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Comparative Aspects of Development and Differentiation in Actinomycetes

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INTRODUCTION

Aims and Scope

This paper is not going to suggest a new model for studies in prokaryotic cell development. It is believed that the *Actinomycetales*, taken as a group, would present difficulties, given the current state of our knowledge, although they do pose intriguing and sometimes unique questions closely related to the area of developmental research.

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The authors of recent reviews, dealing specifically or nonspecifically with particular problems of actinomycete development (69, 136, 235, 485), agree almost unanimously that developmental studies with these forms lag behind those with viruses, aerobic sporeforming bacteria, and fungi. Whatever the reason, current trends of research with various organisms are inconsistent, not only in terms of laboratory techniques used, but also in the kinds of questions asked, goals formulated, and ideas exploited. Thus, periodic attempts to apply approaches becoming conventional with different organisms appear merited. It is hoped that by so doing, the area of "lags" and potential breakthroughs might become more precisely defined and comparative knowledge will be made more meaningful and thought provoking.

Now that the differences between eukaryotes and prokaryotes are understood, the old debate about whether the *Actinomycetales* are bacteria or fungi is definitely over. However, there are still reasons for comparing them with other bacteria and fungi.

Chemical techniques, extensively employed over the last two decades for characterization and identification purposes in microbiology and based mainly on determinations of the presence or absence of certain compounds, most often confirmed the relatedness of the *Actinomycetales* to other bacteria ("true bacteria"). Using more complex features (wall structure, photosynthesis, gliding motility, endospore formation, etc.) one can document differences between gram-positive and gram-negative bacteria. But what are the characteristic features of the *Actinomycetales*, easily sensed by an observer, which support the idea of placing them in a separate order, but which are not so easily expressed in precise chemical terms? Are they not, to a large extent, the results of cellular differentiation in these organisms?

Currently, *Actinomycetales* and fungi are believed to belong to different kingdoms of the living world; it is inferred that their evolutionary separation is greater than that of fishes and whales. Yet, striking examples of supposed convergent evolution include not only mycelial organization, but also general trends in reproductive events. By comparing examples of analogous differentiation, one might hope to learn about specific limits attainable at the prokaryotic level. Relevant here also is the need to understand the mechanisms allowing adaptation to a different (terrestrial?) environment, in which respect the *Actinomycetales* seem the most capable of the prokaryotes.

There is one field that has involved actinomycetes more extensively than other microbes during the last three decades, and this is the screening for antibiotics and other products of secondary metabolism. However, the very interesting hypotheses linking secondary metabolism with differentiation (58, 449) were formulated using either fungi or endosporeforming bacteria. These hypotheses, in our opinion, offer very interesting perspectives for research, and consequently they merit extensive testing, specifically with actinomycetes. Therefore, an attempt to examine the situation "from within the *Actinomycetales*" seemed timely (at least in helping some of our industrial colleagues to realize that actinomycetes do not just grow and produce everything possible, but they also somehow develop).

It is believed that the plan of this review will fit its aims without: (i) stating the basic problems, only to find supporting evidence insufficient, and (ii) describing organisms, one by one, and reporting all relevant information, thus losing sight of the problems in the wealth of less relevant facts.

Whenever there were no questions in our minds, we used the generic names adopted in the eighth edition of *Bergey's Manual*. In doubtful cases, generic names given in the original publications were retained.

Suitability of Various Types of Cultures for Studies of Development and Differentiation

Information pertaining to development of actinomycetes currently stems mainly from studies that employ either submerged or surface cultures. Morphological and, very probably, physiological and biochemical, manifestations of differentiation in these types of cultures differ quite markedly.

To begin with, it is characteristic that most differentiated representatives of the *Actinomycetales* form hydrophobic aerial mycelia and spores in surface cultures. Although spore formation has repeatedly been reported to occur in submerged cultures, the spores and spore-bearing structures formed under these conditions seem to differ morphologically and physiologically from these of surface cultures (cf. Spores, below). Surface and submerged cultures might differ also in the relative areas of cell contacts, these tending to be more extensive in surface cultures. Cell crowding may well influence differentiation within hyphae, as seen, for instance, when submerged cultures grow in the form of pellets. This influence is rather clearly linked with changing gradients of nutrient supply, accumulation of toxic substances and, much less clearly, with the interchange of more specific signals between cells. Physiological differences between surface and submerged cultures appear to be illustrated by the sometimes encountered differences in their antibiotic pro-

ductivity. They are further illustrated by the effect of C-factor (see section on specific factors in spore regulation, below), manifested only in submerged cultures of strains of *Streptomyces griseus*.

The profound character of differences that might be found between surface and submerged cultures of actinomycetes are further documented by studies of morphology during growth of *Nocardia corallina* and other nocardiae. Here, growth on solid media takes the form of a mycelium that later fragments. When shaken in liquid medium of the same composition, the cell form changes to rods, which are much shorter than the filamentous forms. Branching is rare. The rod-shaped cells divide by binary fission. Fragmentation division follows as growth ceases (136). It also is a common observation of industrial microbiologists that fragmentation in several *Streptomyces* spp. is markedly increased under conditions of submerged fermentation, as compared with surface culture on solid media. Thus, different possibilities are offered for studies in development.

Surface cultures are more suitable for studies on differentiation within colonies, aerial mycelium formation, etc. Because the growing hyphae are attached to agar surface, their position might give some information on chemotactic responses and interactions (48).

Although evidence of differentiation, both macro- and microscopic, is more clearly expressed in surface cultures, most physiological studies with actinomycetes have been conducted with submerged batch cultures. These studies include important ones on nutritional limitations, on age-dependent biochemical changes, and on production of some secondary metabolites. Considerable caution is in order when interpreting morphogenetic changes observable in surface cultures with the aid of physiological and biochemical data obtained with submerged shaken cultures.

The value of findings obtained with submerged shaken cultures is limited, among other things, by the marked heterogeneity of the mycelial mass obtained. This heterogeneity can be demonstrated simply by using centrifugation in density gradients and differential staining (495). Thus, submerged hyphae of *S. griseus* were stained with toluidine blue to reveal nuclear elements, with Schiff stain to reveal polysaccharides, and with methylene blue to reveal metachromatic bodies. Each staining procedure tended to distinguish several "types" of hyphae that appeared sequentially in the course of actinomycete cultivation. The hyphae thought to belong to a certain type using one procedure appeared to belong to a different type when another staining procedure was used. Moreover, different portions of one and the same hypha appeared infrequently to belong to different types. Different fractions of the mycelium, obtained by centrifugation in sucrose gradients, appeared to be endowed with different streptomycin-synthesizing capacities in short-term experiments. In experiments with a related organism it was found (110) that the mycelium from a submerged shaken culture could be separated into six fractions by density gradient centrifugation. The fractions differed in stainability with methylene blue, in the rate of colony formation when plated on solid medium, and in ultimate streptomycin yields in transfers on liquid medium (shake cultures). The heterogeneity of mycelia that is characteristic of submerged shaken cultures of actinomycetes probably accounts for the substantial difficulties encountered in attempts to correlate such parameters of growing submerged cultures as mycelium weight, biomass volume, and protein content (206), or fluctuations in deoxyribonucleic acid (DNA) content reported in an earlier paper (103).

Clearly, the continuous culture of actinomycetes might offer solutions to some of the above difficulties. This, however, has seldom been attempted (38, 39, 469). Use can also be made of the fact that rather dense suspensions of actinomycete spores, under proper conditions of nutrition and aeration, can be germinated synchronously and kept in synchronous growth for several hours.

Growth of Actinomycetes

How does the growth of actinomycete mycelium proceed? Some aspects of this process are not yet quite clear.

According to cytological observations with stained preparations (423), nuclear elements in growing *Streptomyces* hyphae divide not only in their apical regions, but in other regions as well. These observations led the author to assume that both intercalary and apical growth is possible. In submerged cultures of *Streptomyces streptomycini*, at a certain stage, a marked increase in the extension of hyphae was observed (111), which was not accompanied by a corresponding increase in the rate of multiplication of nuclear bodies. This was reflected in a marked decrease of the nucleocytoplasmic ratio along the whole length of the hypha and tends to support the possibility of intercalary growth, at least at some stages of culture development. Manometric experiments (352) established a linear relationship between the growth of several mycelial organisms (streptomycetes and fungi) and the cubic root of the oxygen volume consumed. Be-

cause a similar relationship was found with other bacterial cultures, a three-dimensional mode of growth for the mycelial organisms studied was suggested. The interpretation of the physical aspects of the three-dimensional growth model is not quite clear, however.

Time-lapse microphotography experiments with growing *N. corallina* cells (54) indicated the mycelium grows predominantly at the apices and in regions where transverse septa are formed. In the latter case, growth is confined to the opposing ends of the cells that are just separated by the newly formed septa. It is known (203) that the new wall is formed at the equatorial regions, accompanying septum formation in certain gram-positive cocci. It is possible that a similar phenomenon takes place during septation of the mycelium in actinomycetes.

According to Gottlieb (171), the growth of actinomycete mycelium, like that in fungi, is almost entirely confined to apical regions. Recent extensive light microscopy observations of *Streptomyces hygroscopicus* hyphae growing in surface culture (456) showed that growth is restricted to apical regions about 20 μm in length. Nuclear bodies were seen dividing along this region, and no side branches were seen to be formed here. In the next 80 to 110 μm, there is no growth, but side branches are formed. Nuclear bodies divide at those sites where side branches emerge. Still further from the apex, the "old" portion of the hypha begins, which does not grow and does not form side branches. Observations with submerged cultures of *S. streptomycini* (111) also demonstrated the inverse relationship between the ability of individual hypha to extend longitudinally and to form side branches. It seems likely that more decisive information on the relationships that exist during various growth phases between the ability of actinomycete hyphae to elongate and to branch might be obtained by using the "growth unit" concept. As suggested originally for fungi (516), this unit relates total hyphal length to the number of apices found along it. From the observations on the growth of actinomycete hyphae on solid media, one gets the impression that the rates of their growth and frequency of branching are only partly genetically determined and depend heavily on cultivation conditions. These preliminary observations clearly await substantiation by more exact techniques.

Some evidence of indirect and preliminary character indicate that in actinomycetes a kind of mechanism might be operating that results in the so-called apical dominance—a well-known phenomenon in mycelial fungi (32, 573). So far, along with observations on growth of actinomycete hyphae cited above, this evidence stems from data illustrating the ways in which the apices differ from the rest of the hyphae. These include differences in isoelectric points (183) and increased levels of activity of such enzymes as alkaline phosphatase, catalase, and peroxidase (162). Apical regions seem also to be more responsive to alterations in the temperature of the medium. In an attempt to analyze the reasons for "ring" formation in giant colonies of *Thermoactinomyces vulgaris*, it was shown, for example, that certain kinds of rings were formed in response to brief drops in temperature. According to microscopy observations, the apices of growing hyphae underwent autolysis when the cultures were subjected briefly to decreased temperatures (46). Germinal tubes of the same organism responded similarly, and a maximal effect was noted at 20 C, as observed by one of us (N.S.A.). This event is reminiscent of hyphal tips of fungi bursting in response to environmental (including temperature) changes (33). The latter event is thought to reflect increased sensitivity of the apical cell wall to autolytic enzymes (408). It should be emphasized, however, that subcellular structures (vesicles and "Spitzen-Körper"), whose presence is thought to be characteristic for fungal apices (181), have not been reported in the hyphal tips of actinomycetes. Although an observer of developmental patterns in bacteria often meets with phenomena of "polarity" (207, 483), the biochemical basis for such phenomena is far from clear. Perhaps the growing hyphae of an actinomycete might eventually become an interesting object for study and control of polarity and dominance in procaryotes.

LIFE CYCLES IN THE *ACTINOMYCETALES*

Generalized Scheme of Events Accompanying Reproduction

Developmental events in actinomycetes, as in other organisms, are most fully manifested in the course of their reproductive cycles. Figure 1 shows several examples of the latter and is aimed at giving an overall picture of the main modes of reproduction reported in representatives of this group of microorganisms. A number of organisms belonging to *Actinomycetales* and related groups do not form specialized reproductive cells. Vegetative reproduction in these organisms is achieved by cell septation (this process has several modifications) and budding.

As far as the mycelial stage is concerned, the simplest forms are represented by mycococci

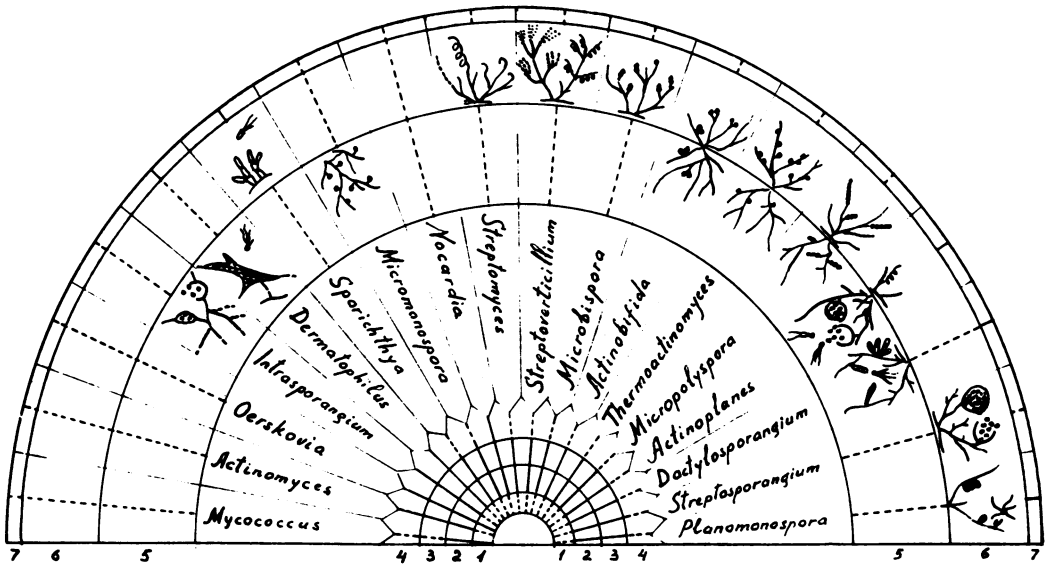

FIG. 1. *Reproduction cycles in selected representatives of Actinomycetales. Numerals refer to certain stages in the life cycles. Solid lines denote presence of the corresponding stages; dotted lines denote absence of a stage. (1-4) Vegetative stages; (5-7) spores. (1) Motile cells; (2) nonmotile cells; (3) mycelium; (4) fragmenting mycelium; (5) spores formed on substrate mycelium; (6) spores formed on aerial mycelium; (7) motile spores. Schematized according to information given in descriptions of: Mycococcus (112); Oerskovia (417); Intrasporangium (241); and other genera (42).*

(112, 278). These organisms form neither mycelial nor rodlike forms; their cells undergo irregular division (Fig. 2) that results in the formation of elements of uneven shape and size. Occasionally, motile, flagellated cells are formed.

Rodlike and coccoid cells in representatives of *Mycobacteriaceae* (474) and *Actinomycetaceae* (439) can develop into a transient mycelium, which is quite unstable in most forms and is not detectable in some others. Along with septation, *Actinomyces* spp. were reported to be able to reproduce by budding (128; see also Fig. 2). Enlarged cells of unknown reproductive value are sometimes observed ("clubs" in *Actinomyces* spp.; "swollen cells" in *Arachnia* sp.). Motile forms are unknown in representatives of these families, except in the genus *Mycoplana*, whose suggested affiliation (87) is not quite certain at the moment because of its gram negativity.

In *Nocardia* (361) and *Oerskovia* (417), the mycelial stage persists considerably longer then in the above-mentioned organisms.

Mycelial fragmentation can be regarded as a special form of vegetative reproduction. It is observed in many actinomycetes and is fairly characteristic for those called "nocardioform" by some authors (416). The process of fragmentation in *Nocardia* spp. involves formation of multiple septa that divide the growing hyphae into more or less regular elements. Contrary to some earlier suggestions (1), the process of septum formation and genophore segregation in *Nocardia* seems to be sufficiently well coordinated (136), so that no nonviable cells are formed as a result of the process.

Flagellated, motile, sometimes branching elements are formed on fragmentation of mycelia in *Oerskovia* spp.

Septa are formed in two perpendicular planes during mycelial fragmentation in *Dermatophilaceae* (167; see also Fig. 2). This process is distinctly different from that in *Nocardia* because of an additional plane of fragmentation and shows greater regularity than is seen in mycococci. Separated cells are often flagellated, actively motile, and sometimes called zoopores. In *Geodermatophilus* spp., which do not form specialized spores, reproduction involves cell septation and budding (221; see also Fig. 2).

The actinomycetes that retain unfragmented (although seldom unseptate) mycelium during long periods of their life cycle usually reproduce by forming asexual spores. Earlier proposals concerning autogamy associated with sporulation (23, 267) seem not to be warranted (see below). As one may see in Fig. 1, there are many types of reproduction involving asexual sporulation. Spores may be formed on substrate and/or the aerial mycelium as single cells or in chains of various length, or harbored in special vesicles (sporangia), and may be endowed with flagella.

The initial steps of spore formation in several

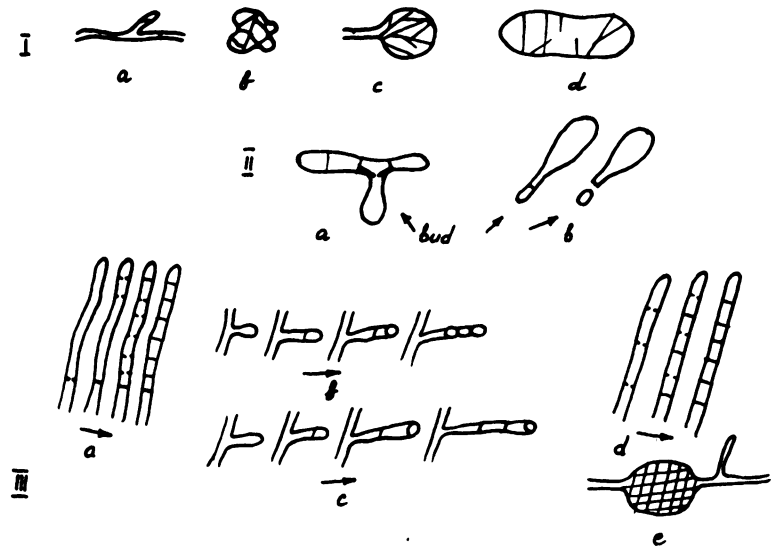

FIG. 2. *Septation in actinomycetes. (I) Septation in vegetative cells. (a) Transverse septation common for majority of actinomycetes; (b–d) septation in diverse planes as exemplified in: (b) Mycococcus spp. cells; (c) vesicles of Frankia spp.; (d) chlamydospores of M. chalcea. (II) Types of budding encountered. (a) Side bud formation in Actinomyces spp.; (b) polar bud formation in Geodermatophilus sp. (daughter cells are budded from a stalk of the mother cell). (III) Septation in sporulating hyphae. (a) Septation in sporulating hyphae of Streptomyces spp.; (b, c) sequential division in sporulating hyphae: (b) basipetal in Micropolyspora spp.; (c) acropetal in Pseudonocardia spp.; (d) "cascade" septation in Actinomadura dassonvillei; (e) septation in two perpendicular planes characteristic of Dermatophilaceae.*

oligosporic actinomycetes can be viewed as budding, since the characteristics of the process meet the main criteria for budding (207) in other bacteria.

So far, it has not been possible to grow representatives of *Frankia* spp. in laboratory cultures. Their reproduction cycles were inferred and reconstructed from observations made on slices of infected plant tissues. Two main forms are seen: branching septate mycelium (usually associated with young nodules) and spherical vesicular bodies (up to 5 μm in diameter). Vesicles are found on the mycelial hyphae in terminal, subterminal, or intercalary positions (37, 344). Vesicles are abundant in nodules that actively fix molecular nitrogen. In dead tissues, irregular polygonal endophyte cells are seen (0.5 to 1.0 μm in diameter), which are believed to represent a resting stage capable of survival in soil after autolysis of the plant cells (437).

Multiplicity of Reproductive Patterns

The species of actinomycetes, like the fungi, are often endowed with more than one potential reproductive route. Mention has already been made of the ability to reproduce by mycelial fragmentation and sporulation. The additional reproductive unit is sometimes different from the mere fragment of the mycelium.

The ability of *Streptomyces* spp. to form some specialized cells along with aerial conidia on substrate mycelium was mentioned and illustrated long ago (278, 326, 536, 537). They were pictured as enlarged, thick-walled cells variously called "chlamydospores" or "arthrospores." Their exact structure and origin have not been reexamined since the advent of electron microscopy. Chlamydospores and microcysts were also sometimes observed in *Nocardia* spp. (361). Some *Streptomyces* spp. carry single spores or chains of spores on substrate mycelium (36, 192, 465, 466), in addition to the characteristic chains of arthrospores on aerial mycelium.

In a study on an unusual species of *Actinoplanes*, *A. armeniacus*, mention was made of the ability of this organism to form two types of spores: (i) motile, peritrichously flagellated zoospores in sporangia on substrate mycelium, and (ii) chains of *Streptomyces*-type arthrospores on aerial mycelium (245). These varied in proportion to the cultivation conditions employed. A similar situation is also met with in other species of *Actinoplanaceae* (563, 565). Species of *Kitasatoa* (358) and *Pilimelia* (249) also form motile spores in vesicles and nonmotile spores in chains.

Micromonospora spp. usually have single

spores but seem to form occasional spores in pairs; they also form terminal, intercalary, and interminate chlamydospores (335). *Pseudonocardia* were reported (193) to produce three types of spores, according to their origin: (i) terminal or lateral chains of spores arising on the mycelium, formed in acropetal fashion; (ii) fragmentational spores irregularly formed on the mycelium; (iii) blastospores, produced in the basipetal fashion and seen in twos and threes in short chains on mycelial segments.

Actinomonospora lusitanica was reported to form large spores (2.5 to 3.0 μm in diameter), which are positioned terminally or laterally on the hyphae, but formation of chlamydospores, arthrospores, and endoconidia was mentioned by the authors who first described the organism (336).

Descriptions of life cycle traditionally serve as one of the milestones in the systematics of actinomycetes. In view of the above, considerable patience is required to provide conditions that allow for expression of as many of the reproductive patterns as possible. The conditions permitting less conventional routes of differentiation in actinomycetes, as well as physiological events accompanying them, are almost untouched by experimental studies.

Rare and Unconfirmed Modes of Reproduction

There are hints that at least some actinomycetes possess much more complex life cycles than is usually believed. A word of caution is in order, since some of these were inferred from microscopy observations on highly heterogenous (cf. above) cell preparations and stained smears. This seems to be particularly applicable to the formation of the much-debated "initial cells" (267), or the complex life cycles of *Streptomyces* spp., which are supposedly exhibited in submerged liquid cultures (400, 401). The scheme of the life cycle of some freshwater streptomycetes (433) also seems insufficiently documented. According to the latter, the change from haploid to diploid state of the mycelium is accomplished by a sexual process, involving fusion of zoogametes.

Nakazawa (379, 380) described a complex life cycle in *Streptomyces sindenensis*, which involves formation of special amycelial "fruiting bodies" carrying motile isogametes, which fuse and form zygotes. The latter develop into mycelium or special vesicles, which can be cultivated separately or together, the latter conditon being inductive for the eventual formation of fruiting bodies. To complicate matters further, in the culture of this and some other streptomycetes, "living crystals" were reported to occur (381), which are able to germinate to give rise to the mycelium on fresh media. In the hands of other authors (331) *S. sindenensis* yielded supposed zoogametes, along with usual mycelium, fruiting bodies, and motile elements.

Insufficiently explored is the interesting suggestion (279) that some actinomycetes form, in the course of autolysis, submicroscopic bodies that may pass through conventional bacteriological filters and regenerate under certain conditions into initial cultures.

STATIC ASPECTS OF DIFFERENTIATION

Differentiation Within Colonies

The colonies formed by actinomycetes in surface cultures may be roughly assigned to one of the following three types. (i) The first type consists of pasty rough or smooth colonies that can be easily detached from the solid media. These are seldom covered with aerial mycelium and usually are formed by actinomycetes with a transient mycelial phase. (ii) The second type includes colonies like those of *Sporichthya*, which are almost devoid of substrate mycelium and consist of aerial hyphae attached to the medium through special holdfasts. (iii) The third type consists of compact, leathery colonies usually bearing aerial hyphae and firmly attached to the substrate by hyphae that penetrate the substrate. The latter usually are found in actinomycetes that have a persisting mycelial stage. One may usually distinguish in the colonies of the type (iii) those portions that lie beneath the level of substrate and those that lie above it in the form of either "substrate" and/or "aerial" hyphae.

Since substrate and aerial hyphae seem to differ in some ways (see below), one may question whether or not submerged cultures of actinomycetes are physiologically equal to surface cultures devoid of aerial mycelium. This question is largely unsolved. However, it might be noted that the terminology sometimes used by those who study streptomyces, which involves "vegetative" and "generative" mycelium (meaning substrate and aerial mycelium, respectively), is hardly justified, because many actinomycetes form reproductive structures on the substrate or primary mycelium. Formation of spores in shaken cultures by *Streptomyces* spp. was noted long ago (63), as was the formation of sporangia by *Streptosporangium sibiricum* under similar conditions (223). Considerations of conditions and stimuli that are responsible for the tendencies of growing submerged

hyphae of actinomycetes to form tight clumps or to repel each other remain largely speculative; research in this field clearly lags behind work done in the same area with fungi (405).

The real picture of differentiation in colonies of several *Streptomyces* spp. (423, 550) seems to be much more complex than is usually depicted (e.g., the scheme of Hopwood et al. [213]). The difference might be explained by the fact that actual colonies of actinomycetes usually involve the interaction of several generations of both primary (substrate) and secondary (aerial) hyphae, which sometimes overgrow each other. Strain- and medium-dependent differences are prominent in actinomycetes. It was shown, for example, that in contrast to the parent culture the surface layers of colonies of one of the variants of *Streptomyces variabilis* are actually composed of primary hyphae (532). Besides local gradients in supply of nutritional factors, lytic enzymes, etc., the picture of differentiation within colonies is further complicated by the local accumulation of antibiotics and probably other factors that limit growth. At least for some antibiotics (heliomycin), quite distinct sites of accumulation within colonies of the producer were revealed (531).

Obvious age-dependent differences among populations of cells constituting a colony are reflected in such well-known phenomena as the fragmentation of the mycelium. This (if it does take place at all) spreads from the center of a colony. Much less obvious are the reasons for changing patterns of sporulation in colonies of *Streptomyces massasporeus* (464), in which coiled aerial sporophores predominated in the center of the colonies, while single spores or short chains of spores were seen at the periphery.

An observer of actinomycete colonies is tempted to assume, with Pollock (409), but on different grounds, that an individual cell, exemplified by a fragment of a hypha, is not equal to the whole "living organism." The fungal colony was likened (179) to a city, populated with nuclei. By analogy, an actinomycete colony might be likened to a students' dormitory, with rules and regulations that are incompletely defined and only half-understood.

Differentiation of the Mycelium: Primary versus Secondary Mycelium

The width of mycelium in actinomycetes usually varies from 0.5 to 1.5 μm. Mycelium becomes thicker in some nutritionally unbalanced cultures. Some authors have reported the occasional occurrence of cells that were 0.1 μm (237, 288) or 0.1 to 0.2 μm thick, but systematic studies of "thin" mycelium of this kind are lacking.

Actinomycete mycelium shows one of the following types of branching, which are also known for mycelial fungi: (i) monopodial, (ii) dichotomous, and (iii) verticillate. Monopodial branching is most common. Dichotomous branching is characteristic of species of *Actinobifida*, whereas species of the genus *Streptoverticillium* are noted for the verticillate branching of their aerial sporogenous hyphae. Although dichotomous and verticillate branching is found in fungi of the genera *Thamnidium* and *Spicaria*, respectively, only the overall analogy seems to be justified at present, since the underlying mechanisms are virtually unknown in actinomycetes. In the actinomycete genus *Dermatophilus*, the side branches of the mycelium have a tendency to grow perpendicularly to the parent hyphae. As already mentioned, many, but not all, actinomycetes form two kinds of mycelium: primary (substrate) and secondary (aerial) mycelium. The latter is currently believed by most investigators to arise directly from the primary mycelium.

The following points (taken from the work of Higgins and Silvey [205]) briefly summarize the differences between the aerial and substrate mycelium in the *Streptomyces* sp. studied by the authors mentioned above. (i) The aerial mycelium is slightly thicker. (ii) It usually has a dark, insoluble pigment associated with its outer envelope and looks gray if viewed in reflected light. (iii) It shows less tendency to branch. (iv) The mycelium shows almost no tendency to penetrate the medium. (v) It forms spores through fragmentation. (vi) In contrast to the substrate mycelium, the aerial layer is hydrophobic. These points of difference are also evident from studies of many other authors.

Cytological observations on young aerial hyphae of *Streptomyces antibioticus* suggest that they contain nuclear elements that are richer in DNA as compared with substrate hyphae (423). Data on metabolic activities of aerial hyphae are sparse. Cytochemical techniques (162) applied to young, emerging aerial hyphae of *Streptomyces* spp. showed increased levels of sulfhydryl compounds as well as increased activity of enzymes such as alkaline phosphatase, catalase, and peroxidase. As the cultures entered the sporulation phase, the levels of activity of these enzymes decreased.

In the respirometric experiments of Erikson and Webley (145), the cells of *Thermoactinomyces vulgaris* aerial mycelium, sampled before the onset of sporulation, were shown to have a respiration rate higher than that of my-

celial cells sampled from the bottom of a liquid culture. A study of a mesophilic *Streptomyces* sp. (probably *S. griseus*) (154) concluded that the substrate mycelium may be regarded as facultatively aerobic, whereas the aerial mycelium is obligately aerobic. This conclusion was based on experiments employing respiratory poisons and cultivation in changing atmospheres.

The aerial mycelium of actinomycetes might be considered as belonging to those rare or even unique procaryotic vegetative cells that are kept functional in the absence of water in their immediate surroundings. One can speculate that such a capability might be associated with the first steps of some ancestral procaryotes from a predominately aquatic to a terrestrial environment. In this connection, it is worth noting that actinomycetes that are currently isolated from aquatic environments (e.g., species of *Micromonospora, Actinoplanes,* and some others) are usually represented by forms devoid of aerial mycelium.

The hydrophobic nature of the outer envelope of aerial cells might be relevant to the initial orientation of these mycelial cells and their emergence through the gas-liquid interphase. What maintains the growth of aerial hyphae out of the medium is unclear, but negative geotropism is certainly excluded. Presumably, some barrier exists that protects the cytoplasm of aerial hyphae from rapid desiccation, and this may be related to specific structures, variously called "surface sheath," "surface membrane," or a "microcapsule." The use of term "membrane" seems inappropriate because of confusion with the outer membranes of gram-negative bacteria.

Some characteristics of surface sheath structures are given in Table 1.

Cellular Differentiation

The structure of actinomycete cells is similar to that of other gram-positive bacteria.

The formation of septa is observed more or less frequently in the hyphae of all actinomycetes (Fig. 2). These are thought to be formed in a manner analogous to that of septa in other gram-positive bacteria (133, 204).

Transverse septa are the type found most often in mycelium of actinomycetes (Fig. 2). Occassionally, possibly during regulatory imbalance, the growing septa are split and divide the cytoplasm into several regions (Fig. 2). Examples of this are seen during chlamydospore formation in *Micromonospora chalcea* (337) and are regularly observed in vesicles of *Frankia* (37, 152). Formation of septa in two perpendicular planes, considered to be usual for *Dermatophilus* spp., is also known to occur in *Actinomyces* spp. (128). In vegetative hyphae of *Nocardia* and *Streptomyces* spp., transverse septa seem to start in their "older" portions, and then the activity spreads toward hyphal apices. It is worthwhile comparing this sequence of events with those observed during sporulation (see below). In *Streptomyces megasporus* (121) and *Mycobacterium tuberculosis* (21), cells separate by constriction in addition to septation.

A similar process was described also in *Arthrobacter simplex* (303). The cells that separated by constriction were said to have a gram-negative cell wall, whereas those dividing by septation had a gram-positive cell wall.

Septa in the vegetative hyphae of the majority of actinomycetes studied (561) look single or triple layered, depending on whether a line of electron-dense material in the middle of the septum is revealed (Fig. 3). It was suggested (298) that the failure of septate *Streptomyces* hyphae to separate into individual fragments might be due to local deficiencies in the activity of lytic dechaining enzymes.

The growing transverse septa in fragmenting *Nocardia* hyphae usually are split longitudinally, starting from the ends adjacent to hyphal walls (Fig. 3). Cytologically, the process of cell septation in *Nocardia* is said (136) to be reminiscent of that in *Arthrobacter crystallopoietes*. The persistence of the outer-wall component and its rupture in later stages of daughter cell elongation has been implicated in the formation of characteristic V-form cells. (Compare this with events accompanying spore deliminationin *Actinomadura* spp.). However, light microscope observations (360, 522) point to possible existence of variant modes of fragmentation.

Along with the indispensable and ever-present intracellular structures, one finds in the hyphae of actinomycetes some variable elements, as well as some rare and unusual structures. As in other procaryotes (467), these components may be found enclosed within membranous sacs or lying free in the cytoplasm, forming various inclusions. Localization of polysaccharides, polyphosphates, and lipids was demonstrated cytochemically (5, 107, 561), and the presence of poly-β-hydroxybutyric acid might be inferred from chemical determinations (250). Vacuoles in the actinomycete cells may have widely differing configurations. Some of the vacuoles, enclosed in a nonunit membrane, look very similar to gaseous vacuoles in other bacteria (561).

Rodlike and tubular structures reminiscent of raphidosomes (440), viruses, or defective

TABLE 1. *Aerial mycelium and spores of actinomycetes: some distinct characteristics of their surface structures*

Organism studied and material examined[a]	Reference	Methods applied	Results and comments[b]
Aerial mycelium outer surface: some distinguishing features			
S. coelicolor, *Streptomyces* spp.: SH, AH, spores	138	LM of Sudan black-stained preparations prior to and after washings with solvents	Differential staining of AH; washings with acetone, ethanol, ether, chloroform, and some other solvents prevents staining; presence of lipids associated with AH surface inferred
S. violaceus, *Streptomyces* spp.: SH, AH, spores	234, 285	LM in incident and incident polarized light; Sudan black B staining; exposure to solvent washings	AH and spores distinctly differ from SH in associated demonstration of birefringence, yield interference coloration, and stainability; these distinctions are abolished after acetone, methanol, and ethylacetate washings and are reduced following exposure to water vapors; regular organization of AM surface layers with a likely participation of lipoidal material suggested
S. griseus, *S. viridochromogenes*, *S. finlay*; spores	123	Electrophoresis following treatments with enzymes, surface-active agents, and chemicals specific for certain functional groups	Changes in electrophoretic mobility following lysozyme but not SDS and lipase treatment; surface location of lipids questioned; dominance of carboxyl and amino groups on surfaces inferred
Surface sheath: elementary structures and their features			
S. coelicolor: SH, AH, spores	214	TEM of carbon replicas	Surfaces of AH and spores distinctly different from SH in possessing a delicate envelope inlaid with rodlike elementary structures
S. venezuelae: AH, spores	51, 135	TEM of thin sections, carbon replicas; exposure to solvent washings	Elementary structures present: their localization altered following treatments with ethanol, xylol and benzene; rods removed by acetone washings followed by dehydration with ether; sodium hydroxide probably solubilizes elementary structures
S. coelicolor: AH, spores (smooth)	555	TEM of negatively stained preparations, freeze-etching; exposure to solvent washing	Rodlike elementary structures (12 to 20 by 40 to 250 nm) tend to be assembled in pairs; those seen on preparations probably represent parts of longer structures; not removed by solvent washing
S. glaucescens, *S. viridochromogenes*, *S. spadius*, *S. violaceoruber*: AH, spores	551	TEM of negatively stained preparations, carbon replicas	
S. viridochromogenes: AH, spores	552	TEM of negatively stained preparations, freeze-etching	Elementary structures of slightly differing shape and size invariably present on AH and spores; kinds of ornaments formed on either AH or spore surfaces tend to be similar in species with smooth spores and different in species with spiny spores
S. glaucescens, *S. arcimycini*: spores	553	TEM of negatively stained preparations, freeze-etching	
S. griseus, *S. venezuelae*, *S. viridochromogenes*, *S. finlay*, *S. glaucescens*: AH, spores	557	TEM of negatively stained preparations, freeze-etching, carbon replicas	
Micropolyspora spp.: AH, spores	9	TEM of negatively stained preparations	Position and integrity of elementary structures not changed after treatments with solvents
S. roseoflavus var. *roseofungini*: AH and spores	71	TEM of negatively stained preparations prior to and after acetone extraction	Elementary structures destroyed by brief acetone washings; closely similar structures reassembled in vitro from acetone extracts of AH.

phage particles (298, 509) have been observed.

Electron-transparent regions of irregular configuration containing no membranes are often seen in the cytoplasm of hyphae from the idiophase stage of *Streptomyces* cultures. The formation of these structures is linked with autolysis (8, 550), accumulation of antibiotics (75, 298), or phage infection (432).

Actinomycetes share with fungi not only mycelial organization per se, but also some struc-

TABLE 1—*Continued*

Organism studied and material examined[a]	Reference	Methods applied	Results and comments[b]
Spore appendages: origin, involvement of elementary structures			
Streptomyces spp.: AH, spores (with differing surface architectures)	530	TEM of intact spore chains	Spores with appendages are formed inside AH wall
S. togocaensis: AH, spores (spiny)	12	TEM of spore thin sections	The outer-wall component probably involved in formation of spore appendages
S. violaceus: AH, spores (spiny)	425, 548	TEM of intact spore chains and thin sections	Origin of spore appendages not related to AH walls. Surface sheath, as a distinct structure, directly involved in origin of appendages; kinds of appendages produced do not reflect differences in mode of spore formation
S. venezuelae AH, spores (smooth)	51	TEM of thin sections, carbon replicas	
S. griseus: AH, spores (smooth)	560	SEM of intact spore chains and TEM of thin sections	
S. finlay: AH, spores (hairy)			
Streptomyces spp.: AH and spores (with different surface architectures)	551, 552, 553	TEM of negatively stained and freeze-etched cells, thin sectioning, carbon replicas	Spore appendages consist of elementary tubular, grooved, and similar structures found also in the surface sheath; conelike bundles of elementary structures form spiny appendages; hairy ones represented by bundles of intertwisted elementary structures
Environment and formation of spore appendages			
S. violaceus: AH, spores (spiny)	321	TEM of intact spore chains following cultivation on different nutrient media	Presence and general structural features of appendages almost unaffected by nutritional conditions
S. hygroscopicus: spores (rugose)	104, 105	TEM of carbon replicas	Presence and structure of appendages varies depending on relative distance of spores formed from the agar surface (relative humidity gradient important?)
Streptomyces spp.: spores (spiny)			
Spore appendages: response to treatments			
Streptomyces spp.: AH and spores (with differing surface appendages)	151	TEM of spore chains (intact and after washing with solvents)	Spore silhouettes not affected by washings with chloroform and some other solvents
Streptomyces spp.	341	TEM of carbon replicas and intact spore chains (prior to and after lysozyme treatment)	Appearance of spore appendages grossly changed following lysozyme treatment

[a] SH, Substrate hyphae; AH, aerial hyphae; LM, light microscopy; TEM, transmission electron microscopy; SEM, scanning electron microscopy; SDS, sodium dodecyl sulfate.

[b] Our comments are given in parentheses.

tural features that might result from it. Among these one might list the intrahyphal hyphae found in *Streptomyces roseoflavus* var. *roseofungini* (70), *Micropolyspora* spp. (8), and *Microellobosporia flavea* (561). As in fungi (333, 517), the intrahyphal hyphae in actinomycetes are found not only in ageing cultures; they are thought to assume an important role in the reproduction of over-wintering cells of *Frankia* spp. (345).

The motility of actinomycete cells, whether specialized (zoospores) or unspecialized (mycelial fragments), is always linked with posession of flagella, and all types of flagellation are to be found. Peritrichous flagellation is rather rare. The flagella of *Actinoplanes* spp. were said to be reminiscent (317) of those in *Pseudomonas*, whereas the flagella of *Dermatophilus congolensis* were different in that they were only 8 to 9 nm thick, much less than most flagella (431).

Amycelial Structures

Some actinomycetes form structures that arise from mycelial cells but differ markedly from them. The role of these structures in reproduction is not quite clear; in any case their formation does not represent the sole way of multiplication.

Sclerotia persistently formed by *Chainia* spp. (242, 505) belong to this category. In the formation of sclerotia, the mycelial hyphae thicken and become septate, and many lipid-containing vacuoles are formed. Intercellular cementing material is formed, which contains L-2,3-diaminopropionic acid. The lipids are mainly tri-

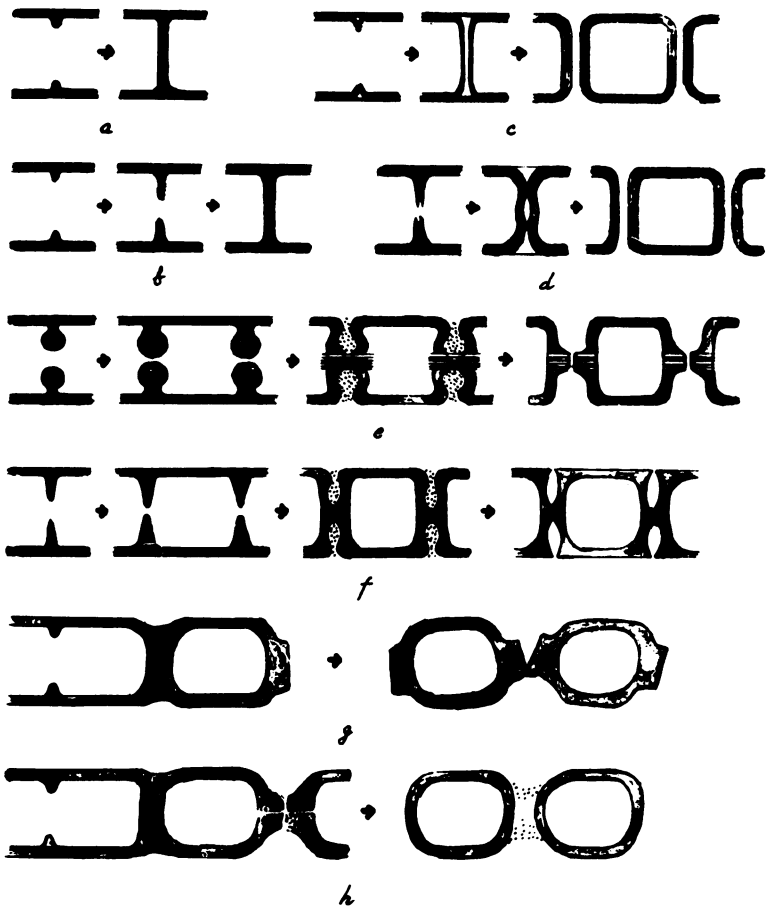

FIG. 3. *Diagrammatic presentation of structural organization and formative events in septae of actinomycetes. (a, b) Single- or triple-layered septa found in the majority of vegetative cells studied. (c, d) "Split" septae: (c) found in fragmenting hyphae of Nocardia spp. and in sporulating hyphae of many actinomycetes; (d) revealed in S. coelicolor (554). (e, f) Multicontour sporulation septae: (e) found in S. viridochromogenes; bear several layers of double unit membranes (568); (f) found in S. ribosidifuscus (568). (g, h) Sporulation septa with intersporal pad: (g) the pad persists in mature spore chain (A. dassonvillei [562]); (h) intersporal pads destroyed during later stages of spore formation in M. rectivirgula (117); at a certain stage of spore formation a plasmodesm is distinguishable within the intersporal pad. Solid areas, cell wall and septal material; dotted areas, intersporal material.*

glycerides of branched fatty acids of the iso/anteiso series having 15 to 16 carbon atoms. These transformed and fattened cells are packed in hyphal mats (322). It is thought (561) that the structure and functions of sclerotia in *Chainia* are reminiscent of those in such fungi as *Sclerotinium rolfsii* and *Verticillium alboartrum*.

Hyphal aggregates formed by certain *Streptomyces* spp. on some media (160) are probably not related to sclerotia (242), nor are the "granules" described by Baldacci et al. (26). The intriguing feature of these granules is that they yield, upon inoculation on fresh media, colonies of actinomycetes that might differ from the parent colonies in ability to produce aerial mycelium and pigments.

Close associations of hyphae are met with in coremia, produced by some *Streptomyces* spp. (278, 326). No signs of special transformations of hyphae in coremia were reported so far. It is interesting to note, however, the ability of neighboring coremia to extend hyphae that form "bridges" through the air and fuse (180). The vesicles formed by *Frankia* spp., already mentioned, may be surrounded with capsular material, with a thickness that depends on the age and condition of the plant host (345).

Some actinomycetes excrete slimy substances, which might contribute to the formation of cell agglomerates. Some are polysaccharides (403), probably enriched in ferric hydrox-

ide (278). The specific glycolipid—"wax D"—produced by some mycobacteria (170) contains components that are common to wall murein and probably originate from transformations of the latter. Coating the cells with wax D probably offers additional protection (124). Immunochemical evidence was said (308) to suggest excretion of membrane complexes, consisting of mucopolysaccharides and a phospholipid, during the growth of mycobacteria, nocardiae, dermatophili, and streptomycetes. The "sulfur granules" formed by *Actinomyces* spp. in vivo consist of mycelial hyphae cemented with a polysaccharide-protein complex. The mineral portion of granules includes apatite and calcium phosphates (155, 404). Central regions of *Streptomyces scabies* colonies appear to be covered with a "blanket" of some extracellular material (126).

Occasionally, round or clublike vesicles of increased size (up to 3 to 5 μm in diameter) are found in submerged *Streptomyces* cultures (529); swollen cells of *Dactylosporangium thailandense* are thought (462) to represent reactions to unfavorable growth conditions. Sporangia of *Intrasporangium calvum* (241) have been placed (318) in the same category.

Modes of Spore Formation

The spores formed by actinomycetes fall within two groups: endogenous and exogenous, according to the mode of their formation.

Endogenous formation of thermoresistant spores inside the cytoplasm of parent hyphae was studied in the thermophilic actinomycetes *Thermoactinomyces* and *Actinobifida* (86, 89, 116, 118, 310). Structurally, it follows essentially the same sequence depicted for spore formation in other gram-positive bacteria (150). Some differences in the regulation of spore formation probably exist, as judged from the final location of spores on the mycelium. In *T. vulgaris*, spores often reside in the hyphae and are partially extruded in the course of formation and maturation; in *Thermoactinomyces sacchari*, most spores are found terminally, and in *Actinobifida dichotomica* only terminally.

Spore formation in *Planomonospora* and *Dactylosporangium* was also said to be endogenous (463, 561), but the process seems to be very different from what is believed to represent "true" endogenous sporulation in bacteria. The difference is especially pronounced in the fate of presporal and sporangial membranes and their participation in the formation of spore coats following "engulfment." According to these authors, the initial steps of spore formation in the above-mentioned actinomycetes include splitting of the parental hyphal wall into two layers. The protoplasmic regions enveloped by the inner layer of the hyphal wall then become condensed, leaving some electron-transparent space between the sporangial and spore walls. Spores, shaped like sporangia, are liberated as soon as sporangial wall (bearing a surface sheath that is characteristic for aerial mycelium of actinomycetes) is broken.

Spores in most actinomycetes are formed exogenously. Formation of spores in *Streptomyces* spp. starts by division of nucleoids and changes in the membranous structures. The latter show in thin sections as thickening of the peripheral plasma membrane and the appearance of a number of mesosomes. Later on, sporulation septa accompanied by mesosomes start to form (51, 163, 425, 554, 568). The wall of the developing exospore seems to be formed with help from the cytoplasmic membrane of the corresponding region of the sporogenous hyphae, in contrast to the situation with endogenous sporulation. The subsequent processes transform the thickening and stratifying hyphal wall into spore wall (121, 164). However, it seems more probable that the sporal walls, or at least their inner layers, are synthesized de novo on the cytoplasmic membrane (337, 554).

Intracellular transformations accompanying sporulation are manifested also by the nucleoid and membranous bodies assuming a more compact form. Spore nuclear areas remind one of those in *Streptococcus faecalis* cells in which protein synthesis has been blocked (91). The consequences of all these transformations become evident on examining fixed preparations of sporogenous cultures of actinomycetes by electron microscopy: spores are usually much less damaged by fixation and sectioning than vegetative hyphae. Also, the internal structures of spores are often less resolved, which probably reflects incomplete penetration of the fixatives.

In some actinomycetes, like *Micropolyspora flavea* (424) and *Micropolyspora viridnigra* (7), an unusual central and granular mass, absent in vegetative cells, is found in spores and is termed the "central body." Spores of these organisms also contain large vacuoles enclosed in a unit membrane.

The variations in exogenous spore formation among actinomycetes mainly involve the origin and fate of sporulation septa, which, contrary to the situation found in vegetative mycelium, seems to be under more stringent control. These temporary and spatial controls bear upon the frequency and the character of the transformations that newly formed septa undergo. Nascent spores in chains (Fig. 2) are delimited by septa appearing in sequential order, which may

be different in various actinomycetes (compare, for instance *Micropolyspora* spp. [283, 320] and *Pseudonocardia* [194]). The sequence reported for *Pseudonocardia* seems especially interesting, since the nascent spores seem to retain vegetative functions (transport of metabolites from hyphae to apical buds) for rather extended periods of time.

In *Streptomyces* spp. (51, 164, 425, 554, 560) and some sporangium-forming actinomycetes (317, 319), septa seem to be formed in two stages. First, the sporophore is separated by a single septum from the corresponding hyphae. Then the sporogenous hyphae is itself divided by a number of septa almost simultaneously into approximately equal-sized cells that eventually are transformed into spores. The simultaneous character of multiple septation in *Streptomyces* sporophores reported by a majority of investigators probably needs further experimental proof. This might be not easy to obtain, since the process is rapid enough to obscure time-lapse observations. If the septation actually proceeds in a basipetal manner, such a sequence would be interesting to compare with an opposite one, found in fragmenting vegetative hyphae.

Occasionally, in *M. viridinigra* (7) and *Actinomadura dassonvillei* (562), septa are formed not quite regularly, dividing the sporogenous hyphae into unequal compartments. In *A. dassonvillei,* the extended cells resulting from the first round of septation may be divided by secondary septa.

Once formed, sporulation septa in actinomycetes may develop further and undergo rather complex transformations that are usually not observed in vegetative cell division. The main types of sporulation septa are diagrammatically presented in Fig. 3. They differ in shape and as to whether special wall thickenings and intersporal material are present (these structures also show various patterns in development).

Functional implications of the differences mentioned above are not clear. The structure of the sporulation septa seems not to affect the final form of the sporophore (i.e., straight, flexuous, spiral, etc.). The latter is thought to be acquired before extensive development of septa, during the first sporulation stage (215). Scanning microscope observations (25, 27) are in agreement with this view, so it is believed that the sporophore architecture is governed by events that occur in the mycelium before the onset of sporulation. The zig-zag form acquired by the *A. dassonvillei* spore chains during one of the stages of sporogenesis is thought (562) to result from spore disjunction starting from one side of the septa. At which stage (if at all) the spores formed become efficiently separated from the mycelium is not clear. The presence of plasmodesms has been reported only for spores of *Micropolyspora rectivirgula* (117) and *Streptomyces viridochromogenes* (568).

Actinomycetes that form exogenous spores fall within two groups according to whether the surface sheath is present on the sporulating hyphae (561). Surface sheath is absent on sporulating hyphae of *Micromonospora,* on sporulating substrate hyphae of actinomycetes, and on fragmentation spores of *Nocardia* spp. The ornamentation on spores of *Micromonospora chalcea* results from projections, present on the outside layer of the parental hyphal wall (337). When present, the surface sheath envelops the sporulating hyphae as a kind of "stocking." In the course of late stages of sporulation, the surface sheath behaves as a separate entity, seemingly independent of the transformations affecting the sporophore wall. The sheath behavior, however, seems to be intimately connected with the resulting spore surface architectures (Table 1).

The coat of vesicles in actinomycetes is thought to be homologous to the surface sheath of aerial mycelium. The use of the term "sporangium," as suggested (77) for the former structures, seems not to be quite justified if one considers the fine details of spore formation within vesicles. It has been suggested (82, 563) that the term sporangium be retained exclusively for actinomycetes with endogenously formed spores and thus establish closer links with the common bacteriological nomenclature.

It seems worthwhile to name a few anomalies in spore formation, since they may help to find eventually the "weak points" in the regulation of sporulation. The number of spores in chains seems usually not to be under a stringent control. Probable exceptions are represented by *Microbispora* (390) and *Microtetraspora* spp. (504). Formation of pairs of spores in *M. chalcea* was interpreted (337) as an indication of imprecise regulation of septum formation.

Anomalous and badly germinating spores were found in some *Micropolyspora* spp. (7), and these showed much-thickened walls, absence of the central body and vacuoles, and accumulation of electron-dense (presumably polyphosphate) granules. Three types of spores differing in ultrastructure were revealed in thin sections of *Streptomyces megasporus* (121). The defective nature of sporulation is here suggested by low germinability, unusual spore size, and location of the nucleoid, etc.

Summarizing this section, we would like to point out that the wide spectrum of structural

modifications during the basic process of cell septation for exospore formation in actinomycetes fits well with the hypothesis (209) that sporulation is a modified procaryotic cell division. The hypothesis fits even closer than it does for sporulation in bacilli, for which it was originally suggested. One reason for this is the possibility of observing some of the likely "intermediate steps" that are preserved and functioning. On the other hand, cellular differentiation in actinomycetes is so rich in terms of resulting forms, it is no wonder that one finds difficulties in describing them in bacteriological terms and seeks refuge in the more developed mycological classification of spore types (82).

Spores

We now turn to the characteristics of spores produced by actinomycetes and the structural diversity that they exhibit. They fall within the broad definition of a spore (488) and the formation of these cells may represent the main means of reproduction in actinomycetes; there is no doubt that they are important.

More information is available on spores produced on the aerial mycelium than on spores produced in submerged cultures and within agar. It is known that *Streptomyces* spp. do produce sporelike structures in submerged cultures (63), which probably differ slightly from mycelial cells in thermoresistance (131). They also differ from the aerial spores of the same species in hydrophobicity and requirements for germination (240). So far, studies on submerged spores have been hindered by technical difficulties in obtaining uniform material. Their further investigation would be interesting in connection with possible attempts to observe the "microcycle sporulation" in actinomycetes.

Since the properties of spores of actinomycetes were the subject of some of the recent reviews (81, 83, 235, 248, 485), what follows is a condensed summary of some of the relevant earlier works, with the emphasis on less-developed and newer areas.

Structure. The structural differences between endo- and exospores are pronounced.

Endospores produced by thermophilic actinomycetes are prominent among actinomycete spores because of polygonal outlines on the outermost surfaces (3, 79). On the surface of spores the pentagonal and hexagonal areas are slightly curved inwards. Similar surface architecture was earlier revealed in some *Bacillus* spores (211). Scanning electron microscopy and application of the freeze-etch techniques enabled investigators (329, 365, 556) to reveal that the outer coat of *T. vulgaris* spores actually consists of parallel rows of fibrils each measuring about 5 nm. In this respect, these spores resemble those of *Bacillus coagulans* (176). The internal structure seems not to differ in any significant respect from that of spores in *Bacillus* and *Clostridium* spp. They have a cortex and an elaborate system of outer coats. Details of sporoplast are not easily seen in thin sections of dormant spores. In contrast to *Thermoactinomyces* spores, those of *Actinobifida dichotomica* possess an exosporium.

In their fine structure, spores of *Planomonospora and Dactylosporangium* resemble exogenous spores of actinomycetes, which show a rich diversity of structure. For the purpose of rather superficial and pragmatic classification, one might distinguish here the following main types.

(i) Spores that do not contain novel internal structures that are completely absent in vegetative cells. One would place into this category the spores of *Streptomyces* spp. (most often studied) and several other actinomycetes, which form chains of spores and vesicles with spores (43, 164, 317, 319, 463). No substantial differences were recorded in the structure of spore nucleoid, ribosomes, membrane structures, and vacuoles compared with analogous structures of vegetative cells. However, the corresponding spore components often look more densely packed, the number of mesosomes being reduced. The spore wall is usually 1.5 to 2.0 times thicker than the vegetative wall, and two to three layers differing in electron density can be revealed within it.

Similar internal structure but thicker walls and more elaborate multilayered external coats characterize the spores of *Micropolyspora* spp., *Saccharomonospora* spp., *Thermomonospora curvata, Actinobifida chromogena,* and *Dermatophilus congolensis* (119, 195, 431). The spores of *M. chalcea* (337) are similar in that they have much-thickened walls, which probably account for rather high refractility characteristic of spores of *Micromonospora* spp. The middle wall layer in spores of *M. chalcea* and *A. chromogena* (342) was termed "cortex." The use of the term in the above cases seems inadequate because of the distinctly different origin of the cortex of true endospores and internal-wall layer of the above spores.

The question of how many nuclear elements may reside in a *Streptomyces* spore is still unresolved. There are indications that most spores of this type contain one nucleoid (50, 111, 114, 257, 258, 423), although claims of finding two nuleoids are also known (147, 445, 446).

(ii) Spores that do contain structures absent in vegetative cells. These structures may be represented by large vacuoles and the forma-

tion of a structure of unknown origin and composition termed "central body." Spores of this category may differ in the structure of their envelopes, and the presence of marked intersporal pads is characteristic for spores of *M. flavea* (424). An extensive fine, granular coat delimited by a triple-layered sheath distinguishes spores of *M. viridinigra* (7, 120). The surface sheath, which covers aerial mycelium in many actinomycetes, usually persists on spores. The exceptions are represented by spores formed on submerged mycelium and spores of *Micromonospora* spp. even in surface culture (561).

Some characteristics of subunit morphologies encountered in surface sheath of various actinomycetes are listed in Table 1. Spore surface architecture (smooth or bearing various appendages) varies among the species of *Streptomyces* and is extensively used for identification purposes (552, 553).

Elementary structures which are contained in the surface sheath form a kind of ornament that might differ among organisms. Potentially, this provides an additional opportunity for identification purposes (551, 172).

Similarity in ornament, however, does not prove the similarity of organisms, since similar ornaments were found in spores of *S. griseus* (557), *A. dassonvillei* (562), *Streptoverticillium* (84), and sporangia of *Planomonospora* (463). Moreover, morphologically similar ornaments were also revealed on spores in several aerobic sporeforming bacteria (211) and conidia of *Aspergillus* and *Penicillium* spp. (196, 197).

Possible functions of both spore appendages and elementary structures are rather obscure. Suggested hypotheses range from enhancement of hydrophobicity and floating abilities for the spores (555) and enhancement of dissemination by insects, to a more extravagant one implying genetic exchange between the spores through microtubules (357). An alternative hypothesis will be formulated in the section on secondary metabolism.

Composition. Information on the chemical composition of actinomycete spores is fragmentary compared with that on bacterial and some fungal spores. Again, spores of *Streptomyces* spp. have been a little more thoroughly investigated.

The amount of water in these spores varies widely according to environmental conditions, 2 to 4% being represented by what may be called "firmly bound" water (314). Although "total" infrared spectra of mycelium and spores seem not to differ appreciably (419), some significant differences in the contents of individual components were found.

The spores of *S. streptomycini* and some thermophilic *Streptomyces* spp. contain increased amounts of Ca and Mg (236), and these spores also seem to accumulate Mn and Ni (411). According to electron spin resonance signals, there is a possibility that Mn is present in both the free and the bound state (412), the last form giving a signal similar to that in bacterial spores which is ascribed to the Mn-dipicolinic acid (DPA) complex (567). According to conventional analysis, however, DPA has not been found in any *Streptomyces* spores examined so far (227).

In contrast, the endospores of thermophilic actinomycetes were shown to contain amounts of DPA and Ca (89, 236, 309) equivalent to those in some of the bacterial spores (375). Traces of DPA were found in spores of *A. chromogena* and *M. rectivirgula* (236).

The DNA content of *Streptomyces* spores seems to be slightly higher, whereas the content of ribonucleic acid (RNA), according to different authors (see section on germination), is lower or higher than in vegetative cells. Cytological data (461) show figures of 94 and 50 to 69% DNA and 19 and 51 to 75% RNA for nuclear bodies of spores and vegetative mycelium, respectively.

There are indications that *Streptomyces* spore DNA differs from that of mycelium. The differences, revealed in studies of *S. venezuelae*, include (i) thermal denaturation temperature; (ii) buoyant density (1.722 and 1.730 g/cm^3 in cesium chloride for spores and mycelium, respectively), and (iii) homology with respective vegetative DNA (134, 135). Interestingly enough, the buoyant density of DNA was shown to fall gradually as spores matured, this process being accompanied by lowering the degree of annealing with denatured vegetative DNA from 100 to just 30%. It has been suggested that altered properties of spore DNA might result from complexing with some unique spore product (135), possibly a pigment (458).

The relative amount of protein in mature spores of *S. venezuelae* reaches 10 to 15% (Folin phenol assay method). Total-protein hydrolysates seem to contain increased relative amounts of arginine and leucine (51, 503).

Spore ribosomes of *S. griseus* were shown to be more stable in solutions of the same ionic strength than vegetative ribosomes (527). This was also shown to be the case with spore and vegetative ribosomes of *Streptomyces granaticolor* (367). On dialysis against 10^{-2} M tris(hydroxymethyl)aminomethane buffer containing Mg^{2+}, the majority of spore ribosomes formed aggregates, whereas vegetative ribosomes per-

sisted as 70S monomers. Both types of ribosomes were shown to be able to dissociate into 50S and 30S subunits. Qualitative differences were said to be found in nucleotide composition of spore and vegetative ribosomal ribonucleic acids.

The murein components of spore and vegetative cells in *Streptomyces* seem not to differ significantly in the relative amounts of constituting monomers (90), although an increased amount of aspartic acid residues was recorded in spore murein hydrolysates (96).

Some possible differences in structure and perhaps arrangement of simple monomeric molecules in spores are reflected in their decreased staining ability with methylene blue, altered ability to stain with a fluorescent brightener (559), and ability to stain selectively (according to Corti [76]). In some *Streptomyces* spp. the ability to stain gram positively was reported to increase during spore delimitation and maturation (472), whereas in others it was reported to decrease drastically (448). Distinct differences were also found in susceptibility to phage attack, although specific phages are absorbed on resting spores (243, 430). The intact spore wall of several *Streptomyces* spp. is not sensitive to lysozyme digestion and is not destroyed by treatment with a 15% KOH solution (96, 476), in contrast to the vegetative cell wall.

The spores of *Streptomyces* spp. are marked by their coloration. The color of "sporulating aerial mycelium" was indeed employed for several decades in descriptions and classification of cultures. Surprisingly, we know very little about the pigments responsible for this coloration—practically nothing in comparison with the abundant information on pigments that diffuse into the medium or are associated with mycelium (44).

Some of the spore pigments are probably associated with spore coat(s), as evidenced by changes in coloration of aerial mycelium in the course of sporulation (215, 494) and loss of pigmentation in the course of spore germination (see below). A distinct correlation was reported between spore color en masse and the surface ornamentation of spores (see Table 2). In spore homogenates, some of the pigments were reported (367, 527) as being firmly associated with ribosomes. It is not clear, however, whether these observations reveal the chemical potentialities of the pigments or their true localization in situ.

The electron spin resonance technique indicated the presence of melanine in spores of *S. streptomycini* (413). The brownish pigment also present in these spores, according to ultraviolet spectra and paper chromatography, was not identifiable with vegetative actinomycete pigments of the same color (18). The brownish pigment of *Streptomyces venezuelae*, which accumulated in the course of sporulation, was shown to possess indicator properties with absorption maxima at 420 and 520 nm in butanol at pH 3 and 12, respectively (458). Traces of streptomycin were detectable in the spores of a strain of *S. streptomycini* of low antibiotic activity (18).

The antigens of actinomycete spores differ from those of vegetative cells and are recognized as a separate group of antigens (307). The relative amount of spores in the actinomycete biomass might thus influence its antigenic and chemical composition (306). The antigens associated with spores of thermophilic actinomycetes and responsible for the allergic condition called "farmer's lung disease" are among those most intensively studied (541). It is not clear, however, whether these antigens are associated only with spores or with the aerial mycelium as well.

The available information on chemical composition of the spore surface sheath was mentioned in Table 1.

During sporulation, some actinomycetes excrete and accumulate in their cultures slimy substances that might hold sporulating hyphae

TABLE 2. *Aerial mycelium color, spore, and sporophore morphologies in Streptomyces spp.*[a]

Sections	Color of sporulating aerial mycelium	% Strains studied	
		Sporophores	Spore surface
Azureus	Bluish, bluish-green	Spiral (93)	Appendages (90)
Cinereus	Gray	Spiral (80)	Appendages (70); spores with hairs found almost exclusively in this section
Roseus	Rosy	Straight (50)	Smooth (90) Appendages (10)
Helvolo-flavus	Yellow, buff	Straight (76)	Smooth (100)
Albus	White	Straight (77)	Smooth (100)

[a] Schematized according to data of reference 418.

together and otherwise modify the appearance of sporophores (217, 287, 438, 464). Staining of slimy substances produced by *S. hygroscopicus* (438) seems to indicate the presence of polysaccharides.

Physiology. Metabolic activity of intact, dry *Streptomyces* spores is low, as shown by respirometric experiments. Respiration of ungerminated spores is rather resistant to cyanide inhibition (245). These observations correlate with diminished amounts of cytochromes *b*, *c*, and *a* (500). However, a number of active dehydrogenases, as well as the enzymes of phosphorus and nitrogen metabolism, were detected in *Streptomyces* spores, the level of the former sometimes exceeding that in 2-day-old vegetative mycelium (239, 459). An increased level of catalase was also reported in spores of *S. streptomycini*; a portion of this activity could be removed by washing with water (477). Photoreactivation of *Streptomyces* spores is well documented in Kelner's (253) pioneering work.

Spectrometry revealed electron spin resonance signals in dry *S. streptomycini* spores (411) characteristic of metabolically active yeast, bacterial, and animal cells (106, 219, 328), but absent in the innately dormant bacterial spores (567). When kept under suitable temperature and aeration conditions, the spores of *S. streptomycini*, whose germination is prevented by elevated salt concentration, are able to incorporate exogenous ^{32}P mainly into their RNA fraction (238). Calorimetric measures of their activities (247) bring to mind certain plant seeds during the so-called dead phase of their germination (61). Therefore the senescent spores of *Streptomyces* seem to be attractive objects for studies of this reversible state and the maintenance of viability in procaryotic cells.

The spores of *T. vulgaris* were shown to be devoid of measurable respirometric activity (145) and deficient in cytochrome *a* (500).

Duration of viability. The maximal duration of viability for spores of *T. vulgaris* surviving in lakes at 5 to 6 C has been reported to be 100, even 1,500, years (88), although earlier workers (326, 389, 455) mentioned rather rapid loss of viability in cultures of the thermophilic actinomycetes studied by them. Duration of viability of actinomycete endospores is thus comparable to that of other bacterial endospores (475).

The maximum value for maintenance of viability by air-dried *Streptomyces* spores so far reported is 14 years (248). No viable spores were found in dry cultures stored for 42 years (422).

Successful storage of *Streptomyces* and *Nocardia* spores in distilled water for 4 years has been reported (364). Cytological observations (423) suggested that many of the spores found in colonies of *Streptomyces* spp. were not viable. The duration of viability in actinomycete exospores is thus comparable to that of asexual conidia in fungi (435).

Resistance to deleterious agents. Most of the actinomycete spores studied share a high resistance to dessication (41, 407, 478) which probably has important ecological implications. It might be responsible, at least in part, for the relative abundance of actinomycetes in arid soils. The biochemical basis for this enhanced resistance is especially intriguing in *Streptomyces* spores because their structure and chemical composition is not different from vegetative cells. Vegetative mycelium of most actinomycetes seems to be less resistant to desiccation and lyophilization (198, 299, 506, 515). When dried spores are stored in air at different relative humidities, the most deleterious for preservation of viability appeared to be the range of relative humidity from 60 to 90% (314).

Compared to vegetative cells, *Streptomyces* spores appear to be more resistant to mechanical abrasion (473), diluted acids, Formalin, hydrogen peroxide, and some alcohols (17), probably also phenol (315) and chloroform (60). Increased resistance to radiation was found to correlate with hydration level, number of nucleoids per cell, and the stage of gemination (383, 423). Nucleic acids were shown to be the radiation-sensitive target in both dry and wet spores (224, 225, 377). Actinomycete (*Streptomyces* ?) populations in soils were reported (66, 549) to be more resistant to soil treatment with methylbromide, 1,3-dichlorpropane, and some other chemicals, as compared with fungi and some groups of bacteria.

According to most authors, who have been discussing the subject for six decades, exospores of *Streptomyces* spp. are just slightly more thermoresistant than vegetative cells. As with vegetative cells of other bacteria, resistance of these spores to dry heat is much higher than that of wet spores (16, 149, 226). In a set of typical experiments, it was shown (355) that viable fragments of vegetative mycelium were still recoverable after exposure of suspensions in water at 45 C for 15 min. Heating spore suspensions of various *Streptomyces* spp. at 60 C for 15 to 45 min left no viable cells. Rod- and coccus-like vegetative fragments of nocardiaform *Streptomyces* variants (378) were shown to possess slightly enhanced heat resistance compared with ordinary fragments of vegetative mycelium in parent cultures, whereas similar fragments of *A. viscosus* (57) showed no such difference.

Studies of heat resistance of *Streptomyces*

spores usually rely on conventional techniques involving suspensions of heated cells plated on solid nutrient media. Deviation from such techniques appeared to be instrumental in revealing the ability of such spores to regain their viability after heating. If the spores were kept in a humid atmosphere or in distilled water after heating at 99 C for 10 min, an appreciable proportion of them recovered viability (313), thus opening prospects for the investigation of mechanisms involved in repair of heat damage. Studies of such mechanisms employing bacterial cells or fungal spores (29) are usually done with suspensions subjected to less drastic heating.

The remarkably increased resistance of dormant actinomycete endospores toward moist heat can be correlated with their structure and composition. As with bacterial endospores, however, it is still uncertain whether increased Ca and DPA content is primarily responsible for increased thermoresistance, and whether the cortex is likely to exert pressure on the sporoplast through contraction (325) or expansion (174).

The spores of other *Actinomycetales* studies occupy an intermediate position between the extremes exemplified by *Streptomyces* and *Thermoactinomyces*. This was shown to be the case with *Nocardia, Micromonospora, Pseudonocardia, Micropolyspora,* and some others (144, 149, 236, 538). Since many of these spores contain no DPA or only traces, have no cortex, and possess walls of different degrees of thickness, it has been suggested (83; Cross, personal communication) that these spores might be arranged in a row in which each succeeding member of the series would be characterized by increased heat resistance and wall thickness. They would thus resemble a row of cortex-less mutant *Bacillus* spores in which at least a portion of the functions supposedly carried by the cortex would be transferred to spore wall layers.

Motility. Active motility of zoospores of *Actinoplanes* spp. was stimulated in the presence of several amino acids (L-arginine being especially efficient) and urea (78). Disruption of the sporangium and release of motile zoospores in *Actinoplanes* sp. was stimulated by a wetting solution, Tween-80 (202), and additives that affect mobility. Spores, although possessing flagella, were not motile when released from old sporangia if suspended in water or solutions containing phosphate and amino acids. Addition of a suitable carbon and energy source to the solution restored the motility of zoospores. Deflagellated zoospores (after ultrasound treatment at 4 C) showed resynthesis if the spores were placed in nutrient solution containing amino acids and glucose. Presence of inhibitors of nucleic acids biosynthesis induced formation of nonmotile flagella.

DYNAMIC ASPECTS OF DIFFERENTIATION

Age-Dependent Changes in Actinomycete Cells

Age-dependent changes were most often noted in submerged cultures of actinomycetes. To summarize observations with several *Streptomyces* spp., one may conclude that during the trophophase the majority of cells in populations are basophilic (high RNA content), with increased rates of protein synthesis and DNA synthesis as well as replication. During the idiophase, these rates and the stainability of the cytoplasm decrease (62, 63, 423, 457). During the periods of a maximal rate of biomass accumulation, enlarged nucleoids rich in DNA were observed in *Streptomyces* hyphae and termed "polyenergid nucleoids" (423). Their functional significance and possible relation to the concept of changing gene dosage (316) await further studies. Anyway, the fact remains that the relative amount of DNA (per unit of dry weight) fluctuates significantly in developing submerged cultures of actinomycetes.

Age-dependent changes were revealed in the plasma membrane of the mycelium of *Streptomyces albus* (372), the difference in membrane-bound nicotinamide adenine dinucleotide, reduced form, oxidase levels being especially prominent in young and older cells. In the course of *S. antibioticus* development in submerged culture, a redistribution of lysine and DAP isomers in the cell walls was reported (427). Marked changes in age-dependent cell response to Gram stain were noted in a study of *Streptomyces erythreus* (426), as well as in various representatives of *Actinomycetales* (165).

Accumulation of volutin granules in actinomycetes cells was shown to be associated not only with ageing, but also with nutritional imbalances (182). As in other organisms, volutin granules in actinomycetes contain high-molecular-weight complexes of orthophosphate with various ions and they are believed to play an important role in regulation of phosphate and cation levels within the cell (395).

In *N. rubra* cultures (366), mycelioid, bacillary, and coccus-like cells predominated during logarithmic, stationary and autolytic stages, respectively. Accordingly, the relative amount of DNA (per biomass unit) varied in the range 2.2 to 3.0 to 1.5%; RNA, 20.1 to 10.0 to 4.0%. Protein content fell from 50 to 45% during the early logarithmic stage and then was constant

until autolysis, when it fell to 31%. The total lipid content rose from 9.9 to 20.0% during logarithmic phase, remained constant, and then reached 30.85% during the autolytic phase.

The growth of *Arthrobacter globiformis* cultures freshly inoculated into a complex medium was preceded by a 4-h lag period (482). RNA synthesis began immediately after inoculation; synthesis of protein and DNA began an hour later (compare with data on arthrospore germination, see below). The differential rate of synthesis of the above macromolecules appeared to be the same before the onset of cell division and during a brief period of growth as rods. It was concluded from similar experiments with *A. crystallopoietes* that morphogenesis in these organisms might involve transient incoordination of macromolecular syntheses and cell division (136).

Environmental Conditions and Morphogenesis

Environmental variables seem to exert a pronounced effect on morphogenetic events in actinomycetes. So far, most of the conclusive evidence, reveiwed by Ensign (136), stems from work done with forms producing transient or multiseptate mycelium. These are suitable for such studies, as pointed out by Heinzen and Ensign (190), because the predominantly mycelioid growth and regular septation appear to be separated in time sequence.

An example of the effect of complex nutrient additives is given in studies with *Nocardia* sp. 721-A, which formed clublike cells on a medium with meat extract. These cells underwent irregular septation; they did not appear on nutrient agar and mineral synthetic media with glucose or saccharose (35).

Among the effects of more specific variables one might cite (i) the limitation of manganese, which causes inhibition of septation in *N. opaca* (545); (ii) the promotion of mycelium development and inhibition of motile-cell formation in *Dermatophilus* by sodium and potassium ions (436); and (iii) induction of R (motile rods) to C (irregular cocci) transition in *Geodermatophilus* sp. by mono- and divalent cations and amines (222). Gas and temperature variables were also shown to be important in determining predominantly mycelioid or diphtheroid growth, as shown by work with *Actinomyces* sp. (232) and *Arthrobacter* sp. (468), respectively.

The utilization of particular carbon and nitrogen sources by growing actinomycete cultures frequently appears to be correlated with predominating cell morphologies. Addition of several carbohydrates to the growth medium of *N. corallina* tended to reduce mycelial growth; and on a medium with fructose the organism grew predominately in the coccoid form (542). Some *Streptomyces* spp. formed septate nocardioforms on synthetic media with fructose; with certain cultures the effect was reversible (384, 386).

A. crystallopoietes grew in coccoid form on a mineral synthetic medium with glucose, and the transition to rodlike forms was controllable by addition of peptone or by one of the following individual compounds: succinate, butyrate, lysine, asparagine, arginine, phenylalanine. The rodlike forms persisted in cultures until these compounds were completely exhausted (137). Two important differences were found in the sphere and rod peptidoglycans (290, 291): (i) the polysaccharide backbone averaged 40 hexosamines in the spheres and 114 to 135 hexosamines in rods; (ii) the peptide cross bridges of the rod peptidoglycans were formed by single L-alanine residues; the spheres contained some alanine cross bridges, but more than half of the bridges were comprised of L-alanyl-glycyl-glycine. The significance of these differences to morphogenesis is not quite clear at present, but they are discussed (136) in connection with the possible changes in wall flexibility, accompanying morphogenetic transitions.

Cell septation early in the mycelial growth of *N. turbata* appeared to be stimulated by decreased temperature of incubation, decreased relative humidity (surface cultures), and by addition of Tween 80. On synthetic media with nitrates, the vegetative growth was rather scanty and was accompanied by frequent septation. With ammonium salts, growth was promoted, the frequency of septation being reduced. Some individual amino acids stimulated growth of rodlike cells, whereas casein hydrolysate caused the appearance of various irregular dividing cells. Among the carbon sources tested, starch, glycogen, and, to a certain degree, glucose inhibited fragmentation and promoted extension of the mycelium (142). Sphere-rod transition in *N. corallina* growing on synthetic medium was markedly promoted by the addition of acetate, propionate, 3-hydroxybutyrate, butyrate, succinate, Krebs cycle intermediates, tyrosine, and peptone (190).

The processes of crystallization are likely to be involved in formation of some surface structures of actinomycetes, and such processes also depend heavily on environmental variables. Thus spiny quasi-crystalline projections on sporangia of *Microechinospora grisea* appeared when the organism was cultivated at 37 C (but

not 20 and 26 C), probably under conditions of enhanced desiccation (273).

As one may see from Table 1, the only environmental variable that looks likely to affect the formation of the surface sheath is the relative humidity of the atmosphere, to which the nascent aerial hyphae are exposed.

Some unspecified variables believed to be related to the host defence mechanisms induce pathogenic anaerobic and possibly aerobic actinomycetes to form "clubs" and some other structures while in the host body (351, 433). It is unclear whether vesicles of *Frankia* spp. belong to this category. Formation of clublike bodies was sometimes mentioned to occur in vitro (34, 233) but on highly complex media.

Metabolic Activity and Morphogenesis

The possible relationship of the growth rate and morphogenesis was noted in *N. corallina* and *A. crystallopoietes* because many (but not all) nutritional compounds implicated in the sphere-rod morphogenesis in these organisms (see above) enhanced the growth rate (190, 338, 339). The connection between the growth rate and cell differentiation might be of a more general nature and was implicated in the sporulation of bacilli (94). According to the hypothesis, the primary effect of starvation is to reduce the growth rate, this reduction being involved in triggering of morphogenesis.

The cells of *N. corallina* during the transition state from spheres to rods lost the ability to oxidize glucose. Interestingly enough, the likely explanation was found in altered cell permeability toward this sugar (55), and the utilization of fructose and caproate was not altered. Ability to oxidize glucose was restored on fragmentation of rods into coccoid cells. Later on, using labeled glucose, it was shown that utilization of glucose by rodlike cells is in fact inhibited incompletely (190). Individual substances that induce and support the growth of *A. crystallopoietes* in the form of rods also repressed glucose assimilation and catabolism. Inhibition of glucose permease was thought to occur. Those substances (e.g., glutamate) that promoted increased growth rate without affecting morphogenesis did not affect glucose metabolism (283). The level of wall-bound *N*-acetylmuramidase activity correlated positively with the change in the length of the polysaccharide backbone in the murein of *A. crystallopoietes*: it remained low when the cells grew as rods and then increased sharply with depletion of peptone when the rods were fragmenting into spheres. The changes in enzyme activity and murein structure accompanying morphogenesis might be causally related. It is not clear, however, which factor(s) might control the enzyme activity itself (136).

As exemplified by the use of metabolic inhibitor, morphogenetic events in actinomycetes might be controlled at several levels. Thus, vacuolization in *A. viscosus* was shown (57) to be markedly sensitive to inhibitors of protein synthesis, while being rather unsusceptible to inhibitors of nucleic acid (except of rifampin), cell wall, and membrane biosynthesis.

Environmental Factors in Control of Sporulation

Nutritional limitations and sporulation. Desiccation and other triggers. There are many reports documenting enhancement of sporulation in *Streptomyces* on media containing mineral rather than organic sources of nitrogen, on "diluted" or "starvation" media (133, 168, 169, 277, 278, 282, 376, 454, 507). Variants of *S. griseus*, either ultraviolet induced or obtained in the course of spontaneous variability and devoid of aerial mycelium and spores (11), retained their phenotypes through many passages if they were transferred on complex media with peptone or glycerol, but rapidly reversed to parental phenotypes if transferred on media with mineral sources of nitrogen. It was also shown with submerged cultures of several *Streptomyces* spp. that exhaustion of nutrients (in particular, phosphorus) might be among conditions favoring sporulation (122, 572). The role of specific limitations, however, was very seldom studied systematically. One would note in this context some studies, showing retardation of sporulation in *Streptomyces* spp. by addition of a carbon source to a medium, in which this source was exhausted but the nitrogen source was still present (171). Future studies of this kind are likely to meet with some technical difficulties because the majority of *Streptomyces* spp. freshly isolated from soil might be considered oligotrophic in that they can be successfully transferred without losing their capacity to sporulate for a rather long time on solid media containing just agar and its impurities.

Unlike the majority of procaryotes, but much like fungi, actinomycetes may form aerial mycelium, which in many instances, at least in surface cultures, serves as a prerequisite for sporulation. Whereas nutritional limitation might be regarded as a kind of a general stimulus directly or indirectly inducing sporulation both in bacteria and fungi, there are some other environmental stimuli that induce aerial my-

celium formation and hence sporulation in mycelial fungi but are not on the list of common inducers of spore formation in bacilli. The case in point is desiccation, which, according to Sussman (487), is likely to act under natural conditions on a rather extended time scale. This characteristic delay might be incompatible with the growth rate of most bacteria but quite compatible with the growth rates of fungi and many actinomycetes. Several observations suggest, indeed, that aerial mycelium formation in actinomycetes is responsive to this stimulus (237). As with fungi (14, 153), sporulation in submerged cultures of *Streptomyces* was found (460) to be enhanced by an increased concentration of ions. It is possible that this variable exerts its effect by lowering water activity in the media and thus mimicking desiccation.

Lower agar concentrations in solidified media tend to favor vegetative growth, whereas higher agar concentrations tend to enhance the onset of sporulation in *Streptomyces* spp. (189).

There is some indirect and chance evidence that aerial mycelium formation in actinomycetes is enhanced in heteroplasic systems. Resumption of aerial mycelium formation and sporulation by actinomycetes subjected to soil culture might perhaps be relevant here (139, 140, 230, 293, 302; see next section for alternative explanation). A likely explanation for similar phenomena in fungi involves increased pO_2 in surface films matched to the obligatory aerobic metabolism associated with conidiogenesis (520). Anaerobiosis and a sufficiently increased CO_2 tension inhibit sporulation in *Streptomyces* spp. (189).

Nutritional requirements for sporulation. Belief in a requirement for additional nutritional factors for aerial mycelium formation and sporulation in some actinomycetes stems from not infrequent observations of stimulation of these events in impure cultures of actinomycetes mixed with bacteria (191) and the effect of unspecified metabolites of other actinomycetes (115). Systematic efforts to dissociate the effect of nutrients on growth and sporulation have not been consistent. As a result, according to Freeze (156), it is often difficult to say whether the condition studied is necessary, pleiotropic, or irrelevant for sporulation.

The "maintenance" media for streptomycetes are usually those that ensure abundant sporulation, and these media often include complex plant materials such as tomato paste, yeast extract, oatmeal extract, etc. (294, 421). Six species of *Streptomyces* (including strains like *S. griseus* and *S. lavendulae*), deliberately chosen as "bad spore producers," produced their best sporulation on solid medium containing peptone, urea, glycerol, and sodium chloride (189). Different batches of peptone varied significantly in their effect on sporulation; and meat extract was found to be useless. Addition of Mg, Fe, and Mn had a slight positive effect. In synthetic media, glycerol and urea appeared to be the best among C and N sources tested, respectively, and the addition of $CaCo_3$ promoted sporulation.

Specific examples of mineral requirements include the stimulation of sporulation in *Streptomyces* spp. by cobalt (201) and sporulation in thermophilic actinomycetes by mixtures of trace elements (501). Extracts of soils differing in their relative trace-element content stimulate sporulation of *Streptomyces* spp. at various rates (302). The requirement for cobalt was said to be specific, and this ion could not be replaced by iron. There is an obvious possibility that orthophosphate-metal complexes (mentioned above), accumulating in the volutin granules of actinomycetes, might serve as depositories, masking ionic requirements for sporulation (should specific ones really exist).

Sporulation of *Actinoplanes* spp. in laboratory culture was stimulated by several amino acids, as well as by preparations of humic and fulvic acids (564, 566). Aerial mycelium formation by *T. vulgaris* on a complete synthetic medium including 18 amino acids and several vitamins was blocked by excluding methionine from the component amino acids (544). In a variant of *S. coelicolor*, production of aerial mycelium and associated activities were not observed on synthetic Czapek agar but reappeared after the addition of biotin to the medium. Culture filtrates of several microorganisms had the same effect (284), probably because of the presence of biotin.

Sporulation in *Streptomyces* spp. seems to cease to depend, at a certain stage, on exogenous nutrients and proceeds endogenously (e.g., in water) both in submerged (382) and in surface cultures (47). In the former case, spore formation was supposedly observed in the first generation of vegetative cells formed following spore germination. In the latter case, sporulation in aerial hyphae of a definite age was not supressed by addition to the medium of glucose and growth-prohibiting concentrations of penicillin.

pH values might be critical for sporulation in actinomycetes (470), because slightly alkaline conditions often favor sporulation, as shown, for instance, in a study with *A. alba* (330).

An intriguing but insufficiently documented effect on aerial mycelium formation in *Streptomyces* spp. 63 to 67 might derive from the nutritional status of the culture used as inoculum

(231). An inoculum from soil agar ensures a more abundant formation of aerial mycelium on synthetic Czapek agar. Whatever the real reasons, it is interesting to recall the findings of Davies et al. (93) with *B. subtilis* which indicate that chromosome packing and position are dependent on the nutritional status of the cell. In its turn, the chromosome state is likely to affect cell behavior at some stages of the developmental cycle.

It would also be interesting to correlate the above observation with the common practice in the antibiotic industry of using empirically developed media to grow the material for subsequent use as inoculum for fermentation.

Suppression of sporulation. Aerial mycelium formation in the mesophil *S. albus* (151) and some thermophilic actinomycetes (81, 141) appears to have narrower temperature optima compared with vegetative growth. Replacement of air with pure oxygen in the gas phase inhibited aerial mycelium formation by *Micromonospora (Thermactinomyces) vulgaris* growing as a surface film on liquid medium, the effect being attributed to inactivation of some thiol enzymes (543). In *Streptomyces* spp., aerial mycelium formation was severely inhibited by adding to the medium 0.01% anionic detergent (143) or lecithin (139).

Illumination with visible light appeared to suppress aerial mycelium formation in several *Nocardia*, the effect being dependent on the incubation temperature (362).

It is a common laboratory observation, mentioned by several investigators, that the inclusion of meat extract in nutrient media (as contrasted with peptone in low concentration) retards aerial mycelium formation and sporulation in many different actinomycetes (59, 276, 569; to cite only a few). In fact, severe difficulties encountered in the systematics of actinomycetes encountered before the introduction of synthetic mineral media are at least partly explainable by almost exclusive use of media containing meat extracts, sera, blood, and similar materials commonly used in medical diagnostic media.

The general suppressive effect of these media on aerial mycelium formation and sporulation is still unexplained. One might speculate on the possible inhibitory effect of the polyamines and some special kinds of proteins present in the extracts. Polyamines are thought to assume some histone-like functions in bacterial cells (444). Their regulatory functions in vivo were shown infrequently (220), whereas their regulatory activity in vitro extends to the level of DNA-dependent RNA polymerase (22). Some of the basic proteins, present in the medium, might function as more than just a substrate for protease action. A case in point is histone, which was shown to penetrate the cells of *Escherichia coli* and *B. cereus* and affect protein synthesis (possibly at the level of transcription) and morphology of the respective bacterial cells (356, 398, 399, 481).

Prolonged cultivation of actinomycetes on laboratory media leads to a poorly understood condition termed "degeneration," which is most frequently associated with loss of the ability to form aerial mycelium, spores, antibiotics, and pigments (299, 429). The proportion of dormant spores in the population of degenerated *T. vulgaris* cultures has been shown to increase (6).

Sporulation under natural conditions. Direct optical microscope observations in situ by earlier workers (139, 292, 479) suggested that actinomycetes (probably *Streptomyces* spp.) form aerial mycelium and sporulate in soil. Krassilnikov (280) noted a reduced formation of the extensive mycelial network of actinomycetes in soil. More recent observations with the help of pedoscopes (13), direct electron microscopy of soil suspensions (388), and fluorescent microscopy (359, 575) suggest that the type of sporulation characteristic of actinomycetes in laboratory cultures is indeed observed in soil. The frequency of sporulation is probably lower than would be deduced from the widespread occurrence of these organisms. Some earlier data (268) might be interpreted to suggest that spores and probably other cells formed by actinomycetes in soil are more resistant to heating. This property is probably utilized in some of the techniques for preferential isolation of actinomycetes from soil, employing heating at elevated temperatures (90 to 100 C) for rather extended periods of time (up to 2 h) (2, 80, 397, 518).

Some patterns of sporulation in actinomycetes were observed occurring in situ in cotton fibers (453) and soil (4) which were not seen in laboratory cultures. Whether these can be attributed to some still uncultivable forms (like *Frankia* spp.) or represent alternative modes of reproduction in some already known forms is unclear.

Internal Factors in Control of Sporulation

The developmental program of all the living organisms is thought to be determined by the sequential expression of genetic activity, the latter being regulated by specific activation and repression of the corresponding genes. The repression hypothesis (348) as applied to microorganisms has been less thoroughly investigated experimentally, which does not make it any less attractive or disprove it.

From the biochemical viewpoint, the distinction of control mechanisms operating on transcriptional, translational, and post-translational levels is important. The scarce information on transcriptional and translational controls available with actinomycetes will be presented in the following section, as well as in the one dealing with secondary metabolism. Mounting evidence stemming from the work with sporulating bacteria (275, 441, 480) suggests that at least some spore proteins might be products of vegetative genes. Besides emphasizing the importance of post-transcriptional controls, these facts might also reflect the economy of information stored in chromosomal genes. The last principle seems to be especially relevant when differentiation and morphogenesis in procaryotes is considered. Possibilities for the involvement of epigenetically regulated processes in morphogenesis are further exemplified by the ability of several large molecules to associate into supramolecular complexes using the conformational specificity stored in these same molecules. Well-studied examples include the assembly of flagella, pili, poly-β-hydroxybutyrate granules (308), and possibly also some surface structures in gram-negative bacteria (508), spore coats, and more complex cell organoids (308). The importance of elucidating the possible matrix activity of the preformed structures also emerges from these types of studies.

Finally, we must identify and discover the nature of chemical signals generated within the developing cells which are probably involved in triggering some phases of developmental events and play a role in coordinating metabolic activities within the differentiating cell. These offer interesting and little-explored prospects for research with actinomycetes.

Variants and mutants with impaired differentiation. Reports on the occurrence of cacogenic (e.g., blocked in the differentiation program itself or mechanisms allowing it to be fulfilled [156]) variants in actinomycetes appeared very early. Papers on selection are flooded with references to variants, depicted in such terms as "morphological" with changed color of aerial mycelium and even as "bald." A strict distinction was not always made between stable and unstable, including conditional, mutants.

A systematic study of a group of stable developmental mutants of *Streptomyces coelicolor* was made by Hopwood and his colleagues (213). These seem to belong to the category of mutants in which the activity of genes absolutely necessary for the synthesis of corresponding enzymes and structures is blocked (e.g., with the exception of protogenic revertants, they fail to make the structures in question at all). These were subdivided as follows: (i) "bald" (no aerial mycelium formed); (ii) aerial mycelium appears but the spiralization of sporophores does not take place; (iii) spiralization is initiated but not completed; (iv) spiralization is completed, but sporal septa are not formed; (v) sporal septa are formed, but spore walls do not thicken and round off; (vi) spore rounding-off initiates but is not completed; (vii) final stages of sporogenesis are blocked, with accumulation of the grey spore-associated pigment released from sporophores.

The above categorization is also a good summary of the sequence of events that occur during sporogenesis in *S. coelicolor*. In other species of the genus, some modifications are likely to be encountered, taking into consideration morphological differences in the last steps of sporogenesis (Fig. 3). Less clear on the scale is the position of the so-called nocardioform mutants, whose origin is frequently mentioned in the literature. Usually, the complete loss of aerial mycelium production and sporulation is accompanied in these mutants by the tendency of the colony mycelium to develop septa and fragment, much as in *Nocardia* spp. (see, for instance, 70, 378, 386). These mutants have not been subjected to genetic analysis, which might present additional difficulties, because some of these mutants are reminiscent of the "abnormal" sporulation mutants of bacilli (449) i.e., they present morphological features not found in the process of normal sporulation. In this case, the cascade of events leading to sporulation could possibly be blocked here at some early step that is still associated more closely with cell septation. It is interesting to recall in this context the hypothesis of Schaeffer et al. (450), which states that the majority of Spo mutants in bacilli are in fact membrane mutants. Apparently, a back mutation in some "early" genes might release a normal sporulation process. Mutation from sporeless state to abundant sporulation was said to be possible with *Streptomyces mediterranei* (146).

An example of conditional mutants is seen in a *S. coelicolor* strain requiring biotin for sporulation (284). Mutants requiring C and A factors for sporulation (which will be dealt with in more detail below) in a certain sense also belong to this category, and they certainly fit into the second category of cacogenic mutants—those in which the genetic program for differentiation is not missing, although the possibility for its fulfillment is impaired.

Less interesting is the group of mutants that exhibit impairment of both differentiation mechanisms and those involved in vegetative growth. These are probably represented by the

"dwarf" colonies not infrequently noted during selection of mutants. The physiology of these mutant strains is almost unstudied.

Besides being a result of the application of physical and chemical mutagens, mutants and variants with impaired differentiation among the *Actinomycetales* frequently arise in the course of spontaneous variability (537). Some complex media are reported to provoke this variability (and are used in studies of its limits), whereas others reduce it (and are recommended for storing of the strains) (300, 301). *Streptomyces* variants with possible blocks in differentiation, most often encountered in selection work, were categorized (301) into the following groups; (i) nocardioform; (ii) asporogenous, or "white"; (iii) oligosporic; and (iv) "dwarf-colony" type. Others were affected in pigment production. Among the effective environmental influences that were reported to induce developmental variants, one should note increased CO_2 tension (378), undefined components in certain batches of meat extract medium (Oxoid) (26), and growth on fructose-containing media (385).

Streptomyces variants with impaired sporulation so far studied were not found to possess alterations in cell wall components which are used in taxonomy (304, 486).

Sporulation and chromosomal genes. Since the genetic aspects of differentiation in actinomycetes were extensively dealt with in several recent competent reviews (69, 215) we shall briefly mention some points that seem to us important for the general picture.

It was shown (63, 213) that such *S. coelicolor* mutant phenotypes as *bld* and *whi* are blocked in the activity of chromosomal genes. The position of some of these genes on the circular *S. coelicolor* chromosome has been mapped. The total number of genes participating in the developmental program in this organism was estimated to be about one dozen, which is significantly less than the number thought to participate in *Bacillus* sporulation according to some estimates (24), but not significantly less according to others (216). As shown, however, by studies on genetic aspects of sporulation in *Aspergillus nidulans*, the number of genes implicated in sporulation is very likely to increase as the subtleties of behavior of a particular system become better understood (see 73, 354). As in *Bacillus* spp., sporulation genes in *S. coelicolor* do not seem to come from a single region, but rather are found to be scattered throughout the circular chromosome with occasional clustering.

Although the application of genetic analysis to studies on differentiation in actinomycetes has already revealed several interesting facts and clarified the formulation of certain problems, a word of caution as to the probability of success in achieving ultimate goals using only this approach might be in order. As pointed out by Dworkin (130), the main hindrance to pursuing genetic analysis beyond the present level is, even with *Bacillus*, a poor understanding of what the products of sporulation genes might be. The situation with actinomycetes is certainly even more difficult.

Indeed, similar ignorance of *Streptomyces* further hampers the tentative assignment of specific functions to individual sporulation genes of *S. coelicolor* A3 (2) suggested by Chater (68). Based on experience with double sporulation mutants, the author discusses the possibility that *whiG* and *whiH* gene products might be involved in the cell wall changes required for the initiation and localization of sporulation septation. The coiling is thought to be a secondary consequence of these changes. The *whiA* and *whiB* gene products are supposed to be directly involved as structural or catalytic elements in the development of sporulation septa.

Sporulation and plasmids. Plasmids were implicated in the control of some phenotypic characters in *Streptomyces* spp. including tyrosinase inheritance in *S. scabies* (177, 178) and fertility types in *S. coelicolor* A3(2) (213). Since plasmid-associated genes are believed to be more sensitive to certain treatments than chromosomal genes, an attempt was made to eliminate plasmids from several *Streptomyces* spp. by treating the cultures with heat and acridine orange (394). The treated cultures lost the ability to form aerial mycelium and spores. The results were interpreted as indicative in terms of plasmid involvement in determination of sporulation. It would be pertinent to say that a similar hyphothesis regarding sporeforming bacteria was widely discussed about a decade ago and abandoned on several grounds. One of these was a demonstration that the results of acridine treatment (not to mention heat treatment) are probably not confined to elimination of plasmids (49). Interestingly enough, use of acridines in experiments with *Nocardia* sp. (56) did not lead to results expected from the earlier experience with *E. coli*. Results from experiments on mapping sporulation genes on the chromosome of *S. coelicolor*, mentioned above, also make positive control of sporulation in actinomycetes by plasmids rather unlikely. To our knowledge, the possibility of negative control has not been tested.

Proteolytic activity and sporulation and the question of RNA polymerase modification. Several observations suggest that total (108,

286, 295) or a limited (e.g., that of aerial mycelium; 10, 509) autolysis accompanies or coincides (at least in time) with sporulation in actinomycetes. Electron microscope pictures suggest that controlled autolysis plays a part in later stages of spore formation in some *Streptomyces* spp. (Fig. 3) and especially in *M. rectivirgula* (117). For modification of the developing spore wall (rounding: stage III of Wildermuth and Hopwood [554]), a number of autolytic enzymes are probably responsible, including proteases, peptidases, amidases, and muramidases.

Although actinomycetes are used for industrial production of some proteolytic complexes (e.g., Pronase and others), specific conditions leading to "oversynthesis" or derepression of extracellular and, especially, intracellular proteases have been studied insufficiently. Some observations (65, 324) suggest that increased production of extracellular proteases is stimulated by nutritional limitations. It is difficult to say at the moment whether there is a specific kind of limitation that favors protease production or derepression. Recent results with *A. nidulans* suggest that limitation in either carbon, nitrogen, or sulfur source might stimulate synthesis and the secretion of extracellular neutral and alkaline proteases (74). In most instances so far reported (with the probable exception of *Clostridium* sp. [343]), one observes a general correlation between such events as onset of nutritional limitation, lowering of the growth rate, and sporulation. The causal type of relationship between the above events, although shown in *Bacillus*, awaits experimental determination in actinomycetes.

Actinomycetes also produce several substances called protease inhibitors (373, 524). These compounds are mainly represented by rather low-molecular-weight peptides (158, 524, 525), and their spectrum of inhibitory activity is rather wide (514). Although aimed at regulation of protease activities in other organisms, these inhibitors have not been looked upon as potential regulators of protease activities in the actinomycete cultures producing them.

Many *Streptomyces* spp. produce serine proteases, which differ in some properties from bacterial and fungal proteases and are related to some animal proteases (40% homology with bovine trypsin was reported by Hartley [188]). It was the serine protease of *B. subtilis* which was thought (323) to be responsible for modification of DNA-dependent polymerase; this event constitutes an important transcriptional control mechanism of sporogenesis in bacilli (332).

Although the exact cause (374, 396) and nature (50, 510) of RNA polymerase modification in vivo seem not to be understood in full detail, the potential significance of the event prompted some initial studies with actinomycetes. The approach taken by Chater (67) made use of a correlation that exists (546) between rifampin resistance and changes in the RNA polymerase of bacteria. It was also reported that rifampin-resistant mutants of *B. subtilis* show an abnormal sporulation process (113). However, work with *S. coelicolor* A3(2) seems to suggest that the RNA polymerase of the actinomycete is less sensitive to rifampin than is the corresponding enzyme of other bacteria tested. Not a single *rifA* mutant among the approximately 100 tested was without morphological abnormalities that might have been associated with the mutation. A substantial number of "bald" colonies were obtained; in all cases, however, this latter marker appeared to segregate in crosses from rifampin resistance. It was concluded that, if some changes in the RNA polymerase of the actinomycete that affect its matrix specificity do occur during sporulation, they are confined to that portion of the enzyme molecule that is not essential for the antibiotic to exert its effect.

Involvement of specific factors in regulation of sporulation. Some observations suggest that developing cultures of actinomycetes may produce and sometimes excrete substances that probably participate in regulation of differentiation. Examples from earlier works include diffusible substances that cause aerial mycelium production in old surface colonies of *Streptomyces* spp. (115) and some substances in spent broth filtrates reported to enhance septation in *N. corallina* (54). Occurrence of the latter was recently questioned (190). Three types of fertility variants revealed in *S. coelicolor* were found to differ in their possession of the plasmid called SCP1, which is found only in IF ("initial fertility") and NF ("normal fertility") but not in UF ("ultrafertile") strains (69, 112). When cultivated on agar medium, IF and NF strains inhibited production of the aerial mycelium by UF strains. The inhibition was ascribed to a chemical factor diffusing through agar; in certain concentrations, it inhibited only aerial mycelium production.

Experiments on the so-called cosynthesis of some antibiotics firmly established the ability of several nonproductive strains of actinomycetes to complement each other, probably in the early stages of antibiotic precursor synthesis, thus allowing the complex biosynthetic pathway to be successfully finished (363). Particular mutants might bear deficiencies not only in their ability to synthesize secondary metabo-

lites, as exemplified by several antibiotics, but also in their ability to synthesize secondary structures, exemplified by spores. These lesions are also repairable in some instances, and studies on metabolic complementation have helped to reveal some interesting examples.

Factor A. The existence of this factor was revealed in the course of studies on streptomycin biosynthesis by parent and mutant cultures of *S. streptomycini* (255). Among the mutants, a number of cultures were detected that lost not only the ability to synthesize streptomycin, but also lost the ability to form aerial mycelium and spores ("bald" colonies in surface cultures). Both abilities were simultaneously restored on addition of culture filtrates from normally sporulating and antibiotic-producing cultures. The effect was traced to a rather low-molecular mass (242 daltons) compound, called "factor A" and having the tentative formula of $C_{13}H_{22}O_4$ (406). The molecule contains hydroxy-, carbonyl-, and isopropyl groups. Its diacetate and 3,5-dinitrobenzoate derivatives retain the biological activity.

The substance was produced by parent cultures and was effective with sporulation mutants, in very low concentrations. The effect of factor A on sporulation and streptomycin biosynthesis was observable both in surface and submerged cultures. A possible clue to the reactions that might be affected in the presence of factor A is given by studies showing the interference of factor A with glucose 6-phosphate dehydrogenase activity (535). It is interesting to correlate this finding with the changing pattern of glucose catabolism in developing *Nocardia* and *Arthrobacter* cultures mentioned earlier in this review.

In another study on metabolic complementation in streptomycin biosynthesis and sporogenesis of *S. streptomycini*, it was shown that some of the mutants blocked in sporulation might produce a diffusible substance(s) that restores the normal differentiation and antibiotic production by the others. The chemical nature of the active principle was not elucidated (109).

Factor C. Comparative studies on two strains of *S. griseus*, of which one was a streptomycin producer (N52-I) whereas the other (N45-II) was not, revealed only some minor differences in the characteristics of their surface cultures (490, 534). Among the biochemical features studied later, one would mention differences in hexose and pentose content in walls of hyphae, the strain 52-I being also less resistant to heating (30). Variants were identical in the relative guanine plus cytosine content of their DNA. The ability to synthesize streptomycin could be passed from 52-I to 45-II by bringing the former into contact with DNA from the latter during several passages (526).

Significant differences were found between the strains in submerged cultures on complex media. Whereas the 52-I strain was characterized by an "attenuated" life cycle (ending in autolysis without sporulation), the development of 45-II under similar conditions was accompanied by apparently normal sporulation. In the cultural broth of 45-II, an active complex was found to accumulate, called "factor C" ("cytodifferentiation factor"; 491, 492, 493). On addition to the developing culture of the strain 52-I (defective in sporulation) of the partially concentrated factor C, a marked stimulation of septum formation, dividing hyphae in sporelike segments, occurred (533).

The factor C has not yet been purified sufficently to regard it as an individual substance. Rather, it is a complex, which is thermolabile (inactivated by heating for 5 min at 45 C), and non-dialyzable. It is inactivated by trypsin treatment, but not by deoxyribonuclease, ribonuclease, papain, pepsin, lysozyme, and diastase treatments. Some information points to the possibly important connection between factor C activity and RNA synthesis in the developing hyphae. It was thus shown (496) that C factor interfered with the endogenous biosynthetic activity in vitro of DNA-protein complexes from *S. griseus*, as well as their ability to bind purified RNA polymerase from *E. coli*. The activity was not observed, however, with DNA-protein complexes from *E. coli* K-12. Factor C interfered also with the activity of *E. coli* RNA polymerase if calf thymus DNA was used as a matrix. Prior and separate incubation of both DNA and *E. coli* RNA polymerase with factor C resulted in similar interference with the enzyme activity. Heating of factor C in solution destroyed every bit of its activity.

The studies of various chemical factors supposedly playing important roles in the coordination of differentiation in *Streptomyces* spp. are thus at different levels of advancement as far as their chemical nature and mechanisms of action are concerned. Comparison of data on factors A and C seems to emphasize, as an additional prospect for study, an attempt to correlate more precisely the events occurring in the differentiation of surface and submerged cultures of actinomycetes.

Spore Germination and Types of Dormancy Encountered

Two extreme types of spores in actinomycetes, exemplified by the endospores of thermophilic actinomycetes and the arthrospores of

Streptomyces spp., respectively, are characterized by distinctly different levels of dormancy, According to terminology accepted by Sussman and Halvorson (488), these may be called "constitutive" and "exogenous" dormancy.

Constitutive dormancy of thermoresistant endospores and an overall picture of germination. Like the majority of bacterial endospores, fresh, intact endospores of *Thermoactinomyces* spp. and *A. dichotomica* would fail to germinate if placed under environmental conditions that are otherwise favorable for development. The main steps involved in the overall germination process (e.g., activation, initiation, and outgrowth) are similar to those of spores in *Bacillus* and *Clostridium* spp. (19, 235). Species differences in the fate of cortical material revealed by structural studies (116, 310) correspond to those encountered earlier with different species of *Bacillus* (187, 311).

As with most bacterial endospores (325), the dormant condition of actinomycete spores might be enhanced by suitable choice of temperature and nutritional conditions for spore formation and the relative humidity of the atmosphere in which spores are stored. Thus, populations of *T. vulgaris* spores formed at 55 C were found to be more heat resistant and contain a higher proportion of dormant cells than populations formed at 37 C (266). Populations of spores formed on synthetic medium were richest in dormant spores, the degree of dormancy being markedly influenced by amino acids and Ca^{2+} and Mg^{2+} levels. An inverse correlation was found in dormancy and thermoresistance of spores formed on synthetic medium. In contrast to the situation with many bacterial spores, thermoresistance of *T. vulgaris* spores was found to be most critically influenced by Mg^{2+} but not Ca^{2+} ion levels (264, 265).

Some special features characterized the activation and initiation stages in germinating endospores of *T. vulgaris*. These spores could be conditioned for germination not only by heat shock but also by exposure to decreased temperature (6); they are markedly sensitive to physical activating agents (259), whereas the list of possible initiators is remarkably wide, including sugars, amino acids, ribosides, and mono- and divalent ions (260). It is the initiation stage of these spores that shows the most exacting requirement for high temperature and is probably critical for the cultures developing at temperatures close to the minimal cardinal point (261; see Fig. 4). **Outgrowing spores of** *T. vulgaris*, in contrast to spores of most *Bacillus* spp., appeared to be insensitive to rather high novobiocin concentrations (20).

Possibility of an alternative way for release from dormancy. Temperatures that were found to be optimal for releasing spores of *T. vulgaris* and *A. dichotomica* from dormancy appeared to be lower than those limiting vegetative growth and seemed to be close to 20 C in either species (262). If the temperature at which spores were produced was lowered by 20 C, this change in its turn lowered by about 20 C the maximal temperature to which these spores were resistant, as well as the temperature optimally suited for heat shocking them in the routine procedure for activation. The temperature range optimal for low-temperature activation, however, was not affected by changes in temperatures at which spores were produced, nor by the composition of the medium on which spores were produced (265).

The critical environmental variables that allow low-temperature activation to proceed include certain levels of humidity (≥10%) and presence of molecular oxygen in the gas phase—this requirement is not usual for activation or initiation of most bacterial endospores so far studied (263). Cooling appeared to be indispensable in conditioning the *T. vulgaris* endospores for germination; it was irreplaceable by such routine activators as heat shock and mild mechanical abrasion.

Only those spores subjected to limited cooling for rather short periods (<60 min) were found responsive to the heat shock. If the cooling was extended, the spores underwent activation without heat shock. In this case, heat shock was found to kill the spores, which lost their thermoresistance (259; see also Fig. 5). These findings probably explain the controversial reports on the possibilities of heat activation of *Thermoactinomyces* endospores (19, 20, 89, 152, 309) to a certain extent.

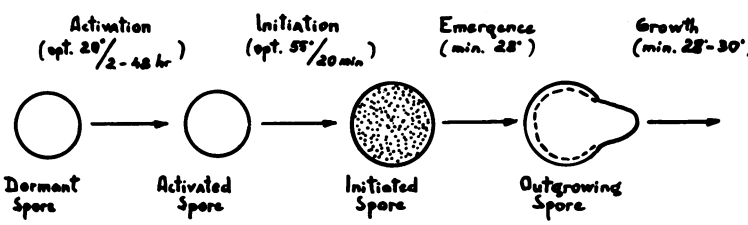

FIG. 4. *Minimal temperatures allowing germinal events in T. vulgaris spores.*

Although cooling appeared to activate intact actinomycete endospores in situ, even in chambers with controlled humidity, the suspension of spores in water significantly facilitated the process, as did all treatments that enhanced wetting of spores. In suspensions, activation by limited cooling was markedly enhanced in the presence of Mg^{2+}, followed by Ca^{2+} ions, whereas K^+ and Na^+ showed no activity. The effect of ions during cooling could not be ascribed to their activity as potential initiators, since the initiation phase required elevated temperatures to proceed.

Low-temperature activation seems not to fit the current concept (254) of bacterial spore activation. Besides the rather specific conditions necessary for it to proceed, the results of the treatment seem to be appreciably "broader," since the spores so activated lose thermoresistance and resistance to many toxic chemicals. The effect of limited cooling was found to be irreversible (263). It thus seems possible that the cooling triggers and then helps to maintain a series of events that bring the dormant spore into conditions that would more correctly be described as the early stages of initiation. It is therefore suggested (262; see also Fig. 5) that low-temperature activation might function as a

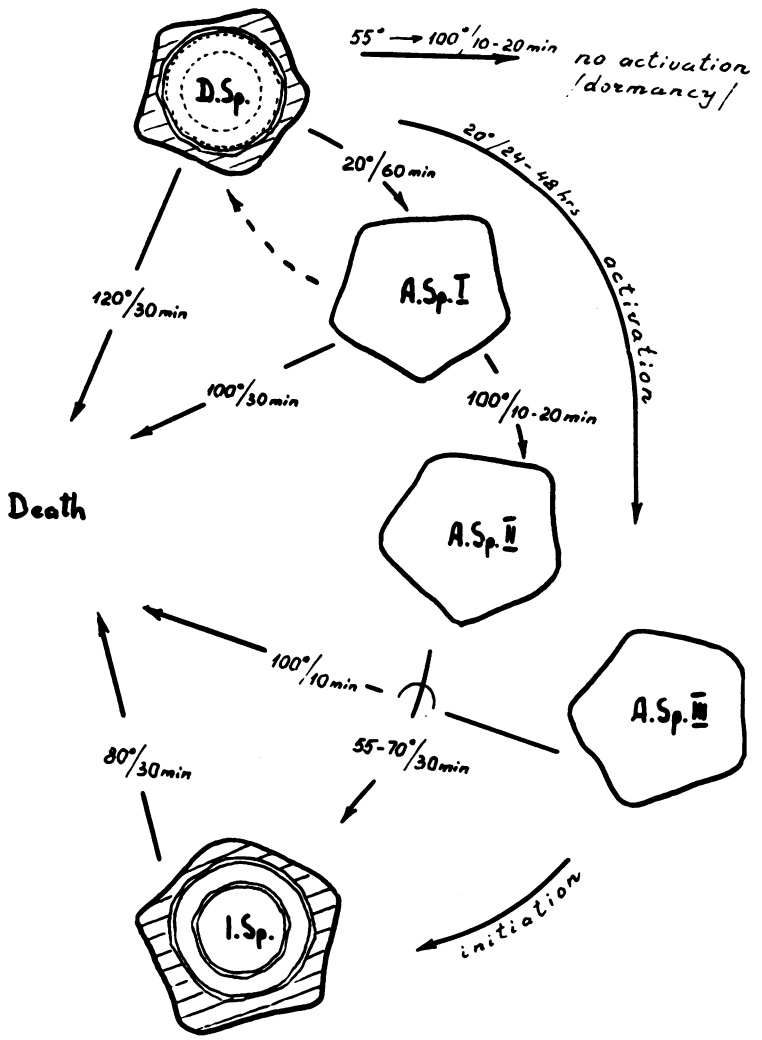

FIG. 5. *Temperature regimes and early germinal events in T. vulgaris spores (D. Sp., dormant spores). Activated spores: (1) AS_p-I, spores subjected to brief (up to 60 min) cooling; (2) AS_p-II—as AS_p-I, but additionally activated by heat shock; (3) AS_p-III, spores activated by prolonged cooling. These do not need heat shock for full activation. This stage is irreversible. IS_p, Initiated spores. Schemes of internal organization of AS are not presented since no reliable data are yet available. Conditions (temperature/time) of treatments are shown beside arrows pointing to final results of treatment.*

partial alternative to the usual endospore activation pathway. The irreversibility of the process resembles the results of ageing of bacterial spores; its results and temperature characteristics also resemble the so-called "after-ripening" encountered with some fungal spores (488).

The question of why spores of thermophilic actinomycetes appeared to be so sensitive to limited cooling, and whether they are unique in this respect, offers interesting prospects for future research. Preliminary speculations might take note of the possible structural rearrangements of water molecule aggregates at temperatures of 15 to 20 C (92, 125), to which the spore membrane next to a water-rich and "expanded" cortex (174) in a thermophilic organism would be especially sensitive. The possibility of an enzymatic reaction(s) being derepressed and proceeding below the growth temperature minimum seems also feasible and is supported by the oxygen requirement for completion of low-temperature activation.

Finally, one probably should not be content with a silent acceptance of the notion that the development of a thermophilic sporeforming organism such as *T. vulgaris* would depend, under natural conditions, on the presence of temperatures close to optimal. The drawing in Fig. 4 presents a scheme that emphasizes the minimal temperatures allowing development to proceed. There is a high probability that neither in soil nor in the infected tissues of animals do the spores of *T. vulgaris* remain dormant, even under conditions that do not allow vegetative growth.

Germination of exospores. Unfortunately, the several studies made on germination of *Streptomyces* spores employed different species of the genus. A look at the diagram (Fig. 3) illustrating structural aspects of sporulation in several *Streptomyces* spp. would strengthen the expectation of differences in germination. Species differences might be pronounced regarding the duration of certain germinal stages, nutritional requirements, the fate of outer-wall component(s) during the emergence stage, etc. Nevertheless, an attempt to correlate structural and physiological findings of various authors seems to be justified and is presented in Fig. 6.

As one can see, it was decided that the terminology for the earlier stages of *Streptomyces* spore germination be retained for the present, despite variance with that commonly adopted (173) in studies of *Bacillus* spores. Besides the reasons for doing so already discussed by Atwell and Cross (19), we think that even temporary retention of different terminologies might serve the purposes of analysis better than premature adoption of a common set.

It should be noted that assisted wetting of *Streptomyces* spores (an operation unnecessary and hence unstudied with the majority of bacterial spores) leads to some activation, causing additional difficulties in attempts to operationally define "intact" spores. Dried spores from freshly grown cultures are sometimes presumed to be intact. But drying inself might inflict some dormancy, as shown recently with vegetative cells of certain gram-positive bacteria (327), rendering them dependent for subsequent "activation" on mild heat shock. It is also clear that extensive washing of spores might potentially alter their nutritional and ionic requirements for germination. At least some of the changes inflicted on the surface sheath of spores by washing seem to be reversible (Table 1, Fig. 6).

Since germ tube formation in several *Streptomyces* spores is significantly affected by the nutritional environment, whereas the wetted spores are endowed with several enzymatic activities, it was thought that their limited metabolic dormancy is not constitutive but largely exogenous. However, a recent and important study employing *S. viridochromogenes* arthrospores (208) suggests that the situation, at least with some species, might be different, and the process of swelling (Fig. 6) actually may represent a sum total of at least two processes.

The authors devised a simple technique, which enabled them to obtain *S. viridochromogenes* spores in a less altered state prior to the start of germination experiments (although brief sonication of suspensions was still employed). Using these spores, convincing proof that both activation and initiation are involved in the overall germination process was presented. Activation was accomplished by a mild heat shock (55 C for 10 min) and was reversible. Initiation was accomplished in solutions containing L-alanine, adenosine, glutamic acid, *p*-aminobenzoic acid, Mg^{2+} and Ca^{2+} ions in the presence of CO_2. The process resulted in phase-darkening and excretion of some substances from ^{14}C-labeled spores. Although the above events are quite reminiscent of those accompanying germination of bacterial endospores, some marked differences in the behavior of the *Bacillus* and *Streptomyces* spore systems were also revealed. Activation was found to be accompanied by pronounced rise in adenosine 5′-triphosphate level and respiration. Characteristics of the initiation process included a further increase in the rate of endogenous metabolism, rise in adenosine 5′-triphosphate content, rapid

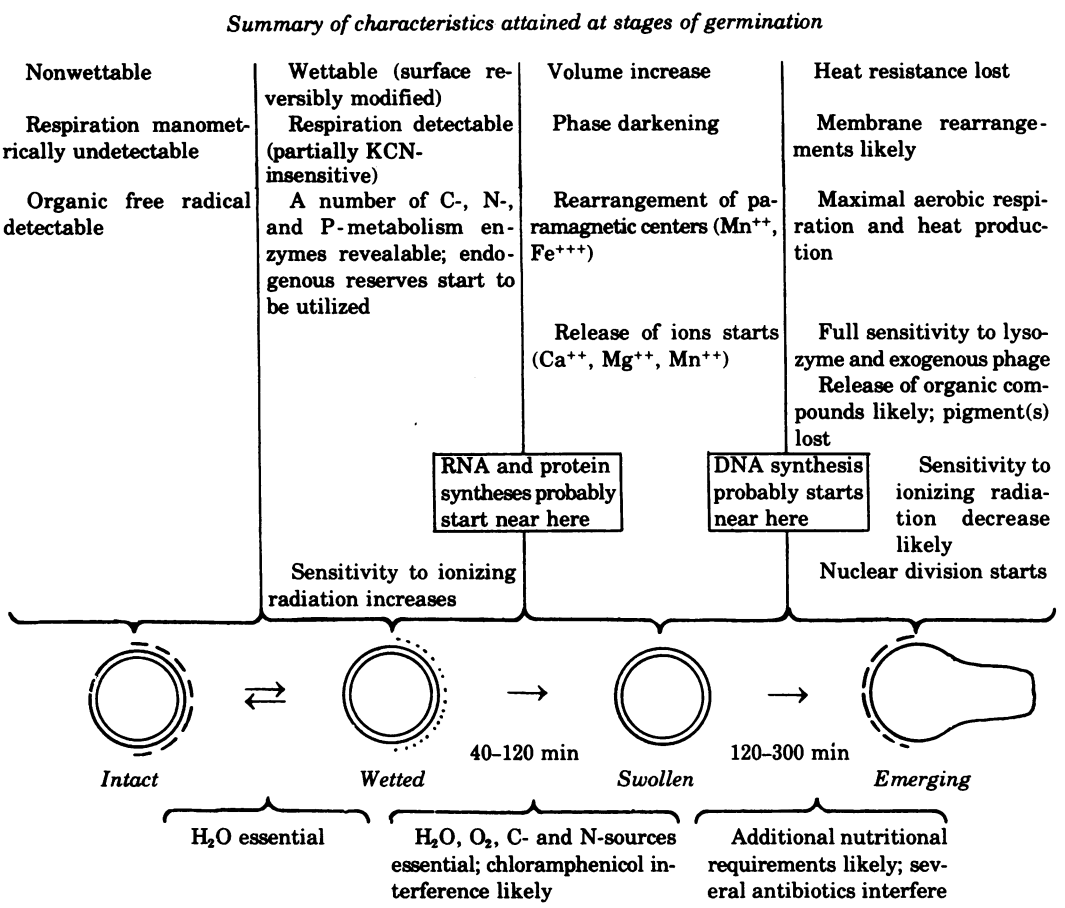

FIG. 6. *Diagrammatic sketch of events accompanying germination of Streptomyces arthrospores (schematized according to data presented in the reviews 19, 83, 248). The drawings emphasize the likely behavior of two spore wall components and the surface sheath.*

incorporation of adenosine or uridine into RNA, and protein synthesis, which started toward the end of the process. The initiation thus does not seem to be confined to a series of primarily degradative processes; if these processes do occur, they seem to be coincident with some early starting events involving energy-yielding and RNA-synthesizing activities. Also, it is not quite clear whether or not the heat activation is an obligatory prerequisite for initiation. Commenting upon early inhibition by rifampin of protein synthesis in *S. viridochromogenes* spores undergoing initiation (followed by chloramphenicol inhibition at some later stages of the process), Hirsch and Ensign (208) point to the possible absence of stable messenger RNA molecules in the spores, an inference which their results make likely.

It is usually thought that DNA synthesis in germinating *Streptomyces* spores is preceded by a considerable lag (Fig. 6). However, conflicting evidence was reported from studies with *S. olivaceus* employing Auramine 00 for measuring DNA levels in germinating spores. This was said (371) to steadily increase immediately after placement of spores in a complex medium favoring germination. It might be interesting to learn whether this finding reflects actual changes in DNA content of spores or alterations in spore permeability and the ability of nucleoids to bind the dye.

Discrimination between some later events, probably associated with outgrowth, becomes feasible with the observation (391) on the influence of mild heat shock on prophage behavior in germinating spores of *S. coelicolor* A3(2). Limited heating of freshly prepared spores neither affected their viability nor induced phage multiplication. Germinated spores (3 to 4 h on a complex medium) were killed as a result of

similar heating without liberating phage progeny. In spores bearing germ tubes (6 to 7 h), the shock induced phage lysis accompanied by liberation of phage progeny.

The nature of endogenous carbon and energy reserves probably utilized by germinating *Streptomyces* spores is likely to become clearer after exploiting the finding that trehalose catabolism is markedly increased in germinating *S. hygroscopicus* spores (200). Interestingly enough, it is this same sugar that constitutes endogenous reserves in many fungal conidia.

DIFFERENTIATION AND SECONDARY METABOLISM

Points of Similarity in Origin and Flow

The features common to the majority of secondary metabolism products are currently categorized (58, 98, 547) as those that: (i) are produced during idiophase; (ii) do not possess a common function in the life of the producing organisms (although they might be endowed with specific functions in the life of particular organisms); (iii) are produced by taxonomically restricted groups of organisms; (iv) are usually produced as mixtures of related compounds that can be grouped in families or series.

These definitions resemble very closely the overall view of the situation encountered with "secondary structures" or the products of cell differentiation in actinomycetes. Some pertinent points should be mentioned.

(i) The onset of differentiation (e.g., aerial mycelium production, sporulation) in actinomycetes is very often associated with the establishment of limiting conditions for vegetative growth (some are cited above in this paper).

Supposed switches for secondary metabolism (99) are indeed very similar to the supposed switches for sporogenesis (184, 449). The biosynthesis of siomycin was shown to be repressed by addition of glucose to the nutrient medium, even though the sugar was quite suitable for supporting vegetative growth (256). Some of the enzyme systems participating in secondary metabolism, like phenoxazinone synthetase, were shown to be catabolite repressed (353).

The term "idiophase" stemmed mainly from studies with submerged cultures. Not all physiological features of these cultures seem to coincide with those of surface cultures, at least with mycelial organisms. Several examples were given elsewhere in this paper to demonstrate how environmental requirements for aerial mycelium formation and sporulation in actinomycetes are more specific and exact than those for vegetative growth (aeration, pH, ionic environment, etc.). Manipulations of the amino acid or ionic composition of the sporulation medium will bring no less appreciable changes in, say, heat resistance or pigmentation of their spores than similar manipulations would cause in the composition of actinomycins (252) or bleomycins (523).

Obligate connections between the onset of secondary metabolism and the idiophase have been questioned in the case of chloramphenicol and tetracyclines. It was shown (346) that, in a batch culture, production of chloramphenicol on a complex medium is observed during idiophase. However, on a mineral synthetic medium the synthesis of antibiotic was also observable during trophophase. It was therefore concluded that a regulatory stress triggering antibiotic formation ensues on a complex medium with the onset of idiophase, whereas a similar stress is experienced by the organism already during trophophase on a minimal medium. No attempt was made in this work to see whether there were some signs of difference in cell differentiation of the actinomycete on complex and minimal medium, which seems to be likely. It would be pertinent to note that it seems logical to regard the onset of secondary metabolism as a probability process, in a manner suggested for sporogenesis (451). Some sporulation in batch cultures of bacilli was not infrequently observed during logarithmic phase (156) and is probably associated with changes in the growth rate of individual cells inflicted by nutritional limitations (94).

With tetracyclines (529), maximal accumulation of antibiotic was found to occur during "production phase." At that time, as shown by these authors, the nutritional environment was changed drastically, because the phosphorus source was completely exhausted. Cytologically, the productive-stage cells differed markedly from those of trophophase stage cells. It was argued, however, that the final yield of the antibiotic heavily depended on some critical regulatory events, which took place significantly earlier than the production itself. It was the relative concentration of the likely precursors within the cell that was held responsible for inducing the cell to produce tetracycline. It was also suggested that the term "secondary metabolites" be abandoned and substituted by the term "excessive metabolites." In our opinion, the strong argument for use of the later form is in the intimate connections that it implies between primary and secondary metabolism. However, its use broadens the category of

substances to be considered almost indefinitely. Under some conditions, regulatory mutants of *E. coli* can probably be induced to overproduce certain amino acids and ribosides. However, the question (97) that asks why the majority of antibiotics are produced not by *E. coli* but, rather, by highly differentiated *Streptomyces* spp. may be more specific and relevant to the situation.

Concerning the importance of very early events for tetracycline production, one would say that an example of the effect of the nutritional state of the inoculum spores on the differentiation of resultant *Streptomyces* colonies (mentioned earlier) would suggest the importance of even earlier events for differentiation in actinomycetes.

(ii) Some common function(s) for spores in bacteria and fungi is occasionally discussed, but certainly not agreed upon, and the situation with actinomycetes is certainly no better. The suggested hypotheses range from the role of spores in dissemination and gene transfer to survival during periods of drought and nutrient limitation or brief exposures to elevated temperatures. All of them might well have a rational basis, but all of them fail to take into consideration the almost unmatched multiplicity of secondary structures (e.g., spores) produced by actinomycetes, as a result of natural selection.

Although industrial strains of actinomycetes most certainly belong to categories of regulatory mutants, some minor quantities of antibiotics are usually produced by cultures freshly isolated from nature. On the other hand, it is a practice to subject actinomycete cultures to soil transfer when they lose ability to differentiate as a result of laboratory manipulations. A number of genes, the activity of which is inferred to be required for tetracycline (528) and streptomycin (101) biosynthesis, lie close to those shown to be required for sporogenesis in *Streptomyces*. Both sets of data seem to emphasize that neither sporulation nor antibiotic biosynthesis is insignificant in terms of the genetic burden for the producing organism. The frequent occurrence in actinomycetes of enzymes that might transform antibiotics, including those produced by the same cultures (101, 402), serves to strengthen this line of reasoning.

Possible specific functions of antibiotics produced by actinomycetes will be dealt with later.

(iii) The recognition of production by taxonomically restricted groups of organisms requires the identification and systematization, which is principally based on characteristic features of their secondary structures. By the latter are meant the specific structures of sporophores, spore-bearing vesicles, spores, and their ornamentation, etc; the last characteristic serves as a kind of "fingerprint" for identification. At least some of these characteristics, like spore wall ornamentation, were shown (172) to be stable and thus reliable for the purposes of systematics.

The number of antibiotics known to be formed by *Streptomyces* spp. exceeds appreciably that known to be formed by representatives of any other genus. This might reflect the effort devoted to screening programs, and the number of species within the genus, as well as their comparable nutritional versatility, which simplifies laboratory cultivation. However, the biosynthetic abilities of species of this genus are matched by the almost unprecedented structural variables involved in differentiation.

The species specificity of antibiotic production in streptomycetes was long debated. Some workers believed, however, that elaboration of particular antibiotics might be helpful for identification at either the species (281) or subspecies (420) levels. This line of thought is apparently contradicted by frequent reports of the production of similar antibiotics by representatives of different genera of actinomycetes. The contradiction seems not to be very deep if one recalls what was said on the possibilities of alternative ways of reproduction in actinomycetes. Besides, actinomycetes usually produce a mixture of antibiotics, and it is the ability to produce a certain mixture which might be specific. There are numerous reports of strains losing the ability to produce a particular antibiotic and almost no reliable information on the production of one type of antibiotic instead of another (say, tetracycline instead of a polyene).

(iv) The "serial" character of secondary structures in actinomycetes is documented with no less certainty than the production of families of antibiotics by them. One would likely point to certain types of spores produced, "sections" recognized within the genus *Streptomyces* according to sporophore morphology (199), and "series," recognized (28, 161) according to the color of the sporulating aerial mycelium.

Although information on sporal pigments is meager, one would be inclined to consider them as allied to secondary metabolites, since their formation was usually not observed during the vegetative growth of corresponding cultures under submerged or surface conditions. Interestingly enough, a kind of correlation seems to exist between the coloration of sporulating aerial mycelia of *Streptomyces* spp. and the resultant morphologies of sporophores and spores

(Table 2). Less correlation is observed between the above characteristics and the colors of colony pigments and those diffusing into the media.

Some of the morphologically peculiar actinomycetes are noted for their ability to produce special types of antibiotics (e.g., *Streptoverticillium* spp. very often produce polyenes (271, 519]). As with bacilli, the possibilities of links between differentiation and antibiotic biosynthesis in actinomycetes are further emphasized by drastic alterations in the ability to produce antibiotics by variants and mutants with impaired differentiation.

Some earlier studies in the area were reviewed by Schaeffer (449). However, possible correlations were not sought consistently, and some of the best papers on selection often contained some rather indecisive formulas of the sort: "... Our superior antibiotics producers were morphologically identical to the parent cultures, except that some of them produced spores of lighter coloration" (129). Since later correlations were sometimes encountered in the antibiotic potency and spore surface ornamentation (114), many of the doubtful cases earlier reported merit reexamination.

Positive correlations between the ability to form aerial mycelium and spores, on the one hand, and some of the secondary metabolite(s), on the other, include the following examples (to mention only a few): (i) pigment and characteristic earthy smell (geosmine?) in a *Streptomyces* sp.; (ii) pigment, smell, and antibiotic in *Streptomyces* sp. (378); (iii) antibiotics in *S. venezuelae* and *S. kasugaensis* (394); (iv) pigment in *S. longispororuber* (148); and (v) pigment and antibiotic in *S. coelicolor* (284). Particularly relevant to those examples is the resumption of streptomycin formation in aerial myceliumless mutants following factor A addition in parallel with the resumption of apparently normal differentiation (225). Also, according to observations of one of us (N.S.A.), antibiotic and extracellular proteases appeared to be synthesized by aerial mycelium-producing variants of *T. vulgaris* var. *calvum*. Workers engaged in the selection and storing of *Streptomyces* strains tend to regard the aerial myceliumless cultures as poor or zero producers of antibiotics (299, 300, 429). However, as shown, in the studies with factor C and its effect on sporulation in submerged cultures and on streptomycin biosynthesis (see above), there are some instances of negative correlations. Some examples of such type correlations with surface cultures were given by Demain (100) and several by Kalakoutskii and Nikitina (246).

Besides reflecting definite chemical and possible physiological individuality of antibiotics for the producing organisms, these apparently controversial examples of correlations suggest that different antibiotics might be linked in their biosynthetic routes to quite different ("early" versus "late") developmental stages. Interruption of development at some medium stage would severely affect the biosynthesis of late-stage-associated secondary products but would not affect, or even stimulate, accumulation of products associated with the early stage. "Abnormal" mutants, as shown with bacilli, are specifically interesting for their inabilities to sporulate normally while overproducing some of the structural entities presumably needed on later stages of sporulation.

However, the present situation is still most closely described by a formula, suggested by Weinberg (547): "changes in abilities to differentiate are usually accompanied by significant changes in levels of secondary metabolites produced."

There are some reasons to think that metabolic machinery of actinomycetes involved in biosynthesis of antibiotics might deviate from that involved in primary metabolism. Peptide moieties of actinomycins (252) and echinomycins (523) are thought to be synthesized without the participation of ribosomes, as earlier shown to be the case with peptide antibiotics of bacilli (340). There are, however, some exceptions (293). In six species of *Streptomyces* studied (229), the first enzyme in the biosynthetic chain leading to aromatic products appeared to be resistant to feedback inhibition by tryptophane, tyrosine, and phenylalanine. Some peculiarities of biosynthetic control mechanisms are probably responsible for the appearance, in antibiotic molecules of actinomycetes, of such rare or even unique compounds and moieties as D-amino acids in peptides, the azide group of azaserine, the vinyl group of sarcomycin and primocarcine, the mitosane of mitomycins and porphyromycin, etc. (275).

There is also an impression that genetic regulation, especially of some final steps in biosynthesis of secondary products in actinomycetes, is not subject to very tight control. This speculation is supported both by the fact that mixture of very similar molecules are often produced and by the frequent occurrence of products that reach their final form after some modifications, depending on environmental conditions. Possible examples of the latter situation would include "protoactinorodine" (53) and, especially, "proviridomycin" (45, 349, 350), a protopigment, the final color of which would depend on availability in excess of a particular (among several

possible) ions in the medium at a certain critical period of time. Even streptomycin seems to be "adjusted" to its final and more active form as a result of LD-mannosidase action, the activity of enzyme being dependent on glucose exhaustion in the fermentation medium (102). Some analogy can probably be drawn between such behavior of secondary metabolites and such events in differentiation as a postulated modification of vegetative into sporal ribosomes as a result of desiccation (269), chemical and physical changes in *B. subtilis* spores undergoing "maturation" in spent medium (95), and self-assembly of sporal coat proteins in vitro into differing structural units depending on pH (15, 176).

Although there are several common points in the pathways of secondary metabolism in such diverse organisms as plants, fungi, and actinomycetes, the latter seem to utilize some of the reactions preferentially or more frequently. Among these, Turner (521) distinguishes the following: (i) incorporation of the whole intact carbon skeleton of glucose (streptomycin) or a portion of it (erythromycin) into a secondary metabolite; (ii) Shikimate pathway for biosynthesis of aromatic moieties of antibiotic molecules (chloramphenicol, novobiocin); (iii) extensive employment of the pentose phosphate cycle reactions for biosynthesis of nucleoside-containing secondary metabolites; (iv) rather extensive conversion (cycloserine) or incorporation (gougerotin) of amino acids into secondary metabolities; (v) extensive employment of polypropionate and less extensive employment of polyketide routes; and (vi) the rather frequent occurrence, among secondary metabolites, of terpene- and sterol-derived compounds.

Prospects for Involvement of Antibiotics and Pigments in Regulation of Metabolism

Earlier investigators noted, first of all, that accumulation of a certain antibiotic above a critical level would inhibit or completely suppress vegetative growth of the producing organism (540). Extensive use of this finding was made in selection for strains with elevated activities. In might be also inferred from these data that, in a physiologically heterogeneous population of actinomycete cells synthesizing an antibiotic, there would be a continuously increasing selective pressure favoring further activities of those cells that are already adapted to certain levels of antibiotic concentration. As shown recently with *S. griseus* producing streptomycin (64), however, the drug caused no change in the proportion of resistant variants, which could be scored on streptomycin-containing media.

The possible regulatory functions of at least some antibiotics are further exemplified by extreme sensitivity to an antibiotic at certain developmental stages. Thus, spore germination in *S. griseocarneus* was inhibited by 5 μg of streptomycin, whereas mycelial growth occurred in the presence of 200 to 300 μg of the antibiotic (40).

Reports on the growth-inhibiting activity of antibiotics were soon followed by studies indicating possibilities for inhibition of particular enzymatic reactions, either in vivo or in vitro. Examples include inhibition (393) or stimulation of endogenous respiration in *S. griseus* by streptomycin, differential inhibitory activity of oxytetracycline on active and inactive strains of *S. rimosus* (392), effect of novobiocin on carbon, phosphorus (511), and nucleic acid (132) metabolism in *S. spheroides*, as well as in vitro inhibition by the antibiotic of succinate (512) and pyruvatedehydrogenases. The polyene nystatin was reported to inhibit in vivo the activity of glucose 6-phosphate dehydrogenase in the mycelium of the producer (513). One might infer from reports on interference of antibiotics with particular enzymatic activities involved in the primary metabolism of the producing cells that at least part of the activities might be concerned with events associated with blocking vegetative growth. Inhibition per se, however, is not the equivalent of regulation. It can be noted that, in one instance at least, the inhibition of respiration and glucose dehydrogenase activity caused by accumulation of a polyene antibiotic in the cell of *S. roseoflavus* var. *roseofungini* mutant appeared to be reversibly abolished by illumination at a certain stage of the actinomycete (499).

Extreme cases of antibiotic interference with the metabolism of producing actinomycetes are probably exemplified by reports on mutations caused by streptomycin (127) and some other antibiotics (218, 519).

The studies on the effects of antibiotics on the producing actinomycetes were, perhaps quite naturally, dominated by the assumptions derived from results of pioneering investigations employing "test" microbial systems for the elucidation of target reactions for antibiotic action. On rather rare occasions, however, it has been possible to demonstrate that appropriate target structures, molecules, or reactions are absent or not employed by antibiotic-producing actinomycetes; e.g., *S. antibioticus* (antimycin A producer) has been shown (428) to be deficient in the target for antimycin A in the respiratory chain. The ribosomes of *S. erythreus* appeared not to bind erythromycin to the extent of even the ribosomes of an erythromycin-resistant

strain of *E. coli* (502). With the *S. griseus* strain producing macrotetralide, however, it appeared to be very difficult (251) to prove the hypothesis (571) involving the ionophore-type antibiotics in ion transport in the producing organism.

There is some evidence that the kind of biological activity expressed by antibiotics in the producing organism might differ from that ultimately expressed in the cells of "test" microorganisms (see also next section). For instance, the protein biosynthesis of a chloramphenicol producer appeared to be completely inhibited in vitro by the antibiotic. The accumulation of antibiotic, however, seemed to induce profound changes in the cell permeability, thus blocking the access of the drug to a sensitive target area (342).

As shown with *S. aureofaciens* and chlortetracycline (166), a minor modification (removal of a water molecule in the "C" ring) of the antibiotic molecule renders it five times more toxic for the producer and less toxic for routine test microbes. Casual observations on profound biological side effects of molecules of polyenes (452) or of penicillin (270), and the suggested enzymatic activities of polypeptides (523), might reveal further facets of their activities irrelevant to the suppression of *E. coli* but relevant to their interaction with other molecules in the differentiating cell of the producing organisms.

The interesting hypothesis on the involvement of polypeptide antibiotics in regulatory events during endospore formation is being widely discussed and developed as applied to *Bacillus* spp. (101, 210, 288, 442, 443, 447). Direct involvement of antibiotics produced by actinomycetes in the regulation of developmental events in these organisms has not been documented.

Indirect evidence suggests that actinomycin D, produced by *S. antibioticus*, might affect in a regulatory manner the RNA synthesis in the producing cell (489, 570). Some of the secondary metabolites might probably enter (or localize in?) such sites within the producing cells that are directly concerned with transcription or translation.

Differences in buoyant density and melting points of DNAs from spores and vegetative cells in *S. venezuelae* were already mentioned. It was shown, however, that washing of spores with ethanol or acetone prior to DNA extraction resulted in DNA preparations that were almost identical as judged by above parameters. It was suggested that the spore DNA is complexed with a spore-specific antibiotic, the phenomenon being directly concerned with the regulation of genetic activity (135). In another study, the pigment associated with sporogenesis in *S. venezuelae* was shown to induce sporulation in vegetative hyphae of the same organism several hours before the sporogenesis started without addition of the pigment (458). The glucoside antibiotic aureovocin was shown to be able to bind to the ribosomes of the producing cell (370). A similar possibility was demonstrated for a melanoid-like pigment and spore ribosomes of *S. granaticolor*. The complexing of the pigment with sporal ribosomes appeared to be sufficiently stable: complexes were not dissociated by treatment with ammonium chloride solutions or other routine purification procedures (367). There is not much certainty, however, in the answer to the question of whether the binding of pigments actually occurs in vivo.

Ribosomes of *S. aureofaciens* mycelium appeared to bind tetracycline molecules, the maximal level of binding reaching 320 molecules per ribosome. Most of the drug molecules were bound reversibly with both ribosomal protein and RNA, whereas 1 molecule per ribosome was bound irreversibly. Although the protein-synthesizing system of *S. aureofaciens* appeared to be more resistant to tetracycline inhibition compared with that of other sensitive bacteria and even nonproductive strains of *S. aureofaciens*, the antibiotic was inferred to play some regulatory role in metabolic changes during development (368, 369). Electron microscopy of ribosomal preparations, obtained during subsequent development stages of *S. aureofaciens* in submerged cultures, demonstrated the increasing tendency of ribosomes to form aggregates; this property correlated with accumulation of tetracycline (334). Other studies point to the accumulation of nucleic acid degradation products in the culture media as a likely consequence of tetracycline accumulation by the producing strain (471, 574).

Antibiotics and Their Components as Possible Building Blocks

Industrial microbiologists usually categorize the antibiotics into those (i) retained in the mycelium; (ii) liberated into the medium; and (iii) accumulating both in the broth and mycelium. Until recently, relatively little attention has been paid to locating the exact places within the actinomycete cell where antibiotics are being synthesized and accumulated and how those that are poorly soluble (or almost insoluble) in water leave the cell and accumulate in an aqueous milieu. Considerations of the "late" consequences of antibiotic production (e.g., killing or not killing potential enemies,

etc.) were, perhaps involuntarily, given priority in speculations on the biological impacts of antibiotics.

Yet there is some information pointing to the suggestion that at least some antibiotics (more correctly, components of their molecules) might assume a structural role within the producing cell, being incorporated into cell organelles. The streptidine moiety of the streptomycin molecule was supposed to be incorporated into cell wall of *S. griseus* (489, 539). The presence of streptidine in acidic hydrolysates of the mycelial wall of *S. griseus* mutant strain 45-H was directly demonstrated (489). Streptomycin could be released on lysozyme treatment of this culture (31). These findings correlate with results from Szabo's group on the developmental characteristics of active and inactive strains, as well as on effect of C-factor on the former. N^6N^6-demethyladenine, a component of the antibiotic puromycin, was shown to accumulate in aerial spores, but not in the vegetative mycelium of *S. alboniger*, which produced puromycin during sporulation (448).

Observations on the localization of antibiotics in developing surface and submerged cultures of active producers often contradict the oversimplified expectations based on diffusion against concentration gradients.

In the colonies of *S. olivocinereus*, an antibiotic of the heliomycin type accumulates on the periphery of the advancing substrate mycelium and in minor quantity in the aerial mycelium (531). Special "vesicles" were revealed in the submerged culture of *S. aureofaciens* which produce tetracycline at the onset of active biosynthesis. No such bodies were seen in cultures of a low-producing strain. A very significant portion of antibiotic activity was shown to be associated with the vesicles, thought to be a mechanism for liberating antibiotic from the producing cell which is analogous with reverse pinocytosis (297).

The cisternae and other cytoplasmic membrane-associated bodies filled with electron-dense material and found in the periplasm of *S. vinaceus* were supposed to represent main sites of viomycin biosynthesis (298).

An interesting picture presented by cultures of actinomycetes producing the water-insoluble antibiotics of the polyene family. Submerged cultures of these actinomycetes accumulate antibiotics in the form of cystals located at the cell surface or in the medium (298, 558) or in gran-

An interesting picture is presented by cultures of actinomycetes producing the water-insoluble antibiotics of the polyene family. Submerged cultures of these actinomycetes accumulate antibiotics in the form of crystals located at the cell surface or in the medium (298, 558) or in granules within the hyphae or in the medium (flavofungin-type polyene; 296, 410). The crystals and granules accumulated in shaken cultures apparently have no relation to the normal morphogenetic process in streptomycetes. A different picture, however, was obtained from a surface culture of a mutant of *S. roseoflavus* var. *roseofungini* with impaired differentiation. This mutant was blocked in aerial mycelium formation and sporulation, but its ability to produce a pentaene-type antibiotic, roseofungin, was simultaneously enhanced severalfold (387). Grown in a solid synthetic medium, the mutant cells were shown by electron microscopy to be surrounded by numerous thin structures having a tubular appearance, external diameter of about 20.0 nm, and an inner channel of about 8.0 nm in diameter (70). These were isolated in pure fraction (72) and later shown to be composed mainly of the polyene antibiotic with the admixture of C_{16}-C_{18} fatty acids and several ions, including Ca^{2+} and Mg^{2+} (415). The tubular structures appeared to be completely soluble in acetone; when the relative proportion of water in solutions was increased, they underwent a self-assembly or crystallization-like process and reappeared in the solutions (72). A structural similarity could be seen in both naturally produced and reassembled tubular structures containing the polyene and those elementary structures that can be revealed in the surface sheath of several actinomycetes, including that of the normally differentiated parent strain of *S. roseoflavus* var. *roseofungini*. Tubular structures in the surface sheath of aerial mycelium of the latter strain appeared to be remarkably sensitive to brief acetone washings; they were almost completely dissolved. Acetone extracts of the aerial mycelium, scraped from surface cultures of this strain, yielded, on subjecting them to dilution with water, a spectrum of different structures, some of which were quite reminiscent of those seen on the aerial mycelium surface (71). Later on, initial "tubular" structures were also recovered from such extracts (498). The conditions that led to self-assembly of differing structures were defined (497) and the similarity of their chemical composition to the original tubules was demonstrated (414). The above facts, although still fragmentary, may be taken to support the hypothesis that a pentaene macrolide antibiotic, superproduced by a developmental mutant and excreted into the solid medium, undergoes there a crystallization process, which mimics the events involved in the formation of surface sheath in a normally differentiating parent

strain. In the latter case, however, one presumes that limited amounts of antibiotic are transported to the cell surface at a certain stage (emergence of aerial hyphae) and utilized there for construction of sheath components, with a likely participation of self-assembly phenomena in the formation of sheath subunits.

An independent study (313) demonstrated reversible reconstruction of surface unit structures on spores of *S. streptomycini*, the process being independent of the viability of spores but dependent upon temperature and relative humidity of the milieu.

Some properties of polyene macrolide antibiotics would seem to be compatible with the hypothetical role discussed for them above. (i) Members of the polyene macrolide family of antibiotics are produced by an unprecedented variety of species within the genera *Streptomyces, Streptoverticillium*, and *Chainia* (185). (ii) Some anomalies in the behavior of these antibiotics in solution (185), as well as their "all or none" effect on susceptible organisms (186), would suggest a definite tendency to form supramolecular structures of a micellar nature. The surface-active nature of polyene molecules is directly related to their structure; conformational and hydration-level changes in these molecules would be reasonably expected to have a significant effect on their behavior at interface. Interaction with ions (those that accumulate during the sporulation of streptomycetes) might influence the solubility of polyenes. (iii) The tendency of polyenes to form granules and crystals in submerged cultures of superproducing strains (already mentioned) might be relevant. Interestingly enough, the empirical fermentation media devised for the production of polyenes almost invariably include oils, fats, insoluble particles (185), etc.; in other words, compounds likely to provide heterophasic conditions also inductive for aerial mycelium formation, provided it has not been supressed by shaking in submerged cultures.

The hypothesis calls for reexamination of some of the data reported earlier on the properties of the surface sheath of actinomycetes (Table 1), including the changing pattern of sheath behavior after exposure to organic solvents and to high humidity. The effect of lysozyme treatment might be related to the interaction of surface structures with a protein, regardless of its enzymatic activity. The tubelike structures found by Stuart (484) to surround the aerial mycelium cells of *S. noursei* (a proved producer of nystatin) might be related not to membrane derangement during sporogenesis (as suggested by the author) but to the derangement of surface sheath structure at the onset of autolysis.

It is tempting to speculate also that incorporation of a polyene antibiotic into a protective film on the generative cells of actinomycetes might be favored by natural selection, since it is the mycelial fungi that are likely to compete with actinomycetes in a heterophasic environment such as the soil. The structurally incorporated surface-exposed antibiotic would thus be looked upon not as a weapon for unlimited chemical warfare, but more as a shield for limited protection against potential aggressors.

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