# WARM-STAGE OBSERVATIONS ON THE DEVELOPMENT OF PSEUDO-MYCELIA IN CULTURES OF AVIAN TUBERCLE BACILLI GROWN IN DILUTE EMBRYO EXTRACT

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(With 3 Figures in the Text)

## INTRODUCTION

Whether the tubercle bacillus can form true 'mycelia' by lateral budding, or whether the branching forms which often appear in tubercle cultures are only 'pseudo-mycelia' consisting of chains of independent organisms, has long been a controversial subject.

'Mycelia' were first described by Metschnikoff (1888), who regarded them as a stage in the normal developmental cycle. Bruns (1895) discussed the question as to whether the ramifications of such 'mycelia' were true side-shoots formed by budding, or 'pseudo-ramifications' due to angular growth from the ends of the bacilli. Coppen-Jones (1895) was uncertain whether 'mycelia' were 'unicellular' or composed of chains of separate elements.

Simple branching forms have often been recorded. They have been described in stained preparations by Nocard & Roux (1887), Malfucci (1892), Klein (1892) and recently by Pryce (1941) in the early growth stages of slide cultures of sputum smears, using a film of diluted blood as medium.

'Mycelial' forms have often been described which, as they bear little resemblance to the well-defined Metschnikoff type, hardly deserve the name. Babes & Levaditi (1897) described giant forms resembling mycelia in human tubercle bacilli injected under the dura of rabbits. Vaudremer (1931) obtained a 'mycelial' network of non-acid-fast filaments on non-glycerinated potato broth. Besançon & Philibert (1924) described a 'mycelial' network as a preliminary growth-phase on the surface of Besredka's fluid egg medium. Moellgard (1931) produced long, convoluted non-acid-fast threads in a medium composed of a mixture of bone marrow and rabbit serum. Karwacki (1934) revived the streptothrix theory by demonstrating non-acid-fast filamentous structures which he thought could be produced either by 'diminishing the vitality of the strain' or by growing bacilli in biological media; his cultures, in which these filamentous forms predominated, were derived from cultures which had remained sealed and untouched for as long as ten years.

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Direct observations of individual living rods grown on artificial media failed to show true 'mycelial' forms. Miehe (1909) observed living human type bacilli grown on glycerin broth and, though unable to demonstrate the formation of mycelia, he saw small side-shoots in a minority of the organisms. Oerskov (1929) demonstrated branching, angular growth in living avian bacilli and considered this to be evidence supporting Jensen's theory that the tubercle bacillus and the actinomyces belong to a separate group of branching bacteria. Kahn (1929), Gardner (1929) and Wyckoff (1934) also failed to see true mycelia in their cultures. MacCarter & Hastings (1934) studied the development of living avian and human tubercle bacilli and of *Bact. phlei* on such media, and saw the formation of branching structures which under low magnification resembled 'mycelia', but careful examination under an oil immersion lens showed clearly that they consisted of chains of individual rods. These authors concluded that the structures previously regarded as 'mycelia' were in fact merely 'pseudo-mycelia', like those in their own preparations.

In a previous communication (Brieger & Fell, 1945) we described various forms of initial development which avian tubercle bacilli may undergo when cultivated in embryo extract prepared as for tissue culture. In this earlier study we observed on the warm stage the development of several typical 'mycelia' like those originally described by Metschnikoff and were able to follow the formation of the complex branching unit from a single filament by a process of lateral budding. In recent experiments we have found that if the embryo extract is diluted to 25–5% of its usual strength, another type of branching colony appears in addition to true 'mycelia'. Under low power these colonies closely resemble 'mycelia' but examination under a high magnification shows that they are 'pseudo-mycelia' and that their branches are independent organisms which adhere to each other, but are not in structural continuity like those of the true 'mycelia'.

Our material was very favourable for studying the living organisms, so we decided to watch the

development of the pseudo-mycelia on the warm stage and compare it with that of the true 'mycelia' and with the other common forms of growth previously seen in the more concentrated extract. We also investigated the behaviour of the pseudo-mycelia when transferred to concentrated extract, to find whether they would revert to the normal type of bacillus under different cultural conditions.

## MATERIAL AND METHODS

Since the material and methods used in this study are identical with those employed in the experiments previously reported, the reader is referred to our earlier paper for a detailed account of the bacterial strains and technique.

Most of the observations were made on what in our previous paper we termed the 'Original' strain of avian tubercle bacilli. This strain, which proved to be highly pathogenic to fowls and rabbits, was obtained from the late Dr Stanley Griffith's laboratory in 1937. The bacilli were incubated in a sealed tube for six years and were then subcultured at monthly intervals on Loewenstein's medium. Control observations were made on the 'Lister' strain which was obtained from the National Collection of Type Cultures, Lister Institute, and maintained by subculture on Loewenstein's medium.

The experimental culture medium consisted of chick embryo extract prepared as previously described and then diluted with Pannett and Compton's saline to 25–5% of its original concentration. The observations were made on hanging-drop cultures, each of which contained 0·03 c.c. of dilute extract to which a droplet of bacterial suspension had been added with a platinum loop.

The cultures were studied on a microscope enclosed in a hot-box kept at 38–40°C., and a 2 mm. Beck apochromatic oil immersion lens with × 10 ocular was used. The organisms were drawn twice daily in the early stages of growth and at longer intervals as the growth rate declined. The drawings were made to a constant magnification with the aid of an eyepiece micrometer and an arbitrary scale marked on a strip of card (see previous paper); the chief dimensions of each group of bacteria and as far as possible each member of the group were measured.

For control purposes many cultures were fixed by osmium vapour and stained by Ziehl-Neelsen's method.

## RESULTS

### A. *The behaviour of bacilli transferred from artificial medium to dilute embryo extract*

(1) *General.* As in the concentrated embryo extract, the bacilli grow differently according to whether they are deposited on the glass or floating in the surface film. In the former situation they merely multiply very slowly by simple binary fission; it is in the surface film that the pseudo-mycelia appear, and they can therefore be studied only at the extreme margin of the drop, as elsewhere the focal depth required is beyond the range of a high-power objective.

The cultures present a remarkable appearance by the fourth or fifth day of incubation, as large areas of the surface film are covered by the strange, moss-like growth of the pseudo-mycelial colonies. True mycelia, though much less numerous, are also common in these tracts.

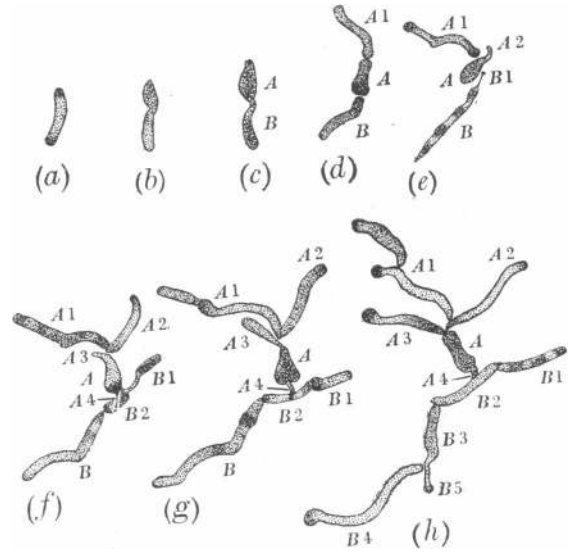


Fig. 1. The development of a simple pseudo-mycelium from a single bacillus (for detailed description see p. 257). *a*, at the beginning of incubation; *b*, after 19 hr. incubation; *c*, after 26½ hr. incubation; *d*, after 43 hr.; *e*, after 51 hr.; *f*, after 74½ hr.; *g*, after 92 hr.; *h*, after 7 days. Note the apical angular growth, the formation of pin-like terminal sprouts which develop into independent filaments (cf. *A*2, *A*3, *B*1, Figs. *e*, *f*, *g* and *h*) and the way in which the daughter filaments stick together after division.

(2) *The development of a simple pseudo-mycelium from a single bacillus.* The development of a pseudo-mycelium from a single bacillus was observed three times, but as all three colonies showed similar characteristics, only one need be described in detail (Fig. 1).

The original bacillus of this colony (Fig. 1*a*) was fairly large, with refractile ends. After 19 hr. incubation (Fig. 1*b*) it had elongated and at one point showed a slight constriction. Seven and a half hours later (Fig. 1*c*) this constriction was reduced to a thin neck connecting a bulbous 'head' (*A*) with a narrower and rather longer 'tail' (*B*). The apex of the 'head' grew rapidly and after 43 hr. (Fig. 1*d*)

it had formed a thick terminal filament (*A* 1) curving to the left; the 'tail' had become separated from the 'head'.

During the next 8 hr. (Fig. 1*e*) the 'head' formed a second apical sprout (*A*2), this time pointing to the right, while *A*1 had lengthened, become sharply bent and acquired a refractile knob at either end. The 'tail' also had elongated, both ends were very refractile and two transverse refractile bands had appeared; protruding from the refractile material at the end next to the 'head' was a minute, pin-like sprout ending in a tiny knob (*B*1).

After 3 days' incubation (Fig. 1*f*) *A*2 had grown into a fairly long, independent organism and the 'head' (*A*) had formed a third apical sprout (*A*3), pointing to the left like *A*1. A delicate, pin-like process (*A*4) had also grown out from the base of the 'head' across one of the constituents of *B*. Meanwhile *B*1 had enlarged greatly; the tiny knob at the end of the process had lengthened and swollen and the hair-like shaft had thickened into a narrow neck. The main body of the 'tail' (*B*) had become sharply bent in the middle and had budded off a second daughter bacillus (*B*2) which appeared as a short, stout rod attached at one end to *B* and at the other to the neck of *B*1. Eighteen hours later (Fig. 1*g*) *A*3 had separated from *A*, and *A*1 had begun to divide in two; *B*1 had developed into a rod of regular shape and *B* had grown into a fairly long filament.

The last drawing of the series (Fig. 1*h*) was made after 7 days' incubation. At this stage the division of *A*1 was complete and the longer of the two daughter bacilli had continued to grow beyond the point of attachment of its sister organism to form a short, thick terminal sprout pointing to the left and ending in a highly refractile bulb. *B* had divided into two at the angle of the bend first seen on the fourth day (Fig. 1*f*) and one of its division products (*B*3) had already formed a thin terminal sprout projecting beyond the point of attachment of the other daughter bacillus (*B*4).

The colony underwent no further change during the remaining 2 days of observation.

(3) *The development of a complex pseudo-mycelium from a group of bacilli.* The development of one complex colony was studied in detail for 10 days. It was derived from a compact, crescent-shaped group of seven rods (Fig. 2*a*). After 19 hr. incubation all the rods had elongated into long filaments (cf. *A*, Fig. 2 *a*, *b*) and some had already divided or begun to do so.

Eight and a half hours later (Fig. 2*c*) the filaments had multiplied considerably partly by ordinary fission and partly by the formation of terminal pin-like sprouts which, as in the simple pseudo-mycelium, developed into independent bacilli. Both types of reproduction were clearly shown by the bacillus *A* (Fig. 2*a*), which divided by binary fission into two unequal daughter rods *A*1 and *A*2 (Fig. 2*c*) and at the same time produced a delicate terminal sprout *A* 3.

During the next 3 days, proliferation continued actively by both processes. Careful comparison of Fig. 2 *d* and *e* reveals many instances of the formation of bacilli from fine terminal sprouts. A good example is provided by the bacillus *B* (Fig. 2*d*), which produced a minute pin-like sprout *B*1; this thickened, elongated and finally separated from *B* to form an independent, though adherent organism (Fig. 2*e*). Many of the filaments were very irregular in shape.

As in the simple colony, the products of division, though structurally independent, tended to remain in contact or stick to each other at the point of cleavage. This behaviour, which caused the single rod to form a branching, tree-like colony (Fig. 1*h*), caused the present group of bacilli to develop into a more complex system resembling an irregular network, the meshes of which enlarged as the filaments multiplied and lengthened.

After 5 days' incubation growth rapidly declined and the development of some of the pin-like sprouts was arrested at this stage. When this happened the minute distal knob enlarged greatly and became intensely refractile (cf. *C*, Fig. 2 *d*, *e*); the sprout remained indefinitely in this state. Many of the full-grown filaments also acquired an increasingly refractile terminal bulb while at the same time transverse bands appeared in more filaments and became brighter and more distinct as the age of the colony advanced.

(4) *Observations on fixed material.* Observations made on cultures fixed with osmium vapour and stained by Ziehl-Neelsen's method confirmed those on living material. In cultures fixed after 48 hr. incubation many of the filaments show pronounced polar staining, apparently corresponding to the refractile terminal bulbs often seen in the living organisms at this stage; they are usually quite strongly acid-fast and very clearly banded throughout their length with alternating red and colourless regions.

In general the staining capacity of the filaments declines with age but it is variable, and in a 25-day culture the same colony may contain every gradation between faintly acid-fast and strongly hyperchromatic individuals. Many of the filaments in the older cultures contain black globules, some being terminal and others beading the whole length of the organism.

B. *The behaviour of pseudo-mycelia formed in dilute embryo extract when transferred to concentrated extract*

(1) *General.* The object of these experiments was to find whether the remarkable branching colonies

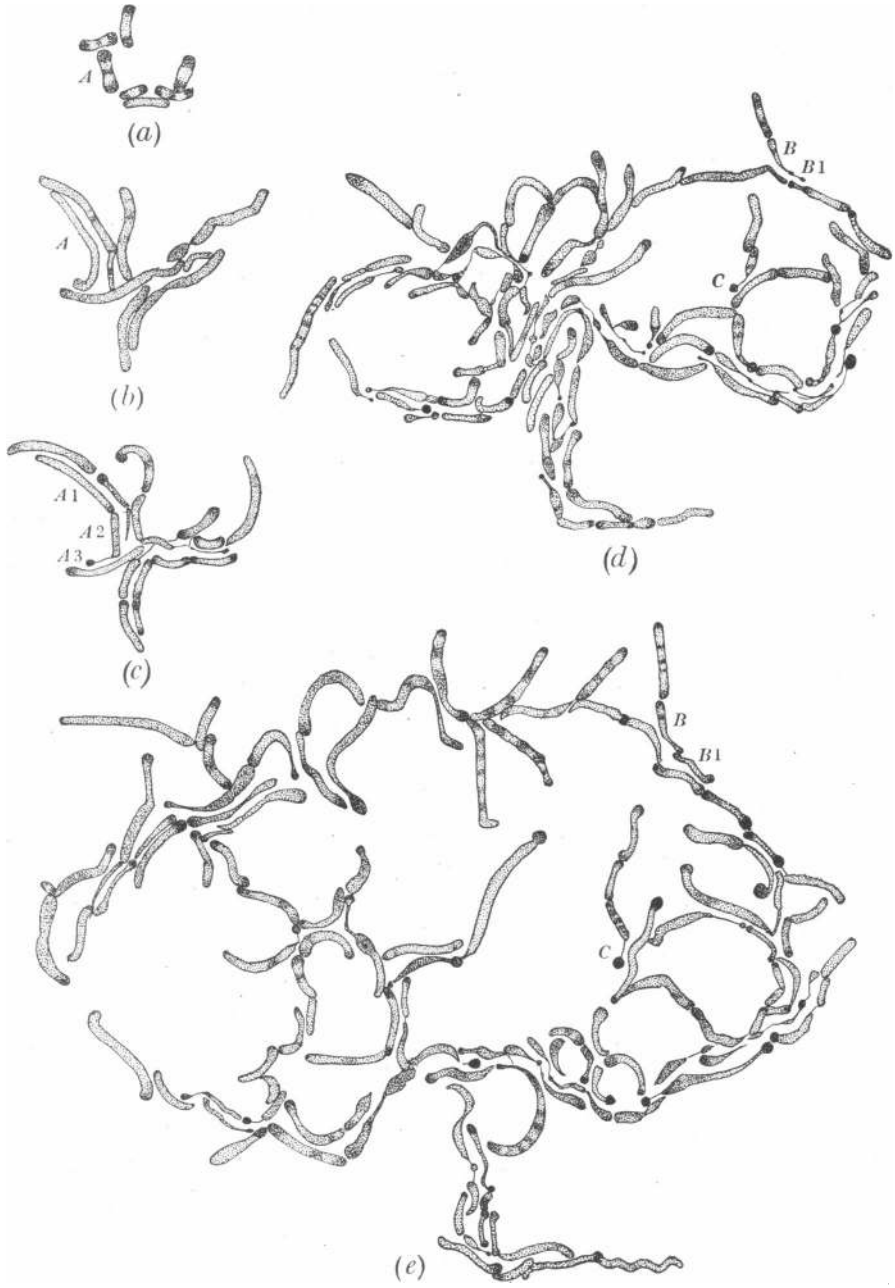


Fig. 2. The development of a complex pseudo-mycelial colony from a group of seven rods (for detailed description see p. 258). *a*, at the beginning of incubation; *b*, after 19 hr; *c*, after 26½ hr.; *d*, after 67 hr.; *e*, after 5 days.

which are formed in dilute extract were capable of further growth when transferred to concentrated extract, and if so what form of reproduction the bacilli would undergo.

Cultures were grown for 23 days in dilute extract (1 part concentrated extract: 20 parts Pannett and Compton's saline), by which time the surface film of the drops contained large tracts of pseudo-mycelial growth. Subcultures were then made by transferring a loopful of the fluid from the old cultures to each of a series of fresh coverslips and adding fresh (concentrated) embryo extract. One subculture was placed on the warm stage for continuous study and the rest were incubated and examined at intervals.

By the third day the subcultures had begun to grow and small colonies had appeared which rapidly enlarged and multiplied. After 12 days' incubation, a profuse growth had formed which, however, bore no resemblance to the pseudo-mycelial colonies of the previous cultures.

(2) *The rejuvenation of a pseudo-mycelium.* As stated above, one subculture was placed on the warm stage and a fragment of a pseudo-mycelial colony was studied for 8 days (Fig. 3). The fragment consisted of eight long and several shorter filaments, many of them sharply banded, and three short rods about one-fifth the length of the longer elements (Fig. 3*a*).

During the first day the bacilli became more refractile but otherwise did not change. By the end of the second day, however (Fig. 3*b*), all with one exception had greatly elongated and several had divided into two; the original banding had almost disappeared but the poles of some of the filaments had become very refractile. The three small rods showed no growth.

During the third day (Fig. 3*c*) several of the filaments had broken up into long rods, some of which dangled loose in the medium, and after 90 hr. incubation (Fig. 3*d*) the whole colony had disintegrated into rods of different lengths. The short filament mentioned above remained an exception since it grew little and failed to divide during the period of observation. The short rods seen on the first day elongated slightly but did not multiply.

As growth continued, larger numbers of the rods became free and were shed into the drop. By the end of the eighth day (Fig. 3*e*), most of the newly formed rods had disappeared in this way; of those still adherent to the glass, many had become sharply banded like the original filaments, several contained highly refractile globules and a few were distorted in shape.

Thus in concentrated extract the pseudo-mycelium reverted to the ordinary 'standard' type of development, though the segmentation of the filaments into short rods was less regular and less sudden than in cultures derived from normal material.

(3) *Observations on fixed material.* A subculture was fixed before incubation as a control and shows many large fragments of pseudo-mycelial colonies as well as scattered rods and filaments. In 3-day cultures the filaments have multiplied considerably and vary widely in length, breadth and degree of acid-fastness; most are characterized by intense terminal staining which probably corresponds to the strong polar refractivity seen in the living organisms at this stage.

By the eighth day large colonies of long and short rods and coccal forms have appeared. Four days later most of the colonies consist of very small acid-fast rods and coccal forms though some groups of short filaments and a few mycelia are also seen. In places the remains of a pseudo-mycelium are still distinguishable, many of the bacilli being distorted and hyperchromatic.

## DISCUSSION

The experiments described above were originally made to test the potency of embryo extract in progressive dilutions on the growth of tubercle bacilli, and the appearance of microscopic pseudo-mycelial colonies in the higher dilutions was an unexpected result.

It is interesting to compare the growth of a pseudo-mycelium with that of the three common types of colony ('mycelial', 'standard' and 'raft') previously seen in the more concentrated extract (Brieger & Fell, 1945).

A pseudo-mycelium develops very differently from a 'mycelium'. Both begin as a single filament, but whereas in the former the branches are formed by the angular terminal growth of independent units which multiply both by terminal budding and by simple fission, in the latter they develop as lateral buds from the main stem with which they remain continuous, and fission is rare.

The development of a pseudo-mycelium also differs widely from the 'standard' type of life cycle in concentrated extract. In both, the original rod first elongates into a filament which then proliferates, but in the pseudo-mycelium the filamentous stage persists indefinitely while in the 'standard' life history the filaments break up after a few days into short rods which multiply slowly by ordinary fission. The products of division in a pseudo-mycelium usually remain in contact at the point of cleavage, but in the 'standard' development they break apart with a snapping movement and usually lie roughly side by side in a loose sheaf. The delicate terminal sprouts of the pseudo-mycelium are not seen in the 'standard' colony where multiplication is by fission only.

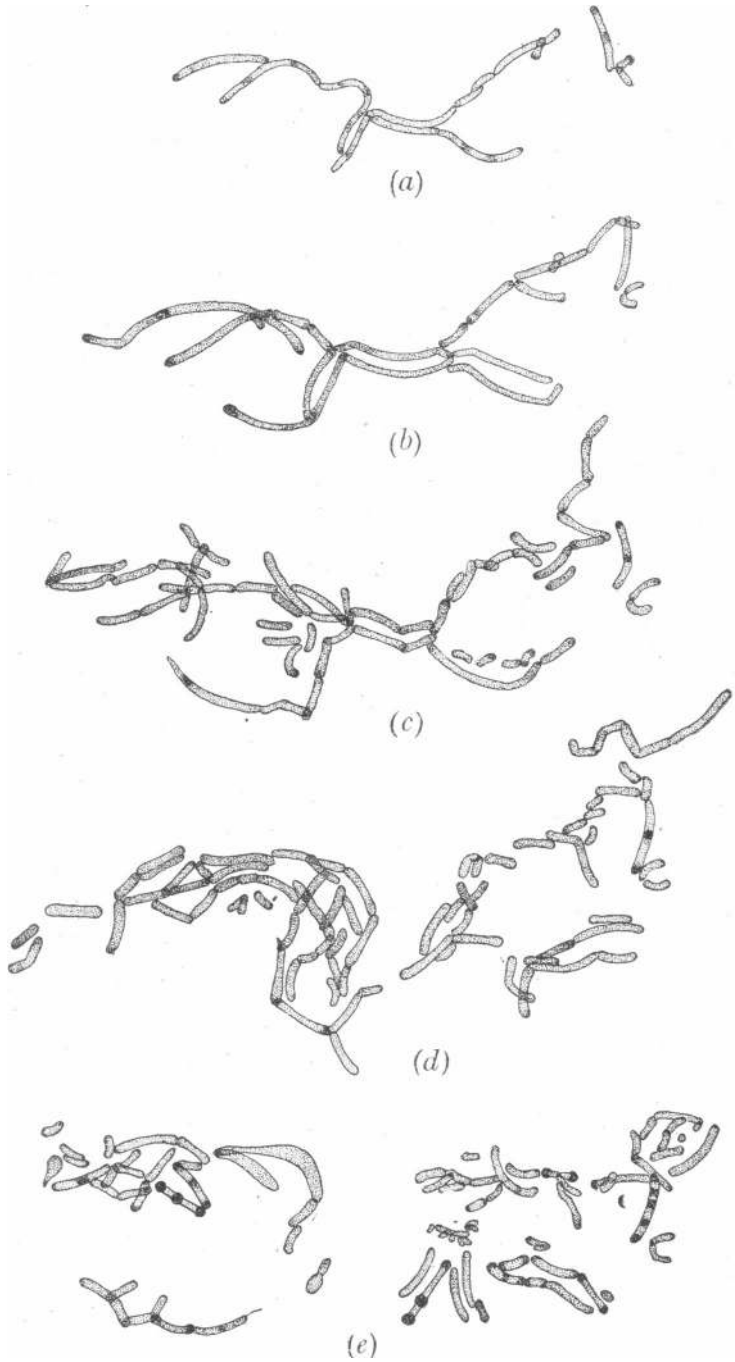


Fig. 3. The behaviour of a pseudo-mycelium when transferred to embryo extract of normal concentration (for details see p. 260). *a*. Part of a pseudo-mycelial colony from a culture grown in dilute extract for 23 days, immediately after transfer to normal extract. *b*. 40 hr. after subculture. The filaments are longer and thicker. Note the refractile ends of several of the organisms. *c*. 72 hr. after subculture. The filaments have begun to break down into short rods. *d*. 90 hr. after subculture. Disintegration into short rods is complete. *e*. 8 days after subculture. Most of the rods have fallen into the medium. Some of those still adherent to the glass are abnormal. Note that the pseudo-mycelium has reverted to the 'standard' type of development when placed in embryo extract of normal strength.

The pseudo-mycelia slightly resemble the so-called 'raft' colonies which develop in concentrated extract near the margin of the drop in the surface film. Thus many of the filaments in a 'raft' colony remain long indefinitely, and although simple fission is the usual form of reproduction, pin-like terminal sprouts are occasionally formed. On the other hand, after division the daughter bacilli fall apart so that 'raft' colonies do not acquire the branching structure so characteristic of the pseudo-mycelia.

Why cultivation in a poor medium should under certain conditions induce this curious branching growth is obscure. Very little organic material is needed for growth of this type and we have seen similar, but smaller and simpler pseudo-mycelial colonies develop in saline where the only organic matter present was that carried over with the original suspension. This lack of suitable nutritive substances may account for the fact that in stained preparations the bacilli of the older pseudo-mycelia, though mostly acid-fast, stain relatively faintly and are conspicuously banded or, especially in saline culture, beaded with black granules producing a very characteristic picture.

It is possible that the pseudo-mycelial form of growth may be due, at least in part, to some deficiency in the structure of the bacterial cell wall rendering the surface of the organisms less rigid and more viscid than normal; this point is being investigated further. That this type of growth is readily reversible is shown by the experiments in which pseudo-mycelial colonies were transferred to extract of normal concentration, when the bacilli reverted to the ordinary 'standard' type of development with the usual short period of filamentous proliferation followed by disintegration into short, discrete rods. The formation of the pin-like terminal buds and the cohesion of the products of division which characterize pseudo-mycelial growth in dilute extract were absent during this rejuvenation process in the more concentrated medium.

We conclude that the results of this study in conjunction with the work already described (Brieger & Fell, 1945) shed further light on the controversial question of whether the 'mycelial' form of the tubercle bacillus is a single individual with branches formed by true lateral budding, or a composite structure whose ramification is due to the terminal, angular growth of the coherent bacilli of which it is composed. Our observations on living cultures have shown that under appropriate experimental conditions both these branched forms are produced: the typical 'mycelium' of Metschnikoff which develops by 'true' lateral budding and could be regarded as a single individual, and the composite pseudo-mycelium whose branches are formed by the angular, terminal growth of independent but contiguous organisms.

## SUMMARY

1. Avian tubercle bacilli were grown in hanging-drop cultures in embryo extract prepared as for tissue culture, but diluted to 25–5 % of its usual concentration.

2. Microscopic branching colonies of pseudo-mycelia developed in the surface film of the hanging drops during the first few days of incubation, after which growth declined.

3. The development of these colonies was studied on the warm stage and proceeds as follows:

(*a*) The original rods elongate into filaments.

(*b*) The filaments multiply by ordinary fission and also by forming fine, pin-like terminal sprouts which grow into independent bacilli.

(*c*) After division the daughter filaments usually remain in contact at the site of fission; their terminal growth continues at an angle to the former cleavage plane and the colony thus acquires its characteristic branching structure.

(*d*) The colony enlarges and ramification becomes increasingly complex until about the fifth day of incubation when growth declines. The bacilli remain filamentous.

4. When transferred to embryo extract of normal concentration the pseudo-mycelial colonies revert to the 'standard' type of development characteristic of the more concentrated medium, i.e. they pass through a phase of filamentous proliferation followed by disintegration of the filaments into short, discrete rods.

5. The development of a pseudo-mycelium is compared with the three common types of growth previously seen in more concentrated embryo extract.

6. The results of this investigation shed light on the controversial question of whether the 'mycelial' form of the tubercle bacillus is a single individual formed by 'true' lateral budding or a composite structure produced by the terminal, angular growth of coherent but independent bacilli. Our observations on the living organisms have shown that under appropriate experimental conditions both these forms may develop.

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