A STUDY OF THE DIPHTHEROID GROUP OF ORGANISMS WITH SPECIAL REFERENCE TO ITS RELATION TO THE STREPTOCOCCI

PART II. CLASSIFICATION OF THE DIPHTHEROID GROUP

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PREVIOUS CLASSIFICATIONS

The difficulties of biologic classification among organisms showing such slight differentiation as the bacteria, have long been noted by bacteriologists. Attempts to arrange assemblages of such organic forms under the captions of species have not met with uniform success, partly on account of overlapping characteristics, and partly on account of the various transitions which this subjective term "species" has experienced from time to time. It is much more practicable to take refuge behind the word "group" as Dr. Prudden (1898) long ago remarked.

Biologically it is doubtful if toxin production on the part of an organism should be considered as a major characteristic, nevertheless from the standpoint of pathology and medicine, it is a quality of first importance, and thus considered indicates a position "sui generis" for the Klebs-Loeffler bacillus. I merely cite this as a concrete difficulty. An adequate classification must conform to the biologic conception, yet it should defer as far as possible to the more utilitarian claims of practical medicine and pathology.

There have apparently been very few attempts to develop a comprehensive, systematic arrangement of the diverse members of the diphtheroid group. The term pseudo-diphtheria.

1 A thesis for the degree of Doctor of Public Health.
bacillus has been inadvertently applied to all atypical diphtheria organisms. This is generally recognized as unfortunate, as the term was already preempted for the organism described by Loeffler (1887) and Hoffman-Wellenhoff (1888) and now commonly known as B. Hoffmanii.

For some years after the discovery of this bacillus there followed a controversy regarding its relation to the Klebs-Loeffler organism. The monists held that the Hoffman bacillus was an attenuated variety of the true diphtheria bacillus, while the dualists regarded them as distinct and separate species. It cannot be said that the question is fairly settled yet, although the present trend of opinion is decidedly opposed to the view that the organisms are separate species in the sense of being totally unrelated. In view of the immense amount of work that has been done, the contention of Flexner and others that B. Hoffmanii or the pseudo-diphtheria bacillus is a true mutant seems logical.

It was soon apparent that the pseudo-diphtheria bacillus was by no means a single race of organisms, and that in all probability there existed a large number of strains of diphtheria-like bacilli, which are identical with neither the Klebs-Loeffler nor the B. Hoffmanii. Although there have been desultory contributions to the literature of this subject for the past twenty years, bacteriologists in general have lapsed into a state of apathy regarding the pathogenic properties of diphtheria-like microorganisms. True, this negligent attitude has been unsuccessfully assailed from time to time, by workers whose experience with the group had convinced them that all its members did not merit a wholesale relegation to the saprophytic scrap-heap. The investigations of Alice Hamilton, Ruediger, Hektoen, Rosenow and others have demonstrated beyond peradventure, that certain strains of this diverse group are capable under certain conditions of showing definitely pathogenic properties.

The lethargic attitude regarding the diphtheroids suffered a shock as the result of a series of investigations begun by Frankel and Much in 1910 and continued by de Negri and Miermet, Bunting and Yates, Rosenow and many others. The first
mentioned authors studied exhaustively thirteen cases of Hodgkin's disease, and were able to recover from the sediment of the lymph glands previously treated with antiformin, certain pleomorphic non-acid-fast bacilli which they did not succeed in cultivating. Frankel and Much, as a result of their studies came to the conclusion that this organism was either an attenuated or a special variety of the tubercle bacillus.

Shortly after this paper appeared, de Negri and Miermet and later Bunting and Yates succeeded in cultivating this organism which had the characteristics of a diphtheroid, and was named by the latter authors *B. corynebacterium Hodgkini*. They claimed to have reproduced with it the characteristic lesions of Hodgkin's disease in monkeys although as yet the contention lacks confirmation. As a result of these investigations a new interest has been awakened in this group, which has manifested itself in the character and number of contributions to the recent literature.

These contributions have been directed mainly toward one of the more pressing exigencies of the situation, namely, the discovery of the *B. Hodgkini* in normal and non-Hodgkin's lymph glands as well as in other locations. As a result a large number of diphtheroids of various types have been found, but owing to the lack of any classification of the group considered as adequate, the various observers state with one accord that they are unable to harmonize their various findings with those of each other. Arbitrary groupings and provisional classifications, based largely on expediency or on some minor characteristic have failed to bring order out of the confusion which at present reigns.

*B. xerosis* also labors under a variety of interpretations. Some make this species identical with the avirulent diphtheria bacillus, while the term is restricted by others to the Kuschbert-Neisser (1884) organism originally thought to be the cause of xerosis, but later found to be a more or less common inhabitant of the normal conjunctival sac.

All through the literature one finds instances of diphtheria-like bacilli receiving specific designations based on a variety
of minor characteristics, such as morphology, or action on gelatin, or perhaps on potato, or pigment production, appearance on serum, or some inadequate combination of these characters, many of which can easily be modified. Another source of confusion has been the results of the sugar reactions in the hands of various observers. For a long time strains were tested on glucose only and naturally it is difficult to correlate such descriptions with those which have been based on six or eight different sugars. Little attention has been paid to immunologic characteristics as a criterion for classification.

The best classification of the diphtheroids that I have encountered is undoubtedly the one made by Morse (1912 a). She divides the entire diphtheria family into two main groups: the Klebs-Loeffler bacillus and the diphtheria-like bacilli or diphtheroids. Of these latter she recognizes four sub-groups which are the following:

*Group A.* This is the largest numerically. The bacilli correspond to the "organism x" described by Hoag from the Danvers State Hospital. It is a medium sized bacillus, showing solid, barred and wedge forms, with abundant but small and imperfect granules. On serum it produces a heavy, confluent, glistening growth with a characteristic salmon-pink color. It ferments glucose and sucrose, but not maltose or glycerin.

*Group B.* The organisms of this group are usually larger than those of Group A, and thick forms with clear cut bars predominate. Neisser's granules are very large and irregular. The growth on serum is heavy and varies in color from white to yellow. It is often noticeably dry and granular. Glucose is always fermented, maltose and glycerin usually, but not sucrose.

*Group C.* This, the smallest group, is differentiated primarily by its slow, scanty, colorless or white growth. Morphologically, the organisms resemble those of Group B. They always acidify glucose, and both sucrose and maltose usually.

*Group D.* Composed of thick, small, straight bacilli, often barred and wedge-shaped, showing no granules. The growth on serum may be scanty or abundant and is white or yellowish white in color. They do not act upon glucose, maltose, glycerin, or sucrose.
Morse names these four groups as follows: “Group A, B. Hoagii: Group B, B. flavidus: Group C, B. xerosis and Group D, B. Hoffmannii.” I have retained this terminology in my own classification, and have confirmed the validity of these various groups as they stand.

**DISTRIBUTION OF THE DIPHTHEROID GROUP**

The diphtheroids are among the most ubiquitous of all bacterial organisms. They are to be found not only in the bodies of animals and man, but also have a wide distribution in nature. R. O. Neuman (1902) and Sudeck (1896) first found them in the air. Wade and Harris (1915) and Torrey (1916) have made definite experiments on this point with positive results. Wade exposed 18 blood-agar plates to the air and recovered 45 colonies of diphtheroids. He has also isolated these organisms from the urine and feces. I have myself isolated them from the air of various laboratories. McNaught (Graham-Smith, 1908) has obtained them from water. McCambell (Graham-Smith, 1908) and Bergey (1904) have found them in milk.

It is of great interest to know that diphtheroids have been found in practically all the organs of the human body in either pure or mixed culture, and in both health and disease. Since the claim of their etiological relation to Hodgkin’s disease, there have been several reports regarding their presence both in normal lymph glands and in gland conditions bearing no relation to Hodgkin’s disease. Harris and Wade (1915) have written a report on the “Wide distribution of diphtheroids and their occurrence in various lesions of the human tissues.” Besides normal and pathological lymph glands (not Hodgkin’s) they have isolated diphtheroids from leiomyomata, fibroma and hepatic cancer, as well as from tuberculosis of lymph glands and lymphosarcoma. Fox (1915 b) has isolated them from the normal and diseased lymph nodes in many cases. Several strains came from lymph glands draining enlarged joints. Torrey (1916) in a special article on lymph gland bacteria, cultured 30 strictly lymph gland conditions and 10 conditions in which
the lymph glands were secondarily involved. He recovered the organisms from one type or other in 22 of the cases. Bloomfield (1915) reports a similar experience. Hoag (1907) finds the organisms in almost every part of the body but mainly in the respiratory tract. Bergey (1904) has found them in spontaneous abscesses in animals, and in cutaneous suppurations and in tumors. Hoag (1907), and Orr, Rows, and Robertson (1910) found these organisms in the central nervous system. J. A. Langford (1914) and Bunting and Yates (1914) found diphtheroids in the spleen in Banti's disease. Hektoen (1901) found them in large numbers in the liver, Axenfeld (1899) and others in the conjunctival sac, Walsh (1899) in the skin, Hall and Stone (1916) in the lymph glands of horses, sheep and calves. They have been found by many observers in the urethra (Hine, 1913) in the vagina (by Voigt, personal communication) and in the bladder (Townsend 1905). Rosenow (1915) has isolated them from the blood, joints, glands, lungs, skin, parotid gland, ganglia of the central nervous system and many other locations. I have myself isolated them from many locations in the body, but especially from the upper respiratory tract, skin, urethra, and lymph glands, less often from joints, blood, lungs and spinal fluid. This list could be indefinitely extended, but as no purpose could be served by so doing, I shall briefly summarize this topic by saying that the most common situations in which diphtheroids may be found in the human body are the mucous surfaces and skin, but a perusal of the references cited shows that no portion of the organism is exempt from them.

MORPHOLOGY

The size and shape of diphtheria-like organisms is subject to an almost infinite number of variations. Some of the subgroups have a fairly constant shape while in others the pleomorphism is so marked that almost any conceivable shape may be assumed as the environmental factors are altered. The detailed classification of diphtheria bacilli by Wesbrook includes all the ordinary and many of the rarer forms encountered in
the throat. The shapes most frequently met with are embraced under his three divisions; first the granular, second the barred and third the solid type. Type I shows spherical or oval metachromatic granules, usually located at the ends of the bacilli although they may be noted elsewhere. The protoplasm stains faintly (Loeffler's). Type II is the barred or segmented type and is characterized by great regularity of staining. The bars may be so arranged as closely to resemble streptococci and indeed may be mistaken for them. Cobbett (1901) designates this form as a separate morphological type. Neisser (1897) mentions a streptobacillus which is not unlike the Klebs-Loeffler organism. This streptococcoid form is not one that has received much recognition in the literature, but in the light of the relation between the streptococcus and the diphtheroid groups to be discussed later on, I believe that such forms merit a more careful consideration. The number of the segments or bars may be from 3 to 20 and they may or may not be metachromatic. The third or solid type is non-granular, may have almost any size and shape, and often appears as a diplobacillus. The different types ordinarily vary in thickness from 0.25 to 2 microns and in length from 1 to 8 microns.

The varied morphology of the diphtheroid group and the conditions which modify it, have been the subject of much discussion in the past. The question has hinged largely on the stability of the morphological and toxic types under varying conditions. A. Williams (1902) makes a thorough revision of this whole subject and in addition repeats the previous experiments as well as contributes new ones with a view to clearing up the question as to the fixity of biological types. Her conclusions are as follows:

Though some cultures change on some media, each changes in its own way and each culture still has its own individuality. Even though the morphology of a culture may be radically changed by alteration of media, etc., nevertheless when transplanted to the same media and under the same conditions that conduced to the establishment of the type, the original morphology will return.
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Morse (1912) also believes that the morphological individuality of a culture is retained tenaciously under good conditions. She studied 295 strains and was able to correlate definitely several morphological types with other fixed characters such as sugar fermentation, etc. Both authors say however that the strain must be observed through several generations and a constant morphology obtained, before attempting cultural changes to induce marked pleomorphism.

My own observations are for the most part in accord with these workers although there is one sub-group which has a pleomorphism so extreme that it will not conform to the above restrictions. Among the strains that I have studied there is a certain percentage described as B. enzymicus in Part I (Journal of Bacteriology, March 1917) which undergo a very remarkable change of form which remains quite constant and which is not brought back to type merely by restoring the original conditions. A barred, fairly long bacillus of this kind can be changed to a typical diplococcus or to a long-chained streptococcus (diplo-streptococcus, Part I, pp. 84-85, Journal of Bacteriology, March, 1917). When this form has developed it remains quite constant and although it is possible to cause it to assume, a bacillary form again, this is accomplished only with great difficulty. These forms are not involution forms in the ordinary sense of that term and are to be clearly distinguished from the ordinary coccoid form of diphtheroids and other bacilli. The coccoid forms are usually single, vary wonderfully in both size and staining characters and are almost always metachromatic. They take the stain very intensely and irregularly, and generally appear under rather adverse conditions. One usually has no difficulty in distinguishing them from true cocci. The streptococcus form is not distinguished from the true cocci by ordinary staining methods. Some of the strains which I have studied showing these characteristics are numbers 1, 3, 11, 13, 14, 16, 26, and 33 (see table 2).

INVOLUTION

This is a very common phenomenon among the different members of the diphtheroid group, being most frequent and
### Table 1

<table>
<thead>
<tr>
<th>Subject</th>
<th>Controls</th>
<th>Test Substances</th>
<th>Reaction</th>
<th>Amount of Gas Produced</th>
<th>Method</th>
<th>Reaction</th>
<th>Therapy</th>
<th>Reaction</th>
<th>Amount of Gas Produced</th>
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<th>Reaction</th>
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<td>+ - + - +</td>
<td>As on glucose</td>
<td>Healthy from first day, rapid growth</td>
<td>Normal</td>
<td>Healthy</td>
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<td>2</td>
<td>Human</td>
<td>+ - + - +</td>
<td>As on glucose</td>
<td>Healthy from first day, rapid growth</td>
<td>Normal</td>
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<td>Healthy from first day, rapid growth</td>
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<td>As on glucose</td>
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*These results were obtained in a series of experiments conducted in the laboratory.
most varied among the granular and barred types, while it is more unusual among the solid types, although some of them show it. Bunting (1913), de Negri and Miermet and many others emphasize the extreme pleomorphism of the so-called *B. Hodgkini*. Club forms and segmented forms are common, while almost all observers agree that the coccoid forms are so confusing that it is only by repeated platings that one may be sure that a pure culture is obtained. Fox (1915) shows photomicrographs of diphtheroids from the lymphatic glands, mostly of Hodgkin’s cases. Some of these develop long clubbed metachromatic forms, others assume very long sinuous filamentous forms. Others occur as threads, some resemble short chains (streptococcoid forms) while some are fused and branched.

Neisser (1884) and Eyre (1896) speak of the marked clubbing of the xerosis bacillus on dried glycerin serum. Rosenow (1915) mentions the remarkable involution forms of the causative diphtheroid of erythema nodosum. In ascitic-glucose broth only bacillary forms appeared, while on blood-agar plates, he recovered small diplococci in short chains.

Strain 1 in the series under investigation in this study showed very similar characteristics. In ascitic glucose broth, growth was slight with no clouding of the media. No coccoid forms developed under these conditions. The bacilli were barred and granular. The granules stained intensely with Loeffler’s stain while the protoplasm stained faintly. Branched forms were numerous after four days, and on several occasions the most extraordinary filamentous network was seen; this branching form of the organism resembled closely certain of the streptothrices. On the nodal points of the network one or more granules of heavily staining chromatin were to be seen. These granules were of varying size and often stained metachromatically. The network itself stained faintly, and resembled debris but on closer examination and differentiation proved to be a direct outgrowth of the organism. The shorter fused forms described by Park and Williams (1910) were also not uncommon in this medium and were usually associated with the more complex structure just described.
On blood-agar slants the ordinary coccocid forms developed. The size of the cells varied between wide limits. They grew singly or in clumps, seldom as diplococci and were spherical or ovoid in shape. They stained intensely in Gram or Loeffler's stains and metachromatism of varying grades was the rule. With careful differentiation or double staining, marked chromatic irregularities appear. A more complete description of this strain will be found in Part I, Journal of Bacteriology, March, 1917.

GROUPING

Most characteristic among the various possibilities of arrangement is the palisade grouping. This was described by the early writers Neisser (1897), Prochaska (1897), and others, and has been emphasized by the more recent contributions to this subject, Park and Williams (1910), Teoumin (1913). V-shapes and diplobacilli are also very common. It is also usual to see diphtheroids in clumps or in tangled masses.

STAINING

As a general thing diphtheria like-organisms stain readily and intensely with the simple anilin dyes. Occasionally one encounters a strain of granular bacilli which reacts faintly to Loeffler's but in such instances Gram's stain produces excellent results.

With Gram's stain one finds all gradations of reaction. It is usually stated that all diphtheroids are Gram-positive. Prochaska (1897) says that "decolorization must not be too energetic." Hamilton (1907) describes a Gram-negative diphtheroid. De Witt (1912) speaks of Gram-variable diphtheroid bacilli. I have referred to the effect of old or dry media, the age of the culture, etc., on the intensity of the Gram stain with the so-called B. Hodgkini, (Mellon, 1915). "In some cultures all grades of reaction to this stain might be seen; in others, the entire culture was finally decolorized." "Coccoid forms usually take the stain more intensely than the bacilli." This has also been observed by Steele (1914) and others.
No matter how pleomorphic a strain may be, the chromatin granules are practically always Gram-positive, while the body of the bacillus or projections therefrom are usually Gram-negative, if the culture is relatively young. The filamentous forms which I have described above as well as in another communication, are always Gram-negative (Mellon, 1915). These variations are much more noticeable in the granular and barred types than in the solid ones. So although the diphtheroids can properly be classed among the Gram-positive bacilli, the limiting conditions must be very strictly adhered to, and even then some strains will be doubtful.

The principle holds equally well regarding the property of "fastness" to acids. Diphtheroids are usually described as being non-acid-fast, and as a general proposition this is true. Nevertheless Wade and Harris (1915) believe that some strains are acid-fast. They class *B. smegma* among the diphtheroids and its reaction in this regard is well known. In addition they have isolated a diphtheria-like organism from the mesoappendix which liquefied blood serum and gelatin, was Gram-positive and acid-fast. Wolbach and Honeij (1914) report "partial acid fastness of a diphtheroid isolated from leprosy lesions." The granular portions of these bacilli are fast to 2 per cent H$_2$SO$_4$ followed by 95 per cent alcohol; and 3 per cent HCl in 95 per cent alcohol, reagents acting for 30 seconds. Similar treatment completely decolorized the Klebs-Loeffler and mixed cultures of cocci and bacilli from the throat.

Kedrowski (1901), Bayon and Williams (Harris and Wade, 1915) have cultivated diphtheroids from leprosy, each regarding his culture as a stage in the life cycle of the Hansen bacillus. Kedrowski assumes a mutation from a non-acid-fast to an acid fast organism because he recovered the latter after injection of the former. Campana and Babes (1910) thought Kedrowski was working with mixed cultures since they could not confirm his results. Duval and Duval and Harris also quoted by Harris and Wade have not been able to repeat these experiments. Dr. Eggers tells me that Kedrowski's work has been recently confirmed. Out of 45 colonies of diphtheroids on blood-agar plates
exposed to the air, Harris and Wade (1915) recovered 7 which were distinctly acid-fast.

I have experimented with quite a number of strains in this regard. I used as a routine 1 per cent HCl in 20 per cent ethyl alcohol and 2 per cent HCl in 50 per cent ethyl alcohol as a decolorizing agent. My results are tabulated in table 2. Out of 37 strains treated with 1 per cent HCl in 20 per cent alcohol for one and one-half minutes following previous treatment with carbol-fuchsin, I found that 13 strains retained the stain very definitely. The remaining strains were either decolorized or doubtful, and seven were completely decolorized. Six strains resisted 2 per cent HCl in 50 per cent alcohol. The granules held the stain tenaciously but often the body of the organism was pink also. The results were fairly constant on Loeffler's medium and 2 per cent glucose-agar but all strains that would grow on potato were either negative or doubtful. More constant results are claimed by Rehr (1915) using alcohol-acetone to decolorize the bacilli previously stained by Gram. He claims to be able to diagnose a much larger percentage of cases of true diphtheria by this means.

Here again is seen a lack of constancy in reaction, depending not only on the individuality of the strain but also on the chemical constitution of the medium. My experience with the staining properties of this group gives me the impression that the diphtheroid protoplasm is very labile, very dependent on factors of which we know little, and which we cannot control. For this reason I am rather skeptical about drawing very definite conclusions of any kind from evidence which has as its basis the retention of a stain by such an ephemeral matrix.

The polychromasia or metachromasia of various members of the diphtheria group has long been known and commented upon. It is quite characteristic for members of the granular group to exhibit reddish or magenta colored chromatin. Under certain conditions these granules may become a very brilliant red even when a pure Loeffler's stain is used. A. Williams (Park and Williams 1910) believes "that the metachromatic masses occurring in involution forms of B. diphtheriae represent
a primitive sexual process, a sort of autogamy." She believes that disturbance of the culture at intervals facilitates this process. I have been able to verify this observation although it occurred as a result of routine examination of a culture in broth. Strain number 16 of my series grew faintly in ascitic broth and only in the bottom of the tube. A transplant was made October 23, and examined October 24, 26, and 29, 1915. No metachromatism was noticed. On October 29, I shook the tube vigorously to try to induce a heavy growth, which just began October 30. At examination at this time showed the large clubbed and branched forms and the immense bright red granules above referred to. When examined on an agar hanging block these forms showed very active growth and a fusion of the metachromatic granules which led Williams to interpret the process as a primitive sexual one.

Strain 29 of my series also showed a remarkable series of pictures covering but two microscopic fields. The transplant was made to fresh potato, which developed a very granular organism from one that had the chromatin concentrated in the ends of the bacillus and which did not resemble the Klebs-Loeffler bacillus. It was stained in carbol-fuchsin and decolorized in the weak acid-alcohol above referred to and very lightly counter stained with weak Loeffler's. The very remarkable forms shown above were found at about one place in the cover-glass, due I believe to the fact that I barely touched the end of the wire on a very moist growth and drew it across the cover-glass but once. These forms are surely very suggestive of the karyokinetic figures so common in the higher forms.

**MOTILITY**

None of the cultures in my series showed motility and I have been able to find only one instance recorded in the literature.

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1 Schaudinn has shown a primitive conjugation in *B. Bützchii*. 
De Witt (1912) describes one such organism which she proved to be the cause of the pathologic condition from which it was isolated.

CAPSULES. SPORES

I have demonstrated capsules in my strain number I, isolated in coccus form from an animal. I have not been able to find any reference to such in the literature. There are many comments on the absence of capsules, spores and motility among the diphtheroids.

CULTURAL CHARACTERISTICS

Glucose-agar

The appearance of the colonies on this medium varies with the subgroup and very often with different strains of a subgroup. B. xerosis exhibits a dry, transparent film which gives the slant a ground glass appearance. This is not changed on prolonged incubation or by standing at room temperature. All observers agree on this point. B. flavidus also has a dry granular growth which may or may not be pigmented, but is usually much more luxuriant than B. xerosis. These are the only two subgroups of this species to give a dry growth.

Typically, diphtheroid colonies are very moist. Prochaska (1897) describes precise, whitish-grey colonies, which enlarge rapidly and form an elevated growth. They are dark in the center and clear on the border, which may be serrated. When pigment is present it varies from light or lemon-yellow, to orange or red. Some have a brown pigment. Hamilton (1904) has described a strain giving a purple color on agar. Prochaska (1897) calls attention to the fact that as the medium dries slightly the center of the colony may fade. My strains 4 and 5 faded out completely, even though the tubes were sealed. The growth became perfectly transparent. This is not a usual feature. Some strains refuse to grow on serum-free media when first isolated but can usually be trained to do so. The more saprophytic subgroups, B. Hoagii or B. diphtheroides-liquefaciens
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give an abundant moist, coalescing growth which proceeds slowly at room temperature.

Blood agar

This medium is conducive to more luxuriant growth of all strains. The typical colony has a dark center and light border. Pigment is more likely to develop on blood-agar. Not infrequently the colonies are stippled. B. flavidus gives a yellowish, spreading, adherent growth which is wrinkled or corrugated radially. Some strains show concentric wrinkling. An occasional race is hemolytic. The bacillus of Preiz-Nocard and of Hall and Stone (1916) was markedly so. My strains 13 and 14 were slightly hemolytic. It is not uncommon for a blood-agar plate to become brown from the acid produced. Not infrequently the colony takes up the changed hemoglobin leaving the surrounding medium colorless.

Blood-agar plates offer a ready means of determining the purity of a culture on account of the various forms of colonies that develop. I have been able to separate different types of organisms by the type of colony produced. This is by no means an infallible criterion, since the variants of a pure strain may show different types of colony. It is often necessary to make several rapid transplants, when the colonies will then be alike in case the strain is pure.

Loeffler's blood serum

The various members of the group grow well on this medium, giving as a rule, moist, transparent or opaque colonies with the fastidious strains, and heavy, opaque, confluent growths with the saprophytic ones. There is little opportunity for colony differentiation with this medium. It is usually the best medium to indicate proteolytic change. De Witt (1912), Hamilton (1904), Wade and Harris (1915) and Graham-Smith (1908) have all described proteolytic strains of this group. One strain of my series (32) was a rapid liquefier of serum.
Gelatin stab

The majority of races will grow in gelatin, some of them quite luxuriantly, although it is the exception to have liquefaction occur. Most of the liquefiers are found in the subgroup *B. diphtheroides-liquefaciens*. Strains 4 and 5 of my series caused a slow liquefaction although they belonged to the *B. Hoagii* subgroup. Pigment formation is not uncommon in gelatin. Viability is well preserved. Strain 3 was alive after eighteen months on this medium. Ordinarily one sees a greyish-white growth which not uncommonly extends along the entire line of inoculation, inasmuch as this group readily adapts itself to anaerobic conditions. Strain 14 developed single, thin, spreading colonies along the upper third of the stab. They were dark in the center and had a lighter serrated border. Strain 21 developed a "nail-head" culture for a few days, then a funnel shaped liquefaction along the entire line of inoculation. Strains 31 and 42 grew mostly in the bottom of the tube showing their preference for anaerobic conditions.

Broth

Here again one finds great variation although there are two main types of growth; first, the formation of scanty, granular, flocculent or nebulous material that collects along the sides or bottom of the tube after twenty-four hours leaving a clear supernatant; second, the heavy, luxuriant, diffusely turbid growth, depositing a finely granular or mucoid sediment. Certain races develop a thin, friable pellicle which is deposited as a flocculent sediment on slight agitation of the tube. *B. xerosis* and *B. flavidus* more often give rise to a pellicle as they are more closely related to *B. diphtheriae* than the others. *B. enzymicus* grows very scantily in broth although it can be made to grow luxuriantly by the method described in Part I, Journal of Bacteriology, March, 1917. The broth has a foul or sour odor depending on whether acid or alkali is produced. De Witt (1912) alone has ascribed a peculiar odor to her cultures. She says that it resembles acetone, and was identical with that coming from the
lesions of the patient. I have observed a very unusual odor with strains 2, 11, 13, and 14. It is always noticed when a carbohydrate is fermented, and it resembles the odor of the esters of the higher alcohols. It resembles amyl alcohol very closely or the heavy sweet odor that comes from lilies. The fact that strains 13 and 14 were isolated from a case of gonorrhoea suggests the idea that these organisms may be a factor in the repugnant odor of a case of this kind. It is known that the odor of the soil is often due to bacterial activity. It is interesting that all these strains belonged to the *B. enzymicus* subgroup.

*Litmus milk*

A majority of my strains caused but little change in this medium. The Ruediger bacillus (Ruediger, 1903) completely decolorizes litmus although no acid is produced. This feature constitutes the most noteworthy cultural characteristic of the organism. This is probably a reduction change. Several of Fox’s (1915) gland diphtheroids reduce litmus. Strains 21 and 1 in my series exhibit this action. Graham-Smith mentions this change in his *B. diphtheroides-liquefaciens*. It most commonly occurs in saprophytic races producing alkali. Lactose is fermented as frequently as are some of the other sugars. This sugar is acted on pretty constantly by *B. diphtheroides-liquefaciens* and *B. enzymicus*. It is rarely acted on by the other sub-groups. When a coagulum is formed it is usually due to the production of acid, although the experiments of Fox (1915 b) and others indicate that certain strains have a coagulating enzyme. In many instances the amount of acid formed was not great enough to coagulate the milk unless it was boiled.

Alternation between red and blue was observed not infrequently in some of my strains which were feeble acid producers. This may perhaps be due to the possibility that there is a small percent of glucose in the milk which undergoes fermentation, the resulting acid entering into combination with some neutral or buffer substance in the milk, only to be thrown out again as the resultant alkaline fermentation displaced it. With two of my strains the litmus cleared and became of a bordeaux-red color.
Potato

The same variation which characterizes the appearance of the various races on other media holds true for this one. In general it may be said that the more luxuriant growers are adapted to this medium. Pigment is readily produced on potato. The growth is generally very moist or slimy and not infrequently is of a dirty grey color. The majority of Fox's (1915 b) strains show no growth on this medium and the same can be said of mine. However when used in the proportion in which it is found in Bordet's medium potato seems to exert a favorable influence on all forms. Particularly has this been true in the isolation of B. Hodgkini, but this form grows as well if not better on glucose-blood-agar.

Mutation

Roux and Yersin and other of the earlier investigators claim to have been able to produce a diphtheroid from a Klebs-Loeffler bacillus by growing it at high temperature. Hewlett and Knight (Park and Williams 1910) Richmond and Salter (1898) claim to have transformed B. Hoffmanii into the Klebs-Loeffler bacillus; the former by culture and passage through guinea-pigs and the latter by passage through gold finches. Bergey (1904), Williams and others have not been able to obtain these results. Fox (1915 a) attempted mutation by culture on different lymph gland media but was unsuccessful.

Thermal Death Point Determinations

Twenty-four hour cultures of salt suspensions of the bacilli were filtered through cotton-glass-wool in order to remove the clumps. The homogeneous suspension was then drawn into thin walled capillary tubes 2 mm. in diameter by 8 cm. long. These were placed in the water bath at varying temperatures for varying lengths of time and then cultured for one week in glucose-serum-broth. The results are indicated in tabular form.
It will be seen that 60°C. was fatal to various strains of this group under the conditions of the experiment. The antiformin-fast strain x-323 was not affected by ten minutes exposure at 55 while the growth of the others was markedly inhibited.

ANTIFORMIN TESTS

Salt suspensions of the various strains after being filtered through cotton-glass-wool were mixed with equal parts of antiformin and placed at 37.5°C. for two hours. They were then washed with NaCl twice, centrifuged in NaCl and the sediment stained by Gram and cultured on blood-agar. The results are shown in table 2. There seems to be the same variability regarding this test that is displayed by the group in other respects.

The B. Hodgkini strains seemed to yield more of the Gram-positive organisms in the sediment than did the other races which showed a tendency to be resistant to antiformin. None of them could be cultured following this treatment. It would not seem that antiformin resistant organisms are limited to any one subgroup of the diphtheroids, although this resistance becomes progressively less as we approach the saprophytic members. Hall and Stone (1916) report their B. flavidus as non-resistant to the reagent and Fox's (1915 b) observations tend to show that the reaction is non-specific.

OXYGEN

Most of the races are facultative anaerobes, as may be inferred from the cultural results on gelatin stabs. A few instances of
strict anaerobiosis have been reported. Dick (1915) describes a Gram-negative and a Gram-positive strict anaerobe isolated from the urine of cases of chronic non-suppurative nephritis. Torrey (1916) has isolated an anaerobe from the lymph glands of Hodgkin's disease which he has called B. lymphophilus. Bloomfield tentatively makes a "partial pressure" group of diphtheroids. He says that they are slow growing and rather anaerobic. Rosenow has often noticed this quality in both diphtheroids and streptococci. Strain 2 of my series was isolated from the blood and urine as a strict anaerobe. Voigt has isolated strict anaerobes from the vagina, (personal communication).

**SUMMARY OF MORPHOLOGY AND CULTURAL CHARACTERS**

The diphtheroids readily adapt themselves to artificial media, this being more noteworthy in the subgroups B. diphtheroides-liquefaciens, B. Hoagii, and B. Hoffmanii. The more fastidious strains usually require the presence of serum in the medium. Viability is prolonged on all media, especially gelatin. The same strains may contain variants giving rise to different types of colonies which increases the difficulties of isolation. Blood-agar is a favorable medium for separating mixed cultures. In general, both cultural and morphological characteristics can be correlated with the sugar and complement-fixation tests.

**FERMENTATION**

Sugar fermentation has been regarded for a long time as one of the best criteria for the separation of bacteria into classes. On reviewing the literature on this subject I was astounded at the number of apparently contradictory results obtained. Further study regarding the large number of factors involved in this reaction, some of which are beyond control, together with the variety of methods used, caused me to accept most gratefully any uniformity that might be discovered.

Before citing the results of the various observers in this regard, a discussion of some of the difficulties regarding fermentative
reactions in this group might be in order. When it was first discovered that the Klebs-Loeffler bacillus produced acid in glucose broth and B. Hoffmanii did not, a distinction between the two organisms was based on this point. Gradually it was found that there existed other diphtheria-like organisms which fermented glucose but which were non-toxic. Accordingly, for a long time various observers described different strains of diphtheroids, making their observations only on glucose and attaching a sonorous name to the organism, which was based very often on some minor characteristics, most commonly, the morphological appearance. The small number of sugars used was the first difficulty in the way of a successful grouping of different strains.

When more sugars were employed, each observer had his own method of determining when fermentation had taken place. Even to the present time a variety of methods is used, many of them being very questionable for a procedure of this kind. Few observations have been quantitative. Litmus has been freely used as an indicator both in liquid media like Hiss serum water and in solid media as well. With scantily growing organisms like some of the diphtheroids it is very difficult to ascertain whether growth has actually taken place in the presence of litmus. It is also subject to reduction decolorization changes which are not infrequent in this group. But its greatest fault, particularly when used with the diphtheroids is its lack of delicacy. Generally speaking these organisms do not violently attack sugars, and many reactions which are positive to phenolphthalein are lost with litmus.

For example: Fox (1915 b) in studies as late as 1915 reports that “observations on carbohydrates were made on litmus-agar-sugar mediums of reaction neutral to phenolphthalein.” In this case the organism would be compelled to produce at least 1.5 percent acid before litmus would register it, a neutral phenolphthalein reaction being alkaline to litmus to the extent of 1.5 to 2.5 percent. Since on many sugars, a variety of strains will not produce this amount of acid it is very easy to see how negative results might be obtained. Hiss serum-water with litmus as an
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**Sub-group B. flavidus**

| STRAIN NO. | T-337, 35 | 0.0 | -0.3 | 1.3 | -0.4 | -0.5 | 1.6 | -0.6 | 0.0 | -0.4 | 0.0 | +++ |

**Sub-group B. Haoctii**

| STRAIN NO. | T-337, 35 | 0.0 | -0.3 | 1.3 | -0.4 | -0.5 | 1.6 | -0.6 | 0.0 | -0.4 | 0.0 | +++ |

**Sub-group B. liquefaciens**

| STRAIN NO. | T-337, 35 | 0.0 | -0.3 | 1.3 | -0.4 | -0.5 | 1.6 | -0.6 | 0.0 | -0.4 | 0.0 | +++ |

**Sub-group B. Hoffmanii**

| STRAIN NO. | T-337, 35 | 0.0 | -0.3 | 1.3 | -0.4 | -0.5 | 1.6 | -0.6 | 0.0 | -0.4 | 0.0 | +++ |

**Sub-group B. Ruedigeri**

**Sub-group B. xerosis**

| STRAIN NO. | T-337, 35 | 0.0 | -0.3 | 1.3 | -0.4 | -0.5 | 1.6 | -0.6 | 0.0 | -0.4 | 0.0 | +++ |

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indicator is often used but its disadvantages can be easily seen, and it should be displaced by more accurate methods. If one wishes to work with a medium of this sort, by far the most delicate and satisfactory is the one devised by Holman (1914) for streptococcic work. Andrade's reagent (basic fuchsin) which is the indicator used in this medium has the advantage of being very sensitive to the presence of acid. It is rather tedious to prepare however. Other observers have used neutral red in solid media which is also more delicate than litmus (Teoumin, 1913).

A third source of diversity is found in the length of time the cultures remain in the incubator. Variation in this regard has ranged from two or three days to an equal number of weeks. With a frankly alkaline or frankly acid producing organism this factor would not be of much moment, but with a group that at one time may produce acid and at another alkali in the self-same medium, it is obvious that it becomes an important factor for control if any uniformity is to be reached. Morse (1912 a) in her biometrical study of the diphtheroids has done more to correct this error than anyone else. She experiments with various strains on various sugars and finds that the sugars have a "time constant" for the production of maximum acidity which is different for every sugar. After this acid limit is reached, the organism attacks the protein of the medium and an alkaline reaction results. This has been shown by Kendall (1911) and Theobald Smith.

It should also be mentioned that adequate care must be taken to be sure that growth has occurred, as in its absence one would not expect fermentation. With doubtful strains this becomes a laborious matter in turbid or colored media. Chemically pure sugars should also be obtained in order to avoid another possible source of error. It is also important that sugars be fractionally sterilized as many of them are broken down by autoclaving.

It seems unnecessary to mention that the cultures tested should be pure. I have obtained a number of cultures from other sources which were free from organisms other than diph-
theroids but which contained more than one type of diphtheroid as was evidenced by the different sugar fermentations of the isolated strains.

Again it seems probable that certain races may at times lose their power of attacking certain sugars. I do not believe that this is a common occurrence especially outside of the body, but we have evidence that it does occur. J. H. Brown (personal communication) has definitely proved that such changes take place among the dysentery strains, and we have reasonable evidence that an organism may lose its power to ferment one sugar or acquire it for a sugar which it did not have, and still retain its other characteristics. *B. xerosis* is usually described as fermenting sucrose, yet I have a strain (number 12) typical in every other way and which on repeated tests did not ferment sucrose. Again Knapp (1904) finds a positive reaction to man-nite while Zinsser is unable to verify the fermentation of that particular carbohydrate. Much more striking is the fact that *B. diphtheriae* is described as never fermenting sucrose, yet Graham-Smith (1908) has described a whole epidemic from which he obtained a sucrose fermenting true Klebs-Loeffler bacillus. The variability of the Klebs-Loeffler bacillus in this respect is well established.

Without doubt the best classification of the diphtheroids on the basis of sugar fermentations is the “Study of the diphtheria group of organisms by the biometrical method” by Morse (1912 a) As noted above she separated the diphtheroids into four main subgroups as follows:

(a) Group A is *B. Hoagii* which she names after Hoag who first described thoroughly a great many strains of this organism. She says that it ferments glucose and sucrose but not maltose. Regarding this last sugar she does not agree with Hoag since in his original article he states definitely that maltose is fermented and his tabular results bear out the statement. However this does not change the grouping. This organism was been described by more observers than any other of the diphtheroids except possibly *B. Hoffmanii*. About half of my *B. Hoagii* strains ferment maltose while the other half do not. On this basis it
CLASSIFICATION OF THE DIPHTHEROID GROUP

will be very easy to place the so called *B. paralyticans* described by Ford-Robertson, (Morse, 1912 a). It ferments glucose, maltose and sucrose, and Morse is unable to place it on account of its maltose fermenting properties. It falls very nicely into one *B. Hoagii* subgroup. It can be very properly compared to the Klebs-Loeffler bacillus in its relation to lactose, and brings out a point which I wish to emphasize viz., that the fermentative history of this whole group shows that no hard and fast lines can be laid down, but that all the characteristics of an organism must condition its allocation.

(b) Group B is the second class of Morse' diphtheroids which she has named *B. flavidus*. Glucose is always fermented, maltose and glycerine usually but not sucrose.

(c) Group C or *B xerosis* always acidifies glucose and usually both maltose and sucrose.

(d) *B. Hoffmanii* is a nonfermenter.

Fox (1915 b) has studied a large number of strains from Hodgkin's glands as well as lymph glands from other conditions, particularly those draining enlarged joints. He tested the different strains on glucose, lactose, sucrose, maltose, mannite, glycerin, dextrin and galactose, but was unable to discover any striking regularities by this method. He did not work quantitatively and the other conditions which I have discussed on page 291 may contribute somewhat to the irregularity. However one seeming generality is noticed. The organisms isolated from the glands draining diseased joints have as a class a wider range of fermentation than the other strains. The possible significance of this fact I shall discuss later.

Hine (1913) attempts to classify the urethral diphtheroids by means of sugar reactions. He uses glucose, sucrose, maltose and lactose. Litmus is used as an indicator and seven days is the incubation period. The first class ferments no sugars (*B. Hoffmanii*); the second ferments glucose, sucrose and often maltose (*B. Hoagii*) and a third which he calls *B. coryza-segmentosus* ferments glucose, or glucose and sucrose. The urethral group proper ferments all sugars but lactose. These do not correspond to any of the other classes.
Torrey (1916) states that since no classification of the diphtheroids exists, he is not able to place accurately the various strains which he has studied, but forms three arbitrary groups for them based partly on sugar fermentation and partly on morphology. The vast majority of his strains fall in two common groups viz., *B. Hoffmanii* and *B. Hoagii*. From his brief description of some of his miscellaneous strains they resemble closely the *B. xerosis* or *B. flavidus* subgroup.

Teoumin (1913) studies the sugar reactions of 20 strains of pseudo-diphtheria. He has placed them in four classes yet they all have the same fermentative reactions, namely positive glucose and maltose. It is very evident that these groups correspond to Morse’s *B. flavidus* and are identical with Hamilton’s Group II.

DeWitt (1912) describes a pathogenic strain closely resembling *B. proteus* and producing both acid and gas in broth. Hamilton (1907) has also described several gas producing strains, and has studied 57 strains which fall into two groups. Her Group I had 40 representatives and fermented glucose and sucrose but was negative to maltose, lactose, and dextrin. This corresponds to Morse’s *B. Hoagii*. Group II ferments glucose and maltose but never sucrose. Dextrin is 60 per cent positive and lactose 10 per cent. This corresponds to Morse’s *B. flavidus*. Group III is the Ruediger bacillus which produces a soluble toxin but ferments none of the sugars.

It would seem that the following sugar fermentation method advocated by Theobald Smith is the best one which we have at present; and I have used it in my work. Broth rendered sugar free by previous treatment with *B. coli* is given a reaction of plus 0.8 to plus 1 to phenolphthalein. Each tube contains 9 cc. One cubic centimeter of a 10 per cent solution of the various sterile sugars is then added to each tube with a sterile pipette, and incubated for forty-eight hours to insure freedom from contamination. The sugars are titrated on the eighth day, as at this time, a maximum acidity is produced in maltose, mannite, dextrin, lactose, raffinose, salicin and inulin. If no acid is formed with glucose, sucrose or glycerin at this time, the
latter is again titrated at the sixteenth day and the former sugars at the twelfth or thirteenth day. This latter procedure was adopted following the suggestion of the Morse article. A control tube was always incubated when the reaction determined on the sugars was titrated, since it was found by actual experiment that a variation of between 0.3 and 0.4 per cent might be produced as the result of evaporation of the medium with concentration of the acid it contained.

Although this method is the most accurate and serviceable of any in use at the present time, it has some faults which it might be well to point out and which stand in a fair way to be corrected in the near future by the application of more accurate physicochemical methods. It has been known for some time that the titratable acidity of a medium is not its actual acidity but only forms a relative and often an inaccurate guide to an estimation of the actual concentration of H-ions contained therein. This idea has been worked out and elaborated very convincingly by Clark (1915). To begin with, he shows that the titrametric method of arriving at the reaction of a medium is inadequate as at present used. He points out that the only correct means of arriving at its true reaction is by an estimation of the H-ion concentration. All nutrient media contain in varying proportion the so called “buffer substances” of which peptone and phosphates are two familiar examples. These substances are able within limits to absorb acid as it is produced by an organism. The organism is thus protected from its action and is enabled to produce further acid. When titration is performed the free H-ions are neutralized and then dissociation of the buffer substance takes place yielding more H-ions. In this way the same organism might produce a much larger amount of titratable acid on one medium than on another. This is one way in which to account for the very large amount of acid produced by a number of Morse’s strains.

Clark also criticizes severely the titration of media while hot. He claims that the enormous amount of dissociation resulting causes a variable reading.

W. T. Bovie (1913) has recently devised an ingenious method
for measuring the H-ion concentration. It will still be some time however before one is able to make such determinations with facility, but it indicates a great advance toward the solution of a problem which has given much trouble to all who have wished for a really accurate notion of the reaction of bacterial culture media.

I have used ten fermentable substances with the various strains in my series. The monosaccharide glucose; the disaccharides, sucrose, maltose and lactose; the higher saccharides, raffinose, dextrin, inulin and salicin; and the alcohols, glycerin and mannite. The results in the appended table 3 represent the percentage amounts of normal NaOH required to neutralize the acid developed by the organism. Phenolphthalein was used as an indicator. A minus sign preceding a number indicates alkali production. A special column for growth is shown which shows that any negative results were not due to the fact that insufficient growth was obtained. Plus three indicates a luxuriant growth plus two, moderate; and plus one a scant growth.

ANALYSIS

From a survey of the sugar reactions, it is seen at the outset that it will be neither possible nor necessary to exclude from a subgroup an organism which does not ferment precisely the same substances as the original strain after which the subgroup was christened. However desirable such a clean cut grouping might be, there is no criterion of classification among these organisms which will be rigidly constant. I have already indicated a great many reasons why this must be so, and have substantiated my contention with evidence collected from the literature.

None of my strains were aerogenic. Some strains produced no acid on any of the sugars, while others had a wide fermentative range. Between these extremes one finds all gradations. Quantitatively the diphtheroid group does not produce a high percentage of acid, although some of Morse's strains compare favorably
with the amount produced by the streptococci. Torrey (1916) also describes an anaerobe producing a very high amount of acid. Six of the strains were B. Hoffmannii, 10, 24, 25, 31, 43, and 44, producing no acid from any sugar, but often yielding an alkaline reaction. Strains 4, 5, 7, 8, 19, 34, acidified glucose and sucrose constantly and in some cases salicin or inulin. Strain 34 also fermented maltose. These strains correspond to B. Hoagii of Morse. One strain, 12, fermented glucose and dextrin and was obtained from Dr. Wolbach's laboratory as B. xerosis. This organism usually ferments sucrose also but did not do so in this case. It was typical in all other respects. Strain 32 might be considered a variant of B. Hoagii. Strain 35 fermenting maltose and glucose was typical in all respects of Morse's B. flavidus.

Strains 1, 2, 3, 9, 11, 13, 14, 16, 33, 37, 38, 39, 41, 42, and 45, although isolated from a variety of sources, I have placed in one group which I have called B. enzymicus on account of the wide range of sugar fermentation which they possess. It is certain that these organisms fall into a group which is different in many ways from the other groups. They ferment from six to ten sugars which is a phenomenon that has been commented on only once before to my knowledge. Rosenow (1915) mentions that his erythema nodosum strains have a wide range of fermentation although he does not say how many sugars are fermented.

It is possible that this group may have to be further subdivided at a later time. Strains 37, 38, 39, and 40 were given me by Dr. Bunting and were isolated from Hodgkin's glands and in addition were strains with which he had produced results in animals. It is noteworthy that qualitatively and quantitatively they are practically identical. Strain 3 is a strain of my own isolated from Hodgkin's glands which has nearly the same reactions as Bunting's strains. The remaining characteristics of this subgroup have been discussed in Part I, Journal of Bacteriology, March, 1917. Strain 45 (one of Bunting's strains from pseudoleukemia) ferments all ten sugars and is thus fairly closely related to the Hodgkin's strains in that it...
is a powerful fermenter. However the Hodgkin's strains do not ferment inulin and salicin although strain 37 attacks raffinose slightly. The chronic leukemia strain number 44 and the Banti strain number 43 ferment none of the sugars, and on this basis must fall into the B. *Hoffmanii* class or the B. *Ruedigeri* class. The latter however has a soluble toxin. These strains are both from Dr. Bunting.

In Chart I, I have indicated diagrammatically the interrelationships of the various subgroups and their connection with the Klebs-Loeffler bacillus, as well as the relation of the entire diphtheria group to other micro-organisms. The broken curved line bounds a segment of a circle which represents the
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domain of the pathogenic forms. Since there can be no hard and fast line separating the pathogenic and saprophytic fields, the division is pictured by a broken line. The larger area surrounding the arc of this circle represents the domain of the saprophytic organisms. The entire diphtheria group is shown by the medium sized circle occupying the border line position, part lying in the saprophytic field and part in the pathogenic.

The individual subgroups of diphtheroids are shown by small circles, and are shaded or not according as they fall within or without the pathogenic field. A double solid line connecting the subgroups indicates a close relationship; a single solid line indicates a moderate linking and a dotted line a distant relationship. A curved line indicates a mutation.

Beginning at the small area indicating the *B. diphtheriae* there is shown a close direct relationship between it and *B. flavidus*. If there is any organism which could properly be called an avirulent diphtheria bacillus this is certainly the one. I believe that this designation should be applied to an organism similar in most respects to the Klebs-Loeffler bacillus but lacking the specific toxin formation of the latter. In the dry growth on blood and serum media, the firmness of the colonies and their adherence to the media in old cultures, *B. flavidus* is much more like the diphtheria bacillus than are most of the diphtheroids. It also forms a very distinct, friable pellicle on glucose-broth which falls to the bottom on slight agitation of the tube. Involution forms appear in the older cultures. It ferments glucose, maltose, and glycerin, but not sucrose. It is pathogenic for guinea-pigs but does not produce the lesions of the diphtheria bacillus. Most noteworthy was the evidence of complement fixation tests, (see Part III). The fact that this strain was practically alone in its separation from the other strains was noticeable. It is quite closely related to *B. xerosis* (strain 12), a fact that Morse's complement fixation work has also brought out. Hamilton (1907 b) also believes this glucose-maltose fermenter to be closely related to the true diphtheria bacillus. Four of the seven strains which she studied killed guinea-pigs. She describes it as Group II of her classification of the diphther-
oids, but refers to it as *B. xerosis*. Morse has identified Hamilton's Group II as *B. flavidus* of her own classification. The strain which I have studied is one that Teacher (1915) has shown to be the causative organism in an epizootic of infective abortion in guinea-pigs. To reiterate briefly: One finds the cultural, morphological, immunological and sugar reactions of *B. flavidus* to be strikingly similar to those of the true diphtheria bacillus, but inasmuch as it produces no specific toxin it must be classed among the diphtheroids.

Closely related to the diphtheria bacillus as well as to *B. flavidus*, is *B. xerosis*. It also has a scant, dry, adherent growth and a diphtheria-like morphology. It is described as characteristically a sucrose fermenter, although the strain which I have studied has no action on this sugar. Its complement-fixation and agglutinin reactions show that it is closely related to *B. flavidus*. It has only been described by one observer as having been pathogenic for guinea-pigs (Eyre, 1896), producing only local edema, decided loss of appetite and weight. Escherich and C. Frankel (1896) and F. Schanz (1896) have all noted the strong resemblance between the avirulent Klebs-Loeffler bacillus and *B. xerosis*. The only reference to its pathogenicity in man is the report of Eyre (1896) who isolated it from 15 cases of chalzion, and reproduced the condition in the eyes of animals. Accordingly I have given it a very close relationship to both the Klebs-Loeffler bacillus and *B. flavidus*.

*B. Hoagii* is an entirely different type of organism from the previous two described. It is a luxuriant grower on almost any kind of media, under almost any conditions, and has the widest distribution of any of the subgroups. Hoag describes it as fermenting glucose, sucrose, and maltose. He has isolated 150 strains of it. The so called *B. paralyticans*, supposed by Ford-Robertson and others to have an etiological relationship to paresis is merely the saprophytic *B. Hoagii*. It has the same sugar reactions. Hamilton (1907) has also studied 40 strains of this organism wrongly calling it the pseudo-diphtheria bacillus. She also finds that it ferments only glucose and sucrose.

My strains 4, 5, 19, and 34 were representatives of this sub-
group. Strains 4 and 5 fermented glucose, sucrose and inulin, while 19 fermented salicin in place of inulin; strain 34 in addition to glucose, sucrose and maltose fermented salicin and dextrin. A study of the characteristics of these organisms shows that they all belong to the same group, despite the fact that the fermentation varies somewhat. They have in common the fermentation of glucose and sucrose. In this connection, I wish to mention strain 32 which has the same fermentative powers as strain 34 except for a doubtful reaction with sucrose. Even glucose is fermented only to 0.6 per cent. In addition it liquefies serum agar (Loeffler's). In spite of these differences I consider it to be merely a variant of \textit{B. Hoagii} and not deserving of some special name. Recourse to the immunological reactions of the two strains seems to bear out the contention. \textit{B. Hoagii} is also related to strains 1, 2, 3, and x-323 which subgroup is located at the other extreme. For these reasons I regard this subgroup as occupying an intermediate position between \textit{B. flavidus} on one hand and \textit{B. enzymicus} on the other. When pathogenic, it is usually found in connection with suppurations, usually not initiating a pathologic process, but sustaining it when once started by a more virulent organism, Hamilton (1907), Bergey (1904). Experimentally I have produced local subcutaneous abscesses in guinea-pigs with \textit{B. Hoagii} after the animal had previously received an intraperitoneal injection of the same organism.

I have next to consider an organism which I believe merits a distinctive position. Ruediger (1903) and later Hamilton (1904) have described a very important bacillus, which morphologically resembles the diphtheria bacillus, but culturally and fermentatively seems more closely allied to \textit{B. Hoffmanii}. It grows heavily on agar, and gives diffuse cloudiness in broth. Its most noteworthy cultural characteristic is its decolorization of litmus. This is probably due to its reducing action which is possessed to a greater or less degree by most members of the group, although complete decolorization of litmus is not usual especially in the absence of acid formation. This form ferments no sugars but produces a soluble toxin which is very pathogenic
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for guinea-pigs. The toxin is not neutralized by anti-diphtheritic serum. I was not able to obtain a culture of this organism but Hamilton's immunological work indicates that it is distinct from B. Hoagii and B. flavidus. I have given it a separate place closely related to the diphtheria bacillus, and more distantly related to B. Hoffmannii and have named the subgroup B. Rue-digeri after the author of the original description of this organism.

B. Hoffmannii, I have considered as a mutant in accordance with the opinion expressed by Flexner and others. Its total fixing value is only eight, showing that the cross-reactions with the other sub-groups are not great. It has slight cross-fixation with B. enzymicus. Kolmer (1916) has shown that there is a definite cross reaction between this organism and B. diphtheriae. Strains 10, 24, 25, 31, 43, and 44 of this series belong in this group.

A very interesting bacillus was described by Graham-Smith (1904) in 1904 which he has called the B. diphtheroides-liquefaciens. He first isolated it from the mouth of a patient supposed to be suffering from diphtheria. It is characterized by the following features. The organisms are very long and curved and may be motile. There is practically no segmentation or involution produced; slow liquefaction of serum and gelatin; slow but abundant growth on potato. Glucose, or glucose and lactose are fermented and milk usually coagulated. Litmus is decolorized. Nitrates are reduced and much indol is formed. No gas is produced.

Hamilton (1904) has described an organism which can be placed in this subgroup but which produces gas in glucose broth. Klein (1903) has described a B. diphtheroides which also liquefies both serum and gelatin, produces hepatization in rat's lungs and is pathogenic for guinea pigs. De Witt's (1912) proteus-like organism may also be included in this group. Strain 21 of my series must also be placed here. It liquefies gelatin but not serum, ferments glucose and lactose and coagulates milk with decolorization of the litmus. This assemblage of organisms is very closely related to B. Hoagii (strain 34) but particularly to strain 32 which is a variant of strain 34. These sub-
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groups have a distinct relationship to the saprophyte B. proteus a fact which I have shown in the diagram.

I now come to a subgroup which I have designated B. enzymicus on account of the range of its sugar fermentation. Its distinctive characteristics can be learned in detail by a study of strain 1 which I take to be a representative member of the subgroup.

It has three main characters not common to the other subgroups: first, a remarkable pleomorphism; second, a wide range of sugar fermentation; and third, a distinct biological relation to the streptococci.

On account of these qualifications I have represented this subgroup as being more intimately connected with the non-hemolytic streptococci than with the other members of the diphtheroid group. This particular strain produces in addition a poisonous substance in the broth filtrate which resembles in many ways a true toxin. Strain 1 from a purely morphological standpoint can be called either a diphtheroid bacillus or a non-hemolytic streptococcus. It can be made to grow in long chains of diplo-streptococci. Biologically it has the features of a streptococcus of a moderate grade of virulence in that it constantly produces suppurative arthritis, cholecystitis, and myositis in animals. Reference to the immunological reactions shows how definite is its relation to the non-hemolytic members of this group. The viability of this strain is much more prolonged than that of the streptococcus and its virulence is also lost much sooner on artificial media. It then occupies an intermediate position between the remainder of the diphtheroid group and the streptococci.

Rosenow's erythema nodosum strain appears to show almost identical cultural and morphological features, as well as pathological, although he has not attempted to demonstrate its relation to the streptococci. However he has suggested it. It can be placed with certainty in the subgroup B. enzymicus. Likewise, I believe that the so called B. Hodgkinii can be placed in this subgroup, although if it is demonstrated to have the etiologic relation to Hodgkin's disease that is now claimed, it will no
doubt merit a more specific ranking. The strains in this series which I have studied and placed in this group are strains 1, 2, 3, 9, 11, 13, 14, 16, 33, 37 (x-323), 38, 39, 41, 42. Without exception the strains in the bacillary form were very scant growers, even on blood or serum media. I have been able permanently to alter the morphology of most of them, and in every case to develop quickly a very luxuriantly growing strain from one cultivated with difficulty.

I shall later describe the relation of this group to the enterococcus of Thiercelin and the complement-fixation tests show some cross-reaction with the staphylococcus. This work in connection with that of Frankel and Much who interpret B. Hodgkinii as a special form of the tubercle bacillus, is evidence that the group touches on the more definitely acid-fast organisms such as the B. tuberculosis. Gordon (Graham-Smith 1908) has shown the relation between certain streptothrices and the diphtheria group. As a result of this evidence, I have placed as contiguous, the B. tuberculosis and the streptothrices, as well as the enterococcus, the streptococcus, the staphylococcus and B. proteus.

Any of the diphtheroid subgroups under favorable conditions may become pathogenic, although this proclivity is much more marked in some than in others. B. enzymicus containing as it does examples of the erythema nodosum, streptococcoid, and B. Hodgkinii strains would seem to represent an adaptable assemblage which can easily become pathogenic. Likewise we have many examples of the pathogenicity of B. flavidus and B. Ruedigeri. On the other hand it is very rare to find B. Hoffmannii pathogenic, although Fox (1915a) and others have reported it as the probable cause of certain benign unilateral types of pharyngitis.

I shall now consider the various diphtheroids which have been reported from time to time under various names and relate them to this classification so far as possible. Cautley (1894) described an organism which he calls B. coryza-segmentosus which he believed to be the cause of some of the common colds. Gordon (1901), Benham (1906) and Graham-Smith (1908) have
isolated various strains of it. It ferments glucose, galactose, lactose, levulose, sucrose and maltose. Benham has renamed it *B. septus*, on account of its morphology at the particular time when he happened to observe it. It corresponds well to strain 16 of my series isolated from the nasal discharge of a fresh cold, and strain 11 isolated from a normal throat. It falls under *B. enzymicus* or the group of active fermenters. We know that it is not related to coryza except as a secondary invader. Neumann (1902) from a study of 206 cases of nasal colds, believes that diphtheroids have no etiological relation to the condition. He found them in 98 per cent of diseased noses, and 100 per cent of normal noses.

Graham-Smith (1908) has isolated a diphtheroid which he calls *B. maculatus*. Its poor growth on most media is its most noteworthy characteristic. It ferments glucose. From this general description it probably falls into the *B. xerosis* or *B. flavidus* subgroup. He says that it resembles closely a non-virulent diphtheria bacillus, in which case it would naturally fall in the latter group. I see no reason for giving it a separate name. *B. muris* described by Klein (1903) is an interesting organism. It was isolated from the hepatized lung of a white rat, and he was able to reproduce this lesion in other rats. Nothing but a large local abscess was produced in guinea-pigs. Culturally and morphologically this form has a strong resemblance to the true diphtheria bacillus. In the absence of a more complete description it could be placed in the *B. flavidus* subgroup. Bergey (1904) and Dean (Graham-Smith 1908) also isolated organisms from abscesses in a leprosy-like disease of rats which have the same characters as Klein's *B. muris*. MacFadyean and Hewlett (Graham-Smith 1908) have isolated the same bacillus from the mouth of healthy and diseased pigeons.

Nakanishi (1900) and Brown (Graham-Smith 1908) isolated an organism from cases of vaccinia and variola which they called *B. lymphae-variabilis*. It is easily identified as belonging to the subgroup *B. Hoffmanii*. Galli-Valerio (Graham-Smith 1908) has called his organism recovered from vaccine lymph, *Corynebacterium vaccinae*. This also is *B. Hoffmanii*. Klein
describes the *B. xerosis variola*, and *B. albus variola* from glycerin emulsions of small-pox crusts which are in all probability the ordinary *B. xerosis*. The descriptions are inadequate. De Simoni (Graham-Smith 1909) isolated an organism from the same sources which corresponds to *B. Hoagii*. Levy and Fickler (1900) isolated a *Corynebacterium lymphae-vaccinalis* which is very probably *B. flavidus*.

Graham-Smith's *B. xerosis canis* is merely the ordinary *B. xerosis*. His *B. diptheriodes gallinarum* is very probably *B. flavidus* and his *B. ceruminous* is the common *B. Hoagii*. His *B. diptheroides-brevis* and *citreus* also have nothing distinctive and can be grouped under *B. Hoagii*. Graham-Smith has also isolated a *B. auris* from the ear. De Simoni (Graham-Smith 1908) has found the same organism there, while Griffith has isolated it from the normal eye. Bergey has isolated an identical organism from the vagina which was pathogenic for guinea-pigs in large doses. All these organisms can be grouped under *B. Hoagii* although the sugar reactions are only given in part. Certainly there is nothing distinctive about any of them.

Bloomfield (1915) divides the diphtheroids of lymphatic glands into three arbitrary groups, although there is really no biological classification made. Group I he correlated with surface saprophytes, probably meaning *B. Hoagii*, *B. Hoffmanii* and *B. xerosis*. In Group II he describes a short pleomorphic bacillus mildly anaerobic, and another type resembling a coccus with the same characters as the short pleomorphic bacillus. They are probably identical, one being the coccus form of the other. It probably would be placed with *B. enzymicus*. Group III is heterogenous.

Hine (1913) has classified urethral organisms most of which he has identified with *B. coryza-segmentosus*. As I have already suggested, all these organisms can be grouped under *B. enzymicus*. His Group I is *B. Hoffmanii* and his Group II is *B. Hoagii*.

Torrey studied the lymph gland diphtheroids and placed them in three arbitrary groups. The majority of the strains which he places in Groups I and II can be identified with *B. Hoagii* and *B. Hoffmanii* from their action on sugars. Some
strains in class I belong to \textit{B. enzymicus}. In class III \textit{B. xerosis} or \textit{flavidus} predominates. He describes a \textit{B. lymphophilis} which I have not had opportunity to study. It is an anaerobe obtained mostly from Hodgkin's glands and ferments glucose, glycerin and sucrose. He believes it to be a distinct species.

The classification of Teoumin (1913) based on sugar reactions and agglutination tests I have already referred to on page 294. These organisms fermenting maltose, levulose and glucose plainly belong to the \textit{B. flavidus} subgroup. Wolbach and Honeij (1914) describe a diphtheroid bacillus which they believe to be identical with the majority of those described from leprosy. It is a very typical example of \textit{B. flavidus} in its morphological and cultural characteristics and in its sugar reactions.

De Witt (1912) has described a very unusual pathogenic diphtheroid organism which she thinks is closely related to \textit{B. proteus}. It liquefies both serum and gelatin, is actively motile, produces gas in glucose broth, coagulates milk and develops indol. This form is closely related to \textit{B. diphtheroides liquefaciens} of Graham-Smith and undoubtedly will fall in this subgroup.

(To be continued)