Differentiation of the Vegetative and Sporogenous Phases of the Actinomycetes

2. Factors affecting the Development of the Aerial Mycelium

By DAGNY ERIKSON

Rothamsted Experimental Station, Harpenden, Herts

SUMMARY: When first isolated on soil extract agar, soil actinomycetes consistently produce aerial spores in surface colonies. They retain this property when maintained in sterile soil, or when grown on washed suspensions of common soil bacteria, living or dead, in a water agar medium. In soil, when the composition, moisture content and temperature are kept constant, the initial stimulus towards the production of aerial mycelium is free access of air: the quantity and nature, vegetative or sporogenous, of the inoculum, and, within a broad range, the pH of the soil are of minor importance. Once growth is established, the next most important factor stimulating sporulation in the soil is also physical, namely dehydration. In natural and sterilized soils of different origins, and in a 'synthetic' soil containing 250 p.p.m. of nitrogen as nitrate, the modes of growth of different actinomycetes strains are similar, and generally uncharacteristic of their species.

The stress laid upon the variability of the actinomycetes in general, as reflected in their remarkable responsiveness to changes in media, in the extensive studies of Waksman (1919) and of Lieske (1921), has been echoed by most succeeding workers in the field. The two outstanding gross variations are in pigment production and the appearance of the aerial, spore-bearing apparatus. Soluble pigment production, whether of the yellow-brown (Plotho, 1940) or of the red-blue (Oxford, 1946) type has been shown in mass cultures to depend on the pH reached in the varying stages of metabolism of different nutrient substances. The striking difference between the smooth, cartilaginous, dense-textured colonies composed of vegetative hyphae only, and the 'powdered' growth characteristic of an abundant production of aerial mycelium arising from ramified substratum filaments (see Pl. 1, figs. 1*a*,*b*), is not so readily attributable to single factors. Nevertheless, the ability of the organism to reproduce itself by means of spores is biologically one of its most important properties. This paper reports an investigation of some of the factors which affect this property.

Composition of the medium

The effect of readily available nitrogen. Afanasiev (1937), working with parasitic and saprophytic strains of *Actinomyces scabies*, attempted to find the C : N ratio which favoured the development of the vegetative growth only. In a synthetic solid medium containing glucose as the main source of carbon, a high proportion of nitrogen, supplied either as KNO_3 or asparagine, partly suppressed the development of aerial mycelium. The organic nitrogen compound was particularly effective, a phenomenon noted also by Erikson (1935) in a group of actinomycetes isolated mainly from human pathological material. In

twenty of twenty-six strains no aerial mycelium developed on complex nitrogenous media such as inspissated serum, blood agar or Dorset egg, and only very occasionally a sparse aerial mycelium developed on heart broth and glucose heart broth agars. Aerial growth was obtained only on simple synthetic media like Czapek's sucrose or glycerol or Ca malate agars, or starvation substrates like Ørskov's tap-water agar; and with some four or five strains not even on these. In the present work fifty out of seventy-two strains of saprophytes selected at random from routine soil platings developed the exclusively vegetative growth of the kind pictured in Pl. 1, fig. 1*a* on a peptone Lemco agar with the addition of 0·5 % lecithin, although all except five of the fifty produced aerial growth on the same peptone agar without the lecithin; and all yielded an abundant development aerial growth like that in Pl. 1, fig. 1*b*, when starch was substituted for lecithin. On Czapek's sucrose nitrate agar almost every one produced an aerial growth which was abundant in comparison with the thinly spreading substratum mycelium.

The exclusively vegetative growth formed on the complex phospholipin-containing media is almost always considerably bulkier than that produced on the simpler organic or inorganic substrates. It is also less viable. This was generally true of about 300 strains of actinomycetes belonging to Ørskov's Group I. Readily available nitrogen in excess, therefore, favours the multiplication of the vegetative hyphae at the expense of the biologically more economical and reproductively more efficient aerial spores.

Bacteria as a source of nitrogen. This is not the case with nitrogen in the form of dense suspensions of washed bacteria, living or heat-killed, in tap-water agar. The vegetative growth is not enhanced, not even for those strains of actinomycetes with considerable bacteriolytic activity, that produce a clear area in the vicinity of their growth. All of 124 strains, which when tested against fifteen varieties of soil bacteria, were lytic in varying degrees and grew on the bacterial agar with only a moderate substratum mycelium that soon gave rise to aerial sporogenous hyphae. Both phases of growth, however, were similar in morphology and bulk to those of the same strains grown on bacteria insusceptible to lysis. It would appear, therefore, that the amount of nitrogen rendered accessible to the growing actinomycete by the lysis of the bacteria that it induces must be small and little more than that found in the autolysates normally present in a suspension of insusceptible bacteria. In this connexion it is noteworthy that a definite stimulation of growth is observed when bacterial cells are added to a water-agar medium, but that no better growth of the actinomycete occurs where the bacteria are visibly lysed than where they are not.

Soil extract. When soil dilutions are plated directly on soil-extract agar the actinomycetes appearing as surface colonies invariably develop some aerial mycelium. This proved to be the case with several hundred strains isolated from differently treated soils, and emphasizes the fact that freshly isolated actinomycetes grown on a medium whose nitrogen content approximates to that of their natural habitat commonly display their normal powers of reproduction by aerial spores.

Influence of penultimate medium

Jones (1946) described the cultivation of actinomycetes in moist sterile soil as a means of maintaining their native properties. The method has been effective in the present work, though it was found unnecessary to keep the soil moist. Several strains were sown into sterile soil, allowed to grow, and left to dry in the medium for periods exceeding one year. All were subsequently recovered in their original form by plating on 'synthetic' agar. All such cultures developed normal aerial mycelium on starch tryptone, Czapek and other simple media, whereas subcultures of the same strains, kept in the vegetative phase in nutrient glucose broth for prolonged periods and then plated on similar media, occasionally produced variant sectors or colonies that for at least three or four generations were devoid of aerial growth. It is very probable that the sparse sporulation found for most of the twenty-six strains of pathological origin investigated by Erikson (1935) was in great part due to their continuous cultivation over a period of years on rich laboratory media.

Minimal requirements for growth

The identification of the substances in a medium which favour the development of aerial mycelium is difficult by reason of the simplicity of media that support the growth of the saprophytic soil actinomycetes. The trace elements present in tap water and the impurities in commercial agar make a medium which, since its introduction by Ørskov (1923), has been found sufficient for a thin substratum and aerial growth of many strains. As the following experiment shows, very little need be added to a purified agar to obtain similar growth.

To a well-washed agar made up in distilled water were added varying amounts of sterile glucose in distilled water. Plates were made and seeded with a row of droplets of a spore suspension from each of the strains tested. After 3 days' incubation at 33° two of seven strains produced aerial mycelium in the presence of 0·0625 and 0·0312 % glucose, and vegetative growth with only 0·0019 % glucose. After a fortnight, fair growth developed throughout the series with 0·0312 % glucose, while all strains yielded a very thin growth with a delicate but perceptible aerial mycelium, quite visible to the naked eye in the presence of 0·0019 % glucose.

The limiting concentration of glucose for growth in liquid media also was 0·002 %, using sodium nitrate or ammonium phosphate as the nitrogen source. From eight and ten similar strains, minute colonies were produced as bottom and surface growth, the surface colonies all giving rise to aerial hyphae after 2 days. With 0·001 % glucose, only two or three colonies were produced from two strains. The same liquid media without added nitrogen did not support visible growth.

Nature of inoculum

Millard & Burr (1926) pointed out that to secure early surface growth and sporulation on liquid cultures, inoculation of spores is essential. The lipid nature of the spore membrane (Erikson, 1947) enables it to remain floating and to germinate in the most favourable conditions for aerobic growth; vegetative growth sinks. The diagnostic criterion of primary vegetative bottom growth in liquid media, which characterizes Group I actinomycetes, enunciated by

Ørskov and confirmed for the most part by Erikson (1935), is by no means universally applicable and is indeed limited to subcultures (*a*) from substratum mycelium on agar cultures; (*b*) from bottom-growth colonies in liquid cultures; (*c*) from good sporulating growth to media in which there is a sufficient concentration of surface-active substances to cause the spores to sink; and (*d*) from poorly sporing material in which the few spores remain attached to the vegetative growth and fall with it to the bottom. Unless spores are made to sink, surface growth may occur. In fact, Ørskov (p. 47) ensures that the spores do not float by drying them first on sterile filter-paper, and states that if the inoculated spores remain on the surface, they quickly form a mycelium.

The growth phase of the inoculum used for sowing liquid cultures may be of great practical importance. For example, an asporogenous variant of *A. griseus*, producing only submerged vegetative growth in stationary flasks was found by Schatz & Waksman (1945) to produce no streptomycin. Furthermore, when the substratum mycelium only of active, sporing strains was inoculated, a similar type of submerged growth resulted, devoid of antibiotic substance.

Factors influencing growth in natural and sterile soils

Kubiena & Renn (1935), examining undisturbed, naturally developed soils in New Jersey, U.S.A., with the aid of a special vertically illuminated microscope, found actinomycetes growing particularly well in the soil spaces opening to the surface. All their illustrations show the same type of growth, predominately aerial tufts of hyphae in more or less compact colonies with long twisted strands bridging the gulfs between soil crumbs. Similar growth was obtained when soils from different Rothamsted plots, treated or untreated, from Marlborough chalk-down arable or from Thames-side pasture lands were left undisturbed in sterile Petri dishes. In such soils a surface growth of actinomycetes is frequently visible to the naked eye after 3 months or more as a faint greyish veil round the rim of the dish, where aeration is greatest and there is an accumulation of nutrients consequent upon the evaporation of water. This growth is almost entirely aerial, and uniformly colourless, although the cultivation of samples on artificial media yields various chromogenic strains.

Growth on buried coverslips. Coverslips buried beneath the surface of natural soils that still retain their natural moisture content become covered with growth, commonly in the form of loose, straggling, vegetative filaments, from which occasional sporing branches may arise wherever there is a minute air-space (Pl. 2). Lutman's (1945) criticism of the very similar findings obtained by Starkey (1938) as the result of using Cholodny slides—that the slide disturbs the soil-particle arrangement and introduces unnatural air pockets—is not valid in the present instance. For similar results were obtained by burying fragments of coverslips about one or two microscope fields in diameter, which can justifiably be claimed to cause no greater disturbance than many of the foreign particles commonly present in soils.

Sterilization. Sterilization of the soil does not affect the mode, abundance or the appearance of the growth of various actinomycetes. Sterile soil moistened with sterile distilled water, and evenly inoculated with a watery suspension of

washed, lightly ground, vegetative mycelium derived (*a*) from bottom growth in liquid cultures, (*b*) from non-sporing surface growth on rich agar media, developed in 6 days at 25° a copious aerial mycelium covering almost every surface crumb. As good, but no better, results were obtained with an aqueous suspension of spores of the same strain. Successful inoculations were also made with the smallest possible soil crumb which could be lifted on a needle from an already grown plate, placed in the centre of a fresh plate of sterile dry soil and subsequently moistened by a fine spray of water.

Site of inoculations. The importance of the site of inoculation for growth in undisturbed soils is shown by the following experiment.

Discrete colonies, approximately 3 mm. in diameter, of *Actinomyces* strain 6513 Al, 20 days old, with well-developed aerial mycelium, which had been grown on cellophan over a synthetic agar, were cut out and inoculated (*a*) in the centre of the bottom of the dish, cellophan downwards, and the soil carefully replaced over the colony, (*b*) in the centre of the surface of the soil, cellophan uppermost, and

Table 1. *Showing the influence of site of inoculation on spread of actinomycetes in plates of sterile soil*

Mode of inoculation	No. of colonies developing from the Central sample	Peripheral sample
One colony, buried	0	0
One colony, on top	500	0
One colony, dispersed	P	P
Strip, buried	70	0
Strip, on top	300	0
Strip, dispersed	P	P

P = profuse growth.

(*c*) dispersed in sterile water and thoroughly mixed with the soil. In (*a*) and (*b*) 25 % water by weight was added to the soil (Barnfield, unmanured) before inoculation. The three tests were repeated using as inocula large 2 × 1 in. strips of cellophan bearing uniform growth of the same strain. After 2 days' incubation at 25° samples were taken from the soil plates with a sterile cork-borer, both from the immediate vicinity of the centre and from the periphery of the plate, diluted 1000-fold, and plated.

Table 1 shows that with a surface inoculum growth in the immediate vicinity of the site of inoculation is abundant and unrelated to the size of the inoculum (one colony or a large strip); with a buried inoculum of either size growth is poor; and only when the inoculum is dispersed does growth extend to the periphery of the plate.

Coverslips buried halfway between centre and periphery of replicate plates, when examined a fortnight later, showed growth of the typical vegetative filamentous pattern with the occasional sporing branches portrayed in Pl. 1, fig. 2, in every instance when the inoculum was dispersed, in three of five cases with the strip on top, in two of five cases with one colony on top, in two of five with the buried strip, and in one of five with the buried colony. At this time a surface development of aerial mycelium could be detected microscopically in all soil plates, and was visible to the naked eye wherever the inoculum had been introduced from above.

Thus, when the composition of the soil, moisture content, and temperature are kept constant, the initial stimulus towards the production of sporogenous hyphae is free access of air.

Size of soil crumb. To test the influence of the size of soil crumb and corre-

sponding differences in air-space, allotment soil was graded through three sieves of 0–1, 1–3, and 3–4 mm. mesh and a series of plates made up with different moisture contents obtained by spraying the water on with an atomizer, smoothing the surface as far as possible, and then autoclaving. The moisture content after sterilization was determined. A central plug was removed with a sterile cork-borer from each plate, and varying amounts from 0·01 to 0·5 ml. of an aqueous suspension of spores of *Actinomyces* strain '*g*' were introduced into the hole, the soil being carefully restored and pressed flat. The plates were incubated for 15 days at 25°. In all dense aerial growth was macroscopically visible in the central area extending from the site of inoculation irregularly for 0·5–5·0 cm. No correlation could be observed between extent of growth and the varying moisture contents (15–25 %) or the size of inocula. But whereas with the large crumb size (3–4 mm.) a delicate greyish veil of diffuse aerial growth could be seen almost all over the plate (see Pl. 2, fig. 3), with the finer crumbed soils (especially 0–1 mm.) growth was limited outside the central zone to discrete colony masses which sometimes reached as much as 10 mm. in diameter and which resembled growth on solid artificial media (see Pl. 2, fig. 4). A similar set of plates were stacked in piles in a large glass container with a little water at the bottom, and held in a greenhouse subject to fluctuating day and night temperatures. Here, after the same period, growth was of the types described above, but noticeably less in extent in every case. It was particularly noteworthy that the top plate of each pile showed no visible growth, an effect probably due to the adverse effect of bright sunlight on the development of aerial mycelium already noted by Ørskov (1923). In this country the ordinary diffuse daylight reaching a laboratory bench in front of a window has not been found to have any such effect; the greenhouse experiment, however, was carried out during a bright spell in the spring of 1945.

Hydrogen-ion concentration. All the sterile soils supporting growth were maintained at a pH of about 7·0. The natural soils ranged from pH = 4·2 to 8·7. It has already been mentioned that none of the naturally occurring or inoculated actinomycetes growing in the different specimens of soils examined produced pigmented growth. An attempt was made to see whether pigment production could be induced by varying the pH. A vigorous strain of *A. coelicolor* was sown in massive doses on the smoothed surface of sterile soil plates adjusted to pH = 4·3 with acetic acid, and to pH 8·6 with sodium bicarbonate. Growth was fair, but identical in both cases, colourless aerial mycelium appearing in the uncharacteristic cottony tufts observed throughout this work, with a sparse development of undifferentiated vegetative filaments that became increasingly fragmented as the soil dried. However, the spirals characteristic of the aerial hyphae of this strain could be seen in many places irrespective of pH of soil.

Growth in synthetic soils

A 'synthetic' substrate physically similar to soil has been described by several investigators. The sand-bentonite mixture of Madhok (1937) has been found most satisfactory for growth experiments.

It was prepared in the proportion of 47·5 g. washed, ignited quartz sand to 2·5 g. bentonite per plate. To this was added cellulose in the form of shredded Swedish filter paper or cotton-wool, and 0·05 g. of the dried insoluble residue left after extracting 5 g. of dried autolysed yeast three times in 100 ml. distilled water. The plates were dry-sterilized. An artificial soil solution based on the average figures quoted by Russell (1927) was made up as follows: $Ca(NO_3)_2$...0·33 g., $CaSO_4$...0·8 g., $MgSO_4, 7H_2O$...0·7 g., K_2SO_4...0·025 g., K_2HPO_4...0·005 g., $NaHCO_3$...0·2 g., water to 1 litre. This gives total solids, in p.p.m., of Ca 310, SO_4 850, Mg 70, Na 55, K 13, HCO_3 140, PO_4 3, NO_3 250: total = 1691. This solution, sterilized separately, was used as the suspending fluid for the inoculum of spores and added to the sand plates.

Growth was visible in 5 days at 25° in plates containing 8–24 % by weight of the solution and, although sparse in the lower concentrations, was of the same type throughout—minute tufts of colourless aerial mycelium, which in many strains were attached directly to the quartz particles (see Pl. 2, fig. 5) and not to the filter paper or cotton-wool fibres. On buried coverslips fragments of the initial vegetative mycelium could be discerned, but often with difficulty owing to the obscuring effect of the colloidal bentonite. Enhanced growth was obtained with extra nutrients, but the mode of growth was the same in all cases—a sparse development of vegetative mycelium followed by an early and relatively abundant production of aerial sporogenous hyphae on the surface and in the air-spaces below the surface. The type of growth was substantially similar to that in natural soils, native or sterile.

DISCUSSION

Any ready evaluation of the factors stimulating aerial growth of soil actinomycetes can only be roughly qualitative because of their ability to develop on a minimal supply of nutrients. Thus, 0·002 % glucose in a washed, but not necessarily nutrient-free, agar supplied the requirements for complete growth. The provision of nitrogen in excess, however, particularly in the form of phospholipins in artificial media, favours the growth of the vegetative at the expense of the aerial mycelium in a large number of soil actinomycetes. This effect of rich media can to some extent be imposed on the organisms, for strains maintained in the vegetative phase in nutrient glucose broth tend to produce sporeless variants when subcultured on to solid media.

In liquid cultures the primary factor ensuring sporulation is the flotation of an inoculum of spores upon the surface, made possible by reason of the lipid nature of their external surface.

This work was carried out by the author as a member of the scientific staff of the Agricultural Research Council.

I wish to thank Dr H. G. Thornton, F.R.S., for helpful suggestions and criticism, and, together with Mr Stansfield, for taking the photomicrographs; Mr A. V. Garcia for many cultures and for showing me the results of his large-scale isolations from different soils; Dr A. R. Martin (Imperial Chemical Industries Ltd.) for a supply of detergents; Miss Mabel Dunkley and Miss Mary Williams for technical assistance.

REFERENCES

AFANASIEV, M. M. (1937). Comparative physiology of *Actinomyces* in relation to potato scab. *Bull. Neb. agric. Exp. Sta.* no. 92.

ERIKSON, D. (1935). The pathogenic aerobic organisms of the *Actinomyces* group. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 203.

ERIKSON, D. (1947). Differentiation of the vegetative and sporogenous phases of the actinomycetes. I. The lipoid nature of the outer wall of the aerial mycelium. *J. gen. Microbiol.* **1**. 39.

JONES, K. L. (1946). Further notes on variation in certain saprophytic actinomycetes. *J. Bact.* **51**, 211.

KUBIENA, W. & RENN, C. E. (1935). Micropedological studies of the influence of different organic compounds upon the micro-flora of the soil. *Zbl. Bakt.* (2. Abt.), **91**, 267.

LIESKE, R. (1921). *Morphologie und Biologie der Strahlenpilze.* Leipzig: Borntraeger.

LUTMAN, B. F. (1945). The spread of potato scab in soil by potato plant humus. *Bull. Vt. agric. Exp. Sta.* no. 528.

MADHOK, M. R. (1937). Synthetic soil as a medium for the study of certain microbiological processes. *Soil Sci.* **44**, 319.

MILLARD, W. A. & BURR, S. (1926). A study of twenty-four strains of *Actinomyces* and their relation to types of common scab of potato. *Ann. appl. Biol.* **13**, 580.

ØRSKOV, J. (1923). *Investigations into the Morphology of the Ray Fungi.* Copenhagen: Levin and Munksgaard.

OXFORD, A. E. (1946). Note on the production of soluble blue pigment in simple media by *Actinomyces coelicolor*. *J. Bact.* **51**, 267.

PLOTHO, O. VON (1940). Beiträge zur Kenntnis der Morphologie u. Physiologie der Actinomyceten. *Arch. Mikrobiol.* **11**, 33.

RUSSELL, E. J. (1927). *Soil Conditions and Plant Growth*, 6th ed. London: Longmans, Green and Co.

SCHATZ, A. & WAKSMAN, S. A. (1945). Strain specificity and production of antibiotic substances. IV. Variations among actinomycetes, with special reference to *Actinomyces griseus*. *Proc. nat. Acad. Sci., Wash.*, **31**, 129.

STARKEY, R. L. (1938). Some influences of the development of higher plants upon the micro-organisms in the soil. VI. Microscopic examination of the rhizosphere. *Soil Sci.*, **45**, 407.

WAKSMAN, S. A. (1919). Cultural studies of species of *Actinomyces*. *Soil Sci.* **8**, 71.

EXPLANATION OF PLATES

PLATE 1

Fig. 1*a*. Colonies of actinomycete '*g*' on peptone-lecithin medium, showing only vegetative mycelium—14 days.

Fig. 1*b*. Actinomycete '*g*'—single colonies, on starch tryptone agar, 2 weeks, showing abundant aerial mycelium.

Fig. 2. Coverslip buried in garden soil 9 days, showing long straggling filaments of vegetative mycelium of wild *Actinomyces*—fixed osmic acid, stained haematoxylin by Dr B. N. Singh.

PLATE 2

Fig. 3. Soil, 3–4 mm. crumb, 1 month, showing faint greyish veil of growth over soil crumbs.

Fig. 4. Soil, 0·1 mm. crumb, 1 month, showing macroscopic colonies.

Fig. 5. Pure culture of *A. coelicolor* in 'synthetic' soil—yeast residue, 2 weeks. Growth attached to quartz particle.

(*Received* 23 *July* 1946)

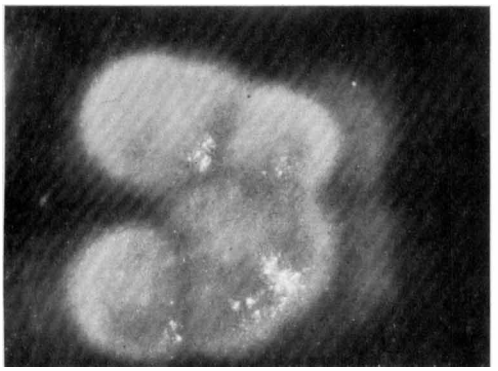

Fig. 1*a*

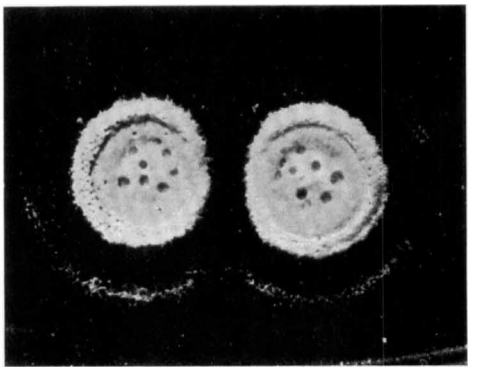

Fig. 1*b*

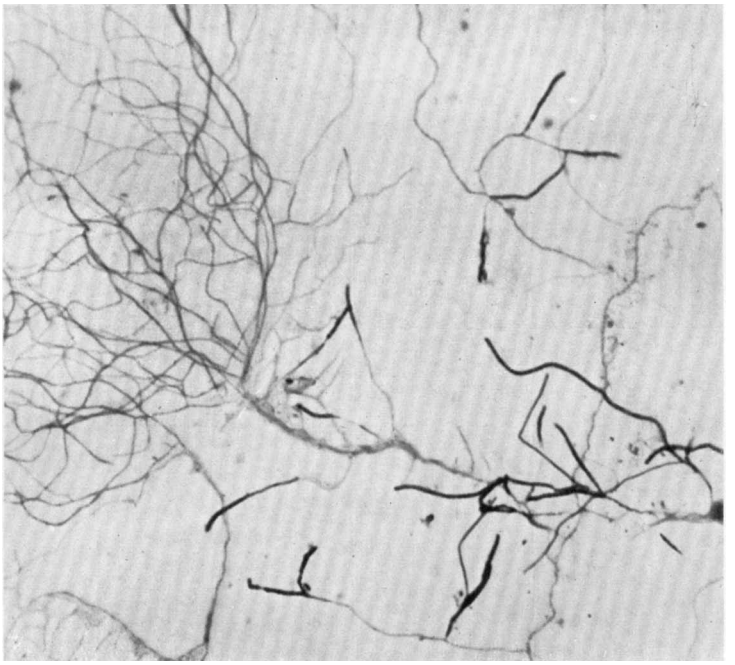

Fig. 2

D. Erikson—Development of aerial mycelia in actinomycetes. Plate 1

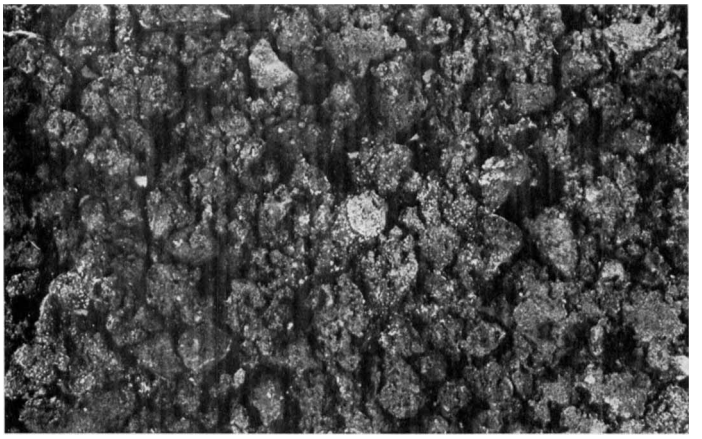

Fig. 3

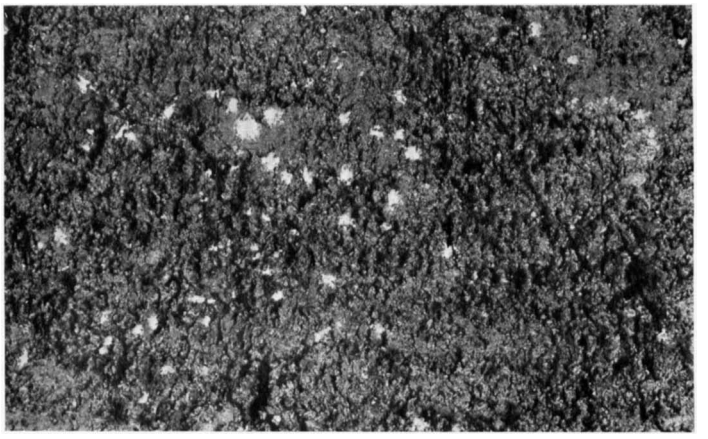

Fig. 4

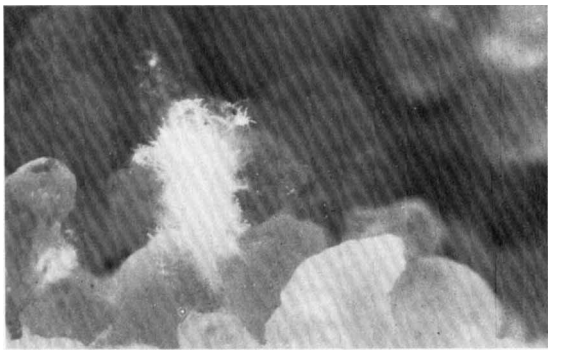

Fig. 5

D. Erikson—Development of aerial mycelia in actinomycetes. Plate 2