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I hereby recommend that the thesis prepared under my supervision by Thomas Legran Snyder

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THE FUNCTION OF PROTOHEMIN

FOR THE GROWTH OF *HEMOPHILUS INFLUENZAE*

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by

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I INTRODUCTION

During the fifty years that have passed since the discovery by Pfeiffer that blood was required for the cultivation of Hemophilus influenzae the problem of the nature and function of the accessory growth factors has been the subject of almost continuous study. This sustained interest has been due in part to the supposed etiological relation of the organism to epidemic influenza, and a great need has been felt for suitable methods of isolation and cultivation. To many, however, the biology of the bacterium possessed an intrinsic interest by virtue of its peculiar relation to other bacteria and because of the light it promised to throw on the fundamental biological processes of living forms. Today, when it has become obvious that the organism is of but little significance in the production of disease, this other phase has become increasingly important and it is highly probable that in the future Hemophilus influenzae and related bacteria will serve as useful biochemical tools for the study of enzyme chemistry and the chemistry of respiration. Before this can be done, however, a more detailed knowledge of the biochemical characteristics of these bacteria is required.

The "vitamin" or accessory growth requirement of Hemophilus influenzae was early resolved into two components, and, within certain limits, each of these may be studied separately. The nature of both of these factors is now known and the function of the heat-labile "V-factor" may be considered

largely understood, but controversy still exists concerning the function of the heat-stable "X-factor", protohemin. It is with the function of the latter factor in relation to the process of growth that the present work is concerned. The investigation has been confined to a single strain of Hemophilus influenzae, but since there is no evidence that protohemin serves different functions for different bacteria, it is expected that the conclusions will be applicable to other bacteria that require this factor.

Of the several functions that have been suggested for protohemin, some have been later eliminated. It is now known, for example, that protohemin is not required for its content of inorganic iron as such, and that neither hemoglobin nor protohemin is active by virtue of an ability to transport oxygen. Three theories concerning the function of protohemin as the X-factor remain in good repute at the present time. These suggest that protohemin is used by the cell for the synthesis of some essential mechanism, which may be either a catalytic respiratory system (cytochrome and cytochrome oxidase), catalase, or peroxidase. The available evidence does not permit a choice to be made from these possible functions, nor their relative importance for growth to be evaluated. It has been observed that proportional augmentation of oxygen consumption occurs when protohemin is added to cultures of Hemophilus influenzae, which shows that protohemin does have a respiratory function, yet the fact that growth

may be obtained under anaerobic conditions seems to indicate that this function is not essential for growth. The evidence for the catalase theory is not strong and consists of the observations that compounds possessing catalase activity support the growth of bacteria requiring the X-factor, and that in the absence of oxygen the X-factor is dispensable. No evidence has been presented concerning the formation of a true peroxidase. It is highly desirable, therefore, that additional information be obtained in respect to these questions.

In the present work methods are employed whereby the respiratory catalytic function and the catalase function may be studied separately and the influence of each alone on growth determined. These methods consist of substituting for protohemin, which may possess both functions, substances that can provide only one of the two functions. It was found that substances supplying the respiratory catalytic function alone did not permit growth but, on the contrary, were growth inhibiting, and this inhibition was traced to the accumulation of hydrogen peroxide formed as a result of oxygen consumption. It was also shown that hydrogen peroxide was formed in the absence of protohemin or other added catalysts and that substances preventing its accumulation permitted growth. Finally, it was demonstrated that the organism is able to produce catalase from protohemin. From these results it was concluded that the only essential function of protohemin for growth is the production of catalase and perhaps of peroxidase.

II REVIEW OF THE LITERATURE

The bacterium now known as Hemophilus influenzae was first observed by Pfeiffer in influenzal sputum during the grippe epidemic of 1889-1890. In his preliminary publication (Pfeiffer, 1892) he described the morphological characteristics of the organism, but was unable to obtain it in pure culture. In the same year, however, Pfeiffer and Beck (1892) reported successful cultivation on a nutrient medium containing hemoglobin. Coagulated hemoglobin was also found satisfactory.

At this time and for many years after it was not clearly realized that the accessory growth requirement of the organism was of a dual nature. Although it had been reported by Gohn and v. Preyss (1902, 1904) and Luerssen (1904) that hemoglobin alone was insufficient for growth, it was not until 1917 that Davis (1917) demonstrated thattwo substances were required. These were differentiated on the basis of their resistance to heat. The heat-stable factor withstood autoclaving for 20 minutes at 20 pounds and was supplied by protohemin as well as by hemoglobin; the other factor was inactivated by autoclaving and occurred in other bacteria, in fresh plant tissue, and it was later claimed by Davis (1921 a, 1921 b) that it was present also in pure hemoglobin. Separation of the factors was independently obtained by Fildes (1920, 1921), who discovered the heat-stable factor in the pigmented deposit of his peptic digest blood

medium, and the heat-labile factor in the colorless supernatant. The terms "X-factor" and"V-factor" were proposed by Thjøtta and Avery (Thjøtta and Avery, 1920-21; Thjøtta, 1921) to designate the heat-stable and heat-labile factors respectively. They also showed that fresh plant tissue could supply both factors, and were thus the first to demonstrate that the organism was not dependent on blood for growth.

The elucidation of the dual accessory growth requirements of Hemophilus influenzae led to the discovery of other bacteria with like and differing relations to the same growth factors. Examples of all the possibilities were found, so that the "hemophilic" bacteria may be separated from other bacteria by the need of one or both factors, and may be classified as follows according to their exact requirements in this respect:

Group I, requiring the addition of both the X-factor and V-factor to the culture medium, and including:

Hemophilus influenzae Pfeiffer, of which there are several strains differentiated mainly on the basis of indol production and the formation of an hemolysin.

Hemophilus conjunctivitidis Koch and Weeks, which is considered by many to be the same organism as Hemophilus influenzae.

Hemophilus gallinarum Eliot and Lewis, placed in this group by Schalm and Beach (1936).

Group II, requiring the addition of only the V-factor to the medium, and including:

Hemophilus hemolyticus Bergey et al.

Hemophilus parainfluenzae Rivers.

Group III, requiring the addition of only the X-factor to the medium, and including:

Hemophilus canis Rivers.

Hemophilus ducreyi Bergey et al. (Lwoff and Pirosky,1937).

Hemophilus influenzae murium Kairies and Schwartzer (Iványovics and Iványovics, 1937).

Pasteurella lepiseptica Bergey et al. (Webster, 1925).

It appears that all other known bacteria require the addition of neither growth factor to the medium. This method of classifying the "hemophilic" bacteria was introduced by Davis (1921 d), Fildes (1923, 1924), and Valentine and Rivers (1927).

It is now generally conceded that the "hemophilic" bacteria owe their accessory growth requirements to the inability to synthesize these substances from the usual nutrient media, and that they differ from many other bacteria in this way. This explanation was presented largely as the result of the study of commensal growth of the "hemophilic" bacteria.

The influence of other bacteria on the growth of Hemophilus influenzae was noted early. Grassberger (1898) observed the growth of this organism on ordinary nutrient

media in the presence of either living or dead Staphylococcus aureus. Meunier (1898) noted that colonies of Hemophilus influenzae grew abundantly in the neighborhood of colonies of other bacteria on hemoglobin-free solid media, and he termed this phenomenon "satellitism". These observations were confirmed by Neisser (1903), who showed that some species of bacteria were better "nurses" than others, and by Luerssen (1904). It was reported that when killed cells were used, only certain species induced growth of Hemophilus influenzae.

It has been shown that commensal growth of Hemophilus influenzae may be related to either the X-factor or the V-factor. It was demonstrated by Davis (1921 c) that the typical satellite phenomenon might be observed on media containing an abundance of the X-factor, and thus must be due to the production of the V-factor by the foreign bacterial colonies. The V-factor was apparently diffusible through agar and might exert a stimulating influence on Hemophilus influenzae colonies at a distance of as much as one centimeter. In media containing a sufficiency of V-factor but no X-factor, growth of the organism may also be induced by foreign bacteria, but in this case there is no formation of satellite colonies. According to Kollath (1925 a) this is because the X-factor is not readily diffusible and there need by a more intimate relation between the cells of the two species. These results suggested that

Hemophilus influenzae differs from other bacteria in having a lesser synthetic ability, since it is unable to produce from ordinary media two growth essentials that are formed from such media by other bacteria.

The question then arose, whether those "hemophilic" bacteria requiring the addition to the medium of only one or the other growth factor differ from Hemophilus influenzae in that they require only the one mechanism for growth, or in that they are able to synthesize the factor that need not be added. The answer was readily obtained by observing the effect of the various types of "hemophilic" bacteria on the growth of the other types. Fildes (1924) was able to show that Hemophilus canis, which does not require the addition of the V-factor, was able to support growth of Hemophilus influenzae on media lacking this factor. In the same way Valentine and Rivers (1927) demonstrated that Hemophilus parainfluenzae synthesizes the X-factor. They obtained, in fact, true symbiotic growth of Hemophilus canis, requiring the X-factor, and Hemophilus parainfluenzae, requiring the V-factor, on a medium containing neither growth factor. These results throw the weight of probability on the view that the actual growth mechanisms are the same for "hemophilic" and for other bacteria, and that the former differ from other bacteria and among themselves only in regard to their synthetic abilities. This is quite in harmony with the observation of Andre Lwoff (1936 b) that all the chemically known

growth factors are fundamental and general constituents of living organisms, and with the theory that the need of growth factors is secondary, being due to a loss of synthesis and not to the gain of new growth mechanisms. In connection with the "hemophilic" bacteria, this view was first expressed by Knight (1936), who suggested that these organisms are parasites that have been constantly associated with the two growth factors preformed in the animal body, and have consequently lost the ability of synthesizing one or both of them from simpler substances.

Since the present work is not concerned with a study of the V-factor, it will suffice here simply to note its probable nature and function. This problem has been elucidated principally by Lwoff and Lwoff (1937 a). It was noted that certain properties of the V-factor were similar to those of cozymase, and tests showed that either cozymase or Warburg's coferment was able in high dilutions to replace the V-factor. These codehydrogenases are known to act as mediators in certain oxidation-reduction reactions, and it was shown that Hemophilus parainfluenzae possesses some of the specific enzymes catalyzing such reactions. It is probable, therefore, that the V-factor consists of the codehydrogenases, and that these substances are growth promoting by virtue of their well-known function. There is some evidence indicating that they may have additional functions, and their function as mediators in oxidation-reduction reactions has not been

shown to be essential for the growth of those bacteria requiring the V-factor.

It was demonstrated by Lwoff and Lwoff (1937 a) that the component parts of the codehydrogenases could not replace the V-factor, either singly or severally, which suggests that the V-factor requirement is due specifically to an inability to unite the parts to make the whole codehydrogenases.

The fact that the dual nature of the growth requirement of Hemophilus influenzae was not at first recognized delayed the discovery of the identity of the X-factor. Attempts to substitute a single pure substance for blood were naturally not successful. Hemoglobin has been an exception in this respect (Pfeiffer and Beck, 1892; Davis, 1921 a , 1921 b) undoubtedly because of the difficulty experienced in purifying this compound. Since coagulation did not inactivate hemoglobin as the X-factor, Pfeiffer believed iron to be the essential constituent, but he was unable to substitute iron albuminate. Gohn and v. Preyss (1904) were unable to substitute potassium ferro- or ferricyanide, sodium nitroprusside, iron phosphate, iron tartrate, or iron heated with urea until the biuret test was obtained, although they observed "symbiotic" growth on media containing iron compounds, as did Kollath (1925 a, 1925 b, 1926). They also claimed that iron was a necessary component of a bile salt medium used successfully by Cantani (1901). Davis (1907b)

was unable to substitute hemocyanin, hemerythrin or echinochrom. With the discovery that two substances are required for the growth of Hemophilus influenzae, Davis (1917) was able to show that one of these is the prosthetic group of hemoglobin, protohemin. Olsen (1920) demonstrated that the iron-free tetrapyrrol, protoporphyrin, is inactive, as is bilirubin, chlorophyll and pyrrol. Fildes (1921) confirmed the observation that while protohemin is active, the related porphyrin is not. Protohemin has been shown to be much more active in the free state than when combined with a protein as in hemoglobin.

Several theories have been suggested to explain the growth promoting function of the X-factor. It was pointed out by Davis (1917) that hemoglobin does not act as an oxygen carrier in a manner comparable to its function in the animal, which had, of course, already been demonstrated by Pfeiffer's use of coagulated hemoglobin. It was noted early that only very small amounts of blood are required for growth (Czaplewski, 1902; Gohn and v. Preyss, 1902), and Davis (1907 a, 1907 b, 1910) showed that growth might occur in dilutions of hemoglobin as high as 1/180,000. More recently, Lwoff and Lwoff (1937 b) have claimed that limiting dilutions of hemolyzed blood represent about 10^{-9} grams/ml. of protohemin. The efficacy of such small amounts suggests a catalytic function (Davis, 1921 b) and this view was supported by the observation of Olsen (1920,

1921) that media permitting growth of Hemophilus influenzae gave the guaiac or benzidine test for "peroxidase". As a result of this observation, Olsen suggested that the hemin fraction of hemoglobin has some peroxidase function that enables the organism to obtain required oxygen. There has been some controversy concerning the correlation between the "peroxidase" activity of certain preparations and their activity as the X-factor. Both the observation and the theory of Olsen were supported by Fildes (1921), who attributed the relative inactivity of hemoglobin as the X-factor to its ability to take up oxygen liberated under the influence of the peroxidase. Davis (1921 b) found, however, that not all compounds exhibiting a peroxidase test possessed the biocatalytic X-activity, and Thjøtta and Avery (1921 a, 1921 b) claimed that banana was active as the X-factor but gave no peroxidase reaction, and Kent (1923) obtained similar results for certain other plant tissues. Later Fildes (1922) demonstrated that the failure to obtain a peroxidase test with these tissues was due to faulty technique, and this was confirmed by Anderson (1930). On the other hand, Baudisch (1932) has shown that certain ferric oxide preparations having catalase activity but no peroxidase activity function as the X-factor for Hemophilus canis and Hemophilus influenzae, but it has been argued that the bacterial test may be more sensitive than the peroxidase reaction, and that the failure to demonstrate the latter is not proof of the absence of per-

oxidase activity. Hemophilus parainfluenzae, which synthesizes the X-factor, gives a peroxidase test (Valentine and Rivers, 1927), and Anderson (1930, 1931) has shown that Hemophilus influenzae cultivated anaerobically without the X-factor does not give a peroxidase reaction. Thus, it appears that nearly all substances supporting the growth of bacteria requiring the X-factor give a positive peroxidase test with guaiac or benzidine. However, it has been pointed out by Stephenson (1939) that while these substances are oxidized by hydrogen peroxide in the presence of the enzyme, peroxidase, the oxidation is also catalyzed by many hemin compounds present in blood and tissues (including cytochrome c), and that while the reactions given by the latter are likely to be confused with those caused by the enzyme when only qualitative tests are used, they are quantitatively insignificant. Thus, the guaiac, benzidine, and paraphenylenediamine tests may not be considered conclusive evidence of the production of a peroxidase by the organisms.

At the time this peroxidase theory was proposed to explain the function of protohemin for the growth of bacteria requiring the X-factor, the mode of action of peroxidase was not clearly understood. It is now conceded that peroxidase catalyzes the oxidation of substrates by hydrogen peroxide and, while it is true that hydrogen peroxide is reduced and thus removed, it was not this aspect that was

stressed by the earlier proponents of the peroxidase function of protohemin. It was considered, rather, that Hemophilus influenzae and the related bacteria were obligate aerobes and peroxidase was essential because of its oxidative or "oxygen transferring" ability. It is now known that only in certain cases (e.g., Acetobacter peroxydans) is a peroxidase used as the main respiratory mechanism, and that its more important function is probably the removal of hydrogen peroxide. The older peroxidase theory of the function of protohemin has little in common with the peroxidase theory suggested more recently.

Lwoff and Lwoff have developed the theory that protohemin is used by bacteria requiring the X-factor for the formation of a respiratory catalytic system. This theory is similar to the original peroxidase theory in that oxygen consumption is again stressed. Marguerite Lwoff (1933 a) noted that certain trypanosomes require protohemin, catalase, or vegetable peroxidases in the medium in order for growth to occur, and suggested that the need was connected with a deficiency of respiratory catalysts. Later (1933 b), she reported that protohemin might be replaced by protoporphyrin but not by related compounds such as deuterohemin, mesohemin, pheophorbide b-hemin, or the active iron preparations of Baudisch (1932). The similarity in this respect of these protozoa to the influenza group of bacteria was noted. Andre Lwoff (1934) demonstrated that the addition of protohemin to

hemin-deficient cultures of these trypanosomes caused a marked increase in both oxygen consumption and growth, and suggested that protohemin and protoporphyrin are used by these organisms for the formation of cytochrome c and of the respiratory ferment (cytochrome oxidase). Similar experiments were carried out with Hemophilus influenzae and protohemin, with similar results (Lwoff and Lwoff, 1937 b). When an excess of protohemin was added to cultures of Hemophilus influenzae depleted of X-factor, minimal oxygen consumption was increased by 150 to 400 per cent, and with limiting doses of protohemin the effect was proportional to the amount of protohemin added. In these experiments, hemolyzed defibrinated blood was used as the source of protohemin and the augmentation of oxygen consumption began in less than seven minutes. This seems a remarkably short time for the complex changes probably concerned in the transformation of protohemin into cytochrome c and the respiratory ferment.

However, other evidence of the transformation of protohemin into hemin c (the prosthetic group of cytochrome c) has been presented. André Lwoff (1936 a) showed that, while Hemophilus canis is unable to grow in the presence of cytochrome c alone, the addition of this compound to cultures decreases the amount of protohemin required. It was suggested that the hemin c supplied by cytochrome replaces that amount of protohemin which would otherwise be transformed into this

substance. Cytochrome c alone is inactive probably because protohemin is necessary as such and because the reaction, protohemin ——→ hemin c may not be reversible.

The catalase theory of the function of protohemin differs significantly from the respiratory catalyst theory and the original peroxidase theory. While the latter are concerned with establishing the necessity of oxygen consumption for growth, the catalase theory suggests that protohemin may be required for protection against the harmful effects of oxygen consumption. This protection is supposed to be effected through the removal of growth-inhibiting hydrogen peroxide formed as the result of oxygen consumption. This theory has received some support from several sources. When Thjøtta and Avery (1921 a, 1921 b) reported their inability to obtain a peroxidase reaction with banana, they noted that the extract contained a markedly active catalase. Likewise, certain inorganic iron preparations have been reported to possess catalase activity and the biocatalytic X-activity but give no peroxidase reaction. Webster (1925) found that Pasteurella lepiseptica required for growth an element present in blood which was apparently the X-factor, and Webster and Baudisch (1925) observed that certain chemically defined iron compounds, particularly sodium pentacyanoaquoferroate and to a less extent magnetite, could function as the X-factor for this organism. These compounds showed catalase and peroxidase activity and absorbed oxygen. The biocatalytic activity was

destroyed by heat under the same conditions as the oxygenase and peroxidase activity. These observations were confirmed by Bourn (1927). Later, Baudisch (1932) reported that certain ferric oxide preparations having catalase activity but giving no peroxidase reaction could replace protohemin for the growth of Hemophilus influenzae and Hemophilus canis, but the suggestion that the biocatalytic activity was related to the decomposition of hydrogen peroxide (catalase activity) was not expressed. Baudisch and Dubos (1932) showed that these compounds prolonged the aerobic life of Diplococcus pneumoniae cultures, probably by destroying peroxide, and recently Baudisch (1937) has demonstrated that inorganic iron compounds possessing only the peroxidase activity will not replace the X-factor for the growth of Pasteurella lepiseptica. It is necessary to note that Marguerite Lwoff (1933 b) was unable to substitute these active iron compounds for protohemin or protoporphyrin in the cultivation of the "hemophilic" trypanosomes, so that there may be a fundamental difference between the "hemophilic" bacteria and protozoa as regards the function of the X-factor. It has already been noted that protoporphyrin may be used by the protozoa but not by the bacteria.

Anaerobic cultivation of the "hemophilic" bacteria has a very important bearing on the catalase theory. Pfeiffer (1893) was unable to cultivate the original strain of Hemophilus influenzae in an atmosphere of carbon monoxide or of hydrogen.

Fildes (1921) also experienced difficulty, and attributed the short-lived growth he did obtain anaerobically to loosely combined oxygen not removed from the medium by the anaerobic methods that he used. It is only fair to state that he obtained growth with the best anaerobic methods available, and that he was forced to conclude that growth could not be dependent upon dissolved oxygen. Kopp (1927) obtained anaerobic cultures, and claimed that not only was the X-factor dispensable under these conditions but that it was even inhibitory. He concluded that in aerobic culture the X-factor protects the organism from the harmful action of oxygen, but he was unable to explain in what manner this was effected. Eirund (1929) confirmed Kopp's anaerobic cultivation and the inhibitory effect of the X-factor anaerobically. He obtained anaerobic growth for five passages with certain strains. Kleineberger (1930) observed anaerobic growth of the organism and noted that growth was less abundant under these conditions. As a rule, these authors report anaerobic growth with some strains and not with others. Anderson (1930, 1931) found the X-factor dispensable anaerobically, but did not consider its presence detrimental to growth. She suggested that the X-factor neutralized some growth inhibiting substance formed in the presence of oxygen. Apparently, Knight (1936) was the first to point out the possibility that this harmful substance might be hydrogen peroxide, and he compares this theory with the respiratory catalyst theory of Lwoff, admitting the possibility that the X-factor might be

dispensable anaerobically because the organism may then be using metabolic processes not involving the cytochrome respiratory system. Lwoff and Lwoff (1937 b) have suggested, without adducing any evidence on the point, that protohemin may be used for the formation of catalase and peroxidase by Hemophilus influenzae, as well as for the synthesis of cytochrome c and cytochrome oxidase.

Although the catalytic respiratory function of protohemin for Hemophilus influenzae has been demonstrated since Knight's review, this function was not shown to be necessary for growth, so that it is still not possible to express a definite opinion concerning the relative importance of the respiratory catalytic function and the catalase function of protohemin. Consequently, the function of protohemin as an essential growth factor remains to be determined.

For further details concerning the history of the accessory growth requirements of the "hemophilic" bacteria, the reviews of Scott (1929), Anderson (1931), Thompson and Thompson (1933), Knight (1936), and Lwoff (1937, 1938) may be consulted. Recent information concerning the nature and mode of action of respiratory mechanisms is reviewed by Stern (1937), Clark (1938), Stephenson (1939) and Barron (1939).

III METHODS AND MATERIALS

1. Stock and inoculating cultures

The strain of Hemophilus influenzae used for the present study was isolated from the human throat.* It is not hemolytic, and produces considerable quantities of indole from Difco Proteose Peptone. It has been maintained on laboratory culture media as described below for about two years. Frequent attempts made during this time to detect any variation in the basic cultural requirements of the organism showed that none has occurred.

Originally, the cultures were maintained on chocolate agar (10% rabbit blood added to meat extract agar and heated at 75° for 15 minutes) and the inocula for experimental purposes were prepared by suspending a small amount of growth from an 18 to 24 hour slant culture in 0.8% sodium chloride solution. It was found that inconsistent results were obtained by this method. Accordingly, a liquid medium containing 10^{-6} grams/ml. of protohemin and a 1/500 dilution of yeast extract (see Sections 4 and 5) was adopted both for maintenance of the stock culture and for use as the experimental inoculum. Abundant growth occurred in this medium, but transfer of 0.1 ml. of an 18 to 24 hour culture did not permit growth in the fresh medium unless both growth factors were present in suitable concentrations. Since this method results

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* It gives us great pleasure to express our thanks to Dr. Merlin Cooper of The Children's Hospital for the gift of this culture.

in the transfer of small amounts of growth factors with the inoculum, controls containing each of the growth factors alone accompanied all experiments.

Later, anaerobic cultures containing only the V-factor were used for maintenance and for inoculation. In this way the possibility of transferring even subminimal amounts of protohemin to the experimental cultures was eliminated, and it was also found that stock cultures remained viable for much longer periods in this condition.

It was shown that washing of the bacterial suspension was not necessary in order to prevent significant amounts of growth factor being transferred with the inoculum. This has been amply demonstrated by comparison with washed suspensions.

Throughout the work the standard inoculum consisted of 0.1 ml. of an 18 to 24 hour culture, which was either an aerobic culture containing both protohemin and yeast extract in the concentrations described above, or an anaerobic culture containing only yeast extract.

2. The basal medium

The basal nutrient medium used for maintenance of stock cultures and for experimental work consisted of 2% Difco Proteose Peptone and 0.6% sodium chloride in distilled water. This medium was adjusted to pH 7.3 to 7.4 with normal sodium hydroxide, bottled in 300 ml. quantities and autoclaved at 15 pounds for 15 minutes. The medium was not further

buffered except where noted. It contained neither V-factor nor X-factor in detectable amounts. Experimental cultures were always made up to a final volume of 5 ml. with this medium.

3. Examination of cultures for growth

All cultures were incubated at 37° and examined at intervals for the presence of turbidity. The degrees of turbidity that were produced under different conditions were found to differ considerably. In the tables this difference is indicated by the symbols, -, +, ++, and +++. It is recognized that these symbols are arbitrary and that observations made on different experiments are not strictly comparable, but the following approximate meanings may be attached to the symbols:

-, no turbidity detectable.

+, the slightest degree of turbidity that can be considered definite.

++, an intermediate degree.

+++, turbidity comparable to that produced by a 10^{-6} protohemin, 1/500 yeast extract culture after 24 hours.

Turbidity due to growth of the organism was usually distinguished with ease from that due to other causes. Crucial experiments were always checked by microscopic examination of smears stained by Gram's method.

Cultures were discarded after 72 hours incubation since it is very probable that the concentration of V-factor would have become subminimal by this time (see Section 5).

4. Preparation and titration of protohemin

Protohemin was prepared from defibrinated, filtered dog's blood by the method of Schalfeiev (see Gatterman and Wieland, 1932). For use, the dry material was dissolved in distilled water with the aid of a minimum of normal sodium hydroxide and was sterilized by autoclaving.

In order to determine the minimal quantity of protohemin required for growth, 0.5 ml. quantities of protohemin dilutions ranging from 10^{-4} to 10^{-11} grams/ml. and 0.5 ml. quantities of a 1/10 yeast extract dilution were added aseptically to sterile basal medium and inoculated with 0.1 ml. amounts of a saline suspension of Hemophilus influenzae from a 24 hour chocolate agar slant culture. These tubes were incubated 24 hours and the lowest concentration of protohemin showing turbidity was used to inoculate a second set of dilutions. The transfers were thus continued for five subcultures. While growth occasionally appeared in the 10^{-7} protohemin, it was consistently present and was more abundant in the tubes containing 10^{-6} grams/ml. The latter concentration of protohemin was consequently adopted as the minimal concentration for the present work. This concentration was insufficient to permit transfer of significant amounts of protohemin with the inoculum, since 0.1 ml. of this type

of culture did not initiate growth in fresh media containing no protohemin. Nevertheless, all experiments were controlled with protohemin-free tubes, and crucial experiments were always confirmed by series transfers or by the use of protohemin-free inocula.

It was later found that growth might be considerably delayed or entirely absent in 10^{-6} grams/ml. of protohemin when inoculations were made from anaerobic cultures containing only the V-factor. In this case, a concentration of 2 x 10^{-6} gave more satisfactory results.

5. Preparation and titration of yeast extract

Yeast extract was prepared according to the method described by Lwoff and Lwoff (1937 a), but with a single modification. During heating the hydrogen ion concentration of acidified yeast suspensions falls with some rapidity, so that it was found preferable to determine beforehand the amount of acid required to give the desired final reaction.

470 grams of fresh, pressed baker's yeast were emulsified in 260 ml. of distilled water and the reaction adjusted to pH 4.5 with concentrated hydrochloric acid. The suspension was then added slowly to 210 ml. of distilled water at 80°, the temperature being maintained above 75° during the addition by means of a water bath. At regular intervals during this time the calculated amount of acid was added. After addition was complete, the suspension was kept at 80° for 20 minutes. It was then centrifuged and

and the supernatant filtered by means of a Seitz filter. It was found that filtration proceded more rapidly if the supernatant was previously refrigerated over night. The reaction of the filtrate was approximately pH 4.5. It was tubed aseptically and stored in the refrigerator. No decrease in activity was detectable after a period of two years.

For use, the extract was diluted 1/10 with distilled water. Yeast extract was always added to the medium shortly before inoculation.

It was desired to work with minimal concentrations of the V-factor as well as with the X-factor, so that a titration of the yeast extract was attempted. The instability of the codehydrogenases in alkaline solution is well known and it was shown by Lwoff and Lwoff (1937 a) that the codehydrogenases are also specifically removed from the medium during the growth of the bacterium. It might be supposed, then, that not only the initial yeast extract content of the culture used for inoculation, but also the age of this culture would influence the results obtained in the fresh medium. In the present work it was found that this effect was registered not only by the presence or absence of growth, but also by the time required for its appearance. For this reason, as the following results show, accurate titration of the yeast extract would require rigorous standardization of several factors.

Fifteen tubes containing 10^{-5} grams/ml. of protohemin

were divided into three sets and to each set was added one of three dilutions of yeast extract. All tubes were inoculated with 0.1 ml. of a 24 hour 10^{-5} protohemin 1/500 yeast extract culture and were incubated. At intervals, tubes containing each dilution of yeast extract were centrifuged and the bacteria resuspended in an equal volume of 0.8% sodium chloride solution. 0.1 ml. of this suspension was inoculated into media containing 10^{-5} protohemin and various dilutions of yeast extract. The approximate time at which turbidity appeared is shown in Table I. It is seen that the age of the inoculum, as well as the initial concentration of yeast extract in both the inoculum and the experimental culture, conditions the time of appearance of turbidity, and that turbidity may appear after as long as 46 hours. It is probable that other factors, such as slight differences in the reaction of the medium and variation of the synthetic ability of the organism, may affect this delicate balance, since it was shown that with experimental conditions supposedly constant, the time of appearance of turbidity could vary within rather wide limits. It is not to be expected that titrations based on either the presence of growth after a certain period of time, or on the time of appearance of turbidity, will have any real value until the exact influence of these various factors has been determined.

Similar results were obtained with anaerobic cultures containing no protohemin.

The difficulties thus encountered in attempting to define a minimal concentration of yeast extract have led us to select an arbitrary dilution (1/500) which permits successful transfer from an 18 to 24 hour culture, providing a similar concentration is present in the inoculated medium, but which does not allow the transfer of sufficient V-factor to permit growth in media lacking this factor.

6. Preparation and Use of cozymase solution

In certain of the experiments, cozymase * was substituted for yeast extract. Since it was not possible to weigh out the very small amounts of purified cozymase required, a minute amount was dissolved in phosphate buffer at pH 6.0 and sterilized by Seitz filtration. This solution was found to support growth in very high dilution. In crucial experiments similar results were obtained with either cozymase or yeast extract, so that it may be concluded that yeast extract introduces no significant factor other than the codehydrogenases.

7. Preparation of solutions

Cysteine hydrochloride Pfanstiehl was dissolved in distilled water and autoclaved. Immediately after autoclaving sufficient normal sodium hydroxide was added to bring the reaction to pH 7.0 (as previously determined by titration with bromthymol blue) and to bring the concentration of cysteine to

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* It gives us great pleasure to express our thanks to Prof. K. Lohman and to Prof. P. Ohlmeyer for supplying us with a specimen of purified cozymase.

0.06 molar. Required amounts of this solution were used immediately, not more than 30 minutes being allowed to elapse between the autoclaving of the cysteine and the incubation of the cultures. A fresh solution was prepared for each experiment. It was shown, by comparison with solutions sterilized by Seitz filtration, that autoclaving in this way had no detectable effect on the cysteine. Cystine in similar concentrations and 0.1 M. thioglycollate were prepared in a like manner from crystalline cystine and technical thioglycollic acid. Autoclaving was shown to have no deleterious effect. 0.05 M. neutralized ascorbic acid and 0.1 M. 8-hydroxyquinoline were prepared in the same way from crystalline ascorbic acid and 8-hydroxyquinoline sulfonate, but were sterilized by Seitz filtration.

Sodium thiosulfate and sodium sulfide were dissolved in distilled water and autoclaved. Sufficient normal hydrochloric acid was added to bring the reaction to pH 7.0 (as previously determined by titration with thymol blue) and the concentration to 0.5 or 0.05 M.

Potassium ferricyanide and sodium diethyldithiocarbamate were dissolved in distilled water in concentrations of 0.05 M. and 0.1 M. respectively, and sterilized by filtration.

Analyzed samples of the dyes * were dissolved in distilled water in a concentration of 0.001 M. actual dye content, and were sterilized by autoclaving.

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* It gives us great pleasure to thank the National Aniline and Chemical Company, Inc. for a generous supply of dyes.

Potassium cyanide was dissolved in distilled water and the reaction adjusted to pH 7.6 with potassium dihydrogen phosphate. Hydrogen peroxide was prepared in the desired concentrations by addition of sterile distilled water to "30% hydrogen peroxide" known to contain 25% hydrogen peroxide. Fresh solutions of potassium cyanide and hydrogen peroxide were prepared for each experiment, and were not sterilized.

8. Methods of obtaining anaerobiosis

For the most part the pyrogallol-sodium hydroxide method was used on individual tubes. After inoculation, the cotton plug was pushed down each tube to within one or two centimeters of the surface of the medium, and a shorter cotton plug placed about a half centimeter above this. About 0.1 gram of pyrogallol and 2 ml. normal sodium hydroxide were added, and the tube closed with a rubber stopper. It was found that as long as 30 minutes might be required for nearly complete absorption of the gaseous oxygen in the tube, in so far as this was indicated by the complete reduction of methylene blue in a drop of alkaline glucose solution, and that one to two hours might be required for complete reduction of indicators dissolved in 5 ml. of medium.

In addition, evacuation together with the pyrogallol-sodium hydroxide has been employed for certain purposes, but this is described, together with the results obtained, in the section on anaerobic cultivation. (Experimental, Section 5).

9. Determination of hydrogen ion concentration

Hydrogen ion determinations were made with the Coleman and Coleman pH Electrometer employing the glass electrode. The results are reported to two decimal places on the pH scale, but it is recognized that the last figure is approximate only. Determinations were made on 5 ml. quantities of material. When anaerobic cultures were studied it was necessary to withdraw the medium with pipettes in order to avoid alteration of the reaction by the sodium hydroxide adhering to the mouth of the tube.

The reaction of all cultures showing growth inhibition was determined in order to eliminate the possibility that inhibition might be due to an alteration of the reaction.

10. Qualitative chemical tests

The presence of cysteine was determined by the nitroprusside reaction for the detection of the sulfhydryl group (-SH). To the solution to be tested was added one crystal of sodium nitroprusside, ammonium sulfate to saturation, and a few drops of concentrated ammonium hydroxide. The appearance of a reddish-purple color was taken as positive evidence of the presence of sulfhydryl sulfur in the cysteine-cystine system.

For the detection of catalase, 0.2 ml. of a 5% hydrogen peroxide solution was added to 0.2 ml. of the culture to be tested in a small serological tube. The test was not regarded as definitely positive unless the evolu-

tion of gas occurred at the rate of several bubbles per second or greater.

For the determination of the presence of hydrogen peroxide, the phenolphthalein test (Schales, 1938) was employed. This reaction depends on the oxidation of colorless reduced phenolphthalein (phenolphthalin) by hydrogen peroxide in the presence of the cupric ion. According to Schales, the dilute reagent gives a definite color within 30 seconds to 3 minutes in the presence of a dilution as high as 1/100 million of hydrogen peroxide (0.000,0003 molar) while a 1/200 million dilution (0.000,000,15 M.) gives a doubtful test. This has also been our experience using known dilutions of hydrogen peroxide in distilled water.

One drop of Schales' phenolphthalein reagent (Schales, 1938) diluted 1/4 with distilled water was added to 5 ml. of the solution to be tested. This was followed by one drop of 0.02 M. copper sulfate, which was found preferable to the 0.01 M. solution recommended. Development of a pink color within 30 seconds to 3 minutes was regarded as a positive test.

Certain precautions must be observed. The medium must be sufficiently alkaline to show the presence of phenolphthalein if it is formed. The peroxide reagent contains alkali, but when diluted this may not be sufficient to neutralize the buffer action of 2% peptone. Depending

upon the reaction of the medium, additional alkali may or may not be required. Excess alkali, however, will give a biuret test, especially if excess copper sulfate is added, and this is easily confused with a positive test for peroxide when both are faint.

Before peroxide tests were performed on media containing dyes, the dye was removed by absorption with kaolin, and it was sometimes necessary to repeat this procedure. It is obvious that this manipulation may have introduced an error by removing a portion of the hydrogen peroxide present.

11. Recrystallization of catalase

Three ml. of an ammonium sulfate suspension of Sumner's catalase crystals (Sumner and Dounce, 1937)* were centrifuged and the crystals washed five times with distilled water. The crystals were then shaken for 1 hour with 1 ml. distilled water plus a few drops of 0.3 M. phosphate buffer at pH 7.4, centrifuged, and the remaining crystals shaken 1 hour with the same amount of fresh water and buffer. This suspension was centrifuged and the two supernatants pooled. Acid potassium phosphate was added until the solution was orange to methyl red by the spot plate method, and saturated ammonium sulfate added to complete crystallization. The crystals were removed by centrifugation and dissolved as before. Crystallization was repeated twice again in a similar manner, and the crystals finally dissolved in 3 ml. 0.3 M. phosphate buffer at pH 7.4.

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* It gives us great pleasure to express thanks to Prof. James B. Sumner for the gift of a specimen of his crystalline catalase.

This solution was filtered through a small, sterile Seitz filter and the pad washed twice with 1 ml. quantities of distilled water. In this way, there was obtained 4 ml. of solution of such concentration as to correspond colorimetrically to about 10^{-5} grams/ml. protohemin solution.

IV EXPERIMENTAL

1. The effect of reversible oxidation-reduction systems on growth

It is generally accepted that protohemin is essential for the growth of Hemophilus influenzae. It has been suggested by Lwoff and Lwoff (1937 b) as a result of the observation that addition of protohemin (hemolyzed, defibrinated blood) to cultures containing a minimum of protohemin caused a proportional increase in oxygen consumption, that one function of this substance is to supply a respiratory catalyst necessary for the growth of the organism. It has also been suggested, however, that protohemin may supply another function, namely the formation of catalase in order to remove hydrogen peroxide which may be toxic for the organism (Knight, 1936). Since it is probable that protohemin serves of both these functions it is difficult to draw any conclusions concerning the relative importance of these two possibilities from the use of protohemin alone. It is possible, however, to select substances that are capable either of destroying hydrogen peroxide or of acting as respiratory catalysts, but which cannot supply both functions.

It has been shown by Barron and Hoffman (1930) that reversible oxidation-reduction systems of suitable potential can function as efficient respiratory catalysts. Dye systems may supply this function if their potential (E_o') is such that the dye will be reduced by the substrate and the reduced dye will be oxidized by oxygen. Thus, the dye may oxidize

the substrate either directly (Equation 1) or through the intervention of the codehydrogenases (Equations 2 and 3). The reduced form of the dye is oxidized by oxygen with the formation of hydrogen peroxide (Equation 4). If the essential function of

(1) substrate + dye $\underset{\text{dehydrogenase}}{\overset{\text{substrate}}{\rightleftharpoons}}$ oxidized substrate + reduced dye

(2) substrate + codehydrogenase $\underset{\text{dehydrogenase}}{\overset{\text{substrate}}{\rightleftharpoons}}$ oxidized substrate + reduced codehydrogenase

(3) reduced codehydrogenase + dye $\underset{\text{codehydrogenase}}{\overset{\text{diaphorase}}{\rightleftharpoons}}$ + reduced dye

(4) reduced dye + O_2 $\rightleftharpoons$ dye + H_2O_2

protohemin is to supply a respiratory catalytic system it should be possible to substitute such systems for protohemin.

Accordingly, tubes of the basal medium containing yeast extract with or without 10^{-4} or 10^{-6} grams/ml. of protohemin and dye systems of various potential were inoculated with 0.1 ml. of a Hemophilus influenzae culture containing a minimum of protohemin and incubated aerobically and anaerobically. The relative turbidity due to growth of the organism after 48 hours incubation is shown in Table II.

It is apparent from these results that under aerobic conditions none of the dye systems used was able to replace protohemin as the X-factor. It is also to be noted that those dye systems known to be most efficient in respiratory catalysis (E_o' at pH 7 and 30°C. = + 0.047 to -0.252 volts) actually inhibited aerobic growth in the presence of minimal amounts of protohemin. In the presence of greater amounts (10^{-4} grams/ml.) or under anaerobic conditions these dyes did not inhibit growth (with the exception of nile blue: see below). Thus, a system providing only the respiratory catalytic function was shown to prevent rather than to promote growth of Hemophilus influenzae.

The results given in Table II are similar to those obtained by Broh-Kahn and Mirsky (1938) with cyanide treated Escherichia coli. Cyanide, which inhibits the activity of catalase and of the cytochrome respiratory system (cytochrome oxidase) had little apparent effect on the growth of Escherichia coli in aerobic culture. In the presence of both cyanide and an added respiratory catalyst (dye systems of suitable potential), however, growth was markedly inhibited. This inhibition was traced to the effect of hydrogen peroxide formed under the influence of the added respiratory catalyst. Growth occurred in the presence of cyanide alone since Escherichia coli possesses no cyanide insensitive respiratory mechanism and hydrogen peroxide could not be formed. This type of growth, which occurred in the presence of air but without oxygen consumption, was termed anoxytrophic. It appears probable that

Hemophilus influenzae possesses a respiratory mechanism in addition to the cytochrome system, since in the presence of oxygen anoxytrophic growth of protohemin-free cultures does not occur.

The potential range of the dyes found inhibitory for Hemophilus influenzae is similar to that inhibiting growth of cyanide treated Escherichia coli. Dyes of the highest potential did not inhibit probably because these were less efficient respiratory catalysts. While these dyes are readily reduced by the substrate, reoxidation by oxygen is slow and little hydrogen peroxide is formed. Dyes of the lowest potential are also less efficient since, while oxidation by oxygen must be rapid, they are not readily reduced by the substrate.

The results of numerous experiments with nile blue have been variable. This dye may inhibit growth even under anaerobic conditions in the absence of protohemin, and later (Table V) it will be shown that cysteine does not always protect the organism against the inhibitory activity of this dye in aerobic culture. It is possible either that nile blue is the most efficient respiratory catalyst for these experimental conditions of any that were tested, or that it possesses toxic properties not related to its catalytic respiratory function.

2. The effect of added hydrogen peroxide on growth

It is well known that hydrogen-peroxide is formed as a normal product of oxygen consumption and that many forms of life are sensitive to this substance. It appeared most probable from the results given above that inhibition of growth under the influence of dye systems was due to an increased accumulation of hydrogen peroxide. In order to test this possibility, the sensitivity of Hemophilus influenzae to peroxide was investigated.

Dilutions of "30%" hydrogen peroxide were prepared aseptically and added to sterile basal medium containing yeast extract and 10^{-6} grams/ml. of protohemin. These were inoculated with 0.1 ml. of a culture containing yeast extract and a minimum of protohemin. The final theoretical concentrationsof hydrogen peroxide ranged from 0.3 molar to 0.000,03 M. Growth occurred within 24 hours in 0.000,03 M.hydrogen peroxide and within 48 hours in 0.000,15 M. but not at all in concentrations of 0.0003 M. or greater.

It appeared probable that under these conditions the organism might be expected to form, after a period of incubation, sufficient catalase to destroy the hydrogen peroxide added, or that it might produce additional hydrogen peroxide by its respiratory activity, and that a more exact knowledge of the limiting dilution could be obtained by adding dilutions of hydrogen peroxide to the basal medium containing only yeast extract, inoculating this with a hemin-free strain of the

organism, and incubating anaerobically. It was expected that a markedly lower concentration of hydrogen peroxide would be found inhibitory in this case, since in the absence of protohemin the formation of catalase and the consequent decomposition of hydrogen peroxide would be impossible. The difference was found not to be marked, however. Growth occurred in the presence of 0.000,03 M. of added peroxide and not in the presence of 0.00015 M. The addition of 10^{-6} protohemin permitted growth in the latter dilution after 24 hours. The results of both experiments are summarized in Table III. It may be shown, however, that these figures, also, do not represent the lowest limiting concentration of hydrogen peroxide for growth inhibition, because a considerable proportion of added hydrogen peroxide is reduced immediately upon addition to the basal medium.

A rough estimation of the limiting growth inhibiting concentration of hydrogen peroxide may be obtained in the following manner. Schales' (1938) qualitative test for hydrogen peroxide is the most sensitive at the present time, and gives a definite reaction with 0.000,0003 M. hydrogen peroxide in distilled water. It was demonstrated that when the basal medium is used as a menstruum, a concentration of 0.003 M. must be added to give a definite test. This indicates an immediate reduction of at least 0.9999 of the added peroxide. Since it was noted that the anaerobic growth of the organism in the absence of protohemin was inhibited by

0.000,15 M. of added hydrogen peroxide, an actual concentration of 0.000,000,015 M. or less is probably growth inhibiting. Inasmuch as Schales' reagent is insensitive to 0.000,000,15 M., it is probable that the accumulation of hydrogen peroxide in cultures must reach at least ten times the inhibiting concentration before it is detectable. These amounts are so much smaller than those previously recognized as growth inhibiting as to appear incredible, but it must be remembered that previous observations may have neglected to take into account the reducing activity of the medium and have considered the added peroxide representative of the actual peroxide concentration. It must be emphasized that the figures here reported cannot be considered very accurate since the experimental errors are probably large and since certain assumptions that were made may not be valid.

Since the difference between limiting dilutions of added peroxide for growth inhibition as obtained anaerobically with and without protohemin is only five-fold (Table III), it is questionable whether it can be considered significant. If the difference is significant, it would seem to indicate that catalase formation is at first inhibited by peroxide but, perhaps as a result of reduction of part of the hydrogen peroxide by the medium, is later resumed and ultimately results in the decomposition of all the added hydrogen peroxide and growth of the culture.

It is interesting to note that concentrations of added hydrogen peroxide as low as 0.000,15 M. were shown by Broh-Kahn and Mirsky (1938) to inhibit the growth of cyanide treated Escherichia coli. This was the lowest concentration tested, and reduction of the peroxide concentration by the medium was not taken into account; it is possible that the limiting dilution may be similar for both cyanide treated Escherichia coli and anaerobic, hemin-free Hemophilus influenzae, since evidence to be presented later (see Discussion) will show that in these situations both organisms are lacking catalase and a functioning oxygen consuming mechanism.

The cause of growth inhibition by hydrogen peroxide is not known. It may be shown that in the present case inhibition is not due to the high potential of the medium caused by the presence of peroxide, since growth was obtained both aerobically and anaerobically in the presence of 0.001 M. and 0.0001 M. potassium ferricyanide which has a higher potential (E_o' at pH 7.0 = + 0.43 volts) than hydrogen peroxide (E_o' = + 0.25 volts). Although the sensitivity to peroxide differs considerably among the various bacterial species, the phenomenon of peroxide inhibition is of rather wide occurrence so that it is probably concerned with mechanisms essential to living tissue in general.

3. The accumulation of hydrogen peroxide

Although Hemophilus influenzae has now been shown to

be extremely sensitive to hydrogen peroxide, it is necessary to demonstrate that hydrogen peroxide is produced under the conditions in which growth inhibition occurs before a causal relationship can be considered established. In addition, it would be desirable to demonstrate the absence of hydrogen peroxide under all conditions in which growth is not inhibited. However, it is apparent from the results given above that chemical detection of inhibiting concentrations may not be possible, and a perfect correlation between chemical detection and growth inhibition cannot be expected a priori.

Cultures containing yeast extract, or yeast extract plus protohemin and dye systems of the upper, lower and middle oxidation-reduction potential range previously used were inoculated with 0.1 ml. quantities of a yeast extract-protohemin culture and tested with Schales' diluted reagent after 24 hours incubation. The results of the tests and the occurrence or absence of growth are indicated in Table IV.

It is seen that the presence of dye systems intermediate in the oxidation-reduction potential range caused the formation of detectable amounts of hydrogen peroxide under aerobic conditions but not anaerobically. This is correlated perfectly with growth inhibition with the exception of the anaerobic hemin-free culture containing nile blue, which may have some unrelated toxic activity, and the aerobic hemin-free culture to which no dye was added. The

latter is susceptible of explanation. It may be expected that in the absence of a supplementary dye system less peroxide would be produced and that this might reach an inhibiting concentration but not be sufficient for chemical detection. That all the mechanisms required for the formation of hydrogen peroxide are present is indicated by another experiment using a heavy inoculum and testing for hydrogen peroxide after 30 minutes (Section 9 and Table IX). In this case a strong test for peroxide is obtained. It must also be pointed out that the method of testing for peroxide after 24 hours incubation is open to criticism. Hydrogen peroxide may be formed early in the incubation period and so damage the bacteria that when the peroxide concentration is later lowered below the inhibiting level as a result of partial reduction by the medium the bacteria may be incapable of multiplying or of forming catalase. In this case a test for hydrogen peroxide would be negative in the absence of growth.

In order to explain the observed production of hydrogen peroxide in the absence of protohemin it is necessary to postulate the existence of a non-hemin respiratory system. Hydrogen peroxide is very unlikely to be formed under conditions that allow the existence of living forms, except as a product of oxygen consumption (see Broh-Kahn and Mirsky, 1938), and hemin-free Hemophilus influenzae is presumably unable to form a cytochrome system in the absence of protohemin. It is most likely that this additional respiratory

system is of the flavoprotein type since this is very common among bacteria. This question will be treated in more detail in the Discussion.

Since yeast cells contain flavoproteins, the possibility suggests itself that the additional respiratory system may have been supplied preformed in the yeast extract, which was markedly fluorescent. Dye inhibition was demonstrated, however, when yeast extract was replaced with purified cozymase, so that it is necessary to conclude that Hemophilus influenzae normally synthesizes the additional respiratory system from the constituents of the basal medium. The flavoprotein present in the yeast extract is probably without effect since the large molecule cannot be taken into the cell.

Table IV shows that neither growth nor production of detectable amounts of hydrogen peroxide occurs in the absence of the V-factor. It is to be expected that no peroxide is formed since the codehydrogenases are probably essential parts of the respiratory system (Lwoff and Lwoff, 1937 a). There is some evidence that this factor is essential for growth aside from its role in dehydrogenations because lactic and succinic dehydrogenations appear to be effected by Hemophilus para-influenzae in the absence of the V-factor, but growth does not occur without V-factor even when lactate and succinate are present in aerobic (Lwoff and Lwoff 1937 a) or anaerobic conditions, so that

growth may not be expected in its absence quite apart from the question of inhibition by peroxide.

4. The production of catalase

It has now been established that growth inhibition of Hemophilus influenzae in the absence of the X-factor is causally related to the production of hydrogen peroxide by the organism. It remains to show what means are possessed by the organism for the removal of hydrogen peroxide, or for rendering it innocuous. That catalase is produced for this purpose has already been suggested, but there appear to be no reports in the literature of the demonstration of catalase production by this bacterium. The presence of catalase in appreciable quantities is readily determined by the addition of hydrogen peroxide to the culture, since the decomposition of hydrogen peroxide under the influence of catalase is highly specific and is easily recognized by the formation of bubbles of oxygen. It must be recognized, however, that under the usual cultural conditions only small amounts of catalase would be required for the decomposition of the minute amounts of peroxide produced, so that unless the organism produces catalase far in excess of its needs, this enzyme might not be detectable. It must also be noted that where minimal amounts of hemin are used, catalase production must also be minimal.

It was shown b y the addition of hydrogen peroxide to cultures of Hemophilus influenzae that catalase is pro-

duced. An equal amount of 5% hydrogen peroxide was added to portions of cultures and the occurrence of bubbling noted. The test was always negative in the case of anaerobic cultures containing no protohemin. In the presence of minimal amounts of protohemin (10^{-6} grams/ml.) the test was very weakly positive and was sometimes negative, even after the appearance of turbidity. In cultures containing an excess of protohemin (10^{-4} grams/ml.) a moderate degree of foaming always occurred, and a positive test was frequently observed before turbidity was apparent. Although the test cannot be considered accurate in a quantitative sense, the rapidity of foaming appeared to decline in older cultures (30 to 60 hours incubation).

The observation that greater amounts of catalase are formed in the presence of 10^{-4} protohemin than in cultures containing only 10^{-6} is quite in agreement with the results of the experiments on the inhibition of growth by dyes. It was noted there (Section 1 and Table II) that the greater concentration of protohemin protected the organism against the inhibitory activity of the dyes. It appears most probable that in the presence of 10^{-4} protohemin sufficient catalase is formed or is formed with sufficient rapidity, to decompose the additional hydrogen peroxide resulting from the supplementary oxygen consumption promoted by the dye systems. Later, (see Discussion) evidence will be presented to show that the protective effect of excess protohemin is due to

an increased rate of formation as well as to the formation of larger amounts of catalase. The rate of formation of catalase may be especially significant when autoclaved broth is used because, as it has been shown, the hydrogen peroxide formed early in the period of incubation must be reduced by the basal medium and some time might be expected to elapse before a growth inhibiting level of peroxide is attained.

5. Anaerobic cultivation

Supplementary evidence indicating the importance of catalase for the growth of Hemophilus influenzae is presented by the results of anaerobic cultivation. In the absence of oxygen respiration does not occur, hydrogen peroxide is not likely to be formed, and catalase is thus not required. If the formation of catalase is the sole essential function of protohemin, the X-factor should be dispensable in anaerobic culture. The dispensability of the X-factor in anaerobic culture has been reported (Kopp, 1927) and confirmed (Eirund, 1929; Anderson, 1930, 1931) but it was not claimed that all strains studied were able to grow anaerobically. Other investigators (Pfeiffer, 1893; Fildes, 1921) have preferred to regard the organism as an obligate aerobe.

In the present work a single strain was studied but the results of anaerobic cultivation have so varied under different conditions as to suggest that part at least of the differences between strains reported by earlier workers was

due to the technical methods employed rather than to actual differences in the bacteria. It is not claimed, however, that strains of Hemophilus influenzae which are obligate aerobes do not exist.

At the time of writing the strain of Hemophilus influenzae used in the present work has been maintained anaerobically without protohemin for more than 90 transfers. In this form it is used as a stock inoculating culture. The pyrogallol-sodium hydroxide method is used to secure anaerobiosis and the medium is the basal medium containing yeast extract. This strain has also been carried anaerobically for 5 transfers on the basal medium plus purified cozymase. Transferred to aerobic culture growth does not occur unless protohemin (10^{-6} or, preferably, 2×10^{-6} grams/ml.) is present. Thus there appears to be no question that this strain is capable of anaerobic growth and that catalase is not essential in this condition.

It must be recognized that the anaerobic methods used in the present work and by most other observers do not produce an immediate condition of strict anaerobiosis. With the pyrogallol-sodium hydroxide method a significant period of time is required for depletion of the oxygen in the atmosphere of the culture tube. With certain other methods this depletion may be effected almost immediately, but in either case the removal of dissolved oxygen is relatively slow and traces may remain in the medium for an indefinite period after inoculation. Consequently, it is not possible to state that

growth has occurred in a condition of complete anaerobiosis, and it is possible that a minimum consumption of oxygen is necessary for growth. This is a purely theoretical possibility and has never received any experimental support. In connection with the present problem it is important to note that oxygen consumption through flavoprotein systems has been shown to be proportional to the partial tension of oxygen so that under "anaerobic" conditions respiration must be slight and insignificant amounts of hydrogen peroxide formed. Furthermore, while the anaerobic method employed in the present work may not secure strict anaerobiosis, the condition is definitely different from free access to air since only the anaerobic condition permits growth of the organism in the absence of protohemin.

The occurrence of growth in the absence of protohemin under anaerobic conditions is a strong indication that the respiratory catalytic function of protohemin is not an essential part of the growth mechanism, nor could the respiratory function be expected to play even a supplementary role in anaerobic culture. It was frequently observed that the presence of protohemin in anaerobic cultures had no detectable effect on the rate of growth or on the degrees of turbidity finally attained.

In the presence of oxygen, however, hemin might be expected to supply the respiratory catalytic function, and this supplementary function should provide additional energy

for the cell. It is well known that the free energy of reactions that may be used to obtain energy for cell synthesis under anaerobic conditions is considerably less than that obtainable when oxygen is the hydrogen acceptor. In addition, since anaerobic oxidoreductions depend upon the reactions of two substances in the medium, organisms growing anaerobically frequently require more complicated media than those growing aerobically. Thus, it is possible that some of the energy sources utilized by Hemophilus influenzae growing aerobically in peptone solution are not available under anaerobic conditions. It might be predicted, therefore, that the rate of growth and the final amount of cell material obtained would be less when oxygen consumption is prevented. Such has been found to be the case, turbidity appearing later and not reaching as great a final degree in anaerobic culture. Using the standard inoculum of 0.1 ml. and the usual amounts of protohemin and yeast extract, numerous observations have shown that aerobic protohemin cultures develop a visible turbidity in as short a time as 5 hours, whereas anaerobic cultures usually require 8 hours or more. The degree of turbidity finally attained is definitely superior in aerobic cultures. These observations indicate that the respiratory function of protohemin, while supplementary, is of some importance in aerobic culture.

In aerobic culture, pH 7.4 to 7.6 has usually been considered optimum for the growth of Hemophilus influenzae. It may be shown that under anaerobic conditions the reaction of the medium has little effect over a rather wide range.

Quantities of the basal medium were adjusted to various reactions in the pH range 6.5 to 7.5 by the addition of molar dihydrogen phosphate/disodium hydrogen phosphate buffer solutions to a final buffer concentration of M/15. Media adjusted to each hydrogen ion concentration were distributed in 5 ml. quantities in eight tubes, half of which were autoclaved. Yeast extract and 0.1 ml. of an anaerobic yeast extract culture was added to each, anaerobiosis was secured by the use of the pyrogallol-sodium hydroxide method, and the tubes were incubated. At intervals, tubes were removed, the degree of turbidity noted, and the reaction of the medium determined. The results are presented in Table V.

It is seen that growth occurs in the autoclaved broth at a hydrogen ion concentration as low as pH 7.4 and as high as pH 6.5. In broth that was not autoclaved, growth did not appear at pH 7.4. It is also to be noted that the hydrogen ion concentration increases whether turbidity finally appears or not. Kristensen (1922) observed that Hemophilus influenzae is able to produce acid on protein media in the absence of added carbohydrate. It is probable that some of the reactions catalyzed by the organism proceed whether growth occurs or not. That pH 7.4 is near the lower limiting hydrogen ion concentration is indicated by another experiment in which growth failed to occur at pH 7.6.

It has been found necessary to autoclave the basal medium before using in order to insure the occurrence of

growth in anaerobic culture without protoheмin. The results obtained in this connection have been inconsistent. Usually growth has failed if the medium was not autoclaved, but occasionally growth has occurred in broth that was not known to have been recently autoclaved. In this case it was usually delayed, appearing after 24 hours as compared to the usual appearance of turbidity in 8 to 12 hours (see, for example, Table IV). It is possible that the observed inconsistencies in this respect which are on our records are due to the fact that bottles of basal medium were frequently re-autoclaved after part of the contents had been used, and a history of each bottle not recorded. Thus, broth that was regarded as not autoclaved may have been autoclaved on the previous day.

Attempts to determine the factor responsible for growth inhibition in broth that was not autoclaved have been hampered by the inconsistency of its occurrence. Two of the more probable explanations may be suggested. In the first place, it is known that autoclaving is a very efficient means of depleting water solutions of gases, and it was thought that the oxygen dissolved in unautoclaved basal medium might be consumed by the inoculated bacteria and an inhibiting concentration of hydrogen peroxide produced before anaerobic conditions were attained in the culture. Since solution of oxygen in water attains equilibrium rather slowly, less oxygen might be contained in media inoculated and subjected to pyrogallol as soon as possible after autoclaving (approximately 5

minutes), so that hydrogen peroxide might not reach an inhibiting concentration in this case. This explanation was made improbable by the observation that growth occurred to the same extent in autoclaved media that were aerated with bubbling compressed air for 20 minutes and for 2 hours before inoculation and subjection to anaerobic conditions as in the same autoclaved media inoculated immediately and without aeration.

An alternative explanation appears more probable. It is well known that hydrogen peroxide is formed in bacteriological culture media under the influence of irradiation with light, especially of the shorter wave lengths (ultraviolet). The basal medium used in the present work was stored on open shelves exposed to diffuse daylight, and it is possible that hydrogen peroxide accumulated under these conditions. Hydrogen peroxide could not be detected by the use of Schales' dilute reagent, but it has already been shown that this test may not permit the detection of a growth inhibiting concentration. Thus, hydrogen peroxide might be present in the unautoclaved basal medium. It is volatile and may be completely removed by autoclaving. Under the conditions of storage it is not to be expected that peroxide would accumulate at all rapidly, so that broth autoclaved one or a few days before use might permit growth without additional autoclaving, and this may account for the discrepancies that have been recorded. Growth has always appeared early when substances known to reduce hy-

drogen peroxide were added to anaerobic culture. This phase of the problem is still under investigation, but it appears probable that similar phenomena may account for some of the conflicting reports on anaerobic cultivation of Hemophilus influenzae in the absence of the X-factor that may be found in the literature.

Preliminary autoclaving of the basal medium does not permit aerobic growth of the organism in the absence of protohemin, because hydrogen peroxide is formed as a result of oxygen consumption early in the incubation period.

A few experiments indicate that a minimum concentration of carbon dioxide is required for growth. An apparatus was devised with which dissolved gases could be removed from the inoculated basal medium containing yeast extract and the culture maintained under anaerobic conditions. The culture tube was attached to a rotary suction pump through a second tube plugged with cotton soaked with pyrogallol-sodium hydroxide solution. The culture was exposed to low pressure for 15 minutes with continuous shaking, after which the vacuum was either maintained during incubation or else was released by very cautious admission of air through the pyrogallol-sodium hydroxide soaked cotton. All connections were well greased and it was shown by manometric measurement that leakage during the 24 hour incubation period was negligible. Under these conditions growth did not occur. The organism was able to grow if the preliminary period of evacuation with shaking was less prolonged (5 minutes) or if the pyrogallol-sodium hydroxide system

which absorbs carbon dioxide, was replaced by Rockwell's solution (1924) and pyrogallol, which does not absorb carbon dioxide. It appears probable from these results that a minimum concentration of carbon dioxide is required for growth and that this gas is removed by prolonged evacuation with shaking. If this is so, it is certain that the minimum concentration required is very low, since the ordinary pyrogallol-sodium hydroxide method of anaerobiosis must absorb some carbon dioxide even without preliminary evacuation. If a minimum carbon dioxide requirement does actually exist for Hemophilus influenzae, those anaerobic methods previously used (Pfeiffer, 1893; Fildes, 1921), which depend upon the replacement of air by an inert gas such as hydrogen or nitrogen, are subject to criticism.

6. The substitution of cysteine for protohemin

It was shown that substances, such as dye systems, that are capable of supplying only the respiratory catalytic function are unable to replace protohemin for the growth of Hemophilus influenzae. It is now proposed to study the effect of substances that reduce hydrogen peroxide but which probably do not act as respiratory catalysts.

Cysteine reduces hydrogen peroxide with the formation of cystine and water (Equation 4). Quastel and Stephenson (1926) have shown that cysteine protects cells from the

$$(4_a)\quad 2\ CySH + H_2O_2 \rightleftharpoons CyS\text{-}SCy + 2\ H_2O$$

inhibitory activity of added hydrogen peroxide and Broh-Kahn (unpublished) has observed that the inhibitory action of the

hydrogen peroxide produced by cyanide treated Escherichia coli in the presence of dyes is neutralized by cysteine. In the latter case, the peroxide is produced as a result of cellular activity and might be expected to continue to be formed as long as oxygen consumption continues. If the catalase function is the only essential function of protohemin in the case of Hemophilus influenzae, it should be possible to replace protohemin by cysteine or other substances reducing hydrogen peroxide.

Tubes of the basal medium containing yeast extract and concentrations of cysteine ranging from 0.\006 M. to 0.0006 M. were inoculated with 0.1 ml. of a culture containing yeast extract and a minimum of protohemin. Turbidity appeared in the presence of 0.006 M. cysteine but not in 0.003 M. or less, nor in the absence of cysteine. This observation has been repeatedly confirmed using a series of five transfers without protohemin, and also using hemin-free inocula. It was also shown that yeast extract may be replaced by purified cozymase in such experiments.

It was noted, however, that growth in cysteine cultures was not as abundant as in cultures containing protohemin. Although turbidity appeared early (8 hours or less), the final degree of turbidity attained was comparable to that found in anaerobic cultures. It was also observed that viable bacteria did not persist for more than 18 or 24 hours in cultures containing, cysteine alone, as shown by loop

transfers to chocolate agar. Cysteine is readily oxidized under these experimental conditions and it could be demonstrated by means of the nitroprusside reaction that the disappearance of viable bacteria was preceded by the disappearance of cysteine from the culture. Further information on this point was shown by a bacterial dilution plate count made on cysteine and protohemin cultures. One hundred ml. of basal medium containing yeast extract and 0.006 M. cysteine or 10^{-6} grams/ml. of protohemin were inoculated with 2 ml. of an 18 hour anaerobic yeast extract culture and incubated. At intervals 1 ml. samples were withdrawn and dilutions plated on the basal medium containing 1/100 yeast extract, 10^{-4} protohemin, and 1.5% agar-agar. Plates were counted after 48 hours incubation. The results are illustrated in Figure 1.

It may be seen from Figure 1 that the bacterial count of the cysteine culture increases logarithmically during about the first nine hours, after which there is a logarithmic decrease. The maximum count approximates the disappearance of cysteine as measured by the nitroprusside reaction. The absence of any prolonged stationary growth phase and the occurrence of a logarithmic degrease in numbers in place of the usual growth phase of slow decline is highly significant when considered in relation to the disappearance of cysteine.

In the hemin culture the smooth growth curve does not appear until later (10 to 14 hours) and the interim is

characterized by rather wide fluctuations in the count. It is not known whether the fluctuations observed are significant or not, since the dilution plate count of Hemophilus influenzae on this medium has not been used extensively enough to allow evaluation of the experimental error. In cultures containing both cysteine and protohemin, growth appears early and the viable bacteria persist.

Thus, although the maximum turbidity attained in cysteine cultures is comparable to that of anaerobic cultures, it is probable that the cause differs in the two cases. While the low degree of turbidity attained in anaerobic cultures may be related to the available energy, in cysteine cultures it appears to be due to a cessation of growth when the protective action of cysteine disappears and allows the accumulation of inhibitory concentrations of hydrogen peroxide. Anaerobic cultures remain viable for much longer periods than do cysteine cultures.

It is also possible to demonstrate that cysteine protects the organism against the inhibitory effects of added dye systems. This is shown by Table VI. It is seen that cysteine affords protection in the case of all dyes except nile blue. In this it is comparable to an excess (10^{-4} grams/ml.) of protohemin (Table II). The results obtained in the cultures containing both cysteine and protohemin are surprising. When dye systems of the intermediate potential are used, cysteine appears to exert an inhibitory

effect on growth even in the presence of protohemin. After 12 hours incubation, all tubes containing protohemin plus cysteine showed the same turbidity as the corresponding tubes containing cysteine alone, so that undoubtedly cysteine is capable of protecting the organism in the presence of all dyes except nile blue until there has been time for considerable growth and, presumably, for catalase formation if protohemin is present. Catalase is extremely efficient in the removal of hydrogen peroxide, so that if catalase is formed growth should continue and maximum turbidity might be expected in the presence of cysteine plus protohemin with all dyes that permit any growth at all. For this reason, the partial inhibition that is observed is probably not due to direct inhibition of growth by hydrogen peroxide. It may be noted, however, that the degree of turbidity observed after 36 hours incubation in cysteine plus protohemin increases as the potential of the dye system present recedes in either direction from that of nile blue. It is probable, then, that the partial inhibition observed in these tubes is related to the rate of oxygen consumption since this is plausibly the only factor that varies in precisely this manner. Furthermore, turbidity is greater in the presence of protohemin alone in the case of naphthol sulfonate indophenol and phenosafranine than in the presence of both protohemin and cysteine. The observations make it seem very probable that the partial inhibition observed when cysteine is present with protohemin is due to the result of increased respiration on the cysteine.

This may tentatively be referred to the formation of inhibitory oxidation products of cysteine.

Although it is questionable whether cysteine is able to act as a respiratory catalytic system as in Equations (5) and (6), the objection may be raised that it performs this function in relation to the growth of Hemophilus influenzae. This mechanism involves the reduction of cystine

(5) $2\ CySH + 1/2\ O_2 \rightleftharpoons CyS\text{-}SCy + H_2O$

(6) $\text{substrate} + CyS\text{-}SCy \rightleftharpoons \text{oxidized substrate} + 2\ CySH$

under the influence of the organism and the oxidation of cysteine by oxygen. If Hemophilus influenzae is able to cause the reduction of cystine and if growth depends only on respiration through this system, it should be possible to replace protohemin with either cystine or cysteine. Numerous attempts to substitute cystine in concentrations comparable to those found suitable with cysteine have been made without success. Obviously, this explanation will not account for the effect of cysteine on growth. In addition, since cystine does not protect against the inhibitory action of dyes, it is apparent that Hemophilus influenzae is incapable of reducing this compound to cysteine which does exert a protective action.

It may also be objected that in aerobic culture growth of Hemophilus influenzae is dependent upon an increased oxygen consumption resulting from the oxidation of cysteine to cystine. At the present time no evidence is

available to determine whether oxygen consumption is increased in the presence of cysteine. However, the total respiratory capacity of 0.006 M. cysteine (0.1% cysteine hydrochloride) may be calculated. From Equation (5),

$$\frac{\text{11200 ml. (volume of 1/2 mol } O_2)}{\text{315 grams (2 mols CySH·HCl)}} \times 0.001 \times 1000 =$$

35.6 cu. mm. O_2 reducible by 1 ml. 0.006 M. cysteine.

This total quantity of oxygen consumed is of the same order as that consumed by many bacteria in a few minutes, so that it is improbable that the oxidation of cysteine will provide the organism with a very large supply of energy. It has already been shown that this system cannot effect the oxidation of additional substrates since the cystine cannot be reduced by the organism.

The inability of cystine to substitute for cysteine would appear to eliminate several possible functions of cysteine in the growth of Hemophilus influenzae. Since cystine is not reducible by the organism cysteine may not serve a respiratory catalytic function nor as a significant oxidizable source of energy, and for the same reason the cysteine-cystine system may not fulfill a "peroxidase" function suggested by Equations (4a) and (6). Since cystine may not be substituted, it is improbable that the amino acid structure or any part of it is required for synthesis of cellular material. Almost the only function that may be

attributed to cysteine alone of the two compounds is its reducing activity. These results lend very strong supporting evidence in favor of the view that formation of catalase is the only essential function of protohemin for the growth of Hemophilus influenzae.

7. The substitution of thioglycollate for protohemin

Thioglycollic acid also reduces hydrogen peroxide so an attempt was made to replace protohemin with this compound. Since thioglycollate is considerably more stable under the experimental conditions it was hoped that it might offer several advantages over cysteine, and might even be of some practical use in the cultivation of bacteria requiring the X-factor.

Various concentrations of neutralized thioglycollic acid were added to the basal medium containing yeast extract with or without protohemin and were inoculated with an anaerobic yeast extract culture. Several such experiments have been performed and the results were either negative or else of the sort shown in Table VII. Concentrations of thioglycollate of 0.01 M. or greater inhibit growth even in the presence of protohemin or in the absence of oxygen, so that they could not be expected to support aerobic growth in the absence of protohemin. This inhibition was shown not to be due to any alteration of the reaction of the medium. Concentrations of 0.004 M. or less were not inhibitory if other means were taken to prevent the accumulation of peroxide, but

thioglycollate alone in these concentrations did not permit aerobic growth. It was noticed several times, however, that slight growth occurred aerobically in the absence of protohemin if approximately 0.005 M. thioglycollate was added to the medium. It may be seen that the range of concentrations permitting growth was not wide. This concentration is lower than that used successfully with cysteine, but data are not available for comparison in all concentrations (see Table VIII). Thioglycollate appears to exert an inhibitory action at a concentration of 0.006 M., which in the case of cysteine permits moderate growth, but it is possible that at 0.005 M. the effect of cysteine would compare with that of thioglycollate.

It was not considered necessary to confirm these experiments by means of series transfers, since anaerobic cultures carried many transfers in the absence of protohemin were used for inoculation.

Since thioglycollate inhibits growth of Hemophilus influenzae under conditions which do not permit the formation of hydrogen peroxide, it is obvious that the absence of growth in this case cannot be referred to an inability to protect against peroxide. Unless thioglycollate is toxic per se, which appears unlikely because of the action of the closely related cysteine, it is probable that inhibiting substances are either present in the technical grade of thioglycollic acid used, or are formed from it during incubation of the culture. There remains the necessity of explaining

why thioglycollate, which is much the more stable under the experimental conditions, is not effective in significantly lower concentrations than is cysteine. It is possible that this phenomenon is related to the rate of diffusion of these substances into the cell. This question is given further treatment in the Discussion.

Protection against the inhibitory effect of dye systems could not be demonstrated in the case of thioglycollate, probably because the proper concentrations of thioglycollate were not used. However, thioglycollate does cause an acceleration of growth in aerobic cultures containing protohemin. Under conditions in which turbidity appeared in 2×10^{-5} protohemin only after 22 hours, cultures containing the same amount of protohemin plus 0.005 M. thioglycollate were definitely turbid in 6 hours. This growth-accelerating effect of thioglycollate was frequently observed.

The possibility that thioglycollate permits growth by virtue of another capacity than its reducing activity may be met by arguments similar to those given in the case of cysteine.

8. The effect of cysteine and thioglycollate on growth inhibition by added hydrogen peroxide

In order to show that the growth promoting effects of cysteine and thioglycollate are indeed due to the ability of these compounds to reduce hydrogen peroxide, it would be desirable to demonstrate that cysteine and thioglycollate protect *Hemophilus influenzae* against the inhibitory action of added hydrogen peroxide.

Dilutions of "30%" hydrogen peroxide were prepared aseptically and added to sterile basal medium containing yeast extract and 0.006 M. cysteine or 0.002 M. thioglycollate. These were inoculated with 0.1 ml. of a hemin-free anaerobic culture and incubated aerobically. The appearance of the tubes after 24 hours is given in Table IX. The appearance of the tubes remained the same after 48 hours incubation, with the exception that turbidity was observed in the tube containing 0.00015 M. hydrogen peroxide plus hemin alone. It is apparent that both cysteine and thioglycollate exert a marked protective action against added peroxide, permitting growth in about ten times the concentration of peroxide in which it appears with protohemin alone. Aside from this observation it is difficult to assess the significance of these results. At first glance it would appear that 0.002 M. thioglycollate permits growth in about an equivalent concentration of hydrogen peroxide (0.0015 M.) and that cysteine need be present in about three times this concentration in order to give the same effect. The excess cysteine required may be related to the ease with which it is oxidized by oxygen. However, it was shown in Section 2 that at least 0.9999 of the added peroxide is reduced immediately by the basal medium so that it is obvious that both sulfhydryls are present in considerable excess. Since the protective action of these compounds in all probability depends upon their diffusion into the cell, it might be ex-

pected that an excessive extracellular concentration would be required. Also, oxygen is present to permit the formation of additional hydrogen peroxide.

The situation is further complicated by the presence of protohemin, which may give rise to catalase. However, since the same limiting growth inhibiting concentration of added peroxide (0.003 M.) is observed with cysteine plus protohemin as with cysteins alone, no supplementary protective action can be attributed to protohemin in this case. This demonstrates that the formation of catalase from protohemin is inhibited by hydrogen peroxide. In lower concentrations of hydrogen peroxide (0.0015 M. or less) the presence of protohemin causes the production of a greater final turbidity, indicating that later in the incubation period, after cysteine has disappeared, catalase is present and permits the continuation of growth. This conclusion depends upon the assumption that the growth rates are approximately constant.

9. The effect of cysteine and thioglycollate on the accumulation of hydrogen peroxide

It was demonstrated in the previous section that cysteine and thioglycollate protect Hemophilus influenzae against the inhibitory action of rather large quantities of added hydrogen peroxide, and it is now proposed to show that these compounds also prevent the accumulation of hydrogen peroxide when this is produced under the influence of the organism. Since Schales' reagent is insensitive to low concentrations of hydrogen peroxide that are yet growth in-

hibiting, it was desired to use a method that would exaggerate the rate of production of peroxide. This was effected by replacing the usual inoculum with a heavier suspension of the bacteria and testing for hydrogen peroxide after a short incubation period. The only effect of this alteration in procedure should be to increase the velocity of reactions that normally occur, and hence it is a valid method of determining the respiratory mechanisms present under various conditions of culture.

The basal medium containing yeast extract and 0.006 M. cysteine or 0.002 M. thioglycollate with or without 2×10^{-5} M. methylene blue or 10^{-6} grams/ml. of protohemin was inoculated with 0.1 ml. of an 18 hour protohemin-yeast extract culture that had been concentrated 20 times by centrifuging 200 ml. of culture and resuspending the bacteria in 10 ml. of physiological saline. After incubation for 30 minutes the cultures were tested with Schales ' (1938) dilute reagent. The results are given in Table X.

It is to be observed that while moderate or strong tests for hydrogen peroxide were obtained in the absence of cysteine and thioglycollate, the test was weak, negative or questionable when these substances were present. It is surprising that the test for peroxide was no more marked in the presence of methylene blue than in its absence, since this dye system has already been shown to be an efficient respiratory catalyst, but it is possible that this may be accounted for by the fact that it was necessary to treat

dye cultures twice with kaolin, and that even after this treatment the solutions were still bluish. Thus, it is doubtful if the results obtained after treatment with kaolin are strictly comparable with those of solutions not so treated. The marked difference still remains between the cultures with and without cysteine or thioglycollate.

In this experiment protohemin was added in the belief that it might exert only a respiratory catalytic function since Lwoff and Lwoff (1937 b) have shown that augmentation of respiration begins in less than 7 minutes after addition of hemolyzed blood to cultures containing a minimum of protohemin and it appeared unlikely that appreciable catalase formation could take place in the incubation period of 30 minutes. The results do not indicate any such effect, however, since the concentration of hydrogen peroxide, as nearly as can be judged by this method, is lower in the presence of added protohemin than in its absence (Table X, Column 1). This result suggests either a rapid formation of catalase or else a slower rate of formation of the cytochrome system than was reported by Lwoff and Lwoff. Since a protohemin-free inoculum was not used in the present experiment, some catalase may be expected to have been added to the tubes, but this factor should have been equal in all tubes.

10. The substitution of ascorbic acid for protohemin

Ascorbic acid is capable of reducing hydrogen peroxide, and it was found that this compound could replace protohemin for the growth of Hemophilus influenzae. Tubes of the basal medium containing yeast extract and concentrations of neutralized ascorbic acid ranging from 0.01 M. to 0.0005 M. with and without 2×10^{-6} grams/ml. of protohemin were inoculated with 0.1 ml. of a protohemin-free anaerobic culture. The results are given in Table XI. It may be seen that while concentrations of ascorbic acid of 0.01 M. and 0.005 M. were able to replace protohemin, they also had an impeding effect on growth both alone and in the presence of protohemin. With lower concentrations neither protection nor the impeding effect was observed. It is not probable that this impeding effect is due to a decrease in the growth rate since turbidity appears as early in the presence as in the absence of ascorbic acid. It is more likely that growth is halted before maximum turbidity is attained. Furthermore, since this effect occurs also in the presence of protohemin, it is apparently not due to exhaustion of the protective agent as in the case of cysteine. It may be suggested that growth inhibiting factors are produced from the ascorbic acid and that the situation is analogous to the case of thioglycollate in this respect. It was shown that the impeding effect was not due to an alteration of the reaction of the medium.

This experience has been repeatedly confirmed using anaerobic cultures as inocula. Since, for this reason, protohemin could not have contaminated the cultures, it was not considered necessary to repeat the experiment using series transfers.

The effect of ascorbic acid on growth inhibition in the presence of dye systems is shown briefly in Table XII. The results are similar to those obtained with cysteine and need no additional comment. The effect of ascorbic acid on the accumulation of hydrogen peroxide and on growth inhibition by added peroxide has not been studied.

Ascorbic acid is capable not only of reducing hydrogen peroxide, but also of acting as an oxidation-reduction system. Whether this function plays any role at all in the present experiments is not known, but it has already been shown that respiration is probably not essential for the growth of Hemophilus influenzae, so that the essential function of ascorbic acid cannot be its ability to act as a respiratory catalyst. In addition, the substitution of ascorbic acid for protohemin eliminates the possibility that the compounds previously used are effective because they provide the organism with a source of sulfur.

11. The effect of thiosulfate and sulfide on growth

Sodium thiosulfate and sodium sulfide also reduce hydrogeh peroxide. An attempt was made to replace protohemin by these substances.

Sodium thiosulfate was employed in concentrations ranging from 0.1 M. to 0.001 M. No turbidity developed either in the presence of thiosulfate alone or with the addition of protohemin in any concentration of thiosulfate tested. Inhibition of growth was shown not to be due to any alteration of the reaction of the medium.

Sodium sulfide, in concentrations ranging from 0.5 M. to 0.005 M., was tested alone and in the presence of protohemin. Turbidity appeared in all tubes containing sulfide, including those not inoculated, and was proportional to the concentration of sulfide added. It was shown by microscopic examination that the turbidity was due to colloidal precipitation, and that no growth of Hemophilus influenzae was apparent either in the presence or absence of protohemin. Inhibition of growth was shown not to be due to any alteration of the reaction of the medium.

It appears that the results of these experiments contribute no evidence bearing on the problem at hand. While both thiosulfate and sulfide are unable to replace protohemin, it is obvious from the observation that these compounds inhibit growth in the presence of protohemin that some other growth inhibiting mechanism is concerned.

12. The effect of cyanide, diethyldithiocarbamate and 8-hydroxyquinoline on growth

It is well known that cyanide inhibits the action of enzymes in which a heavy metal, particularly iron, is the active group. Growth of Hemophilus influenzae would

not be expected to occur in aerobic culture with protohemin in the presence of cyanide, since this compound poisons both catalase and cytochrome oxidase. The removal of these systems is more easily effected, however, by depletion of protohemin and it has already been shown that when this is done aerobic growth does not occur. It was not expected, therefore, that cyanide would inhibit growth of the organism when growth did not depend on the presence of enzymes derived from hemin (e.g., in anaerobic culture or aerobically with cysteine), but such inhibition was found to occur. Tubes of the basal medium containing yeast extract and 10^{-6} grams/ml. of protohemin or 0.006 M. cysteine plus 0.005 M. or 0.00125 M. potassium cyanide were inoculated with 0.1 ml. of a protohemin-yeast extract culture. These were incubated aerobically and anaerobically with the results shown in Table XIII.

The results show that low concentrations of cyanide inhibit the growth of Hemophilus influenzae not only in aerobic tubes containing protohemin, but also in anaerobic culture and to a less extent in aerobic culture containing cysteine. It is obvious that inhibition in anaerobic culture and in aerobic cysteine culture cannot be attributed to inhibition of the catalase or cytochrome oxidase systems, since these are not required for growth under the conditions used.

It was also observed that sodium diethyldithiocarbamate in a concentration as low as 0.001 M. and 8-hydroxyquinoline in even lower concentrations inhibited the growth of Hemophilus influenzae in the presence of 10^{-6} grams/ml. of protohemin, 0.006 M. cysteine, or 0.005 M. ascorbic acid.

It is known that cyanide, diethyldithiocarbamate and 8-hydroxyquinoline inhibit the action of heavy metal-containing enzymes, so that it is probable that growth inhibition was due to the effect of these compounds on "anaerobic mechanisms" essential for either aerobic or anaerobic growth. A similar explanation may ultimately be found for the impeding or inhibitory effects found with thioglycollate, sulfide, and thiosulfate, since in cultures containing these compounds the odor of hydrogen sulfide was always detectable.

13. The effect of crystalline catalase on growth

It was expected that preparations of catalase might replace protohemin for the growth of Hemophilus influenzae. Even if the added catalase does not act as such, it might be active by virtue of its protohemin content, as is the case with hemoglobin. For this study, a small amount of Sumner's (Sumner and Dounce, 1937) crystalline catalase suspended in ammonium sulfate solution was washed and dissolved in phosphate buffer at pH 6.0. This solution was comparable colorimetrically to about 10^{-5} protohemin and could replace protohemin at a further dilution of 1/1000. Growth appeared about as rapidly in this concentration as in 10^{-5} protohemin.

Since the catalase solution was not prepared from weighed, dry material, and since the activity of the crystals was not known, any comparison of the X-factor activity of this solution with the activity of protohemin could not be considered strictly quantitative, but the results suggested that the crystalline catalase was active as the X-factor in a higher dilution than was protohemin. It was later found however, that the catalase solution could also replace the V-factor (yeast extract) for the growth of the organism, and augmentation of growth may be attributed in part to the excess V-factor present, since lower concentrations of protohemin may be used when a great excess of the V-factor is present. It was found that the ability of crystalline catalase to supply the V-factor was not removed by three additional recrystallizations, but was destroyed by heating to 70° in phosphate buffer solution at pH 7.4 for 5 to 10 minutes, or by autoclaving, The apparent activity of catalase as the V-factor was shown not to be due to yeast extract, introduced into the test along with the inoculum by carrying out a series of 5 transfers in the basal medium containing catalase but no yeast extract.

It was noted that in many cases a concentration of catalase sufficient to give a qualitative catalase test on addition of hydrogen peroxide did not permit the growth of Hemophilus influenzae, which seems to indicate that a relatively high concentration of added (extracellular) catalase does not protect against hydrogen peroxide. This is to be

expected since the large catalase molecule must be unable to penetrate the cell, where in the absence of intracellular catalase, hydrogen peroxide may be produced and kept at a growth inhibiting concentration. This question is given further treatment in the Discussion.

V DISCUSSION

1. The essential function of protohemin for growth of Hemophilus influenzae

At the present time there is no evidence available to indicate that cytochrome c and cytochrome oxidase are not formed from protohemin by those bacteria requiring this factor for growth. The respiratory augmentation observed by Lwoff and Lwoff (1937 b) upon the addition of protohemin to cultures of Hemophilus influenzae containing only a minimum of protohemin lends support to this view, especially since it was noted that augmentation was proportional to the amount of protohemin added. The cytochrome system is found in many bacteria as well as in plant and animal tissue, and acts as the final mediator in oxidations by oxygen. In the presence of the specific dehydrogenases cytochrome may oxidize various substrates either directly (Equation 7) or through the mediation of the codehydrogenases (Equations 8 and 9). In either case, the reduced cytochrome is reoxidized by oxygen in the presence of cytochrome oxidase

with the formation of hydrogen peroxide as the primary reduction product of oxygen (Equation 10). This peroxide may either be removed under the influence of catalase, peroxidases or other mechanisms, or it may accumulate.

(7) substrate + cytochrome $\underset{\text{dehydrogenase}}{\overset{\text{substrate}}{\rightleftharpoons}}$ oxidized substrate + reduced cytochrome

(8) substrate + codehydrogenase $\underset{\text{dehydrogenase}}{\overset{\text{substrate}}{\rightleftharpoons}}$ oxidized substrate + reduced codehydrogenase

(9) reduced codehydrogenase + cytochrome $\underset{\text{codehydrogenase}}{\overset{\text{diaphorase}}{\rightleftharpoons}}$ + reduced cytochrome

(10) reduced cytochrome + O_2 $\underset{\text{oxidase}}{\overset{\text{cytochrome}}{\rightleftharpoons}}$ cytochrome + H_2O_2

Further evidence indicating that the cytochrome system is formed from protohemin by the bacteria requiring this factor is supplied by the observation of André Lwoff (1936 a) that the presence of hemin c has a sparing effect on the amount of protohemin required for the growth of Hemophilus canis. Present knowledge indicates (Stern, 1937) that the prosthetic group of neither cytochrome c nor of cytochrome oxidase is protohemin, so that the formation of the cytochrome system from protohemin involves not only a combination of a hemin with a protein but also an alteration of the hemin structure. For the formation of hemin c, the prosthetic group of cytochrome c, this probably means the addition of a base with tertiary nitrogen to an unsaturated side chain;

and in the case of pheohemin, which is probably the prosthetic group of cytochrome oxidase, the oxidation of a vinyl side chain to a formyl group. Lwoff found it impossible to completely replace protohemin by hemin c for the growth of Hemophilus canis, and suggested that this might be because protohemin is necessary as such and because the reaction, protohemin ——→ hemin c, may not be reversible. Considering the chemical changes concerned this appears quite likely.

Whatever weight may be given the cytochrome-cytochrome oxidase theory of the function of protohemin as a result of the demonstration that protohemin causes a proportional augmentation of oxygen consumption, equal weight must be given the catalase theory as a result of the demonstration of a catalase test by cultures of the organism, since this test is highly specific. There was even a rough indication that the amount of catalase produced depended on the amount of protohemin present. On theoretical grounds, also, the formation of catalase from protohemin might be expected, since the prosthetic group of catalase is protohemin itself (see Stern, 1937).

It may be submitted, however, that the demonstration of the presence of either catalase activity or of respiratory augmentation on the part of cultures of Hemophilus influenzae is insufficient evidence for the evaluation of the importance of either of these functions for growth. This evaluation

has been attempted in the present work by determining whether the reactions catalyzed by the respective systems are essential for growth.

Several observations show that respiration through the cytochrome system is not essential for the growth of Hemophilus influenzae. It would appear that successful anaerobic cultivation is sufficient to demonstrate that oxygen consumption is not an essential function of the cell and that, accordingly, mechanisms catalyzing oxygen consumption cannot be essential as such. Anaerobic cultivation has been reported (Kopp, 1927) and confirmed (Eirund, 1929; Anderson, 1930, 1931) and the present work contains suggested explanations for the reported failures (Pfeiffer, 1893; Fildes, 1921). It was admitted that "anaerobic cultivation" rarely signifies the absolute exclusion of oxygen but it is obvious that under the conditions specified sufficient oxygen was not present to provide a significant respiratory energy source. In addition, it may be shown that the cytochrome system need not even be present to permit cultivation of the organism. It is generally conceded that protohemin is required by Hemophilus influenzae and certain related bacteria because they are unable to synthesize this structure from its components, and because this structure is part of some essential mechanism of the cell. It may be shown that under certain conditions (anaerobically, and also aerobically if means are taken to prevent the accumulation of hydrogen peroxide) Hemophilus influenzae is

able to grow without the addition of protohemin, and that this ability is not due to a suddenly acquired mechanism for synthesizing protohemin, since this compound remains essential in aerobic media lacking agents that reduce hydrogen peroxide. Obviously, then, the cytochrome system is not indispensable for the growth of Hemophilus influenzae and there is no reason to believe that the same conclusion may not be applied to related organisms requiring protohemin.

The available evidence indicates, in fact, that the respiratory function of protohemin, if it were supplied to the organisms without the usually accompanying catalase function, would prevent growth. Since it is not possible to inhibit specifically the catalase function of Hemophilus influenzae, this was shown by the inhibitory activity of certain oxidation-reduction systems capable of supplying only the respiratory catalytic function. In the presence of these systems growth was lacking entirely unless means were taken to prevent the accumulation of hydrogen peroxide.

On the other hand, the evidence supporting the essential nature of the catalase function is rather complete. The enzyme catalase specifically catalyzes the decomposition of hydrogen peroxide (Equation 11), so that attention was focused on the relation of growth to hydrogen peroxide.

(11) $H_2O_2 \xrightleftharpoons{\text{catalase}} H_2O + 1/2\ O_2$

It was shown, by the addition of known amounts and by testing for peroxide, that the growth of Hemophilus influenzae was inhibited by extremely low actual concentrations of hydrogen peroxide. It was demonstrated that in the absence of protohemin hydrogen peroxide accumulated in aerobic culture, and it was shown that growth occurred only when peroxide did not accumulate (anaerobic culture, or aerobically with protohemin or substances reducing peroxide). It was concluded that in the usual culture or in the natural habitat, where agents reducing hydrogen peroxide are probably lacking, the growth of Hemophilus influenzae is dependent on the formation of catalase from protohemin.

It might be objected that these results apply equally well to the essential nature of peroxidase. This enzyme or group of enzymes catalyzes the reduction of hydrogen peroxide by various substrates (Equation 12). It is probable that those peroxidases which have been studied

(12) substrate $+H_2O_2$ $\xrightleftharpoons{\text{peroxidase}}$ oxidized substrate $+ H_2O$

(horseradish and fig sap peroxidases) contain protohemin as the prosthetic group (see Stern, 1937). Satisfactory evidence is not available to determine whether Hemophilus influenzae produces peroxidase or not. It must be recalled that the "peroxidase test" frequently used on cultures of Hemophilus influenzae with positive results cannot be considered valid in this respect, since cytochrome and related

substances also give the test and can be distinguished from the peroxidase enzymes only by quantitative methods. Even if peroxidase is produced it must be noted that it is relatively inefficient in the removal of hydrogen peroxide as compared to catalase and, since the latter was shown to occur, it is obvious that peroxidase cannot play a significant role in any case.

After continued anaerobic cultivation without protohemin it might be expected that the organism would be depleted of the cytochrome respiratory system. If no other means of respiration and formation of hydrogen peroxide is possessed by the organism, aerobic culture without protohemin should then be possible. This did not occur, even when freshly autoclaved media containing no preformed peroxide was used. In addition, it was noted that hydrogen peroxide accumulated when such media were inoculated heavily. These results demand the possession of a non-hemin respiratory system by Hemophilus influenzae, in order to account for inhibition and peroxide accumulation in aerobic hemin-free culture. The nature of this mechanism is not known, but it may well be a flavoprotein, since these are of common occurrence. The phosphoriboflavin proteins containing the alloxazin-adenine-dinucleotides are known to act as mediators in respiration. The flavoprotein isolated by Warburg and Christian is not reduced directly by any dehydrogenase although it may be reduced by either of the codehydrogenases (Equation 13).

At the reduced oxygen tension occurring in tissues and in the presence of electroactive systems of higher potential, such as the cytochrome system, reduced flavoprotein is oxidized by cytochrome (Equation 14) which is in turn oxidized by oxygen in the presence of cytochrome oxidase, with the formation of hydrogen peroxide (Equation 10). However, in the absence of such electroactive systems, as probably in the present case, the flavoprotein may act as the final mediator in the series and reduce oxygen with the formation of hydrogen peroxide (Equation 15). In the absence of catalase, peroxidase or substances reducing peroxide, hydrogen peroxide will accumulate and inhibit growth. If Hemophilus influenzae produces such a system, it must be capable of synthesizing it from peptone plus cozymase.

(13) reduced codehydrogenase + flavoprotein $\rightleftharpoons$ codehydrogenase + reduced flavoprotein

(14) reduced flavoprotein + cytochrome $\rightleftharpoons$ flavoprotein + reduced cytochrome

(15) reduced flavoprotein + O_2 $\rightleftharpoons$ flavoprotein + H_2O_2

2. The relation of growth to the kinetics of peroxide formation and removal

In the present work catalase has not been quantitatively measured. Under conditions in which the accumulation of hydrogen peroxide would be possible except for the presence of catalase, the occurrence of growth has been considered evidence of the production of catalase, and the failure of growth as evidence of its non-production. Consequently, it is difficult to consider separately the effect of hydrogen peroxide on growth and on catalase production. It was shown directly, by the addition of hydrogen peroxide to protohemin-free cultures, that hydrogen peroxide inhibits growth (Table III). Also, from the fact that measurable quantities of hydrogen peroxide accumulate in the presence of living bacteria and of protohemin (Table IV), it may be concluded that hydrogen peroxide inhibits the production of catalase, although this may be indirectly (e.g., by inhibition of growth). It is probable that about the same concentration of hydrogen peroxide inhibits both growth and catalase production. It was noted (Table III) that anaerobically 0.00015 M. added hydrogen peroxide secures permanent inhibition of growth in the absence of protohemin, but only delays growth in its presence. This appears to show that catalase can be formed in concentrations of peroxide that inhibit growth *per se*, but it may be noted that double this concentration (0.0003 M) secures permanent inhibition in either case. If this difference is not attributable to experimental error, it is yet not

great. It was also noted that cysteine permitted growth in the same concentrations of peroxide whether protohemin was present or not (Table IX). Since the production of catalase depends on the reaction,

$$\text{protohemin} + \text{protein} \rightleftharpoons \text{catalase},$$

and since the supply of protein depends on cell synthesis, inhibition of growth should prevent the formation of all but very small amounts of catalase. It is true that even small amounts in particular cells might clear them of peroxide and permit growth and additional catalase formation with the ultimate production of maximal turbidity, but the results no more than suggest this.

It may be shown that the production of catalase also depends on the concentration of protohemin. Growth may appear earlier in the presence of excess protohemin, and 10^{-4} grams/ml. of protohemin permitted growth in the presence of dye systems that caused inhibition when only 10^{-6} grams/ml. was present (Table II). It appears, therefore, that the reaction,

$$\text{protohemin} + \text{protein} \rightleftharpoons \text{catalase},$$

depends on the concentrations of both reacting substances, and probably on other factors.

It is now possible to consider from a kinetic viewpoint the conditions existing in various types of cultures.

Probably three principal reactions are concerned; (1) the formation of hydrogen peroxide, (2) the decomposition of hydrogen peroxide, and (3) the reduction of hydrogen peroxide.

(1) $2\,H^{+} + O_2 \rightleftarrows H_2O_2$ This depends on the presence of respiratory catalysts and of oxygen. In the present work probably three catalysts have been involved; (a) a non-hemin respiratory system (called flavoprotein for convenience), (b) the cytochrome system, and (c) dye systems of suitable potential.

(a) The non-hemin respiratory system was present in the cells under all conditions, although it could be made non-functional by the exclusion of oxygen.

(b) The cytochrome system was probably always present when protohemin was supplied the cell, but could be removed by depletion of this compound. It could not be studied in the absence of catalase. The factors influencing the formation of the cytochrome system are probably similar to those concerned in the formation of catalase, although there is some evidence that this system is functionally less active.

(c) The dye systems are "artificial" catalysts and may be supplied at will. They are accompanied by no complicating accessory functions, so that their effects may be readily studied. The effect of respiratory cata-

lysts of various efficiencies may be determined by using dye systems of various potential.

(2) $H_2O_2 \rightleftharpoons H_2O + 1/2\ O_2$ By removing hydrogen peroxide produced during respiration, this reaction acts to neutralize the inhibiting tendency of the first reaction. The occurrence of these two reactions in nature is so frequent that they have not long been regarded as distinct. The latter is catalyzed specifically by catalase, which is extremely efficient in this respect. It is probable that only very small amounts of catalase are required. This reaction may be supplied at will, but it is always accompanied by the cytochrome respiratory function.

(3) $2\ H^+ + H_2O_2 \rightleftharpoons 2\ H_2O$ This reaction requires no catalyst not present in peptone solution. It depends on the presence of an active reducing agent. For the present purpose these may be considered under two heads; (a) added reducing agents, and (b) those already present in the medium.

(a) The reducing agents added (cysteine, etc.) reduce hydrogen peroxide rapidly, and in the quantities used may be effective for a relatively long period. Cysteine appears not to be oxidized completely for about 9 hours (Figure 1), which is sufficient time for the appearance of a definite turbidity if growth occurs.

(b) Reducing agents present in the peptone solution appear to reduce hydrogen peroxide rapidly but, probably because they are present in low concentration, the protective effect does not last long. The rate of the reaction is probably rapid at first, then gradually becomes progressively slower. The occurrence of delayed anaerobic growth may be due to this effect, the concentration of hydrogen peroxide not becoming low enough in certain cases to permit growth for 24 to 48 hours. In this way, autoclaved peptone solutions exert a sort of "buffer" effect and must be "saturated" with peroxide before active hydrogen peroxide begins to accumulate.

It is possible to consider here the question of a peroxidase, which catalyzes the third reaction and permits the use of less actively reducing substrates, but this enzyme would occur only along with catalase which would be accountable for almost the entire effect.

The factors just described are of very different significance under different cultural conditions. Three typical cultures may be considered; (1) aerobic culture containing protohemin, (2) aerobic culture containing cysteine, and (3) anaerobic culture.

(1) If augmentation of respiration may begin within 7 minutes of the addition of protohemin to aerobic cultures of Hemophilus influenzae, it is probable that the production of hydrogen peroxide in this type of culture begins almost imme-

diately. If a protohemin culture is used for inoculation, some catalase is undoubtedly present initially. The hydrogen peroxide produced is thus removed and growth with the production of more catalase soon occurs if more than a limiting dilution of protohemin is also present. If catalase is not present in the inoculum, the broth may lower the hydrogen peroxide concentration to below an inhibitory level until catalase is formed; this may require many hours (Figure 1). It is probable that the reaction producing catalase needs to proceed more rapidly here, since it was frequently observed that a greater concentration of protohemin was required when protohemin-free inocula were used (2×10^{-6} as compared with 10^{-6}). After a very short time the effect of reduction of peroxide by the broth must become insignificant and continued growth will then depend on the rates (k_1, k_2) of the reactions,

$$2\,H^{+} + O_2 \underset{\text{respiratory catalysts}}{\overset{k_1}{\rightleftarrows}} H_2O_2 \overset{k_2}{\underset{\text{catalase}}{\rightleftarrows}} H_2O + 1/2\,O_2.$$

Sufficient catalase must be present to keep the overall rate (k_2) of the second reaction greater than that of the first (k_1). This represents the limiting concentration of catalase, and while the limiting concentration per cell may or may not remain constant, the limiting concentration for the culture must increase as growth continues and the number of respiring cells increase. For any limiting catalase concentration there must exist a limiting protohemin concentration of the

medium that will permit the formation of this amount of catalase. Undoubtedly this concentration of protohemin is considerably in excess of that actually converted into catalase. Presumably this limiting protohemin concentration increases as the limiting catalase concentration increases with growth of the culture. In order to permit the development of a maximum turbidity, sufficient protohemin must be present to allow the formation of enough catalase to decompose the amount of hydrogen peroxide formed at the height of the metabolic activity of the culture; lesser amounts should stop growth before this point is reached. It is to be expected that amounts of protohemin permitting the development of "definite" and "maximum" turbidities would differ considerably. Lwoff and Lwoff (1937 b), calculating from the protohemin concentration of hemolyzed blood, have found a concentration of 10^{-9} grams/ml. required for the former, while in the present work 10^{-6} has been used for the latter; these results may not be strictly comparable, since different strains and sources of protohemin were used.

The overall rate of the first reaction (k_1) probably depends on two factors: the non-hemin respiratory system and the cytochrome system. Decreasing the protohemin concentration below a limiting dilution for growth may or may not affect this rate, since the flavoprotein system remains unchanged; but increasing the concentration has been shown (Lwoff and Lwoff, 1937 b) to increase the respiratory rate. In this case, however, the concentration of catalase should

be correspondingly increased. If dye systems are added, respiration is increased without an accompanying increase in catalase concentration, so that k_1 may exceed k_2 causing an accumulation of hydrogen peroxide and the resultant growth inhibition. This respiratory increase may be compensated by the addition of more protohemin (Table II), which appears to indicate that the respiratory function of protohemin is less efficient than the catalase function.

In the presence of insufficient protohemin (10^{-6} grams/ml.) it is probable that growth is finally stopped in old cultures as a result of depletion of nutrients and of the V-factor, rather than by the accumulation of hydrogen peroxide, although tests for catalase appear to become increasingly feeble in old cultures (after about 36 hours).

(2) The situation is rather different in cysteine cultures. A considerable concentration of reducing substances is present from the beginning, hydrogen peroxide cannot accumulate at this time, and growth may start immediately (see Figure 1) whether or not a protohemin-free (catalase-free) inoculum is used. Consequently, turbidity always appears early. In this type of culture the limiting concentration of protohemin is zero, since no catalase is required. In the absence of protohemin the non-hemin respiratory system is the only catalyst promoting the production of hydrogen peroxide. However, in the concentrations used in the present work, cysteine does not remain in the cul-

ture for more than about 9 hours. Since many cells (considerable amount of the non-hemin system) are present at this time and the rate of peroxide production must be high, the disappearance of cysteine marks the maximum turbidity attained and death of the cells occurs rapidly (Figure 1) unless protohemin is also present to exert a continued protective effect, in which case the culture continues as a protohemin culture. If dyes of intermediate potential are present the usual final turbidity of protohemin cultures is not attained. The reason for this is not known but it appears to depend upon the presence of the cysteine and upon a high rate of oxygen consumption.

(3) The type of inoculum used makes considerable difference in the case of anaerobic cultures. If inoculation is made from a protohemin culture, so that some catalase is transferred, cultivation is accomplished without difficulty, and several transfers may be made before this protective effect is lost. Presumably, preformed peroxide present in the medium, and any that may be formed before "complete" anaerobiosis is initiated, is decomposed by this catalase and no more can be formed. Since the concentration of peroxide is not great and since no more is formed, only very minute concentrations of catalase must be required. In addition, since hydrogen peroxide in this concentration is inhibitory rather than toxic, decomposition of this peroxide may extend over a period of several hours, so that the rate of

decomposition (concentration of catalase) may be very low without affecting the final result.

If a protohemin-free inoculum is used, very small amounts of hydrogen peroxide inhibit growth completely, so that this type of organism is one of the strictest of anaerobes. Two sources of hydrogen peroxide are possible; there is some evidence that preformed peroxide is present in the medium if it has been stored for some time, and it is possible that peroxide is formed through the non-hemin respiratory system of the organism before complete anaerobiosis is attained. It has not been possible to detect peroxide in stored media by chemical tests, so it may be merely that the reducing capacity of stored media is lessened and it no longer acts as a "buffer" for the peroxide formed before anaerobiosis is attained. To obtain growth consistently with a hemin-free strain of the organism it is necessary to autoclave the medium before inoculation. In this way the accumulation of inhibitory concentrations of peroxide is prevented and growth occurs. Aside from this point, the same result is obtained whether catalase, the cytochrome system, cysteine or other reducing agents, or dyes be present or absent. Without oxygen respiration does not occur and no method of removing hydrogen peroxide is required. Growth appears to persist for longer periods in anaerobic culture, but it is probable that this is related to the stability of the V-factor under these conditions. Turbidity is never as marked in anaerobic cultures but this is probably related to the

available energy sources.

The preceding description of the history of various types of cultures is fragmentary and highly speculative, but it is hoped that it may introduce some sort of order and suggest points of attack for further experimentation. The elucidation of certain points will require laborious and expensive quantitative methods, and were for this reason not undertaken in this preliminary work.

3. The site of formation of hydrogen peroxide

It may be shown that the chemical reactions with which this work is concerned are largely intracellular, so that it is impossible to avoid some consideration of the effect of diffusion across the cell membrane. Some of the results that have been obtained appear explicable only when the importance of this factor is recognized.

Respiration is in general an intracellular process. Flavoprotein and the cytochrome system are normally found within the cell and it is undoubtedly here that they cause the reduction of oxygen. The dye systems, which were added as artificial respiratory catalysts, were placed in the external medium, but it is known that dye systems act as respiratory catalysts only after absorption, since the acid dyes, which are incapable of penetrating the cell, are unable to exert this function. Inasmuch as the respiratory systems just described are the final mediators with oxygen it is obvious that the site of formation of hydrogen peroxide

is within the cell. Consequently, any substance that is to prevent efficiently the accumulation of peroxide must also be located intracellularly.

The relative efficiency of intra-and extracellular protective agents is readily demonstrated in the case of catalase. Catalase formed by the culture from protohemin is located entirely or almost entirely within the cell and exerts a protective action in concentrations that are not demonstrable by the addition of hydrogen peroxide to the culture. Since hydrogen peroxide and its products, water and oxygen, are readily diffusible, this appears to indicate that a very low concentration of intracellular catalase prevents growth inhibition. On the other hand, when crystalline catalase is added to the culture, the size of the molecule undoubtedly retains it in an extracellular position, and in this case growth inhibition may occur even when sufficient catalase is added to give a moderately strong test. Concentrations of added catalase sufficient to prevent inhibition give a very strong catalase test. It may be suggested that such extracellular agents exert a protective effect by lowering the extracellular concentration of hydrogen peroxide and thus increasing the rate of diffusion of peroxide out of the cell. It is possible to show that this effect alone cannot protect the cell. Two concentrations of added catalase may be selected, both of which give a demonstrable catalase test upon addition of hydrogen peroxide, but only one of which permits aerobic growth of the organism. Both

concentrations probably reduce the concentration of extracellular peroxide to zero almost immediately and maintain it there, so that both should cause the same rate of diffusion of peroxide out of the cell, but only the higher concentration protects the organism. For this reason, the protective action of added catalase cannot be referred entirely to the removal of extracellular peroxide, and must be largely due to the utilization by the organism of protohemin formed through the decomposition of the added catalase.

It is probable, however, that extracellular agents exert some protective action by the removal of extracellular peroxide. It was observed that concentrations of added catalase or of thioglycollate that are too small alone to prevent growth inhibition may exert some protective action when a minimum of protohemin is present. Turbidity appears much earlier in protohemin cultures when these substances are present, and this observation has been made within single experiments, where the experimental conditions were presumably constant. It may be suggested that, by the complete removal of extracellular peroxide, these agents increase diffusion of peroxide out of the cell, and also prevent the entrance of any peroxide into the cell from the medium. By thus lowering the effective concentration of intracellular peroxide, these agents might permit the more rapid formation of catalase and the resultant earlier appearance of turbidity. In addition, the lowered intracellular peroxide concentration would decrease the limiting concentration of intracellular peroxide-reducing agents required for growth.

It is to be expected that in the case of respiratory catalysts, the rate of diffusion into the cell might be of minor importance, since very small amounts should exert a significant effect. The situation is quite otherwise in the case of reducing agents, however. These are oxidized as a result of the reaction with peroxide and are thus proportionally inactivated, so that a fresh supply must always be at hand. In a given time an equivalent of the reducing agent must be absorbed by the cell in order to neutralize the production of an equivalent of hydrogen peroxide. Since the hydrogen peroxide is produced under the influence of a catalyst, the reducing agent must be absorbed at a very much higher rate than the respiratory catalyst. Cysteine, for example, might diffuse into the cell much more rapidly than nile blue and still be unable to prevent rapid accumulation of peroxide. In addition, in all the present experiments, at least one respiratory system was always present (non-hemin system) and usually others were added.

The significance of these conclusions to the present work is obvious. Those substances known to reduce hydrogen peroxide, but which were found to be relatively ineffective as protective agents, may well owe their relative inefficiency to slow absorption by the cell. There is some ancillary evidence for this in the case of thioglycollic acid. Rapkine (1937) has shown that while monoiodoacetic acid (Ch_2I-COOH) and monobromoacetic acid (CH_2Br-COOH) inhibited the fermentation of glucose by yeast juice at pH 7, they

had little effect on fermentation by intact cells and that the rate of inhibition in the latter case was increased with increasing hydrogen ion concentrations. It was concluded that the ionic species present at pH 7 did not readily penetrate the cell. Thioglycollic acid (CH_2SH-COOH) is more closely related to these compounds than to cysteine (CH_2SH-$CHNH_2$-COOH) and the influence of an amino group adjacent to a carboxyl group on the ionic structure of the compound is well known. In the present work a reaction slightly on the alkaline side was used, and it is not at all improbable that thioglycollate is less readily absorbed by the cell at this reaction than is cysteine. It is apparent, at any rate, that attempts to replace the X-factor by reducing agents must be made with the diffusion factor in mind.

4. The respiratory structure of Hemophilus influenzae compared with that of other bacteria

Finally, it should be interesting to compare the respiratory structure of Hemophilus influenzae with representative bacteria possessing a like or different structure. For this purpose it will be most convenient to consider the organism in two forms that are functionally quite different: (1) hemophoric Hemophilus influenzae, containing catalase and the cytochrome system, and (2) anhemophoric Hemophilus influenzae, which lacks these factors.

(1) Hemophoric Hemophilus influenzae.

This is the common form. It contains both a non-hemin respiratory mechanism and the cytochrome system, and produces

catalase and perhaps peroxidase. In this it probably resembles many of the common aerobes, although some of the aerobes may lack a non-hemin respiratory system (e.g., Escherichia coli, Broh-Kahn and Mirsky, 1938), and in others the cytochrome system is incomplete (e.g., Staphylococcus, Burnet, 1927). Presumably, it may be differentiated from Shigella dysenteriae Shiga on the basis of catalase formation, but the exact position of the latter organism is not yet known. In this form Hemophilus influenzae is a facultative anaerobe.

(2) Anhemophoric Hemophilus influenzae

In this form Hemophilus influenzae possesses a non-hemin respiratory system, but not a complete cytochrome system, or catalase or peroxidase. It is a strict anaerobe, but differs from the obligate anaerobes (Clostridium) in that oxygen is not toxic per se, and from most of the Clostridia (except, e.g., Cl. butyricum) in that it possesses a non-hemin respiratory system. It may be differentiated also from Diplococcus pneumoniae, Lactobacillus acidophilus and bulgaricus, and from certain Streptococci in that, while a similar respiratory structure is possessed by all, the latter group is relatively insensitive to hydrogen peroxide and, consequently, may grow to a certain extent in the presence of oxygen.

This form of Hemophilus influenzae does resemble certain "artificial" bacteria, such as cyanide treated Staphylococcus (Burnet, 1927) and Escherichia coli in the presence of cyanide plus an added respiratory catalytic (dye) system (Broh-Kahn and

Mirsky, 1938). These organisms, also, are capable of only anaerobic growth, since a respiratory system (non-hemin) is present and no catalase is produced to remove hydrogen peroxide to which the organism is sensitive.

Anhemorphoric Hemophilus influenzae has been observed only under quite artificial conditions, but it is not certain that natural occurrence is impossible. Since the V-factor is not synthesized, association with living tissue would probably be required under natural conditions and this, as a rule, provides also the X-factor. However, anaerobic commensal growth with the Clostridia or with facultative anaerobes not possessing any part of the cytochrome system or of catalase and peroxidase should lead to the development of anhemophoric strains, since such bacteria could supply only the V-factor. Association with living tissue would not be required for those species of Hemophilus which synthesize the V-factor. If the present work may be applied also to Hemophilus canis, this organism might readily come to exist as an anhemophoric saprophyte, provided deprivation of the X-factor occurred anaerobically. Its nutritional requirements are probably as simple as those of Hemophilus influenzae, which is able to use any of several kinds of peptone. Since there are many naturally occurring anaerobic situations which would support bacterial growth but which might not provide the X-factor, it is probable that anhemophoric Hemophilus canis actually does occur in nature. Thus, it is by no means certain that this organism is naturally an obligatory parasite.

VI SUMMARY

(1) It was shown that Hemophilus influenzae is sensitive to extremely small concentrations of hydrogen peroxide. This substance was found to accumulate in aerobic cultures lacking protohemin or reducing agents. The accumulation of hydrogen peroxide was correlated with inhibition of growth. Cysteine, thioglycollate, ascorbic acid, or catalase, which reduce or decompose hydrogen peroxide, permitted aerobic growth of Hemophilus influenzae.

(2) Hydrogen peroxide was found not to accumulate under anaerobic conditions and growth occurred in the absence of agents that remove hydrogen peroxide. Anaerobic growth in the absence of such agents was inhibited or delayed unless the medium was autoclaved before inoculation. It is suggested that this inhibition was due either to the presence of preformed peroxide in the medium, or to the production of peroxide by the organism before anaerobiosis was attained.

(3) Oxidation-reduction systems of various potential would not replace protohemin for growth of Hemophilus influenzae. Oxidation-reduction systems of suitable potential caused an increased accumulation of hydrogen peroxide under aerobic conditions in the absence of agents removing peroxide. In the presence of these systems higher concentrations of protohemin were required for aerobic growth. Substances removing hydrogen peroxide permitted growth in the presence of these systems.

(4) Catalase was produced by Hemophilus influenzae in the presence of protohemin. Inhibition of growth and of catalase formation was found to be intimately related.

(5) Since growth was obtained without protohemin under both aerobic and anaerobic conditions, it was concluded that the cytochrome respiratory system is not essential for the growth of Hemophilus influenzae. Since hydrogen peroxide accumulates in aerobic cultures lacking protohemin, it was concluded that Hemophilus influenzae possesses a non-hemin respiratory system in addition to the cytochrome system.

(6) Since hydrogen peroxide was found to inhibit growth and since growth inhibition was correlated with the accumulation of hydrogen peroxide, it was concluded that the essential function of protohemin is concerned with the removal of hydrogen peroxide, principally through the formation of catalase. The significance of the possible production of a peroxidase is discussed.

(7) The relation of growth to the kinetics of peroxide production and removal is discussed, and the site of hydrogen peroxide formation is indicated.

(8) The respiratory structure of Hemophilus influenzae as depicted by these results is compared with that of other bacteria.

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FIGURE 1

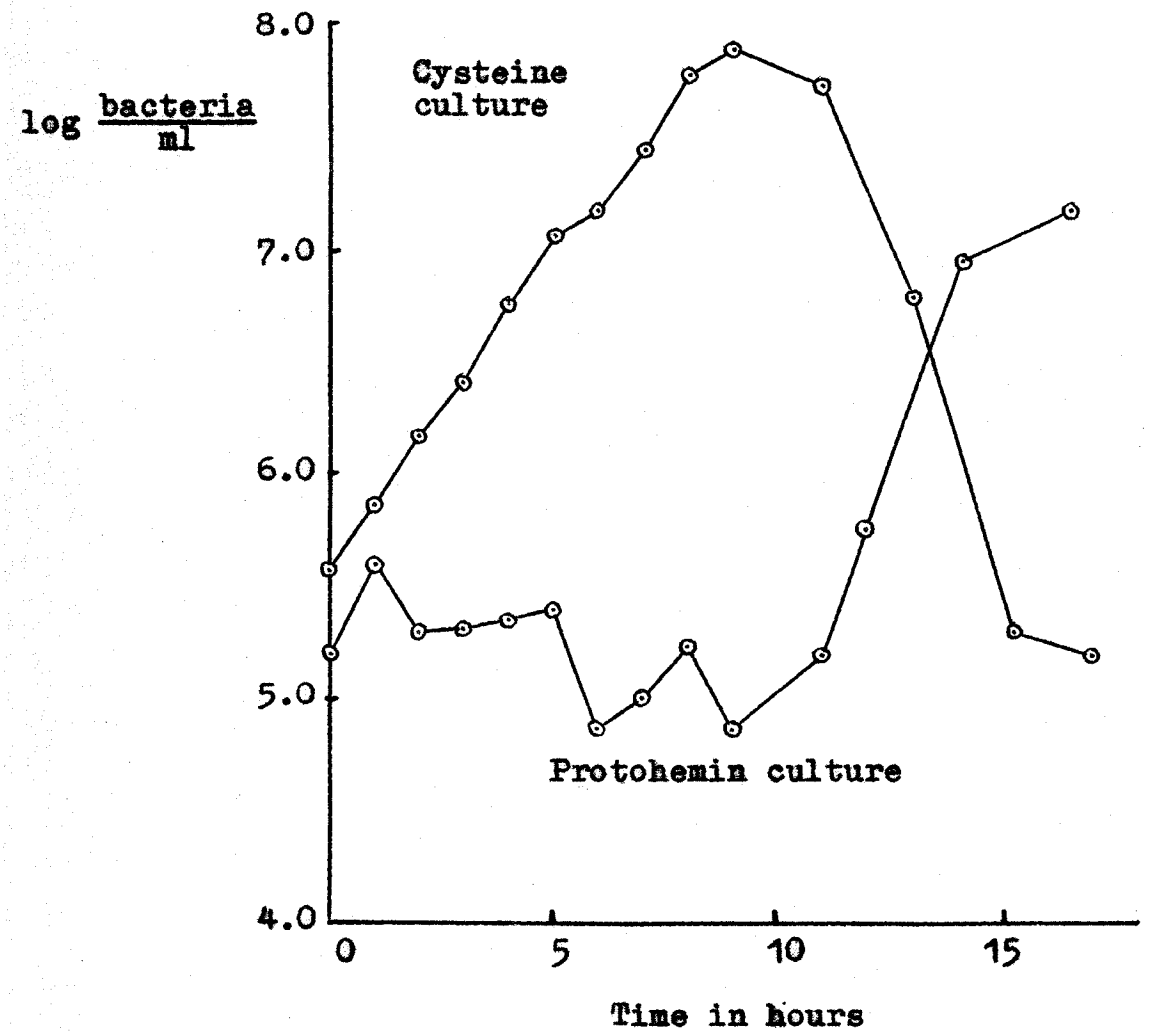

TABLE I

The effect of age of inoculum and of initial concentration of yeast extract in both inoculum and culture on the time of appearance of turbidity in aerobic cultures of Hemophilus influenzae

Age of culture used as inoculum (hours)	Dilution of yeast extract added to inoculum	Time (hours) of appearance of turbidity in hemin (10^{-5}) + yeast extract						yeast extr. 1/100
		1/50	1/100	1/500	1/1000	1/5000	0	
19	1/100	<10	<10	<10	<10	<10		
	1/500	<10	<10	<10	14-25	14-25		
	1/1000	<10	<10	10-14	14-25	14-25		
23	1/100	<9	<9	<9	<9	<9		
	1/500	<9	<9	<9	<9	21		
	1/1000	<9	<9	9-21	9-21	21-46		
43	1/100	<9	<9	<9	9-26	9-26		
	1/500	<9	9-26	9-26	26-58	26-58	144<	144<
	1/1000	9-26	9-26	26	26-58	26-58		
55	1/100	14-28	14-28	14-28	28-46	28-46		
	1/500	28	28	46-61	121<	46-61		
	1/1000	28-46	144<	144<	144<	144<		
81	1/100 1/500 1/1000	144<	144<	144<	144<	144<		

TABLE II

The effect of oxidation-reduction systems of various potential on the growth of Hemophilus influenzae

Dye systems added (2×10^{-5} M.)	E_o' of system at pH 7.0 in volts	(Aerobic) Basal medium + 1/500 yeast extract +			(Anaerobic) Basal medium + 1/500 yeast extract +	
		0	10^{-6} hemin	10^{-4} hemin	0	10^{-6} hemin
2,6-dichlor-indocresol	+0.181	-	+++	+++	++	++
Naphthol sulfonate indophenol	+0.123	-	+++	+++	++	*
Brilliant cresyl blue	+0.047	-	-	+++	++	*
Methylene blue	+0.011	-	-	+++	++	++
Nile blue	-0.122	-	-	+++	-	++
Pheno-safranine	-0.252	-	-	+++	++	*
Neutral red	-0.325	-	+++	+++	++	++
0	·	-	+++	+++	++	++

*not observed

TABLE III

Inhibition of the growth of Hemophilus influenzae by added hydrogen peroxide

Hydrogen peroxide added to medium (mols)	(Aerobic) Basal medium + 1/500 yeast extract +		(Anaerobic) Basal medium + 1/500 yeast extract +			
	10^{-6} hemin		0		10^{-6} hemin	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
0.0003	-	-	-	-	-	-
0.00015	-	+++	-	-	-	+++
0.00003	+++	+++	+++	+++	+++	+++

TABLE IV

The accumulation of hydrogen peroxide in aerobic cultures of Hemophilus influenzae

Gaseous tension	Dye systems added (2×10^{-5} M.)	E_o' of system at pH 7.0 in volts	0	1/500 yeast extract	1/500 yeast extract + 10^{-6} hemin
Aerobic	2,6-dichlor-indocresol	+0.181	-	?	-*
	Methylene blue	+0.011	-	+	+
	Nile blue	-0.122	-	+	+
	Neutral red	-0.325	-	?	-*
	0	·	-	-	-*
Anaerobic	2,6-dichlor-indocresol	+0.181	-	-*	-*
	Methylene blue	+0.011	-	-*	-*
	Nile blue	-0.122	-	-	-*
	Neutral red	-0.325	-	-*	-*
	0	·	-	-*	-*

(Tested for hydrogen peroxide with Schales' reagent after 24 hours incubation)

*indicates tubes in which growth occurred

TABLE V

The effect of the reaction of the medium on the anaerobic growth of Hemophilus influenzae

Treatment of basal medium	Period of incubation (hours)	pH of medium in Series				
		A	B	C	D	E
Autoclaved	0.66 (Tube #1)	7.58	7.41	7.10	6.80	6.51
	8 (Tube #2)	7.51	7.37	7.11	6.81	6.51
	32 (Tube #3)	7.40*	7.22*	6.99*	6.71*	6.46*
	72 (Tube #4)	7.38*	7.29*	6.98*	6.69*	6.43*
not autoclaved	0.66 (Tube #1')	7.58	7.42	7.11	6.79	6.51
	8 (Tube #2')	7.51	7.37	7.06	6.74	6.48
	32 (Tube #3')	7.38	7.27	7.04	6.73	6.49
	72 (Tube #4')	7.46	7.24*	6.98*	6.70*	6.43*

*indicates tubes in which growth occurred. Growth data given for all tubes remaining at time of reading.

TABLE VI

The effect of cysteine on aerobic growth of Hemophilus influenzae

Dye systems added (2×10^{-5} M.)	E_o' of system at pH 7.0 in volts	Basal medium + 1/500 yeast extract +			
		0	0.006 M. cysteine	10^{-6} hemin	10^{-6} hemin + 0.006 M. cysteine
2,6-dichlor-indocresol	+0.181	-	+	+++	+++
Naphthol sulfonate indophenol	+0.123	-	+	+++	++
Brilliant cresyl blue	+0.047	-	+	-	++
Methylene blue	+0.011	-	+	-	+
Nile blue	-0.122	-	-	-	-
Pheno-safranine	-0.252	-	+	-	+
Neutral red	-0.325	-	+	+++	++
0		-	+	+++	+++

(Readings made after 36 hours incubation)

TABLE VII

The effect of thioglycollate on the growth of Hemophilus influenzae

Thioglycollate added to medium (mols)	(Aerobic) Basal medium + 1/500 yeast extract + 0	(Aerobic) Basal medium + 1/500 yeast extract + 2×10^{-6} hemin	(Anaerobic) Basal medium + 1/500 yeast extract
0.05	-	-	-
0.01	-	-	-
0.006	?	++	+
0.005	+	++	++
0.004	?	+++	++
0.002	-	+++	++
0	-	+++	++

(Readings made after 44 hours incubation)

TABLE VIII

The effect of various concentrations of cysteine and thioglycollate on the aerobic growth of Hemophilus influenzae in the absence of protohemin

Concentration of sulfhydryl added to medium (mols)	Basal medium + 1/500 yeast extract +	
	cysteine	thioglycollate
0.01	*	-
0.006	++	?
0.005	*	+
0.004	*	?
0.003	-	*
0.002	*	-
0	-	-

(Readings made after 44 hours incubation)

*not observed

TABLE IX

The effect of cysteine and thioglycollate on inhibition of aerobic growth of Hemophilus influenzae by added hydrogen peroxide

Hydrogen peroxide added to medium (mols)	Basal medium + 1/500 yeast extract +				
			10^{-6} hemin +		
	0	0.006 M. cysteine	0	0.006 M. cysteine	0.002 M. thioglycollate
0.03	-	-	-	-	-
0.015	-	-	-	-	-
0.008	-	-	-	-	-
0.003	-	-	-	-	-
0.0015	-	±	-	++	+++
0.0008	-	+	-	++	+++
0.0003	-	+	-	++	+++
0.00015	-	+	-	++	+++
0.00003	-	+	++	++	+++
0	-	+	+++	++	+++

(Readings taken after 24 hours incubation)

TABLE X

The effect of cysteine and thioglycollate on the accumulation of hydrogen peroxide in heavy suspensions of Hemophilus influenzae

Respiratory catalysts added	Basal medium + 1/500 yeast extract +			
	0	10^{-6} hemin	0.006 M. cysteine	0.002 M. thioglycollate
0	+++	*	-	±
2×10^{-5} M. methylene blue	+++	++	±	±
10^{-6} hemin	++	*	-	+

(Tested for hydrogen peroxide with Schales' dilute reagent after incubation for 0.5 hour with a heavy suspension of Hemophilus influenzae)

*not observed

TABLE XI

The effect of ascorbic acid on aerobic growth of *Hemophilus influenzae*

Neutralized ascorbic acid added (mols)	Basal medium + 1/500 yeast extract +	
	0	2×10^{-6} hemin
0.01	+	+
0.005	++	++
0.001	-	+++
0.0005	-	+++
0	-	+++

(Readings made after 48 hours incubation)

TABLE XII

The effect of ascorbic acid on growth inhibition of *Hemophilus influenzae* by oxidation-reduction systems of various potentials

Dye systems added (2×10^{-5} M.)	E_o' of system at pH 7.0 in volts	Basal medium + 1/500 yeast extract + 0.005 M. ascorbic acid	
		Aerobic	Anaerobic
2,6-dichlor-indocresol	+0.181	++	++
Methylene blue	+0.011	-	+
Neutral red	-0.325	++	++
0	·	++	++

(Readings made after 48 hours incubation)

TABLE XIII

The effect of cyanide on the growth of Hemophilus influenzae

Potassium cyanide added to medium (mols)	(Aerobic) Basal medium + 1/500 yeast extract +			(Anaerobic) Basal medium + 1/500 yeast extract +	
	0	10^{-6} hemin	0.006 M. cysteine	0	10^{-6} hemin
0.005	-	-	-	*	-
0.00125	-	-	±	*	-
0	-	+++	++	++	++

(Readings made after 28 hours incubation)

*not observed