formed around this type of colony is probably due to some metabolic product of the bacteria, perhaps hydrogen sulphide.

**EFFECT OF THE PHYSICAL AND CHEMICAL ENVIRONMENT ON THE PRODUCTION OF THE GREEN COLORATION**

The effect of the physical and chemical environment on a biological reaction does not seem to be fully appreciated. It is well known that chemical reactions are modified and at times completely changed by changing the environment. This is even more true in the case of biological reactions. Not only must we consider the effect of the environment upon the chemical reactants and resultants, but also the effect of the environment upon the living matter producing the chemicals. The environment is especially important in cases where it forms, or may form, one
BACTERIA ON BLOOD AND CHOCOLATE AGAR

of the reactive agents. Such is the case with bacterial oxidations or reductions in which the atmospheric oxygen may play an important rôle. The formation of a green coloration by alpha bacteria in blood agar is partly an oxidation reaction and depends for its completion upon atmospheric oxygen.

As stated above, the alpha-H$_2$S positive type of colony results from the formation of sulfhemoglobin and the subsequent oxidation of the sulfhemoglobin to a green compound. Any change of environmental conditions affecting the production of hydrogen sulphide would also affect the production of the green coloration. In a medium consisting of 0.2 per cent ammonium sulphate, 0.1 per cent disodium phosphate, 0.5 per cent sodium chloride, 0.5 per cent glucose, and 2 per cent agar the alpha bacilli examined produced no hydrogen sulphide as determined by lead acetate. On blood agar plates made with this medium without the glucose the same alpha bacilli produced no green coloration and the colonies had the gamma appearance (see fig. 2, plate 1). In this same blood medium a number of strains of alpha streptococci and pneumococci also failed to produce a green coloration, although some hemolysis was produced (see fig. 4, plate 1). On chocolate agar made with this medium a beta-H$_2$O$_2$ positive streptococcus and some strains of alpha streptococci produced no green coloration, indicating that they were unable to produce any hydrogen peroxide on the medium. Addition of peptone to the synthetic medium generally restored the ability of the alpha bacilli to produce hydrogen sulphide, and of the alpha cocci to produce hydrogen peroxide. Different bacteria differ greatly in their ability to produce hydrogen sulphide or hydrogen peroxide on different media. The same can be said for the production of a green coloration. *Salmonella schottmülleri*, for example, will produce a good green coloration in extract blood agar, while *Eberthella typhosa* ordinarily will not do so. If infusion agar is used *E. typhosa* produces a green coloration. Many strains of *E. typhosa* which fail to produce a blackening of extract lead acetate agar may be found to blacken infusion lead acetate agar. If cysteine or sodium thiosulphate is added to infusion blood agar
the alpha type of colony is formed by many bacilli which have the gamma type of colony on the standard infusion blood agar. In some cases even a change of peptone concentration from 1 to 2 per cent may be sufficient to change the appearance of an organism from that of a non-green-producer to a green-producer, the higher peptone concentration producing more green. The nature of the blood used, the concentration of salts, and the hydrogen ion concentration may materially affect the type of colony produced. Tunnicliff (1930) showed that some hemolytic streptococci will produce a green coloration on chocolate agar incubated at 32°C. but not at 37°C. The kind of agar used, and the manner in which the chocolate agar is produced also greatly influence this reaction. If the action of beta bacteria on chocolate agar is to be of any differential value, a standard technique for the preparation of chocolate agar must be adopted.

SUMMARY

A study of the effect of various bacteria on blood agar and on chocolate agar has resulted in the differentiation of five distinct types. The cause of the green coloration of blood agar has been determined with a fair degree of probability. The effect of various media and environmental conditions on the formation of the different types of colonies is discussed.

REFERENCES

Paulson, M., and Brown, J. Howard 1931 In press.
PLATE I

Fig. 1. Deep colonies of an alpha-colon bacillus in pork infusion blood agar after incubation at 37°C. for forty-eight hours. The green coloration is not apparent in the photograph but it is very strong. The hemolysis seems more prominent in the photograph than it appears to the naked eye.

Fig. 2. Same organisms as in figure 1 in synthetic blood agar. The colonies show no green coloration and practically no hemolysis.

Fig. 3. Deep colony of a pneumococcus (alpha type) in pork infusion blood agar after incubation at 37°C. for forty-eight hours. To the naked eye the colony appears very hemolytic with a touch of green coloration.

Fig. 4. Same organism as in figure 3 in synthetic blood agar. The colony shows no green coloration but decided hemolysis.

Fig. 5. Same organism as in figure 1 in pork infusion blood agar to which was added 1 per cent sodium thiosulphate. The green coloration is much increased due to the addition of the thiosulphate.
(Einar Leifson: Bacteria on Blood and Chocolate Agar)
PLATE 2

FIG. 1. Surface culture on chocolate agar of the same organism as in figure 1, plate 1. There is no green coloration.

Fig. 2. Surface culture on chocolate agar of a pneumococcus (alpha type) showing a strong green coloration around the colonies.

Fig. 3. Surface culture on chocolate agar of a strain of Str. epidemicus (beta-\textsubscript{H}\textsubscript{2}O\textsubscript{2} positive type) showing a green coloration around the colonies.

Fig. 4. Surface culture on chocolate agar of a streptococcus of the beta-\textsubscript{H}\textsubscript{2}O\textsubscript{2} negative type showing no green coloration around the colonies.

The chocolate agar plates were all incubated at 37°C for forty-eight hours.

For the photographs in plates 1 and 2 the writer is indebted to Dr. J. Howard Brown.
Einar Leifson: Bacteria on Blood and Chocolate Agar