



A study of corynebacteria isolated
by B Bayliss

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree
of Master of Science in Bacteriology
Montana State University
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Abstract:

The history of the disease, diphtheria, is discussed, especially in the light of recent epidemics. Throat cultures from 200 Montana State College students were studied to determine the number carrying diphtheria or diphtherialike organisms. Thirteen organisms were isolated and compared morphologically and physiologically with 14 known cultures of *Corynebacterium diphtherias*, submitted by Frobisher and Galbraith, that had been cultured from the throats of patients with diphtheria infections. All these organisms varied in some characteristic, so no serious attempt to classify them was made. Five strains appeared very similar, to known cultures of *C. diphtherias* type *minimus*. The other eight differed in at least one character from any of the corynebacteria described in Bergey's Manual. The advantages and disadvantages of Loeffler's and Pai's media for primary culturing, of the tellurite medium employed and of "chocolate" agar for purification are presented. The Schick reactions of the students from whom these cultures had been obtained were determined, but being negative, no further work was done. Cultures were made also from the throats of students with pharyngitis, but no diphtheria or diphtherialike organisms were found. Possible reasons for this are discussed.

A STUDY OF CORYNEBACTERIA ISOLATED FROM
HUMAN THROATS

by

BERENICE G. BAYLISS

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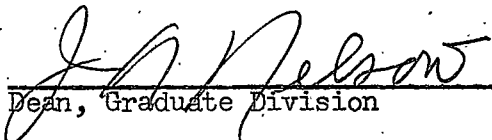
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ABSTRACT

The history of the disease, diphtheria, is discussed, especially in the light of recent epidemics. Throat cultures from 200 Montana State College students were studied to determine the number carrying diphtheria or diphtherialike organisms. Thirteen organisms were isolated and compared morphologically and physiologically with 14 known cultures of Corynebacterium diphtheriae, submitted by Frobisher and Galbraith, that had been cultured from the throats of patients with diphtheria infections. All these organisms varied in some characteristic, so no serious attempt to classify them was made. Five strains appeared very similar to known cultures of C. diphtheriae type minimus. The other eight differed in at least one character from any of the corynebacteria described in Bergey's Manual. The advantages and disadvantages of Loeffler's and Pai's media for primary culturing, of the tellurite medium employed and of "chocolate" agar for purification are presented. The Schick reactions of the students from whom these cultures had been obtained were determined, but being negative, no further work was done. Cultures were made also from the throats of students with pharyngitis, but no diphtheria or diphtherialike organisms were found. Possible reasons for this are discussed.

A STUDY OF CORYNEBACTERIA ISOLATED FROM HUMAN THROATS

INTRODUCTION

Diphtheria was once considered to be a disease whose chief sufferers were children, particularly those of preschool age. In some of the more recent epidemics, however, attention has been drawn to the number of those afflicted who were of college age or older. Although several surveys have been made to determine whether children were carriers of diphtheria organisms, very few such investigations have been conducted on college students. Therefore, it was felt that in view of the shift in age of those contracting diphtheria, it would be valuable to determine the percentage of an unselected group of freshmen students at Montana State College who harbored diphtheria or diphtherialike organisms.

As a part of their Freshman Week physical examination, each student is given a Schick test. Those showing a positive reaction are given the standard immunization inoculations against diphtheria. Since the information concerning the results of the Schick reaction was available, it was planned to reculture the throats of those students showing a positive Schick reaction, and from whom corynebacteria had been isolated to determine the effect of their immunization on the flora of the throat. This was to be done approximately sixty days after the completion of the immunization.

In the cultural diagnosis of diphtheria, Loeffler's blood serum has commonly been employed since it was first described by Loeffler in 1884. McGuigan and Frobisher (1936) mentioned the use of Pai's medium as a substitute. In their opinion the latter was as reliable a medium as Loeffler's

and simpler to prepare. It was, therefore, decided to compare these media when culturing the organisms from the students' throats.

Some contradictory statements are contained in the literature as to whether the diphtheroids, organisms morphologically resembling Corynebacterium diphtheriae (Flugge) Lehmann and Neumann, but with different physiological properties, are inhibited by the potassium tellurite employed in the media now widely used for the isolation and identification of C. diphtheriae. There is also a question as to whether the C. diphtheriae population of apparently normal throats differs from that of throats showing pathological conditions. The present study was undertaken to obtain further information on these points.

REVIEW OF LITERATURE

Historical Background

Diphtheria was established as a clinical entity in 1826 by Bretonneau of Tours, France (Smith and Martin, 1948). It was not, however, until 1883 that the diphtheria organism was described by Klebs in smears from the membrane in the throats of patients with the disease. Loeffler isolated the organism in a pure culture in the following year. For this reason Corynebacterium diphtheriae (Flugge) Lehmann and Neumann is often referred to as the Klebs-Loeffler bacillus. Formerly there was some doubt as to the relationship of these organisms to diphtheria, since they were also occasionally found in the throats of normal people and were not always present in pseudomembranes. Some of the systemic manifestations of the infection which are now known to be due to the action of the diphtheria

toxin were also a cause of confusion. The discovery of the exotoxin by Roux and Yersin in 1888 eliminated these doubts and established the etiological relationship of C. diphtheriae to diphtheria.

A malignant form of diphtheria appeared in France sometime between 1850 and 1860 (Smith and Martin, 1948) and spread over the world, reaching a peak in London in 1874. This virulent form was found in Boston in 1872, where the death rates increased from 35 per 100,000 in 1872 to 218 per 100,000 in 1881. Smaller epidemic waves recurring at five to ten year intervals were recorded throughout the world from 1880 to 1930. Since then the death rate from diphtheria has declined, even in the countries where prophylactic immunization has not been employed, as in Norway. In New York the death rate dropped from 1,175 and 1,005 per 100,000 in 1881 and 1887, respectively, to almost none in 1935. This decline in the number of deaths was attributed to diphtheria antitoxin which was made available to every diphtheria patient by 1900 and to mass immunization which was started in 1915. (A more detailed history of diphtheria can be found in Smith and Martin, 1948.)

Description of Corynebacterium diphtheriae

Corynebacterium diphtheriae is a non-motile, gram positive rod, occurring singly, and varying greatly in size (Breed, et al., 1948). The arrangement of the cells is especially characteristic. When the cells divide, the cell membrane gives way on one side. This results in a snapping post-fission movement and in the formation of a palisade or chinese letter arrangement. Frobisher (1949) feels this to be "an especially useful

diagnostic feature". However, Bisset (1950) refers to this post-fission movement as being "an interesting but quite unimportant artifact". Usually the rods are straight, but often they appear to be slightly curved and frequently are swollen at one or both ends. The rods do not, as a rule, stain evenly with methylene blue, but show alternate bands of stained and unstained material. Very often metachromatic granules appear. These are referred to as polar bodies when they are located at the ends of the organism. These granules are also termed artifacts by Bisset (1950) as they are found only in dried and stained preparations. He believes them to be "dried aggregates of nuclear and, probably, reserve food material".

Just as the organisms vary greatly in morphology, so the colony forms vary. These variants have been described as follows:

1. Smooth (S) with a glistening surface and even edges.
2. Rough (R) having a dull surface and uneven margins.
3. Intermediates, which include
 - a. Sr, which more nearly resembles the pure S form.
 - b. rS, which are very similar to the R.
 - c. SR colonies which show properties that are not like either S or R.
4. Dwarf (D) with colonies so small that magnification is required to make them visible to the human eye.

Based upon these variations in morphology of the organism itself and the different colony forms, several types of C. diphtheriae have been described and named. The four types most universally recognized are briefly described in table I.

Table I
Types of Corynebacterium diphtheriae

Type	Morphology	Colony forms
<u>gravis</u>	short rods uniformly staining	SR
<u>mitis</u>	long rods metachromatic granules	S
<u>intermedius</u>	long rods barred forms clubbed ends	SR
<u>minimus</u>	long rods barred forms clubbed ends	D

Serological investigation has shown that these types are also antigenically distinct from one another (Jordon and Burrows, 1947). Some authorities regard the gravis, mitis, intermedius and minimus, not as types but rather as subspecies of C. diphtheriae. Both virulent and avirulent strains have been isolated in all of the types mentioned. However, Frobisher et al., (1951) reports that strains of the minimus type produce only faint intradermal reactions in rabbits when their virulence is tested by standard procedures, but that in the agar-plate method of testing virulence, not one minimus strain has ever been found to be non-virulent.

The virulence of the diphtheria organism is closely correlated with the production of a potent exotoxin. Studies by Povitsky, Eisner, and Jackson (1933) showed that all diphtheria toxins are similar. If they

differed, it was only in the degree of toxicity, not in the kind. The gravis strain isolated during the diphtheria epidemic at Halifax, Nova Scotia in 1941, when grown in a medium containing four micrograms of iron per ml of culture fluid, synthesized ten times as much toxin as other strains tested.

Malignant diphtheria

There is a possibility that in the future a type of diphtheria may be encountered against which our present methods of protection will not prevail. This severe type of diphtheria has already appeared on the European continent, and spread to the British Isles about 1931. This malignant, hypertoxic, or grave diphtheria differs from the ordinary diphtheria by the presence of marked cervical swelling, extreme toxemia, and albuminuria. There is frequently a development of neuropathies and a very high death rate, in spite of early and large doses of antitoxin (Updyke and Frobisher, 1947).

The first outbreak of this malignant diphtheria to appear on the North American continent, occurred in Halifax in 1940 and 1941. It has been suspected that this outbreak was caused by a Norwegian sailor from a whaling vessel. Wheeler and Morton (1942) report that in this epidemic a high percentage of cases were in the older age group. They suggest that this might be the result of the immunization of most of the school children.

A somewhat similar outbreak of diphtheria occurred in Baltimore, Maryland in 1944. During the first six months of that year the mortality rate from ordinary diphtheria was six percent, while that from the

malignant diphtheria was 44 percent. It was felt by some that this severity might be due, not to the diphtheria organism alone, but to its synergistic action with some other organism, possibly streptococci. Early work by Updyke and Frobisher (1944) seemed to point toward hemolytic streptococci of the Lancefield Group B, associated with diphtheria organisms of the mitis type. However, further work, with animals, also done by Updyke and Frobisher (1947) did not support this supposition, and they felt that the etiology of this malignant type of diphtheria was still uncertain.

A few investigators have tried to determine whether a certain type of C. diphtheriae might not be responsible for this grave or malignant diphtheria. Anderson, Happold, McLeod, and Thomson (1931) felt that in the epidemic which occurred in Leeds, England, C. diphtheriae type gravis was associated with the severe and toxic cases of the disease and the mitis type with the milder cases. Frobisher (1943) has found, however, many strains of C. diphtheriae type gravis in the United States which were completely atoxigenic.

Kellogg and Wende (1946) suggested the possibility that returning troops from Europe might bring back strains of these more virulent forms of diphtheria organisms that are prevalent in Northern Europe, and that the potential threat still made diphtheria a problem that warranted attention.

Corynebacterium diphtheriae type minimus

In 1947 and 1948 an epidemic of diphtheria appeared in Utah with several unusual features. The first was that the rather rare

C. diphtheriae type minimus was isolated from 35 percent of the cases (Jenkins, 1948). The second was the large number of cases among school children and older adults. It was found that the carrier rate of this minimus type was as high as 21.5 percent in a college in Utah from which no previous cases had been reported.

Bramhall, Galbraith, and Fraser (1948) in their report of the Utah epidemic, state that C. diphtheriae type minimus seems to be occurring more commonly in the United States, having been found also in Idaho, Georgia, Maryland, and Kentucky.

A third feature of this epidemic was that of these minimus cases reported, 58 percent had been immunized against diphtheria.

Diphtheria and immunization

Frobisher (1940) points out that diphtheria epidemics of a fulminating nature, which seem to progress regardless of immunity, are not uncommon. This may be because diphtheria antitoxin immunity, regardless of the immunizing procedure, is not permanent as reported by Volk et al. (1942), and that as time goes on, the antitoxin content of the blood is reduced. Volk advocated the reimmunization of children five to six years after the immunization of infancy. This loss of immunity could be, in part, the cause of the high percentage of cases in the older age group in the Utah epidemic. The percentage of those previously immunized, whose diphtheria was caused by C. diphtheriae type gravis was much lower.

Diphtheria carrier surveys

Grossmann (1940) did a Schick test and diphtheria-carrier survey of white school children in Virginia. He found that in the age group from five to fourteen years, C. diphtheriae carriers were five times as great in the Schick negative children as in those who were Schick positive.

Gill (1940) conducted a carrier survey on white children in Alabama during the years 1937 to 1939 and found a carrier incidence of toxigenic C. diphtheriae of 0.87 percent for children from the ages of five to fourteen years. Schuman and Doull (1940) in their study of carriers in Cleveland during the same time, found a lower rate, 0.56 percent, for the same age group. The latter estimated the ratio of carrier infections to clinical attacks as being 533:1. They attributed this high ratio to the loss in pathogenicity of the C. diphtheriae of that period.

From a study of the surveys made in the United States, it appears that the diphtheria picture as regards incidence, susceptibility, and carrier status is not uniform throughout the country. Frobisher (1942) states that carrier surveys have shown that many types of diphtheria organisms, e.g. gravis or mitis, virulent or avirulent, may predominate and at times may be the only type present in some communities but not in others. This phenomenon may be localized or general and conclusions in regard to prevalence or rates are not valid beyond the confines of a county or state.

Frobisher (1940) suggests that the study of the type of C. diphtheriae found in carrier surveys and the number of positive cultures obtained

might be useful guides in diphtheria control by indicating the trends and changes.

Types of media used for the cultivation
and isolation of C. diphtheriae

Although Loeffler's blood serum medium has been the standard used for the isolation of C. diphtheriae since it was first introduced in 1884; it has been responsible for many errors in the diagnosis of diphtheria. These mistakes have been caused by the scarcity of growth of C. diphtheriae and the overgrowth of other organisms of the nose and throat. Medalia et al. (1931) described a modification of Loeffler's medium that made it more selective. This was done by adding N/1 NaOH to give a pH of 7.6 to the final mixture. However, because of its opacity it is difficult to measure the pH of this medium without using a pH meter. Laybourn (1935) proposed a method by which the pH could be determined by using a very small amount of the water of hydration of a culture.

In a paper published in the Chinese Medical Journal in 1932, Pai described an egg medium for the cultivation and isolation of C. diphtheriae. McGuigan and Frobisher (1936) found that this medium produced C. diphtheriae morphologically and toxigenically similar to those cultivated on Loeffler's, and in addition was easier to prepare.

Tinsdale (1947) has formulated a medium which uses the production of hydrogen sulfide as a differentiating characteristic. Different organisms produce definite types of colonies and certain organisms form characteristic haloes. The medium has the advantage of giving definite results in a

comparatively short period of time. On the other hand, the composition of this medium is very detailed and complicated and its keeping qualities are poor.

The use of potassium tellurite as a means of inhibiting the growth of other organisms and for differentiation in the culturing and isolation of C. diphtheriae has been widely investigated and used with varying degrees of success. It was first used by Conrade and Troch in 1912. It had been discovered as early as 1885 that some members of the plant kingdom, among them the "infusoria", could reduce the tellurites in small amounts to metallic tellurium without its becoming toxic to them. About 1900 Klett and others found that some bacteria were colored by the reduced metal. In Klett's study, using numerous molds and bacteria, it was found that some organisms could tolerate the tellurites much better than others. It was also noticed that some organisms were not affected at all by concentrations that would completely inhibit other organisms. Bacillus diphtheriae (C. diphtheriae) was mentioned as one of the organisms used in this study that was able to tolerate the tellurites and was colored by them. Joachimugle and Hirose in studies to determine the concentration of tellurites tolerated by C. diphtheriae found, for example, that C. diphtheriae could tolerate a solution containing 4,000 times as much of the metal as could Salmonella typhosa (Gilbert and Humphreys, 1926).

Many workers have experimented with media containing potassium tellurite. Among them are McGuigan and Frobisher (1936), Hall (1939), Levin (1943), and Kellogg and Wende (1946). A comparative study of the tellurite

plating media was carried out by Frobisher, Parsons, Yeates, and Gay (1948) using Frobisher's own cystine-tellurite medium (1937) as a basis for comparison, since he had used it for many years and knew its properties. The results of this study showed that the tellurite medium of Kellogg and Wende (1946) gave as high or higher percentage of positives. Kellogg and Wende (1946) also claimed a very high percent of positives with it, having checked it carefully and finding that it seldom failed to demonstrate C. diphtheriae if present. Hall, whose medium differs very little from that of Kellogg and Wende, lists the advantages of her medium as the ability to inhibit organisms such as micrococci, streptococci, Neisseria catarrhalis, pneumococci, etc., while the C. diphtheriae grows with typical colonies ranging from grayish-white to black in color. She also claims that the plates can be prepared and kept for two or three weeks in the refrigerator. This is a decided advantage as most of the tellurite media must be prepared only a day or so before their use.

Virulence testing of C. diphtheriae

There has been some doubt as to whether animal inoculations reveal all the pathogenic potentialities of C. diphtheriae manifested in human beings. Frobisher et al. (1947) found that nontoxigenic strains will produce partial or complete protection against virulent organisms in rabbits, and also that the so-called avirulent diphtheria organisms contain endotoxins. He further states that numerous cases of clinically typical diphtheria have been encountered in which the etiological agent has proved to be only strains of C. diphtheriae nontoxigenic to guinea pigs. This is particularly

true with the minimus strains of the organism, as these tend to produce only faint intradermal reactions in rabbits when their virulence is tested by standard procedures (Frobisher et al., 1951).

Guinea pigs are frequently employed for testing the virulence of C. diphtheriae. Two animals are usually used, the first being inoculated subcutaneously with a suspension of the organism to be tested. The second serves as a control and is given diphtheria antitoxin plus the same amount of bacterial suspension in the same manner. If the organisms are virulent, the test animal will die in three to five days, but the control animal will survive.

Schaub and Foley (1947) describe a method employing only one guinea pig which serves both as testing animal and control. This has the advantages of being more economical and eliminating the variation in sensitivity of individual animals. A heavy suspension of the culture is inoculated intradermally in the shaven side of the guinea pig. At a later specified time an intraperitoneal injection of diphtheria antitoxin is given. A short time later the other side of the abdomen is injected with the same amount of the culture previously used. A reaction occurring on the side inoculated first, but not on the opposite side is considered positive, and denotes a virulent strain of C. diphtheriae. A reaction on neither side signifies a nontoxigenic strain. A reaction on both sides is said to be due to some other factor than diphtheria toxin. Another important advantage of this method is that several cultures can be tested on the same animal, depending upon its size.

Frobisher et al. (1942) describes a method in which he used seven to twenty day old chicks. He felt that these could be recommended as a substitute for rabbits or guinea pigs in testing the virulence of C. diphtheriae, especially when conducting a small number of tests, if rodents were not readily available.

Although the need for an in vitro method for testing C. diphtheriae for virulence has long been recognized, it was not until recently that such a test has been described (King, Frobisher and Parsons, 1949). The principle of the test is based on the toxin-antitoxin flocculation as devised by Nicolle in 1920. The test as devised by Elek in 1948 consists of the preparation of an agar base which must be clear and which must contain 0.3 gram of maltose and 0.07 ml of lactic acid per 100 ml. To this melted, cooled base is added 20 percent of normal horse serum. The mixture is then poured into petri dishes. A sterile filter paper strip is dipped in diphtheria antitoxin containing 100 units per ml, drained, and placed in the center of the dish where it is allowed to sink into the warm agar. After solidification, the plates are dried in the incubator and inoculated on the same day. Four organisms may be tested on each plate. A known virulent strain should be included. A white line developing at an angle on each side of the inoculum near the filter strip denotes a toxigenic agent.

King, et al. (1949) noted the sources of error in this in vitro test and outlined methods by which these errors could be minimized. The most important factor necessary to assure correct results, besides the correct adjustment of the pH, the amount of antitoxin, and others as obvious, is the collection and preparation of the serum used in the agar base. Many

lots of serum are not satisfactory for the minimus type of C. diphtheriae. The reaction in minimus strains is inhibited by serum only faintly colored by the lysis of the erythrocytes. Other strains are not so readily affected by this. It has also been found that some unknown substance or condition which interferes with the in vitro virulence test appears in the serum when it remains in contact with the clot for a day or longer.

Using this method, King et al. (1949) ran a long series of tests employing virulent and avirulent cultures of C. diphtheriae in comparison with animal inoculations. Complete agreement with the in vitro and animal inoculations was obtained in 290 cases, of which 123 were avirulent and 167 were virulent.

A positive reaction with the in vitro test seems to be an entirely reliable indication of virulence of diphtheria organisms. However, a negative reaction can be falsely obtained unless the conditions for that strain are provided. They suggest that all negative reactions be checked by animal inoculations. They also concluded that the test needed further study.

However, in a more recent paper these same investigators, after further study of this method, recommend it to be "wholly reliable, simple, rapid, and inexpensive, if properly and carefully performed". They mention other difficulties and sources of error. For example, certain commercial antitoxins are unsatisfactory for this use, although perfectly capable of functioning as prophylactic and therapeutic agents. Once again they stress the care that must be taken in preparing the serum used as nutrient enrichment of the agar when testing strains of the minimus type.

They suggest using as a control a known positively reacting strain of minimus when plating unknown minimus strains (Frobisher et al., 1951).

METHODS AND MATERIALS

Preparation

Before the actual survey on the students for the determination of diphtheria carriers was begun, known virulent and avirulent cultures of Corynebacterium diphtheriae were obtained from Frobisher and from Galbraith that included strains of the gravis, mitis, intermedius, and minimus types. These were used to become familiar with the morphology, staining reactions, and cultural characteristics of the organisms.

The gram stain was employed only to be certain that all of the organisms were gram positive. All morphological studies were made on slides stained with methylene blue. Loeffler's alkaline methylene blue and a methylene blue stain made without the addition of the alkali gave similar staining results. Since the latter was simpler to prepare, it was used through the remainder of the study.

A comparison of the morphology of C. diphtheriae on Pai's egg medium and Loeffler's serum medium was made. The granules and barring of the organisms were better demonstrated on Pai's medium although the Loeffler's medium produced a more luxuriant growth. Therefore, it was decided to inoculate half of the throat cultures obtained during the survey onto Pai's medium and the other half onto Loeffler's medium in order to obtain information concerning the usefulness of the former as a substitute in routine throat cultures of suspected cases of diphtheria.

The Loeffler's medium used throughout this study was prepared from Bacto-Loeffler blood serum.

Pai's medium was prepared according to the formula given by McGuigan and Frobisher (1936).

Both the Loeffler's and Pai's media were coagulated and sterilized as follows:

a. The baskets were wrapped in absorbent cotton and slanted by leaning on a wooden support in the center of the autoclave chamber.

b. All the exhaust valves were tightly closed and the steam was admitted at a fairly slow but even rate.

c. At the end of 90 minutes, the steam valve was closed, and the pressure allowed to go down without opening the exhaust valves.

d. After the pressure reached zero, the exhaust valves were opened for a few minutes before the sterilizer door was opened.

Smooth slants free of gas bubbles were obtained. These were stored at 5 C until used.

Kellogg and Wende's tellurite medium was used for the isolation and identification of the organisms obtained on the Pai's and Loeffler's media. Human blood was substituted for the recommended rabbit blood. This medium was not stored for more than a day or two before use.

Survey

The survey was carried out as suggested by Frobisher (1942 and 1949).

Throat cultures were made from 200 apparently healthy students, 100 men and 100 women, during the Montana State College Freshman Week,

September, 1949. One hundred of these, selected at random, were cultured on Pai's medium and one hundred on Loeffler's medium. The distribution of sexes cultured on each medium was about equal. After 24 hours' incubation at 35 C the cultures were transferred to the tellurite plates previously prepared and again incubated at 35 C.

At the end of 48 hours the plates were observed for colonies. No growth appeared from 78 of the transfers. From the 122 plates on which growth appeared, representative colonies were picked and transferred to Loeffler or Pai slants. Smears were made from these slants at the end of 24 hours and stained by Gram's method. All cultures showing gram positive rods in the typical palisade and chinese letter formation were saved for purification and identification.

During the winter quarter of 1950 the throats of all students coming to the Health Service with severe sore throats were swabbed and subsequently handled in the same manner as the first 200 cultures. Thus, 35 other students were included in the survey.

Purification

A great deal of difficulty was encountered in purifying the mixed cultures. The contaminating organisms were micrococci, except for two cultures which were contaminated with streptococci. The micrococci grew well on the tellurite medium, producing colonies which were more shiny and mucoid in appearance than those of C. diphtheriae. The streptococci grew very poorly on the tellurite medium, producing at best a very small gray colony that was difficult to see. For the most part, they just remained

dormant but viable, and ready to grow if the proper environment was again presented. The tellurite inhibited the diphtherialike organisms to the extent that a very heavy inoculation was necessary to obtain growth. This, then, made it difficult to find isolated colonies. Even though a colony did appear to consist of only one species, upon transfer to Loeffler's medium, or especially to a liquid medium, it was often found to be impure.

After much experimentation it was found that a "chocolate" medium made according to the formula for the tellurite but, without the potassium tellurite, made a better substrate for isolation. This medium gave good growth of all of the organisms, permitting the use of a smaller inoculum. The diphtherialike bacteria produced small transparent to grayish colonies, the micrococci formed large white colonies, and the colonies of the streptococci were surrounded by definite yellow zones.

After streaking the cultures and picking isolated colonies on several occasions, 13 cultures were finally obtained in a pure state. These were maintained on Loeffler's or Pai's media and stored at 5 C.

Cultural studies

The morphology of the organisms was ascertained by studying slides made from 24-hour cultures on Loeffler's or Pai's media and stained with methylene blue. Measurements were made employing a previously calibrated ocular micrometer. Motility was checked by the hanging drop method, using a 24-hour broth culture.

All physiological tests were run in duplicate and if any doubt arose as to their agreement, further checks were made. Several different types of broth were tried, in an effort to find one that would give a sufficiently

good growth for the physiological tests. Difco's veal infusion medium containing 0.1 percent agar was found to support the best growth. This medium was also used for making the agar plates and slants by increasing the agar to 1.5 percent.

The tests for nitrate reduction and indol production were made on the same tube of veal infusion medium to which had been added enough tryptone (Bacto) and potassium nitrate to give a 0.1 percent concentration of each.

The organisms were tested for their ability to ferment glucose, galactose, fructose, sucrose, maltose, lactose, dextrin, mannitol, and glycerol. All these substrates were sterilized by filtering through a sterile fritted glass filter, and a sufficient quantity of the sterile solution added to the basal medium to give a final concentration of one percent. Brom cresol purple was used as an indicator. Every possible precaution was taken to prevent contamination and all tubes were incubated for 24 hours at 35 C before being examined and stored at 5 C until used. Less than one percent of these tubes of media was discarded because of contamination.

Gelatin liquefaction was determined in veal infusion medium to which had been added 12 percent gelatin. Stab inoculations were made and the cultures incubated at 35 C for at least two weeks.

Catalase production was demonstrated by adding a few drops of three percent commercial hydrogen peroxide to colonies on an agar plate.

Cultures in the veal infusion broth were studied in 24 and 48 hours for type of growth, pellicle formation, sediment and area of heaviest growth.

Colonies on some of the plates and slants could not be observed without a 4x hand lens.

The medium for determining hydrolysis of urea was made by adding aseptically 10 ml of a concentrated urea solution (Difco) to a solution consisting of 90 ml of water and 1.5 percent agar. This medium was then poured into sterile tubes and slanted before solidification took place. These slants were streaked with a heavy inoculum, incubated and watched for the development of a pink color that denoted urea hydrolysis.

Virulence

Virulence of the organisms isolated and purified was determined by intracutaneous skin tests on guinea pigs as described by Schaub and Foley (1947). A heavy suspension of the organisms in physiological saline was made by washing the growth from a 24-hour culture on Loeffler's slants. One-tenth milliliter of this suspension was injected intracutaneously into the shaven side of the abdomen of a guinea pig. By staggering the sites of the injections, seven cultures were injected into one side of a large guinea pig. After inoculation, the remaining suspensions were placed in the refrigerator. Five hours later the animal was given 100 units of diphtheria antitoxin intraperitoneally, care being taken not to puncture the intestines. Thirty minutes later another 0.1 ml of the suspension was injected intracutaneously into the opposite shaven side of the guinea pig's abdomen. Precaution was taken to be sure that each organism was injected exactly opposite the first injection. This second injection served as a control. A chart of the sites of the injections was made so that there could be no mistake as to the position of each organism.

A record was made of the reactions of each at 24, 48, and 72 hour intervals. Erythema, edema, and necrosis at the site of the test injection and the absence of such a reaction on the control side meant a positive skin test, and indicated the production of toxin by the organism in question. Known virulent and avirulent strains were also tested so as to check the technique.

EXPERIMENTAL RESULTS

Number of cultures obtained

From the 200 cultures taken from seemingly healthy students during Freshman Week, 122 were found to give black colonies on the tellurite medium. Although many of them were much too shiny and mucoid in appearance to be Corynebacterium diphtheriae, Loeffler and Pai slants were inoculated with representative colonies from all plates. After 24 hours each culture was examined by gram staining. Most of them were found to consist of micrococci and were discarded. Some of the cultures were a mixture of gram positive rods and other organisms, principally micrococci. A few appeared to be seemingly pure cultures of gram positive rods.

No cultures containing gram positive rods were obtained from the 35 students reporting to the Health Service with sore throats during the winter and spring quarters of 1950. A very rapidly growing streptococcus generally predominated. Unsuccessful attempts were made to obtain gram positive rods by transferring throat cultures inoculated on Loeffler slants, to the tellurite plates after an incubation period of 14 to 18 hours as well as at the end of 24 hours. Since no diphtherialike organisms

were obtained from these 35 students, the culturing of pathological throats was discontinued.

Comparison of the Pai's and Loeffler's media

A record was kept as to the medium on which the original throat cultures were inoculated. Of the 13 strains obtained in pure culture, eight were originally placed on Pai's medium and five on Loeffler's medium. However, it was noted that of the five cultures which were isolated from the tellurite agar plates in an apparently pure state, four were originally cultured on Loeffler slants and only one on a Pai slant.

Grouping of the organisms

All of the 13 organisms obtained had several morphological and physiological characters in common. They were all gram positive rods showing chinese letter and palisade grouping, they all produced catalase, but failed to form indol from tryptophane, or to liquefy gelatin. With but one exception, culture 113, all guinea pig tests for toxin production were negative.

By placing together those which resembled one another in the other morphological and physiological characters for which they were tested, four groups were established. Group four, or the last group, consists of three miscellaneous organisms which do not show the similarity that is found in the other groups. The morphological characteristics of the organisms in these groups are listed in table II and the physiological in table III.

Table II

Morphological characteristics of diphtherialike organisms isolated from student throats

<u>Group</u>	<u>Culture</u>	<u>Size</u> (microns)	<u>Cellular appearance</u>	<u>Colonies</u>	<u>Broth</u>
1	101, 102, 103, 104, 111	1.2-3.8 x 0.6-1.2	Distinct barring, oc- casional club shapes, some polar bodies.	Minute, translu- cent, shiny, con- vex and smooth.	Uniform turbi- dity upper half inch of culture.
2	107, 109, 110	1.2-3.6 x 0.4-1.2	Barring and polar bodies prevalent, mo- derate number of club shaped cells.	1-2 mm, white, opaque and shiny, soluble brown pigment produced after 24 hours.	Pellicle formed. Turbidity; upper half inch, slight sediment.
3	108, 114	0.6-1.7.x 0.2-0.6	Very small rod. Varia- bility of staining.	1-2 mm, smooth, shiny, convex and opaque.	Uniform turbi- dity upper half inch.
	(105 (((1.2-2 x 0.6-1.2	Some barring.	1-2 mm, smooth, shiny, opaque, orange-pink pig- ment.	Uniform turbi- dity upper half inch, pink sediment.
4	(112 ((1.2-1.9 x 0.4-0.7	Polar bodies but no barring.	1-2 mm, white, opaque, dry and granular.	Granular turbi- dity upper half inch.
	(113	1.2-3.0 x 0.6-0.9	Some barring.	1-2 mm, white, shiny, smooth, convex.	Uniform turbi- dity upper half inch.

Table III

Physiological characteristics of diphtherialike organisms isolated from student throats

<u>Group</u>	<u>Cul- ture</u>	<u>Glu- cose</u>	<u>Fruc- tose</u>	<u>Galac- tose</u>	<u>Mal- tose</u>	<u>Suc- rose</u>	<u>Lac- tose</u>	<u>Dex- trin</u>	<u>Gly- cerol</u>	<u>Manni- tol</u>	<u>Ur- ease</u>	<u>NO₃ re- duction</u>
1	(101	+	+	-	-	+	-	-	-	-	-	+
	(102	+	+	-	+	+	-	+	-	+	-	+
	(103	+	+	+	-	+	-	-	-	-	-	+
	(104	+	+	+	+	+	+	+	-	+	-	+
	(111	-	-	+	-	-	-	+	-	-	-	+
2	(107	+	+	+	-	-	-	-	-	-	-	+
	(109	+	+	-	-	+	-	-	-	-	-	+
	(110	+	+	+	-	+	-	-	-	-	-	+
3	(108	+	+	-	-	-	-	-	-	-	-	-
	(114	+	+	+	+	+	+	+	-	-	-	-
4	(105	-	-	-	-	-	-	-	-	-	+	+
	(112	+	-	-	-	-	-	+	-	-	+	-
	(113	+	+	-	+	-	-	+	-	-	-	-

Classification of groups

A comparison of the morphological and physiological characteristics of the organisms isolated with those of C. diphtheriae as given in Bergey's Manual of Determinative Bacteriology (Breed et al., 1948) is presented in table IV.

The morphological and physiological characteristics of the organisms obtained from Frobisher and from Galbraith were also determined. All were gram positive rods with frequent angular and palisade formations. Likewise, all produced catalase and reduced nitrates to nitrites. None, however, produced indol from tryptophane or liquefied gelatin. The other characteristics determined are presented in tables V and VI.

It will be noted in table IV that Group 1 and Group 2 present a picture very similar to that of C. diphtheriae as described in Bergey's Manual. Group 2 organisms were separated from Group 1 only on the basis of the production of the brown soluble pigment in solid media. This ability is not mentioned in connection with any of the corynebacteria and seems to place these three organisms in a group apart. Group 3 differs from C. diphtheriae in that nitrates are not reduced to nitrites. The miscellaneous organisms in Group 4 show slight variations in nitrate reduction, urea hydrolysis, and sugar fermentation. Culture 105 rather closely resembles Corynebacterium pseudodiphtheriticum except that it produces a very decidedly orange-pink pigment instead of being "grayish to cream-colored" as described in Bergey's Manual.

It is interesting to note that the organisms of Group 1 very closely resemble two strains of C. diphtheriae type minimus obtained from

Table IV

Morphological and physiological characters of organisms isolated from throats of students compared with C. diphtheriae as described in Bergey's Manual

Character	<u>C. diphtheriae</u> *	Group 1	Group 2	Group 3	Group 4		
Shape	Rods, sometimes clubs.	Rods, few clubbed.	Rods.	Small rods.	Rods.		
Size (microns)	1.0-8.0 x 0.3-0.8	1.2-3.8 x 0.5-1.2	1.2-3.6 x 0.4-1.2	0.6-1.7 x 0.2-0.6	1.2-3.0 x 0.4-1.2		
Appearance	Barring, metachromatic granules.	Barring, a few polar bodies.	Barring and metachromatic granules.	Unevenly staining.	Bar-	Gran-	Bar-
Group formation	Angular and palisade.	Same.	Same.	Same.	Same.		
Gram reaction	+	+	+	+	+	+	+
O ₂ requirement	Aerobic.	Aerobic.	Aerobic.	Aerobic.	Aerobic.		
Motility	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-	-	-
Indol	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	-	+	-	-
Urea	-	-	-	-	+	+	-
Sugar fermentation							
Glucose	+	+	+	+	-	+	+
Fructose	+	+	+	+	-	-	+
Galactose	Variable.	Variable.	Variable.	Variable.	-	-	-
Maltose	Variable.	Variable.	-	Variable.	-	-	+
Sucrose	Variable.	Variable.	Variable.	Variable.	-	-	-
Dextrin	Variable.	Variable.	-	Variable.	-	-	-
Glycerol	Variable.	-	-	-	-	-	-

*According to Bergey's Manual

Table V

Morphological characteristics of *C. diphtheriae* cultures obtained from Frobisher (F) and from Galbraith (G)

<u>Culture</u>	<u>Size (microns)</u>	<u>Cellular appearance</u>	<u>Colonies</u>	<u>Broth</u>
F 1	1.0-2.4 x 0.4-0.6	Little variation in staining, few granules.	White, opaque, smooth, shiny, convex.	Turbidity upper $\frac{1}{2}$ inch, slight pellicle formation.
F 2	0.6-2.2 x 0.4-0.6	Uniform staining, occasional granule.	"	"
F 3	1.2-3.0 x 0.4-0.5	Occasional barred rod, metachromatic granules.	"	"
F 4	1.8-3.6 x 0.5-0.6	Occasional barred rod, metachromatic granules.	White, opaque, rough, irregular, dull.	"
F 5	0.4-1.8 x 0.4-0.6	Uniformly staining rod, club forms present.	White, opaque, smooth, shiny, convex.	"
F 6	0.9-2.4 x 0.4-0.7	Variation in staining, occasional clubbed form.	"	"
F 7	0.6-3.8 x 0.3-0.9	Barred rods, club forms present.	Minute, translucent, smooth, shiny.	Uniform turbidity upper $\frac{1}{2}$ inch.
F 8	0.6-4.2 x 0.5-1.0	Barred rods, club forms present.	"	"
F 9	1.0-2.4 x 0.4-0.6	Barred rods, metachromatic granules.	White, opaque, smooth, shiny, convex.	Turbid upper $\frac{1}{2}$ inch, slight pellicle formation.
G 1	0.6-1.8 x 0.4-0.5	Barred rods, occasional metachromatic granules.	Minute, translucent, shiny, convex.	Uniform turbidity upper $\frac{1}{2}$ inch.
G 2	1.2-1.8 x 0.4-0.7	Barred rods and metachromatic granules.	"	"
G 3	0.4-1.8 x 0.4-0.8	Uniformly staining rods of varying sizes.	"	"
G 4	0.4-1.4 x 0.3-0.5	Barred rods, occasional club shape.	"	"
G 5	0.5-1.2 x 0.3-0.5	Uniformly staining rods, occasional club shape.	"	"

Table VI

Physiological characteristics of C. diphtheriae cultures
obtained from Frobisher (F) and from Galbraith (G)

<u>Culture</u>	<u>Glucose</u>	<u>Fructose</u>	<u>Galactose</u>	<u>Sucrose</u>	<u>Lactose</u>	<u>Maltose</u>	<u>Dextrin</u>	<u>Mannitol</u>	<u>Glycerol</u>	<u>Urease</u>
F 1	/	/	/	-	-	/	/	-	/	-
F 2	/	#	/	-	-	-	/	-	/	-
F 3	/	/	/	-	/	-	/	-	-	-
F 4	/	/	/	-	-	/	/	-	/	-
F 5	/	/	/	-	-	-	-	-	/	-
F 6	/	-	/	-	-	-	/	-	-	-
F 7	/	-	/	-	-	-	/	-	/	-
F 8	/	/	-	-	-	-	-	-	/	-
F 9	/	/	/	/	-	-	/	-	/	-
G 1	/	-	-	/	-	/	/	-	-	-
G 2	/	-	/	-	-	-	-	-	/	-
G 3	/	-	-	-	-	/	/	-	-	/
G 4	-	-	-	-	-	-	-	-	-	-
G 5	-	-	-	-	-	-	-	-	-	-

Table VII

Comparison of Group 1 organisms isolated from students' throats with C. diphtheriae type minus furnished by Frobisher

	<u>Known Minimus</u>	<u>Group 1 Organisms</u>
Size: (microns)	0.6-3.8 x 0.3-1.0	1.2-3.8 x 0.5-1.2
Appearance on stained smear	Barred forms, club shapes, few polar bodies.	Barred forms, club shapes, few granules and polar bodies.
Colony characteristics		
a. Agar slant	Transparent, beaded, dull.	Transparent, beaded, dull.
b. Agar plate	Minute, smooth, convex, shiny, translucent.	Minute, smooth, convex, shiny, translucent.
c. Blood agar plates	Gray, minute, smooth, shiny, translucent.	Gray, minute, smooth, shiny, translucent.
d. Loeffler slants	Small, colorless.	Small, colorless.
Growth in broth	Uniform turbidity upper $\frac{1}{2}$ inch.	Uniform turbidity upper $\frac{1}{2}$ inch.
Urea	No hydrolysis.	No hydrolysis.
Nitrate reduction	/	/
Indol production	-	-
Catalase	/	/
Gelatin	No liquefaction.	No liquefaction.

Frobisher and studied simultaneously. This similarity is shown in table VII.

Results of Schick tests

The Schick reaction of the students from whom these 13 cultures were isolated were all found to be negative, hence no follow-up work was done. It had originally been planned that throat cultures would again be studied from those who showed positive reactions to see if the same type of organisms could be reisolated from the throat after immunization.

Bacterial growth on the tellurite medium used

As was stated earlier, the most frequently encountered organism on the tellurite medium used, was a gram positive micrococcus growing apparently uninhibited. The colonies were very shiny, smooth, and convex. When touched with the inoculating needle, the growth was found to be rather sticky and mucoid.

Streptococci, if they grew at all, produced a very small transparent colony. These were difficult to see and it was easy to pick them up with the other colonies. However, contamination with streptococci presented a problem in only a few of the cultures obtained.

One plate showing flat gray colonies proved when subcultured on another medium to be an organism with the characteristic appearance of the yeasts. This colony could not be confused with C. diphtheriae, but did grow well enough and rapidly enough to overgrow the slower growing corynebacteria.

A small gram negative rod was encountered occasionally, but the colonial characteristics were not observed.

