Technic for pathogenicity test. Organisms to be tested for pathogenicity were inoculated over the entire surface of suitable solid media, usually blood agar, incubated 24 to 48 hours and then washed off the media with small amounts of sterile infusion broth. The growth from one blood-agar slant was injected subcutaneously or intraperitoneally into each guinea pig of approximately 250 grams in weight. Usually a control guinea pig which had received at least 1000 units of diphtheria antitoxin was injected at the same time.

Results. Intraperitoneal injections of the S, SR, R and D forms proved fatal. Injections of the G forms brought about paralysis and death only after a period of several weeks, as previously mentioned. Control animals with antitoxin were not inoculated with the G cultures.

Upon two occasions avirulent variants were isolated from cultures of a Park 8 strain. About 25 broth cultures in test tubes of a Park 8 strain producing SR colonies were sealed off as ampoules after the cultures had shown good growth, usually after 48 hours incubation. These ampoules were stored at room temperature and in the dark. From time to time an ampoule was opened and a few drops of the contents transferred to the surface of a fresh agar plate. In the case of ampoule 096, which had aged 140 days, only one colony appeared on the surface of a glycerol agar plate. This colony was white, moist, round, convex and shiny, decidedly smoother than the colony type of the strain at the time it was sealed in the ampoule. The organisms were agglutinated by anti-Park 8 serum, produced the "mitis" type of colony on potassium tellurite chocolate agar but failed to kill guinea pigs weighing 285 and 369 grams, respectively.

Of greater interest is another variant of the Park 8 strain which was produced under similar conditions. Ampoule 097 was opened after 132 days and a few drops spread over the surface of a glycerol agar plate. Four colonies appeared on this plate. The colonies were distinctly smoother than the colonies produced
at the time the Park 8 culture was sealed in the ampoule. One of the colonies was transferred to a glycerol agar slant and maintained on either glycerol agar or blood agar slants as variant strain 097. The organisms gave the typical fermentations of the parent Park 8 strain, i.e., acid from glucose and dextrin and no action on sucrose, and were agglutinated by anti-Park 8 serum. On December 6, 1933, the entire 48-hour growth from the surface of a blood agar slant was inoculated subcutaneously into a 574-gram guinea pig. The animal remained well. The test was repeated on January 13, 1934, using a guinea pig weighing 240 grams and this animal remained normal. It was concluded that these two variants of the Park 8 strain, 096 and 097, respectively, represented avirulent forms.

On November 20, 1934, a 326-gram guinea pig was inoculated with all the 24-hour growth from a blood agar slant of 097 and remained normal. On November 27, 1934, when the animal received a second and similar dose, the animal weighed 402 grams. The animal remained normal for ten days but was found dead on December 18 (20 days). On December 8, 1934, the 12th generation of the strain was inoculated intraperitoneally into two guinea pigs weighing 278 and 282 grams respectively, and both animals were dead within 48 hours.

This experience suggests that the Park 8 strain of the diphtheria bacillus became avirulent upon aging in ampoules but regained its virulence, as judged by guinea pig inoculations, after propagation on ordinary blood agar slants.

**TOXIGENICITY OF THE ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONY PHASES**

*Technic for toxigenicity test.* For testing the toxigenicity of a culture, the organisms were grown for 7 to 10 days in a Roux flask containing about 100 ml. of infusion broth, pH 7.8, containing Bacto-proteose peptone. The flask was shaken to break up the pellicle, allowed to stand for a short time to allow the large clumps to settle to the bottom and the supernatant liquid decanted to a sterile filter assembly. Filtration was made through either Berkefeld or Chamberland filters by a technic which has
been described in detail elsewhere (Morton, 1938). The filtrate was placed in suitable sterile containers, a preservative, 0.3 per cent tricresol, added, and then placed in the cold room or refrigerator for a week or 10 days. After aging, the filtrate was injected in various amounts into normal 250-gram guinea pigs. The smallest amount of sterile filtrate killing a 250-gram guinea pig in 96 hours was taken as the minimum lethal dose (M.L.D.) of the filtrate.

Results. The S, SR, R and D forms elaborated a soluble toxin, as evidenced by the fact that a sterile cell-free filtrate of the cultures produced death in guinea pigs. There was, however, a quantitative difference, the D forms being much less toxigenic. Toxins from the S, SR and R forms had an M.L.D. of about 0.02 ml., whereas 1 ml. of a similar filtrate from the D form killed a 240-gram guinea pig on the eighth day. Filtrates of the G forms were not tested for toxicity since it was not possible to prepare toxin under the usual standard conditions with this culture type.

The toxin produced by two serological types was neutralized by commercial antitoxin.

AGGLUTINATION, OR AGGLUTININ ABSORPTION, OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, DWARF AND GONIDIAL COLONY PHASES

Technic for agglutination test. Whenever comparative tests were made the original dilution of serum in saline was prepared in sufficient amount to serve in all of the tests. The concentration of the NaCl solution was varied so as not to cause spontaneous clumping of the strains. Usually 1 per cent NaCl was employed. Saline and normal serum controls were included. The rack of tubes was placed in a water bath at 55°C. for 4 hours and then kept at room temperature overnight. Readings were made as soon as the tests were set up, after one and four hours and also after the tubes had stood overnight.

Technic for agglutinin absorption. A measured amount of serum was absorbed with generous quantities of packed, living organisms in a graduated centrifuge tube at 37°C. on three oc-
casions, which was found to be sufficient when working with sera of a titer not over 1:5120. It is not as easy as with many other organisms to produce agglutinating sera in rabbits.

Results. This part of the work has thus far only been carried far enough to establish that there are serological relationships between the several variants. The question of whether or not serological differences exist is reserved until more comprehensive experiments have been made.

The S, SR and D variants of the Park 8 strain have all been agglutinated in Park 8 antiserum to similar titer.

The two G (gonidial) strains which were tested after they had become large enough to be grown in sufficient amounts for antigens, were not found to be agglutinated by serum prepared against the parent culture.

It was impossible to employ the R variants for agglutination because of the tendency to spontaneous agglutination. However, the rough variant of the no. II strain absorbed all the agglutinins from the immune serum prepared by immunizing rabbits against the smooth form of no. II strain.

HEMOLYTIC ACTIVITY OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, DWARF AND GONIDIAL COLONY PHASES

Technic for hemolysis test. Defibrinated rabbit or human blood was mixed with 10 volumes of saline and centrifuged. The supernatant fluid was pipetted off and another 10 volumes of saline added, thoroughly mixed and the suspension recentrifuged. The supernatant fluid was pipetted off and the washed blood cells resuspended in saline to make a 5 per cent suspension. For the test, itself, 0.5 ml. of fresh 5 per cent suspension of washed red blood cells was mixed with 0.5 ml. of a 48-hour broth culture of the organisms and the mixture incubated in a water bath at 37°C. for two hours. A reading was then made, the tubes kept overnight at room temperature and the final reading made next morning.

Table 5 summarizes the results obtained by testing the various colony types against the washed red blood corpuscles of rabbit and human origin.

The results shown in table 5 indicate that the greatest hemo-
lytic power is associated with the S and Sr colony types. The extreme rough colony type is slightly hemolytic and the D colony type non-hemolytic. This might account for the variations in hemolytic power of diphtheria cultures obtained by earlier workers as very little, if any, attention was paid to the colony form of the strains when tested for hemolysis.

**TABLE 5**

*Results of testing members of the diphtheria group and different colony types of the same strain of C. diphtheriae against red blood cells*

<table>
<thead>
<tr>
<th>STRAIN AND COLONY TYPE</th>
<th>RABBIT RED BLOOD CELLS</th>
<th>HUMAN RED BLOOD CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.85 per cent saline (control)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Streptococcus hemolyticus (control)</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Streptococcus viridans (control)</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Corynebacterium hofmanni (control)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Corynebacterium xerosis (control)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C. diphtheriae, O3-64 &quot;S&quot;</td>
<td>++++</td>
<td>++++−</td>
</tr>
<tr>
<td>C. diphtheriae, O3-64 &quot;D&quot;</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C. diphtheriae, II &quot;Sr&quot;</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>C. diphtheriae, II &quot;R&quot;</td>
<td>++−</td>
<td>±</td>
</tr>
<tr>
<td>C. diphtheriae, II &quot;S&quot; derived from II &quot;R&quot;</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>C. diphtheriae, L8 &quot;Sr&quot;</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>C. diphtheriae, L8 &quot;D&quot;</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4 strains C. diphtheriae &quot;mitis&quot;</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>2 strains C. diphtheriae &quot;gravis&quot;</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>2 strains C. diphtheriae &quot;gravis&quot;</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>5 strains C. diphtheriae &quot;G&quot;</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

= no change in the appearances of the red cells.
± = doubtful change in the appearances of the red cells.
+++++ = complete hemolysis (beta type) of the red cells.
++++− = not quite complete hemolysis but more than ++++, etc.
A = Alpha type of hemolysis.

Fermentation reactions of organisms in the smooth, intermediate, rough, dwarf and gonidial colony phases

All the diphtheria cultures tested for fermentation reactions, and this included *gravis* and *mitis* strains as well as S, SR, R and D strains, were found to produce acid from glucose and dextrin but not from sucrose. The S, SR, R and D colony types had the same fermentation reactions. There was, however, slight variation in the rate at which the acid was produced. The
SR appeared to produce acid slightly faster than the S form, but the difference was slight. There was a noticeable difference between the rate of acid production of the R as compared with the S and the SR strains, the R being much slower. The D type produced acid very much more slowly than the S, SR and R strains. The important point is that the S, SR, R and D colony variants of a strain have the same qualitative fermentation reactions, there being only difference in the rate in which acid is produced.

The G strains which were tested for fermentation reactions either failed to produce acid or else produced only a questionable amount.

RESISTANCE TO HEAT AND COLD OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, AND DWARF COLONY PHASES

Heat. Text-books vary considerably in their statements as to the thermal death point of C. diphteriae. It is generally stated that broth cultures of the diphtheria bacillus are killed by ten minutes exposure at a temperature of 58° to 70°C. One observes numerous statements in the literature which imply that the rough stage of a culture is usually more resistant to unfavorable conditions than the smooth stage. To verify that point, determinations of the resistance to a temperature of 56°C. were made on 24-hour-old broth cultures of several strains of the diphtheria bacillus as well as on different variants of the same strain.

Technic for thermal death point determination. Sterile Pasteur pipette stock was drawn into long capillary tubes of about one millimeter inside diameter and of uniform bore and walls. After flaming the tip of the capillary and allowing it to cool, the bacterial suspension was allowed to flow up into the tube by capillary attraction to a height of one inch. Air was then drawn into the capillary tube until the 1-inch column of bacterial suspension reached the 2-inch mark, then the end of the capillary was sealed in a gas flame. The capillary tube was then broken off one inch above the bacterial suspension and that end sealed in the gas flame. The sealed capillary tube, which contained a column of bacterial suspension in the middle with a column of air at
CORYNEBACTERIUM DIPHTHERIAE

Corynebacterium diphtheriae each end, was dropped into disinfecting solution to destroy any organisms which might be on the surface. A series of tubes prepared in this manner were then placed, completely submerged, in an electrically heated water bath at 56°C. and at various intervals of time a tube was taken from the water bath, immersed for a few seconds in tap water at room temperature and then placed in a rack. After the last tube in the series had been removed from the water bath the contents of each tube were transferred to a tube of sterile broth. This was accomplished by wiping the outside of the capillary tube with alcohol, allowing the

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>COLONY TYPE</th>
<th>CON-</th>
<th>TIME IN MINUTES AT 56°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TROL</td>
<td>1</td>
</tr>
<tr>
<td>C. diphtheriae, L8</td>
<td>Sr</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, L8</td>
<td>D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, O3</td>
<td>Sr</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, O3</td>
<td>D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, II</td>
<td>Sr</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, II</td>
<td>R</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, O5</td>
<td>Sr</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, IX</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, XII</td>
<td>Sr</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, VIII</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. diphtheriae, XI</td>
<td>Sr</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = growth.
- = no growth.

alcohol to evaporate, breaking one end of the tube close to the column of bacterial suspension and forcing the suspension into a tube of sterile infusion broth by gently warming the end of the capillary tube which contained the entrapped air. The tubes inoculated with the heated suspension were incubated for 96 hours, with readings being made at the end of each 24-hour period. Usually four series of tubes were carried through the operations at one time, so that all the factors in the experiment except the culture under test were as uniform as possible.

Results. Table 6 summarizes the results obtained in the ther-
mal death point determination of various diphtheria strains and also of the various colony forms of the same strain.

It can be seen from this table that the diphtheria bacillus is not very resistant to heat, being killed by one to three minutes exposure at 56°C. The slight variations in resistance to heat appear to be associated with the individual strains rather than with the colony forms of the strains. These findings are not in agreement with those of Weiland and Leinbrock (1938).

Cold. Since it is fairly generally known that the diphtheria bacillus is less affected by cold than by heat it was decided to try the S and R variants of the same strain against low temperature for varying periods of time. A knowledge of resistance of diphtheria bacilli to cold is also essential for the preservation of the organisms, since recent trends in the preservation of bacterial cultures are to employ methods of drying cultures from the frozen state.

A freezing mixture of solid carbon dioxide (Dry-Ice) and methylcellosolve was employed. This freezing mixture gave a temperature of −60 to −75°C. One milliliter of bacterial suspension was deposited in separate sterile Pyrex glass vials. Estimation of the number of organisms in the bacterial suspensions used was made by means of agar pour plates. The Pyrex glass vials containing the suspension were immersed in the freezing mixture and after varying periods of time two vials (one containing smooth organisms and the other containing rough organisms) were removed. After the suspensions had thawed out at the room temperature, 15 ml. of agar, melted and cooled to 45°C, were added to each vial, the contents were well mixed and poured into a sterile Petri dish. It was not necessary to make serial dilutions of the suspensions after being frozen and thawed as the number of viable bacteria had been greatly reduced. The number of organisms surviving the freezing and thawing, as evidenced by the number of colonies on the agar plates, were counted and the percentage of destruction calculated (table 7).

It can be seen from the results described in this section that the rough phase of the diphtheria bacillus was no more resistant to cold under these circumstances than the smooth phase.
RESISTANCE TO AGING OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONY PHASES

During the course of the studies many of the cultures were inoculated into tubes of infusion broth, incubated (usually overnight) until there was good growth, and the tubes were then sealed as ampoules. It was found that the diphtheria bacillus did not live as long in the ampoules as did many other organisms. Test tubes of practically the same diameter were used and they contained practically the same amount of infusion broth. Usually 15 or more tubes were employed in a series. Each tube in a series was inoculated with the same size inoculum. The ampoules contained approximately 5 ml. of culture and about the same volume of air above the cultures. The ampoules were stored at room temperature and in the dark. From time to time an ampoule was opened and some of the contents was seeded to an agar plate. Whenever growth occurred it was definitely identified as C. diphtheriae. Table 8 summarizes the results.

**TABLE 7**

*Destruction of C. diphtheriae by low temperature*

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>DESTRUCTION IN MINUTES AT (-75^\circ C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>per cent</td>
</tr>
<tr>
<td><em>C. diphtheriae</em>, II, S</td>
<td>76.6</td>
</tr>
<tr>
<td><em>C. diphtheriae</em>, II, R</td>
<td>76.8</td>
</tr>
</tbody>
</table>

**TABLE 8**

*Resistance of the various colony types of C. diphtheriae to aging in ampules*

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>COLONY TYPE</th>
<th>ALIVE*</th>
<th>DEAD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. diphtheriae</em>, II</td>
<td>Very nearly S</td>
<td>90</td>
<td>107</td>
</tr>
<tr>
<td><em>C. diphtheriae</em>, II</td>
<td>R</td>
<td>224</td>
<td>267</td>
</tr>
<tr>
<td><em>C. diphtheriae</em>, 03</td>
<td>SR</td>
<td>171</td>
<td>231</td>
</tr>
<tr>
<td><em>C. diphtheriae</em>, 03</td>
<td>D</td>
<td>467†</td>
<td></td>
</tr>
</tbody>
</table>

* This time represents the maximum number of days in which the organisms were ever found viable.
† End of series.
It can be seen in this experiment that there was a distinct difference in the resistance to aging between the colony forms of the same strain.

REDUCTION OF NITRATES

Contrary to Bergey's Manual (1939) diphtheria cultures produced nitrites when grown in the presence of \( \text{KNO}_3 \).

CHROMOGENESIS

Various observers have reported pigmentation in certain diphtheria cultures. It has been our experience during these studies that chromogenesis took place seldom and irregularly. Upon different occasions throughout these studies it was noticed that certain strains of diphtheria bacilli developed a pigment which was somewhat out of the ordinary. A lemon-yellow pigment was observed in several cultures of one of the Park 8 strains which was in the Sr phase. In one instance the pigment was produced in a stock culture of the Park 8 strain being carried on Loeffler's medium and in the cold room. This pigmentation appeared in a few generations after receiving the strain. In another instance the lemon-yellow color developed in a single-celled strain of the Park 8 culture kept on plain infusion agar at room temperature and in diffuse daylight. It appears that the production of pigment is not necessarily associated with a change in any other characteristic of the culture. The pigmentation seems to appear more often and to a greater extent in the S or Sr culture phase.

CELL MORPHOLOGY

More significance is attached to the morphology of diphtheria organisms than in the case of any other microorganism; yet the diphtheria organism is most pleomorphic. Since attention has been directed to the study of bacterial colonies, it has been found that certain morphological forms can be associated with the various colony types. This is also true in the case of the diphtheria species.

Organisms from the S colony are fairly long and slender, sometimes slightly bent and showing the typical V, L and pallisade
arrangements. They show the classical irregular staining and granules by methylene blue. They are gram-positive.

Organisms from the SR colony are longer than the organisms from the S colonies. They show a greater tendency toward irregular staining and irregularly swollen forms. They show the typical arrangements and are gram-positive.

Organisms from the R colony are characterized by the definite tendency for the organisms to form chains or threads. They are gram-positive and usually stain more intensely than do organisms from the other colony types. The cells are usually thicker than the organisms in the S and D colony types.

Organisms from the D colonies are very short, and somewhat thick, rods. They are gram-positive and usually stain solidly with the gram and methylene blue stains. They show the typical arrangements of cells and some tendency towards the irregularly swollen forms.

Organisms from the G colonies are short rods, sometimes being nearly spherical. They show the typical arrangement of cells, are variable towards the gram stain and stain irregularly with methylene blue.

MORPHOLOGICAL FORMS NOT ASSOCIATED WITH COLONY FORM

A. Coccoid forms. At various times during these studies the bacilli were observed to assume coccus-like appearances. It was found possible to cause this change to appear or disappear at will. The change, either from rods to coccoids or vice versa, was in many cases practically complete. "Coccus," "coccal," "coccoid" and "staphylococcus" forms of the diphtheria bacillus have been described in the literature. Many of the circumstances bringing about these forms have been listed elsewhere (Morton).

The coccoid forms were encountered under the following conditions: In the course of the isolation of a single cell of the Park 8 strain by the method of Kahn (1929), a very rich medium was employed, consisting of infusion broth, pH 7.6, containing 5 per cent glycerol to which was added sterile glucose solution and

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1 I wish to express my appreciation to Dr. Arthur Bernstein for his assistance in the study of the coccoid forms.
sterile normal rabbit serum to the concentrations of one and 20 per cent, respectively. The single cell picked was of Wesbrook's type C. Culturally and serologically the single-cell and parent strains were identical. When the single-cell strain was grown in this rich medium, only spherical cells were present; the organisms appearing very similar to staphylococci. When these spherical cells were transferred to ordinary blood agar, typical diphtheria bacilli resulted.

The single-cell culture showing typical diphtheria bacilli was seeded into glycerol-glucose-serum broth and after 24 hours the growth showed only an occasional granular rod, a few short, plump, solid-staining rods and numerous spherical cells or slightly elongated spheres. Seeded simultaneously into glycerol-glucose-serum broth and onto a Loeffler slant the resulting cultures after 48 hours incubation showed spherical cells and typical rods, respectively. The culture on Loeffler's medium contained granular, barred and solid-staining rods, whereas on blood infusion agar and glycerol agar, the culture showed the granular type of rods almost entirely.

To ascertain, if possible, which ingredient in the glycerol-glucose-serum broth was producing these variations in morphology, the following media were seeded from a Loeffler slant culture and incubated 48 hours.

- Infusion broth, pH 7.3
- Infusion broth + 20 per cent serum
- Infusion broth + 1 per cent glucose
- Infusion broth + 1 per cent glucose + 20 per cent serum
- Infusion broth + 5 per cent glycerol
- Infusion broth + 5 per cent glycerol + 20 per cent serum
- Infusion broth + 5 per cent glycerol + 1 per cent glucose
- Infusion broth + 5 per cent glycerol + 1 per cent glucose + 20 per cent serum

The media are listed according to their ability to change the rod forms into coccoid forms, the first named medium being the least effective and the last named, most effective. Ten-per-cent normal rabbit serum could be substituted for the 20 per cent with about the same results. Stained preparations were made
after the incubation period (fig. 1), then a subculture was made from each type of medium onto a Loeffler slant. The cultures on Loeffler's slants were examined after 48 hours incubation and in all cases the morphology was found to be identical with that of the Loeffler culture of the stock strain.

To determine the effect of normal serum of different species of animals on the morphology of the diphtheria bacillus, the following media were inoculated from a blood agar slant culture and incubated 48 hours.

![Figure 1. C. diphteriae, Park 8, Single Celled Strain, Grown 48 Hours at 37°C. Methylene blue stain, X 2650.](image)

**Infusion broth + 20 per cent guinea pig serum**

**Infusion broth + 20 per cent rat serum**

**Infusion broth + 20 per cent human serum**

**Infusion broth + 20 per cent rabbit serum**

**Infusion broth + 20 per cent horse serum**

**Infusion broth + 20 per cent beef serum**

Practically no change in the morphology of the culture was noticed in the case of the guinea pig serum. Rat and human sera produced a slight change to the solid-staining rod and coccoid
forms, there being no difference in the effect of serum from Schick + and Schick – individuals; rabbit serum caused a distinct change to the coccoid and solid-staining forms while the horse and beef sera produced a very marked change to the coccoid forms.

No agglutinins could be demonstrated in the sera which caused the production of coccoid forms. Normal horse serum was separated into its albumin and globulin fractions by the addition of an equal volume of saturated solution of ammonium sulfate. After filtration, dialysis and sterilization by Berkefeld filtration the albumin and globulin fractions were added to the culture medium. When either fraction was added to the culture medium, coccoid forms resulted.

Best coccoid formation was obtained when the sera were added to the culture medium under aseptic conditions. If the serum and medium were autoclaved, there was very little tendency for the production of coccoid forms. Heating at 56°C for 30 minutes had no noticeable effect on the ability of a serum to produce the coccoid forms. Normal horse serum from four different sources was tried and, while the extent of coccoid formation varied, none of the sera failed to give coccoid forms.

To test the observation of Yarisawa, that the amount of heat employed for sterilization of the Loeffler's medium had an effect on the morphology of the organisms, a batch of the medium was prepared; part of it being sterilized in the autoclave as described previously and the remainder being sterilized in the usual manner by heating in the Arnold sterilizer on three successive days. Both types of media were then inoculated from the same stock culture and incubated 48 hours. The autoclaved medium showed long, slender, pleomorphic, granular bacilli, whereas the medium which had been sterilized in the Arnold gave shorter and more solidly staining organisms.

In summary, it was concluded from these experiments that the morphology of the diphtheria bacillus may be changed to that of coccoid cells under the influence of normal sera from various species of animals. The serum from individuals of a given animal species varies somewhat in its ability to transform the
Corynebacterium diphtheriae

morphology to that of coccoid forms; the globulin and albumin fractions appear to work equally well in bringing about the transformation. There were no demonstrable agglutinins in sera which brought about the production of coccoid forms. The heating of a serum at 56°C for 30 minutes did not alter its ability to transform the morphology of the diphtheria bacillus. Prolonged heating, such as autoclaving at 120°C for 20 minutes, reduced, but did not completely destroy, the ability of serum to change the morphology of the diphtheria bacillus to that of coccoid forms.

To determine whether or not the "coccal" forms, which had been produced by Pope and Pinfield with the aid of copper sulphate, were in any way similar in appearance to the coccoid forms produced in glycerol-glucose-serum broth, the following experiment was performed.

Sterile beef infusion broth, pH 7.3, (sterilized in the usual manner) was dispensed into sterile test tubes in 5 ml. amounts. A 0.04 per cent solution of copper sulphate in distilled water was prepared and sterilized by filtering through a Berkefeld W filter using a negative pressure of 10–15 cm. of mercury. The sterile copper sulphate solution was dispensed in varying amounts into the tubes of broth to give final concentrations of 17 to 38 mgm. of copper per liter. To make sure that any change occurring in the morphology of the culture was due to the presence of the copper sulphate in the medium and not to dilution, a similar series of broth tubes was set up to which were added corresponding amounts of sterile distilled water. All the tubes were incubated 24 hours to insure sterility of the medium, then two drops of a suspension of the single-cell strain, prepared by washing off a blood-agar slant, were added to each tube. The tubes were then incubated 48 hours and stained preparations made.

In all the tubes containing the copper sulfate, the morphology of the organisms was that of coccoid and diplococcoid forms, with a few solid staining and barred forms present. The control series of tubes showed only typical, granular bacilli.

The stained preparations made of cultures from glycerol-glucose-serum broth and from copper sulphate broth appeared
practically identical. The fine granular types of growth in the two media also appeared very similar. The growth from a flask of broth containing copper in the concentration of 23 mgm. per liter was centrifuged and resuspended in 0.85 per cent solution of NaCl. The resulting suspension of coccoid forms was agglutinated by rabbit serum produced against the granular forms of Park 8 rods to the full titer of the serum. Transplants of these coccoid forms were as virulent for guinea pigs as the stock strain which was maintained on blood agar.

Pope and Pinfield attributed the "coccoid" formation as being due to the presence of the copper ion in the medium. Some observations suggested that sulphate and magnesium ions as well as copper ions might produce the change in morphology. The final concentration of copper sulphate in the broth which gave good coccoid formation was N/1000. Various other salts were made up in aqueous solution, sterilized by Berkefeld filtration and added in 2 ml. amounts to tubes containing 5 ml. of broth to give a final concentration of N/1000. The plan of the experiment was essentially the same as that described for the previous experiment. Table 9 summarizes the experiment.

Cultures consisting of the coccoid forms were inoculated into plain Hiss serum water + brom-cresol purple and also into tubes of the same medium containing, respectively, glucose, sucrose, and dextrin in the amounts of one per cent. The fermentation

<table>
<thead>
<tr>
<th>SALT</th>
<th>MORPHOLOGY OF CELLS WHEN 48 HOURS OLD</th>
</tr>
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<tbody>
<tr>
<td>CuSO₄</td>
<td>Coccoids</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Coccoids</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>Coccoids and a few very short rods</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>Short, solid rods in pairs and some coccoids</td>
</tr>
<tr>
<td>Cu(NO₃)₂</td>
<td>Coccoids and some very short rods</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>Typical rods, very granular</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Coccoids and some very short rods</td>
</tr>
<tr>
<td>NaCl</td>
<td>Typical rods</td>
</tr>
</tbody>
</table>

Control: 5 ml. of broth and 2 ml. of distilled water. Typical rods.
CORYNEBACTERIUM DIPHTHERIAE

801

reactions were typical for *C. diphtheriae*. As reported by Parish, the coccioid forms persisted in the Hiss serum water. When the stock culture of *C. diphtheriae* was inoculated into a similar set of tubes of Hiss serum water + brom-cresol purple containing the three carbohydrates and a control tube of plain Hiss serum water and indicator, the growth in the control tube and in the tube containing sucrose (which was not fermented) was made up of a large number of coccioid forms and short solid-staining rods. The coccioid forms were even more numerous in the glucose and dextrin tubes in which the carbohydrates had been fermented. A tube of infusion broth pH 6.2 gave small forms, but not coccioids.

In addition to the influence of the normal sera of various species of animals upon the morphology of the diphtheria bacillus, it was concluded from these experiments that the presence of very small amounts of various salts to the culture medium also had a marked effect. In extension of the views of Pope and Pinfield, who believed that it was the copper ion in copper sulphate which caused the transformation to the coccioid forms, the sulphate ion, as well, appears to be able to bring about the transformation. It appears, then, that either the anions or the cations may influence morphology. The coccioid cells appear very much alike, whether produced by the addition of various normal sera or salts to the culture medium.

Discussion. The purpose of this section of the work has been to investigate these recorded instances in which the diphtheria bacillus assumed the coccioid form so that these transitory forms will not be confused with coccus-like variants, which appear to be entirely different in nature. The term " coccioid" is given these forms because they are very transitory, and do not take on any properties that are strikingly different from the rod forms from which they are derived and to which they readily revert. Although they may appear to be perfect spheres, it is also possible to see, mixed with them, cells which are slightly elongated and which are almost impossible to distinguish as either short, swollen rodlets or slightly elongated spherical cells.

Yarisawa’s (1926) conclusion that the occurrence of coccioid
forms of *C. diphtheriae* was a transitory phenomenon in accordance with conditions, variety and surroundings of the medium apparently holds true and is borne out by the experiments in this section and literature cited previously. The change from rods to coccoids, or *vice versa*, takes place in one generation and, in many cases, is practically complete. This does not parallel the production of such dissociative variants as the rough and smooth colony types, for which a number of generations are usually required and, in which the transformation is often more gradual. Another feature in which the coccoid forms differ from actual dissociants, is that the former are not stable. Toxicity tests could not be performed because as soon as the culture was placed in a plain bouillon, suitable for toxin production, the cells assumed the rod form. Even when kept in contact with the agent which brought about their formation, the coccoids gradually reverted to the rod form. There was thus no way of maintaining a pure culture of coccoid forms suitable for prolonged study.

The fact that certain animal products, when brought into contact with the culture, exert a marked change in the morphology of the culture might explain the staphylococcus forms which Killian was able to re-isolate from guinea pigs, the change in morphology which Gins and Jermoljewa and Malcherek noticed when a sterile emulsion of guinea pigs' liver or kidney was added to the medium, and the coccoid forms which Crowell observed with the peritoneal fluid of normal guinea pigs, *in vivo*. That insufficient heating does not destroy the power of animal products to alter the morphology is in agreement with the observation that Loeffler's medium gave a variety of morphological forms (Yarisawa), depending on the amount of heat to which it had been subjected during the sterilization process; that Grubb and Koser obtained coccoid forms on liver infusion agar; that Hadley obtained coccoids by means of ascitic fluid, and that occasionally the coccoid forms appear in ordinary media (Heinemann, Parish, Parker). Pope and Pinfield's observation that the presence of a certain ion may exert a profound influence on the morphology of *C. diphtheriae* was verified. The copper ion,
however, is not the only one which can bring about this change to coccoid forms. It appears that magnesium and sulphate ions may produce this change as well. This perhaps also accounts for Maver’s observations of coccoids in synthetic media.

The colony type of a culture of coccoid forms remains the same as when the culture is composed of rods. The tendency of the cells of *C. diphtheriae* to adhere to one another after fission is very striking with the coccoid forms, giving diplo and tetrad forms. Maver (1931) shows some characteristic tetrad forms from digested Loeffler’s serum, which are identical to the tetrad forms observed in media containing raw serum. Plain agar plates streaked from cultures showing these tetrad forms show only colonies of typical diphtheria bacilli.

With methylene blue the coccoid cells may stain solidly or, occasionally, show one metachromatic granule within the cell. By Beck’s stain the majority of organisms take the counter stain while a very few of them stain solidly or partially with gentian violet.

Grubb and Koser observed rod forms to “contract” into coccoid cells on liver infusion agar. All of these observations indicate that these coccoid forms merely represent another morphological type of the diphtheria bacillus. These coccoid forms do not assume characteristics which would place the culture, necessarily, in a colony phase different from that of the rods from which they were derived.

There are, however, instances in the literature in which the change to coccus-like cells suggests a phenomenon which is different from the production of coccoids as herein described, namely, the A-forms (Pettenkofer) of Kuhn (1924), the C-forms (coccus) of Kuhn and Sternberg (1931) and the G-forms of Hadley, Delves and Klimek (1931). In these cases the culture has become changed and stabilized in such a manner as to indicate a definite culture phase.

The C-forms of Kuhn and Sternberg and the G-forms of Hadley, Delves and Klimek are now thought to be one and the same culture phase and to represent the filterable form of the organism (Hadley, 1933). The A-forms (Pettenkofer) of Kuhn have not been
explained on the basis of colony phases. In one instance when a strain of the diphtheria bacillus was being propagated in infusion broth plus as much LiCl as the organisms would tolerate and still produce visible growth, morphological forms were encountered which were suggestive of the Pettenkofer forms. These forms were not investigated further and reverted to typical rod forms during their propagation on plain agar slants.

Kuschnarjew (1930) and more recently Stone and Hobby (1934) described "coccus" forms of the diphtheria bacillus which may be different from the coccoids just described. Cultures of these "coccus" forms in broth showed turbidity, but no pellicle. The growth near the surface was ropy. Colonies on solid media were mucoid in consistency. Stone and Hobby found their "coccoid" forms to be more susceptible to bacteriophage than the rod forms. It might be that these workers were dealing with a stage of the diphtheria culture quite different from the rod forms.

B. Changes in the morphology depending upon the youth and age of a culture. A few of the early workers were of the opinion that there was a normal evolution in the morphological forms of the diphtheria bacillus. In this connection the works of Deny (1903), Clark and Ruehl (1919), Powell (1923) and Henrici (1928) may be mentioned. All of these workers found that there was a definite series of changes in diphtheria cultures when planted on fresh media and observed over a fairly long period of time. To lend further support to the findings of the workers mentioned above, the observations which were made on the Park 8 strain during these studies might be briefly mentioned.

The strain was inoculated into glycerol infusion broth and then stained preparations were made hourly for the first 24 hours (fig. 2). Additional observations were made at the end of 36, 48, 72 and 96 hours as well as at the end of one, two, three, four, five and seven weeks. During the first few (two to five) hours of growth the cells were small and solid staining, usually appearing as very short rods or as spheres. After the 5th hour the cells elongated. Granules appeared at about the 13th hour. The
length of the rods remained fairly constant from about the 22nd hour to the end of the first week; thereafter they elongated somewhat for a period of a few weeks, but at the end of the 7th week they had shortened to coccoid forms. Club forms were first observed at 24 hours. Barred forms were first noted at the end of the second week. About the end of the first week two general types of rods were present. One type was long and slender and stained a very faint blue with Loeffler's methylene blue, whereas the other general type of rod was short and thick and more solid staining. The granules also assumed a different appearance.
when stained by methylene blue. Until the first week the granules had stained an intense blue, afterwards they were somewhat smaller and stained a bright red. These observations were made when an actively growing culture was transplanted to fresh glycerol broth and are in agreement with the observations of the workers mentioned above.

No record could be found of observations upon the series of events when an old, dormant culture was transplanted to fresh media. For these observations a broth culture of the Park 8 strain, which had aged in an ampoule for 144 days was used. Upon opening the ampoule, a Loeffler's methylene blue stain revealed that about one-half of the organisms were moderately long, slender, typical diphtheria rods of a uniform light-blue tint. The remaining rods were granular. Some of the rods possessed small granules of a deep blue color while a few of the rods contained granules of a reddish color. Sometimes these red granules appeared well separated from any bacterial cell and at other times some of the granules appeared to have a small amount of bluish debris adhering to them. The isolated granules varied in size from the limit of visibility to 0.5 of a micron in diameter. Occasionally the red granules gave a very clear-cut picture of a small diplococcus, suggesting that they might be undergoing division.

Three drops of this 144-day-old culture were spread over the surface of a glycerol agar plate and the plate incubated. Nothing appeared on the plate during the first 48 hours. At the end of the 5th day, about 300 well separated colonies were present on the plate. These colonies varied in size from 0.2 to 0.5 mm. in diameter. The colonies were convex, opaque, of a creamy color and the margins entire. As far as colony appearances were concerned, these colonies were no different from any other colonies of the Park 8 strain, being typically Sr. All of the various-sized colonies appeared to be composed of the same type of organisms.

The cells from several of the typical diphtheria colonies were stained by methylene blue. The organisms were variable in size and shape. There were solid-staining blue spheres and some
CORYNEBACTERIUM DIPHTHERIAE

elongated spheres. Some of the elongated spheres had a light-staining band in the center so that they appeared like a diplococcus. Some of these blue cocci contained red granules within them, in other cases the small red granule protruded from the surface. In addition to these various spherically shaped cells there were a few short solid-staining rods and sometimes these short rods stained very lightly and contained a reddish granule at each end of the rod. These organisms did not at all resemble diphtheria organisms. One of the colonies showing these peculiarly shaped organisms was transferred to a glycerol agar slant. There was good growth after 48 hours and the organisms in a

methylene-blue stained preparation still showed a very few of the spherically shaped cells, but for the most part the cells were of the rod form. There were long thick rods, much larger than ordinary diphtheria bacilli, which contained both bars and granules and short rods which were thicker than the usual diphtheria rod. The width of these short rods was about the same as the diameter of the cocci in the previous culture so that they appeared as if they were elongations of the spheres. Third and fourth generations of this strain showed the organisms in practically their normal morphology, as shown in figures 2 and 3. The growth from a 24-hour-old blood agar slant culture killed a 300-gram guinea pig in less than 45 hours. The organisms were

FIG. 3. C. diphteriae, Park 8 Strain
Aged 144 days in an ampoule at room temperature then transferred to glycerol infusion agar. Methylene blue stain. × 2650.
agglutinated by an agglutinating serum which had been prepared against the parent Park 8 strain.

At the same time that the glycerol agar plate was inoculated with three drops of the aged culture from the ampoule, three drops were also inoculated into glycerol infusion broth. The culture was propagated serially in glycerol infusion broth and showed the same transition in morphology as the culture did on glycerol agar. Only one difference was noted and that was that about twice as many generations were required in broth as on agar before the organisms returned to their usual morphology.

Thus, the granular-barred form described by Schultz was encountered. Upon other occasions it was observed in stained preparations of the diphtheria organisms, but it is not as common as Wesbrook's types. The fact that this form does occur warrants its inclusion in any table of morphological types of the organism. Since coccoid forms also have been observed by numerous workers, this form, too, should be included among the morphological forms.

**DISCUSSION**

Inasmuch as smooth and rough colony variants exist for practically all species of microorganisms which have been studied at all extensively and since the terms smooth and rough are descriptive terms for such colony variants, have been found applicable to bacterial species in general and have priority to the terms mitis and gravis in reference to the diphtherial species, it is logical to attempt to explain the mitis and gravis variants in terms of the older and more generally used terminology. From the literature reviewed and the experimental work cited it appears that the mitis colonies are similar to the previously described smooth colonies for the diphtherial species, and the gravis colonies are similar to the larger, intermediate colony form. The group of colonies not classifiable as typically mitis and gravis appear to be nearer the rough phase than the larger intermediate or SR type colony.

A special medium containing potassium tellurite is not necessary for differentiation of the colony types. Unquestionably the
special tellurite medium may be very helpful when working with clinical material where one has present a mixture of microorganisms, some reducing the metal and the colonies thus becoming blackened, and others not reducing the metal and the colonies remaining uncolored. Anderson, Happold, McLeod and Thom-son stated as one of the characteristics of the mitis type of colonies that the organisms are partially inhibited in their growth on the special potassium tellurite chocolate agar. This inhibition of growth of the mitis and of the S colony types has been observed—sometimes complete inhibition even when large inocula were used—so the question arises as to how many mitis or S strains might be missed when employing this medium to the exclusion of all others when searching for the diphtheria organism. In the study of 57 bacteriologically proven cases of diphtheria, Cooper, Peters and Wiseman (1939) reported the isolation of 4 strains of C. diphteriae on Loeffler's medium which failed to appear on the potassium tellurite agar medium, Perry and Petran (1939) also report similar experiences. Their recommendation of the use of both media for the isolation of the diphtheria organism from clinical material is logical. A medium, in order to be perfectly satisfactory for the growth of the diphtheria organism and especially for isolating the organism from clinical material, should not exert an inhibitory action on any culture phase of the organism.

From the meager descriptions of the small colony forms in the literature it appears that the organisms are much smaller than those which are usually considered normal for the diptherial species, and in the few instances in which the cultural reactions have been investigated, these organisms appear to require a somewhat longer period of time to bring about the various reactions which are usually considered characteristic of the diptherial species. The observations on the strains of small colony variants described in this paper substantiate these earlier observations.

In 1935 the term “D,” or dwarf, was employed (Morton, 1935b) to denote small colonies of the diphtheria bacillus because the organisms in these dwarf colonies had not been shown to be filterable, had not gone through a stage in which their growth
was invisible, had not been obtained by any special bacteriological technics and did not show a great variation from the parent culture of normal, large size except for their relatively small colony size and slowness in bringing about biochemical reactions. It was considered best to discontinue the use of the term "small" as sooner or later an abbreviation would be used, and since the letter S already signified the smooth colony type. The small colonies sometimes observed in other species of microorganisms have been designated as midget, minute or dwarf colonies. The term midget and minute, while descriptive terms for the colonies, are unsatisfactory as sooner or later they will be abbreviated to the letter M which already denotes the mucoid colony form. Neither are these small colonies the C forms of Kuhn and Sternberg (1931) as the morphology of the cells does not resemble a coccus. Moreover, Hadley (1933) announced that his G colonies were similar to the C colonies of Kuhn and Sternberg. Since the size of the colonies and the physiological activity of the organisms within the small colonies are dwarfed in comparison to that which has been commonly recognized for the species, the term D or dwarf was selected. It signifies that the size and activity of the organisms in this culture phase appear small or slight in comparison to the colony sizes and activities of those organisms commonly regarded as the normal size for the diphtherial species. Also the letter D was not being employed for any other culture phase. A later search of the literature for evidence of similar small colony forms for other bacterial species revealed that Eisenberg in 1914 also employed the term dwarf to designate a small colony form of the typhoid bacillus. This ability to exist in an extremely small colony form in addition to the more common large colony forms, is not a characteristic of the diphtherial species alone but is shared by about twenty other bacterial species (Morton).

In 1931 Hadley, Delves and Klimek described at some length the filterable form or G culture phase of the Shiga dysentery bacillus and analogous forms of culture in 11 other species, namely: Escherichia coli, Eberthella typhosa, Salmonella paratyphi A and B, Salmonella enteritidis, Salmonella cholerae-suis, Sal-
monella typhimurium I, Salmonella typhimurium II (Bacillus pestis-caviae), Corynebacterium diphtheriae (Park 8 strain), Lactobacillus acidophilus and Vibrio cholerae. In the same year Kuhn and Sternberg (1931) described several culture phases for bacteria, some of which were similar to the culture phases previously described by other workers. Hadley (1933) pointed out this similarity, stating that the C (Kokken formen) seemed definitely related to the G forms. In addition to producing C forms for Escherichia coli, Eberthella typhosa, Salmonella typhimurium, and Corynebacterium diphtheriae, for which Hadley, Delves and Klimek had described G forms, Kuhn and Sternberg produced C forms also from Vibrio metchnikovi, Spirillum volutans, Bacillus anthracis, Bacillus pseudo-anthracis, Proteus vulgaris and Proteus X-19, Mycobacterium tuberculosis, Salmonella suipstifer, "Schweineseuche" and the dysentery bacillus of Shiga-Kruse.

Since 1931 small colonies and G colonies have been described for many bacterial species. Many of the small colonies referred to in the literature as G colonies are so named solely because of their small size; however, smallness of size is not the only criterion for the G culture phase. The small colonies of the diphtheria bacillus herein described as G colonies are so named because they fulfill the 5 postulates of Hadley (1931) for filterability and the G culture phase. This work is a confirmation of the experiments of Hadley and Richardson on the Park 8 strain which were described in the monograph by Hadley, Delves and Klimek. Haddow (1938) described G forms for Salmonella paratyphi B (Tidy) and reported the appearance of similar G colonies in cultures of Eberthella typhosa (Cole), Salmonella paratyphi A (Schottmüller) and a recently isolated strain of Escherichia coli. The G colonies for Salmonella paratyphi B (Tidy) fulfill the postulates of Hadley.

GENERAL CONCLUSIONS

A study of the diphtherial organism from many angles of approach reveals that its colonies on agar are characterized by at least four distinct, stable colony types and are not necessarily "small, grayish, granular, almost transparent, lace-like, margin
irregular" as described in Bergey's Manual for Determinative Bacteriology. As in other bacterial species, these colony types have been described as smooth (S), intermediate (SR), rough (R) and dwarf (D), according to their appearance on plain infusion agar.

Organisms from these four main colony types are true diphtheria bacilli, as judged by their tinctorial, cultural, and serological reactions and by their pathogenicity for guinea pigs.

With these differences in colonial appearances on plain agar can be associated variations in the manner of growth in liquid media, in stability in saline solutions of varying concentrations of sodium chloride, differences in appearance on potassium tellurite agar and certain quantitative variations in such properties as fermentation, toxigenicity, hemolysis, resistance to aging, etc.

There is order in the variation process within the diphtherial species. It is possible to predict, with a reasonable degree of certainty, the cultural reactions and the morphology of the organisms when a strain passes from one colony form to another. There is less certainty, however, which colony form will be the first to appear when a strain is undergoing forced dissociation. In contrast to some bacterial species, the variations in the characteristics of the organisms from the various colony forms are quantitative rather than qualitative.

In addition to the four stable colony forms of the diphtheria organism mentioned above, another phase, namely the G, was encountered. This G culture phase differs from all the other colony variants in that it is filterable, usually passes through a period of invisible growth, and when G colonies are obtained, by special culturing technics, the organisms differ from the more common colony forms biochemically, serologically and in pathogenicity. The characteristics of the G colonies of the diphtheria organism in relation to the larger and more common colony forms are not at all unlike the characteristics of the G colonies in relation to the larger colony forms of other species for which the G culture phase has been produced.

Unlike most other bacterial species the morphology of the diphtherial organism is very alterable without there being a
concomitant change in colony form. Because of its practical importance in diagnosis this aspect has been investigated quite extensively. The morphology of the cells differs with the physiological youth and age of the culture, the pH and composition of the medium, the manner in which the medium was sterilized, oxygen tension, symbiotic relationship with other organisms, and in different sections of the same colony.

Many discrepancies within the species reported by earlier workers can be explained by the quite normal variations of the diphtherial organism. These variations within the species are usually associated with the S, R, and D colony variation.

I am very grateful to Dr. Philip Hadley for his guidance and assistance in the earlier dissociative studies and to Dr. Stuart Mudd for many helpful suggestions.

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PLATE 1

THE FOUR MAIN COLONY TYPES OF THE DIPHTHERIA BACILLUS

FIG. 1. (upper left). S, smooth colony, C. diphtheriae, Park S strain, grown 48 hours on infusion agar at 37°C. Reflected light. ×12.

FIG. 2. (upper right). SR, intermediate colony, C. diphtheriae, Park S strain, grown 48 hours on infusion agar at 37°C. Reflected light. ×12. These two colonies S and SR were growing side by side on the same infusion agar plate.

FIG. 3. (lower left). R, rough colony, C. diphtheriae, II strain, grown 48 hours on infusion agar at 37°C. Transmitted light. ×18.8.

FIG. 4. (lower right). D, dwarf colony, C. diphtheriae, Park S strain, grown 48 hours on infusion agar at 37°C. Transmitted light. ×18.8.
(Harry E. Morton: Corynebacterium Diphtheriae)