BRANCHING IN BACTERIA WITH SPECIAL REFERENCE TO B. DIPHTHERIÆ.

HIBBERT WINSLOW HILL, M.D.
(*Director; Boston Board of Health Bacteriological Laboratory.*)

The term "branching" is used in this article to mean apparent union between two more or less elongated bacterial bodies, or parts of bodies, such that one end of one body lies at any point in the length of the other, except an extremity. This definition is adopted to avoid any à priori implication as to the nature of such union, which the use of the term in its ordinary sense might seem to involve.

The chief problems of the subject divide themselves into those of the nature of the branching, the actual morphological mechanism; and those of the origin of the branching, the physiological or developmental relationships. Both are very complex. To place the whole matter of branching as it occurs in different species on a definite basis is as yet impossible. It is not safe to reason closely from one species to another nor can the branching even in one species be regarded as yet well worked out. In this article, attention is confined to the diphtheria bacillus and the results are suggestive rather than satisfactory.

The writer believes the general subject one of fundamental biological importance to bacteriology and, as it affects the diphtheria bacillus, of considerable practical importance in public health work. Indeed theory and practice are more intimately interwoven in hygienic bacteriological work than in any other department of medicine. An intimate knowledge of the morphology and biology of the diphtheria bacillus is peculiarly requisite to meet the demands of every-day work in the diagnosis and release of diphtheria cases. To the failure to recognize this is due some at least of the difficulties met with in practice.

The writer, interested in branching diphtheria since 1895, has of late been more strongly impressed with the importance of the whole subject. Wesbrook[10] in 1899 made public the results of a laborious and painstaking investigation into the morphology of the diphtheria bacillus, passing over the branching forms it is true, but placing the various morphological types encountered in cultures on the most definite basis yet set forth. That his morphological types, purely as such, do exist, no one familiar with diphtheria diagnosis can deny. What the relationships of these types may be to each other, whether or no the types are directly convertible, how they are related, if at all, to clinical types of the disease, to toxicity, to virulence, and to the distribution of the bacillus in nature are problems not yet on a satisfactory basis. Much attention has been paid to dried and stained preparations from cultures in these studies, but the information thus gained must always be largely inferential, although its aid is indispensable. Recognizing this, the writer has studied the morphology and development of individual diphtheria bacilli directly under the microscope. Although attention was given largely to the study of the relationships of Wesbrook's types, branching bacilli, when encountered, were carefully observed also.

Elsewhere[13] the writer's technique is described in detail. Here only the rather sparse results so far achieved connected with the branching itself are dealt with.

The principal hypotheses relating to the nature of the branching of B. diphtheriæ which have been offered up to date are:

Hypothesis one: That the branching is apparent only, due to accidental apposition, and merely an optical illusion.

Hypothesis two: That the single unbranched bacillus, whatever its size, is a single-celled organism; that the branch arises by a pushing out at one side of the cell contents; that this continues until the projection assumes more or less the general size and shape of the original mother rod; constriction at the base of the daughter rod may follow until it separates entirely; or without separation either rod may again branch, giving rise to complicated figures; or either rod may divide further by fission only. (See Figs. 9 to 15, 27 to 30.)

Hypothesis three: That the bacilli are single-celled rods only when very small; that the large (often clubbed) rods, in cultures of which branching seems to be most often found, are really short chains of cells with little or no interval between adjacent members; that these rods elongate usually by the axial elongation and fission of their components, and that branching results by occasional extra-axial elongation of one or more of these. The cell thus elongating sidewise rather than endwise is supposed to continue to elongate and subdivide in the direction of the new axis, forming a new chain of coherent cells at an angle with the mother chain. (See Figs. 6, 16 to 21 and 31, 32.)

Hypothesis four: That whether Hypothesis two or Hypothesis three be correct, the branching is connected in some undefined way with the large granules, sometimes polar, sometimes medial, so characteristic of *B. diphtheriæ*. The particular granules meant are, the writer believes, those which take the reddish tint when Lœffler's methylene blue alone is used as a stain, probably the same granules which with Neisser's stain stand out prominently as a deep blue or black. (See Figs. 17 to 24.) This hypothesis, referred to by the writer in his first article [13] (1898), has not been, so far as he is aware, yet placed on a definite basis. It has been suggested to the writer in a private communication that these granules may be intimately connected with the reproduction of *B. diphtheriæ* in all cases; that new rods develop always from or in connection with such granules in another rod; that such development is usually in the axis of the mother rod and so gives rise to appearances quite compatible with the accepted ideas of multiplication by ordinary fission; that occasional extra-axial development from a polar terminal granule results merely in a bent or V-shaped rod, while extra-axial development from a medial granule gives the appearance of branching. (See Fig. 18 and compare Figs. 16 to 21 with Figs. 25 to 30.)

Hypothesis five: That the branching is merely an exaggeration, accidentally symmetrical, of the swellings, projections, and general irregularities of outline well known to occur in degenerate or dying cultures of many bacterial species. (See Fig. 8.)

The physiological or developmental origin of the branching is accounted for on hypotheses connected intimately with the above hypotheses of its nature.

The first two of these hypotheses of origin hold that branching is a part of the normal active life history of the organism, contributing to its struggle for existence, but of only occasional occurrence under conditions not well understood; that it is a development in an irregular direction of forces similar to those usually resulting in simple fission; that the irregularity of the operation of these forces in this direction indicates their relative weakness and lack of organization, and that they are therefore either remnants of forces once much more constant in operation, perhaps even the most prominent ones (the reversionary hypothesis) or that they are evidences of a now developing tendency towards a more complicated existence (the evolutionary hypothesis).

The third hypothesis of origin, based on the belief that the branching is purely degenerative, traces it to the auto-intoxication by excretory products, loss of oxygen, exhaustion of food, water, etc., occurring in old cultures — in short, to the usual conditions supposed to account for involution in general.

Before giving the writer's results and conclusions, attention must be directed to the work of Nakaniski,[5] on which the third hypothesis of the nature of branching is based. He claims to have determined the presence of a nucleus or nucleus-like body in many bacterial cells, including those of *Bacillus diphtheriæ* and *Bacillus variabilis lymphæ vaccinalis*. He maintains that the smaller forms of these bacilli are single cells, the larger are chains. It seems that some of his work was done as mentioned, not on *Bacillus diphtheriæ* itself, but on the closely allied species, which he at one time[11] considered to be the bacillus of vaccinia, a claim which was afterwards withdrawn.[11] It is probable, however, that his conclusions are as applicable to one as to the other. He describes fission as resulting, before complete separation, in the well-known "double-header" or cuneiform bacilli, which he regards as two cells. He describes and figures also the enlargement and further separation of these cells, and the gradual development of parallelism of their axes, explaining the "box of cigars" formation so often described in stained preparations. (See Figs. 31, 32.)

The writer's observations were made partly on ordinary dried and stained preparations, partly upon unstained organisms while in the process of development on the surface of an agar "hanging block." They deal only with the gross morphology. Whether the larger diphtheria bacilli are composed of one or several cells the writer cannot say from personal observation. One branching form observed seemed to carry out, so far as it went, Nakaniski's view, and the other observations neither confirm nor confute it. (See Figs. 6 and 7.)

The figures in Plate VI. represent some reproductions from sketches made to scale by the writer directly from measurements with a micrometer eye-piece upon developing diphtheria bacilli of ordinary morphology, culture reactions, and virulence to guinea-pigs.

Figs. 1 to 5 indicate the growth and division, by suddenly snapping across, of a single rod, resulting in new rods lying at an obtuse angle. The subsequent enlargement, increasing parallelism, and repeated division of the new rods is shown. The sudden snapping across of the rods and their subsequent angular positions are very characteristic features of ordinary multiplication as seen in these preparations.

Figs. 6 to 8 indicate the development of "branching." As the most satisfactory example yet seen by the writer, the conditions of development of Fig. 7 are described in detail.

The "hanging block" actually observed was made at 11 A.M., December 13, 1901, from an agar culture then five days old which had been grown in the 37° C. incubator for forty-eight hours and thereafter at room temperature. Active multiplication under the microscope began at 11.20 A.M. and continued until the observation ceased. The branching form (Fig. 7A) was stumbled upon accidentally at 4.40 P.M. and at once drawn. Thereafter this form was constantly under observation until 8.30 P.M., when the writer was called away.

The following deductions from these observations and others on stained preparations seem to be in order:

First. — Branching of *Bacillus diphtheriæ* may occur on agar and may begin within five or six hours of inoculation under the conditions described.

Second. — It may occur when active multiplication in the ordinary way is going on all over the same preparation.

Third. — Active multiplication by apparent fission may be going on in a different part of the same rod which is also branching.

Fourth. — After the branch is formed, it may separate from the parent stem.

Fifth. — That portion of the parent stem from which the branch originates may become faint, shrunken, and passive.

Sixth. — Branching is not necessarily a matter of accidental optical apposition, for its gradual development may be watched; in Fig. 7 the branch snapped over (D, E) from the stem, the snapping occurring under the eye of the observer; obviously it must have been attached or it could not have snapped off.

The final oval form of the branch recorded was exactly similar to several oval forms seen by the writer to undergo development, and it is not unfair to assume that this body might undergo such changes. Fig. 4 represents observations on a somewhat similar body, previous developmental history unknown, made some weeks previously.

Fig. 8 indicates a form of apparent degeneration, giving rise to projections simulating branches, in the sense defined in this article.

Here it would seem that a dead or dying rod was under observation. No appreciable increase in length occurred. The separation into the two portions shown (B) occurred gradually, without change in the relative positions of the separated portions. The bacillus (A) was at first fairly definite in outline and density. It became (B) swollen, granular, and faint, some of the granules being very fine. Only the larger granules are figured. Then it became shrunken (C), breaking up slightly, and developing two distinct projections, granuled at their extremities. The granules were refractive points, bright or dark as the focus changed. Their relation to the granules staining reddish with Loeffler's methylene blue was not determined. Active multiplication was proceeding in other parts of the same "hanging block" preparation.

These observations, few and imperfect as they are, so far confirm, explain, and add to the observations already made by various observers that the following conclusions seem justified.

First. — Passive degenerative changes in dead or dying bacilli may give rise in this species to slight irregular projections which distantly simulate branches, using this term in the wide sense already defined in this article.

Second. — As a part of the active development of the diphtheria bacillus, active branching by apparent budding, ending in the production of an oval or elliptical body, probably capable itself of further development and the production of new rods, may occur in very young cultures, the parent stem then degenerating.

Third. — As a part of the active development of the diphtheria bacillus, branching similar to that described, but terminating in an ordinary diphtheria rod-like body, and without any degeneration of the parent stem at the point of origin, may occur within twenty-four hours of inoculation; and this new rod may segment in the ordinary way, or itself produce branches, terminating in rods similar to itself or in oval bodies such as are described above.

Fourth. — Various modifications of all the processes of branching described probably exist.

Fifth. — The origin of the active multiplicative branching may be reversionary or evolutionary or merely due to special conditions of growth not understood at present. According to the latter view, the active forces usually resulting in fission may at times undergo a lessening of tension or some other modification which results in a change in direction of their activities. Whether such a change is regressive, " a degeneration of forces " as phrased by Sedgwick, or progressive, an " ascension of forces " has not yet been determined.

[Whether the force manifested in branching proper indicates progression or regression in the individual does not affect the question as to whether the possession of such a force indicates in itself reversion or evolution in the species. Two distinct problems are involved. It may be possible to determine that problem relating to the individual; that relating to the species must probably remain speculative.]

Review of Literature. — In May, 1900, Hektoen reviewed exhaustively the literature relating to all branching bacteria without coming to any very definite conclusion, and since that time the writer has been able to find very few articles relating to the subject. In 1900 Skschivan[2] described branching plague bacilli at some length, and Galli-Valerio[8] and Conradi[9] described branching glanders bacilli. Reichenbach[3] in 1901 described branching in spirilla, and recently Meyer,[4] after a study of the *Bacillus cohærens* and other branchers, concludes that branching is a " rudimentary and infrequent reversion to ancestral types." He finds branching in young cultures, agreeing in this with Gorham and the writer, and he feels justified on this ground in believing that the bacteria in general furnish in this particular an example of correspondence between phylogeny and ontogeny, the development of branches in the young cultures being analogous to the development of branches in the species itself at an early stage in its history. His view of the branching process itself apparently corresponds with that of the second hypothesis already given. Nakaniski, in 1901,[5] described for *Bacillus diphtheriæ* and allied forms the process referred to in Hypothesis three. He says that further investigation is required. A. Fischer and others have maintained Hypothesis four. With the exception of Gildersleeve,[6] who, according to Bergey, maintains somewhat Hypothesis three, and the writer, no other Americans have devoted much attention to the subject, so far as the writer is aware, except Craig in 1898 and W. H. Smith in 1900, who both describe branching tubercle bacilli.

Branching other than purely involutionary has now been recorded, according to Chester,[7] for twenty organisms previously supposed to be ordinary bacilli (in the sense of unbranched cells), including those of actinomycosis, tuberculosis (human and avian), glanders, leprosy, diphtheria, etc. Plague bacilli also branch (the earliest reference which the writer has been able to find being in an article by Klebs in 1898). The writer also found branching in a diphtheria-like organism, non-virulent and growing with luxuriance on agar, isolated from the nose of a horse and perhaps identical with Nakaniski's *Bacillus variabilis lymphæ vaccinalis*.

In a preliminary note[13] the writer called attention to a possible connection between the branching of diphtheria bacilli and the metachromatic granule so often found in the parent stem at the base of the branch. But, as was then pointed out, such a granule is by no means always present. Further observation leads to doubt that the connection is very close. Granules, indistinguishable from these, are very common in many types of diphtheria cultures, and are as common in those which do not branch as in those that do. In the branched forms the granules may not be present at all, or may be present in some other situation than at the base of the branch. A granule may be present at the base of a constricted branch, sometimes at the bases of two branches springing from a single point, and no granule may exist at all at the base of a non-constricted branch. The observations of Nakaniski[5] also seem to negative this view. Finally, although the second or third hypothesis may suffice for the diphtheria bacillus, the branching of other bacilli like *Bacillus pestis* remains to be cleared up.

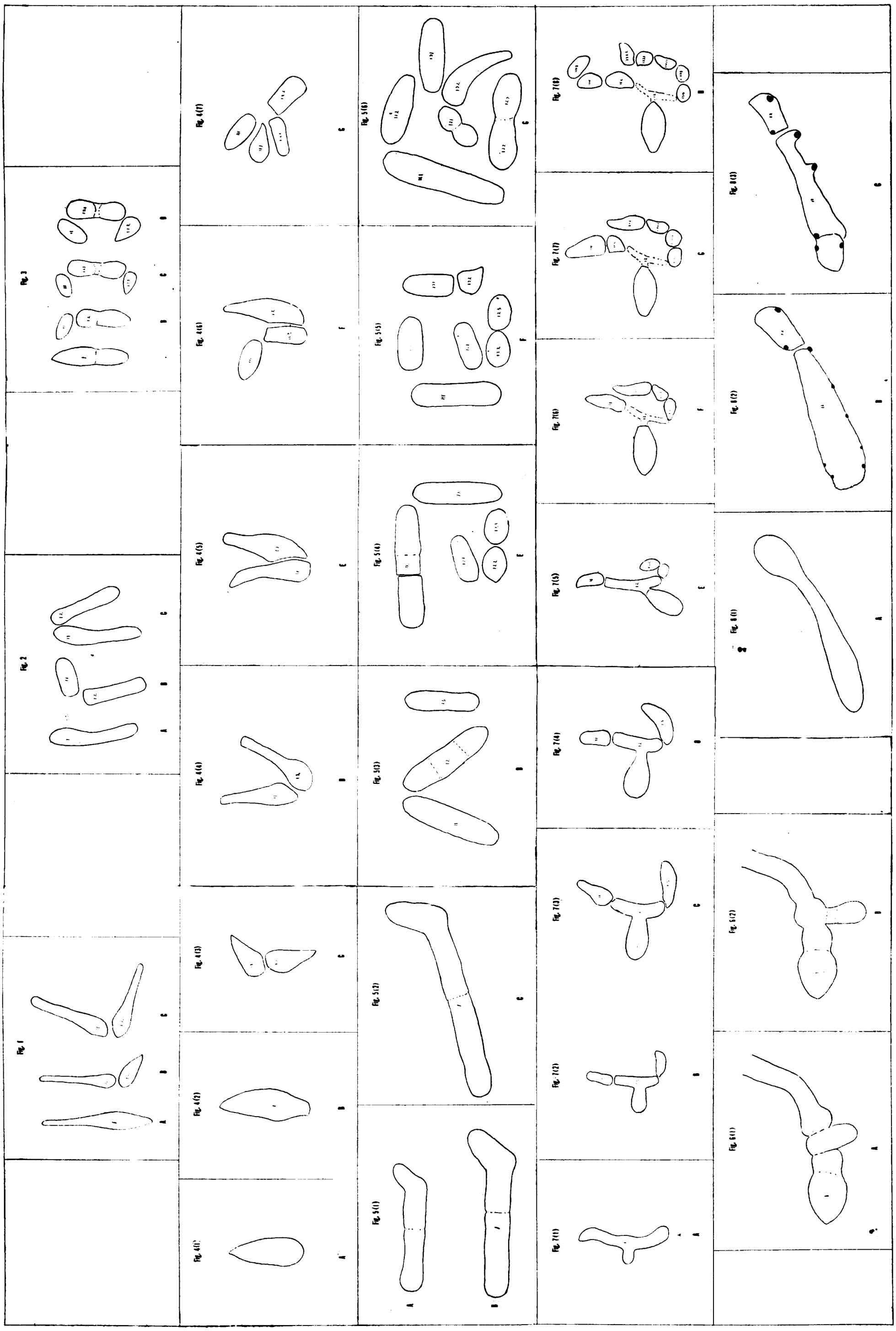
Nomenclature. — Following various previous writers, the writer, in 1899, advocated the name streptothrix for the diphtheria organism, but feels now that in common with others at the time his ideas were far astray. Bacterial nomenclature should follow as far as possible the canons of the botanist. It is easy to see that the whole subject needs much development before consideration of the names to be employed is necessary. Names, indeed, cannot be intelligently selected until the facts themselves are thoroughly established and correlated. Moreover, even the advisability of placing branching bacteria in any class distinct from the forms not yet known as branching seems questionable. It is not improbable that it will ultimately prove simpler, as Meyer maintains,[4] to readjust the definition of the term bacillus, since so many of this group show branching at times, rather than to make any more radical revision. Chester,[7] in his recent work, classes diphtheria bacilli as myco-bacteria, a step which, while proper in so far as it recognizes the branching peculiarities as of importance, is perhaps a little premature.

DESCRIPTION OF PLATES.

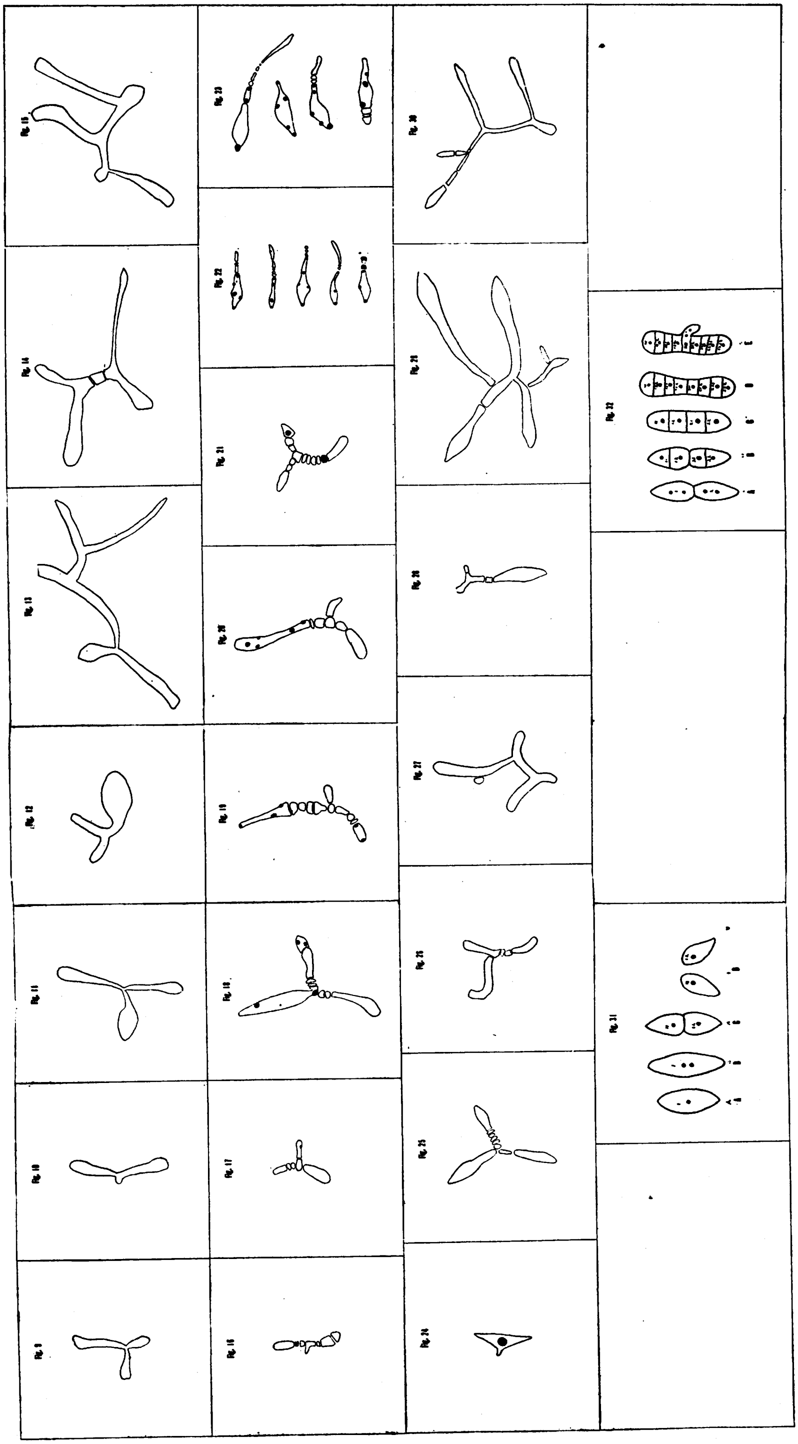
Plate VI. shows drawings to scale from micrometer measurements of individual diphtheria bacilli during the process of growth on the surface of a nutrient agar "hanging block" in a warm stage under the microscope; Zeiss, ocular five, objective one-twelfth, oil immersion; Welsbach light, concentrated by a four-inch lens of seven-inch focus. The individuals grouped under each figure were all drawn to the same scale; the scale was not the same, however, for all the different figures. Figs. 1 and 3 are comparable to each other; Figs. 2 and 4; and Figs. 5, 6, and 8. Fig. 7 is drawn to one-half the scale of Figs. 5, 6, and 8.

Plate VII. shows drawings, not to scale, of dried and stained preparations of diphtheria bacilli found in serum and agar cultures at various times. Figs. 31 and 32 are copies from Nakaniski's plates.

The bacilli observed came from cultures of *Bacillus diph-*



HILL. DIPHTHERIA BACILLI.



theriæ obtained in the routine diagnostic work of the Boston Board of Health.

Figs. 1 to 3, 27, 29, and 30 were from McKenna case (virulent bacillus in mild clinical case).

Figs. 4 to 7 were from Dias case (virulent bacillus in well person).

Fig. 8 was from Raymond case (virulent bacillus in well person).

Figs. 16 to 26, and 28 were from Charak case (virulent bacillus in mild clinical case).

The agar "hanging block" preparations in which development was actually observed under the microscope (Figs. 1 to 8) were never more than ten hours old from the time of inoculation. Some were inoculated from serum cultures, some from agar; these cultures being of various ages up to several days. The cultures from which came the dried and stained preparations represented in outline (Figs. 9 to 30) were not more than twenty-four hours old at the time of making the preparations. Figs. 9 to 15 were from serum cultures about twenty-four hours old. Figs. 16 to 26, and 28 were from agar cultures seventeen hours old. The bacilli, in all cases, were grown at 37°C. The genealogical relationships are indicated in Figs. 1 to 8 by an adaptation of Rickards' system of genealogical culture record.[12] The time occupied in the various stages of development was not noted in all cases. Sometimes a number of different individuals or groups were under observation at the same time, changes occurring in one group while attention was devoted temporarily to another, so that only approximate time relations could be recorded. The changes in Fig. 1 occurred in from 30 to 60 minutes, the change from A to B occurring suddenly under the observer's eye, as did also the change Fig. 3 from B to C; in Fig. 4, D became G in forty-five minutes; in Fig. 5 the times recorded beyond indicate only the times at which the changes were first noted after they had occurred, usually within a few minutes. In Fig. 6 development beyond B occurred, but was obscured to some degree by the overlapping of other multiplying bacilli and was therefore not recorded. In Fig. 8 the passive disintegration of the bacillus gave no definite points in time for record, the drawings being made simply at those intervals when it became apparent that the changes had distinctly advanced. In Fig. 7 alone a fairly complete record was obtained, a new drawing being made whenever the smallest change could be definitely detected. Only certain of these drawings have been reproduced. The time relations for Fig. 5 were: A to B = sixty minutes; B to C = thirty minutes; C to D = fifty-five minutes; D to E = twenty minutes; E to F = seven minutes; F to G = sixty-eight minutes. Total, four hours and fifteen minutes.

The time relations for Fig. 7 were: A to B = thirty-seven minutes; B to C = thirty-two minutes; C to D = sixteen minutes; D to E = eight minutes (this change (D to E) in the relation of the branch to the parent stem occurred suddenly under the eye of the observer, the developing branch having then been under observation one hour and thirty-three minutes); E to F = one hour two minutes; F to G = thirty-seven minutes; G to H = thirty-eight minutes (about). Total, three hours and fifty minutes. The agar "hanging block" under observation had been inoculated five hours and forty minutes when the branched form was first seen; H therefore represents the condition nine hours and thirty minutes after inoculation. Active multiplication was continuous in other parts of the same preparation, being noticed twenty minutes after first focussing on the preparation, or a total of nine hours and ten minutes.

Figs. 9 to 15 are selections from drawings by the writer representing cultures of branchers observed from 1900 to date.

Figs. 16 to 30 are selections from drawings illustrating some of the relations of "granular," "barred," and "solid" types of *Bacillus diphtheriæ* to red granules and to branching. Figs. 16 to 21 seem to support Nakaniski's theory of branching, already described. Rough copies of some of his diagrams illustrating this theory are given in Figs. 31 and 32. Fig. 18 shows a red granule at the base of a branch.

Figs. 22 to 24 show the presence of red granules without branching. Figs. 16 and 25 to 30 show branching without red granules. Figs. 16 to 26, and 28 were all from the same agar cultures inoculated from the same original culture and grown under the same conditions at the same time, both granuled and non-granuled branchers being found in the same tube.

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