MORPHOLOGY OF THE GENUS ACTINOMYCES. I[1]

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While the genus *Actinomyces* has received a large measure of attention in its relations to soil biology and to human, animal, and plant pathology, the natural affinities of the congeneric organisms that it has been customary to include in the group have been the subject of diverse opinions. Under a variety of synonyms, among which *Cladothrix*, *Nocardia*, *Discomyces*, and *Oospora* have been used nearly as frequently as *Actinomyces*, the group has been placed with the bacteria, with the Hyphomycetes, or assigned to an intermediate position. In the earlier publications on the ray fungus, including the papers by Bostroem (1)[2], and by Wolff and Israel (24), this organism was referred to the pleomorphic bacteria. The belief was seriously entertained that cocci, bacteria, and spirilla were produced by the plant, and in such regular succession that a number of investigators were led to draw up detailed ontogenetic schemes of considerable complexity. It is frequently not easy to determine the exact nature of the structures that were interpreted as pleomorphic stages. There are plenty of indications that contaminating bacteria were often present as secondary invaders; but more frequently aërial spores, segments of spiral or sinuous hyphae, and degenerative bodies of metachromatic substance were mistaken for schizomycetous types of nearly every description.

More recently *Actinomyces* has been frequently associated with the tubercle and diphtheria organisms on the assumption that they may represent a transition between the Hyphomycetes and the true Schizomycetes. A family of Actinomycetes has thus been erected as a natural group from these diverse components, united chiefly by resemblances in their staining reactions, a usual or an occasional filamentous habit, and the development of clavate elements in the animal body. It has been supposed by adherents of such a taxonomic disposition that either a progressive phylogeny

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[2] The bibliography will appear at the end of Part II, which will be printed in February.

has occurred in the family, with *Actinomyces* at the head of a transition series developing increasingly well marked fungoid characteristics, or else that *Actinomyces* is the probable progenitor of the groups *Corynebacterium* and *Mycobacterium* by degenerative reduction.

The view that the ray fungus represents an organism with hyphomycetous affinities was advanced early by HARZ (7) and DEBARY (2). These authors regarded as conidia the clavate elements of the actinomycotic lesion, which BOSTROEM'S studies later properly degraded to the rank of degenerative structures. SAUVAGEAU and RADAIS (20), DOMEC (3), THAXTER (22), GASPERINI (4), and others placed a number of congeneric forms among the Hyphomycetes on account of their production of aërial spores. It may be mentioned in this connection that an examination of a considerable number of species has convinced the writer that this disposition is the only one which is in harmony with the morphological conditions represented in the genus.

The material used in these studies, with the exception of authentic cultures of the species described by WAKSMAN and CURTIS (23), and of a number of organisms isolated by H. J. CONN from soil collected near Geneva, New York, was largely obtained from soil collected in Cambridge, Massachusetts. By the use of the dilution method more than 1000 plants belonging to the genus were isolated from this source; and of these about 300, representing probably more than 100 species, were selected for morphological examination. Approximately 400 additional individuals were derived from soil collected in Porto Rico, Cuba, Panama, Montana, Wisconsin, and Kansas, as well as from outdoor air, tap water, horse manure, and gross cultures of dung, dead leaves, and other vegetable matter. The potato scab organism was obtained from Mr. M. SHAPAVALOV, who had isolated it from a diseased tuber, and experimentally established its pathogenicity.

The morphology of the vegetative thallus of *Actinomyces*, apart from its astonishing minuteness, the diameter of the filaments ranging commonly from 0.5 to 1.2 μ, presents no features unusual among the fungi. In most species the mycelium is generally sparsely and irregularly septate; and although in other forms transverse walls may appear with somewhat greater frequency, there are none in which septation approaches any pronounced degree of regularity or closeness. Ramifications are abundant, and the branching is altogether of the "true" type. MACÉ (15), who first carefully observed the formation of branches, found it to proceed by the elongation of lateral buds arising some distance back from the growing point of an axial filament, the branch thus produced giving rise similarly to secondary branches by lateral proliferation. LACHNER-SANDOVAL (12) confirmed MACÉ, designating the process as monopodial and denying the existence of true dichotomy in the genus, which had been affirmed by previous investigators. Later NEUKIRCH (18) reported that the branching in *Actinomyces ochroleuceus* was occasionally of the nature of a true dichotomy. From an examination of very young mycelium (fig. 3)[3] it is apparent that, at least in stages following the germination of the spore, filaments are not infrequently terminated by two elements too closely similar in size and angular relationships to be distinguished as bud and axial tip. The branching in such cases must be regarded as dichotomous, although all gradations toward the prevailing well defined monopody may be found. It seems reasonable to suppose, however, that the distinction is one of convenience, not implying any fundamental difference in manner of development.

The cytological structure of *Actinomyces* is equally devoid of bacterial characteristics. The branches forming the periphery of the actively growing pellicle, or the young sporogenous branches attached at intervals to the superficial mycelium, are filled with dense protoplasm, which, with haematoxylin, takes a deep homogeneous stain. Further toward the origin of the hyphae the contents become more attenuated, and vacuoles appear, increasing in number and size until they occupy the larger portion of the filaments. When individual vacuoles become excessively large and extend through a considerable length of filament, the cytoplasm is in a large measure confined to a peripheral layer, a condition which led NEUKIRCH to distinguish a thin, strongly refringent "Aussenplasma" and a less refringent "Innenplasma."

[3] The plates will appear in connection with the second part, to be published in the following number.

The presence of large vacuoles is commonly associated with local distentions of the filament wall. These may occur with such regularity in the degenerate mycelium of some species as to suggest the appearance of *Leptomitus*, each swollen segment being largely occupied by a single elliptical vacuole, separated from the vacuole in the adjacent distention by a protoplasmic partition at the constriction (figs. 47, 48, 106). In other species and quite generally in the nutritive mycelium of all forms, that is, the mycelium immersed in the substratum, no marked regularity in the alternation of inflated portions and constrictions is observable; but pronounced deviations in the diameter of filaments may occur with more or less variable frequency.

A great deal of importance has been attributed by some writers to a variety of abnormalities and products of degenerative changes occurring in the thallus of *Actinomyces*. In the earlier literature on the ray fungus, especially in the publications of ISRAEL (8), JOHNE (9), and MACFADYEAN (16), bodies described as "micrococci," "cocci," or "coccus-like granules" were given minute attention, and assigned an important rôle in the complex ontogeny ascribed to the parasite, then supposed to belong to the pleomorphic bacteria. WOLFF and ISRAEL (24), whose photomicrographs of these bodies leave no reasonable doubt about their identity with structures very frequently observed by the writer (figs. 15, 31, 32, 42, 91), confused them with the spores reported by other authors; and as the structures did not possess the heat resistance common to spores of bacteria, these investigators were inclined to question the production of spores by *Actinomyces*. Since the organism used by WOLFF and ISRAEL was constantly sterile, their conclusion concerning it was undoubtedly correct. BOSTROEM, who experimented with a sporiferous form, did not succeed in avoiding the same confusion, and refers indiscriminately to the unicellular products formed from aërial hyphae, and to the spherical endogenous granules, as "spores."

Round granules, deeply stained in the living filament by very dilute methylene blue, were studied later by NEUKIRCH. He noted in them a variable size, a method of multiplication, and an orientation related to the regions of growth in the thallus. These observations led him to believe that the structures represented nuclei. SCHÜTZE (21), who applied NEUKIRCH'S method of staining, designated the bodies as metachromatic granules. After an examination of their occurrence in the aërial mycelium of a considerable number of species, such an interpretation seems, in the opinion of the writer, to offer the greater degree of plausibility.

The metachromatic material is easily distinguished by a powerful affinity for most of the stains ordinarily employed in laboratories. In material fixed in alcohol, and treated with Delafield's hematoxylin, it retains a nearly opaque stain after all other structures have been completely decolorized. Indications of its presence in the tips of growing filaments, or in sporogenous branches, in general are very infrequent. Some distance toward the origin of the hyphae, associated with a more attenuated or vacuolated protoplasm, the material makes its appearance in the form of rather minute granules widely separated from one another. As the filament is followed still farther back, the granules increase in size and frequency; often their arrangement is one of much regularity, the individual spherical bodies being of nearly equal size, exactly filling the lumen of the filament, and separated by nearly equal spaces (fig. 42). In other cases the granules seem to coalesce and occupy entire segments of hyphae (fig. 32); and in a few species extensive portions of mycelium were frequently found entirely filled with long unbroken masses of metachromatic substance. It is this property of coalescence of smaller granules, to form incomparably larger masses, bearing out the similarity in appearance to a homogeneous liquid with a relatively high surface tension, that makes it difficult to believe that we are dealing here with anything relating to spores or to nuclei.

The function of the metachromatic material in the *Actinomyces* thallus cannot be ascertained with certainty. A number of views have been advanced regarding the rôle of metachromatic substance in the cell, none of which has gained universal acceptance. The best explanation, in the opinion of the writer, seems to be that it represents an occluded waste product. While its presence in small or moderate quantities in the sterile hyphae bearing the sporogenous branchlets is probably more or less normal, its abundant occurrence here, as everywhere else, is an indication of advanced degeneration. In the more mature mycelium of *Actinomyces* VIII (fig. 47) metachromatic granules are usually very conspicuous, often occupying most of the space in the narrowed constrictions between the large vacuoles in the highly inflated mycelial segments. The appearance of such a thallus is not in the least suggestive of the structure of bacteria, and indicates that the resemblance between *Actinomyces* and the true Schizomycetes in the consistency of protoplasm, emphasized by some writers as an important phylogenetic connection, has been unduly overestimated.

While the sterile filaments in the nutrient and in the aërial mycelium are relatively uniform in structure throughout the genus, the sporogenous apparatus of many species exhibits a large degree of morphological individuality. This diversity has not usually been recognized by writers, and has undoubtedly been responsible for a portion of the controversy that has arisen, particularly with regard to the method of spore formation. LACHNER-SANDOVAL (12), from a study of *Actinomyces albido-flava*, distinguished two kinds of propagative bodies: (1) fragmentation spores appearing as spherical to cylindrical segments in old hyphae, formed by a contraction of the protoplasm; and (2) segmentation spores developed by a septation of the tips of aërial filaments. Segmentation was usually found to involve only lateral branches coming from aërial hyphae, but in submerged growths it frequently extended also to the main filament, leading to the development of a dendroidic system of spore chains. LACHNER-SANDOVAL'S figures of these formations, however, are much less striking than might be expected from the description in the text, and do not convey the impression of ramifications approaching treelike proportions.

NEUKIRCH identified the segmentation of LACHNER-SANDOVAL with oidium-spore formation among the fungi, and abandoned the use of a specific term. He vigorously disputed the development of aërial spores by a septation of the mycelium. According to his account the spores are formed as the result of successive contractions of protoplasm until approximately isodiametric portions are separated by regularly alternating empty spaces. This process he identified with the fragmentation of MACÉ, localizing it in a different region from LACHNER-SANDOVAL, and properly relegating the fragmentation of the latter to the category of degenerative changes.

GILBERT (5) found some lateral branches to begin the process of forming spores by becoming differentiated into highly refractive and weakly refractive portions. Constrictions later appear, unassociated with visible changes inside the filament, and soon the spores are completely cut off. GILBERT designated the process as segmentation, following LACHNER-SANDOVAL, who, however, had actually observed septa appearing more or less simultaneously with the constrictions, their appearance being followed by the enlargement and rounding up of the segments to form spores.

MIEHE (17), in his study of *Actinomyces thermophilus*, only incidentally examined the mode of sporulation. He believed spores were produced singly on very short stalks attached laterally to the main hyphae, or possibly by successive contractions in chains. In either case conidia were produced, not by the segmentation of a completed filament, but by the development of a structure which at no time constituted a cylindrical, continuous hypha. This account, in general, bears strong resemblances to the later description by SCHÜTZE (21) of *Actinomyces monosporus*, a form in which the spores are borne singly on delicate stalks in racemose arrangement on a thicker axial filament. It might well be questioned, however, whether forms like this, which depart so widely from the main morphological trend of *Actinomyces*, are properly to be assigned to this genus, even if allowance is made for much liberality in the definition of hyphomycetous form-genera.

The same criticism, however, cannot be extended to the condition described by SCHÜTZE in his strain of *Actinomyces thermophilus*. In his account of this species its author strongly defended NEUKIRCH'S position that the mode of sporulation was one of fragmentation. However, while NEUKIRCH found long filaments converted into spore chains by successions of protoplasmic contractions, the long portions finally becoming resolved into ultimate spores, SCHÜTZE found that only short terminal portions or short lateral branches yielding about 5 spores were involved. According to NEUKIRCH, the slightly refractive spaces between the masses of protoplasm that later develop into spores are entirely empty, and the intervening portions of filament wall merely collapse as the spores mature. SCHÜTZE believed that these intervals were filled with attenuated protoplasm, and that by their constriction the spores were delimited without the evacuation of portions of hyphal wall. The spore of NEUKIRCH, consequently, is a structure possessing its own spore wall, enveloped, except at its ends, by the remnants of the old filament wall; that of SCHÜTZE, on the other hand, is without a separate spore wall, the filament wall constituting the only membrane present, and forming a spherical shell everywhere inclosing the protoplasm.

NEUKIRCH gave much attention to certain structures he designated as oidium-spores. They developed in submerged growths, the transformation of the filament consisting only in more or less close septation, followed by a slight swelling of the segments. Under suitable conditions filaments grew out from them, an occurrence NEUKIRCH regarded as germination. "Aussenplasma" and "Innenplasma," in his opinion, were sharply defined, but a spore wall was absent. The elements did not exceed the filaments in resistance either to heat or desiccation. NEUKIRCH believed their function to be the dissemination of the fungus in liquid media.

LACHNER-SANDOVAL seems to have seen the same structures and regarded them as segmentation spores that had developed in the submerged condition. GILBERT, SCHÜTZE, and KRAINSKY (10) record their failure to find these bodies without, however, denying their existence. According to SCHÜTZE and GASPERINI, sporulation may occur in hyphae which are not truly air hyphae.

It seems questionable whether any desirable end is served by calling NEUKIRCH'S elements spores at all. To apply the term to structures with so little individuality, even though a sort of promiscuous viability may be attributed to them, is approaching very close to the point where all bodies not filaments of uniform thickness are to be regarded as spores. Certainly the distended elements in old mycelium of *Actinomyces* VIII (figs. 47, 48), which represent enlargements of axial filaments developed gradually in the course of time at the junctures with moderately complex systems of sporogenous hyphae, frequently have an equal or greater resemblance to reproductive bodies; and the behavior, under similar conditions, of forms like the smuts, *Mucor*, and *Penicillium*, would make advisable a larger measure of caution in dealing with fungi growing irregularly in a submerged condition.

Although various details associated with the sporulation of *Actinomyces* have thus been dealt with in the literature, the opinion still seems to prevail widely that the process is of an irregular and miscellaneous nature. LITMAN and CUNNINGHAM (14) in recent years have denied the character of spores to the "gonidia" produced by the potato-scab organism; the elements are believed simply to "serve as a segment of the mycelium, which, by increasing the number of segments, may increase the chances for spread and continuous existence." This view seems, in the opinion of the writer, very much at variance with the distinctiveness of the well characterized sporogenous apparatus found in *Actinomyces*.

In pursuing the present studies a method of mounting material was employed which, in view of the exceptional fragility of all species of *Actinomyces*, and the great difficulty ordinarily encountered in attempting to stain undisturbed sporulating conditions, gave exceptionally good results. The plants were grown on a suitable substratum, usually potato or glucose agar. Growth on potato agar, as a general rule, is more prompt and productive of mycelium; but as its use, especially with species exerting a strong tyrosinase reaction, stimulates to excessive guttation and disruption of the sporophores by the extruded droplets, a medium not possessing this property is often found to be advantageous. After the cultures had attained a proper degree of maturity, the whole growth was cut from the agar and removed from the tube as carefully as possible. A slide smeared with albumen fixative was now brought into firm contact with the mycelium and then separated from it, precautions being taken to avoid altogether any sliding of the two surfaces on each other. If the growth is not too young, this procedure will leave the upper portions of the aërial mycelium adhering to the slide without serious disarrangement, and killing and fixation may be at once effected by the use, for example, of strong alcohol. The material was subsequently stained and mounted in balsam. The quality of preparations in which the spore chains have commenced to disintegrate in large numbers is impaired by the presence of large masses of free spores, which retain their staining properties for some time after maturing. Later the spore walls seem to become entirely impervious to stains, and as a result when the secondary mycelium develops beyond a slight clouding effect no difficulty is encountered from this source. The best results are obtained when the print is made soon after the mycelium begins to adhere readily to the smeared slide.

The nature of the killing agent employed was found to have no noticeable effect on the preparation. Flemming's mixtures, both weaker and stronger, picro-formol, picro-acetic, Carnoy's fluid, and 95 per cent alcohol were tried with apparently the same results. Owing to the small diameter of the filaments, the penetration is probably so nearly instantaneous that plasmolysis is effectively prevented by nearly any toxic agent capable of readily wetting the material. In order to obviate the necessity of washing, strong alcohol was used almost exclusively.

Much more depends on the proper choice of a stain. Saffranin, gentian violet, Bismarck brown, and eosin usually fail to bring about a sufficiently deep coloration. Carbol-fuchsin acts powerfully and rapidly, but is poor for purposes of differentiation. Haidenhain's iron-alum haematoxylin is good for protoplasmic structures. The most satisfactory results were obtained with Delafield's haematoxylin, which if allowed to act for 24 hours, with the proper degree of decolorization, yields deeply stained, clear preparations, showing vacuoles, metachromatic, and nuclear structures, as well as septa, with remarkable distinctness.

The spores of all species of *Actinomyces* are developed by a transformation of more or less specialized hyphal branches distinguishable from the sterile hyphae of the aërial mycelium at an early stage in their development. In general, with the exception of such inflated hyphae as are shown in figs. 47, 48, and 106, the diameter of any portion of sterile mycelium is attained at the time it arises through the elongation of the growing filament tip, subsequent increase in thickness being very slight. The sporogenous branches, however, are in the beginning conspicuously thinner than the axial hyphae from which they are derived. Later, when their final linear extension has been nearly attained, increase in thickness generally follows. This increase may be slight, as in some species in which the mature sporogenous hyphae are still somewhat thinner than the vegetative hyphae (fig. 46), or more considerable, as in forms in which they conspicuously exceed the latter in thickness (figs. 4–6). The very simple type represented by *Actinomyces* XIII, in which the aërial mycelium is represented by very long filaments, rarely branching and apparently sporogenous almost to their point of origin in the nutritive mycelium, constitutes the only exception, since in this instance there is no indication of thickening in the young fertile hyphae, nor indeed any variation in the diameter of its vegetative filaments.

In a majority of the species the maturation of the sporogenous hyphae is associated with a peculiarity in growth by which they become coiled in more or less characteristic spirals. The tendency toward the coiled condition is usually clearly manifested before the branch has grown to half its final length through the open flexuous habit of the young filament (figs. 5*c2*, 107). As elongation continues, the turns become increasingly definite, but the contraction leading to the final condition, which ranges from that illustrated by *Actinomyces* XIII with its open, barely perceptible turns, to one in which the spirals are so strongly compressed that its adjacent turns are in continuous contact (figs. 44, 51, 57) in a fashion resembling that of the spores of the hyphomycetous genus *Helicoön*, is usually delayed until the later growth in thickness of the filament. Specific differences may not only be indicated by the obliquity of the spiral, but involve also the number and diameter of its turns, and its construction with reference to the dextrorse or sinistrorse condition. The range in different species extends from the 2 or 3 turns exhibited in forms like *Actinomyces* II and XVI, to over 20 turns in others; but the range in a particular species is always considerably smaller. The writer once observed a spiral with 24 turns, but this probably approximates the extreme maximum; spirals with 14–16 turns (figs. 23, 57, 94*c*) are by no means abundant, and probably no species produces many in which there are more than 12 turns.

The diameter of the spirals as a whole is more or less in inverse ratio to the number of turns characteristic of the species. This correlation is very evident in a comparison of types like *Actinomyces* II and XVI showing spiral sporogenous hyphae with a few wide turns, and types like IV, V, and XVII illustrating spirals of many narrow turns.

Rotation in the formation of spirals is specifically sinistrorse or dextrorse in different species of the genus; and it is interesting to note that here, as in the vegetable world in general, sinistrorse are much more abundant than dextrorse species. Of the 17 species with spiral sporogenous branches figured in the present paper, which have been selected as representative of a much larger number, 5 are dextrorse, 11 are sinistrorse, while the condition of the remaining one could not be ascertained with certainty. In general, the proportion appears throughout the entire genus. As a morphological feature, the absolute constancy with which a species adheres to one kind of rotation is noteworthy, particularly in view of the extremely minute dimensions of the structures concerned.

An examination and comparison of the relation of the sporogenous branches to each other, and to the axial filaments, enables one to recognize several tendencies, the distinguishing characteristics of which are correlated with differences in the sequence of proliferation. Two main types may thus be recognized, approaching each other in apparently intermediate forms, but moderately distinct at the extremes: (1) an erect dendroidic type in which the sequence of development of the sporogenous hyphae is successive; and (2) a prostrate, racemose type in which the development is more nearly simultaneous.

In the erect type, well exemplified in *Actinomyces* I, the development of the fructification starts from a single erect hypha with a spiral termination. Sporogenesis commences at the tip by the insertion of regularly spaced septa, and proceeds downward toward the base of the filament. Usually before much of the hypha has been involved, a single septum will appear well toward its base, and immediately below it the bud anlage of a new sporogenous branch appears. As the latter is attaining its growth in length and thickness, and its spiral disposition, the basipetal septation in the axial filament proceeds to the septum above the insertion of this first branch, the young spores thus delimited undergo maturation processes, the spiral becomes relaxed, and the chain of spores subject to disruption. The branch now passes through the same course of development as the axial filament and in turn gives rise to a sporogenous branch below a septum a little above its own insertion. The number of sporogenous branches developed below a single septum is generally increased to several by proliferations subsequent to the first; and as the initiation and development of successive orders may be indefinitely repeated, complex fructifications are frequently developed, in which a succession of the processes described are simultaneously taking place at many points.

In the second type there is no such clearly defined relation between younger and more mature sporogenous hyphae. Development of a fructification is initiated by the proliferation of branches at irregular intervals on the distal portion of a prostrate axial filament which often exceeds 1 mm. in length. The branches may either stop their more extensive development after forming a spiral, or themselves proliferate a secondary branch a short distance above their own insertion; and this in turn may form a spiral and give rise to a tertiary branch (fig. 43). By a repetition of this process each lateral element may become branched several times, the whole apparatus as well as its insertion on the axial filament being characterized by an absence of septa. Sporulation, instead of beginning in any individual spiral as soon as it is formed, is usually delayed until the branching and growth of spiral hyphae in the same lateral process have come to an end (figs. 42–44, 46), when it will often proceed rapidly and almost simultaneously in all the spirals (fig. 41). The termination of the axial filament itself develops into a spiral, and behaves essentially like a primary lateral branch.

Occasionally the axis of one of these racemose arrangements may be comparatively short, resulting in a rather intricate structure in which the spirals of one lateral branch may be entangled with those of another (fig. 44). The tendencies characteristic of the type, however, are maintained: the absence of a septum above the insertion of branches, and the delay in sporulation in the spirals first formed, until the growth of the last order of sporogenous branches is more or less complete.

In addition, however, to species in which these two types are clearly distinguished, a still larger number of species present a combination of the two features. Frequently the open racemose arrangement of the lateral branches on the main axial filament is associated with a successive order of development in the further ramifications of the branches (figs. 63, 79). The presence of a septum above the insertion of a branch is characteristic of more species than is its absence (figs. 2, 5*c*); and in some species both conditions prevail (figs. 53, 79, 81). In other forms a fructification with successive development may terminate a long prostrate filament.

In a few species, particularly *Actinomyces* X and XVIII, there are formed, in addition to the more regular fructifications, others of a more miscellaneous tendency. The branching axial filaments are relatively thick, densely filled with protoplasm, and bear at very close and irregular intervals a short, thick, unbranched sporogenous hypha with little or no spiral modification (fig. 103). It seems quite probable that this type of development is associated with the excessively rapid growth that characterizes the two forms in which it was most frequently observed.

The degree of completeness to which the aërial mycelium of *Actinomyces* is converted into spores has generally been overestimated. On the contrary, sporulation is quite strictly confined to terminal elements, never as a rule passing beyond the first junction with another element. The proliferation of the branch nearest the end of the axial filament limits spore production in this filament to the portion beyond the insertion of the branch; and in the same manner the proliferation of a secondary from a primary lateral branch results in a sterilization of the portion of hypha below the insertion of the new branch. In one species, *Actinomyces* V, sporulation is even further restricted by the apparent abortion of a number of potential spores at the proximal end of the unbranched lateral branches. The hyphal portion involved first develops as usual, but when the characteristic septation associated with the delimitation of spores in this species appears in the spiral, it is not extended to the base of the branch, although indications of regularly spaced membranes may usually be distinguished (figs. 21, 24, 25). Later the unsegmented portion is gradually evacuated and converted into a sterile stalk devoid of protoplasm (figs. 29, 30). It is interesting to note that the basal septum, which in an allied and very similar form, *Actinomyces* VI, delimits the lowest spore from the axial filament, here also is present as a well developed cross-wall.

The delimitation of the ultimate cells in the process of sporulation occurs usually as the growth in thickness and the contraction of the spiral (where this is present) are approaching a stage of completion. It has usually been believed by investigators that the details connected with spore formation are uniform throughout the genus. This belief, which the writer was at first inclined to share, must be considerably modified in view of the diversity of conditions actually found. In most species the sporogenous hyphae become divided into regular cylindrical cells separated by septa; the latter generally stain deeply with Delafield's haematoxylin, probably as the result of an association with metachromatic or possibly nuclear material. The species which are thus characterized by clearly defined septation may be assigned to three different categories, separated by differences in the disposition of their septa and in the development of their spores.

In the first category, represented by *Actinomyces* I, the cross-walls in the sporogenous hyphae remain without any very pronounced change, continuing to separate the adjacent cells until these have developed into a chain of mature contiguous spores. The insertion of these septa progresses from the tip toward the base, and does not break the physiological continuity of the hyphae; for food material apparently is readily transported through them to the young spores at the termination, since these subsequently increase in size, and may deposit a wall of measurable thickness.

In the second category the septa apparently split into halves, which are then drawn apart by the longitudinal contraction of the individual protoplasts (figs. 5*c1*, 8*a–f*, 59). In *Actinomyces* II the very pronounced growth in thickness of the sporogenous hyphae, following the insertion of septa, indicates that in this species also septation brings about no impediment in the transfer of food material. This is particularly remarkable on account of the extraordinary thickness of the septa characterizing this species. *Actinomyces* XVII, however, while less striking, probably represents more nearly the usual condition prevailing in the second category. The segment of the filament wall evacuated by the contraction of each two successive spores undergoes no change until fractured by the disruption of the chain of mature spores.

In the third category (*Actinomyces* IV, V, VI, VII, and XII) the cross-walls first undergo a deep constriction, which by involving the ends of the young cylindrical spores gives to the latter an elongated ellipsoidal shape (fig. 70*a–d*). The constricted septum now gradually loses its staining properties, and appears to become slightly drawn out in a longitudinal direction (fig. 70*e*). A preparation stained with Delafield's haematoxylin usually shows many old spore chains in which the individual spores are thus connected by hyaline isthmuses. Occasionally an isthmus may be found with a remnant of the old deep staining septum still unchanged in its center (figs. 16, 70*e*).

Beyond these three types of sporulation another must at least be provisionally recognized, in which septa are either absent from the developing sporogenous hyphae, or are not demonstrated by the use of ordinary stains. The protoplast appears to contract at regular intervals, yielding a series of non-contiguous spores, held together for a time by the connecting segments of evacuated filament wall (fig. 73). It is this type of sporulation which NEUKIRCH and his followers, in opposition to LACHNER-SANDOVAL, believed to prevail throughout the genus. NEUKIRCH'S conclusion that septa are never involved in the sporogenesis of *Actinomyces* certainly cannot be extended to the large majority of species; and its application to any forms whatsoever is associated with some reasonable doubt. The writer is inclined to believe that cross-walls appear in the development of the sporogenous hyphae probably throughout the genus, but in some members are too thin to be recognized as distinct septa. Such an interpretation is suggested by the wide range in the thickness of septa found to occur, from the very massive structures of *Actinomyces* II, through those of moderate thickness in *Actinomyces* I, XII, and XVI, to the condition prevailing in

Actinomyces III and VIII, where cross-walls can only rarely be distinctly perceived.

All investigators, with the exception of SCHÜTZE, agree in attributing to the peripheral wall of the filament of *Actinomyces* an extreme thinness. Indeed, KRUSE (11) and others have urged the single contoured character of the membrane as an evidence of the bacterial affinity of the genus. It is only necessary to examine fungus forms like *Chlorosplenium* or *Phoma*, to convince one's self that the single contoured wall is generally characteristic of minute cells, whatever their taxonomic connections may be. Yet while the phylogenetic inference may safely be rejected, it still remains true that the peripheral wall of every species of *Actinomyces*, except possibly those of some old enlarged hyphae, cannot be made out as a distinct structure with double contour. In evacuated portions its location is indicated by only the faintest indication of its outline. Nor is this surprising when we consider that the maximum resolving power of any combination of lenses employing visible light is approximately 0.17 μ. As this magnitude barely equals the widths of the thinnest cross-walls observed, it is not difficult to suppose that, in the type of sporogenous hyphae represented in *Actinomyces* XIII, the dimensions of the partitions, like the filament wall generally, fall below the limit of visibility.

It is pertinent in this connection to emphasize the peculiarity in the nature of the cross-walls, the appearance of which in many species of *Actinomyces* initiates the development of the individual spores. Their unusual relative thickness, even in species in which they can be distinguished only with difficulty, but where nevertheless their thickness must exceed 0.17 μ, in filaments with a diameter of only 0.9 μ, is indicative of a composition essentially different from that of the peripheral wall. This indication is strengthened by the strong affinity for dyes characteristic of the septa, the evident ease with which they permit of the passage of food material, and their apparent plasticity of behavior, resulting in a median split in some species, and in others in a gradual constriction followed by a slow transformation into an attenuated isthmus.

The disappearance of the deep staining derivatives of the septa from the ends of the young spores is in some species accompanied by the appearance of one or several deep staining granules within the spore. Whether the latter represent nuclei or bodies of metachromatic material cannot definitely be determined. It seems not at all improbable, however, that some of the structures that can be differentiated within the more mature spores, particularly those characterized by uniform size and moderate staining properties (figs. 1, 2, 33, 35) are nuclei. In *Actinomyces* IV and XII they may frequently be distinguished comparatively early, before the septa, with which they alternate in regular succession, show any perceptible constriction, indicating that their existence is not related to the subsequent disposition of the partitions (fig. 67). When two of these bodies occur in the same spore they uniformly occupy opposite or diagonal positions (fig. 2, *a*, *d*2). The question arises why these bodies, if they actually represent nuclei and not structures originating *de novo*, cannot be distinguished in the young continuous sporogenous hyphae. The only explanation that can be advanced is that the protoplasm in the earlier stages is too dense to make possible any conspicuous contact between cytoplasm and nucleus. Later, with the attenuation and vacuolization of the cytoplasm that occur with the maturation of the spore, apparently as the result of the deposition of a special wall, the nucleus becomes increasingly distinct, and in some species it constitutes the only spore structure clearly visible in the stained preparation (fig. 41).

It cannot be denied, however, that granules having more the appearance of the metachromatic granules found in degenerate sterile filaments occur in the spores of some species, either alone or together with a nucleus-like body. They differ from the latter in taking a deeper stain; in having an absolutely smooth contour; in offering considerable variability in size; and when present in numbers assuming no definite orientation with reference to each other. They have been noted in those species in which the septa associated with the delimitation of spores is particularly massive; and in *Actinomyces* II (fig. 8*f*) their derivation from the excessive wall material seems reasonably well established. After the septa have separated along a median plane, the deep staining substance at each end may contract, yielding a number of spherical bodies inside of the spore. This process is probably of a more or less pathological nature, since in the usual type of development the wall material is gradually distributed through the inclosed protoplasm, causing the normal mature spore, except for the presence of a vacuole, to take an almost homogeneous stain.

Another indication of the similarity in nature existing between metachromatic material and the deep staining transverse septa of *Actinomyces* is found in the occurrence of both within peculiar large spherical structures. These structures appear generally to occupy nearly the entire lumen of the filament, and not infrequently are related to local enlargements. Occasionally, however, their diameter is considerably smaller than that of the hyphae (fig. 103). In any case they may contain either one or several peripherally located metachromatic granules, or a uniformly thick, well defined, deep staining, transverse septum, exactly median in position. It is interesting to note that whenever granules occur their surfaces in contact with the periphery of the structure represent portions of convex spherical surfaces conforming accurately to the confining surface; and whenever a septum is found traversing one of these structures considerably smaller in diameter than the filament, it does not extend into the protoplasm, but remains in its finished state as a curious partial partition.

The germination of the spores of *Actinomyces* takes place readily in dilute nutrient solutions, such as 1 per cent glucose solution, or nearly any vegetable decoction. During the first few hours of incubation at a moderate temperature they increase considerably in volume by swelling. From 1 to 4 germ tubes are then produced, apparently more or less successfully, the approximate number being, in a measure, characteristic of the species. Specific characteristics are expressed also in the diameter of the hyphae, and in the frequency of branching.

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