WARM-STAGE OBSERVATIONS ON THE INITIAL DEVELOPMENT OF THE AVIAN TUBERCLE BACILLUS CULTIVATED IN EMBRYO EXTRACT

By E. M. BRIEGER (*Papworth Village Settlement*) and HONOR B. FELL*
(*Strangeways Research Laboratory, Cambridge*)

(With 4 Figures in the Text)

INTRODUCTION

In spite of the increasing literature on the life history of the tubercle bacillus, the mode of reproduction of the organism, especially in the tissues and fluids of a host, remains a source of controversy. This is particularly true of the avian tubercle bacillus of which Feldman (1938) remarks: 'Just what the developmental cycle of this...organism may be is problematic.' The usual opinion expressed in the text-books of bacteriology is that the tubercle bacillus 'multiplies by elongation and transversal division' (Stanley Griffith, 1930). This view, however, has been contested by many eminent research workers such as Vaudremer (1931), Much (1931), Karwacki (1934) and Fontes (1939).

Recent investigations on this subject fall into two categories: (1) direct observations on the development of individual living rods, and (2) studies of the initial growth stages in stained preparations made at daily or hourly intervals.

The method of direct observation has been applied to the development of individual tubercle rods in connexion with two problems: first to test Metschnikoff's theory of the 'sclerothrix' nature of the tubercle bacillus (Miehe, 1909) and more recently to re-examine Kahn's observations (1929) on the life cycle of the human bacillus (Gardner, 1929; Oerskov, 1931, 1932; Wyckoff, 1934; MacCarter & Hastings, 1934). The chief obstacle to such investigations, encountered by all previous authors, has been the very slow initial growth of the bacteria in the usual culture media. Oerskov (human and avian), Gardner (human), Wyckoff (cold-blooded type) and MacCarter & Hastings (avian) confirmed Miehe's findings that multiplication occurred only by the transverse fission of elongating rods, but Kahn (1932) thought that transverse division was so rare as to be negligible. He described a life cycle in which the bacillus passed through a coccal, granular and ultragranular (but not filter-passing, Kahn & Nonidez, 1936) phase and which was completed by the regermination of rods from granular particles.

The method of following initial growth and development in stained preparations has been applied by Besançon & Philibert (1924), Groh (1933), Nedelkowitch (1936) and Pryce (1941). Besançon & Philibert described as the first developmental stage in the surface film of Besredka's medium a 'substance cyanophile' without definite structural characteristics, within which appeared a network of mycelial growth with dark granules from which the bacillary phase originated. Nedelkowitch recorded two prevalent types of reproduction in pleural exudates: (1) by transverse division and fission, and (2) by spores (cf. Groh, 1933). Pryce showed that, in sputum smear cultures with defibrinated blood, development proceeded in stages including a branching stage with final subdivision into rods, but he suggested that the starting-point of growth was often a small group of granules.

All these observers used different materials and methods. Unless the conflicting accounts of the development of the tubercle bacillus are due to misinterpretation of observations, it appears that the bacillus varies its mode of development according to its environmental conditions.

Our own observations refer to the avian type whose life cycle has received comparatively little attention. One of us (E.M.B.) had been studying the development of avian and human tubercle bacilli in the chorio-allantoic membrane and amniotic fluid of the fowl embryo after intra-amnial infection, and had difficulty in interpreting the bacteriological pictures obtained. We therefore decided to investigate the early development of the avian bacillus when grown in an extract of embryonic fowl tissue and to see how it compared with development on standard artificial media. It was hoped that the results might shed light on some of the problems previously encountered.

It is known that embryonic mince is a good growth-promoting medium for tubercle bacilli (Soltys, 1942). For our experiments, however, we used a centrifuged saline extract of minced embryo like that employed in tissue-culture. This medium was found to possess two important advantages for

* Foulerton Research Fellow, Royal Society.

https://doi.org/10.1017/S0022172400013358 Published online by Cambridge University Press

our purpose: its optical properties are excellent for direct observation of the living organisms, and it encourages rapid and profuse initial growth.

It should be noted that all the direct observations on living tubercle bacilli quoted above, except those of Gardner who added serum to his glycerine agar, were made on organisms growing in solid or fluid artificial media. Initial growth is known to be poor in such media as compared with that in blood or egg media, and it cannot therefore be assumed that observations made under these conditions necessarily apply to development in biological media.

The profuse growth which was found to occur when avian tubercle bacilli were cultivated in hanging drops of embryo extract, has made it possible to study directly the development of individual bacteria undergoing rapid multiplication, and, since growth was not equally active in all parts of the cultures, to compare this rapid development with various slower types. We were also able to observe in the living material the development of the much disputed branching 'mycelial' forms. The results of these investigations are recorded in the present paper.

MATERIAL AND METHODS

*Bacterial strains**

The observations were made on two avian strains which in this communication are termed the 'Original' and the 'Lister' respectively.

The Original was obtained from Dr Stanley Griffith's laboratory in 1937. The culture tube had been sealed and kept in the incubator for 6 years, by which time the medium (Dorset egg) had dried considerably and the growth, creamy in colour, had assumed a filmy, greasy appearance. When the seal was broken and a smear made, the microscopical picture differed from the normal. There was great pleomorphism, but the most abundant forms were very strongly acid-fast granules or very short rods; non-acid-fast elements were extremely rare.

Subculture on Löwenstein's medium produced a fairly thick, moist, cream-coloured growth which appeared somewhat different from the routine subcultures of stock strains. The growth rate was normal and was only slightly increased by regular monthly subculture on Löwenstein's medium. Microscopical examination of the subcultures showed that they consisted mainly of acid-fast rods with an unusually large proportion of 'branching' forms; very few non-acid-fast elements were present.

The pathogenicity of the Original strain for fowl and rabbit was very high in spite of the old age of the parent culture (cf. Stanley Griffith, 1941; Saenz, 1938).

* We acknowledge with thanks the help received from Mr Boot, senior technical assistant, Papworth.

The Lister strain was obtained from the National Collection of Type Cultures, Lister Institute. It was received on glycerine agar and appeared as a dry, wrinkled, filmy growth, creamy in colour. When subcultured on Löwenstein's medium, it grew slowly at first, but after 3–4 generations assumed the normal growth rate. The cultures contained a large proportion of non-acid-fast elements.

When injected into fowls, both strains proved fatal, but the pathogenicity of the Original was rather greater than that of the Lister.

Unless otherwise stated, the observations recorded in this paper were made on the Original strain, the Lister being used as a control.

In these experiments no attempt was made to dissociate the rough and smooth types.

Culture methods

Suspensions were made in the usual way: a small proportion of the growth was rubbed up in saline and the emulsion was lightly centrifuged to remove any coarse particles. The supernatant fluid was then poured into another tube and was ready for use. No attempt was made to observe any standard of strength, simply a milky suspension being aimed at. The cultures and subcultures of the Original strain were easily emulsified as were the subcultures of the Lister strain, but the Lister culture when first received was difficult to bring into suspension.

To prepare the embryo extract, an 11-day fowl embryo was removed aseptically from the egg, the eyes were rejected to avoid the presence of pigment rods in the medium, and after the chick had been broken into large fragments it was well washed with Pannett and Compton's saline. The tissue was then finely minced with curved scissors, transferred to a $2 \times \frac{3}{4}$ in. diameter centrifuge tube and ground up with a glass rod. Pannett & Compton's saline was added in the proportion of about two parts of saline to one part of embryo pulp; the mixture was well stirred and centrifuged for 5 min. The opalescent supernatant fluid was used as culture medium.

Hanging drop cultures were made by placing a loopful of the bacterial suspension on a $1\frac{1}{4}$ in. square cover-slip, adding 0·03 c.c. embryo extract and then spreading the drop with a platinum wire into a circle about 0·5 in. diameter. The cover-slip was inverted on to a $3 \times 1\frac{1}{2}$ in. hollow-ground slide and heavily sealed with molten paraffin wax. For subsequent warm-stage observations, the culture was turned over and allowed to stand with the cover-slip downwards for about 2 hr. at room temperature, so that sufficient bacteria might be deposited on the glass.

Observation

The inverted culture was turned to bring the cover-slip upwards and firmly clipped to the stage of a microscope enclosed in a hot-box kept at 38·5–

40° C. Observations were made with a Beck 2 mm. apochromatic oil-immersion lens and a × 10 ocular. The objective was lifted and cleaned after each observation without disturbing the culture, and the oil was wiped from the cover-slip with a camel-hair brush dipped in xylol. In the early stages of growth the culture was usually examined twice daily, but later only once a day or on alternate days.

The changes seen were recorded by pencil drawings. At first this was done without measurement, but most of the drawings, including all those reproduced in this communication, were made with the aid of an eyepiece micrometer in the following way. An arbitrary scale of suitable size was made on a strip of paper, each division of the scale being taken to correspond with one division of the micrometer. The organisms were measured with the micrometer and drawn to the same measurements on the arbitrary scale, so that a constant magnification was maintained throughout the investigation. The chief dimensions of each group of organisms and as far as possible each member of the group were measured. Plotting and drawing a complicated field in this way often entail several hours of intensive work.

The figures shown in the plates are from tracings of the original pencil drawings. Unfortunately, limitation of space has made it impossible to reproduce the complete set of pictures in any series.

Fixation and staining

The specimens were fixed in osmium vapour, dried and transferred to 80% alcohol; they were bleached if necessary in a solution of peroxide and 80% alcohol in equal parts until any blackish, brownish colour had disappeared. They were then rinsed with water, dried, and the paraffin wax from the cover-slip was removed by holding it over a hot-plate and wiping off the molten wax. There should be no traces of paraffin wax on the cover-slip and, of course, none on the smear.

The cover-slips were placed face upwards in small watch-glasses on a hot-plate, covered with carbol-fuchsin (Ziehl-Neelsen) and left staining for 5 min. After being rinsed in water they were decolorized by placing them for a few seconds in 15% sulphuric acid until the smear stained a blackish brown; they were then transferred to 70% alcohol for 2 min. After being rinsed in water they were counterstained by Loeffler's methylene-blue solution.

RESULTS

A. *The behaviour of bacilli transferred from artificial medium into embryo extract*

(1) *General.* The bacilli grow much more actively in the surface film and near the margin of the drop than on the glass in the interior of the culture where they are covered by a relatively deep layer of fluid. On the glass, towards the centre of the culture, growth is rather scanty, irregular and diffuse, but it is more abundant near the margin, where the overlying film of fluid is shallower. In the film of fine cell debris deposited at the surface of the drop float many long, twisted, skein-like colonies and also large masses of bacteria not arranged in well-defined groups. The most compact colonies in the cultures are those at the extreme periphery of the drop. Here the amount of fluid varies slightly, so that the bacilli sometimes rest on a solid but moist surface and at other times are temporarily lifted from the glass by the small condensation droplets which often form on this partly dried peripheral zone in response to small temperature changes. At first such colonies grow in one plane to form a sheet of bacteria which drifts about in a condensation droplet as a single raft, but as multiplication continues the organisms become heaped up to form a flattish mound.

'Mycelia' occur throughout the culture, but they too grow more luxuriantly at the surface and periphery of the drop than on the glass in the centre. Some colonies consist entirely of 'mycelia', but most are composed of a central mass of rods or shorter filaments from which mycelial branches protrude.

(2) *The 'standard' life cycle.* Continuous observations on the growth of individual bacilli show that the widely different forms present in the cultures all develop by variations of the same life history. For convenience we have arbitrarily chosen as the standard for comparison the commonest form of development, in which also the different phases of the life cycle are the most clearly defined.

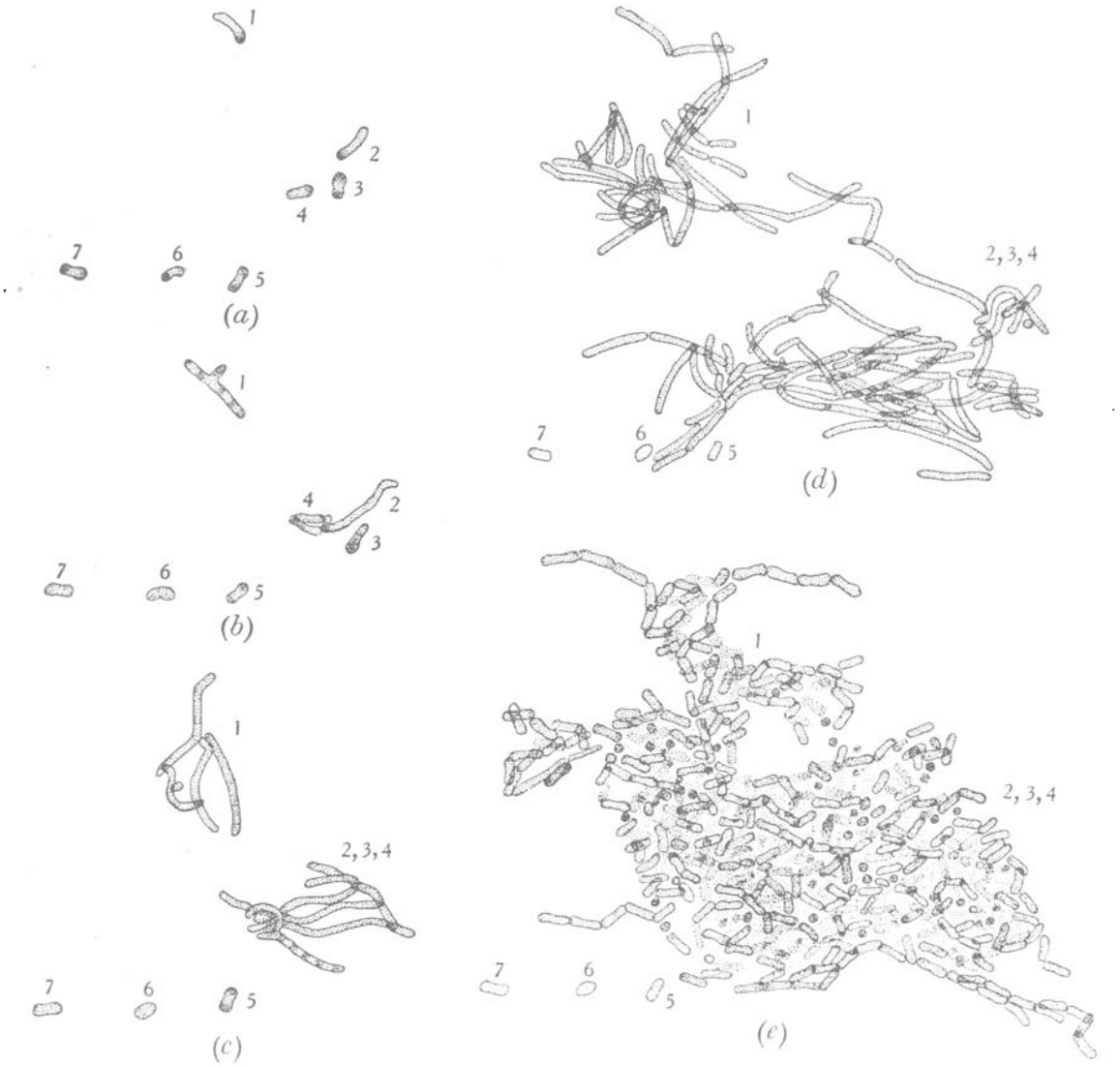
If a small group of bacilli resting on the cover-slip near the margin of the drop, where they are covered by a fairly thin layer of fluid, are periodically studied on the warm stage (Fig. 1), it is seen that development is very rapid during the first few days, although the rate may vary in different members of the same group and a minority do not grow at all (cf. 5, 6, 7, Fig. 1 *a–d*).

The growth cycle begins with a rapid elongation of the bacillus to form a long, curving filament which after 15 hr. incubation (Fig. 1 *b*) may be three or four times the length of the original rod. One of the filaments shown in Fig. 1 *b*, had produced a small side branch at this stage, but this was exceptional. Each filament then bends at one or more points, which sometimes involves the rotation of one end through nearly 180°, and breaks at the angle of the bend. The daughter filaments elongate, bend and break in the same way. During this period a faint transverse banding (Fig. 1 *b*, *c*) is distinguishable in some of the bacteria.

The actual process of division has been seen several times and is surprisingly rapid. The filament becomes sharply bent, the ends approaching each

other quite quickly, a break appears at the angle of the bend and segmentation is completed in 15–30 min. This rapid bending and breaking somewhat resembles the 'snapping' post-fission movements of diphtheria-like bacteria described by previous workers (cf. Diagram 8 H, Graham-Smith, 1910). Curvature of a filament, however, may appear quite slowly and does not necessarily imply that segmentation is imminent, though pronounced curvature is usually followed eventually by fission. The twisting and contortion displayed within a few hours by a group of proliferating filaments are remarkable.

As multiplication continues, the average length



H. B. F. del.

Fig. 1. The 'standard' type of development. This series of drawings shows a group of seven bacilli situated on the glass near the margin of the drop; four of them (nos. 1–4) underwent the 'standard' development, but the remaining three (nos. 5–7) did not grow during the period of observation. (*a*) The seven rod-shaped bacilli at the beginning of observation. (*b*) After 15 hr. incubation. Nos. 1–4 have grown into filaments and no. 1 has formed a small side-branch (this is exceptional). (*c*) After 39 hr. incubation. The filaments are now several times longer than the original rods and have multiplied. (*d*) After 63 hr. incubation. Filamentous proliferation has continued; note the large group derived from a single bacillus (no. 1). (*e*) After 88 hr. incubation. The initial stage of rapid filamentous growth has ended and the threads have broken down into short rods. (Reproduced at $\frac{2}{3}$ of the original magnification.)

https://doi.org/10.1017/S0022172400013358 Published online by Cambridge University Press

of the bacteria tends to lessen. Filamentous growth reaches its height after 2–3 days when a single rod-shaped bacillus may have produced a bunch of thirty or more filaments (Fig. 1, cf. *a* 1 and *d* 1). Then, within a few hours, each organism becomes sharply bent at several points, the average distance between the bends being the average length of the original small rods. Breaks occur at the angles thus formed, and usually by the end of the 4th day the bunch of filaments has disintegrated into a chaotic mass of short rods (Fig. 1 *e*).

From now onwards the growth rate declines. Growing bacilli no longer form long, segmenting filaments but merely elongate to twice their normal length and divide in two. As the mass of rods enlarges, many become detached and fall into the drop, so that the growth on the glass is more active than the actual size of the colonies suggests.

The above observations were repeated on hanging drop cultures from the Lister strain. The bacilli developed almost exactly like those of the Original strain but were much more clearly 'banded' during the stage of filamentous proliferation.

These observations on the living bacilli were confirmed by a study of control preparations of sister cultures fixed and stained at different stages of growth. After 24 hr., cultures of the Original strain consist largely of filaments 2–5 times the average length of the rods in cultures fixed immediately before incubation. The filaments are strongly acid-fast and some show broad, acid-fast bands alternating with lighter bands, which probably correspond with the alternating regions of more and less refractile material seen in some of the living organisms at this stage. By the end of the 2nd day the average length of the filaments has much increased and rod-shaped bacilli are comparatively rare. In 3-day cultures the filaments have multiplied greatly and form micro-colonies, but they are rather shorter than before. Between the ends of the 3rd and 4th days the filaments in most of the colonies have broken down into acid-fast rods, though many filamentous groups remain. In the older cultures big colonies of very small rods and coccal forms appear in addition to masses of larger rods and filaments, but their formation has not yet been studied in the living material.

(3) *The development of slowly growing bacilli.* As stated above, growth is least active on the glass in the interior of the culture where the bacilli are covered by a relatively deep layer of fluid. Three different but related forms of development have been noted in this region.

In one series of observations (Fig. 2) two of these forms could be watched in the same field. For this experiment four small, rather widely separated rods had been selected for study. Two rods (not figured) failed to grow during the period of observation (14 days).

Of the remaining two bacilli one (Fig. 2 *a* 2) elongated rapidly and about trebled its length during the first 16 hr. incubation. After 23 hr. (Fig. 2 *b*) this filament was longer still, and one end had bent downwards at an angle to the glass. By the 41st hour (Fig. 2 *c*) the two arms had broken down into eight small rods of about the same size as the original bacillus. These multiplied slightly by ordinary binary fission and a few fell off the glass into the medium, but proliferation was scanty during the rest of the culture period.

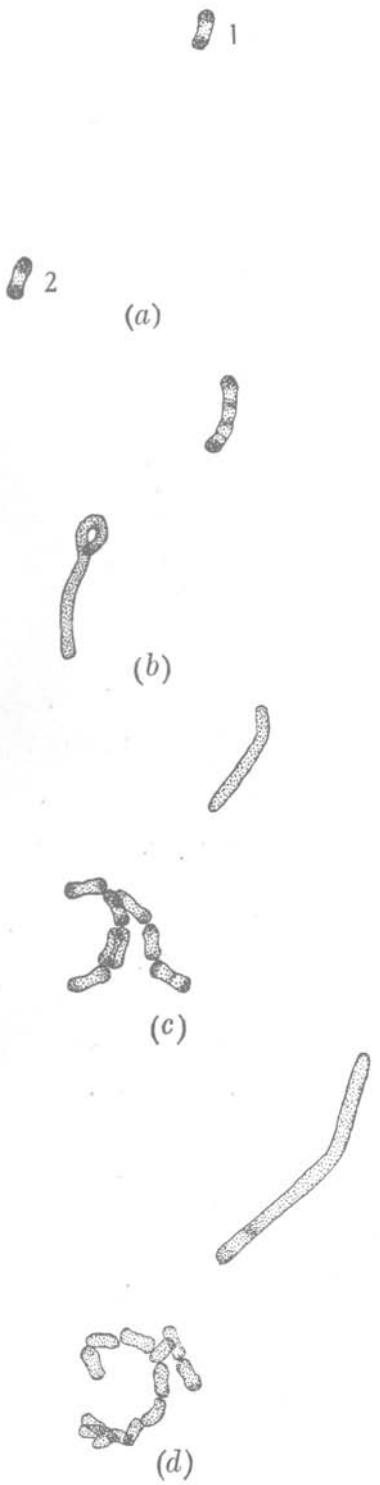
The other bacillus (Fig. 2 *a* 1) elongated but did not divide. It grew much more slowly than the organism described above, and after 23 hr. incubation (Fig. 2 *b*) was less than a third the length of the latter, from which it also differed in showing well-marked banding. The bacillus continued to elongate slowly until by the end of the 7th day (Fig. 2 *d*) it was nearly seven times its original length. Growth ceased soon after this and no further change was seen.

The third type of development is simple binary fission, like that which takes place in the 'standard' life cycle after the segmentation and disintegration of the filaments, but which in our cultures was rare during the first 2–3 days. On one occasion when this was observed, the rod slowly elongated during the first 2 days and then divided into two; though watched for another 4 days it underwent no further change.

It is interesting to compare these three life histories with the 'standard' type. In the first form of slow growth described, the bacillus elongates and later segments as in the 'standard' development, but the intermediate stage of filamentous proliferation is greatly curtailed; in the second type the bacillus elongates, though very slowly, but division is completely inhibited; in the third type there is only slight elongation but one division occurs.

(4) *The development of a marginal 'raft' colony.* As described above, at the extreme margin of the culture a form of colony appears which differs somewhat from the 'standard' type, and which we have termed the 'raft' type. Such colonies are not attached to the glass but float freely in the surface film of the medium; this renders continuous study rather difficult, as the fluctuating depth of the fluid in the peripheral zone (cf. p. 160) sometimes carries the colonies below the working distance of the objective.

One colony (Fig. 3) was observed for nearly 3 days, when it sunk below the range of the lens. It developed from a compact, well-isolated group of five small rods (Fig. 3 *a*). During the first 22 hr. the rods elongated to about twice their original length without multiplication (Fig. 3 *b*), but division



H. B. F. del.

Fig. 2.

began soon after this period and by the 39th hour (Fig. 3 *c*) thirteen filaments had been formed, many of them 4–5 times the length of the original bacilli. The filaments, which lay in a single plane, varied in width, some being quite stout and one almost thread-like, while a few varied in thickness in different regions; the ends of several were slightly bulbous and more refractile than the 'shaft'.

Nine hours later about thirty-four rods and filaments had been formed, and after 63 hr. (Fig. 3 *d*) about eighty-seven. Most of the peripheral organisms in the 63 hr. colony were still several times as long as the original rods, but those in the interior were mostly shorter and thinner than the peripheral ones.

At this stage the colony was lost for the reason mentioned above, but observation was resumed on a similar 3-day-old colony in a sister culture, which was studied for a further period of 15 days.

This colony, at first composed of four filaments, grew rapidly until the 9th day of incubation, after which its area enlarged more slowly. Growth, however, was greater than at first appeared, as by the 11th day a second layer of rods had been formed on top of the tightly packed original layer. The average length of the bacteria declined as the age of the colony advanced, but there was no rapid breakdown into short elements as in the 'standard' life cycle, and throughout the period of observation most of the bacilli remained considerably longer than the short rods of the original suspension. At about the 11th day these long rods began to display a banded appearance which became increasingly distinct. By the 18th day of cultivation some of the organisms had become detached from the interior of the colony and drifted away, leaving gaps in the 'raft'.

As described above, such peripheral colonies sometimes grew to a fair size, forming a rounded, convex mound of rods on the cover-slip.

If the development of a 'raft' colony is compared with the 'standard' life cycle, it is seen that while elongation and filamentous proliferation occur in both, the return to the original short form is more gradual and less complete in the former than in the latter. The habit of growth also differs. In the young 'raft' colony the organisms multiply in one plane and become arranged in a tidy mosaic; they show

Fig. 2. Two forms of retarded growth. (*a*) The two rods at the beginning of observation. (*b*) After 23 hr. incubation. No. 1 has only doubled its length while no. 2 has formed a long, curved filament. (*c*) After 41 hr. incubation. No. 1 has elongated still more, but no. 2 has already segmented into short rods; the intermediate stage of filamentous proliferation has been greatly curtailed. (*d*) After 7 days' incubation. No. 1 now forms a single long filament; in no. 2 the rods have multiplied slightly by ordinary binary fission. (Reproduced at the original magnification.)

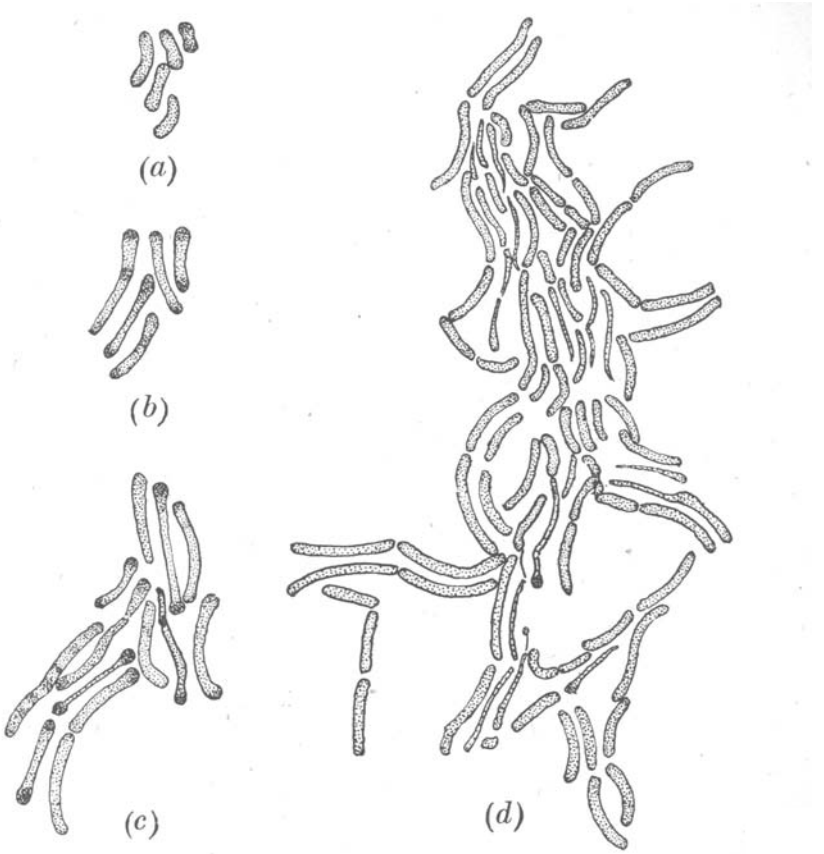
https://doi.org/10.1017/S0022172400013358 Published online by Cambridge University Press

none of the twisting and contortion so characteristic of the 'standard' life cycle, and when dividing their snapping movements, probably for mechanical reasons, are usually less pronounced.

Raft colonies are easily identified in the fixed and stained cultures. In preparations fixed during the early stages of growth they appear as orderly groups of filaments many of which have slightly swollen, deeply stained ends, corresponding to the appearance seen in the living organisms. In rather older cultures the raft colonies are seen as shallow mounds of unusually long rods.

(5) *The development of the 'mycelial' form.* 'Mycelial' forms appeared in cultures of both strains, but, as stated above, in the Original strain the proportion was unusually high so that the development of 'mycelia' could be studied in this material without much difficulty. These forms originate as follows.

The long filaments formed by the elongation of the rod-shaped bacilli do not always break up at about the 3rd day into series of daughter rods, but a minority continue to elongate beyond this critical period and attain several times their normal length. When these hypertrophic filaments are observed on the warm stage they are found to represent the first stage in the development of a 'mycelium'. The subsequent history of three such filaments has been studied in detail.



H. B. F. del.

Fig. 3. The development of a 'raft' colony. (*a*) Five bacilli floating in the surface film near the margin of the drop, drawn at the beginning of observation. (*b*) After 22 hr. incubation. The rods have elongated into filaments. (*c*) After 39 hr. incubation. The filaments are proliferating. (*d*) After 63 hr. incubation. The marginal organisms are still several times longer than the original rods, but those in the interior are mostly shorter and thinner than those at the periphery. There is no sudden breakdown into rods as in the 'standard' life cycle. (Reproduced at the original magnification.)

The organism shown in Fig. 4 was observed from the 48th hour of incubation. When first seen it was a single, sharply banded filament about three times the usual length (Fig. 4 *a*). Sixteen hours later it

https://doi.org/10.1017/S0022172400013358 Published online by Cambridge University Press

had more than doubled its length and produced four branches. The very early rudiment of such a branch was seen during an observation made on the 3rd day of incubation (Fig. 4 *b*, *x*), when a minute, refractile bulge had appeared on the main stem distal to the other branches; during the next 15 hr. (Fig. 4 *c*, *x*) this lengthened into another branch.

At this stage, i.e. after 3¾ days' incubation, the 'mycelium' consisted of a long, curved main stem and eight primary branches. Secondary branches had now begun to form as tiny, refractile buds on three of the primary arms (Fig. 4 *c*, *y*). By the 5th day (Fig. 4 *d*) the 'mycelium' had nearly reached its maximum size and complexity, having formed

Fig. 4. The development of a 'mycelial' form. (*a*) After 2 days' incubation. An abnormally long filament is seen which proved to be a young 'mycelium'. Note the transverse banding. (*b*) After 3 days' incubation. The thread has acquired four primary branches and the bud (*x*) a fifth. (*c*) After 3¾ days' incubation. More primary branches have developed and the bud (*x*) seen in (*b*) has elongated. Secondary buds (*y*) are now seen on three of the branches. (*d*) After 4¾ days' incubation. The 'mycelium' has now reached its maximal development with a complex system of primary, secondary and (*z*) tertiary branches. (*e*) After 5¾ days' incubation. Part of the 'mycelium' is almost transparent and everywhere its refractivity is much reduced. Note the intensely refractile globules which seem to be derived from some of the transverse bands seen at earlier stages. The 'mycelium' underwent no further change. (Reproduced at ⅔ of the original magnification.)

https://doi.org/10.1017/S0022172400013358 Published online by Cambridge University Press

eleven primary and fourteen secondary branches with small tertiary buds on two of the latter (Fig. 4 *d, z*). The banded structure seen in the original filament was still distinct except in the upper part of the main stem which showed traces of two bands only and for part of its length was highly refractile and almost homogeneous. Within a few hours, however, this part of the main stem and its associated branches had almost lost their refractivity and by transmitted light looked much paler than the rest of the 'mycelium'.

Twenty-four hours later (Fig. 4 *e*) the whole 'mycelium' had become less refractile; many of the bands had vanished, and here and there they had been replaced by brilliant globules. The non-refractile part seen at the preceding stage was now almost transparent and in places considerably swollen.

Growth now ceased, and the only visible change was the continued decline in refractivity and the increasing distinctness of the globules. Observation was discontinued on the 17th day of incubation.

Another 'mycelium' was studied for 22 days. Observation began on the 3rd day when the organism was a simple thread about twice the length of a normal filament; the banded appearance was less marked than in the 'mycelium' described above. The first change was seen 7 hr. after the beginning of observation when the filament had become sharply bent. When examined on the 4th day it had broken at the apex of the bend into two daughter filaments lying at an acute angle to each other. These elements continued to thicken and to lengthen and a banded structure became distinguishable.

By the 6th day one filament had developed one primary branch and the other two branches. During the next 4 days the branches multiplied and lengthened and the whole 'mycelium' continually twisted and contorted so that its shape changed considerably every few hours. Such violent changes in form were not seen in the 'mycelium' shown in Fig. 4.

The transverse bands in both the main stem and its branches had become pronounced by the end of the 7th day and certain bands were particularly bright. These refractile areas increased in size and number during the next few days, but in general the banded appearance began to fade and by the 10th day was indistinguishable in some parts of the 'mycelium'.

From the 10th day growth declined and the twisting and turning of the branches diminished in activity. The organism, like that described above, became ever less refractile, and the bright bands appeared as brilliant globules either in the tips of the branches or distributed here and there along their length.

The third 'mycelium' was observed from the 41st hour of incubation, when it was a single filament only a little longer than normal, until the end of the 3rd day when it fell off the glass into the drop. During this period it formed three primary branches and, though it did not divide, underwent much twisting and bending.

When 'mycelial' development is compared with the 'standard' life cycle, it is seen that the stage of filamentous growth has been indefinitely prolonged while division has been completely (first 'mycelium') or partially (second 'mycelium') suppressed.

In the fixed and stained preparations, young mycelia of simple form are present at the end of the 2nd day; most are strongly acid-fast with the typical banded appearance, but every grade of staining reaction from deep red to grey or blue is seen. The 'mycelia' are larger, more complex and more numerous in 3-day cultures and vary widely not only in their staining reaction but also in the diameter of their branches which are usually thickest in the most intensely acid-fast individuals. By the 5th day, though some of the 'mycelia' remain strongly acid-fast, most stain more faintly than before and often have small dark granules spaced along their branches. As the age of the cultures advances, the acid-fastness of the 'mycelia' continues to decline and the dark granules become larger, more conspicuous and occur in more individuals.

These results accord well with the observations on the living 'mycelia'; the gradual loss of acid-fastness probably corresponds with the loss of refractivity described above (cf. Fig. 4 *a–e*), and the deeply stained granules are almost certainly identical with the refractile bodies which appear in the living organisms (cf. Fig. 4 *d, e*).

B. *The behaviour of bacilli grown in embryo extract when transferred to fresh extract*

Cultures which had been grown in strong embryo extract for 24 days were opened and a loopful of the contents transferred to each of a series of fresh cover-slips to which fresh extract was then added.

In another experiment similar subcultures were made from cultures which had been grown for 85 days in rather less concentrated extract than that used in the previous experiment.

(1) *General.* In both experiments the subcultures grew actively, though growth was rather more rapid in the first experiment, in which the parent culture was younger and the embryo extract of the subculture rather stronger, than in the second.

The general appearance of the subcultures resembled that of the parent cultures at equivalent stages of growth except in one particular. 'Mycelial' forms, which were abundant in the parent cultures, were extremely rare in the subcultures and those few that were present appeared to have been carried over from the original stock and not to have developed anew.

(2) *The behaviour of rods and a 'mycelium' in a first subculture.* A first subculture from the 24-day cultures used in the first experiment was placed on the warm stage and a field comprising fourteen rods of various lengths and a small 'mycelium' was studied for 5 days.

After 15 hr. incubation the rods had already lengthened greatly and two had each divided into two daughter filaments. Some of the filaments had a distinct banded appearance. During the next 9 hr. the bacilli continued to elongate and divide and some became very refractile at the ends, which probably corresponded to the terminal staining seen in the fixed preparations. The only visible change in the 'mycelium' was slight growth and alteration in form of some of the branches.

On the 2nd day the filaments began to break up in the usual way into short rods which continued to multiply rapidly. After 5 days' incubation a large mass of rods had been formed which had partly merged with neighbouring colonies, though one well-isolated bacillus had formed a small, independent group of descendants.

The 'mycelium' underwent no further change during the period of observation.

From these observations it is seen that subculture in fresh embryo extract induces a return to the 'standard' type of life cycle which characterized the bacilli when they were originally transferred to embryo extract from Löwenstein's medium.

DISCUSSION

Avian tubercle bacilli obtained from two different strains show two main types of reproduction when cultivated in embryo extract: what we have termed the 'standard' type and a 'mycelial' type.

The 'standard' type of life cycle proceeds in four phases: (1) during the first 24 hr.: elongation of the rods into a filament several times the length of the original bacillus; (2) during the 2nd and 3rd days: the rapid segmentation and proliferation of this filament to form a bunch of daughter filaments (cf. Nedelkowitch's 'colonie rudimentaire'); (3) at about the end of the 3rd day: disintegration of the filaments into short rods forming a typical micro-colony; (4) slow multiplication of the rods by binary fission which continues indefinitely.

The 'standard' type of development fits into the general scheme of bacterial multiplication by transverse fission (Miehe, Gardner, Oerskov, Wyckoff, MacCarter & Hastings); but while in bacteria of the typhoid or coli group the parent organism normally elongates and then divides into two individuals of equal length, the tubercle bacillus behaves more like *Bact. aerogenes* in which, according to the direct observations of Kelly & Rahn (1932), 'one cell divides into four cells during the stage of fastest growth'.

As described above not all the rods develop into micro-colonies, an observation which agrees with Wilson & Schwabacher's recent results (1937). Some organisms remain unchanged, others (Fig. 2, 1) elongate slowly into long filaments which never divide (similar filaments have been described by Nedelkowitch in the initial stages of his exudate cultures), while others again nearly double their length and then divide once.

Another modification of the 'standard' form of reproduction produces a micro-colony, but the component rods are of more than average length. This is seen in the so-called 'raft' colonies (Fig. 3) in which elongation and division both occur but the filaments do not suddenly break down into rods of the original size. Colonies of a similar type have been described by MacCarter & Hastings and by Gardner.

The size of the individual organisms in a colony probably depends on the relationship between rate of elongation and rate of segmentation. Oerskov explains the fact that in general the length of the rod diminishes with age on the biological principle that elongation is predominant and vigorous in youth and then declines. If elongation becomes increasingly restricted while segmentation follows its normal course, smaller and smaller segments will result. Wyckoff considers that the micro-rods and coccal forms seen in his cinema films developed in this way and Kahn & Nonidez (1936) agree that the granules, which form an intermediate stage of the life cycle in micro-droplets of Long's medium, are minute segments resulting from continued cell division after arrested elongation.

The fact that, in the filamentous stage of the 'standard' life cycle, organisms which are about to divide usually become sharply bent at the point of fission (see p. 160) may explain the 'angular' growth which Oerskov (1923) observed microscopically in living cultures of avian tubercle bacilli on glycerine agar. In our embryo extract cultures, a similar configuration was sometimes seen in colonies growing in the surface film of the hanging drop, where the mechanical conditions would most nearly resemble those obtaining on the surface of a solid medium.

Oerskov regards this 'angular' habit of growth as characteristic of a group of 'branching fungi' (*Actinomycetes*), to which he therefore considers that the tubercle bacilli belong.

The fact that the tubercle bacillus may grow not only in a bacillary but also in a 'mycelial' form has caused much speculation about its place in the system of Fungi. It has been classed as '*Sclerothrix*' (Metschnikoff), *Mycobacterium tuberculosis* (Lehmann, Neumann & Breed, 1919) and, as stated above, among the *Actinomycetes* by Oerskov (1923).

An interesting feature of our embryo extract cultures was the presence of many 'mycelial' forms.

They developed during the initial stages of cultivation when a minority of the filaments formed during this period instead of segmenting into separate rods in the usual way, continued to lengthen and produce lateral buds which gave rise to a complex system of primary, secondary and tertiary branches. These 'mycelia' are identical with the forms first described by Metschnikoff (1888) and studied by Coppen-Jones (1895) and Bruns (1895). The appearance of lateral buds is extremely rare in the 'standard' life cycle and our results indicate that the 'mycelial' form is not necessarily a stage in ordinary reproduction.

It is important to distinguish between pseudo-mycelia and true branching forms. In (unpublished) observations on the behaviour of tubercle bacilli in very dilute embryo extract, we saw large colonies of what under low-power appeared to be 'mycelia', but which on closer examination proved to be pseudo-mycelia like those described by Miehe and by MacCarter & Hastings; we have seen similar forms in saline and in Long's medium. These pseudo-mycelia consist of branching colonies of attached but distinct filaments and not of a continuous system like the true 'mycelia'. Their development in living cultures has been studied and will be described elsewhere.

The results recorded in this paper differ conspicuously from those obtained by Kahn (1929) on the human type. This is probably due partly to the versatility of the tubercle bacillus which is able to vary so widely its mode of reproduction and habit of growth according to the environment in which it is growing, and perhaps partly to the fact that our observations refer to the avian and not the human type.

Kahn's medium and cultural conditions were very different from those we employed. Preliminary observations have been made on avian tubercle bacilli cultivated in Long's medium, the same type of large hanging-drop preparation being used as was employed for the embryo extract cultures so that growth in the two media might be compared under identical conditions. The initial processes of growth in these cultures are not yet clear, but there can be no doubt that the mode of reproduction is different from that seen in embryo extract, egg yolk water, blood media, sputum smear cultures, Löwenstein's medium or even glycerine agar. We could find no evidence of what we have termed the 'standard' type of development, which probably occurs only when germination is rapid, or of true 'mycelia', though, as mentioned above, pseudo-mycelia appeared; initial growth was far less profuse than in embryo extract. In Kahn's experiments it is probable that the life history of the bacilli was still further modified by their isolation in micro-droplets, which is not an ideal method of cultivation.

Whether embryo extract has a specific stimulating effect on the initial growth of the bacilli cannot be decided until the behaviour of the organisms in other favourable media has been carefully studied during the earliest stages of cultivation.

The cytological mechanisms of the various forms of reproduction we have described and their relationship to the reproduction of other organisms are now being investigated and will be considered in another communication.

The observations recorded in this paper are by no means a complete survey of all the phenomena which occur in the embryo extract cultures. We have merely described the changes most commonly seen during the early growth period, and further study may show that some of the forms of reproduction described by previous authors but which we have not yet encountered in the living cultures may also occur in our material, possibly in later stages of cultivation.

SUMMARY

1. Avian tubercle bacilli were cultivated in centrifuged embryo extract in large hanging-drop preparations.

2. Small groups of individual bacilli were studied undisturbed on a warm stage for several days.

3. The cultures grew very actively and several types of reproduction were observed, all of which were modifications of what was arbitrarily termed the 'standard' type.

4. In the 'standard' life cycle, which was that most commonly seen, the bacillus elongates during the first 24 hr. to form a filament several times its original length; this divides repeatedly to form a bunch of 20–30 filaments which on the 2nd or 3rd day break down into short rods, which continue to multiply slowly by ordinary binary fission. If the bacilli are subcultivated in fresh embryo extract the cycle is repeated but not otherwise.

5. Three forms of slow growth were seen: (*a*) The bacillus elongates, though very slowly, to form a long filament which does not divide. (*b*) The bacillus elongates to about twice its original length and then divides by simple binary fission. (*c*) The bacillus elongates and segments into short rods, but the intermediate stage of filamentous proliferation is greatly restricted.

6. Bacilli floating freely in the surface film of the drop often form what we have termed a 'raft' colony. In this form of development elongation of the rods and filamentous proliferation occur, but the return to the original short form is gradual and incomplete. The organisms grow in one plane and are arranged in a tidy mosaic.

7. The development of 'mycelial' forms was studied. A minority of the long filaments formed by the elongation of the rod-shaped bacilli do not

break up into strings of daughter rods but continue to elongate rapidly into abnormally long threads; small buds appear at intervals along the thread and grow into primary branches on which secondary and tertiary branches form in the same way: finally a complex branching 'mycelium' is produced.

8. The 'standard' life cycle appears to be characteristic of rapid initial growth. The wide differences between our results and those of Kahn are probably due, at least in part, to the different cultural conditions used in the two investigations.

REFERENCES

BESANÇON, F. B. & PHILIBERT, A. P. (1924). *C.R. Soc. Biol., Paris*, **90**, 475.

BRUNS, H. (1895). *Zbl. Bakt.* **17**, 817.

COPPEN-JONES, A. (1895). *Zbl. Bakt.* **17**, 76.

FELDMAN, W. H. (1938). *Avian Tuberculosis Infections*, p. 35. London.

FONTES, A. C. (1939). *Internat. Congr. for Microbiol., N.Y.*, 1940, p. 157.

GARDNER, A. D. (1929). *J. Path. Bact.* **32**, 115.

GRAHAM-SMITH, G. S. (1910). *Parasitology*, **3**, 17.

GRIFFITH, STANLEY (1930). *A System of Bacteriology in Relation to Medicine*, **5**, 156. London.

GRIFFITH, STANLEY (1941). *J. Hyg., Camb.*, **41**, 530.

GROH, E. (1933). *Zbl. Bakt.* **128**, 353.

KAHN, MORTON C. (1929). *Amer. Rev. Tuberc.* **20**, 150.

KAHN, MORTON C. (1932). *Zbl. Bakt.* **125**, 451.

KAHN, MORTON C. & NONIDEZ, J. F. (1936). *Amer. Rev. Tuberc.* **34**, 3.

KARWACKI, L. (1934). *Proc. Conf. Intern. Union against Tuberc., Varsovie.*

KELLY, C. D. & RAHN, O. (1932). *J. Bact.* **23**, 147.

LEHMANN, K. B., NEUMANN, R. & BREED, R. S. (1919). *Text-book of Bacteriology*. Philadelphia.

MACCARTER, J. R. & HASTINGS, E. J. (1934). *J. Bact.* **27**, 41.

METSCHNIKOFF, E. (1888). *Virchows Arch.* **113**, 63.

MIEHE, H. (1909). *Z. Hyg. InfektKr.* **62**, 131.

MUCH, H. (1931). *Beitr. z. klin. d. Tuberk.* **77**, 60.

NEDELKOWITCH, J. (1936). *Ann. Inst. Pasteur*, **57**, 171.

OERSKOV, J. (1923). *Investigation into the Morphology of the Ray Fungi*. Copenhagen.

OERSKOV, J. (1932). *Zbl. Bakt.* **123**, 271.

OERSKOV, J. (1932). *Zbl. Bakt.* **126**, 580.

PRYCE, D. M. (1941). *J. Path. Bact.* **53**, 327.

SAENZ, A. (1938). *Ann. Inst. Pasteur*, **61**, 662.

SOLTYS, M. A. (1942). *J. Path. Bact.* **54**, 375.

VAUDREMER, A. (1931). *Beitr. z. klin. d. Tuberk.* **77**, 16.

WILSON, G. S. & SCHWABACHER, H. (1937). *Tubercle*, **18**, 161.

WYCKOFF, R. W. J. (1934). *Rev. Tuberc.* **24**, 93.

(*MS. received for publication* 1. IX. 44.—Ed.)